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GCMS Separation of the Bioactive Fraction Obtained from *Aspergillus flavus* isolated from El-Qussair Marine Environments

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DUE TO THEIR diversity and chemo-diversity, marine fungi are renowned for producing structurally distinct secondary metabolites and are regarded as a viable source of novel treatments. This study aimed to evaluate the antimicrobial activity of 26 fungal strains collected from the Mediterranean and Red Seas at Sharm el-sheikh, Marsa matrouh, Damietta, El-ain el-sobhy, and El-Qusair. Using the well diffusion method with ethyl acetate, petroleum ether, methanolic, and chloroformic extracts, the antibacterial activity of each fungal isolate against pathogenic microbes was assessed. Our research revealed that marine fungi, particularly those from El-Qusair, possess potent antibacterial properties. The IC50 values for the cytotoxic activity of natural products extracted with ethyl acetate and petroleum ether against human hepatocellular cancer cell lines (HepG2 cells), human colon carcinoma cell lines (HCT-116 cells), and human breast cancer cell lines (MCF-7 cells) were 62.13, 115.93, and 154.82g/mL, respectively. Using the DPPH free radical scavenging assay in triplicate and average values, the antioxidant activity of the active fraction was determined. Our findings revealed that the ethylacetate extract had the highest percent DPPH scavenging activity compared to petroleum ether extracts. Using GCMS, chromatographic separations of the active ethyl acetate extract were performed to determine the active principles responsible for the activities.

Keywords: Anticancer activity, Antimicrobial activity, Antioxidant activity, GCMS, Marine fungi, Red sea.

Introduction

The production of physiologically active secondary metabolites is crucially dependent on marine microorganisms, particularly fungi. Comparatively to terrestrial fungi, marine fungi have developed secondary metabolic pathways that have proven to be an important source of novel biological natural products.

Been demonstrated that fungi produce bioactive chemicals that can be used to combat cancer and pathogenic microbial infections (Wiese et al., 2011).

Marine fungi produce an abundance of well-known bioactive compounds, including anticancer, antibiotic, antiviral, antiangiogenesis compounds, and antiproliferative molecules. Intriguingly, a large number of antioxidant molecules have been identified that, due to their unique properties, can be utilized in a variety of fields, including food, cosmetics, and pharmaceuticals. The use of products derived from marine fungi would present several advantages over synthetic antioxidants and those derived from nonrenewable sources, such as plants and algae, in terms of cost reduction and process sustainability. In reality, fungal fermentation is an easily scalable process, and fungi can be genetically modified (Vitale et al., 2020).

Material and Methods

Locations of specimens

In this study, various samples were collected in sterile bottles from various locations in the Mediterranean and Red Sea at Sharm el-Sheikh, Marsa matrouh, Damietta, El-ain El-sokhna, and El-Qussair at a depth of 30cm from the surface of

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the sand, as well as marine water.

Isolation of fungi

Subsequently, using the Direct Plating method fungi were isolated on malt agar medium plates with an antibacterial agent (0.5g/L chloramphenicol). After the malt agar medium solidified, 0.5g of soil samples or 1mL of water samples were placed directly on top (Farrag et al., 2017).

The plates were then incubated at 28±2°C for 7–15 days. After the incubation period, each fungal colony was isolated, identified, and preserved by transferring it to a pure slant containing the same medium, and the slants were stored at 4°C for subsequent testing (Guimarães et al., 2014).

Antimicrobial activity

The media used are malt extract agar and nutrient agar. Gram-positive and Gram-negative pathogenic bacteria were grown on a nutrient agar medium. Pathogenic fungal strains were grown on a malt extract agar medium as test organisms. All microorganisms were generously provided by the Regional Center for Mycology and Biotechnology (RCMB) atAl-Azhar University in Cairo, Egypt.

Isolate cultivation for secondary metabolite production

Yeast-extract sucrose (YES) liquid medium was used to produce secondary metabolites. Each 250mL Erlenmeyer flask containing 100mL of the medium was filled with the medium. Adjusting the pH to 6.5 ±0.2. The medium was then autoclaved at 121°C for 15min. Each strain of fungus received 5L. Subsequently, 100mL of YES medium was inoculated with 1mL of spore suspension from the examined fungus and cultured at 25°C for 21 days (Farrag et al., 2017).

Extraction of secondary extracellular metabolites

At the end of each incubation period, inoculated flasks were retrieved. The culture filtrates were separately subjected to solvent extraction, as previously described by Younis et al. (2014), with the following modifications:

For each fungus, 5L of broth was filtered through Whatman®No. 1 filter paper to separate culture filtrate and mycelia. The culture filtrates were combined with petroleum ether in a separating funnel, agitated vigorously, and allowed to settle for at least 6h until complete separation. The petroleum ether layer (the top layer) was then separated. Three portions of the remaining filtrate layer were exposed to three distinct solvent systems: ethyl acetate, chloroform, and methanol. The filtrate was mixed with an equal volume of ethyl acetate, chloroform, or methanol in a separating funnel. After separating the solvent phases of aqueous and organic solvents, they were concentrated to dryness using a rotary evaporator (Buchi) and stored at 5°C until testing.

Antimicrobial activity assay

Next, using the agar well diffusion method, the antibacterial potential of the fungal extracts was evaluated against pathogenic bacterial and fungal strains. The agar plates were prepared, and an inoculum of log-phase bacteria and fungi was generated. In order to create wells, the plates were subsequently pierced with a cork-borer (6mm in diameter) under aseptic conditions. The examined extract (100L) of each sample was placed in a separate well. As a negative control, a solvent-filled well was utilized. Before the growth of microorganisms could commence, all plates were kept at 4±2°C for 2h to allow for the diffusion of substances. For fungi and bacteria/yeast, the agar plates were incubated for 48h at 28±2°C and 24h at 37±2°C, respectively.

Extraction of extracellular secondary metabolites

At the end of each incubation period, inoculated flasks were retrieved. Separate solvent extractions were performed on the culture filtrates, as previouslydescribed by Younis et al. (2014).

