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Evaluation of antimicrobial activity and *in vitro* safety of the methanolic extract of *Streptomyces manipurensis* soil isolate H21 for potential industrial applications

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ABSTRACT

Streptomyces manipurensis isolate H21 was recovered from a soil sample in Cairo, Egypt. Cell-free culture supernatant of *Streptomyces manipurensis* isolate H21 previously showed antifungal and broad-spectrum antibacterial activity against some Gram-positive, Gram-negative, MDR, and ESBL producer pathogens as well as some reference strains. The present study aimed at investigating antimicrobial and cytotoxic activities of its crude methanolic extract. The antibacterial activity of the crude methanolic extract was determined using broth-dilution method against *Staphylococcus aureus* ATCC 43300, *Klebsiella oxytoca* ATCC 700324, *Klebsiella pneumoniae* ATCC 700603, *Klebsiella pneumoniae* ATCC BAA-1705 and 2 MDR uropathogens. The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of the crude extract against the tested bacteria were in the range of 5-10 mg/mL and 10 mg/mL, respectively. The results revealed that the CD₅₀ value of the extract was 1.17 mg/mL, against Caco-2 cell line, indicating *in vitro* safety and low cytotoxicity. Accordingly, *Streptomyces manipurensis* isolate H21 would be an excellent source of relatively safe and potent antibacterial agent.

Keywords: *Streptomyces manipurensis*; agar well diffusion; MIC; MBC; MTT assay.

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1. INTRODUCTION

Microbial infections, with the emergence of multiple drug resistant (MDR) microorganisms, remain the second leading cause of death worldwide [1]. To overcome this crisis, an attempt has been made by multiple researchers to improve the existing antibiotics and/or discover novel antibiotics with improved effectiveness [2].

Screening microorganisms for the production of potent antibiotics have been intensively undertaken for many years by researchers [3]. Among the screened microorganisms, *Streptomyces* which represent an outstanding source of many antibiotics (around 80% of total antibiotic production) and active secondary metabolites [4, 5].

Our previous work demonstrated the richness of antibiotic-producing *Streptomyces* in the Egyptian soil [6]. *Streptomyces manipurensis* soil isolate H21 which was previously recovered from Cairo, Egypt proved to have potent anti-ESBL activity and broad-spectrum antimicrobial activity [6]. The present work aimed to evaluate the antifungal and antibacterial activities of crude methanolic extract of cell-free culture supernatant (CFCS) of *Streptomyces manipurensis* soil isolate H21 against several references and MDR microorganisms and to determine its *in vitro* safety on a specific cell line.

2. MATERIAL AND METHODS

2.1. Microorganisms

2.1.1. Standard bacterial strains

Extended-Spectrum beta-lactamase (ESBL) producer reference strain, *K. pneumoniae* ATCC 700603, a producer of the novel enzyme SHV-18 [7] was used in studying anti-ESBL activity.

2.1.2. Other reference strains

The used reference strains from the American Type Culture Collection (ATCC) were kindly provided by Prof. Dr. Mahmoud Abd El-Megead Yassien, Professor of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University. Other reference strains were provided by the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The reference strains were preserved at -80 °C in glycerol stock vials (Table 1).

2.1.3. Clinical pathogenic isolates

Two multiple drug resistant (MDR) bacterial uropathogens from El-Demerdash hospital were kindly provided by Ann Elshamy, assistant lecturer of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University (Table 2).

2.2. Cell line

Colorectal adenocarcinoma derived from human colon (Caco-2 cell line) was used for determination of cytotoxic activity of methanolic extract of *Streptomyces manipurensis* isolate H21. It was obtained from VACSERA, Cairo, Egypt.

2.3. Preparation of cell-free culture supernatant

This was carried out as previously described [8] with some modifications as follows, one pure colony of *Streptomyces manipurensis* isolate H21, grown on starch nitrate agar for 7 days, was used to inoculate 10 mL of the seed medium contained in 100 mL flask. The flask was then incubated at 28 °C and 200 rpm for 72 h. The culture obtained was used to inoculate the main culture (inoculum size 5% v/v) then incubated at 28 °C and 200 rpm for another 72 h. After incubation, the obtained culture broth was centrifuged at 15938g for 15 min to separate mycelium from the supernatant, and the cell-free culture supernatant (CFCS) obtained was harvested, filtered through 0.22 µm pore size cellulose membrane filters (CHEM LAB, Barcelona, Spain) and used for further study.

