Antioxidant, antimicrobial and antiproliferative activities of fungal metabolite produced by *Aspergillus flavus* on *in vitro* study

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Abstract

Regarding to the defiance came upon scientists to output natural products with potent biological activity from cheap sources and in an attempt to recover the average of antiproliferative agents, the employment of fungal metabolite in creating a cordial proper process was a prerequisite. In the present results, fungal metabolites were detected by thin layer chromatography. One fungal isolate out of ten isolates bioassayed, was showed to have the most potent antioxidant, antiproliferative and antimicrobial activity; this fungal isolate was identified as belonging to *Aspergillus flavus* (*A. flavus*). Fungal extract of *A. flavus* showed a high free radical scavenging activity using Diphenyl-1-picrylhydrazyl (DPPH) scavenging % with low a concentration of IC₅₀ and potent flavonoid content. The result of instrumental analysis using GC/Mass illustrates the presence of different potent products of *A. flavus* extract. In addition, *A. flavus* extract showed a promising antiproliferative activity. Inhibitory activity against Hepatocellular carcinoma cells was detected under these experimental conditions. This strategy can be further used to elicit new age drugs.

Keywords: *Aspergillus flavus*; bioactive compounds; GC; DPPH; antibacterial; antifungal.

Practical Application: The present study confirmed the efficient role of fungal bioactive products as strong in *vitro* antioxidant, antimicrobial and antiproliferative activities. The results indicated that the *A. flavus* had a potent naturalistic constituent in cytotoxicity. In addition, these natural sources constituent have recently shown a promising interest in treating cancer or inhibiting multidrug pathogenic bacteria.

1 Introduction

The existence of numerous issues of cancer disease and antibiotic resistant strains is increased recently and their inhibition by alternative agents are necessary (Shi et al., 2007; El-Gazzar et al., 2020; Abdel-Shafi et al., 2020; Enan et al., 2020; El-Sayed et al., 2020). In addition, the biocontrol of this cancer is of interest to persist study to detect and discover newly solution to prevent such disease in *vitro* (Shi et al., 2007).

In this regard, there is an inevitable and urgent medical need for natural bioactive metabolites with novel anticancer and antimicrobial activity (Abdel-Shafi et al., 2020; Enan et al., 2018) and there is frequently concern in detection protocols to create confident and appreciate-efficient biocidal products (El-Gazzar et al., 2020; El-Gazzar & Ismail., 2020).

Recent perspectives all over the world are to use safe materials as either safe therapy or as a biosorptive tools for anticancer and antimicrobial activity (Enan et al., 2020; Abdel-Shafi et al., 2020; Osman et al., 2021). In this framework, natural metabolites from fungi are also used to create confident and appreciate-efficient biocidal products by applying the material technology, industrial processes and others (El-Gazzar et al., 2020). For a wide variety of secondary metabolites, fungi are the most diverse, where different primary extracts play a powerful role in the biological activity of species (Ronsberg et al., 2013; El-Gazzar & Enan, 2020). Recently,

research procedures have been undertaken for various fungi that have confirmed their function in curative fields (Simmons et al., 2005). Derivative extracts from fungal strains are distinguished by incoming inherent outputs that are subject to characterize new medicine and industries (Suryanarayanan et al., 2009).

Numerous bioactive metabolites isolated from *Aspergillus sp.* were published in several literatures (Huang et al., 2009). These metabolites, such as anticancer, antimicrobial and antioxidant activities have shown therapeutic importance. The biological importance of these fungal species is of great interest to the scientific community. In order to detect compounds with successful anticancer activity, there are criteria to continue testing. This research seeks to detect the function of *Aspergillus flavus* bioactive metabolites as an anticancer, antibacterial and antioxidant agent.

2 Materials and Methods

2.1 Fungal strains used:

The fungal strains used in this study were isolated from soil contaminated with wastes from Photographic Industries (El-Sharkia Governorate, 80 km North Cairo, Egypt) (El-Gazzar, 2015). They were purified and finally grown in slants of Czapeks Dox agar

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medium. The isolates were purified using Potato Dextrose agar (PDA media) (glucose- yeast extract – potato filtrate) as reported by El-Gazzar (2015) and identified according to Raper & Thom (1949); Raper & Fennell (1965) and Booth (1971).

2.2 Preparation and extraction of fungal metabolite:

The fungal cultures used in this investigation were subjected for spore production through the growing of the tested fungi on PDA broth (Oxoid) medium at about one week at $28 \pm 2^\circ\text{C}$. The spore suspension was purified and filtered by cheesecloth. Spores were enumerated by qualified hemocytometer. A convenient attenuation was prepared using the supply spore narrator through sterilized 0.1% (w/v) peptone water as diluents to take out the in demand spores level of 4×10^2 cells/mL (Abdel-Salam et al., 2001; Enan et al., 2013).