Genetic Identification and sequencing of El-Qussair's most active marine fungi

Using forward and reverse primers, the purified PCR product was sequenced in one direction using the forward designed universal primer in an automated sequencer ABI 3730xl (GATC Co., Germany). Geneious Pro 7.3.1 was used to analyze the sequences. The ERG11gene sequences were compared to those of the Genbank and EMBL databases using an advanced BLAST (Megablast) search program to determine the percentage of similarity between the sample and those on the Genbank database. The isolates' phylogenetic relationship was determined.

Anticancer activity

Carcinoma cell lines

This study used human colon carcinoma cells (HCT-116 cells), human breast cancer cells (MCF-7 cells), and human hepatocellular cancer cells ($HepG_2$ cells) as carcinoma cell lines (HepG2

cells). Each and every cell line was obtained from the VACSERA Tissue Culture Unit.

The extract was evaluated against three tumor cell lines, namely human colon carcinoma cells (HCT-116), human breast cancer cells (MCF-7), and human hepatocellular cancer cells ($HepG_2$) using crystal violet viability assay (Saotome et al., 1989; Wilson, 2000).

Antioxidant activity

At Al-Azhar University's Regional Center for Mycology and Biotechnology, the antioxidant activity of active extracts was measured in triplicate using the DPPH free radical scavenging test (RCMB).

The IC_{50} value, i.e., the concentration of the tested chemical that resulted in a 50% inhibition of the DPPH radical, was determined using graphical plots of DPPH radical scavenging versus compound concentrations (Xie & Schaich, 2014).

GCMS analysis

The *Aspergillus* metabolites were identified using an Agilent Model 7890A gas chromatograph and an Agilent 5975C Mass Selective Detector (Agilent Technologies, Palo Alto, CA, USA).

Analytical Statistics were done using a one-way ANOVA test (Castilla-Serna & Cravioto, 1999), Microsoft Excel® was utilized to calculate the percentage of viable cells andthe standard deviation (Castilla-Serna & Cravioto, 1999).

Results and Discussion

As shown in Table 1, the isolated fungi were coded by isolation area and primarily identified as follows:

TABLE 1. Fungi isolated from various locations in the Mediterranean and Red Seas, along with their preliminary identification and percentage of occurrence

No.	Sample source	Preliminary generic identification	Isolate code	Occurrence (%)
1	Marsa Matrouh Sand	*Aspergillus*	F1	10
		Penicillium	F2	20
		Aspergillus	F3	10
		Mucor	F4	60
2	El- ain El-sokhna Sand	*Aspergillus*	F5	30
		Penicillium	F6	70
3	Sharm El-sheikh Sand	*Acremonium*	F8	20
		Aspergillus	F10	50
		Aspergillus	F11	30
4	Sharm El-sheikh Water	*Penicillum*	F7	50
		Aspergillus	F9	50
5	El-kosseir Sand	*Aspergillus*	F17	80
		Acremonium	F18	20
6	El-kosseir Water	*Penicillum*	F12	50
		Aspergillus	F13	10
		Penicillum	F14	10
		Aspergillus	F15	10
		Aspergillus	F16	10
		Aspergillus	F19	10
7	Damettia Sand	*Penicillum*	F24	100
8	Damettia Water	*Aspergillus*	F20	20
		Aspergillus	F21	10
		Aspergillus	F22	10
		Aspergillus	F23	30
		Penicillum	F25	20
		Penicillum	F26	10

Twenty-six fungal isolates (F1–F26) were obtained from two sources (water and sand) in five distinct Mediterranean and Red Sea locations.

The percentage of colonies belonging to the genus *Aspergillus* in samples from the Mediterranean and Red seas was 46%. In contrast, *Penicillia* comprised 41% of colonies counted in the Mediterranean and Red Seasamples. *Mucor* (which was only isolated from Marsa matrouh) and *Acremonium* (which was only isolated from Sharm El-Sheikh and El-Qussair) had a lower proportion of colonies counted in samples from the Mediterranean and Red seas (Fig 1).

The antimicrobial activity of 26 marine fungi isolates extracted by petroleum ether, and ethylacetate in Sharm el-sheikh, Marsa matrouh, Damietta, El-ain El-sokhna, and El-Qusair against pathogenic Gram-positive bacteria, Gram-negative bacteria, fungi, and yeasts is shown in Tables 2, 3, and 4. Number (13) from El-Qussair was found to be the most potent antimicrobial sample, with substantial inhibition zones; therefore, this isolate was chosen for future research. Previously, it was reported (Nadeem et al., 2015) that the Red Sea is a rich source of microbial diversity as well as novel compounds with pharmacological and therapeutic value.

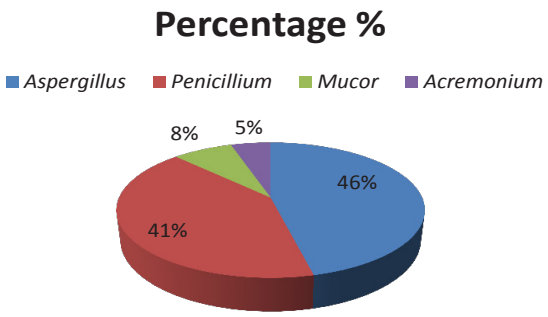

Fig. 1. The percentages of isolated fungal genera were obtained from various Mediterranean and the Red Sea locations

Sequence and genetic identification of the most active fungi (13)

Figure 2 indicates Phylogenetic tree of fungus (13) from El-Quseir is *Aspergillus flavus* and its sequence is:

