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Bioactive Secondary Metabolites from *Aspergillus fumigatus* ON428521 Isolated from Wadi El Rayan, El Fayum Governorate

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FUNGI were isolated from Wadi El Rayan soils, and their secondary metabolites, extracted by petroleum ether, ethyl acetate, and chloroform, were tested for antimicrobial activities. Among the isolates, fungus-F1 extracts exhibited remarkable inhibitory activity against the tested microorganisms; therefore, it was subjected to morphological and molecular identification, and the fungus identified as *Aspergillus fumigatus* ON428521. With an inhibition zone ranging from 7mm to 21.4mm, ethyl acetate extract exerted the greatest activity. However, the petroleum ether extract only reached 16.5mm, and the chloroform extract demonstrated a lower activity level. Interestingly, activity was higher against tested Gram negative than those of Gram positive bacteria. Furthermore, *Escherichia coli, Streptococcus mutans,* and *Cryptococcus neoformans* were treated with ethyl acetate extract to explore possible effects on ultrastructure using transmission electron microscopy. The cell walls of treated cells lost their uniformity, ruptured, and became thicker. Moreover, the antitumor activities were evaluated against hepatocellular carcinoma (HepG2) and prostate carcinoma (PC-3) cell lines using the MTT assay, which revealed a high inhibitory activity with petroleum ether extract (IC_{50}= 61.02 ± 1.64 and 90.31± 2.37µg/mL, respectively). Lower inhibition was detected with ethyl acetate extract (IC_{50} of 84.42±3.36 and 121.98 ±3.46µg/mL against HepG2 and PC-3, respectively). In addition, antioxidant activity was evaluated using the DPPH radical scavenging assay, and ethyl acetate extract demonstrated moderate activity with IC_{50} = 278.24 ± 8.52µg/mL, followed by petroleum ether (581.07 ± 41.95µg/mL). The phytochemical analysis showed that the ethyl acetate extract is rich in phenolics, followed by flavonoids and alkaloids while lipids are the major component of the petroleum ether extract. GC-MS analysis of petroleum ether extract gives dodecanamine, N, N-dimethyl-, lupeol acetate, amyrin, kojic acid, and oleanenol-acetate as the major components. In conclusion, ethyl acetate and petroleum ether extracts of *A. fumigatus* F1 exhibited moderate antimicrobial, antioxidant, and antitumor properties.

Keywords: Anticancer, Antimicrobial, Antioxidant, GC-MS, Secondary metabolites, Soil fungi.

Introduction

There is a critical need for natural bioactive metabolites with diverse antioxidant and anticancer activity (Abdel-Shafi et al., 2020; El-Sayed et al., 2020.; Enan et al., 2018), and establishing the credibility and efficacy of biocidal products is a common concern (El-Gazzar et al., 2020).

Given the devastation caused by highly infectious and noninfectious diseases, it is a significant challenge to identify pharmaceutical candidates that can treat these diseases with minimal or no side effects. However, despite the development of medications to treat and manage diseases like malaria, HIV/AIDS, diabetes, and cancer, these diseases continue to be epidemic in diverse global populations and are associated with mortality. Plants,

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algae, fungi, bacteria, etc., as well as their natural products, coexist in an ecosystem and interact in the production of secondary metabolites, which are produced to increase the fitness of an organism for survival. Each year, numerous natural products are discovered and introduced to the pharmaceutical market. Consequently, the discovery of novel compounds in nature stays an untapped resource (Ramesh et al., 2019).

Plants are considered important natural sources of medicinal properties. Plant-based medications have been used to treat a wide range of pathological disorders including *Cinchona* spp., a source of the antimalarial drug quinine. In addition, anticancer drugs such as taxol from *Taxus brevifolia,* vinblastine from *Catharanthus roseus*, and artemisinin from *Artemisia annua* are also obtained (Thomford et al., 2018).

However, microbial metabolites are considered a competing source. Among microbes, fungi are a special source as they produce inclusive and diverse secondary metabolites with different chemical structures. Among fungi, *Aspergillus* is unquestionably still a major contributor to interesting secondary metabolites due to its diversity. The World of Microorganisms Information Center (WDCM) has received reports of approximately 378 species of *Aspergillus* (Vadlapudi et al., 2017). Moreover, there are approximately 180 fungal species in the genus *Aspergillus* that have medicinal and commercial uses. In addition, *Aspergillus* secondary metabolites are known to exhibit anti-bacterial, anti-inflammatory, anti-cancer, and antioxidant activities (El-hawary et al., 2020). The present study aims to isolate fungi from the soil of Wadi El Rayan, a unique habitat in El Fayum Governorate, and investigate their bioactive compounds in antimicrobial, antioxidant, and antitumor activities.

Materials and Method

Isolation and purification

The isolation of fungi was performed from soil samples collected in Wadi El Rayan, Fayoum Governorate (Latitude 29.2101° or 29° 12’ 36.3” north, Longitude 30.2711° or 30° 16’ 16.1” east). After sample collection, the soil samples were dried, ground, and sieved, then used for the isolation of fungi. Malt medium was used to isolate fungi using the definitive serial dilution-spread plate method. Plates were incubated for 7–15 days at 28°C ± 2°C (Almalki et al., 2020).

Morphological identification

Using Czapek's agar media, the morphological characteristics of the fungus were examined, including growth rate, colony color and color variation over time, colony upside color, surface texture, etc. Additionally, the microscopic characteristics of the fungus, including those of mycelium, conidiophores, conidiogenesis, conidia, etc., were also examined (Atlas, 2006). Identification was based on current universal keys (Raper & Fennell, 1965; Samson et al., 2014) and the Regional Center for Mycology and Biotechnology’s Data Base Identification Program (RCMB).

Cultivation of isolates to produce secondary metabolites

Yeast extract sucrose (YES) liquid medium was used to produce secondary metabolites. The YES medium consists of (g/L) yeast extract 20.0, sucrose 150.0, $MgSO_4.H_2O$ 0.5, and distilled water to a volume of 1.0L. The pH was adjusted to 6.5 ± 0.2. The medium was then autoclaved for 15 min at 121°C. The investigated fungi were cultured and incubated at 25°C for 21 days (Keller, 2019).

Extraction of extracellular secondary metabolites

The culture filtrates were separately subjected to solvent extraction, as previously described by Song et al. (2019), with the following minor modifications as follows: To separate the culture filtrates from the fungal mycelia, the broth media was filtered through Whatman No. 1 filter paper. Then, in a separating funnel, each culture filtrate was first mixed with petroleum ether by the ratio 1:1 V/V, vigorously shaken, and allowed to settle for at least 6h until full separation. The top layer of petroleum ether was then separated. To ensure complete extraction, the residue was re-extracted twice, and the solvent was then concentrated using a rotary evaporator (Buchi RV 4) and stored at 5°C until testing. Further, the filtrates were re-subjected to extraction with the other solvent systems including both ethyl acetate and chloroform each one separately. The filtrates were extracted with each organic solvent three times in succession and then concentrated and stored as previously mentioned.