Fungal metabolites were extracted from the mycelium that inoculated in a sterilized Czapek Dox Broth (100 mL) at 121°C , 15 lbs pressure for 15-20 min in Erlenmeyer flask and incubated in dark (10 d, $25 \pm 1^\circ\text{C}$).

The fresh mycelial mat of each fungal strain was ground in a mortar in the presence of sterile fine sand and methanol: chloroform (1:2 v/v) to extract the secondary metabolites then centrifuged and filtered. The solvent was evaporated, using an air drier and the metabolites were dissolved in methanol and stored at 5°C (El-Gazzar et al., 2020; El-Sayed et al., 2020).

The broth filtrates were defatted with n-hexane, then collected and taken away using methanol: chloroform (1:2 v/v) in a separating funnel, shaken well and left at least for six hours until complete separation from the lower phase. Sediment was re-taken away again for full separation by evaporation of solvents and the metabolites were dissolved in methanol and stored at 5°C (Abdel-Salam et al., 2001).

2.3 Detection of fungal extracts with TLC

The thin layer chromatography was employed successfully for the separation of different bioactive compounds (Dedio et al., 1969). The broth filtrates can be applied on thin layer chromatography TLC plates. Developing processes can be carried out, using chloroform: methanol (9: 1 v/v) system for separation of fungal secondary metabolites.

2.4 Free radical scavenging activity of the fungal extract

The antioxidant efficiency of the methanolic extract was determined with the standard of their scavenging efficiency of the 1, 1- diphenyl-2-picryl hydrazyl (DPPH) free radical (Melo et al., 2008; Baba & Malik, 2015). Briefly, 10 μL of each extract (50 $\mu\text{g/mL}$) were mixed with 3.8 mL of DPPH solution and incubated at 37°C for 30 minutes. The absorbance of the mixture was examined at 517 nm. Ascorbic acid was used as a positive standard. The study was performed in triplicate. Scavenging efficiency rate was determined using the subsequent Formula 1:

$$\text{Scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

2.5 Determination of Total Flavonoid Content (TFC)

The TFC of the fungal extracts was determined by aluminum chloride colorimetric assay. The absorbance of each mixture was determined at 510 nm against the same mixture but without fungal extracts as a blank. TFC was determined as μg quercetin equivalent per mg of samples with the help of calibration curve of quercetin. All determinations were performed in triplicate ($n=3$).

2.6 Determination the IC_{50} of Free radical scavenging activity and TFC for the most active fungal extract

100 μL of diverse concentrations prepared from the stock of the most active fungal extract (800 $\mu\text{g/mL}$). From this stock extract (800 $\mu\text{g/mL}$), different dilutions were made to contain 5, 10, 20, 40, 80, 160, 320 and 640 $\mu\text{g/mL}$, respectively. Serial two-fold dilutions of fungal extract were made in sterile deionized water by taking 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 mL, from the original stock and this equals 5, 10, 20, 40, 80, 160, 320 and 640 $\mu\text{g/mL}$, respectively. The IC_{50} of The antioxidant efficiency of these different concentrations of fungal extract was determined with the standard of their scavenging efficiency of the 1, 1- diphenyl-2-picryl hydrazyl (DPPH) free radical (Melo et al., 2008) as mentioned above in subsection 2.4. In addition IC_{50} of TFC of these different concentrations of fungal extract was determined by aluminum chloride colorimetric assay as mentioned above in subsection 2.5.

The IC_{50} of ascorbic acid (standard antioxidant) determined by taking different concentration from stock ascorbic acid (100 $\mu\text{g/mL}$), different dilutions were made to contain 5, 10, 15, 20, 25, 30, 35 and 40 $\mu\text{g/mL}$, respectively. Serial two-fold dilutions of ascorbic acid were made in sterile deionized water by taking 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4 mL, from the original stock and this equals 5, 10, 15, 20, 25, 30, 35 and 40 $\mu\text{g/mL}$, respectively.

2.7 Antimicrobial assay using fungal extract

The sediment of the most potent fungal extract (*A. flavus* extract) was re-taken away again after extraction of secondary metabolites as mentioned above in subsection 2.2. for full separation by evaporation of solvents and the metabolites were dissolved in DMSO. Stock preparation of fungal extract (200 $\mu\text{g/mL}$) was prepared and then stored in Eppendorf tubes (Gomhuria Co., Zagazig, Egypt) at 5°C until antimicrobial activity tests were performed.