2.4. Extraction of the bioactive metabolite from CFCS

About five hundred milliliters of CFCS were dried by lyophilization. Then the dry lyophilized CFCS was extracted with distilled methanol (HPLC grade) for 2 h to ensure complete extraction. Finally, the extract was completely evaporated *in vacuo* using R3000 Rotary evaporator (Heidolph instruments GmbH and Co. Schwabach, Schwabach, Germany) at 40 °C until complete dryness.

Table 1. List of the standard strains used for studying the *in vitro* antimicrobial spectrum

Character	Strain source
Gram-negative rod	<i>K. pneumoniae</i> , ATCC BAA-1705 <i>K. oxytoca</i> , ATCC 700324
Gram positive cocci	<i>S. aureus</i> , ATCC 43300
Yeasts (unicellular fungi)	<i>Candida albicans</i> , ATCC 10231 <i>Candida tropicalis</i> , ATCC 13803 <i>Candida lusitaniae</i> , ATCC 3449 <i>Candida parasilosis</i> , ATCC 22019 <i>Cryptococcus neoformans</i> , RCMB 0049001
Moulds (multicellular fungi)	<i>Aspergillus niger</i> , RCMB 002005 <i>Fusarium oxysporum</i> , RCMB 001018 <i>Penicillium italicum</i> , RCMB 001004

RCMB: Regional Center for Mycology and Biotechnology, ATCC: American Type Culture Collection

Table 2: List of MDR bacterial pathogens for studying the *in vitro* antimicrobial spectrum

Character	Organism/code	Spectrum of resistance	Detected genes
Gram negative rods	<i>K. pneumoniae</i> / KP14	Penicillin	<i>ctx-m</i>
		Cephalosporins	<i>shv</i>
		Carbapenems	<i>tem</i> <i>aac(6')</i> -
		Fluoroquinolone	<i>Ib/aac(6')</i> - <i>Ib-cr</i>
		aminoglycosides	<i>qnrS</i> <i>qnrB</i> <i>oxa-A</i>
Gram positive cocci	<i>S. aureus</i> / SA51	Penicillin Tetracycline Folate inhibitor Macrolide	ND

ND: No plasmid-mediated resistance genes were detected

2.5. Screening the methanolic extract for its antibacterial activity

2.5.1. Agar well diffusion method

The methanolic extract of lyophilized CFCS was tested for its anti-ESBL activity against *K. pneumoniae* ATCC 700603 using agar well diffusion as described formerly [9,10] with minor modifications. Methanol was used as negative control. In this experiment, 150 μ L of 10 mg/mL dried extract was tested and the obtained zones of inhibition (IZ) were measured in millimeter (mm).

2.5.2. Disc diffusion method

The antimicrobial activity of the crude extract was estimated via comparison with some standard commercial antibiotics using disc diffusion method. The crude extract (40 μ L) was gradually loaded on 6 mm sterile disc (Becton Dickinson, NJ, USA) and air dried under sterile conditions at room temperature. Three standard commercial antibiotic discs (Oxoid[®], Cheshire, UK) were used for the comparative study as positive controls. The used antibiotic discs were meropenem (10 μ g/disc), ampicillin/ sulbactam (20/10 μ g/disc) and amoxiclav (30 μ g/disc). The discs were placed on the surface of Mueller Hinton agar (MHA) plate which was surface inoculated with ESBL producer *K. pneumoniae* ATCC 700603 (0.5 McFarland). The plates were incubated for 24 h at 37 °C and the produced inhibition zone around each disc was measured (mm). A negative control disc was loaded with methanol. The assay was done in duplicate. The antibacterial activity was evaluated by measuring the diameters of zones of inhibition in mm [11].

2.5.3. Minimum inhibitory concentrations (MICs)

The methanolic extract of the lyophilized CFCS was further tested against MDR

uropathogens and some reference strains to find its minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs). The used bacteria were listed in Table 3. According to the Clinical and Laboratory Standards Institute (CLSI) guidelines, the MICs of the extract were determined using the broth microdilution method. Each well was first filled with 100 μ L Mueller-Hinton broth (Difco, NJ, USA). Then, 100 μ L of the extract was added to the first well in the row and two-fold serially diluted. Each well contained 100 μ L extract in the dilution series. Suspension of the test organism (0.5 McFarland) was prepared and 10 μ L of the prepared suspension was added into each well, except for the negative control wells. Column 11 and 12 were used for solvent control and positive control, respectively. The microtiter plate was incubated at 37 °C for 18-20 h and the MIC was determined by selecting the lowest concentration showing no visible growth of the tested organism. All experiments were performed twice [12].