Antimicrobial assay of the crude extracts by Well-Diffusion method

The antimicrobial activity of petroleum ether, ethyl acetate, and chloroform crude extracts was investigated against certain Gram-positive bacteria, Gram-negative bacteria, and pathogenic fungi (Perez et al., 1990). 50µL of each extract at

a concentration of 10mg/mL dissolved in Dimethyl Sulfoxide (DMSO) was added into wells (5mm diameter) in agar plates having either Nutrient agar (NA) for bacteria or Malt agar (MA) for fungi. The inoculated plates with the tested microorganisms were kept for 12h at 2-8°C to allow metabolites diffusion then incubated for 48h at 28°C for fungi and yeast while 24h at 37°C for bacteria. The inhibition zone was calculated in mm (Keller, 2019). Ampicillin was used as a positive control for Gram-positive bacteria, gentamycin as a positive control for Gram-negative bacteria, and amphotericin B as an antifungal for fungi (Mane & ad Vedamurthy, 2020). Then, the minimum inhibitory concentrations were determined using various two-fold dilutions to determine the lowest tested concentration of the active extract that inhibits the microbes. All tested microorganisms were obtained from the Regional Center for Mycology and Biotechnology culture unit at Al-Azhar University in Cairo, Egypt (Table 1).

Molecular identification for the most antimicrobial-effective fungal isolate

Using a DNeasy kit (Qiagen), the DNA was extracted and amplified using universal Primer sequences used for the identification of 18s rRNA in the current study, which were ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-›3) and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-›3). Polymerase chain reaction (PCR) was performed on the extracted DNA (Mohammed et al., 2021; Tilahun et al., 2022). The amplified PCR fragments were sequenced at the Regional Center for Mycology and Biotechnology using the Cy5 /Cy5.5 Dye Primer Sequencing kit from Visible Genetics Inc. for use with the Open Gene automated DNA sequencing system (Sanger et al., 1977; Tabor & Richardson, 1995). The gene sequence was added to the NCBI GenBank database and assigned an accession number http://www.ncbi.nlm.nih.gov.blast.

Transmission Electron Microscope (TEM)

Electron microscopy was used to evaluate the effect of purified antimicrobial compounds produced by *Aspergillus fumigatus* on bacterial and yeast cells. At 37°C, pathogenic bacteria were grown on NA and yeast on Sabouraud Dextrose Agar. During the exponential phase, the cultures were collected by centrifugation at 9000rpm for 7min and then resuspended in peptone water. The samples were treated with the extracted secondary metabolites and incubated at 37°C for 1 day. The collected samples were then fixed with 2% glutaraldehyde in 0.1M sodium cacodylate, pH 7.4, for 2h at 42°C. The cells were then collected, washed twice with PBS (Phosphate-buffered saline) buffer, and postfixed in 1% osmium tetroxide for 1h at 4°C. The cells were dehydrated using ascending ethanol solutions, then treated with propylene oxide. The cells were cut, embedded, and stained with 2% uranyl acetate and lead citrate. The sections were examined using Transmission Electron Microscopy TEM (JEOL 1200 EX II, Japan) at the Regional Center for Mycology and Biotechnology culture unit at Al-Azhar University in Cairo, Egypt (Rizwana et al., 2021).

Anticancer assay

The cell lines derived from humans were acquired from the VACSERA Tissue Culture Unit including both HepG2 and PC-3 cells. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer, and 50µg/mL gentamycin. All cells were subcultured twice per week at 37°C in a humidified atmosphere containing 5% CO_2. The cytotoxic effect of fungal extracts was investigated using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability assay and the absorbance was measured at 490nm. Next, cell viability was determined using the following equation:

% cytotoxicity= Absorbance of control cells − Absorbance of treated cells/Absorbance of control cells × 100.

The IC_{50} values were calculated using linear regression and the test was made in triplicates (Kausar et al.,2021).

Antioxidant assay

Fungal extracts' free radical scavenging activities were assessed using 2, 2-diphenyl-1-picryl-hydroxyl (DPPH) as a model for evaluation of the antioxidant activity of the tested metabolites. The test was made in triplicates. The capacity of each fungal extract to scavenge free radicals was used to determine its ability to inhibit DPPH:

Percentage of radical scavenging activity= $[(A_{control} - A_{sample})/A_{control}] \times 100$.

A is the absorbance reading. The IC_{50} value (µg/mL) was calculated based on the ratio of radical scavenging activity to extract concentrations. (Ayob

et al., 2019).

Phytochemical analysis

The petroleum ether and ethyl acetate extracts of *A. fumigatus* F1 were subjected to qualitative phytochemical screening for the presence of phytoconstituents by submitting to various standard chemical tests for the detection of total phenolics, flavonoids, and alkaloids (Ramamurthy et al., 2017; Thorati & Mishra, 2017; Falade et al., 2017).

GC analysis

Using GC-MS, the constituent compounds separated from the petroleum ether extract of *A. fumigatus* F1 were identified. The extract of petroleum ether (5mg) was dissolved in petroleum ether solvent (HPLC grade) prior to detection. The separated chemicals were identified by means of gas chromatography (GC) and a mass spectrometer selective detector (MSD) (Thermo scientific CA, USA). After optimizing the detection parameters, the following GC and MSD conditions and settings were selected:

Firstly, GC conditions included HP-5 Methyl Siloxane chromatographic column (250µm×30m, 0.25µm); Helium was used as a carrier gas; the injection was performed in the split-less mode, and the injection port temperature was set to 250°C. The oven temperature was set as follows: initial temperature 50°C, hold for 3min, ramp to 120°C at 20°C/min, and then ramp to 250°C at 5°C/min, hold 10min, then raised to 270° at 20°/min and held for 2min. The flow rate of 1mL/min, the injection volume was 2µL, and the gasification chamber temperature was 280°C.

The MS parameters included an EI ionization source, electron energy of 70eV, ionization temperature of 230°C, interface temperature of 280°C and solvent delay of 3min, multiplier voltage of 1964.7 V, and quantity scanning range of 30 AMU to 500 AMU.

The components were identified by comparing the mass spectra to the mass-spectral library maintained by the National Institute of Standards and Technology (NIST 11.0, National Institute of Standards and Technology, Gaithersburg, MD, USA) and Wiley (Wiley, Chichester, West Sussex, UK) library search data during GC-MS qualitative analysis (Almanaa et al., 2021; Olivia et al., 2021).

Statistical analysis

The data were expressed as mean ±S.D. The statistical significance of the difference between mean values was determined by Student's unpaired t-test. Data were considered statistically significant at a significance level of $P< 0.05$. STATA statistical analysis package was used for the dose response curve drawing to IC_{50} calculations.

Results

Isolation and identification

In this study, fifteen fungal isolates obtained from the soil of Wadi El Rayan, Fayoum governorate, were identified by their cultural and morphological characteristic features. After a preliminary screening for the antimicrobial activity of these isolates (unpublished data), the fungal isolate (F1) (Fig. 1) was chosen to continue the study. Based on the morphological characteristics, the fungus was primarily identified as *Aspergillus fumigatus.* The fungus was subjected to further molecular identification, which confirmed the identification. The blast alignment (Fig. 2A) and the constructed phylogenetic tree (Fig. 2B) for the nucleotide sequences showed about 99.82% identity to *Aspergillus fumigatus* strains. The sequence was submitted to the NCBI GenBank and acquired an accession number; ON428521.

