Ghada A. Fawzy et al /J. Pharm. Sci. & Res. Vol.3(1), 2011,980-987

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

In Vitro Antimicrobial and Anti-Tumor Activities of Intracellular and Extracellular extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris*

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Abstract

Fungi are known to produce a vast array of secondary metabolites that are gaining importance for their biotechnological applications. In the present study, the crude methanolic extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* were tested by the disc diffusion method and minimal inhibitory concentration (MIC) determination for detection of antimicrobial activity against ten species of microorganisms: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 31488, *Pseudomonas aeruginosa* ATTC 27853, *Shigella dysenteriae* ATCC 13313, *Proteus vulgaris* ATCC 6380, *Salmonella typhimurium* (LIO, locally isolated organism), *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* (LIO), *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231 and by the sulforhodamine B (SRB) assay method to test their *in vitro* anti-tumor activity. The extracellular extracts of both fungi showed their activity against Gram-negative bacteria only. The antibacterial activity of extracellular extract of *Aspergillus flavus* var. *columinaris* was higher than that of extracellular extract of *Aspergillus niger*. *Candida albicans* was sensitive to the intracellular extract of *Aspergillus flavus* var. *columinaris*. The extracellular extracts of both isolated fungi were having more cytotoxic effect than their intracellular extracts. IC50 (concentration of extract required to reduce cell survival by 50%) of extracellular extract of *Aspergillus niger* was the most reducible one (0.905 µg/ml) followed by the extracellular extract of *Aspergillus flavus* var. *columinaris* (1.48 µg/ml). The IC50 of the intracellular extracts was approximately similar among two isolates (3.50 µg/ml for *Aspergillus niger* and 3.03 µg/ml for *Aspergillus flavus* var. *columinaris*). In conclusion, the extracellular extract of *Aspergillus flavus* var. *columinaris* possesses antibacterial activity against Gram-negative bacteria. While the extracellular extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* have *in vitro* anti-tumor activity, which support their use in medicines. It is hoped that this study would lead to the establishment of some extracts that could be used to formulate new and more potent antimicrobial and anticancer drugs.

Keywords: *Aspergillus niger*, *Aspergillus flavus* var. *columinaris*, antimicrobial activity, *in vitro* anti-tumor activity.

1. INTRODUCTION

There is high demand for innovative lead structures in order to develop novel drugs for the treatment of cancer and other menacing diseases (Newman and Cragg, 2007). Resistance of tumor cells, pathogenic bacteria and viruses is steadily increasing and many established drug therapies are not effective anymore. Natural products have been shown to possess the potential to be excellent lead structures and to serve as a basis for promising therapeutic agents in the area of anticancer treatment (Koehn and Carter, 2005).

Fighting cancer is considered one of the most important areas of research in medicine and immunology. A new direction in cancer treatment has arisen, devoted to the adjuvant use of natural bioactive compounds in conventional chemotherapy. This kind of research is gaining more attention (Lu *et al*, 2004). In particular, fungi can be used not only as strong immunoceuticals but also as a source of potent metabolites, capable of penetrating cell membranes and interfering with particular signal transduction pathways linked to processes such as inflammation, cell differentiation and survival, carcinogenesis, and metastasis (Rocha *et al,* 2001).

Fungi represent an immense source of bioactive substances with immune-stimulating and anti-cancer properties that make them very potent natural supplements in cancer therapy. Thus, the activities of more and various fungal species are worth investigation in order to establish other, more potent fungal substances

that can be reliable therapeutics for humans (Hu *et al*, 2002).

Secondary metabolites are of intense interest to humans due to their pharmaceutical and/or toxic properties. Each species of Aspergillus can produce a range of secondary metabolites associated with fungal growth and development (Hicks *et al.,* 1997, Palanee *et al.,* 2001 and Calvo *et al.,* 2002). In some cases these metabolites have been implicated in disease, since they appear to be virulence factors (Moon, 1998 and Rossano *et al.,* 1999). Interestingly, many of these secondary metabolites have been used in medicine for their antiviral, antibacterial, tumor suppressing, anti—hypercholesterolemic and immunosuppressant activities (Huisman and Gray, 2002 and Pelaez, 2005).