NNNNNNGGATCCTGGCGTAGC-
CATCCTACCACCCGTGTTTACAGTATGTT
AGTTGCTTCGGCGCGCCCGC-
CATTCATGGCCGCCGGGGGCTCT-
GCCCCCGGGCCCGCGCCCGCCG-
GAGACACCACGAACTCTGTCTGATC-
TAATGAAGTCTGAGTTGATTGTATCA-
CAATCATTTAAAACTTTCAACAATG-
GATCTCTTGGTTCCGGCATCGAT-
GAAGAACACAGCGAAATGCGATA-
ACTAGTGTGAATTGCTGAATTCC-
GTGAATCATCGAGTCTTTGAACG-
CACATTGCGCCCCCTGGTATTCC-
GGGGGGCATGCCTGTCCGAGCGT-
CATTGCTGCCCATCAAGCACGGCTT-
GTGTGTTGGGTCGTCGTCCCCTCTCC-
GGGGGGGACGGGCCCCAAAGGCGGC-
GGCGGCACCGCGTCCGATCCTCGAGC-
GTATGGGGCTTTGTCACCCGCTCTG-
TAGGCCCGGCCGGCGCTTGCCGAACG-
CAAATCAATCTTTTCCAGGTTGACCTC-
GGATCAGGTAGGGATACCCGCTGAACT-
TAAGCATATCAATANCCGGAAGAAC-
GATCATTACCGAGTGTAGGGTTCCTAGC-
GTGCCACATCTCCCACCCGTGCTT-
TACTGTACCTTAGTTGCTTCGGCCC-
GCCGTCTTTCAGGCCGCGGGAG-
GCTCTGACCCCCGGGCCCCGCNAAA-
CACACGAATCAATCTATTCTGATGA-
GATATCNGAATCTTGATTGGTATCA-
CAGTCAATCTAATATCTATTCATTCATC-
GAAGCTCTCTGTNGTCTGTCATC-
GATCGAAANAAAGACAGCNAGTGAC-
GTGTGAACTAGTGAAGGATGTGCAT-
GACATACATGTCAATGCAAATATTC-
TAGTCATACGCACACTCGCTCCCC-
GCCCTAGGTCAGGTCGGGCGAGGCAT-
GTCCGATCTANNATTCATTTNTTACT-
CAGGNGGTGTGTTTTTTGTTTTGNN
NNNNNNTCTNNNGGGGAAAGGGCC
CCNNAGAAGCCCCCGCNCCCCNNC-
CATTCTTGCCCCNTANAGANTC-
GGGTCTGCTTCTTTNGCCCAAGC-
CANGNNAGCCCNGCCCNGGTNCACTC-
GAAANNNNNNNNTTTACTCGCTTTN-
ACTTGAGCGNNNNNACTCGGGCGGA-
NANNNANNNNACCTANA

TABLE 2. Antimicrobial activity of petroleum ether extracts of isolated marine fungi from various locations in the Mediterranean and Red Seas against pathogenic microorganisms.

Microbes / Isolated fungi	*Staphylococcus aureus*	*Streptococcus*	*Pseudomonas aeruginosa*	*E.coli*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Penicillium mornefi*	*Cryptococcus neoformans*	*Candida albicans*
Fungus1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus11	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus13	**13**	**15**	**14**	**12**	**13**	**10**	**12**	**9**	**7**	**9**
Fungus14	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus16	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus17	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus18	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus19	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus20	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus21	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus22	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus23	-ve	-ve	**10**	**9**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus24	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus25	**12**	**13**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus26	**15**	**10**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

After subtracting the petroleum ether control from the inhibition zone results in mm, (-ve) indicates negative results of isolated marine fungi extract against pathogenic microorganisms.

TABLE 3. Antimicrobial activity of ethyl acetate extract of isolated marine fungi from various locations in the Mediterranean and Red Seas against pathogenic microorganisms.

Microbes / Isolated fungi	*Staphylococcus aureus*	*Streptococcus*	*Pseudomonas aeruginosa*	*E.coli*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Penicillium mornefi*	*Cryptococcus neoformans*	*Candida albicans*
Fungus1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus11	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus12	**16**	**12**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus13	**30**	**21**	**7**	**9**	**6**	**10**	**9**	**11**	**10**	**7**
Fungus14	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus16	-ve	-ve	-ve	-ve	**16**	-ve	-ve	-ve	-ve	-ve
Fungus17	**16**	**13**	-ve	-ve	-ve	-ve	-ve	**12**	-ve	-ve
Fungus18	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus19	-ve	-ve	**2**	-ve	-ve	-ve	-ve	**9**	-ve	-ve
Fungus20	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus21	-ve	-ve	**24**	**20**	-ve	-ve	-ve	-ve	**9**	-ve
Fungus22	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus23	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus24	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus25	**15**	**16**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus26	**39**	**35**	-ve	-ve	-ve	-ve	**14**	**18**	-ve	-ve

After subtracting the ethylacetate control from the inhibition zone results in millimeters, (-ve) indicates negative results of isolated marine fungi extract against pathogenic microorganisms.

TABLE 4.Antimicrobial activity of aqueous fraction of isolated Mediterranean and Red Sea marine fungi against pathogenic microorganisms

Microbes / Isolated fungi	*Staphylococcus aureus*	*Streptococcus*	*Pseudomonas aeruginosa*	*E.coli*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Penicillium mornefi*	*Cryptococcus neoformans*	*Candida albicans*
Fungus1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus6	-ve	-ve	**15**	**12**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus7	-ve	-ve	**15**	**13**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus8	-ve	-ve	**12**	**14**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus9	-ve	-ve	**15**	**16**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus10	-ve	-ve	**12**	**13**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus11	-ve	-ve	**16**	**18**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus12	**17**	**15**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus13	**31**	**29**	**26**	**21**	**9**	**8**	**8**	**7**	**10**	**6**
Fungus14	-ve	-ve	-ve	-ve	**14**	-ve	-ve	-ve	-ve	-ve
Fungus15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus16	-ve	-ve	-ve	-ve	**18**	-ve	-ve	-ve	-ve	-ve
Fungus17	**29**	**26**	**12**	**10**	-ve	-ve	-ve	**9**	-ve	-ve
Fungus18	**18**	**20**	**18**	**17**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus19	-ve	-ve	**8**	**7**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus20	-ve	-ve	**13**	**14**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus21	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus22	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus23	-ve	-ve	**7**	**5**	-ve	-ve	-ve	-ve	-ve	-ve
Fungus24	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus25	**15**	**17**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Fungus26	**28**	**29**	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Negative results of isolated marine fungi extract against pathogenic microorganisms, as measured in millimeters, are denoted by (-ve) in the inhibition zone results.