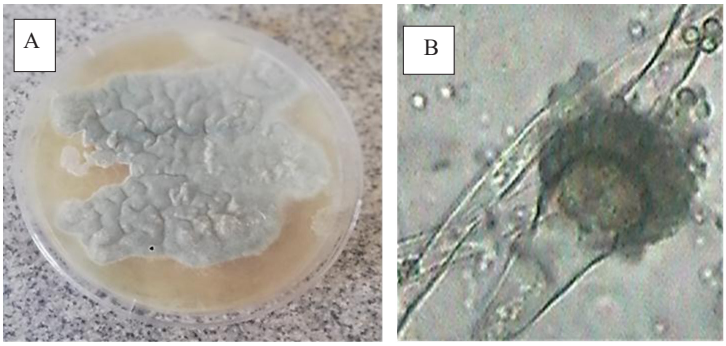


Fig. 1. Morphological identification of fungus *Aspergillus fumigatus* F1 isolated from Wadi El Rayan; (A) Culture characteristic on Malt agar plate, (B) Micropscopic characteristic (40X)

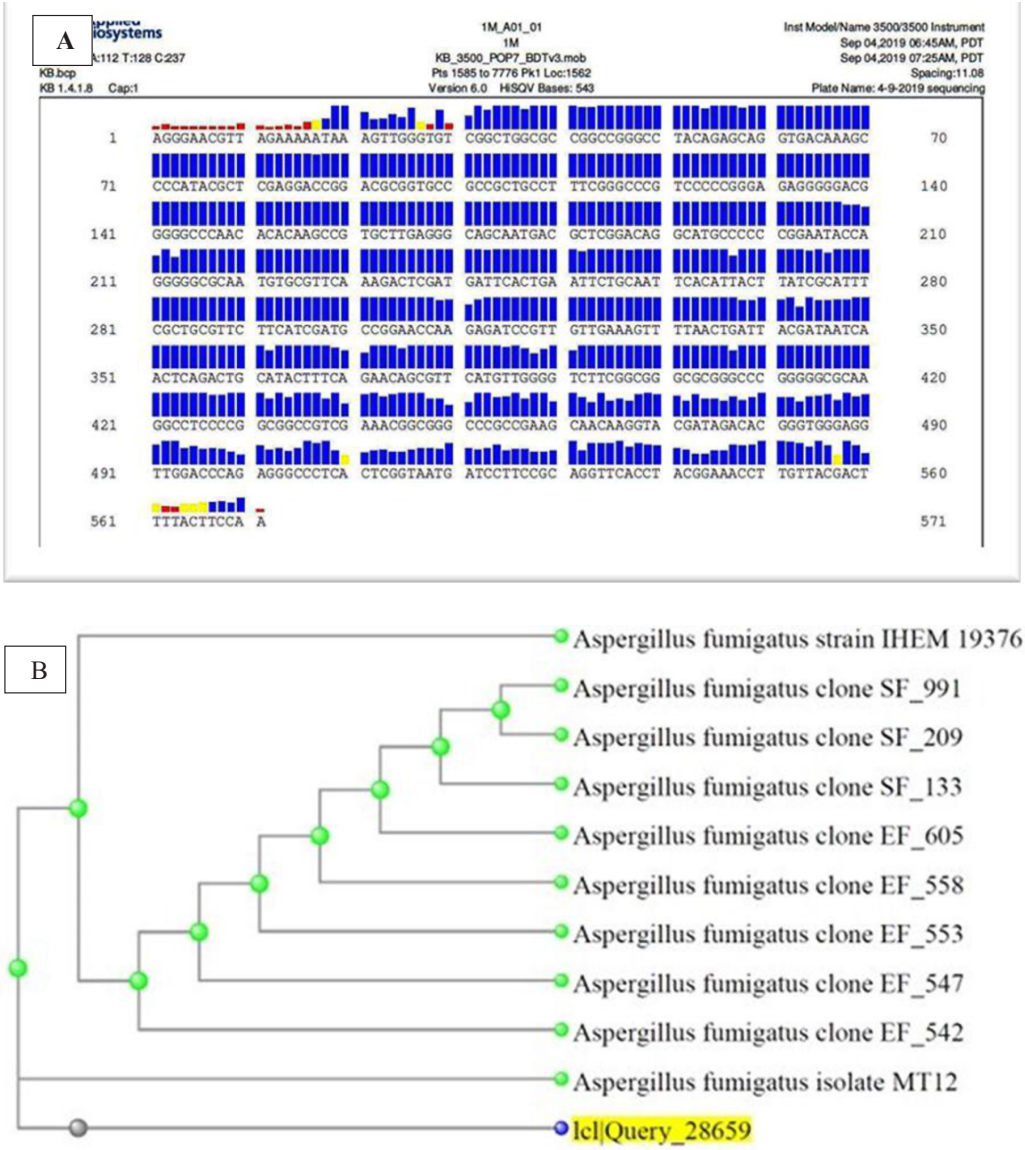


Fig. 2. The molecular identification of the of the fungus *Aspergillus fumigatus* F1 isolated from Wadi El Rayan; (A) The alignment of the gene sequence, (B) The phylogenetic tree of the DNA sequence

Antimicrobial assay of the crude extract by Well-Diffusion method

After a preliminary screening of the antimicrobial activity of 15 fungal isolate, crude extracts of *A. fumigatus* F1 was chosen due to their broad spectrum of antimicrobial activity against human pathogenic microorganisms. The well-diffusion assay of extracts revealed activity against Gram positive and Gram negative bacteria in addition to some of the tested fungi. According to the results, the inhibition zone for the test pathogens was between 7 and 21.4mm for the ethyl acetate extract and between 7 and 16.5mm for the petroleum ether extract. In contrast, chloroform extract demonstrated negligible activity (Table 1). Interestingly, the investigated *A. fumigatus* F1 isolates exhibited the highest antimicrobial activity against *Escherichia coli* ATCC 25922, followed by *Salmonella typhimurium and Proteus vulgaris* ATCC 13315, indicating that the activity was greater against Gram negative bacteria pathogens than Gram positive bacteria (*Streptococcus mutans* ATCC 25175 followed by *Staphylococcus aureus* ATCC 25923). The highest antifungal activity was observed against *Cryptococcus neoformans* (RCMB 049002), followed by *Candida albicans* ATCC 10231 then *Geotrichum candidum* (RCMB 027016).

