*Available online at* www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

**Der Pharma Chemica, 2016, 8(20):121-134**
*(http://derpharmachemica.com/archive.html)*

# Molecular identification of newly isolated non-toxigenic fungal strains having antiaflatoxigenic, antimicrobial and antioxidant activities

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## ABSTRACT

*The aims of this work were to isolate fungal strains from agriculture soil samples, to screen their antimicrobial and antiaflatoxigenic activity, to identify and evaluate the antimicrobial and antioxidant activity of their bioactive potential secondary metabolites. Five fungal strains were isolated from soil samples collected from different areas in Mansoura, Dakahlia governorate, Egypt. Only two of the isolated fungal strains showed high antimicrobial activities against several microbial pathogenic microorganisms and they also showed antiaflatoxigenic activities. These promising isolates were identified using molecular identification technique as Aspergillus niger and Aspergillus fumigatus. Bioactive secondary metabolites were extracted from these fungal species and showed strong antimicrobial activity against several microbial pathogens, as well as variable antioxidant properties and total phenolic content. Twenty-six compounds were identified from the ethyl acetate extracts of the mycelia of the shaken culture broth of Aspergillus niger by Gas Chromatography-Mass Spectrometry analysis. It could be concluded that the two fungal species isolated from the Egyptian environment and their crude extracts may be considered promising candidates for food application due to their antimicrobial, antioxidant and total phenolic content.*

**Keywords**: Fungi; Aflatoxin $B_1$; antimicrobial activity; antiaflatoxigenic activity; antioxidant capacity; total phenolic content; food contaminants

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## INTRODUCTION

Agricultural commodities such as legumes, cereals, spices, vegetables and fruits are usually infested with molds and their associated mycotoxins during harvesting, storage, transporting and processing [1]. Several species of *Aspergillus* including *A. flavus, A. parasiticus, A. nominus* and few other Aspergilli are able to produce aflatoxins (AFs), the well-known toxic, carcinogenic, mutagenic, teratogenic and immunosuppressive compounds [2]. Therefore, extensive researches concerning methods for detoxification and/or degradation of mycotoxins in food and feeds have been established. Several chemical and physical approaches have been developed to remove mycotoxins from food and feed. However, the success of these methods varies and they are not always economical [3]. Therefore, identification of different microorganisms has become an increasing interest to prevent the growth of aflatoxigenic fungi and consequently their mycotoxin production. Thus, the development of biological detoxification methods using different microorganisms is vital to increase the safety of these foods for human consumption [4].

Useful enzymes are produced from a number of non-pathogenic fungi such as *Aspergillus* species which are able to produce a broad range of enzymes [5]. Enzyme production has been described for many *Aspergillus* species including *Aspergillus niger* [6, 7, 8], and *A. fumigatus* [9]. *Aspergillus niger* is one of the most important microorganisms used in biotechnology and has been in use for many decades to produce extracellular food enzymes

such as citric acid. The citric acid produced mainly from *A. niger* is considered Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration [10]. Since ancient times, enzymes have been used in the production of food such as cheese, beer, wine and vinegar [11]. Moreover, *A. fumigatus* is a widespread saprotroph found in nature and has the ability to produce phytase, alkaline phosphatase, and acid phosphatase enzymes, which are of industrial and commercial importance [12]. *Aspergillus fumigatus* which is known to produce xylanase is a principal basic constituent in plant cell walls and could interfere in many industries and biological activities [13].

To accurately characterize microbial communities, PCR-based molecular methods have been used widely over the past two decades [14], and are considered a quick and subtle method for specific amplification of a certain segment of DNA. Regardless of the culture-ability or viability of the microorganisms, the amplification occurs as long as the segment exists in the sample. As in other cells, ribosomal RNA (rRNA) genes in a microbial cell conserve and present multiple copies, thus they are considered popular targets for gene amplification and molecular analysis [15, 16]. To develop a sensitive system for the detection of microorganisms, PCR techniques based on rRNA genes have been widely used [17].

Due to microbial resistance and the side effect of chemical antimicrobial agents research has been forwarded towards antimicrobial agents from natural sources which provide higher safety margins to use in the food industry. Therefore, the aims of the current study were to isolate and characterize soil fungal strains, to screen their ability to prevent and degrade aflatoxin $B_1$ production and further to evaluate the antimicrobial, antioxidant activity and phenolic content for their bioactive potential secondary metabolites.

## MATERIALS AND METHODS

### Microorganisms

Gram-negative bacteria, *Escherichia coli*, *Salmonella typhi* and Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus aureus* MRSA, *Bacillus cereus*; *Pseudomonas aeruginosa*, as well as *Candida albicans*, *Aspergillus parasitics* and *Aspergillus ochraceus* were obtained from the culture collection of the Microbial Resources Centre (MlRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. An aflatoxin $B_1$-producing fungus (*Aspergillus flavus*) was preserved in the Department of Food Toxicology and Food Contaminants, National Research Centre. *Fusarium solani* and *Fusarium oxysporum* were isolated from maize samples in Egypt in a previous study [18].

### Sampling

Soil samples were collected from the agricultural soil located in Mansoura city, Dakahlia governorate (lies on the east bank of the Damietta branch of the Nile, in the Delta region. Its coordinates are Latitude 31° N and Longitude 31° E.), Egypt. Soil samples were taken at 10 cm depth, using a sterile spatula, in sterile plastic bags. The samples were brought to the microbiology laboratory at National Research Center, sieved and air dried for 3-5 days at 25°C. After drying, samples were kept at 10°C until used for the isolation of fungi.

### Isolation of fungi

Fungi were isolated by serial dilution technique on Czapex Dox Agar medium (CZA, DSMZ GmbH, Germany) supplemented with Rose Bengal (as an antibacterial agent). Soil dilution and soil sprinkle plates were used as isolation techniques. Soil dilutions were made by suspending 10 g of each soil sample in 100 mL of sterile distilled water. These suspensions were stirred for 30 min before making 7-fold serial dilution (from $10^{-1}$ to $10^{-7}$) and aliquots (0.1 mL) of each dilution was pipetted onto the medium in the plates. Sprinkle plates were prepared by uniformly distributing the soil directly on the surface of the medium. The plates were incubated at 4, 10 and 25°C for up to 3 weeks. Fungi growing on the agar plates were transferred by subculturing from hyphal tips, colonies or spores on Potato Dextrose Agar (PDA, BD Difco™, MD 21152, USA) slants [19].

### Antifungal activity of the isolates

The qualitative assessment of the antifungal effect was done using plate diffusion method [20] with some modification as suggested by Barakate et al. [21]. In brief, sterilized PDA medium was poured into Petri plates and allowed to dry. Fungal cultures were inoculated over the dried surface of PDA plate. Fungal isolates were removed from their agar using a sterile cork borer (9 mm in diameter) and placed onto agar plate lawn with the pathogenic microbes. The antifungal activity was determined by measuring the diameter of the zone of inhibition.

### Antibacterial activity of the isolates

The bacteria were pre-grown on Nutrient agar (NA, Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 37.0 ± 0.1°C and the qualitative assessment of the antibacterial effect was done using plate diffusion test. Overnight grown

bacterial cultures in nutrient broth were individually lawn cultured on nutrient agar plates. Fungal isolates were inoculated over the dried surface of PDA plate using a sterile cork borer (9 mm in diameter) and placed onto agar plate lawn with the pathogenic bacteria. The antibacterial activity was determined by measuring the diameter of the zone of inhibition.

**Culture filtrate preparation**

Potato dextrose broth was inoculated with fungal strains SH2 and SH5 separately at 28°C under rotary shaken condition (150 rpm) for 7 days. Then, the mycelia were separated and the culture filtrate was sterilized using 0.2 µm filter paper. The prepared culture filtrate was used for the prevention and degradation of $AFB_1$.