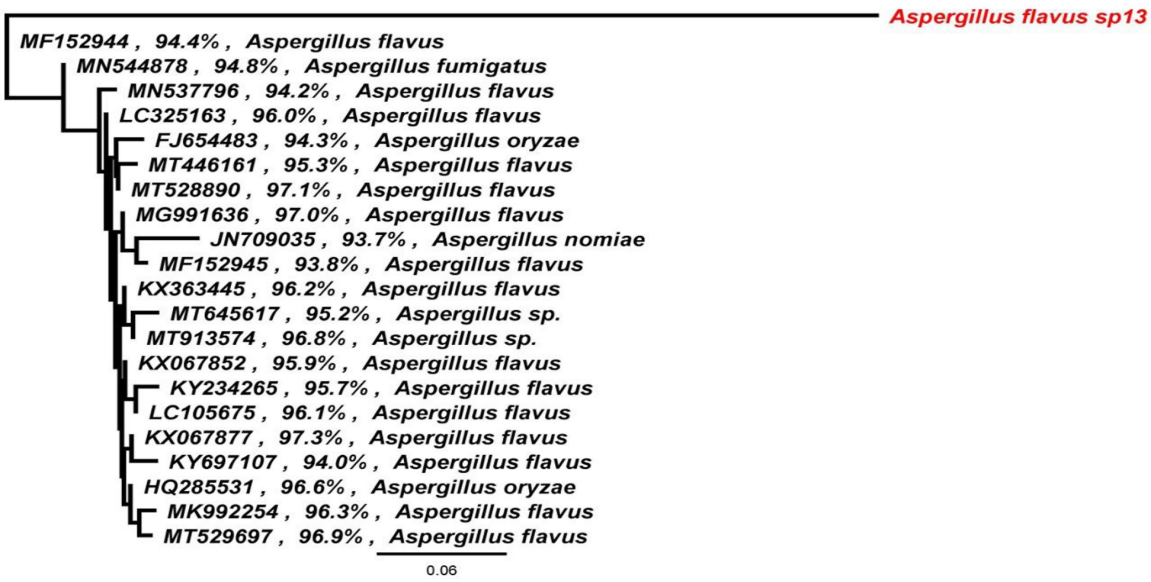

Fig. 2. According to the phylogenetic tree of fungi (13) from El-Qussair, *Aspergillus flavus* is present

Antimicrobial activity of Aspergillus flavus of El-Qussairagainst pathogenic microorganisms

All *Aspergillus flavus* extracts exhibited antimicrobial activity against MRSA, *Klebsiella, Enterobacter cloacae*, and *Enterococcus*, as shown in Table 5. Except for the methanolic extract, these extracts exhibited antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis*, and *Salmonella*; however, the ethyl acetate extract exhibited the highest antimicrobial activity with the largest inhibition zones. Except for petroleum ether extract, *Streptococcus* mutants, *Bacillus subtilis, Micrococcus, Serratia marcescens,* and *Cryptococcus* are sensitive to all extracts. *Proteus vulgaris* and *Fusarium oxysporum* are sensitive to petroleum ether and ethylacetate extracts but resistant to methanol and chloroform extracts. Only ethyl acetate extract is effective against *Escherichia coli*, whereas petroleum ether, methanolic, and chloroform extracts are ineffective. *Candida albicans* is susceptible to methanolic and chloroformic extracts but resistant to petroleum ether and ethyl acetate extracts. *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus,* and *Penicillium mornefi* are resistant microorganisms (Fig. 3).

In other studies (Qin et al., 2015), it was reported that antibacterialor antifungal activity was found in 38% to 59% of marine fungus test extracts. These findings are consistent with these findings.The study of antimicrobial compounds from marine fungi dates back to 1998 when neomangicol B (231) was isolated from *Fusarium heterosporum* CNC-477 grown on driftwood. By the end of 2019, it is expected that 272 new antimicrobial compounds derived from marine fungi will be reported. Since 2010, more secondary metabolites than previously reported have been isolated from marine fungi,except for 2016 and 2017. China, other Asian nations, Europe, and the United States produced 193, 41, and 36 antimicrobial compounds, respectively. Chinese researchers published approximately 71% of all new compounds. More than half of the papers, 52%, were published in March. Alcohol and J. Nat. Prod. The top journals that published antimicrobial compounds derived from marine fungi were published in March. Drugs 14.3%, J. Nat. Prod. 13.6%, whereas J. Antibiot. 11.4%. The majority of novel structures were reported in the journal Org. Lett. and J. Nat. Prod. Fungi isolated from sediments, sponges, algae, and mangroves generatedthemajority ofnew antimicrobial compounds (22.4, 18.4, and 18.015.1%, respectively) (Wang et al., 2021).

While, Abdou et al. (2017) reported that antibacterial activity against Gram-negative bacteria was more effective than against Gram-positive bacteria. In addition, the Red Sea has been identified as a rich source of microbial diversity and novel compounds with potential pharmacological and therapeutic applications. The development of new active metabolites is a mental barrier in evaluating new compounds for therapeutic applications. *Candida albicans*

exhibited a resistant strain in our studies. In accordance with the findings of Kaya et al. (2008), *C. albicans* 845981, The strains *C. crusei* ATCC 6258 and *C. albicans* 90028 were resistant to chloroform, acetone, and methanol extractsof *O..basilicum* leaves, contrary to Al-Ghamdi et al. (2020) assertion that methanol and hexane extracts of aerial parts of *O. basilicum* exhibited antifungal activity against *C. albicans*.

TABLE 5. Antimicrobial activities of secondary metabolities obtained from *Aspergillus flavus* [The results are expressed as inhibition zones (mm)]

Microbes	The isolated fungi	Petroleum ether extract	Ethyl acetate extract	Culture broth	Methanol extract	Chloroform extract
Gram positive bacteria	*Staphylococcus aureus*	1	30	31	-	16
	MRSA	7	21	22	20	7
	Staphylococcus epidermidis	7	20	23	-	2
	Streptococcus mutants	-	18	15	25	22
Gram negative bacteria	*Pseudomonas aeruginosa*	-	-	16	13	-
	Eschericia coli	-	15	18	-	-
	Klebsiella	11	20	27	15	8
	Bacillus subtilis	-	18	25	16	9
	Micrococcus	-	27	33	9	19
	Enterobacter cloacae	12	23	28	2	20
	Salmonella	17	27	26	-	3
	Proteus vulgaris	15	25	27	-	-
	Enterococcus	12	30	30	10	4
	Serratia marcenscens	-	30	26	1	2
Fungi	*Aspergillus niger*	-	-	-	-	-
	Aspergillus flavus	-	-	-	-	-
	Aspergillus fumigatus	-	-	-	-	-
	Penicillium mornefi	-	-	-	-	-
	Fusarium oxysporum	26	30	27	-	-
Yeast	*Cryptococcus*	-	25	30	5	6
	Candida albicans	-	-	-	6	2

The assay was performed using the agar well diffusion method.