TABLE 1. The antimicrobial activities of the tested *A. fumigatus* F1 extracts expressed as a mean zone of inhibition in mm ± Standard deviation on a range of pathogenic microorganisms using (10mg/mL) concentration of tested extracts

Sample / Tested microorganisms	Ethyl acetate extract	Petroleum ether extract	Chloroform extract	Standard drug *Amphotericin B*
Fungi				
Aspergillus niger (RCMB 002005)	NA	NA	NA	26.1± 0.9
Penicillium expansum IMI 28169	NA	NA	NA	22.9± 0.6
Syncephalastrum racemosum (RCMB 014025)	7.2 ± 0.4*	NA	NA	25.4 ± 0.8
Cryptococcus neoformans (RCMB 049002)	14.4 ± 0.8*	11.3± 0.9*	NA	23.6± 1.2
Candida albicans ATCC 10231	10.3 ± 0.6*	7.1 ± 0.7*	NA	21.9 ± 1.3
Geotrichum candidum (RCMB 027016)	7.3 ± 0.5*	NA	NA	26.4 ± 0.8
Gram-positive bacteria				***Ampicillin***
Staphylococcus aureus ATCC 25923	12.5 ± 0.7*	10.8 ± 0.6*	NA	28.9 ± 1.1
Bacillus subtilis NRRL B-543	9.3 ± 0.9*	8.2 ± 0.8*	NA	29.8 ± 1.5
Streptococcus mutans ATCC 25175	14.9±0.4*	10.3 ± 0.9*	NA	24.3± 0.9
Micrococcus sp. RCMB 02801	10.1± 0.9*	8.7± 0.5*	NA	21.8± 1.2
Enterococcus faecalis ATCC 23355	9.8± 0.7*	7.4± 0.6*	NA	26.4± 1.4
Methicillin-Resistant *Staphylococcus aureus* (MRSA)	NA	NA	NA	13.8± 0.7
Gram-negative bacteria				***Gentamycin***
Proteus vulgaris ATCC 13315	17.6 ± 1.1*	14.9 ± 0.6*	9.1 ± 0.8*	21.7± 1.5
Pseudomonas aeruginosa ATCC 27853	NA	NA	NA	17.3± 1.2
Klebsiella pneumoniae (RCMB 0010093)	9.5± 0.9*	8.3± 0.4*	NA	22.4± 0.9
Salmonella typhimurium (RCMB 0010042)	18.3± 1.2*	15.7± 1.1*	9.4± 0.9*	25.7± 1.5
Escherichia coli ATCC 25922	21.4± 0.6*	16.5 ± 0.9*	10.3 ± 0.5*	28.3± 1.8
Porphyromonas gingivalis EMCC 1699	11.7± 0.5*	10.2± 0.6*	NA	19.1± 0.9
Enterobacter cloacae ATCC 23355	NA	NA	NA	27.2± 1.7
Serratia marcenscens RCMB 03902	NA	NA	NA	23.9± 1.1

Data were expressed as means ± SD. Unpaired Student-t-test was conducted using the Open Epi program. The samples were compared with standard drugs and differences between means were considered *significant at *P* values of less than 0.05. NA: No activity.

The results of antimicrobial activities of the tested *A. fumigatus* F1 extracts were also confirmed after estimation of the minimum inhibitory concentrations (MIC values), showing *Escherichia coli* ATCC 25922 was the Gram negative bacteria most sensitive to ethyl acetate extract with a MIC value of 78.12µg/mL. In addition, *Streptococcus mutans* ATCC 25175 had the highest MIC value among Gram positive bacteria, at 312.5µg/mL. In addition, *Cryptococcus neoformans* (RCMB 049002) was a highly susceptible fungus with a MIC value of 1250µg/mL (Table 2).

Transmission Electron Microscope (TEM)

After estimation of the MIC values of the ethyl acetate extract on the tested pathogens, the highly inhibited pathogens including *Escherichia coli* ATCC 25922 (Gram negative bacteria), *Streptococcus mutans* ATCC 25175 (Gram positive bacteria), and *Cryptococcus neoformans* (yeast), were selected for transmission electron microscopy analysis. In order to investigate the potential effects on the microbial structure, the ethyl acetate extract of *A. fumigatus* F1 was applied.

TABLE 2. The minimum inhibitory concentrations showing antimicrobial activities of the tested *A. fumigatus* F1 extracts. The data are expressed as MIC values in µg/mL

Tested microorganisms \ Sample	Ethyl acetate extract	Petroleum ether extract	Chloroform extract
Fungi			
Syncephalastrum racemosum (RCMB 014025)	5000	NA	NA
Cryptococcus neoformans (RCMB 049002)	1250	2500	NA
Candida albicans ATCC 10231	5000	5000	NA
Geotrichum candidum (RCMB 027016)	5000	NA	NA
Gram-positive bacteria			
Staphylococcus aureus ATCC 25923	625	2500	NA
Bacillus subtilis NRRL B-543	2500	5000	NA
Streptococcus mutans ATCC 25175	312.5	2500	NA
Micrococcus sp. RCMB 02801	2500	5000	NA
Enterococcus faecalis ATCC 23355	2500	5000	NA
Gram-negative bacteria			
Proteus vulgaris ATCC 13315	156.25	312.5	5000
Klebsiella pneumoniae (RCMB 0010093)	2500	5000	NA
Salmonella typhimurium (RCMB 0010042)	156.25	312.5	2500
Escherichia coli ATCC 25922	78.12	312.5	2500
Porphyromonas gingivalis EMCC 1699	1250	2500	NA

TEM micrographs demonstrated alterations in the ultrastructure of treated microbes (Figs.3A–5A). Microphotographs of untreated cells revealed distinct membrane and wall differentiation, intact margins, uniform distribution of cell organelles, and intact septal walls. Nevertheless, the treated cells' cell walls lost their uniformity, resulting in cell wall rupture and even severe damage in many areas, as well as a thickened appearance that was more pronounced in polar regions (Figs. 3B–5B).

Anticancer activity by MTT method

The anticancer activities of the tested extracts were also tested to determine the effect of the fungal extracts on cancer cell proliferation using the MTT viability assay. HepG-2 cells and PC-3 cells were used to achieve this goal showing dose-dependent inhibition by the tested metabolites compared with vinblastine that tested as the reference standard (Fig. 6). Under these experimental conditions, the half-maximal inhibitory concentration IC_{50} of petroleum ether extract from *A. fumigatus* F1 against PC-3 cells was detected with IC_{50}= 90.3± 2.9µg/mL. However, under experimental conditions, the IC_{50} of ethyl acetate extracts from *A. fumigatus* F1 activity against PC-3 cells was 122.3 3.5µg/mL. Also, IC_{50} of petroleum ether extracts from *A. fumigatus* F1 against HepG-2 cells showed the best activity with IC_{50}= 61.2 ± 1.6µg/mL. In addition, the IC_{50} of ethyl acetate extract against HepG2 cells was detected with IC_{50} = 84.4 ± 3.4µg/mL.

Antioxidant activity using DPPH Free Radical Scavenging Assay

Extensive use of the stable free radical DPPH has been made to evaluate the ability of antioxidants to scavenge free radicals. The results demonstrated that the DPPH scavenging activities of the investigated metabolites were dose-dependent (Fig. 7). Under these test conditions, the half-maximal inhibitory concentration (IC_{50}) of *A. fumigatus* F1 petroleum ether extracts demonstrated antioxidant activity with a value of 581 41.9 µg/ml. In addition, the half-maximal inhibitory concentration (IC_{50}) of *A. fumigatus* F1 ethyl acetate extracts exhibited antioxidant activity under these experimental conditions, with an IC_{50}= 278.2 ± 8.5µg/mL when compared to the standard ascorbic acid.