Antibacterial activity of the most potent fungal extract was evaluated against *Staphylococcus aureus* DSM 1104, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* LMG 8223 and *Pseudomonas aeruginosa* LMG 8029. Stock bacterial cultures were routinely sub-inoculated in brain heart infusion broth (BHIB) (Oxoid) (Enan et al., 2013; Abdel-Shafi et al., 2016), and then kept at 4°C for uses in this investigation. Brain Heart infusion agar plates (Difco™, Maryland, USA) were prepared and seeded with log phase cells (10^5 CFU/mL) of bacterial inoculants. Each test organism (100 μL) was mixed with cooled Mueller–Hinton agar and poured into 80 mm petri dishes; then the sterile singly stock preparation of fungal

extract (200 µg/mL) was applied to discs (6mm diameter) which were added onto the above plates. All samples were incubated at 37 °C for 24-48 h. Inhibition zone diameters (IZDs) were determined (Abdel-Shafi et al., 2016, 2020; El-Gazzar & Enan, 2020; Aziz et al., 2014). Gentamycin was used as positive control. All experiments were performed in triplicates.

Antifungal activity of stock preparation of fungal extract (200 µg/mL) was evaluated against *Candida albicans* and *Fusarium oxysporium* by agar disc diffusion method. The plate incubated at 37 °C for 5-7 days and the zones of inhibition were measured. Ketoconazole was used as positive control. All experiments were performed in triplicates for statistical analysis.

2.8 Antiproliferative activity of the most potent fungal secondary metabolites by cell lines

The cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 50 µg/mL gentamycin and 10% (v/v) heat inoperative fetal bovine serum (FBS) and developed at 37 °C in 5% CO₂ (Water Jacketed Double door incubator, Shel Lab, Sheldon Manufacturing, Inc., USA) about 48 hrs. Samples were examined with an inverted microscope (CKX41; Olympus, Japan) to check the cultures and to confirm their sterility from contamination by microorganisms. Cells layer rinsed using phosphate buffered saline, pH 7.2 without Ca²⁺/Mg²⁺, a volume equal to semi the cultivated medium size. Trypsin/ EDTA was introduced into the rinsed single layer cell using 1 mL per 25 cm² of surface area and left in 37 °C about 5 minutes. Then, the samples were examined by a microscope to confirm their detached and floated. They were combined with new FBS containing RPMI environment. The count of samples was measured by taking away 100-200 µL from their followed by trypan blue dye exclusion method using a hemocytometer. The demanded count of samples was put in plates supplied with growth environment then kept as recommended for the cell line (Mosmann, 1983; Vijayan et al., 2004).

2.9 Estimation the antiproliferative efficiency

The antiproliferative activity was evaluated on single carcinoma cell lines, namely Liver hepato cellular cells (HepG2). The cell line was originated as mono layers with 10 per cent (v / v) inoperative foetal calf serum and 50 µg/mL gentamycin in the growth medium. The single layers of 10,000 organisms subjected at down of the 96-bore plate kept at 37 °C about 24 h and 5% CO₂. The cells were then rinsed by phosphate buffered saline (0.01 M; pH 7.2); then they were subjected by 100 µL of diverse concentrations prepared from stock extract of *A. flavus* (1000 µg/mL) or pure metabolites new environment and kept at 37°C. From this stock extract of *A. flavus* (1000 µg/mL), different dilutions were made to contain 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500, µg/mL, respectively. Serial two-fold dilutions of *A. flavus* extract were made in sterile deionized water by taking 0.0039, 0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25, and 0.5 mL, from the original stock and this equals 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500, µg/mL, respectively. The control of unhandled cells was applied without strains products.

Then, the count of handled cells was determined after one day of incubation by staining these cells with crystal violet (0.1%, w/v) subsequence with rapture using 33% glacial acetic acid and examining at 590 nm using ELISA (Model: Sunrise, Tecan Inc., USA) after good combination. The readings of controlled cells were pointed as 100% proliferation (23, 24). The rate of cell efficiency was measured by the subsequent Formula 2:

$$\text{Cell efficiency (\%)} = 1 - \frac{\text{ODt}}{\text{ODc}} \times 100 \quad (2)$$

Whereas, ODt is the medium optical density of the strain product handled wells and ODc is the optical density medium of the control sample (Abdel-Shafi et al., 2020).

2.10 Gas chromatography and mass spectrometry analysis of the most active *A. flavus* extract

The stock extract of *A. flavus* (200 µg/mL) was dissolved in DMSO. To determine and identify the bioactive compounds of *A. flavus* extract, Gas Chromatography–Mass Spectroscopic (GC-MS) was used (Trace GC 1310-ISQ Mass Spectrometer, Thermo Scientific, Austin, TX, USA) at Department of fungi, Al-Azhar University, Egypt. A direct capillary column TG–5MS (30 m × 0.25 mm × 0.25 µm film thickness) was used. About 3 µL of *A. flavus* extract was injected automatically to the equipment using Auto sampler AS3000 coupled with GC in the split less mode. The procedure was integrate canicular line (DB1 J&W; 30 m length; 0.25 mm Inner diameter; 1.5 µm film thickness), that synthetically fixed dimethyl polysiloxane. The procedure was adjusted at 40 °C (1 min) then elevated to 250 °C (2 min) at a rate of 5 °C/min and then elevated to 310 °C (2 min) at a rate of 5 °C/min. The temperature for the detector and the injector was set at 300 °C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database (Enan et al., 2020).