**Effect of isolated fungal strains on the ability of *A. flavus* to produce $AFB_1$**

The toxigenic strain of *Aspergillus flavus* was maintained on slants of PDA and incubated at 28°C for 7 days. Fungal conidial suspensions ($10^6$conidia/mL) were prepared in an aqueous solution of 0.1% Tween 80. One mL of each of the culture filtrate for each of the strains (SH2 and SH5) was transferred into 250 mL conical flask containing 100 mL yeast extract broth (2% yeast extract and 20% Sucrose) and inoculated with 1 mL fungal conidial suspension (*A. flavus*). The flasks were incubated for 7 days at 28°C under rotary shaken conditions (150 rpm) and a control without the culture filtrate was also prepared. Aflatoxin $B_1$ was extracted from culture filtrates according to Tosch et al. [22]. The culture filtrates were extracted three times with chloroform and the chloroform extracts were evaporated under nitrogen gas. The residue was dissolved in methanol and then completely passed through immunoaffinity column (AflaTest, VICAM, MA, USA) at a flow rate of about 1-2 drops/second. The immunoaffinity column was then washed twice with 10 mL purified water at a rate of about 2 drops/second. Elution was performed with 1.0 mL methanol and then analyzed by HPLC.

**Effect of isolated fungal strains on $AFB_1$ degradation**

Different concentrations (1, 2 and 3%) of the culture filtrates were added to a PDB containing $AFB_1$ at a concentration of 20 ppb. The flasks were incubated for 3 and 8 days at 28°C under rotary shaker condition (150 rpm). Flasks were taken at each interval and centrifuged at 10000 rpm for 5 min. The remaining $AFB_I$ in the supernatant was extracted using chloroform three times and then evaporated under nitrogen gas. The residue was dissolved in methanol and then passed through immunoaffinity column and analyzed by HPLC as mentioned above.

**HPLC conditions**

The HPLC system used for $AFB_1$ analyses was an Agilent 1200 series system (Agilent, Berks, UK) with a fluorescence detector (FLD G1321A), an autosampler ALS G1329A, FC/ALS therm G1330B, Degasser G1379B, Bin Bump G1312A and a C18 (Phenomonex, Luna 5 micron, 150 × 4.6 mm) column joined to a pre-column (security guard, 4 × 3-mm cartridge, Phenomenex Luna). The mobile phase was water: acetonitrile: methanol (3:1:1, v/v/v) using an isocratic flow rate of 1 mL/min at 360 nm excitation and 420 nm emission wavelengths.

**Molecular identification of the isolated fungal**

Fungal cultures (SH2 and SH5) were identified according to a molecular biological protocol by DNA isolation, amplification (PCR) and sequencing of the ITS region. The primers ITS2 (GCTGCGTTCTTCAT CGATGC) and ITS3 (GCATCGATGAAGAACG CAGC) were used for PCR while ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used for sequencing. The purification of the PCR products was carried to remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean-up kit (Millipore). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied Biosystems, USA) and *Candida* sp. was used as a control. Sequencing results were individually inputted online into the nucleotide BLAST program (BLASTN 2.2.29) through the NCBI database (http://blast.ncbi.nlm.nih.gov/) to identify the isolates [23, 24]. The phylogenetic tree was constructed using ClustalW program editor. The gene sequence of the fungal isolates was deposited in the GenBank database as *A. niger* strain SH2-EGY (Accession No. KP317994.1) and *A. fumigatus* strain SH5-EGY (Accession No. KP317993.1)

**Extraction and isolation of fungal bioactive compounds**

Erlenmeyer flasks (1L-volume) each containing 250 mL of PDB medium were inoculated with a fungal suspension from 7 days old PDA slants inoculated with SH2 or SH5 strain (1 slant for 2 1L-Erlenmeyer flasks). The flasks were kept at 28°C using rotary shaker (150 rpm) and harvested after 7 days. Another set of flasks were incubated static at 28°C. The mycelia were separated from the culture supernatant by centrifugation at 5000 rpm at 4°C then the supernatant was extracted with ethyl acetate. However, the mycelia were extracted with acetone and evaporated then the residual aqueous portion was extracted with ethyl acetate. All the ethyl acetate extracts were evaporated separately in vacuo till dryness. The methods of extraction of the crude extracts and the isolates used are mentioned in Table (1).

**Determination of biological activities of the *A. niger* and *A. fumigatus* isolates crude extracts**

***Antimicrobial activity***

The antimicrobial activities of crude extracts of the bioactive secondary metabolites were tested against four different microbial strains. The obtained extracts were dissolved in methanol and filter paper discs (5 mm diameter, Whatman No.1) were saturated with 10 µL from each extract [20, 25] then dried for 1 h at room temperature under sterilized conditions (final extract conc. was 100 µg per disc). The paper discs were placed on inoculated agar plates with the tested microbes and incubated at the appropriate temperature and time for each microorganism. Both bacterial and yeast strains were grown on nutrient agar (DSMZ GmbH, Germany) medium. After the incubation period, the diameter of the growth inhibition zones was measured averaged and the mean values were recorded.

***Determination of total phenolic content***

The total phenolic content was determined using Folin-Ciocalteu's reagent using Gallic acid as standard. The reaction mixture was composed of 50 µl of crude extracts of the bioactive compounds (200 µg/mL), 250 µl of the Folin-Ciocalteu's reagent and 0.75 mL of sodium carbonate (20%). The mixture was shaken and made up to 10 mL using distilled water then allowed to stand for 2 hrs-and the absorbance was measured at 765 nm. All determinations were carried out in triplicate and the total phenolic content was expressed as mg gallic acid equivalent (GAE) per g dry extract [26].

***Determination of total antioxidant capacity***

The antioxidant activity was determined according to phospho-molybdenum method, using ascorbic acid as standard. In this method, 0.5 mL of each crude extract of the bioactive compounds in methanol (200 µg /mL) was combined in dried vials with 5 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The vials containing the reaction mixture were capped and incubated in a thermal block at 95°C for 90 min. After the samples were cooled to room temperature, the absorbance was measured at 695 nm against a blank. The blank consisting of all reagents and solvents without the sample was incubated under the same conditions. The antioxidant activity of the extracts was expressed as the number of equivalents of ascorbic acid (AAE) per g dry extract [27. 28].

***Determination of Reducing Power Antioxidant Assay (RPAA)***

The spectrophotometric method described by Ferreira et al. [29] was used by mixing 2.5 mL of each of the crude extracts of the bioactive compounds with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide (10 mg/mL). The mixture was incubated at 50ºC for 20 min, then rapidly cooled, mixed with 2.5 mL of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (2.5 mL) of the supernatant was diluted with distilled water (2.5 mL) then ferric chloride (0.5 mL, 0.1%) was added and allowed to stand for 10 min. The absorbance was read spectrophotometrically at 700 nm, ascorbic acid was used as a standard and the percentage of reducing power was calculated using the formula:

$$Reducing\ power\ (\%) = \frac{A\ control - A\ sample}{A\ control}$$

Where: A control was the absorbance of the solution without the extract and A sample was the absorbance with different dilutions of the sample and the reducing power and was reported as ascorbic acid equivalent per gm of dry sample [26, 28, 29].

**Identification of chemical composition of the crude extracts by GC-MS**

The most promising crude extract (II) that showed high antioxidant and phenolic content activities was analyzed by a coupled Varian gas chromatography/ mass spectrometry (Perkin Elmer Auto XL GC, Waltham, MA, USA) equipped with a flame ionization detector to identify their chemical composition. The GC conditions were an EQUITY-5 column (60 m x 0.32 mm x 0.25 µm); $H_2$ carrier gas; column head pressure 10 psi, the oven temperature was maintained initially at 70°C for 2 min, and then programmed from 70 to 250°C at a rate of 3°C/min. The ionization voltage was 70 eV, mass range m/z 39-400 amu. The identification of individual compounds was based on their retention times relative to those of authentic samples and matching spectral peaks available with the published data [30].