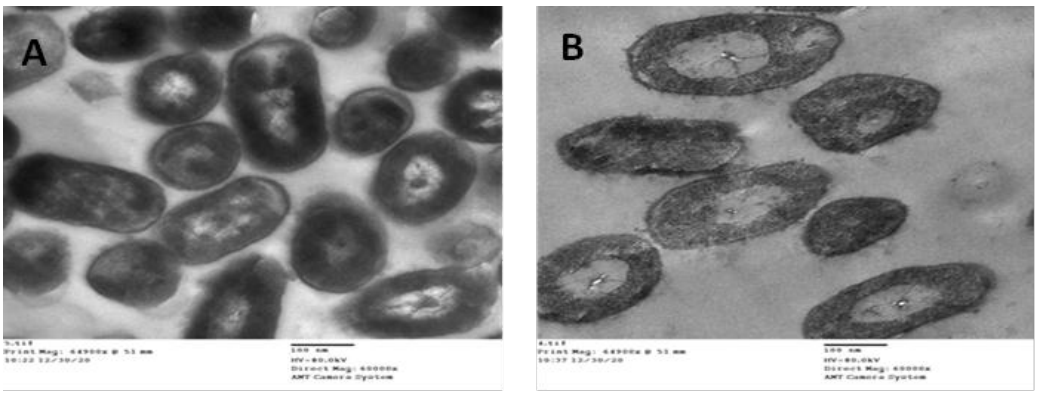


Fig. 3. TEM microphotographs of *Escherichia coli*. A: without treatment. B: treated with *A. fumigatus* F1 ethyl acetate extract

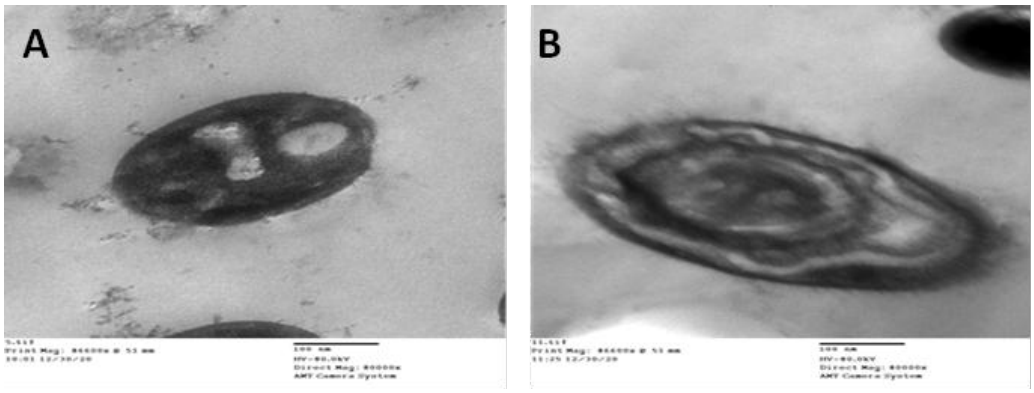


Fig. 4. TEM microphotographs of *Streptococcus mutans*. A: without treatment. B: treated with *A. fumigatus* F1 ethyl acetate extract

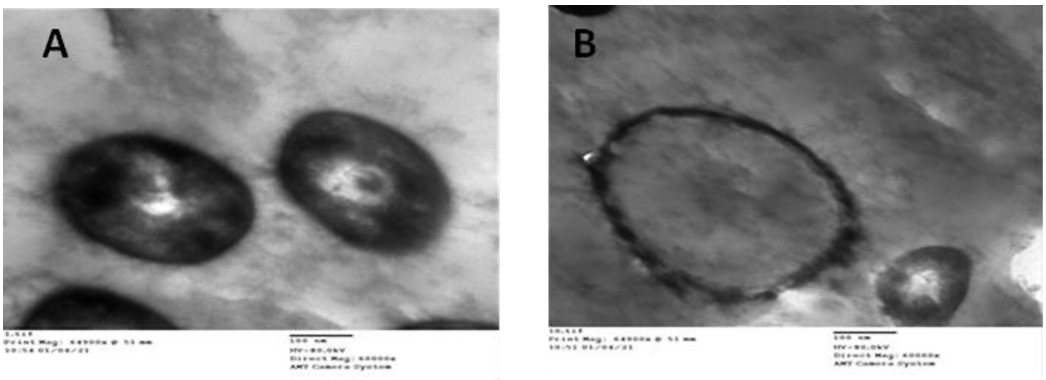


Fig. 5. TEM microphotographs of *Cryptococcus neoformans*. A: without treatment. B: treated with *A. fumigatus* F1 ethyl acetate extract

Phytochemical analysis

The petroleum ether and ethyl acetate extracts of *A. fumigatus* F1 was subjected to qualitative phytochemical screening for the presence of phytoconstituents by submitting to various standard chemical tests for detection of the total phenolics, flavonoids, and alkaloids (Fig. 8). The phytochemical screening of the active ethyl acetate extract was also conducted to identify diverse groups of secondary metabolites responsible for the activities that led to a high concentration of flavonoids, followed by phenolics and alkaloids. Furthermore, petroleum ether extract was abundant in lipophilic substances (Table 3).

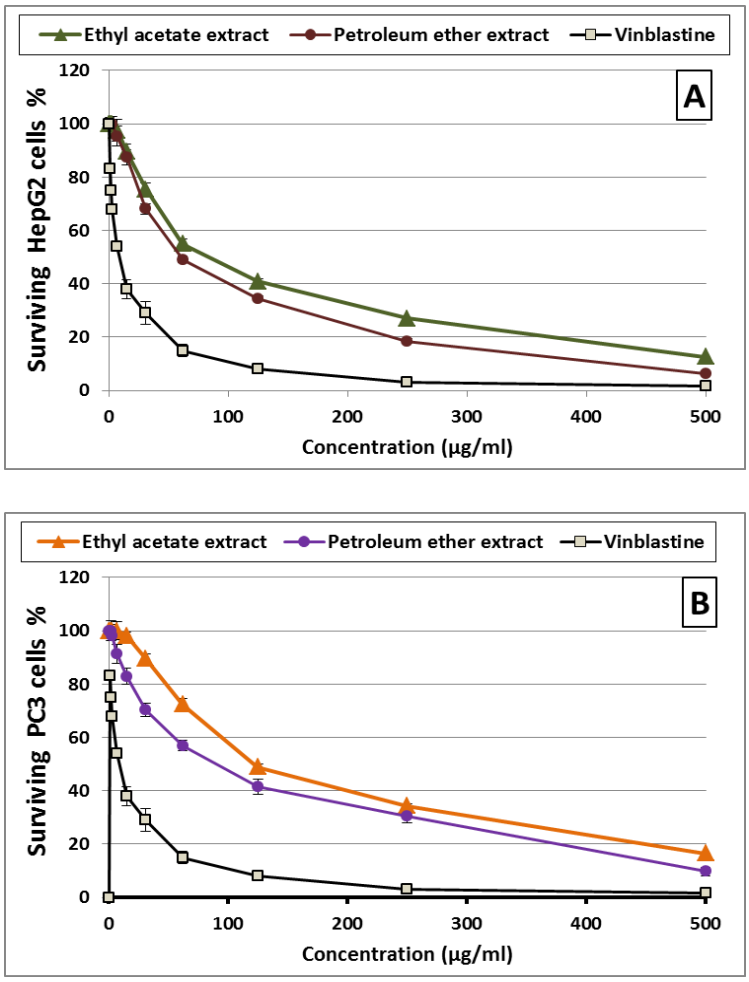


Fig. 6. The dose-response curve showing the in vitro inhibitory activity of ethyl acetate and petroleum ether extracts from the fungus *A. fumigatus* F1 against different cell lines. (A) hepatocellular carcinoma (HepG2), (B) prostate carcinoma (PC-3). Data are expressed as surviving viable cells percentages at various concentrations (µg/ml) ±SD of three replicates