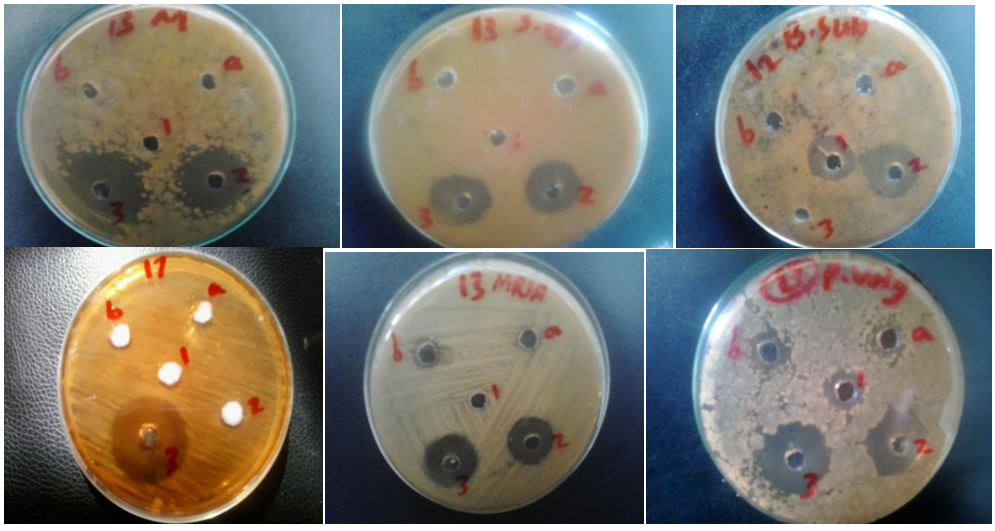

Fig. 3. Antimicrobial activity of the marine fungus extract against *Micrococcus, Staphylococcus epidermidis, Bacillus subtilis, Pseudomonas aeruginosa,* MRSA, and *P. vulgaris* [a: Petroleum ether, b: Ethyl acetate, 1: Extract of petroleum ether, 2: Extract of ethyl acetate, 3: The culture broth]

Evaluation of cytotoxicity of extract of fungus (13)

These results showed that cytotoxicity activity of metabolities of marine fungus against hepatocellular carcinoma (HepG2) cell line is very high with an inhibition rate of 90.16% at a concentration of 500µg/mL and an IC_{50}= 62.13 ± 2.81µg/mL, the cytotoxic activity of marine fungus metabolites against the hepatocellular carcinoma (HepG2) cell line was found to be extremely high. In the case of the colon carcinoma (HCT-116) cell line, the anticancer activity of marine fungus metabolites is greatest with an inhibition rate of 85.43% ataconcentration of 500µg/ml and an IC_{50}= 115.93 ± 3.75µg/mL. In the case of breast carcinoma (MCF-7) cell line, the anticancer activity of marine fungus metabolites is lower than for the other two tested cell lines (IC_{50}= 154.82 5.34µg/mL) (Fig. 4).

These results are consistent with the findings of Nawwar et al. (2011), who reported that the dichloromethane extract of an anamorphic fungal strain MF 014 recovered from a submerged mangrove in Safaga, Egypt, exhibits a significant cytotoxicity activity. These results are also consistent with the findings of Sun et al. (2012) who reported that the marine-derived fungus *Aspergillus* sp. was obtained from the sponge *Xestospongia testudinaria*. In addition collected from the South China Sea. This exhibited *in vitro* moderate cytotoxicity toward HepG-2 and Caski human tumor cell lines with the IC_{50} values of 9.31 and 12.40µg/mL respectively. Also, Prata-Sena et al. (2016) reported that the chemical analysis of the marine-derived fungus *N. siamensis* was isolated from the sea fan *Rumphella* sp. which was collected from the Andaman Sea of Thailand, led to the isolation of chevalone C exhibited moderate cytotoxicity against three tumor cell lines, including colon HCT-116, liver HepG2, and melanoma A375 with IC_{50} values ranging from 24 to 153µM.