2.11 Statistical analyses

The data obtained were conducted in triplicates and results were expressed using subjected to one-way ANOVA analysis for estimating means and standard deviations (± SD). Post hoc: Duncan's multiple comparisons, to determine the significant variations among the various parameters in the experimental groups. All of the statistical analyses were performed using SPSS version 14 (SPSS, Chicago, IL, USA). A P value of < 0.05 was considered statistically significant.

3 Results

The obtained data in Table 1 emphasized that preliminary identification of the ten fungal isolates, by international Reference Key. The results confirmed the presence of different significant antioxidant activities in the most cases between the different tested fungi as shown in Table 1 and Figure 1. The antioxidant activity of the extracellular extracts are higher than the intracellular extracts of the fungal isolates. Whereas, the antioxidant activity of intracellular extracts was recorded only in *A. flavus*, *Trichoderma sp.*, *A. terrus*, *A. oryza* and *A. flavus* at % 61.5 ± 1.15, 49.6 ± 0.55, 21.1 ± 0.55, 19.2 ± 0.60 and 15.9 ± 0.56, respectively. On the

Table 1. Fungal secondary metabolites separated on TLC and its antioxidant activities of conc. (50 µg/mL).

No.	Fungal isolates	DPPH scavenging %		Flavonoid content
		Intra.	Extra.	Flavonoid content (µg/mg quercetin)
1	<i>Aspergillus flavus</i>	61.5 ± 1.15	81.4 ± 0.60	469 ± 1.25
2	<i>Aspergillus fumigatus</i>	-	48.9 ± 1.19	367 ± 1.22
3	<i>Aspergillus terreus</i>	21.1 ± 0.55	68.2 ± 1.19	332 ± 1.2
4	<i>Penicillium chrysogenum</i> .	-	22.9 ± 1.31	182 ± 1.19
5	<i>Fusarium moniliform</i>	-	17.6 ± 0.52	134 ± 0.85
6	<i>Fusarium oxysporium</i>	-	11.8 ± 0.45	129 ± 0.85
7	<i>Penicillium citrinum</i> .	-	21.6 ± 0.16	178 ± 1.19
8	<i>Aspergillus flavups</i>	15.9 ± 0.56	33.3 ± 0.76	166 ± 0.65
9	<i>Trichoderma sp</i>	49.6 ± 0.55	79.1 ± 0.56	221 ± 0.45
10	<i>Aspergillus oryza</i>	19.2 ± 0.60	42.5 ± 0.45	218 ± 0.45

Figure 1. Separation of extracellular secondary metabolites on TLC plate under short wavelength UV light. Where, the numbers from 1 to 10 are the numbers of fungi in the Table 1.

other hand, the antioxidant activity of extracellular extracts was recorded in all tested fungal isolates. However, weak antioxidant action was examined in extracellular outputs % (22.9 ± 1.31 , 21.6 ± 0.16 , 17.6 ± 0.52 and 11.8 ± 0.45) of fungal isolates, *Penicillium chrysogenum*, *Penicillium citrinum*, *F. moniliform* and *F. oxysporium*, respectively. On the other hand, marked antioxidant action was investigated in the extracellular outputs % (48.9 ± 1.19 , 42.5 ± 0.45 and 33.3 ± 0.76) of fungal isolates *A. fumigatus*, *A. flavus* and *A. oryza*, respectively. In addition, strong antioxidant activity was detected in the extracellular extracts % (81.4 ± 0.60 , 79.1 ± 0.56 and 68.2 ± 1.19) of fungal strains, *A. flavus*, *Trichoderma sp* and *A. terreus*, respectively. The data presented in Table 1 showed a different flavonoid contents between the various tested fungi. Whereas, the most potent antioxidant activity is presented with *A. flavus* extract using DPPH at % 81.4 ± 0.60 with flavonoid concentration at % 469 ± 1.25 µg/mg compared the other tested fungal isolates.