Aspergillus is the best known and economically important genus of fungi. It belongs to the family Eurotiaceae (Moniliaceae), order Moniliales in the class Fungi Imperfecti (Deuteromycetes). About 160 species of Aspergillus have been recognized so far. They are found almost every-where on every conceivable type of substratum but are abundant in soil (Webster, 1970).

Aspergillus flavus belongs to the Genus Aspergillus (Alexopoulos *et al.*, 1996). The fungus occurs on many types of organic material in various stages of decomposition. Not all isolates of the fungus produce aflatoxins; thus, the mere presence of *Aspergillus flavus* does not mean that aflatoxins will be present in the substrate (Duncan and Hagler, 1986). Among the at least 16 structurally related aflatoxins characterized, there are some variants of *Aspergillus flavus* which produce aflatoxin B1and B2 (AFB1 and AFB2) (Bennett and Klich, 2004).

Aspergillus flavus var. *columnaris* is studied to modernize the soy sauce fermentation in Thailand in 1978 when a locally yellow-green Aspergillus strain was recommended for use in soy sauce factories because it was a high protease producer, was free of aflatoxin and other toxins, and it produced good taste and aroma in the final fermentation strain. This strain was first labeled as a strain of *Aspergillus oryzae* but was later found to conform well to the description of *Aspergillus flavus* var. *columnaris* (Kalayanamitr *et al.*, 1987). It is generally accepted that strains of *Aspergillus flavus* are variable in their morphology and aflatoxin production (Kamei and Watanabe, 2005).

Aspergillus niger is a member of the genus Aspergillus which is geographically widely distributed and is commonly found as a saprophyte growing on dead leaves, stored grain, compost piles, and other decaying vegetation (Finkelstein, 1989). The primary uses of *Aspergillus niger* are for the production of enzymes as amylase, amyloglucosidase, cellulases, lactase, invertase, pectinases, and acid proteases (Bennett, 1985a and Ward, 1989) and organic acids as citric acid and gluoconic acid by fermentation (Kubicek and Rohr, 1986). *Aspergillus niger* fermentation is "generally regarded as safe" (GRAS) by the United States Food and Drug Administration (US FDA/CFSAN. 2008). Another use for *Aspergillus niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules for NMR analysis (Mulder, 2009).

The aim of the present study is to test the antimicrobial activity and *in vitro* anti-tumor activity of intracellular and extracellular extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris*.

2. MATERIAL AND METHODS

2.1. Collection of samples and isolation of fungi

Soil samples were obtained from Riyadh deserts in April, 2009. About 15mg of soil was mixed with 15ml sterile distilled water and a series of dilutions were made. From the dilutions, 0.5ml volumes were pipetted onto potato dextrose agar (PDA) and incubated at 25ºC for three days. Fungi were isolated from the mixed isolates from each plate and subcultured on PDA. Subculturing was continued until a pure isolate was obtained. Two well identified isolates were obtained, *Aspergillus niger* and *Aspergillus flavus* var.

columinaris. Identification of fungi was based on colony features of the cultures and their morphologies under the microscope according to Rapper and Fennel (1965), Samson (1979) and Guarro *et a*l. (1999).

2.2. Fungal growth

The two fungal isolates were grown in malt extract broth (MEB), (CM0057, OXOID), at 25-27ºC for 7-10 days for collection of 50 gm mat of each isolate from 5Liters of MEB of *Aspergillus niger* and 4 Liters of MEB of *Aspergillus flavus* var. *columinaris*..

2.3. Detection of the absence of aflatoxin-producing *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* by fluorescent agar technique

The two isolates were screened for the absence of aflatoxin-production on PDA plates by using the fluorescent agar technique of Hara *et al.* (1974). The production of aflatoxins coincided with the presence of a bright blue or blue-green fluorescent area surrounding colonies when observed under long-wavelength (365 nm) UV light after 3 days of incubation at 28°C. Absence of fluorescence of agar medium under ultraviolet light reveals that, the strains are non-toxigenic (El-Shiek *et al.,* 2007).