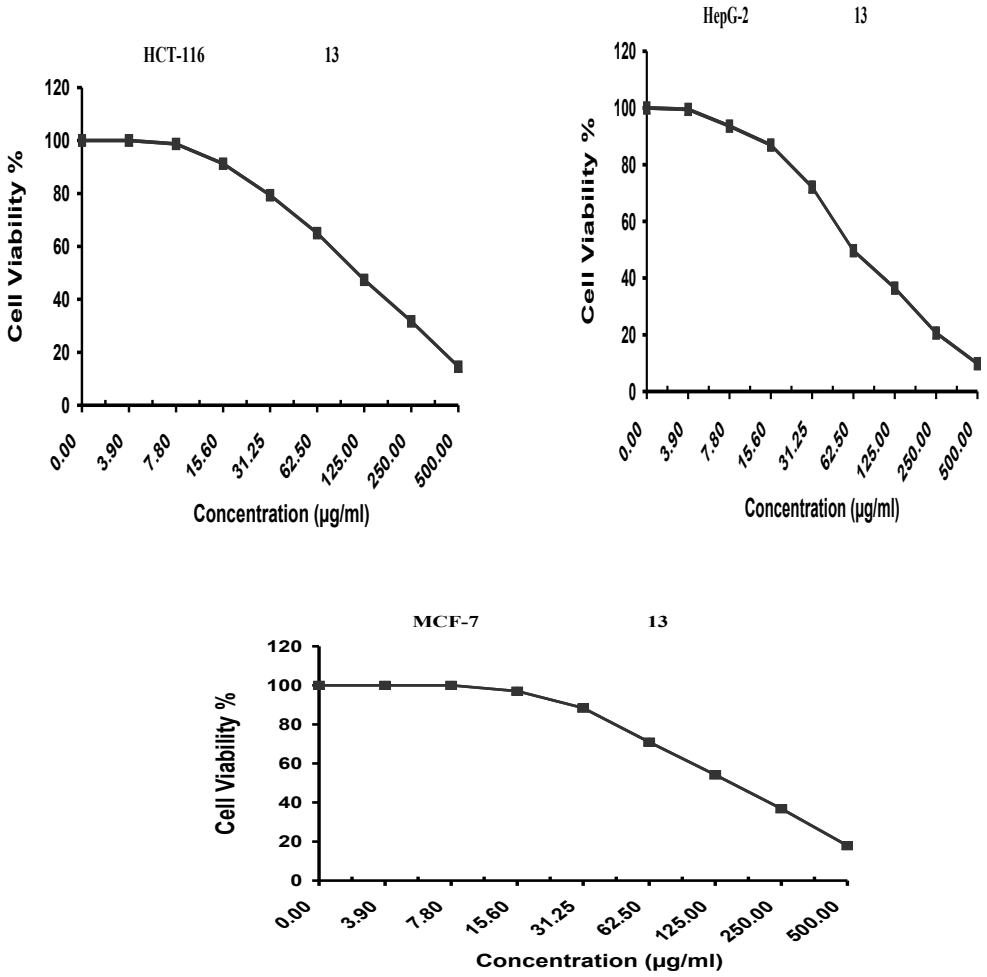

Fig. 4. The dose-response curves demonstrating the cytotoxic effects of the ethyl acetate extract of *A. flavus* were isolated in El-Qussair. A, HepG2, B, HCT-116, and MCF-7 carcinoma cell lines

Evaluation of antioxidant activity of extract of fungus (13) using DPPH scavenging

At a concentration of 3200µg/mL, the ethylacetate extract of metabolites of marine fungus exhibited the highest percent DPPH scavenging activity, 92.44%, with an IC50 value of 291.6µg/mL in the two solvents used. Petroleum ether extract of metabolites of marine fungus exhibited the lowest percent DPPH scavenging activity, 54.07%, with an IC_{50} value of 2749µg/mL (Fig. 5).

GCMS analysis

The GCMS profile of the fraction is shown in Fig. 6 and Table 6 lists the retention time (RT) and percentage of the peak for each compound. The promising ethyl acetate fraction contained eight major compounds, namely, tributylacetylcitrate (22.95%), hexadecane (7.87%), pentadecane (7.81%), 1, 2-benzene dicarboxylic acid, diisoctyl ester (6.43%), 2-methyl benzyl amine, N-heptyl-N-octyl (5.23%) benzene, (1-methyl undecyl) (4.81%), heptadecane (4.56%) and 1, 2-benzene dicarboxylic acid, buty (4.05%). These compounds may be responsible for various biological functions. The *Casimiroa edulis* leaf extract contained tributylacetylcitrate, which exhibited insecticidal activity against *Spodoptera littoralis* larvae (Barakat, 2011). Also, it has been reported that 1,2-benzene dicarboxylic acid to possess anti-inflammatory and antibacterial properties (Modupe et al., 2010). Moreover, Senthilkumar et al. (2011) discovered that 1,2-dicarboxylic acid, diisooctyl ester, andother compounds would have inhibited the growth of *F. oxysporum*.

In contrast tothe Gram-positive bacterium *S. aureus* to the Gram-negative bacterium E. *coli*, according to the results presented in Table 6,the Gram-positive bacterium *S. aureus* was more sensitive to the fractions that were tested. This could be due to the dissimilarity oftheir cell walls (Epand et al., 2016).

Gram-positive bacteria have apeptidoglycan layer. *S. aureus*has much thicker cell walls than *E. coli*, whereas the layer of lipopolysaccharide (LPS) in *E. coli* is significantly thicker. Generally, fungi are more resistant to the examined fractions than bacteria.

Anticancer activity

Twenty-one compounds in the promising ethyl acetate fraction of the marine fungus *Aspergillus flavus*. Tables 6 and 7 list the active ingredients along with their retention time (RT), molecular formula, molecular weight, and concentration (percent).

The spectrum of the unknown constituents of the promising ethyl acetate fraction was compared to the spectra of known constituents stored in the NIST library. Six biologically active compounds were identified in *Aspergillus flavus* extract (Fig. 6 and Table 6). Moreover, two of these compounds (benzenedicarboxylic acid, butyl octyl ester, and 1,2-benzenedicarboxylic acid, diisooctyl ester, with butyl octyl ester) are diisooctyl esters.

Asreported by Senthilkumar et al. (2011), both (at concentrations of 4.05 and 6.43 percent, respectively) exhibited significant antioxidant activity (Shanab et al., 2011).

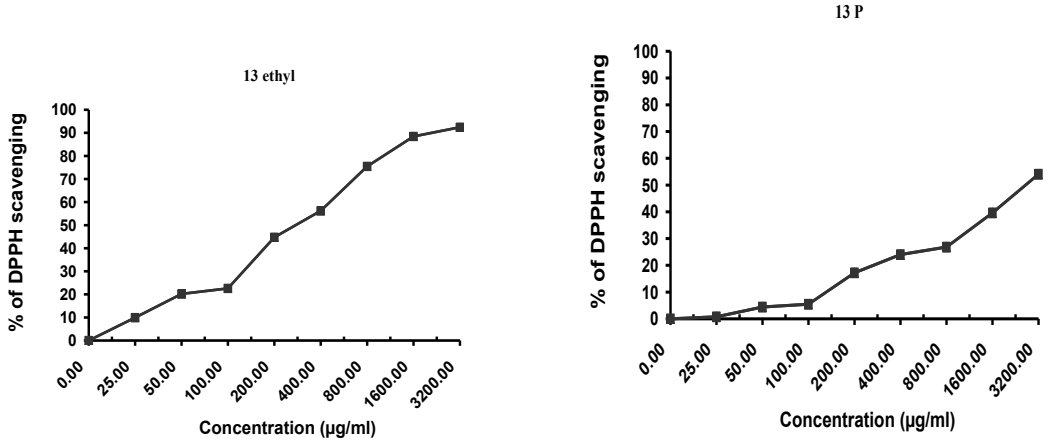

Fig. 5. Evaluation of antioxidant activity of extracts obtained from *A. flavus* was isolated from El-Qussair by means of the DPPH scavenging assay