From Table 1 and Figure 1, it was shown that fungal secondary metabolites constitute from a huge array of low molecular weight natural products. In addition, the presence of a wide range of chemical diversity after application on TLC silica gel plate, Figure 1. Whereas, methanol and chloroform were mandatory

used as they were the best two solvents for extraction of the secondary metabolites from fungi compared with other solvents used in the extraction (data unpublished). Extractability of the tested solvents was arranged in following descending manner: chloroform and methanol > diethyl ether > ethyl acetate > benzene > petroleum ether > hexane > Acetonitrile > toluene. So, the developing solvent system (chloroform and methanol) in the ratio 9:1 v/v optimized for separation of the secondary metabolites into individual metabolites.

The *A. flavus* extract showed an antioxidant activity under these experimental conditions with $IC_{50} = 5$ µg/mL as shown in Table 2. In addition, the IC_{50} of a significant flavonoid activity of the most potent *A. flavus* extract was evaluated at 5 µg/mL (Table 2). Moreover, the sample of ascorbic acid reference standard showed the most powerful natural antioxidant activity under these experimental conditions at % 92.48 ± 2.48 with $IC_{50} = 5$ µg/mL as shown in the Table 3.

From the data given in Table 4, high significant antibacterial activity was noted against some the tested pathogenic bacteria. The extract of *A. flavus* showed the most significant growth inhibition against *Streptococcus pyogenes* and *Pseudomonas aeruginosa*. On the other hand, there was a weak significant growth inhibition against *Staphylococcus aureus* and *E. coli* compared with Gentamycin as a control.

The results obtained from Table 5 confirmed that there is a high significant antifungal activity of *A. flavus* extract against *F. oxysporium* followed by *Candida albicans*. Whereas, the inhibition zone that formed by *A. flavus* extract against *F. oxysporium* is more than that formed by applying the ketoconazole as a control (Table 5).

Inhibitory activity of metabolites extract of *A. flavus* against hepatocellular carcinoma cells was detected under these experimental conditions with $IC_{50} = 109 \pm 3.6$ µg/mL. as shown in Table 6 and Figure 2. Moreover, there is gradual decreasing of tumor cells when the concentration of fungal extract increased as shown in Figure 3a, b, c.

The GC/MS analysis of extracts of *A. flavus* culture filtrate gave us twenty six major compounds with potent antioxidant, antimicrobial and anticancer activities as shown in Figure 4 and Table 7. The bioactive substances were categorized in the following groups viz. flavonoids, phenols, alkaloids, terpenoids,

Table 2. IC₅₀ of antioxidant activity and flavonoid content of *A. flavus* extract.

Sample conc. (µg/mL)	DPPH scavenging %	Flavonoid content (µg/mg quercetin)
640	77.44 ^a ± 7.44	121 ^a ± 1.21
320	64.33 ^b ± 4.33	116 ^b ± 1.12
160	53.11 ^c ± 3.11	101 ^c ± 1.0
80	42.22 ^d ± 1.89	78 ^d ± 1.0
40	36.56 ^d ± 6.00	53 ^e ± 0.85
20	18.33 ^e ± 3.33	33 ^f ± 0.85
10	9.33 ^f ± 0.33	16 ^g ± 1.19
5	7.59 ^f ± 1.59	11 ^g ± 0.65
0	0 ± 0.00	0 ± 0.00

Values with different superscripts within rows are significantly different ($P < 0.05$).

Table 3. IC₅₀ of antioxidant activity of ascorbic acid Reference standard.

Sample conc. (µg/mL)	DPPH scavenging %
40	92.48 ^a ± 2.48
35	87.53 ^b ± 5.00
30	80.65 ^c ± 3.00
25	77.41 ^c ± 2.00
20	70.94 ^d ± 0.94
15	54.86 ^e ± 3.00
10	17.49 ^f ± 0.49
5	11.78 ^g ± 1.78
0	0 ^h ± 0.00

Values with different superscripts within rows are significantly different ($P < 0.05$).

Table 4. Antibacterial activity of *A. flavus* extract.

Tested bacteria	Sample code	Control Gentamycin
Gram Positive bacteria	Inhibition zone (mm)	
<i>Staphylococcus aureus</i> (DSM1104)	16 ^b ± 2.00	23
<i>Streptococcus pyogenes</i> Atcc19615	19 ^{ab} ± 3.00	25
Gram negative bacteria		
<i>Pseudomonas aeruginosa</i> LMG8629	24 ^a ± 4.00	16
<i>Escherichia coli</i> (LMG8223)	20 ^{ab} ± 1.00	29

The test was done using the disc diffusion agar technique, well diameter: 6.0 mm (100 µ was tested), The sample was tested T 200 µg/mL concentration. Values with different superscripts within rows are significantly different ($P < 0.05$).

Table 5. Antifungal activity of *A. flavus* extract.