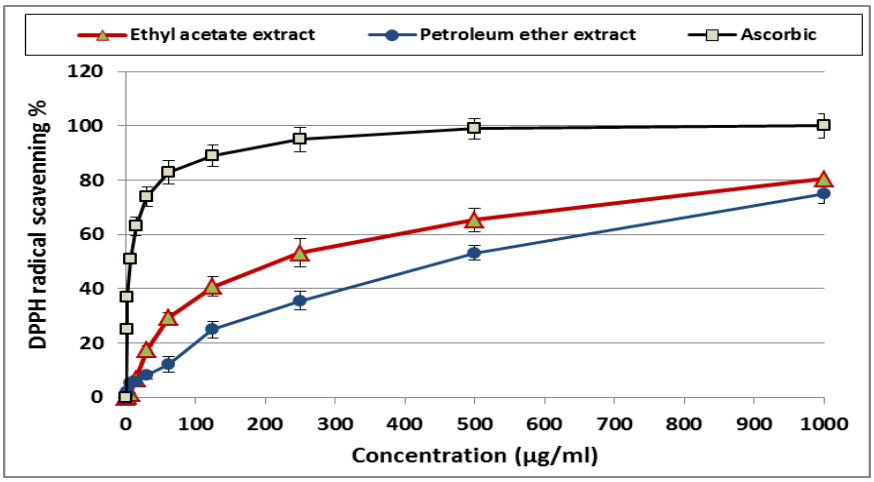


Fig. 7. The dose-response curve showing the in vitro antioxidant activities of ethyl acetate and petroleum ether extracts from the fungus *A. fumigatus* F1 expressed as DPPH radical scavenging activity percentages at various concentrations (µg/mL) ±SD of three replicates

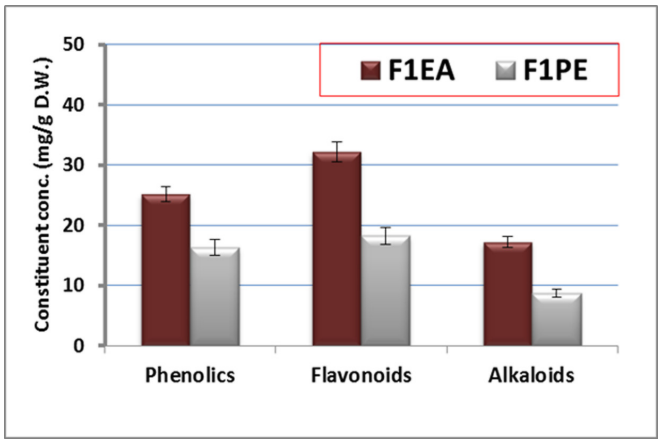


Fig. 8. The concentrations of the total phenolics, flavonoids, and alkaloids contents of *A. fumigatus* F1 active ethyl acetate (F1EA) and petroleum ether (F1PE) extracts

TABLE 3. Qualitative determination Phytochemical Constituents of *A. fumigatus* F1 active extracts

Phytochemicals	F1EA	F1PE
Phenolics	++	++
Flavonoids	+++	++
Alkaloids	++	+
Anthraquinones	-	-
Reducing Sugars	-	-
Glycosides	+	-
Saponins	+	+
Sterols	-	++
Steroids	-	-
Tannins	++	+
Terpenoids	+	+
Proteins	++	-
Lipids	-	+++

GC-MS analysis

Identification of constituent compounds separated from *A. fumigatus* F1 petroleum ether extract using GC-MS was performed to explore the active ingredients that may be responsible for the biological activities (Table 4). Meanwhile, for *A. fumigatus* F1 metabolites detected by GC-MS, dodecanamine, N, N-dimethyl- was the major component, followed by lupeol acetate, amyren, phthalate, and oleanenol acetate with the abundance of 20.39%, 16.9%, 9.08%, 8.52 and 4.28% in GC chromatogram were identified at 19.71, 42.63, 42.2 24.37 and 38.22 min, respectively (Fig. 9).

Discussion

Compared to other fungal genera, *Aspergillus* species is the most prevalent pathogen and possesses a reputation for producing the most physiologically active secondary metabolites (Cairns et al., 2018; Cohen et al., 2021; Orfali et al., 2021).

Multidrug-resistant pathogenic microorganisms are a significant problem for healthcare systems and a global concern (Aksoy & Unal, 2008). In this study, there was an attempt to determine the antimicrobial activity of *A. fumigatus* F1. The results demonstrated the antimicrobial activity of the ethyl acetate and petroleum ether extracts of against *E. coli, S. typhimurium,* and *B. subtilis,* which was consistent with the results obtained by Abdel-Aziz et al. (2018), who isolated fungi from local agricultural soil in Mansoura Governorate and discovered that *A. fumigatus* 3T-EGY ethyl acetate extract has antimicrobial activity against four pathogenic microbial strains, including, *S. aureus, P. aeruginosa, C. albicans,* and *A. niger.*

TABLE 4. Identification of constituent compounds separated from *A. fumigatus* F1 petroleum ether extract using GC-MS

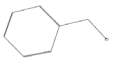
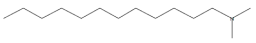
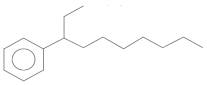
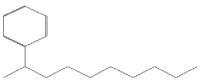
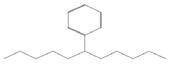
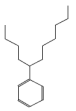
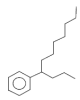
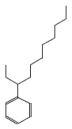
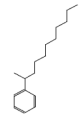
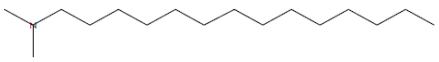
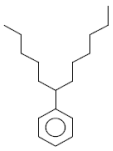
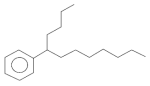
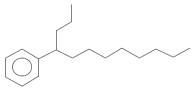
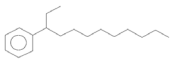
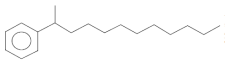
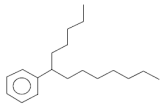
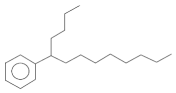
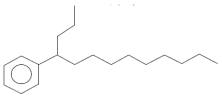
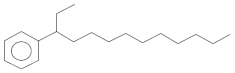
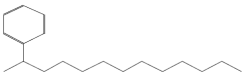
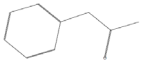
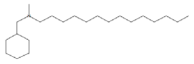
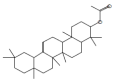
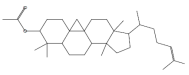
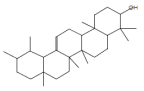
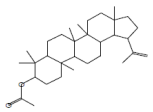
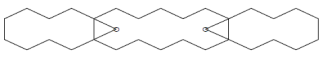
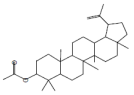
Retention time	Constituents identification	Relative content%	Chemical formula	Molecular weight	Chemical structural
7.03	Benzene, (chloromethyl)-	3.82	C7H7Cl	126	
18.98	1-Dodecanol	0.51	C12H26O	186	
19.71	1-Dodecanamine, N,N-dimethyl-	17.39	C14H31N	213	
21.08	Benzene, (1- ethyloctyl)-	0.45	C16H26	218	
21.95	Benzene, (1-methylnonyl)-	0.74	C16H26	218	
22.69	Benzene, (1-pentylhexyl)-.	0.66	C17H28	232	
22.77	Benzene, (1-butylheptyl)-	1.50	C17H28	232	
22.99	Benzene, (1-propyloctyl)-	1.54	C17H28	232	
23.45	Benzene, (1-ethylnonyl)-	1.88	C17H28	232	
24.28	Benzene, (1-methyldecyl)-	2.41	C17H28	232	
24.37	1-Tetradecanamine, N,N-dimethyl	8.52	C16H35N	241	
24.89	Benzene, (1-pentylheptyl)-	1.77	C18H30	246	
24.99	Benzene, (1-butyloctyl)-	1.91	C18H30	246	
25.25	Benzene, (1-propylnonyl)-	1.85	C18H30	246	
25.70	Benzene, (1-ethyldecyl)-	2.12	C18H30	246	