2.4. Extraction of fungal biomass

Cultivation media and fungal isolates were separately extracted. The extracellular metabolites in the media were obtained by means of Liquid-Liquid extraction technique with ethyl acetate (2×250 mL per 300 mL media). The organic fractions were combined and the solvent removed under reduced pressure at 35°C to yield crude extracellular extracts (*Aspergillus niger* 500 mg and *Aspergillus flavus* var. *columinaris* 400 mg). The fungal isolates (50gm of each) were exhaustively extracted with methanol and the solvent removed under reduced pressure at 35°C to yield crude intracellular extracts (*Aspergillus niger* 800 mg and *Aspergillus flavus* var. *columinaris* 700 mg) (Zain *et al*, 2008). All extracts were subjected to antimicrobial and cytotoxic testing.

2.5. Test microorganisms

The following strains of pathogenic microorganisms were used for the antimicrobial assay: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 31488, *Pseudomonas aeruginosa* ATTC 27853, *Shigella dysenteriae* ATCC 13313, *Proteus vulgaris* ATCC 6380, *Salmonella typhimurium* (LIO, locally isolated organism), *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* (LIO) and *Staphylococcus aureus* ATCC 25923 (Oxoid, England). The yeast strain used in this study was *Candida albicans* ATCC 10231 (Oxoid, England). The micro-organisms were grown overnight at 37°C in Mueller-Hinton Broth (Oxoid, England) at pH 7.4. Their sensitivity to the reference antibiotics was checked using Ampicillin, Gentamicin and Amphotericin B (Sigma, USA) as positive controls. Negative controls were done using paper discs loaded with 20 µl of the solvents. The experiment was performed in triplicates.

2.6. Antimicrobial sensitivity assay

Antimicrobial susceptibility testing of the crude intracellular and extracellular methanolic extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* were determined by the Disc diffusion method (NCCLS (National Committee for Clinical Laboratory Standards), 2005). The filter paper discs (6mm in diameter) (Schleicher and Schuell, ref. no. 10321260, lot. DG0274-1) were impregnated with extract solution and transferred onto the surface of growth medium inoculated with tested organism. The inhibitory zones were determined and measured for each extract upon each tested organisms. An inhibition zone of 14 mm or greater (including diameter of the disc) was considered as high antibacterial activity (Ramzi and Ulrike, 2005).

The Minimal Inhibitory Concentration (MIC) of each extract was determined by Broth dilution methods for the sensitive organisms at a density of 10^5 CFU/ml (table 1) (Van Den Berghe and Vlietinck, 1991). The tubes were incubated at 37°C for 24 h (or 48 h for the yeast). The growth of organisms was observed as turbidity determined by a spectrophotometer (Ultrospec III, Pharmacia LKB, UK) at 620 nm. Control tubes without the tested extracts were assayed simultaneously.

2.7. Cytotoxicity testing using Sulphorhodamine-B (SRB) assay

This method was carried out according to that of Skehan et al. (1990). The sensitivity of the human tumor cell lines to thymoquinone was determined by the SRB assay. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content.