Tested fungi	Sample Inhibition Zone(mm)	Control
<i>Fusarium oxysporum</i>	28 ^a ± 2.00	25
<i>Candida albicans</i>	15 ^b ± 3.00	20

The test was done using the agar disc diffusion technique diameter: 6.0 mm (100 µ was tested), The sample was tested T 200 µg/mL concentration. Values with different superscripts within rows are significantly different ($P < 0.05$).

Table 6. Evaluation of cytotoxicity of *A. flavus* extract against HepG-2 cell line.

Sample conc. (µg/mL)	Viability %	Inhibitory %	S.D. (±)
500	14.69	85.31	1.82
250	26.71	73.29	0.97
125	39.65	60.35	3.41
62.5	79.21	20.79	2.78
31.25	93.82	6.18	0.96
15.6	99.43	0.57	0.43
7.8	100	0	
3.9	100	0	
0	100	0	

All experiments were carried out in triplicate. S.D. (±) for the cell cytotoxic effect; three wells were used for each concentration of the test sample.

Figure 2. Evaluation of cytotoxicity against HepG-2 cell line; Inhibitory activity against Hepatocellular carcinoma cells was detected under these experimental conditions with IC₅₀ = 109 ± 3.6 µg/mL.

alchole, sterioids, terpinoids and silane. In addition, the presence of some compounds that determined herein by Gc.mass analysis as Carophylla and Tricyclo-carboxylic acid. Also, the presence of ether and pyrazole compounds in *A. flavus* extract plays major roles as antioxidant and antimicrobial activities. Moreover, the present results confirmed the presence of other bioactive compounds as Spirosten, Naphthalene, Trinolabdan and hexadecene that characterized by their antiproliferative activity.

4 Discussion

Natural sources have recently shown a promising interest in treating cancer or inhibiting multidrug pathogenic bacteria as a treatment (Abdel-Shafi et al., 2019, 2020; Enan et al., 2020; Uzma et al., 2018). As they are stable officers, they have an enticing future. In the present analysis, the biological efficacy of certain secondary metabolites as antioxidant, antimicrobial and antitumor was suggested for the filamentous fungi; this finding is in accordance with the observations of Chen et al. (2011); El-Gazzar et al., (2021); El-Bahr et al. (2021). In addition, the present investigation found that *A. flavus* was the most activate fungus similar with that reported with the observations of Tenguria & Khan's (2011).

Figure 3. HepG cells treated with *A. flavus* extract at 10µg (A); at 100µg (B); at 500 µg (C).

Figure 4. GC-Mass of *A. flavus* extract.

The characterization of the secondary products of *A. flavus* in this study confirmed the presence of the bioactive compounds similar to other fungal organisms that reported in the previous observations of Frisvad et al. (2008). All metabolites of diverse fungi in the present study showed an antioxidant activity up to varying extent and this concurs with the observations of Kumaresan et al. (2015). In addition, the isolated fungi herein had antioxidant efficiency *in vitro*. Whereas, the estimation of the bioactive compounds from *A. flavus* confirmed the existence

of diverse natural drugs similar to that reported previously (Abubakar & Ndana, 2016). Moreover, the results reported the presence of free radical scavenging power of antioxidant components that coincided with other parallel work related to antioxidant activity by Ghasemzadeh et al. (2010). Based on the DPPH assay in the present study, it was apparent that *A. flavus* exhibited strong antioxidant potential $81.4\% \pm 0.60$ with IC_{50} at $5\mu g$ compared with the IC_{50} values ($5\mu g$) of ascorbic acid which was used as a standard antioxidant $92.48\% \pm 2.48$. Regarding to ascorbic acid, the previous studies confirmed that this acid has commonly been used as a standard antioxidant in DPPH assays, considering that ascorbic acid is one of the most powerful natural antioxidants and prominent representatives of dietary antioxidants for human (Sugiharto et al., 2015).

Fungi themselves could be sources of different compounds associated with antioxidant activities (Arora & Chandra, 2010). In the present study, there are other compounds in the *A. flavus* extract share in antioxidant, antimicrobial and cytotoxicity effects as determined by GC/MS analysis. Several studies have revealed that the antioxidant capacity in filamentous fungi was attributed mainly to their flavonoid and phenolics contents that have been determined as major contributions for the antioxidant capacity in fungi beside other bioactive compounds (Arora & Chandra, 2010).

Herein data suggested that naturalistic potent metabolites isolated from fungi act as sequential provenance for the detection of novel antiproliferative and antibacterial agents and this similar with the previous reports (Jalgaonwala et al., 2017; Tejesvi et al., 2008). Moreover, those effective products possess broad efficiency in medical fields. Thus, the presented results confirmed an exclusive activity of *A. flavus* extract against the HepG2 hepatocellular carcinoma cell line as similar to that confirmed previously by González-Menéndez et al. (2018).