## RESULTS AND DISCUSSION

**Isolation of fungi**

A total of five fungal isolates were identified morphologically as *Aspergillus* genera and given laboratory codes. These results are similar to those of Thompson et al. [31] who reported that the most frequently isolated fungal strains was *A. fumigatus* followed by *A. niger* from the soil of ornamental plants. Similar observation was reported by Ratnasri et al. [32] and Sarkar and Aikat [9] who isolated *A. fumigatus* from soil samples. On the other hand, 104 different strains of *A. niger* were isolated from soil collected from different areas of Lahore [33]. In another study by

Morya and Yadav [34], only one strain of *A. niger* was isolated from the soil of Kusmi Forest; an Indian subtropical Teak forest at Gorakhpur.

**Antimicrobial activity**

The five isolated fungi were tested for their ability to inhibit the growth of fungal and bacterial pathogens (Table 2). These fungal isolates showed antimicrobial activities towards pathogenic fungi and bacteria in variable degrees. Out of these five fungal isolates, only two isolates showed a significant antimicrobial activity against the four pathogenic fungi and were designated as SH2 and SH5 which induced mean values of zone inhibition diameter recording 70.0 and 64.5 mm, respectively against *F. solani* (Table 2). On the other hand, the same two isolates showed low antifungal activity against *F. oxysporum* and the mean values of zone inhibition recorded 25.3 and 26.0 mm, respectively. The greatest inhibition resulting from these isolates was followed by the isolates MO3 and MO4 and the recorded-inhibition zones of *F. solani* were 41.0 and 34.5 mm, respectively. Moreover, the isolate A2 showed relatively low antifungal activity compared to the fungal isolates SH2 and SH5 and did not show any antifungal activity against *F. oxysporum*. However, these two fungal isolates showed antibacterial activity against the four pathogenic bacteria (*Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, and *Bacillus cereus*), whereas the mean values of zone inhibition diameter of *S. aureus* due to the two fungal strains (SH2 and SH5) recorded 15.0 and 13.6 mm, respectively (Table 2). Similar antibacterial activity was recorded for the two fungal isolates (SH2 and SH5) against *B. cereus* and *S. typhi*. It is of interest to mention that isolates A2 and MO3 did not show any antibacterial activity, whereas the isolate MO4 showed antibacterial activity against *S. aureus* only.

In this concern, Abdel-Motaal et al. [35] indicated that eight endophytic fungi (*Alternaria alternata*, *Aspergillus fumigatus*, *Drechslera hawaiiensis*, *Fusarium solani*, *Penicillium citrinum*, *Neoscytalidium dimidiatum*, *Thyrostromella myriana* and *Ulocladium chartarum*) showed antagonistic activities against all the examined fungal strains. Similar observations were reported by Rosa et al. [36] and Shentu et al. [37]. The current results are considered to be in accordance with those obtained by Svahn et al. [38] who revealed that the majority of the *Aspergillus* strains isolated from sediments of Indian River displayed antimicrobial activity against pathogenic bacteria. Moreover, these authors reported that *A. fumigatus* showed stronger antimicrobial activity. In the same trend, Lihan et al. [39] analyzed soil samples for antimicrobial producing fungi and reported that fungal isolates showed strong antibacterial activities against different strains of pathogenic bacteria. The antimicrobial activity of the fungal isolates could be due to the production of enzymes such as amyloglucosidase, cellulases, lactase, invertase, pectinases, and acid proteases by *A. niger* [6], in addition, to the annual production of citric acid [6, 40]. Furthermore, Kelecom [41] added that the antimicrobial activity of fungi may be due to the production of metabolites such as fumid quinazolines, indole alkaloids, halogenated acetogenins and sesquiterpenes from *Aspergillus* genera, depending on the origin of the fungus.

**Effect of the isolated fungal strains on $AFB_1$ production by *A. flavus***

The effects of *A. niger* and *A. fumigatus* culture filtrate designated (SH2 and SH5) on the production of $AFB_1$ by *A. flavus* after 7 days of incubation was shown in Figure (1). These results revealed that both fungal isolates inhibited the production of $AFB_1$ but to a different extent, since *A. niger* and *A. fumigatus* inhibited $AFB_1$ production by 73.40 and 67.70%, respectively. These results are considered lower than those recorded by Cvetnić and Pepeljnjak [42] who reported that *A. niger* isolates inhibited $AFB_1$ production by *A. flavus* NRRL 3251 in a range from 80 to 100%. In the same trend, Xu et al. [43] revealed that *A. niger* FS10 inhibited $AFB_1$ accumulation by 97.2% after 15 days of incubation. More recently Ding et al. [44] isolated *A. niger* from stored peanuts and indicated that all isolates degraded $AFB_1$ with a rate of 44.5-100%.

The present results are considered similar to those obtained by Misra et al. [45] who indicated that *A. niger* and *A. fumigatus* inhibited $AFB_1$ production by 79.38 and 66.84%, respectively. The ability of *A. niger* and *A. fumigatus* to remove other mycotoxins was reported by several authors who stated that *A. niger* among other *Aspergilli* as well as *A. fumigatus* degraded OTA in liquid media [46, 47, 48]. These authors added that *A. niger* degraded OTA to OTα and phenylalanine, whereas OTα was degraded into an unknown compound. A preliminary study was also performed to screen 12 black *Aspergillus* strains for their zearalenone (ZEN) transformation activity where ZEN was completely metabolized in 48 h by the mycelium even with high concentrations of ZEN in the culture medium [49].
Although the mechanism of $AFB_1$ removal by fungal strains is not clear, Horn and Wicklow [50] concluded that $AFB_1$ production was suppressed by *A. niger* which lowered the substrate to acidic pH. Other authors reported that oxalic and gluconic acid secreted by *A. niger* could be responsible for the suppression of $AFB_1$ production [51, 52]. Recently Xu et al. [43] suggested that *A. niger* culture filtrate could cleave $AFB_1$ lactone ring by enzymatic degradation.

**Effect of isolated fungal strains on $AFB_1$ degradation**

The effect of *A. niger* and *A. fumigatus* culture filtrate designated (SH2 and SH5) on $AFB_1$ degradation was

presented in Table (3). These data revealed that after incubation at 3 and 8 days, the concentration of $AFB_1$ residue in the culture was decreased. The degradation process of $AFB_1$ by *A. niger* isolate was relatively high and continuous, with 38.73% of $AFB_1$ removed after 3 days, and 60.70% removed after 8 days. The degradation of $AFB_1$ by *A. fumigatus* was considerably low compared to *A. niger* especially after 3 days of incubation, with 5.29 % of $AFB_1$ removed after 3 days, and 74.54% removed after 8 days. These results also revealed that the concentration of $AFB_1$ residue decreased by increasing the concentration of the culture filtrates.

**Molecular identification of the isolated fungi**

The two fungal isolates that showed antifungal, antibacterial and antiaflatoxigenic activities were identified on the basis of their molecular characteristics. The amplification of 18S rRNA with ITS1 and ITS4 primers has been successfully performed and 18S rRNA gene was chosen as a target for PCR amplification because the sequence data is widely used in the molecular analysis to reconstruct the evolutionary history of organisms. The phylogenetic tree was constructed by the neighbour-joining (N-J) method based on the 18S rRNA sequences. The18S rRNA gene sequence analyses showed that strain SH2 was most closely affiliated with members of the genus *Aspergillus*. In the phylogenetic tree based on the neighbor-joining algorithm, strain SH2 fell within the cluster comprising *A. niger* and *A. awamori* (Figure2). The results revealed that strain SH2 exhibited a high level of 18S rRNA (~605 bp) similarity (99%) with *Aspergillus niger* strain Cumin 1(GenBank accession No. KF221069.1) (Figure3). On the other hand, the second fungal strain SH5 of the sequenced 18S rRNA gene (~575 bp) was identified as *Aspergillus fumigatus*. The 18S rRNA sequence analysis revealed that the isolate is a close relative (99%) of *Aspergillus fumigatus* isolate CBS 121325 (GenBank accession No. KJ175444.1). The gene sequences of fungi were deposited in GenBank database; *Aspergillus niger* strain SH2-EGY, Accession No. KP317994.1; and *Aspergillus fumigates* strain SH5-EGY, Accession No. KP317993.1.