2.5.4. Minimum bactericidal concentrations (MBCs)

To determine the MBCs, samples (10 μ L and 50 μ L) from wells showing no visible growth in the MIC assays were subcultured onto nutrient agar plates. The plates were incubated at 37 °C for 24 h. After incubation, the concentration that caused 99.9% killing of the original count was considered as the minimum bactericidal concentration (MBC) according to CLSI, 2017 guideline. Both MIC and MBC for the test bacteria were determined in triplicate. This was done as described previously [12,13,14]. When the ratio of MBC/MIC is ≤ 4 , the active extract was considered as bactericidal otherwise as bacteriostatic. If the ratio is ≥ 16 , the extract was considered ineffective [15].

2.6. Screening the extract for its antifungal activity

Methanolic extract of lyophilized supernatant was tested for its anticandidal activity using disc diffusion method. Discs were impregnated with 40 μL extract. The concentration of the used extract was 25 mg/mL. Tested species of *Candida* were mentioned in **Table 4**. Antifungal activity was tested using agar well diffusion against some fungi mentioned in **Table 5**. Cups (6 mm diameter) were loaded with 100 μL of 10 mg/mL of extract.

2.7. Cytotoxic activity of extract against Caco-2 cell line using MTT assay

MTT assay was carried out as described by Saliba *et al.* [16].

2.7.1. Maintenance of the Caco-2 cell line

Caco-2 cell line was grown in 50 mL sterile tissue culture (TC) flasks and subcultured every 3 days as follows: The spent RPMI with fetal bovine serum was discarded and cells were washed twice with phosphate buffered saline. The cells were then detached by incubating with 2 ml of the trypsin solution at 37 °C for 5-10 min. After cell detachment, 20 mL of fresh maintenance TC medium were added to the flask and 10 mL aliquot of this cell suspension was then transferred into another new TC flask (ATCC manual). The two flasks were then incubated at 37 °C in the presence of 5% CO₂ and humid atmosphere for 48 h.

2.7.2. Preparation of tissue culture plates

Caco-2 cell line maintained in the tissue culture flasks were trypsinized as described previously and suspended in propagation tissue culture medium to a count of 10⁵ living cells/mL. This count was determined by using a hemocytometer (Shanghai, China) and cell viability was confirmed by staining with trypan blue solution. After gentle pipetting of the cell

suspension for even distribution, aliquots of 100 μL were transferred to the wells of 96 well microplates (flat bottom sterile tissue culture plate). The plate was then incubated at 37 °C in the presence of 5% CO₂ and a humid atmosphere for 24 h to form a confluent monolayer (approximately contains 5 x 10⁴ cells/well).

2.7.3. MTT assay

Caco-2 monolayer was prepared, washed twice with PBS then two aliquots of 100 μL of tissue culture medium (RPMI: fetal bovine serum 9:1 ratio) and 100 μL of provided sample (dissolved in 5% DMSO and RPMI tissue culture medium) of the test isolate were added to each well to reach final concentration 10 mg/mL, followed by two-fold serial dilution. 12 dilutions were used for the extract to calculate its CD₅₀. Control wells contained two aliquots of 100 μL of tissue culture medium (RPMI: fetal bovine serum 9:1 ratio) and 100 μL sterile DMSO (5%) and RPMI tissue culture medium, followed by two-fold serial dilutions. Following the specified incubation period (24 h) at 37 °C, the wells were washed with PBS and the cells were incubated with MTT solution (2 mg/mL) at 100 μL per each well for 1 h at 37 °C. After incubation, solutions were removed by decantation and cells in each well were treated with 100 μL DMSO to dissolve the formed formazan crystals. Elutes of each test isolate in the 12 wells were then collected and their absorbance was measured at 540 nm using Platos R496 Microplate reader AMD diagnostics (Graz, Austria). Control wells were treated similarly. The percentage of cytotoxicity was calculated using the following formula [17].

$$\text{Cytotoxicity}\% = 1 - \left[\frac{A_{540} \text{ of test culture}}{A_{540} \text{ of control culture}} \right] \times 100$$

CD₅₀ is defined as the concentration of compounds that resulted in a 50% inhibition of mammalian cell growth.