TABLE 4. Cont.

Retention time	Constituents identification	Relative content%	Chemical formula	Molecular weight	Chemical structural
26.52	Benzene, (1-methylundecyl)-	3.14	C18H30	246	
26.99	Benzene, (1-pentyloctyl)-	2.27	C19H32	260	
27.13	Benzene, (1-butylnonyl)-	1.58	C19H32	260	
27.39	Benzene, (1-propyldecyl)-	1.63	C19H32	260	
27.86	Benzene, (1-ethylundecyl)-	1.87	C19H32	260	
28.66	Benzene, (1-methyldodecyl)-	2.68	C19H32	260	
32.57	Kojic acid	4.87	C9H10O	134	
36.24	N-Methyl-N-benzyltetradecanamine	1.36	C22H39N	317	
38.22	Olean-12-en-3-ol, acetate, (3á)-	2.28	C32H52O2	468	
39.24	9,19-Cyclolanost-24-en-3-ol, acetate, (3á)-	1.39	C32H52O2	468	
42.20	à-Amyrin	9.08	C30H50O	426	
42.63	Lup-20(29)-en-3-ol, acetate, (3á)-	16.90	C32H52O2	468	
44.11	Tricyclo[20.8.0.0(7,16)] triacontane, 1(22),7(16)-diepoxy-	0.84	C30H52O2	444	
44.50	Lup-20(29)-en-3-yl acetate	3.04	C32H52O2	468	

(-) =absent, (+) = present, > 50 %, compared with control, (++) = 50% < 80%, (+++) =>80%.

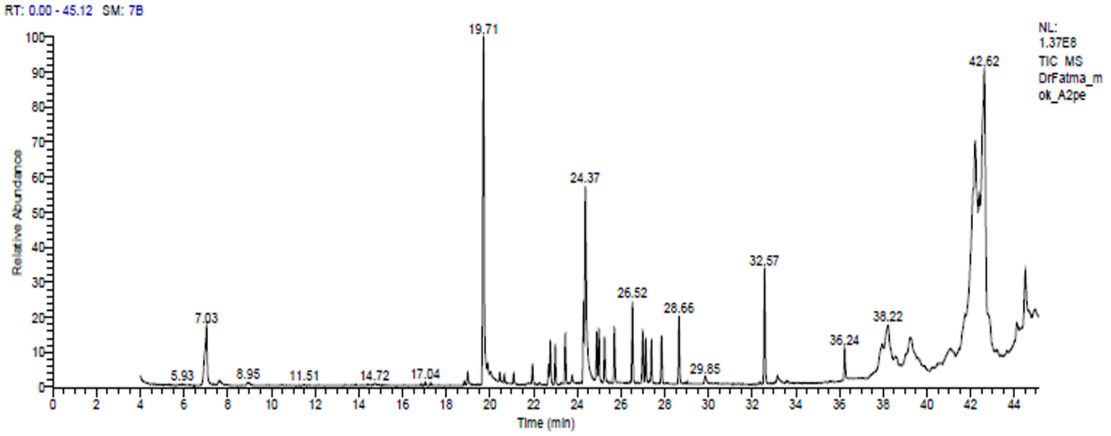


Fig. 9. Chromatographic separation of the semi-purified petroleum extract obtained from *A. fumigatus* F1 showing GC-MS total ion chromatogram with the separated peaks

In addition, Al-jassani et al. (2016) reported that the methanolic extract of *A. fumigatus* produces a broad spectrum of secondary metabolites with significant biological activity against *S. aureus*, *S. faecalis*, *S. pyogenes, Proteus mirabilis,* and *P. eurogenosa*. Moreover, Hussein et al. (2022) reported that the ethyl acetate extract of *A. fumigatus* isolated from *Albizia lucidior* leaves, had antimicrobial activity against *C. albicans*, *P. vulgaris*, and *S. aureus*.

In the present study, according to transmission electron microscope (TEM) microphotographs, the examined pathogenic microorganisms exhibited obvious distortion. Similarly, Jalil & Ibrahim (2021) reported that the endophytic fungus *Lasiodiplodia pseudotheobromae* IBRL OS-64 caused significant damage to the MRSA cells, including cell shrinkage, and breaking. In addition, cytoplasm shrinkage and loss of the spherical structure. There was also evidence of bacterial cell leakage and autolysis.

The use of metabolites derived from natural resources as a therapy for cancer and to inhibit multidrug resistant pathogenic bacteria is promising. Our findings indicated that *A. fumigatus* F1 demonstrated cytotoxicity toward HepG2 and PC-3 cell lines. Similarly, González-Menéndez et al. (20l8) reported that *A. fumigatus* extract has a unique activity against HepG2 cell line.

In a separate study conducted recently, the bioactive metabolites of *A. fumigatus*, which was isolated from polluted rhizosphere soil, demonstrated cytotoxicity against HepG2 cells (Almanaa et al., 2021). In addition, the ethyl acetate extract of *A. fumigatus* strain MF1 which was isolated from marine soil samples found to have anticancer activity on HeLa cell lines (Kalyani et al., 2021).

In this study, the results showed that the *A. fumigatus* F1 had antioxidant activity using DPPH free radical scavenging assay. Numerous studies reported the significant antioxidant capacity of different cultivated fungal species (Cui et al., 2015; Zhou et al., 2018). The highest biochemical antioxidant activity showed by the isolates *Fusarium brachygibbosum*, *A. phoenicis*, *A. proliferates*, *P. chrysogenum,* and *A. niger* which were isolated from various locations in desert soils of Saudi Arabia (Ameen et al., 2021). The fungi of the genera *Aspergillus, Penicillium, Epicoccum,* and *Lecanicillium* that were isolated from Brazilian caves were evaluated in another study. The ethyl acetate extract of *P. flavigenum* (CML2965) exhibited the greatest antioxidant activity (Tavares et al., 2018).

