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Research Article

Effect of Changing Culture Media on Metabolites of Endophytic Fungi from *Halocnemum strobilaceum*

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ABSTRACT

Halocnemum strobilaceum halophyte occupies tidal and coastal environments in Egypt. Endophytes have the potential to yield novel and important natural products. Herein we evaluate the effect of changing media on the production of secondary metabolites by *Halocnemum strobilaceum* endophytes. Endophytic fungal ethyl acetate extracts of different culturing media; corn "*Zea mays*" (CGA), rice "*Oryza sativa*" media (M1), cowpea "*Vigna unguiculata*" (M2), painted pony "*Phaseolus vulgaris*" (M3) and broad bean "*Vicia faba*" (M4), were screened to evaluate their cytotoxic and antimicrobial activities. Media showing the highest antibacterial activities of fungal metabolites were rice culture media with *Penicillium citrinum* E-346 and broad bean media *with Aspergillus flavus* RF-03 with MIC (Minimum inhibitory concentration) value of 0.94 µg/mL for both against *Bacillus subtilis* (ATCC-6633). No antifungal activities against *Candida albicans* or *Aspergillus niger* were noticed with any of the tested fungal strains. The cytotoxicity assays using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method against the breast cancer cell line (MCF-7) manifested a good safety index with all media. This evaluation aims to select the best growth conditions for the isolated endophytic fungi to direct further studies toward the best media for chemical investigation of their antimicrobial metabolites.

Keywords: *optimization of media; antimicrobial activity; cytotoxicity; safety index; Halocnemum endophytes.*

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1. INTRODUCTION

World Health Organization (WHO) estimates that 70 million annual deaths occur due to the rise of microbial multidrug resistance **[1]**. Moreover, From the national perspective, breast cancer is progressively increased in Egypt in the last few years **[2, 3]**. These global health concerns require immediate action by finding purified bioactive metabolites. Fungi, the creative producers of secondary metabolites, produce bioactive and chemically novel compounds with huge medicinal impact; particularly, endophytes associated with halophytes as they are exposed to

harsh growing habitat; thus, enhancing and gearing their genes towards the production of unique natural molecules capable of protecting them against salinity and competitor organisms **[4]**. From this aspect, halophytes-associated fungi were chosen to explore their innovative power to yield antimicrobial new molecules **[5]**.

Screening *in-vitro* cytotoxicity and viability on cellular parameters using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as a fast, cheap, and highly sensitive method **[6]**. Breast cancer cell line (MCF-7) retained mammary epithelium characteristics useful for experimental therapeutics to determine the cytotoxicity or safety of drugs **[7, 8]**.

Halocnemum strobilaceum a wild halophytic plant growing wild in Egypt and reported as a digestive, stimulant as well as a cure for fever and headache in Algeria and Iran folk medicine. Even though some studies revealed the chemical and biological prospects of the aerial part of this terrestrial halophytic plant **[9]**. The aerial part of *H. strobilaceum* revealed interesting phytochemicals compounds such as flavonoids; quercetin 3-O-β-D-glucopyranoside, isorhamnetin 3-O-β-D-glucopyranoside, isorhamnetin, rhamnazin, chrysoeriol, luteolin 7-O-galactoside, quercetin 7-O-rhamnoside, and luteolin. Moreover, coumarins such as 7-hydroxy-3-methylcoumarin, oreoselone, heraclenin, and scopoletin. Besides of caffeic acid esters were identified and isolated from aerial part **[10–15]**.