It is well known that molecular characterization is a fast and quick technique which needs minimal handling of pathogens and also helps in distinguishing morphologically, similar fungal species [53]. Similar applications of PCR technology were used for the identification and detection of fungi, by using internal transcribed spacer (ITS) [18, 54, 55]. The genomic DNA containing 18S rRNA was the right candidate for the detection of fungus as it is a multi-copy gene which evolves slowly and is conserved among fungi. The present study proves that the genomic DNA containing 18SrRNA-based PCR is suitable for probing a large range of significant fungi owing to its higher level of analytical sensitivity and specificity [56].

**Determination of biological activities of the isolated *A. niger* and *A. fumigatus* crude extracts**

***Antimicrobial activity***

The crude extracts of the fungal isolates SH2 and SH5 were used to determine the antimicrobial activity against pathogenic microorganisms (*Staphylococcus aureus*, *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans*). These crude extracts inhibited the pathogenic microorganism growth at varying degrees (Figure 4). The results also showed that crude extract number (III) showed the highest antimicrobial activity against the pathogenic microorganisms followed by extracts II, IV and VII respectively. The data also showed that extract V did not induce inhibition of the pathogenic microorganisms *S. aureus* and *S. aureus* (MRSA), whereas extract I did not inhibit *S. aureus* (MRSA) only. The data presented in Figure (5) showed the inhibition zone of the crude extracts against pathogenic bacteria and yeast. These results are similar to those of Qadri et al. [57] who established that several fungal extracts demonstrated antimicrobial activity against *E.coli*, *S. aureus*, and *Candida albicans* at varying degree. Moreover, Shaaban et al. [58] reported that crude extract of the fungal strain *Alternaria alternate* exhibited high activity against bacteria and yeasts.

***Total phenolic content and total antioxidant activity***

The current results revealed that antioxidant activity of the fungal crude extracts varied from 392.92 mg AAE /g for extract VII to 940.10 mg AAE /g for extract II. The antioxidant capacity was related mostly to the total phenolic content. Data in Figure (6) showed that the total phenolic content of the crude extracts varied according to the method of extraction which in turn was expressed in the antioxidant capacity. It was also noticed that the highest antioxidant capacity and total phenolic contents belonged to the crude extract II. The highest antioxidant capacity of crude extracts could be related to the presence of high total polyphenol contents [59] which are considered to be the major contributor to the antioxidant activity [60]. The data presented in Figure (6) also pointed out that there is a positive relationship between antioxidant activity and the amount of phenolic compounds of the crude extracts [61].

**Table 1: Crude extracts of fungal bioactive secondary metabolites**

| Extract number | Fungal name | Incubation condition | | Extraction from | |
|---|---|---|---|---|---|
| | | Rotary shaker (150 rpm)[a] | Static [b] | Fungal Supernatant [c] | Fungal mycelia [d] |
| I | *Aspergillus niger* | ↔ | | ↔ | |
| II | | ↔ | | | ↔ |
| III | | | ↔ | ↔ | |
| IV | | | ↔ | | ↔ |
| V | *Aspergillus fumigatus* | ↔ | | ↔ | |
| VI | | ↔ | | | ↔ |
| VII | | | ↔ | ↔ | |
| V III | | | ↔ | | ↔ |

[a] *The flasks were kept at 28°C using rotary shaker (150 rpm) for 7 days.*
[b] *The flasks were incubated static at 28°C for 7 days.*
[c] *The culture supernatants were extracted with ethyl acetate*
[d] *The mycelia were extracted with acetone, concentrated, and the remaining water residue was re-extracted with ethyl acetate*

**Table 2: Antimicrobial activity of fungal isolates against various fungal and bacterial pathogens**

| Fungal isolates | Zone inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ***A. parasiticus*** | ***A. ochraceus*** | ***F. solani*** | ***F. oxysporum*** | ***B. cereus*** | ***E. coli*** | ***S. aureus*** | ***S. typhi*** |
| A2 | 29.0±1.1 | 19.3±1.15 | 42.5±3.5 | ND | ND | ND | ND | ND |
| SH2 | 31.5±2.1 | 40.0±0.0 | 70.0±14.1 | 25.3±4.5 | 13.0±0.0 | ND | 15.0±0.00 | 12.5±0.70 |
| SH5 | 43.5±2.1 | 22.0±2.64 | 64.5±24.7 | 26.0±5.2 | 13.0±2.64 | 14.0±0.0 | 13.6±0.57 | 15.0±1.41 |
| MO3 | 27.0±2.8 | ND | 41.0±1.4 | ND | ND | ND | ND | ND |
| MO4 | 28.5±2.12 | ND | 34.5±3.5 | ND | ND | ND | 11.6±0.57 | ND |

*Results are mean ±SD (n=3).*
*ND: Not determined*

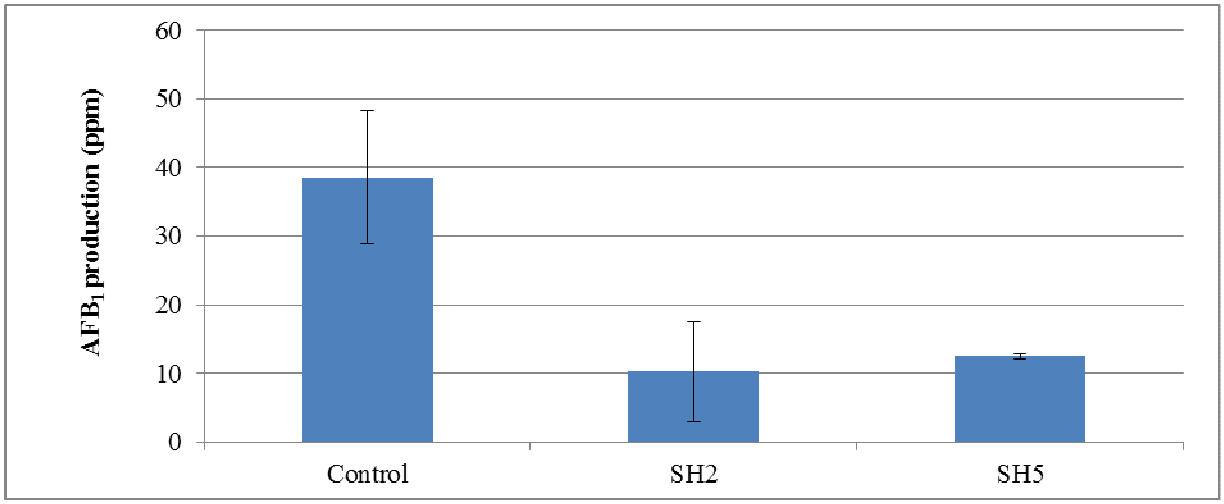


**Figure 1: Effect of fungal isolates on $AFB_1$ production by *Aspergillus flavus*. Results are mean ± SD (n=3). Bars represent the standard errors. Fungal isolate SH2 was later identified as *A. niger* and SH5 identified as *A. fumigatus***

**Table 3: Percentage of inhibition of aflatoxin $B_1$ by different concentrations of culture filtrates**

| Incubation period (days) | SH2 | | | SH5 | | |
|---|---|---|---|---|---|---|
| | 1% | 2% | 3% | 1% | 2% | 3% |
| 3 | 38.73 ± 1.33 | 62.63 ± 0.11 | 69.16 ± 0.25 | 5.29 ± 0.96 | 20.19 ± 1.12 | 39.22 ± 0.56 |
| 8 | 60.70 ± 0.06 | 66.81 ± 0.23 | 74.45 ± 0.45 | 74.54 ± 0.38 | 83.94 ± 0.03 | 84.99 ± 0.06 |