In this connection, the antiproliferative ability of the selected fungal isolate in the present study showed that the fungal secondary extract has antitumor activity due to the presence of bioactive natural compounds. Similarly with the observations of Strobel (2003) who reported the production of diverse forms of bio-effective secondary products combined with most potent sides of flavonoids, phenols, alkaloids, terpenoids and others with their different biological activities.

Gc.Mass analysis in the present study confirmed the presence of some compounds in *A. flavus* metabolites as alchole, phenol, steriods, terpinoids, silane and others that characterized by their antioxidant, antimicrobial and anticancer activities; this concurs with other previous studies (Verma, 2006; Mou et al., 2013; Kim et al., 2015; Govindappa et al., 2017; Tamil et al., 2017; Karakuş et al., 2018; Al-Romaizan et al., 2019; Purnamasari et al., 2019; Al-Mohammadi et al., 2020). In addition, the presence of some compounds determined herein as Carophylla and Tricyclo-carboxylic acid that reported in the previous studies by their potent anticancer activities (Verma, 2006; Jelena & Maja, 2015; Al-Romaizan et al., 2019). In this regarding, presence of ether and pyrazole compounds in *A. flavus* extract play major roles as antioxidant and antimicrobial activities; this concurs with that reported in the previous studies (Jelena & Maja, 2015; Srivastava et al., 2015; Narra et al., 2017; Yadav et al., 2018).

Table 7. Chemical composition of 26 components from *A. flavus* extract when subjected to GC-MS (gas liquid chromatographic mass spectrometry).

No.	Retention Time(Rt)	M.formula	M.W.	Compound name and structure	Area	Parent ion (M ⁺)	Base Peak (m/e) (100%)	Biological activity
1	5.71	C ₇ H ₁₀ O ₂	126.0	2,4-HEXADIENOIC ACID, METHYL ESTER	3.15	126.0	67.00	Anticancer& Antioxidant, Kim et al. (2015).
2	7.31	C ₁₆ H ₁₆ O	224.0	1,4-DIPHENYLBUT-3-ENE-2-OL	0.60	224.0	207.0	Anticancer& Antioxidant, Mou et al. (2013)
3	7.31	C ₁₁ H ₁₅ NO ₂ S	225.0	2-THIOPHENEETHANOL,5-(4,5-DIHYDRO-4,4-DIMETHYL-2-OXAZOLYL-	0.60	225.0	207.0	Anticancer & Antioxidant, Karakuş et al. (2018)
4	17.22	C ₁₄ H ₂₂ O	206.0	Phenol, 2,4-Bis(1,1-DIMETHYLETHYL)_	5.18	206.0	191.0	Anticancer& Antioxidant, Kim et al. (2015) Govindappa et al. (2017)
5	20.89	C ₁₅ H ₂₄ O	220.0	Caryophylla-4(2),8(13)-dien-5a-ol	1.41	220.0	136.0	Anticancer, Qsar (41); Al-Romaizan et al. (2019)
6	21.95	C ₁₈ H ₁₆ O ₇	344.0	4H-1-BENZOPYRAN-4-ONE,2-(3,4-DIMETHOXYPHENYL)-3,5-DIHYDROXY-7-METHOXY-	11.57	344.0	57.0	Antioxidant, Tamil et al. (2017);, Al-Mohammadi et al. (2020),
7	23.53	C ₂₉ H ₄₃ NO ₃ Si	481.0	ANDROSTANE-11,17-DIONE,3-[(TRIMETHYLE SILYL) OXY]-17-[O-(PHENYLMETHYL)OXIME],(3a,5a)	0.83	481.0	73.0	Anticancer & Antioxidant, Purnamasari et al. (2019) ; Jelena & Maja (2015) ; Yadav et al. (2018),
8	23.68	C ₁₂ H ₁₈ O ₂	194.0	Tricyclo[4,3,1,1(3,8)]undecane-3-carboxylic acid	0.84	194.0	149.0	Anticancer, Jelena & Maja (2015)
9	23.87	C ₂₄ H ₃₄ O ₅	402.0	1,2,3,4,4a,5,6,7,8,8a,9-Dodecahydrophenanthren-9-one,2-acetoxy-7-methylene-1,1,4a-trimethyl-8-(2-methoxycarbonylethyl)-	1.40	402.0	43.0	Anticancer & Antioxidant (Purnamasari et al., 2019)
10	24.05	C ₂₃ H ₃₂ O	324.0	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	2.18	324.0	43.0	Anticancer & Antioxidant, Karakuş et al. (2018)
11	24.61	C ₁₇ H ₃₄ O ₂	270.0	Hexadecanoic acid, methyl ester	37	270.0	74.0 & 87.0	Anticancer& Antioxidant, Narra et al. (2017),
12	25.70	C ₁₈ H ₃₆ O ₂	284.0	Pentadecanoic acid, 14 methyl-, ethyl ester	4.23	284.0	88.0	Anticancer& Antioxidant, Narra et al. (2017),
13	26.18	C ₃₂ H ₅₈ OSi	486.0	Silane, trimethyl (stigmast-5-en-3beta-yloxy)-	4.87	486.0	73.0	Antioxidant activity, Yadav et al. (2018),
14	26.18	C ₃₀ H ₅₃ NO ₄ Si	519.0	Glycine, N-[(3alpha,5beta)-24-oxo-3-[(trimethyl silyl) oxy]cholan-24-yl]-, methyl ester	2.82	519.0	131.0	Antioxidant activity, Yadav et al. (2018),
15	26.73	C ₃₀ H ₅₂ O ₂	444.0	2,6,10,15,19,23-hexamethyl-2,6,14,18,22-tetracosapentaene-10,11-diol	5.62	444.0	69.0	Antioxidant, Srivastava et al. (2015),
16	26.73	C ₂₃ H ₃₈ O ₂	346.0	6,9,12,15-Docosatetraenoic acid, methyl ester	2.81	346.0	69.0	Antioxidant (Srivastava et al. (2015),
17	27.34	C ₁₉ H ₃₆ O ₂	296.0	9-octadecenoic acid, methyl ester, (E)-	19.26	296.0	55.0	Antioxidant (Narra et al. (2017)