Changing the media parameters of marine-derived fungi increased the number of novel bioactive metabolites available from a single fungal strain; for instance, rice solid media culture was reported as the best culture media for marine-derived fungi **[16, 17]**. Through the course of our project on halophytes, *Halocnemum* associated fungi revealed the production of bioactive metabolites that prompt this further step of media refinement. Microbial growth, influenced and thrived by extreme environmental conditions, adaptations, and physical factors, affects the secondary metabolite production from different biosynthetic pathways. In the same vein, culturing media can impact the types of chemical molecules and direct their metabolism to certain biological activities, yet there are no optimum media for optimal fungal growth known to date, and factors such as carbon, trace elements, nitrogen, and phosphate sources serve to induce or repress enzymes and controls auto regulators inside fungal cells **[18]**. Because natural products remain the most imperative source of drugs in the face of combinatorial chemistry, in the present study we aim to find the best culturing conditions for the production of active antimicrobial nor cytotoxic secondary metabolites of endophytic fungi namely, *Penicillium citrinum* and *Aspergillus flavus*, to select and utilize the directed bioactive chemical diversity for further chemical and biological investigations.

2. MATERIALS AND METHODS

All chemicals, solvents, and reagents used were of analytical or High-performance liquid chromatography (HPLC) grades.

2.1. Fungi Material

Aspergillus flavus RF-03 (H.S. L1.1) and *Penicillium citrinum* E-346 (H.S. St.35B) endophytic fungi were isolated from *Halocnemum strobilaceum* halophyte in our ongoing project and chosen for this study. The two fungal strains were identified using morphological and molecular identification using DNA it's sequencing.

2.2. Preparation and extraction of endophytic fungi metabolites cultured on different media

Fresh fungal cultures were transferred into 1 flask (1L), each containing 100 g of different nutrient sources, corn "*Zea mays*" (CGA), rice

"*Oryza sativa*" (M1), cowpea "*Vigna unguiculata*" (M2), painted pony "*Phaseolus vulgaris*" (M3) and broad bean "*Vicia faba*" (M4), 6 gm agar-agar media and 50 ml distilled water for solid cultures. Cultures were incubated at room temperature for 21 days in the dark. Ethyl acetate (250 mL) was added to extract the fungal metabolites of each of the 1 L culture flask and left overnight for maceration. Culture media were cut into small pieces to increase the extraction surface and shacked in a water bath shaker at 80 rpm for 3 days at 40 °C, filtered and extracted twice till exhaustion. The ethyl acetate extract was concentrated under vacuum (HeidolphValve-Regulated Vacuum Pumps - Rotavac) at temperature 45 °C of the rotation speed of 120 rpm, the vacuum pressure of 200 mbar **[19–21]**.

2.3. Antimicrobial activity screening using agar well diffusion method

Standard tested microorganisms were obtained from the Fermentation Biotechnology & Applied Microbiology Center (FBAMC), Azhar University, Egypt. The antimicrobial activity was carried out using the agar well diffusion method with 10 mm well diameter enclosing 100 µL of each tested sample dissolved in dimethyl sulfoxide (DMSO); Samples were oily resin with no solvent residue and were dissolved directly in DMSO, with a concentration of 30 mg/mL for sample extracts. Samples activities were tested against; Gram-positive bacterial strains, *Bacillus subtilis* (ATCC-6633), and *Staphylococcus aureus* (ATCC–6538); Gram-negative bacterial strains, *Pseudomonas aeruginosa* (ATCC-9027), and *Escherichia coli* (ATCC-8739); and fungal strains, *Candida albicans* (ATCC-90028), and *Aspergillus niger* (ATTC-7966) **[22, 23]**. The results were reported in terms of inhibition zones around the well in the agar well diffusion method. The minimal inhibitory concentrations (MIC) were determined against Gram-positive bacterial strains, *B. subtilis* (ATCC-6633), and *S. aureus* (ATCC–6538); Gram-negative bacterial strains, *P. aeruginosa* (ATCC-9027), and *E. coli* (ATCC-8739) were compared to the reference antibiotic, chloramphenicol, using a serial dilution of 0.9, 1.8, 3.75, 7.5, 15, 30 µg/mL for *B. subtilis* and *S. aureus*. Ciprofloxacin was the control for *P. aeruginosa*, and cephalexin was the *E. coli* control **[24–26]**.