*Results are mean ±SD (n=3)*
*Culture filtrates were added at different concentrations to a PDB containing $AFB_1$ at a concentration of 20 ppb*
*The flasks were incubated at 28°C in a rotary shaker incubator at 150 rpm for 3 and 8 days*
*Flasks were taken at each interval and centrifuged at 10000 for 5 min, and the $AFB_1$ was extracted from supernatant using chloroform according to the Association of Official Analytical Chemists.*
*Fungal isolate SH2 was later identified as A. niger and SH5 identified as A. fumigatus.*

**Table 4: Partial sequence of 18S ribosomal RNA gene of two fungal isolates**

| Strain | bp | | Aligned Sequence Data |
|---|---|---|---|
| SH2 | 605 | 1 | ggactgggga tcctaccctg atcgaggtca cctgggaaag aatggttgga aaacgtcggc aggcgccggc |
| | | 61 | caatcctaca gagcatgtga caaagcccca tacgctcgag gatcggacgc |
| | | 121 | ggtgccgccg ctgcctttcg ggcccgtccc cccggagagg gggacggcga cccaacacac |
| | | 181 | aagccgggct tgagggcagc aatgacgctc ggacaggcat gccccccgga ataccagggg |
| | | 241 | gcgcaatgtg cgttcaaaga ctcgatgatt cactgaattc tgcaattcac attagttatc |
| | | 301 | gcatttcgct gcgttcttca tcgatgccgg aaccaagaga tccattgttg aaagttttaa |
| | | 361 | ctgattgcat tcaatcaact cagactgcac gctttcagac agtgttcgtg ttggggtctc |
| | | 421 | cggcgggcac gggcccgggg ggcagaggcg ccccccggc ggccgacaag cggcgggccc |
| | | 481 | gccgaagcaa cagggtacaa tagacacgga tgggaggttg ggcccaaagg acccgcactc |
| | | 541 | ggtaatgatc cttccgagcc ccccccccc aaaaaaaggt ctccccgggg gcggattccc |
| | | 601 | ttggg |
| SH5 | 575 | 1 | gaatgggga tcctaccctg atcgaggtca ccttagaaaa ataaagttgg gtgtcggctg |
| | | 61 | gcgccggccg ggcctacaga gcaggtgaca aagccccata cgctcgagga ccggacgcgg |
| | | 121 | tgccgccgct gcctttcggg cccgtccccc gggagagggg gacgggggcc caacacacaa |
| | | 181 | gccgtgcttg agggcagcaa tgacgctcgg acaggcatgc cccccggaat accaggggc |
| | | 241 | gcaatgtgcg ttcaaagact cgatgattca ctgaattctg caattcacat tacttatcgc |
| | | 301 | atttcgctgc gttcttcatc gatgccggaa ccaagagatc cgttgttgaa agttttaact |
| | | 361 | gattacgata atcaactcag actgcatact ttcagaacag cgttcatgtt ggggtcttcg gcgggcgcgg |
| | | 421 | gcccgggggc gcaaggcctc cccggcggcc gtcgaaacgg cgggcccgcc |
| | | 481 | gaagcaacaa ggtacgatag acacgggtgg gaggttggac ccagagggcc ctcactcggt |
| | | 541 | aatgatcctt ccgcaggttc accctacgag aaggg |

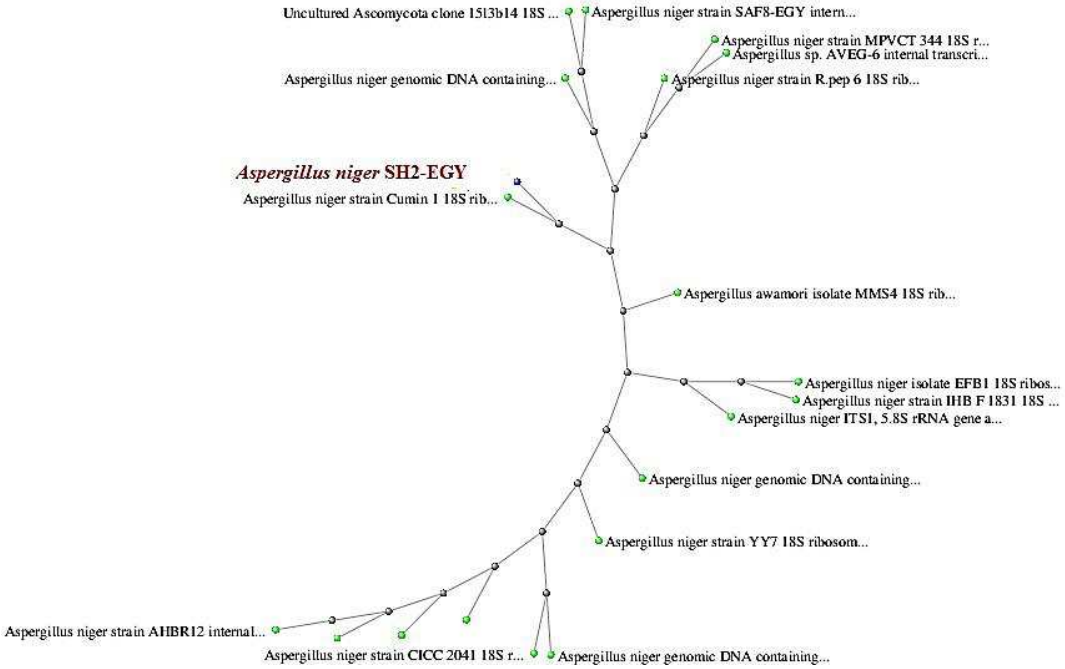


**Figure 2: Phylogenetic tree showing the relationship of strain SH2 with other related fungal species retrieved from GenBank based on their sequence homologies of 18Sr RNA**

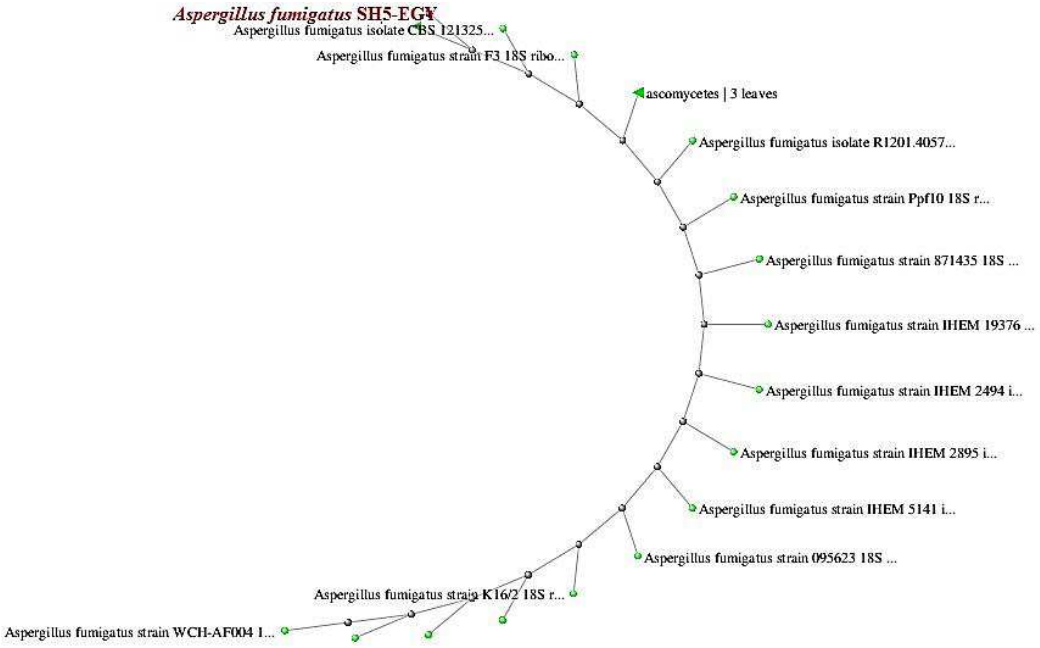


**Figure 3: Phylogenetic tree showing the relationship of strain SH5 with other related fungal species retrieved from GenBank based on their sequence homologies of 18Sr RNA**