Hepatocellular cell line (HepG2) (obtained frozen in liquid nitrogen -180 ^{0}C) from the American Type Culture Collection. The tumor cell line was maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing). The cell lines were grown in monolayer cultures in RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo, U.S.A) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma Chemical Co., St. Louis, Mo, U.S.A), Penicillin/Streptomycin (Sigma Chemical Co., St. Louis, Mo, U.S.A): 100 units/ml Penicillin and 2 mg/ml Streptomycin and maintained at 37°C in a 5% CO_2/95% air atmosphere, with 95% humidity. Potential cytotoxicity of tested extracts was done using the method of Skehan and Storeng (1990). Cells were plated in 96-multiwell plate (104cell/well) for 24 hours before treatment with the tested extracts, to allow attachment of cell to the wall of the plate. Different concentrations of the tested extracts (0, 1, 2.5, 5 and 10 µg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the extracts for 48 hours at 37˚C in 5% CO_2 atmosphere. After 48 hours, cells were fixed, washed and stained with sulforhodamine B (SRB) stain (Sigma Chemical Co., St. Louis, Mo, U.S.A. 0.4 % SRB dissolved in 1 % acetic acid was used as a protein dye). Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer . Color intensity was measured in an ELISA reader (Meter tech. Σ 960, U.S.A.) at λ max 564 nm. The mean background absorbance was automatically subtracted and means values of each drug concentration was calculated. The relation between surviving fraction and extract concentration was plotted to get the survival curve of the tumor cell line after the specified tested extracts. Also, the IC50 (Dose of the extracts which reduces survival to 50%) for each tested extracts was calculated by using Graph-Pad PRISM program (Graph-Pad, UK) (Peter *et al,* 2007). The standard antitumor drug used was doxorubicin (Skehan and Storeng, 1990).

3. RESULTS

In this study, 500 mg and 400 mg crude extracellular extracts were obtained from 5 Liters of MEB of *Aspergillus niger* and 4 Liters of MEB of *Aspergillus flavus* var. *columinaris* respectively. We also extracted 800mg and 700mg of crude intracellular extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* respectively from 50gm mat extract for each strain.

3.1. Antibacterial activity of the extracts

The results of antimicrobial activity assay showed that, the two extracts possess antimicrobial activities against some of the tested organisms at a concentration of 150 mg/ml for *Aspergillus niger* and 100 mg/ml for *Aspergillus flavus* var. *columinaris*) (Table 1). The two extracts compared favourably with the standard antibiotics Ampicilin, Gentamicin and Amphotericin B.

In the agar diffusion assay, the negative control did not show any inhibition, while antibiotic control showed mean zones of inhibition ranging from 16 to 30 mm. The mean zones of inhibition against bacteria were observed more with extracellular extract of *Aspergillus flavus* var. *columinaris* than other extracts. The extracellular extract of *Aspergillus niger* showed inhibition activity against *Escherichia coli* only (20 ±0.00mm) while the extracellular extract of *Aspergillus flavus* var. *columinaris* showed the highest inhibition zone against *Escherichia coli* (34±0.017mm) followed by *Shigella dysenteriae* and *Salmonella typhimurium* (32±0.051mm and 27±0.087mm respectively). *Candida albicans* was inhibited

by the intracellular extract of *Aspergillus flavus* var. *columinaris* (17± 0.106 mm).

Table 1 showed also the results of the MIC of two tested extracts against the microorganisms which showed inhibition zone in the agar diffusion assay: *Escherichia coli* ATCC 25922, *Shigella dysenteriae* ATCC 13313*, Salmonella typhimurium* (LIO)*,* and *Candida albicans* ATCC 10231. For *Escherichia coli*, the extracellular extract of *Aspergillus flavus* var. *columinaris* showed more sensitive MIC than the extracellular extract of *Aspergillus niger* (6250 µg/ml and 9375 µg/ml, respectively). *Salmonella typhimurium* showed the same sensitivity as *Escherichia coli* to the extracellular extract of *Aspergillus flavus* var. *columinaris* (6250 µg/ml) while *Shigella dysenteriae* showed more sensitivity than both microorganisms (3125 µg/ml). For antifungal activity, the intracellular extract of *Aspergillus flavus* var. *columinaris* showed MIC at 1563 µg/ml against *Candida albicans*.

3.2. In vitro anti-tumor activity of the extracts

The SRB assay is sensitive, simple and more rapid reproducible method. It relies on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B (SRB) by basic amino acids in the cells. The greater the number of cells, the greater amount of dye is taken up and, after fixation, when the cells are lysed, the released dye will give a more intense colour and greater absorbance (Peter *et al,* 2007).