2.4. Cytotoxic activity screening using MTT assay

The breast cancer cell line (MCF-7) was provided from the Fermentation Biotechnology & Applied Microbiology Center (FBAMC), Azhar University, Egypt. Cells were added to a 96-well plate with a concentration of 1 X 10^5 cells/mL (100 µL/well) and incubated at 37 °C for 24 h in presence of 5% CO_2 to be treated with different samples, using doxorubicin as a control. Roswell Park Memorial Institute (RPMI) medium with 2% serum, as a maintenance medium, was added to the wells of each tested plate. Subsequently, eight serial dilutions were prepared from each sample of 78.125, 156.25, 312.5, 625, 1250, 2500, 5000, 10000 µg/mL, and 0.1 mL of each dilution was added to 93 wells leaving three wells as the control on each plate before incubation was conducted at 37 °C. Each sample's effect was observed on each cell line after incubation periods of 24 h. Cells were observed for any physical signs of toxicity such as partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. 20 µL MTT solution was added to each well using a shaking table at 150 rpm for 5 min to mix the MTT into the media. Furthermore, cells were incubated at 37 °C with 5% CO_2 for 1-5 h to allow the MTT to be metabolized. After rinsing the media, formazan dye is resuspended and dissolved in 200 µL of DMSO using a shaking table at 150 rpm for 5 min to add it to all the wells. Optical density was measured using a spectrophotometer at 560 nm and the background is subtracted at 620 nm **[27, 28]**.

% cytotoxicity was calculated using the equation:

% cytotoxicity =100- (100 × (sample absorbance)/ (control absorbance)) **[29]**.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial Activity

The control antibiotics showed different activities on the tested microorganism such as chloramphenicol for *B. subtilis* and *S. aureus*, which showed inhibition zones of 35 mm, 33.5 mm, and MIC of 7.81 and 62.5 µg/mL respectively. While ciprofloxacin was used as a control for *P. aeruginosa* with 39mm inhibition zone and MIC of 7.81 µg/mL, cefalexin was used for *E. coli* and revealed an inhibition zone of 24 mm and MIC of 125 µg/mL. The results of the inhibition zones equal to 10 mm were considered negative. *Penicillium* sp. cultured on M1, M3, and M4 showed the best inhibition zones of 30.5, 25, and 25 mm and favorable MIC values of 0.94, 1.88, and 0.94 µg/mL against *B. subtilis.* Regarding *S. aureus,* M1 media manifested an inhibition zone of 30 mm and MIC of 15 µg/mL due to the presence of antibacterial agents. *P. aeruginosa* inhibition zones were finest with M4 media of 30 mm with MIC of 15 µg/mL **(Table 1& 2, Fig. 1 & 4)**. However, with *E. coli all* inhibition zones were very comparable, and 15 mm revealed the best microbial inhibition zone. *Penicillium* sp. cultures on M1 *and* M3 showed the greatest potential for the production of antibacterial activity on Gram-positive bacteria, *B. subtilis* (ATCC-6633) and *S. aureus* (ATCC-6538), with MIC values of 0.94 and 15 µg/mL, respectively, while its effect on the Gram-negative bacteria, *P. aeruginosa* (ATCC-9027) and *E. coli* (ATCC -8739), was 15 µg/mL for both. The evaluation of *Penicillium* sp. ethyl acetate extracts showed that M1 is the media of choice for the production of Gram-positive and Gram-negative antibacterial metabolites. *Aspergillus* sp. cultured on M1 and M4 indicated inhibitory zones of 25 and 30 mm and MIC values of 1.88 and 0.94 µg/mL on M2 and M4 against *B. subtilis*. Concerning *S. aureus,* only M4 was effective as well as M1 and M4 with *P. aeruginosa and E.coli* **(Tables 1 & 2, Fig. 1)**. None of the two endophytic cultures demonstrated antifungal activity against *C. albicans* or *A. niger*. M4 showed the greatest potential for producing antibacterial activity against Gram-positive bacteria *B. subtilis* (ATCC-6633) and *S. aureus* (ATCC-6538) with MIC of 0.94 and 30 µg/mL. Moreover, Gram-negative bacteria, *P. aeruginosa* (ATCC-9027), and *E. coli* (ATCC-8739) manifested MIC of 3.75 µg/mL for both. The antibacterial screening showed that M4 is the media of choice for the production of potent antibacterial metabolites of *Aspergillus* sp. While endophytic fungi use low concentrations of host plant carbohydrates more efficiently, higher concentrations slowed their growth **[30]**. Media with the least sugar concentrations were reported to stimulate secondary metabolite production **[31]**. By analyzing the glucose and amino acid content in the utilized media, it was evident that M1 has the highest carbohydrate carbon source of 79.34 g % followed by CGA, 74.26 g %. M2 shows the highest amount of proteins, 26.12 g %, followed by M3 and M4 whose percentage of amino acids recorded 22.33 and 23.85 g %. The least amount of proteins was shown in CGA and M1 media of 9.42 and 6.61 g % respectively. Also, total minerals were the highest in M4, 2.31 g %, and total fats reported the greatest quantity in GCA, 4.74 g %. Consequently, M1 and M4 that revealed the best microbicidal activities were the richest in carbon and minerals, a factor that should be considered in future studies formulating fungal media for achieving the best results in fungal extract yield and bioactivity **(Table 4)**.