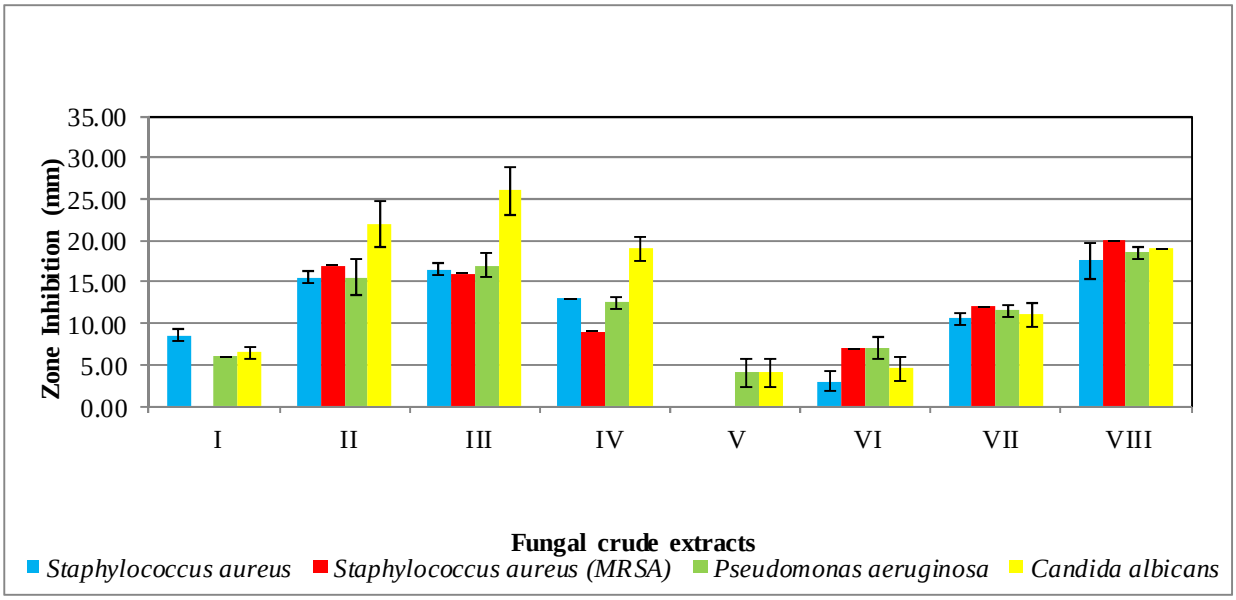


**Figure 4: Antimicrobial activity of fungal crude extracts. Results are mean ± SD (n=2). Bars represent the standard errors**

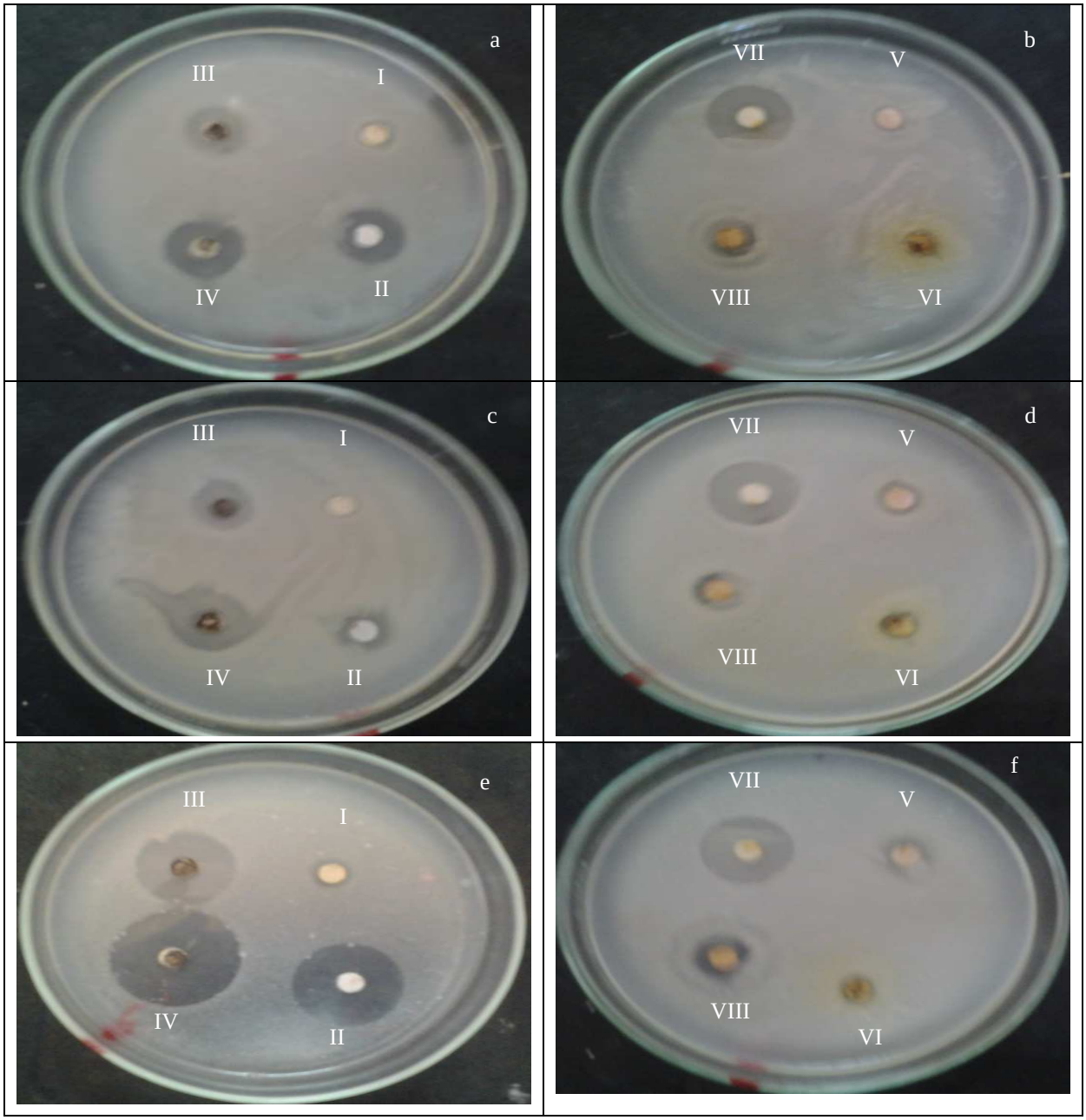


**Figure 5: Zone Inhibition of the fungal crude extracts against pathogenic microorganisms. Pictures a and b) *Staphylococcus aureus*; c and d) *Pseudomonas aeruginosa*; e and f) *Candida albicans*. Crude extracts I to IV extracted from *A. niger* and crude extracts V to VIII extracted from *A. fumigatus***