TABLE 6. The identification of constituents of the separated metabolites obtained from the ethyl acetate extract of *A. flavus* was isolated from El-Qussair using GC/MS Chromatography

RT	Area %	Suggested compound name	Molecular formula	Molecular weight
4.81	1.95	2(5H)-FURANONE, 3,5-DIMETHYL-	C6H8O2	112
6.29	1.24	METHYL 4-OXOPENTANOATE	C6H10O3	130
7.39	1.17	Z-10-Pentadecen-1-ol	C15H30O	226
9.84	33.22	2-PROPYL-2-PENTENAL	C8H14O	126
11.57	4.60	2-Piperidinone	C5H9NO	99
12.62	0.77	2-Coumaranone	C8H6O2	134
12.85	0.38	8-Nonenoic acid	C9H16O2	156
14.10	0.54	3-Decyn-2-ol	C10H18O	154
14.15	0.67	3(2H)-Isoquinolinone, octahydro-, (4ar-trans)-	C9H15NO	153
14.59	0.59	Cyclopentanone, 2-methyl-3-(1-methylethyl)-	C9H16O	140
14.78	2.85	2-Pentenal, 2-methyl-	C6H10O	98
15.73	2.84	3,7-Diazabicyclo[3.3.1]nonane, 9,9-dimethyl-	C9H18N2	154
17.34	1.37	5-METHYL-1,3-BENZENEDIOL	C7H8O2	124
17.50	1.02	2H-Oxecin-2-one, 3,4,7,8,9,10-hexahydro-4-hydroxy-10-methyl-, [4S-(4R*,5E,10S*)]-	C10H16O3	184
17.71	0.93	3-METHYL-6-OXO-9-OXABICYCLO[3.3.1]NON-2-YL ACETATE	C11H16O4	212
18.13	6.03	1-PROPANONE, 1-(2-FURANYL)-	C7H8O2	124
18.46	0.54	3-Hepten-2-one, 3-ethyl-4-methyl-	C10H18O	154
18.94	3.29	Bicyclo[2.2.2]octane-1,4-diol, monoacetate	C10H16O3	184
19.02	2.11	3-Heptyne-2,5-diol, 6-methyl-5-(1-methylethyl)-	C11H20O2	184
19.17	2.29	Cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-2-propenyl)-, ethyl ester, cis-	C12H20O2	196
19.64	6.45	2,2,7,7-TETRAMETHYL-3,6-OCTANEDIONE	C12H22O2	198
19.86	3.48	Furan, tetrahydro-2-isopentyl-5-propyl-	C12H24O	184
19.99	0.34	2-Cyclohexen-1-one, 5,5-dimethyl-3-(2-methylpropoxy)-	C12H20O2	196
20.52	0.41	Furan, 4,5-diethyl-2,3-dihydro-2,3-dimethyl-	C10H18O	154
20.62	1.76	(-)-Mellein	C10H10O3	178
21.16	2.95	ACETHYDRAZIDE, N2-(5,5-DIMETHYL-3-OXOCYCLOHEXYLIDENO)-	C10H16N2O2	196
21.43	0.81	2(3H)-Benzofuranone, hexahydro-4,4,7a-trimethyl-	C11H18O2	182
21.71	0.42	2(3H)-Benzofuranone, hexahydro-4,4,7a-trimethyl-		182
21.84	3.59	4H-THIENO[3,2-B]INDOLE, 5,6,7,8-TETRAHYDRO-	C10H11NS	177
21.99	1.69	3,5A,9,9-TETRAMETHYLDECAHYDROBENZO[2,3] CYCLOHEPTA[1,2-B]OXIREN-3-OL	C15H26O2	238
22.66	0.99	2,5-Hexadienoic acid, 3-methoxy-5-methyl-4-oxo-	C8H10O4	170
24.31	1.78	1-Tetradecanamine, N,N-dimethyl-	C16H35N	241
24.55	0.41	2,6-Dimethylhex-4-enamidine, 2-(3-methylbut-2-enyl)-	C13H24N2	208
24.84	3.25	Benzaldehyde, 3-ethoxy-	C9H10O2	150
32.32	0.48	9-Octadecenoic acid (Z)-, methyl ester	C19H36O2	296
32.52	0.38	Phenethylamine, N-benzyl-à-methyl-	C16H19N	225
35.32	0.49	Tributyl acetylcitrate	C20H34O8	402
37.22	0.44	Hexadeca-2,4,15-trienoic acid, ethyl ester	C18H30O2	278

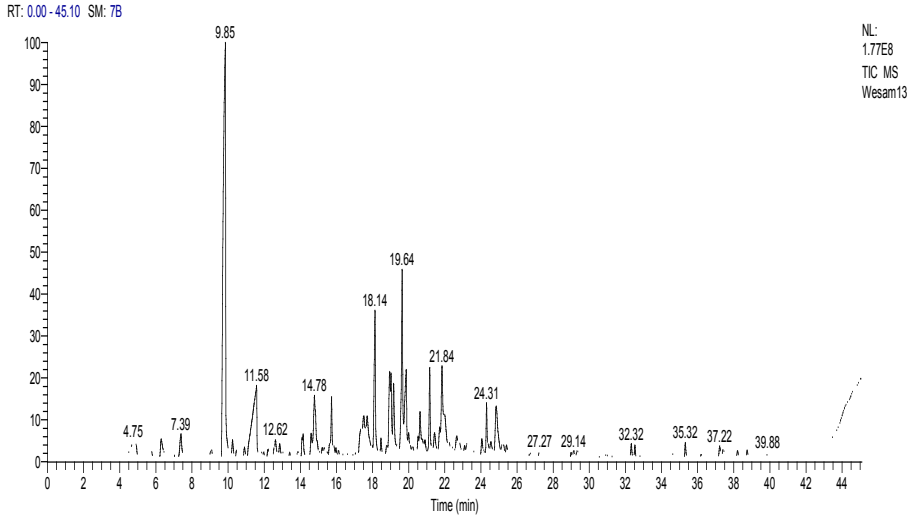

Fig. 6. Chromatographic separation of the purified ethyl acetate extract obtained from *A. flavus* isolated from El-Qussair with a GCMS total ion chromatogram displaying distinct peaks