Table 1. Results of antimicrobial screening activity in term of zone of inhibition in mm of fungal extracts cultured on different media

Media	Antibacterial												Antifungal					
	B. subtilis			*S. aureus*			*P. aeruginosa*			*E. coli*			*C. albicans*			*A. niger*		
	AE	***PE***	***C***	***AE***	***PE***	***C1***	***AE***	***PE***	***C***	***AE***	***PE***	***C***	***AE***	***PE***	***C***	***AE***	***PE***	***C***
CGA	16	19		10	13		14	14		11	10		10	10		10	10	
M1	25	30.5		10	30		26.5	18		20	14		10	10		10	10	
M2	19	20	33.5	10	10	35	21	16	39	14	13	24	10	10	n.d.	10	10	n.d.
M3	18	25		10	15		21	29		19	15		10	10		10	10	
M4	30	25		20	15		32	30		21	11		10	10		10	10	

AE, *Aspergillus* sp. ethyl acetate extract; PE, *Penicillium* sp. ethyl acetate extract; C, Control Chloramphenicol Control for *B. subtilis* and *S. aureus*, Ciprofloxacin control for *P. aeruginosa*, Cefalexin control for *E. coli.;* n.d., Not done. The well diameter is 10 mm therefore results of 10 mm inhibition zone is negative result. CGA, Corn "*Zea mays*" girt agar media; M1, Rice "*Oryza sativa*" media; M2, Cowpea "*Vigna unguiculata*" media; M3, Painted Pony "*Phaseolus vulgaris*" media; M4, Broad bean "*Vicia faba*" media.

Table 2. Results of antimicrobial activity of Minimum inhibitory concentration (MIC) of fungal extracts cultured on different media in µg/mL

Media	***B. subtilis***			***S. aureus***			***P. aeruginosa***			***E. coli***		
	AE	*PE*	*C*	*AE*	*PE*	*C*	*AE*	*PE*	*C*	*AE*	*PE*	*C*
CGA	3.75	3.75		> 30	> 30		30	30		30	> 30	
M1	3.75	0.94		> 30	15		3.75	15		3.75	15	
M2	1.88	1.88	7.81	> 30	30	62.5	15	30	7.81	15	15	125
M3	3.75	0.94		> 30	30		15	15		15	30	
M4	0.94	3.75		30	30		3.75	15		15	30	

AE, *Aspergillus* sp. ethyl acetate extract; PE, *Penicillium* sp. ethyl acetate extract; C, Control Chloramphenicol Control for *B. subtilis* and *S. aureus*, Ciprofloxacin control for *P. aeruginosa*, Cefalexin control for *E. coli*. CGA: Corn "*Zea mays*" girt agar media; M1, Rice "Oryza sativa" media; M2, Cowpea "*Vigna unguiculata*" media; M3, Painted Pony "*Phaseolus vulgaris*"; M4, Broad bean "*Vicia faba*" media.