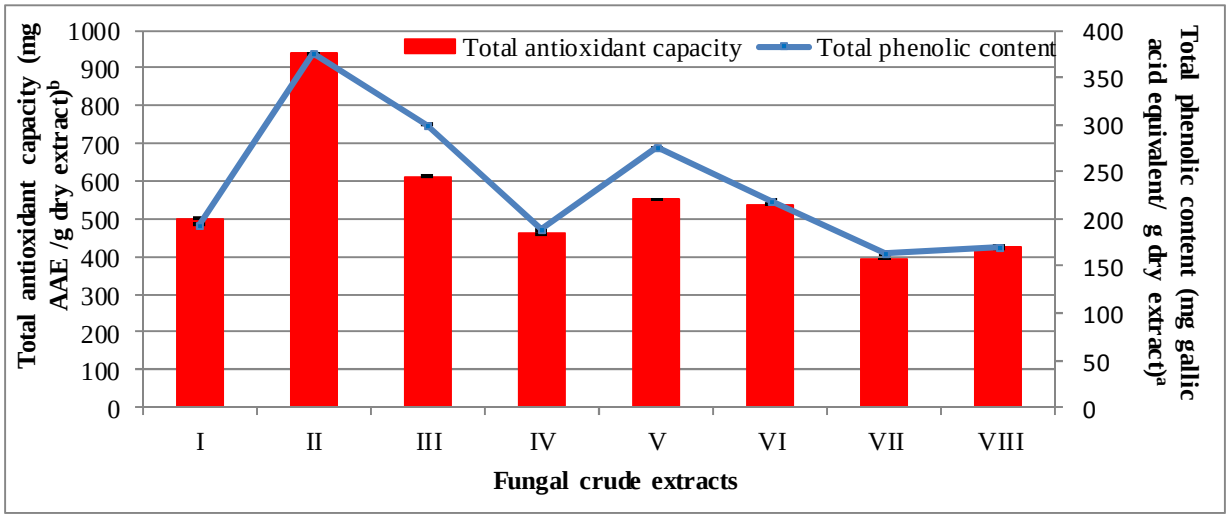


**Figure 6: Total phenolic content and total antioxidant capacity of fungal bioactive secondary metabolites. Results are mean ± SD (n = 3). Bars represent the standard error. Total phenolic content values are expressed as mg Gallic acid equivalent/g extract (mg GAE/g dry extract). [b] Total antioxidant capacity values are expressed as mg ascorbic acid equivalent/g extract (mg AAE/g dry extract)**

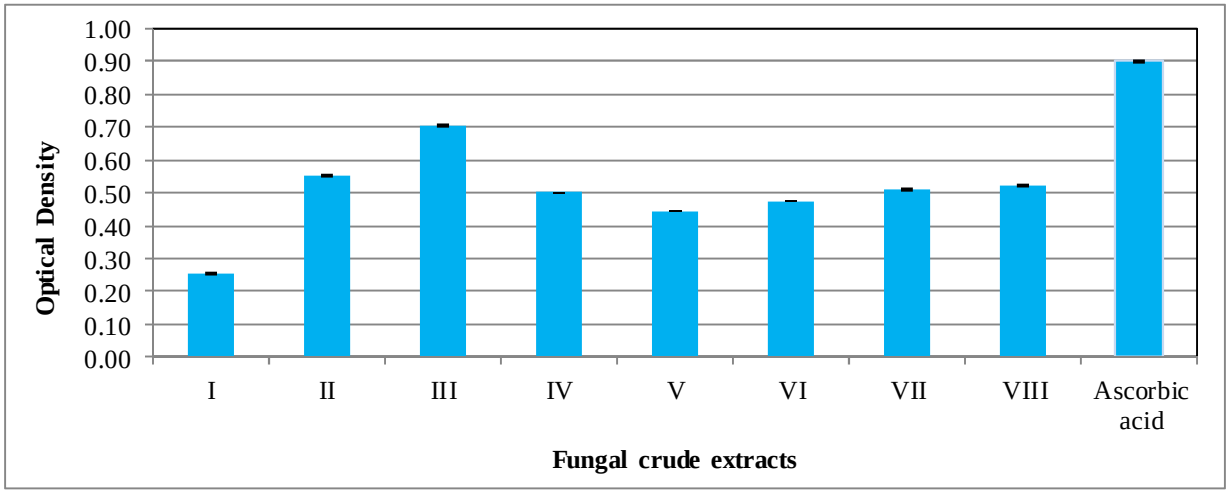


**Figure 7: Reducing power antioxidant activity of fungal bioactive secondary metabolites against ascorbic acid. Results are mean ± SD (n = 3). Bars represent the standard error**

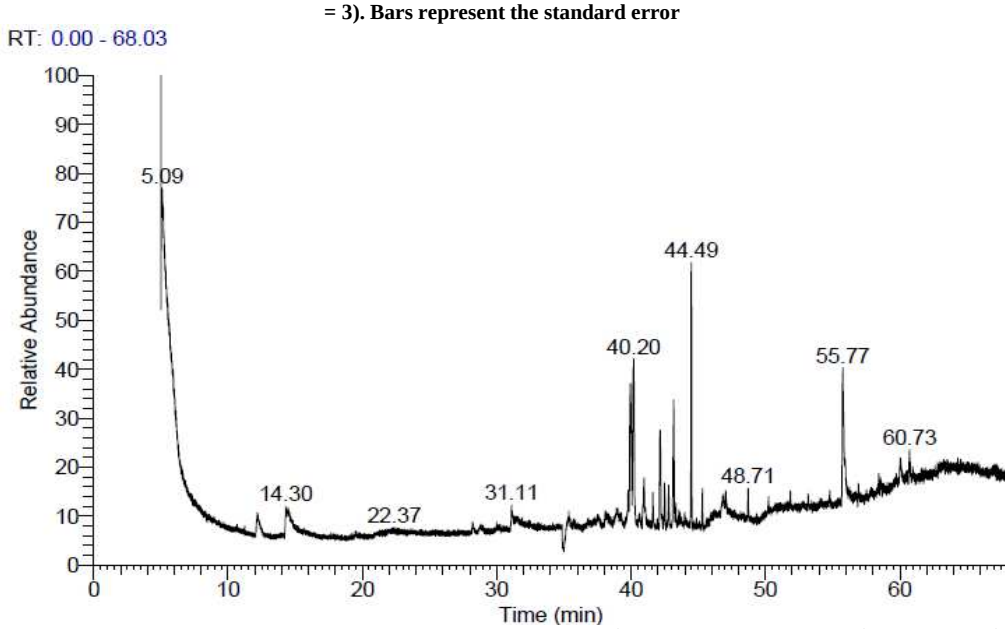


**Figure 8: Gas chromatography-mass spectrometry (GC-MS) chromatogram of the ethyl acetate extract of the mycelia of the shaken culture broth of *Aspergillus niger* (Accession No. KP317994.1)**

**Table 5: Chemical compositions of the of the ethyl acetate extract of the mycelia of the shaken culture broth of *Aspergillus niger* (Accession No. KP317994.1)**