TABLE 7. List of major components and their biological activities of a promising ethyl acetate fraction obtained by GCMS chromatographic separation from *Aspergillus flavus*

RT	Compound name	Relative concentration	Reported activities
5.0-12.02	Alkane derivatives	22.83	No activity
12.78	Benzene, (1-pentylhexyl)-	1.72	No activity
12.95	Benzene, (1-butylheptyl)	3.71	No activity
13.34	Benzene, (1-propyloctyl)-	3.77	No activity
14.08	Benzene, (1-ethylnonyl)-	2.01	No activity
15.31	Benzene, (1-pentylheptyl)-	3.79	No activity
15.50	Benzene, (1-butyloctyl)-	3.69	No activity
15.88	2-Methylbenzylamine, N-heptyl-N-octyl	5.23	Antimicrobial
16.72	Benzene, (1-ethyldecyl)-	2.36	No activity
17.83	Benzene, (1-pentylheptyl)-	3.54	No activity
18.08	Benzene, (1-butylnonyl)-	2.12	No activity
18.53	Benzene, (1-methylundecyl)-	4.81	No activity
21.05	Naphthalene, 2,3,6-trimethyl-	3.12	Antimicrobial, Anticancer
29.47	Octadecanoic acid, ethyl ester	1.88	Antioxidant, Anticancer
38.22	1,2-Benzenedicarboxylic acid, butyl octyl ester	4.05	Antimicrobial, antifouling
43.09	Tributyl acetylcitrate	22.95	Antimicrobial activity
44.65	2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-ethylidene-	2.57	No activity
46.43	1,2-Benzenedicarboxylic acid, diisooctyl ester	6.43	Antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant, chemo preventive, lipoxygenaseinhibitor and pesticide

Our findings are consistent with those of Abd El-Rahman et al. (2020). They discovered that both hard and softcoral reefs in the Red Sea are home to a variety of microorganisms with antibacterial, antioxidant, and anticancer properties. In addition, our findings are consistent with those of Thirunavukkarasu et al. (2012), who stated that marine-derived fungi that live in a symbiotic relationship with marine invertebrates produce bioactive metabolites such as antibiotics, antioxidants, antitumors, antifungals, antiinsect, and acetylcholine esterase inhibitors.

Conclusion

In comparison to terrestrial fungi, marine fungi play an essential role as a source of physiologically active secondary metabolites with potent antibacterial activity against Gram-positive and Gram-negative bacteria, fungi, and yeasts. Additionally, they have established distinct secondary metabolic pathways.

Furthermore, marine fungi from El-Qussair, in particular, other Mediterranean and Red Sea locations, El-Qussair fungi possess the highest antibacterial activity.

Marine fungi have been a significant source of highly effective conventional drugs for the treatment of a variety of cancer cell lines, including colon carcinoma cell lines, hepatocellular carcinoma cell lines, and breast carcinoma cell lines, with the most effective fungal extract originating in the El-Qussair region of the Red Sea. *Aspergillus flavus*, a fungus isolated from the Red Sea, possesses significant antioxidant activity.

Due to the aforementioned, significant secondary metabolites can be extracted from the marine fungus *Aspergillus flavus*. These metabolites are advantageous because they possess distinct antioxidant, anticancer, and antibacterial properties.

Competing interests: The authors report no conflicts of interest regarding this work.

Authors' contributions: Dr Om Kalthoum participate in writing and revision of the results. Dr Amany abo El-nasr participate in following up the work and in writing. Dr Mahmoud El-aasser participate in the practicle work, writing and revision. Wesam Samir (the author) participate in the whole work, researches publishing and in writing.

Ethics approval: Not applicable.

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فصل GCMS للجزء النشط بيولوجيًا المتحصل عليه من *Aspergillus flavus* المعزول من بيئات القصير البحرية

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تشتهر الفطريات البحرية بإنتاج مستقلبات ثانوية مميزة هيكليًا وتعتبر مصدرًا حيويًا للعلاجات الجديدة. الهدف من هذه الدراسة هو اختبار النشاط المضاد للميكروبات لـ 26 سلالة فطرية تم جمعها من البحر الأبيض المتوسط والبحر الأحمر في شرم الشيخ ومرسى مطروح ودمياط والعين السخنة والقصير. تم فحص الفعالية المضادة للبكتيريا لكل عزلة فطرية ضد الميكروبات المسببة للأمراض باستخدام طريقة انتشار البئر باستخدام خلات الإيثيل، الإيثر البترولي، الميثانول، ومستخلصات الكلوروفورميك. أظهرت النتائج التي توصلنا إليها أن الفطريات البحرية، وخاصة تلك الموجودة في القصير، لها نشاط مضاد للجراثيم. أسفر النشاط السام للخلايا للمنتجات الطبيعية المستخلصة من أسيتات الإيثيل والأثير البترولي ضد خط الخلايا السرطانية للخلايا الكبدية البشرية (خلايا HepG2)، وخط خلايا سرطان القولون البشري (خلايا HCT-116)، وخط خلايا سرطان الثدي البشري (خلايا MCF-7) عن IC50 قيم 62.13 و 115.93 و 154.82 جم / مل على التوالي. تم تحديد النشاط المضاد للأكسدة للجزء النشط باستخدام مقايسة مسح الجذور الحرة DPPH في قيم ثلاثية ومتوسطة، وكشفت النتائج التي توصلنا إليها أن مستخلص أسيتات الإيثيل لديه أعلى نسبة من نشاط الكسح DPPH مقارنة بمستخلصات الإيثر البترولي. للتحقيق في المبادئ النشطة المسؤولة عن الأنشطة، تم إجراء عمليات الفصل الكروماتوجرافي لمستخلص أسيتات الإيثيل النشط باستخدام GCMS.