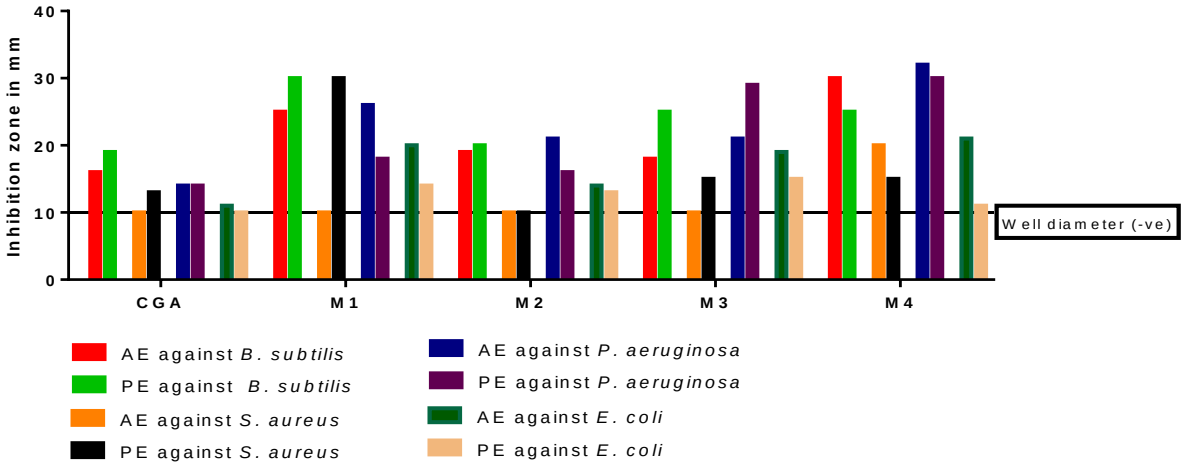

Fig. 1. Antibacterial activity against Gram-positive and Gram-negative bacteria in term of zone of inhibition in mm of fungal extracts cultured on different media

3.2. Cytotoxic Activity

The cytotoxic effect of the various media used with *Penicillium sp.* and *Aspergillus sp*. metabolites were evaluated and showed safe profile index with all of the IC_{50} values above 200 µg/mol; accordingly, these endophytic extracts present good candidates to be used as safe antimicrobial agents in human cells **(Table 3, Fig. 2 & 3)**.

Table 3. IC_{50} of MTT Assay for *Aspergillus* sp. and *Penicillium* sp. ethyl acetate extracts from different media culture

Media	AE	PE	D
CGA	278.51	397.95	23.52
M1	1039.32	452.80	
M2	573.03	500.23	
M3	608.99	830.51	
M4	415.44	240.54	

AE, *Aspergillus* sp. ethyl acetate extract; PE, *Penicillium* sp. ethyl acetate extract; D, Doxorubicin control; CGA, Corn "*Zea mays*" girt agar media; M1, Rice "*Oryza sativa*" media; M2, Cowpea "*Vigna unguiculata*" media; M3, Painted Pony "*Phaseolus vulgaris*" media; M4, Broad bean "*Vicia faba*" media.