The results of the GC-MS analysis showed that the major components in the petroleum extract were dodecanamine,N, N-dimethyl, lupeol, kojic acid and amyrin. Similarly, Abd El-Latif et al. (2021) reported that in the GC-MS analysis, the predominant component of the ethyl acetate extract ingredients of *A. terreus* SHE05 isolated from Alexandria was the dodecanamine, N, N-dimethyl with the formula $C_{14}H_{31}N$. In addition, this substance possessed antibacterial properties and was previously produced by Many fungi including *Citrullus colocynthis* (Idan et al., 2015). Moreover, according to Li & Xiao

(2018), lupeol derivatives exhibited anticancer activity against HepG2 cells, which explains our petroleum ether extract's activity against HepG2 and PC-3 cells. Experiments on lupeol derivatives have revealed that these substances possess various pharmacological properties, such as anticancer and antibacterial properties (Saini et al., 2019; Liu et al., 2021; Phan et al., 2022).

In accordance with the present study kojic acid has anticancer and antimicrobial activity against K562 (leukemia) and MDA MB435S (breast cancer) cell lines produced by *A. flavus.* This explains the anticancer activity of petroleum ether extract due to kojic acid as determined by GC analysis (Devi et al., 2016). Additionally, amyrin from GC analysis has been reported to have anticancer properties (Neto et al., 2021).

Conclusion

The fifteen fungal strains isolated from Wadi El Rayan, the *A. fumigatus* F1 showed remarkable inhibitory effects against tested pathogenic microorganisms. Also, TEM electron micrographs showed the cellular alteration formed after a treatment with this active fungal extract.

Petroleum ether extract showed higher inhibitory activity against HepG2 and PC-3 tumour cells than ethyl acetate extract. In addition, the ethyl acetate extract showed moderate antioxidant activity followed by petroleum ether extract. The major chemical constituents of the extracts that would be a responsible of these biological activities were detected belonging to phenolics, flavonoids and terpenes along with sterols. In conclusion, ethyl acetate and petroleum ether extracts of *A. fumigatus* isolated from the sandy soils of Wadi El Rayan exhibited moderate antimicrobial, antioxidant, and antitumor activities. Further pharmacological and in vivo studies are recommended to explore the mechanism of these activities.

Conflicts of interest: The authors declare no conflict of interest.

Authors' contributions: Fatma Yehia participated in the whole work, research writing and publishing process. Prof. Dr. Amany Abo El Nasr participated in the revision. Prof. Dr. Mahmoud Mohamed Elaasser participated in the practical work, writing and revision. Dr. Yasmin Mohamed Elsaba participated in following up on the work, writing and revision.

Ethics approval: Not applicable.

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النشاط الحيوى لنواتج الايض الثانوية من فطر *Aspergillus fumigatus* ON428521 المعزول من منطقة وادى الريان بمحافظة الفيوم

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تزايدت جهود العلماء في العقود الماضية سعياً وراء اكتشاف عقاقير جديدة لاستخدامها كأدوية لعلاج السرطان والأمراض الميكروبية واسعة الانتشار خاصة مع اكتساب الميكروبات مقاومة للمضادات الحيوية المتداولة حالياً. وتلعب منتجات الأيض الثانوية المنتجة من الفطريات دوراً رئيسياً ضمن مجموعة كبيرة من المنتجات الطبيعية لما اكتشف لها من أنشطة بيولوجية متنوعة وفائقة الفعالية وبعضها وصل إلى مراحل التجارب السريرية، وقد اتجهت هذه الدراسة إلى الحصول على منتجات الأيض الفطرية الثانوية لفطريات معزولة من التربة من بعض الأماكن بمحافظة الفيوم والبحث عن الفعالية البيولوجية لهذه المركبات الأيضية. تم عزل الفطريات من عينات من التربة من منطقة وادي الريان وجبل المدورة وتم اختبار مستقبلاتها الثانوية، والتي تم استخلاصها باستخدام مذيبات عضوية (الأثير البترولي، وخلات الإيثيل والكلوروفورم)، وتم بعد ذلك اختبار منتجات الأيض للأنشطة المضادة للميكروبات. ومن بين العزلات، أظهرت مستخلصات الفطر F1 تأثيرا ملحوظا ضد الكائنات الحية الدقيقة التي تم اختبارها، وبالتالي، تم تعريف هذه العزلة النشطة بيولوجيا بدراسة السمات المزرعية المميزة لها ودراسة صفاتها المورفولوجية وكذلك الدراسات الجزيئية والتى أشارت إلى أن الفطر هو اسبرجلس فيوميجاتس. وأظهر مستخلص خلات الإيثيل لأسبرجليس فيوميجاتس أعلى نشاط مع منطقة تثبيط تتراوح من 7مم إلى 21.4مم. بينما اظهر المستخلص البترولى منطقة تثبيط بمعدل 16.5مم، أما مستخلص الكلوروفورم أظهر نشاطا أقل تأثيرا.

تم استخدام مستخلص خلات الإيثيل لاستكشاف التأثيرات المحتملة على التركيبات الدقيقة لخلايا البكتريا الأكثر تأثرا بالمستخلص باستخدام المجهر الإلكتروني النافذ. أظهرت الخلايا المعالجة أن جدران الخلايا فقدت شكلها المميز وتمزق جدار الخلية وقل سمكها. علاوة على ذلك، تم تقييم الأنشطة المضادة للأورام أيضا ضد خلايا سرطان الخلايا الكبدية وسرطان البروستاتا بطريقه اختزال أملاح التترازوليوم.

حيث أظهرت النتائج نشاطا مثبطا عاليا باستخدام مستخلص الأثير البترولي وقد كان التركيز المثبط النصفى 1.64 ± 61.02 و 2.37 ±90.31 ميكروجرام لكل ملليلتر ضد خلايا سرطان الكبد وخلايا سرطان البروستاتا، على التوالي.

وفى حين كانت قيم التركيز المثبط النصفي لمستخلص خلات الإيثيل التركيز المثبط النصفي ±84.42 3.36.و 3.46 ± 121.98 ميكروجرام لكل ملليلتر ضد خلايا سرطان الكبد وخلايا سرطان البروستاتا على التوالي. بالإضافة إلى ذلك، تم اختبار النشاط المضاد للأكسدة وأظهر مستخلص خلات الإيثيل نشاطا معتدلا وكانت قيم التركيز المثبط النصفي=8.52±278.24 ميكروجرام لكل ملليلتر متبوعا بالأثير البترولي 41.95±581.07 ميكروجرام لكل ملليلتر. وأظهر التحليل الكيميائي لمستخلص خلات الإثيل على انه يحتوي على كمية عالية من الفينولات تليها الفلافونيدات ثم القلويدات بينما أظهر مستخلص الايثير البترولى وجود كميات عالية من الدهون. وفى ضوء النتائج السابقة تم عمل تحليل الفصل الكروماتوغرافى الغازي واستخدام مطياف الكتلة للتعرف على المواد الفعالة لمستخلص الأيثر البترولي. وكانت المركبات الرئيسية فى المستخلص هى دوديكانامين، أسيتات لوبيول، أميرين، حمض كوجيك، وأسيتات أولينينول. وبالتالي يمكن القول أن مستخلصات خلات الإيثيل والأثير البترولي من فطر اسبرجلس فيوميجاتس قد أظهرت أنشطة معتدلة مضادة للميكروبات ومضادة للأكسدة ومضادة للأورام.