Table 3. Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs) of methanolic crude extract of lyophilized CFCS from *Streptomyces manipurensis* isolate H21

Bacterial strain	MIC mg/mL	MBC mg/mL	R
<i>K. oxytoca</i> ATCC 700324	5	10	2
<i>K. pneumoniae</i> ATCC 700603	5	10	2
<i>K. pneumoniae</i> ATCC BAA-1705	10	10	1
<i>K. pneumoniae</i> KP14	5	10	2
<i>S. aureus</i> ATCC 43300	5	10	2
<i>S. aureus</i> SA51	5	10	2

R= MBC/MIC ratio

Table 4. The activity of *Streptomyces manipurensis* isolate H21 extract against different *Candida* species (25 mg/mL)

ID (species)	Mean IZ diameter(mm)
<i>Candida albicans</i> ATCC 10231	NA
<i>Candida tropicalis</i> ATCC 13803	NA
<i>Candida lusitanae</i> ATCC 3449	10±1
<i>Candida parasilosis</i> ATCC 22019	NA

NA: no activity.

Table 5. Antifungal activity of methanolic extract of *Streptomyces manipurensis* isolate H21

Pathogenic microorganism	Mean IZ diameter (mm)	
	<i>Streptomyces manipurensis</i> isolate H21	Ketoconazole (control) 100 µg/mL
<i>Aspergillus niger</i> RCMB 002005	NA	15
<i>Cryptococcus neoformans</i> RCMB 0049001	NA	25
<i>Fusarium oxysporum</i> RCMB 001018	NA	18
<i>Penicillium italicum</i> RCMB 001004	NA	19

Cup diameter= 6 mm. Volume/cup= 100 µL. NA: no activity

3. RESULTS AND DISCUSSION

3.1. Extraction of the bioactive metabolite from CFCS

Whenever different methods of extraction were used, all extracts should be tested for their activity. The best suitable recovery method to be used depends on the extraction method cost, which adds to the final product price, the required level of purity of the final agent and the adaptability of the method in the industry [18]. Results of the present study revealed that lyophilization is a good step in the extraction of the required antimicrobial agent.

3.2. Screening the methanolic extract for its antibacterial activity

3.2.1. Agar well diffusion method

The results obtained showed that methanolic extract of lyophilized CFCS of *Streptomyces manipurensis* isolate H21 exhibited inhibitory activity against reference ESBL producer, *K. pneumoniae* ATCC 700603. Extract of 10 mg/mL produced IZ of 17 mm.

3.2.2. Disc diffusion method

The anti ESBL activity of *Streptomyces manipurensis* isolate H21 crude extract against *K. pneumoniae* ATCC 700603 was recorded in the form of inhibition zone diameters surrounding the loaded discs. An extract that produced an inhibition zone ≥ 15 mm against tested strain was recorded as active. The methanolic extract of lyophilized CFCS of *Streptomyces manipurensis* isolate H21 had demonstrated anti-ESBL activity. Extract of *Streptomyces manipurensis* isolate H21 showed 18 mm inhibition zone. These results were comparable with amoxiclav (11 mm) and ampicillin/sulbactam (18 mm) discs as shown in (Fig. 1). Therefore, the methanolic extract of the lyophilized supernatant was further studied.

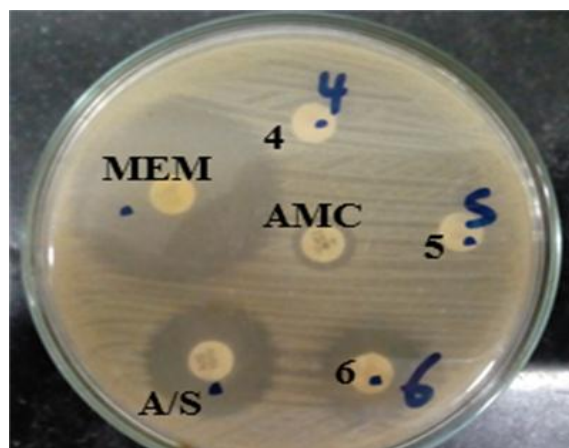


Fig. 1. The anti ESBL activity of *Streptomyces manipurensis* H21 methanolic extract against *K. pneumoniae* ATCC 700603 in comparison with some standard commercial antibiotic discs. Diameters of inhibition zones were as following: 4) control loaded with methanol (0 mm), 5) H21 ethyl acetate extract of CFCS (0 mm), 6) H21 methanolic extract of the lyophilized CFCS (18 mm), Meropenem (MEM; 30 mm), amoxiclav(AMC; 11 mm) and ampicillin/ sulbactam (A/S ;18 mm).