Table 4. Biomass yield of different endophytic extracts cultured on different media

Agar Media	*Aspergillus* sp. extract weight (gm)	*Penicillium* sp. extract weight (gm)
CGA: Corn "*Zea mays*"	5.636	3.671
M1: Rice "*Oryza sativa*"	5.072	2.606
M2: Cowpea "*Vigna unguiculata*"	3.024	3.399
M3: Painted Pony "*Phaseolus vulgaris*"	4.759	3.712
M4: Broad bean "*Vicia faba*"	2.574	3.121

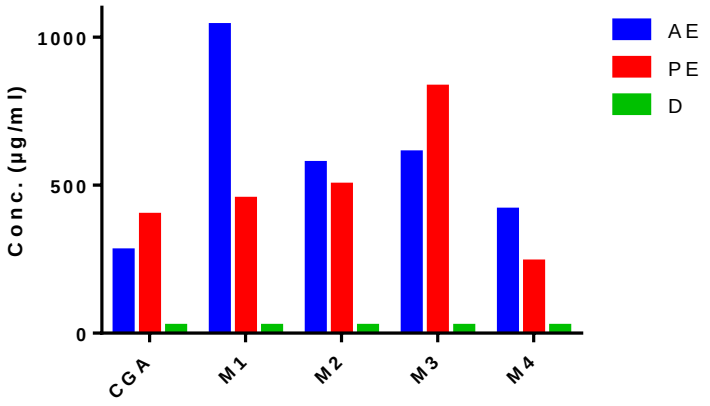

Fig. 2. IC_{50} cytotoxicity of different media extracts of endophytes on MCF-7 cell line. AE, *Aspergillus* sp. ethyl acetate extract; PE, *Penicillium* sp. ethyl acetate extract; D, Doxorubicin control; CGA, Corn "*Zea mays*" girt agar media; M1, Rice "*Oryza sativa*" media; M2, Cowpea "*Vigna unguiculata*" media; M3, Painted Pony "*Phaseolus vulgaris*" media; M4, Broad bean "*Vicia faba*" media.

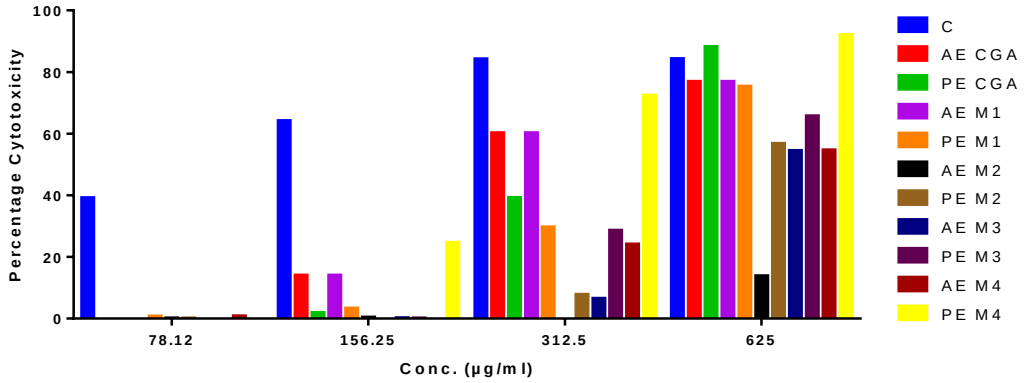

Fig. 3. Percentage cytotoxicity of different media extracts of endophytes on MCF-7 cell line. AE, *Aspergillus* sp. ethyl acetate extract; PE, *Penicillium* sp. ethyl acetate extract; D, Doxorubicin control; CGA, Corn "*Zea mays*" girt agar media; M1, Rice "*Oryza sativa*" media; M2, Cowpea "*Vigna unguiculata*" media; M3, Painted Pony "*Phaseolus vulgaris*" media; M4, Broad bean "*Vicia faba*" media.