Table (1): Antimicrobial sensitivity assays of tested extracts (150 mg/ml for *Aspergillus niger* and 100 mg/ml for *Aspergillus flavus* var. *columinaris*) comparing to reference standards

Micro-organism	Inhibition zone (mm) [a] & MIC (µg/ml)										
	Aspergillus niger				Aspergillus flavus var. columinaris				Gentamicin (10mg /disc)	Ampicilin (10mg/ disc)	Amphotericin B (20µ/ disc)
	Intracellular extract		Extracellular extract		Intracellular extract		Extracellular extract				
	Inhibition zone	MIC	Inhibition zone	MIC	Inhibition zone	MIC	Inhibition zone	MIC			
Pseudomonas aeruginosa	0	-	0	-	0	-	0	-	18	0	-
Escherichia coli	11±0.029	-	20 ±0.00	9375	13±0.011	-	34±0.017	6250	20	0	-
Klebsiella pneumoniae	0	-	0	-	0	-	0	-	0	0	-
Proteus vulgaris	8±0.036	-	7±0.037	-	10±0.045	-	12±0.023	-	30	24	-
Salmonella typhimurium	9±0.032	-	7±0.036	-	12±0.342	-	27±0.087	6250	27	19	-
Shigella dysenteriae	8±0.000	-	8±0.00	-	13±0.087	-	32±0.051	3125	25	23	-
Staphylococcus aureus	5±0.046	-	4±0.035	-	0	-	0	-	22	0	-
Enterococcus faecalis	9±0.033	-	10±0.267	-	6±0.044	-	7±0.032	-	16	19	-
Bacillus cereus	13±0.027	-	12±0.342	-	10±0.134	-	10±0.271	-	19	0	-
Candida albicans	7±0.000	-	8±0.00	-	17±0.106	1563	12±0.023	-	-	-	19

(mm) [a] = Mean of three replicates (±) standard deviation

0 = Resistant

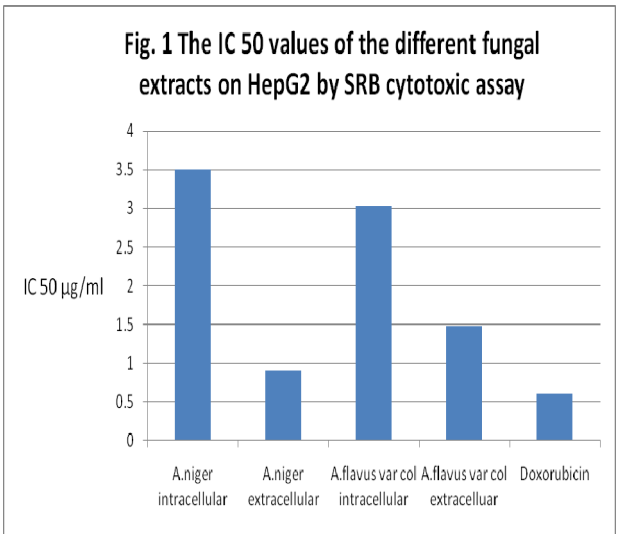
- = not determined

Table (2): Potential cytotoxicity on HepG2 of the extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* by SRB assay

Mean of surviving fraction ± S.D[a].						
Tumor cell line	***Compound Conc. µg/ml***	***Aspergillus niger***		***Aspergillus flavus var. oluminaris***		***Doxorubicin***[b]
		Intracellular	Extracellular	Intracellular	Extracellular	
HepG2	0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000
	1.000	0.847±0.115 [d]	0.462±0.074	0.795±0.102 [d]	0.631±0.122 [d]	0.347 ± 0.117
	2.500	0.597±0.149 [d]	0.422±0.061	0.609±0.168 [d]	0.353±0.089	0.350 ± 0.136
	5.000	0.358±0.117	0.279±0.051	0.338±0.099	0.348±0.108	0.359 ± 0.124
	10.000	0.361±0.136	0.255±0.048	0.354±0.117	0.311±0.081	0.345 ± 0.115
IC 50[c]		3.50 µg/ml	0.905 µg/ml	3.03 µg/ml	1.48 µg/ml	0.60 µg/ml

[a] Mean of surviving fraction ± S.D: Mean of three assays ± standard deviation.
[b] Doxorubicin: standard antitumor drug.
[c] IC 50: concentration of extract required to reduce cell survival by 50%.
[d] Significantly different from standard drug at $P < 0.05$, using student T-test.