Table 7. Continued...

No.	Retention Time(Rt)	M.formula	M.W.	Compound name and structure	Area	Parent ion (M ⁺)	Base Peak (m/e) (100%)	Biological activity
18	27.44	C ₂₇ H ₁₀₄ O ₆	884.0	9-Octadecenoic acid,1,2,3-propanetriyl ester, (E,E,E)	1.77	884.0	55.0	Antioxidant (Srivastava et al. (2015),
19	27.64	C ₂₇ H ₄₀ O ₄	428.0	Spirost-8-en-11-one,3-hydroxy-, (3a,5a,14a,20a,22a,25R)	1.99	428.0	314.0	Anticancer, antibacterial and antioxidant (Lu et al. (2015)
20	27.74	C ₁₉ H ₃₈ O ₂	298.0	Heptadecanoic acid, 16-methyl-, methyl ester	13	298.0	74.0	Antioxidant (Naganna et al.,
21	28.34	C ₂₉ H ₅₀ O ₂	430.0	Cholestan-3-ONE,Cyclic 1,2-Ethanediyl Aetal,* (5 a)-	2.62	430.0	99.0	Anticancer & Antioxidant (Sisa et al. (2003)
22	31.28	C ₁₈ H ₂₄	240.0	Cyclohexane, hexaethylidene-(isomer 1)	2.62	240.0	196.0	Anticancer, Zheng et al. (2018); Karakuş et al. (2018),
23	31.28	C ₁₉ H ₂₄ N ₂	280.0	Curan, 16,17-didehydro-, (20.xi.)-	2.62	280.0	55.0	Anticancer (Mou et al. (2013)
24	35.36	C ₂₄ H ₃₈ O ₄	390.0	Bis(2-ETHYLHEXYL)PHTHALATE	6.68	390.0	149.0	Antioxidant Lemoreaux
25	38.25	C ₂₁ H ₁₈ O ₆	366.0	9-Carbomethyl-6,11-DIMETHOXY-5-OXO-9,10-DIHydroXANTHO[3,2-G] NAPHTHALENE	29.72	366.0	307.0	Anticancer, Zheng et al.,2018)
26	38.25	C ₁₉ H ₃₃ NO ₂	307.0	12-ACETYLAMINO-15,16,17-TRINORLABDAN-8-ONE	59.44	307.0	292.0	Anticancer and antimicrobial, El-Sayed et al. (2020), ; Popova et al. (2009),

Moreover, the present investigation confirmed the presence of other bioactive compounds as Spirosten, Naphthalene, Trinolabdan and hexadecene that characterized by their antiproliferative activity, similarly with that reported previously (Huang et al., 2003; Sisa et al., 2003; Popova et al., 2009; Lu et al., 2015; Lemoreaux, 2017; Zheng et al., 2018).

5 Conclusion

The present study confirmed the efficient role of fungal bioactive products as strong *in vitro* antioxidant, antimicrobial and antiproliferative activities. Performing the GC-MS analysis of *A. flavus* extract, with potent phytochemicals confirmed its ability to induce the antiproliferative mechanism. The results indicated that the *A. flavus* had a potent naturalistic constituent in cytotoxicity. Additional researches are in demand for purification the bioactive products which can be discussed as efficient antiproliferative drugs.

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