3.2.3. Minimum inhibitory concentrations (MICs)

The data obtained, through the determination of MICs of the extract are shown in (Table 3). The results revealed variability in the inhibitory concentrations of the extract against different organisms. The MIC values of methanolic extract of lyophilized supernatant produced by *Streptomyces manipurensis* isolate H21 against *K. pneumoniae* ATCC 700603 was 5 mg/mL (results represent the mean of MIC values).

The tested methanolic extract was active against a panel of the tested bacteria. The MIC values of methanolic extract of *Streptomyces manipurensis* isolate H21 were in the range from 5 to 10 mg/mL. The lowest activity of the extract was observed against *K. pneumoniae* ATCC BAA-1705. On the other hand, the highest antibacterial activity was recorded against *K. oxytoca* ATCC 700324, *K. pneumoniae* ATCC 700603, *K. pneumoniae* KP14, *Staphylococcus*

aureus ATCC 43300 and *Staphylococcus aureus* SA51.

However, it is difficult to compare the data with other researchers because several variables influence the results, and the choice of the extraction method and antibacterial test. Moreover, there are no standard criteria for the evaluation of crude extract activity.

3.2.4. Minimum bactericidal concentrations (MBCs)

The MBCs were established by subculturing the samples with no visible growth in the MIC assays. Most of the MBC values of the crude extract were double the MIC values in case of *Streptomyces manipurensis* isolate H21. Identical values were shown against *K. pneumoniae* ATCC BAA-1705 (Table 3).

For bactericidal agents, the MBC values are usually ≤ 4 times the MIC values. On the other hand, the MBC values of bacteriostatic agents are > 4 times higher than the MIC values. Such properties of antimicrobial agents can be influenced by the infecting bacterium as the antimicrobial agent may be bactericidal to one organism but bacteriostatic to another [15]. Therefore, the antimicrobial agent produced by *Streptomyces manipurensis* isolate H21 revealed to be bactericidal in nature for all tested bacteria.

3.2.5. Screening the extract for its antifungal activity

Methanolic extract of lyophilized CFCS from *Streptomyces manipurensis* isolate H21 showed positive anticandidal activity against *Candida lusitanae* ATCC 3449 (Table 4). However, no antagonistic antifungal activity of the tested extract was observed against other tested fungi (Table 5).

The extract of *Streptomyces manipurensis* isolate H21 exhibited various degrees of activities against all tested Gram-positive and Gram-

negative bacteria. In addition, it exhibited antagonistic activity against reference MRSA strain *Staphylococcus aureus* ATCC 43300. Similar results reported previously [19]. The tested extract lacks antifungal activity except for *Candida lusitanae* ATCC 3449. Although the CFCS showed anticandidal activity, extraction of the bioactive compound(s) with methanol revealed limited anticandidal activity. These finding envisaged that the extraction method had a definite effect on the isolation of bioactive principles. Similar results were published previously [20].

3.3. Cytotoxic activity of extract against Caco-2 cell line using MTT assay

According to the screening program of American National Cancer Institute (NCI), a crude extract is generally considered to have *in vitro* cytotoxic activity if the CD_{50} value is ≤ 30 $\mu\text{g/ml}$. Methanolic extract of lyophilized CFCS of *Streptomyces manipurensis* isolate H21 showed no *in vitro* cytotoxic activity ($CD_{50} = 1.17$ mg/mL). Results indicated that lyophilized supernatant of *Streptomyces manipurensis* isolate H21 had no cytotoxic activity may be considered as relatively safe based on the *in vitro* assay.

4. CONCLUSION

Methanolic extract of *Streptomyces manipurensis* isolate H21 showed a broad spectrum of antibacterial activity. To the best of our knowledge, this is the first report that addressed the production of the antimicrobial agent by *Streptomyces manipurensis* isolate H21. The antibacterial activity was assigned to the presence of bactericidal compounds with activity against MRSA and ESBL producer. No cytotoxic activity was detected indicating *in vitro* safety of the bioactive extract. The promising features of this bioactive extract can pave the way towards profitable large-scale production of antimicrobial

drugs from *Streptomyces manipurensis* isolate H21.

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Conflict of Interest

The authors declare no conflict of interest.

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