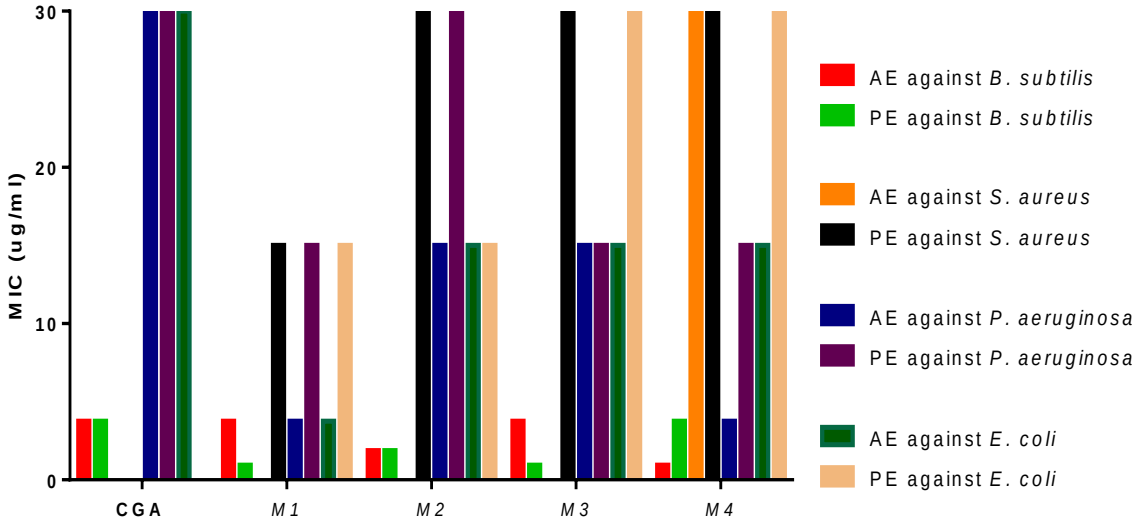

Fig. 4. Antibacterial minimum inhibitory concentration (MIC) of fungal extracts cultured on different media against Gram positive and Gram-negative bacteria. AE, *Aspergillus* sp. ethyl acetate extract; PE, *Penicillium* sp. ethyl acetate extract; CGA, Corn "*Zea mays*" girt agar media; M1, Rice "*Oryza sativa*" media; M2, Cowpea "*Vigna unguiculata*" media; M3, Painted Pony "*Phaseolus vulgaris*"; M4, Broad bean "*Vicia faba*" media, media. control; chloramphenicol for *B. subtilis* (7.81 µg/mL) and *S. aureus* (62.5 µg/mL), ciprofloxacin control for *P. aeruginosa* (7.81 µg/mL), cefalexin control for *E. coli* (125 µg/mL)

Conclusion

Penicillium citrinum and *Aspergillus flavus* cultured on rice (M1) or broad bean (M4) media revealed significant antimicrobial properties against Gram-positive and Gram-negative bacteria as well as a minimal cytotoxicity profile on MCF-7 human cell line, which encourages further investigation of the constituting chemical molecules for future biological studies.

Ethics approval and consent to participate

Not applicable

Consent to publish

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article in the main manuscript.

Competing interests

The authors declare that no competing interests exist

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Authors' contributions

M.M.A. conducted the experimental part, collecting data, writing a draft of the manuscript, A.Y.M., supervised, writing, and editing the manuscript, M.A.E. revised and supervised the manuscript; A.B.S. revised and supervised the manuscript. All authors have read and approved the final manuscript.

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List of abbreviations

A-549, human lung carcinoma; AE, *Aspergillus flavus* ethyl acetate extract; CGA, Corn "*Zea mays*" media; D, Doxorubicin control; DMSO, Dimethyl sulfoxide; HPLC, High-performance liquid chromatography; M1, Rice "*Oryza sativa*" media; M2, Cowpea "Vigna unguiculata" media; M3, Painted Pony "*Phaseolus vulgaris*" media; M4, Broad bean "*Vicia faba*" media; mbar: Millibar; MCF-7, Breast cancer cell line; MIC, minimum inhibitory concentration; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye; PC-3, Human prostate carcinoma; PE, *Penicillium citrinum* ethyl acetate extract; Rpm, Revolutions per minute.

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