| No. | RT | Rate % | M.W. | M.F. | Identified compounds |
|---|---|---|---|---|---|
| 1 | 12.18 | 2.30 | 364 | $C_{20}H_{28}O_6$ | Oridonin[1] |
| 2 | 14.30 | 2.04 | 302 | $C_{20}H_{30}O_2$ | 5,8,11,14,17-Eicosapentaenoic acid |
| 3 | 14.44 | 1.75 | 416 | $C_{27}H_{44}O_3$ | 24, 25-Dihydroxycholecalciferol |
| 4 | 31.11 | 1.58 | 220 | $C_{15}H_{24}O$ | Butylated hydroxytoluene[3] |
| 5 | 34.86 | 2.34 | 331 | $C_{13}H_{26}BNO_6Si$ | à-D-Glucopyranoside, methyl 2-(acetyl amino)-2-deox y-3-O-(trimethylsilyl)-, cyclic methylboronate[4] |
| 6 | 35.22 | 1.78 | 385 | $C_{22}H_{27}NO_5$ | Thalicpureine |
| 7 | 35.29 | 0.94 | 534 | $C_{28}H_{30}O_{10}$ | 3-Desoxo-3,16-dihydroxy-12-desoxyphorbol 3,13,16,20-tetraacetate |
| 8 | 35.36 | 1.65 | 344 | $C_{18}H_{16}O_7$ | Quercetin trimethyl ether |
| 9 | 37.56 | 1.17 | 520 | $C_{27}H_{36}O10$ | (2R,3R,5S,2'R,5'R,8'abeta)-2-Methoxy-5-(3-furyl)-2'alpha-methyl-4'aalpha-(acetoxymethyl)-4,5-dihydrodispiro[furan-3(2H),1'-decalin-5',2''-oxirane]-4'alpha,6'beta-diol diacetate |
| 10 | 38.20 | 0.91 | 416 | $C_{22}H_{36}N_6O_2$ | 1,2-Bis[1-(2-hydroxyethyl)-3,6-diazahomoadam antantydene-9] hydrazin[2,3,4,5,6,7] |
| 11 | 39.79 | 2.34 | 648 | $C_{27}H_{52}N_2O_{10}Si_3$ | á-D-Glucopyranosiduronic acid, 3-(5-ethylhexahydro-2,4 ,6-trioxo-5-pyrimidinyl )-1,1-dimethylpropyl 2,3,4-tris-O-(trimethylsilyl)-, methyl ester[4] |
| 12 | 39.96 | 11.51 | 540 | $C_{12}H_{52}O_6Si_5$ | D-(-)-Fructopyranose, pentakis(trimethylsilyl) ether[1, 4] |
| 13 | 40.20 | 12.75 | 437 | $C_{28}H_{23}NO_4$ | Fmoc-L-3-(2-Naphthyl)-alanine [3, 4] |
| 14 | 40.97 | 3.79 | 482 | $C_{19}H_{46}O_6Si_4$ | alpha-D-Glucopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)- |
| 15 | 41.62 | 3.44 | 532 | $C_{16}H_{48}O_6Si_7$ | Heptasiloxane, Hexadecamethyl- |
| 16 | 42.16 | 6.78 | 368 | $C_{22}H_{44}N_2O_2$ | Octadecanamide, N- (2- methyl propyl)-N-nitroso |
| 17 | 42.80 | 2.27 | 306 | $C_{20}H_{34}O_2$ | 5Z,8Z,11Z-eicosatrienoic acid |
| 18 | 43.17 | 7.60 | 614 | $C_{24}H_{62}O_6Si_6$ | Trimethylsilyl ether-glucitol |
| 19 | 44.49 | 14.78 | 540 | $C_{21}H_{52}O_6Si_5$ | 1,2,3,4,6-Penta-trimethylsilyl Glucopyranose[1, 4] |
| 20 | 46.83 | 1.53 | 514 | $C_{30}H_{58}O_4S$ | Didodecyl 3,3'-thiodipropionate[3] |
| 21 | 47.05 | 0.88 | 243 | $C_{12}H_{10}FN_5$ | 1H-Purin-6-amine, [(2-fluorophenyl)methyl] |
| 22 | 48.71 | 1.41 | 384 | $C_{12}H_{36}O_4Si_5$ | Pentasiloxane, dodecamethyl[2, 3] |
| 23 | 55.76 | 9.20 | 390 | $C_{24}H_{38}O_4$ | Bis (2-ethylhexyl) phthalate |
| 24 | 56.92 | 0.83 | 597 | $C_{32}H_{39}NO_{10}$ | 3-Pyridinecarboxylic acid |
| 25 | 60.04 | 1.52 | 464 | $C_{24}H_{32}O_9$ | 5H-Cyclopropa[3,4]benz[1,2-e]azulen-5-one, 9,9a-bis(acetyloxy)-1,1 a,1b,2,4a,7a,7b,8,9,9 adecahydro-2,4a,7b-trih ydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl |
| 26 | 60.72 | 1.96 | 594 | $C_{27}H_{30}O_{15}$ | Flavone 4'-OH,5-OH,7-Di-O-Glucoside[4] |

*M.F.: Molecular formula; M.W.: Molecular weight; RT: Retention time*

*[1]Cytotoxic; [2]Antimycotic; [3]Antioxidant; [4]Anticancer; [5]Antiaflatoxin; [6]Antifungal; [7]Antimicrobial (Data obtained from National Center for Biotechnology Information, PubChem Compound Database; https://pubchem.ncbi.nlm.nih.gov/; and from Dr. Duke's Phytochemical and Ethnobotanical Databases)*

***Reducing power antioxidant activity***

The reducing power of the fungal crude extracts was studied by the reduction of $Fe^{3+}$ to $Fe^{2+}$, and in the presence of ascorbic acid. The measured values of absorbance varied from 0.252 for extract I to 0.704 for extract III, whereas extract III gave the highest reducing power. The reducing activity was considered lower than ascorbic acid standard which recorded 0.900. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [62].

**Identification of chemical composition of the crude extracts by GC/MS**

Based on the above-mentioned results, the crude extract of *A. niger* isolate SH2 which showed the highest antioxidant effects was studied. Consequently, this extract was subjected to the identification of its active components based on the peak area, molecular weight and molecular formula. The chemical analysis of these crude extracts by GC/MS indicated the identification of twenty-six components representing 99.05% of the total extract composition (Table 5). The major components were 1,2,3,4,6-Penta-trimethylsilyl Glucopyranose (14.78%), Fmoc-L-3-(2-Naphthyl)-alanine (12.27%), D-(-)-Fructopyranose, pentakis(trimethylsilyl) ether (11.51%), bis (2-ethylhexyl) phthalate (9.20%), trimethylsilyl ether -glucitol (7.60%), and octadecanamide, N- (2- methylpropyl)-N-nitroso (6.78%), as well as other abundant components such as methyl alpha-D-Glucopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)- (3.79%), and Oridonin (2.30%). These results are in accordance to those obtained by Qi et al. [63] who isolated the fungus *Cladosporium* sp. F14 and found that the chemical analysis of its fermentation broth yielded nine compounds, one of which was bis (2-ethylhexyl) phthalate. Similarly, Debbab et al. [64] reported that *Aspergillus* genera are considered frequently distributed and are well-known for their ability to produce a vast amount of structurally unique bioactive metabolites. Several authors reported that bioactive compounds produced by several *Aspergillus* species showed antibacterial, antiviral and cytotoxic activities [65, 66, 67]. In this concern, Trisuwan et al. [68] and Gamal-Eldeen et al. [69] indicated that the secondary metabolites from the genus *Penicillium* are considered as one of the most important sources of a wide range of biological and pharmacological activities. The present results suggested that potential antimicrobial and antioxidant activity of crude extracts II and III could be due to the bioactive compounds identified such as butylated hydroxytoluene (1.58%), Didodecyl 3,3'-

thiodipropionate (1.53%), Pentasiloxane, dodecamethy (1.41%), and 1,2-Bis[1-(2-hydroxyethyl)-3,6-diazahomoadam antantydene-9] hydrazine (0.91%). Meanwhile, Yehye et al. [70] indicated that butylated hydroxytoluene is a most commonly used antioxidant and is recognized as safe for use in foods containing fats, pharmaceuticals and oil industries.

## CONCLUSION

Isolation of fungi from soil samples and the rapid screening by plating on Czapex Dox Agar medium led to the finding of five fungal isolates showing variable degrees of antifungal, antibacterial and antiaflatoxigenic activity. Molecular characterization confirmed that the isolates SH2, SH5 as *Aspergillus niger* and *Aspergillus fumigatus*, respectively. The fungal secondary metabolites crude extracts II and III were found to have excellent biological activities. The total phenolic content of the crude extracts varied and related mostly to the antioxidant activity. The results obtained were promising and thus further studies concerning purification of the active crude extracts are necessary.

**Acknowledgment**
This work was funded by the National Research Centre, Cairo, Egypt, under grant No. 10070208.

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