Color intensity was measured in an ELISA reader and the IC50 for each tested extracts was calculated.The extracellular extracts of both isolated fungi were having more cytotoxic effect than the intracellular extracts of them (Table 2). IC50 of extracellular extract of *Aspergillus niger* was the most reducible one (0.905 µg/ml) followed by the extracellular extract of *Aspergillus flavus* var. *columinaris* (1.48 µg/ml). The IC50 of the intracellular extracts was approximately similar among the two isolates (3.50 µg/ml for *Aspergillus niger* and 3.03 µg/ml for *Aspergillus flavus* var. *columinaris*).These results are illustrated in Figure 1.



4. DISCUSSION

There were no published reports dealing with bioactivity of extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris*. No information was known about their antimicrobial and cytotoxic activity prior to our investigation.

This study reports the antimicrobial activity of four extracts from two isolated soil fungi against ten different bacterial isolates comprising of both Gram negative and Gram positive organisms. In general, the extracellular extracts of both fungi showed their activity against Gram-negative bacteria only while the intracellular extracts did not show any antibacterial activity. The antibacterial activity of extracellular extract of *Aspergillus flavus* var. *columinaris* was higher than that of extracellular extract of *Aspergillus niger*. *Candida albicans* was sensitive to the intracellular extract of *Aspergillus flavus* var. *columinaris* only.

Aspergillus niger extract exhibits a potent activity against *Escherichia coli* while, *Aspergillus flavus* var. *columinaris* extract exhibits more degree of antibacterial activity towards *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteriae* and *Candida albicans*. Thus, it shows that some of the soil fungi may be used in medicine as potentially effective antimicrobial agents. Investigation of

the antimicrobial compounds in *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* is now underway. The resulting information will contribute to a better understanding of the antimicrobial activity of the fungi.

In this study, regarding to the cytotoxic activity of four fungal extracts, both intracellular extracts exhibited nearly similar cytotoxic effect on HepG2 cell-line, with IC50, 3.50 and 3.03 µg/ml for *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* respectively. While, a significant cytotoxic activity was shown by the extracellular extract of *Aspergillus niger* (IC50 was 0.905 µg/ml) ($P < 0.05$), followed by that of *Aspergillus flavus* var. *columinaris* (1.48 µg/ml) when compared with the standard antitumor drug (Doxorubicin) (0.60 µg/ml).

Our study is similar to other studies, (Johannes *et al* 2009, Preecha *et al* 2006, Ning *et al* 2003, Ranieri and Calton 1978, Glasby 1976, Beard and Walton, 1969, and Saito 1907) aimed to test the antimicrobial and anticancer effects of some soil fungi extracts, but differ from them in the type of isolated fungi. In the study of Zain *et al,* 2008, they studied the antifungal activity of intra- and extra-cellular metabolites of *Aspergillus terreus* cultured on two types of media against some unicellular and filamentous fungi and they concluded that the extracts of *Aspergillus terreus* that were cultured on yeast extract sucrose medium remarkably inhibited the growth of *Aspergillus fumigatus.*

In conclusion, the extracellular extract of *Aspergillus flavus* var. *columinaris* possesses antibacterial activity against Gram-negative bacteria and does not show any activity against Gram-positive bacteria. While the extracellular extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris* have *in vitro* anti-tumor activity, which support their use in medicines.

It is hoped that this study would lead to the establishment of some extracts that could be used to formulate new and more potent antimicrobial drugs. Further studies must be in progress to further evaluate the mechanisms of action of these extracts on some organisms associated with human diseases.

ACKNOWLEDGMENT

This research was supported by a research fund from Deanship of Scientific Research, Research Center for female scientific and medical college, King Saud University.

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