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Antimicrobial Activity of Fungi Isolated from Manikaran Hot Spring

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ABSTRACT

To overcome the problem of multidrug resistance, the most serious concern is developing new drugs with different modes of action by exploring potent microorganisms from different sources. The present study was carried out to isolate potential fungi with the ability to produce antimicrobial compounds. A total of eight fungal isolates were isolated from Manikaran hot spring and screened for antimicrobial activity. Out of them, three were found to exhibit antimicrobial activity against one of the three test organisms tested viz., gram-positive- *Staphylococcus aureus* (MTCC No. 3160), gram-negative- *Escherichia coli* (MTCC No. 1687) and the fungus *Candida albicans* (MTCC No. 183) during primary screening. Fungal isolates MKH 4 and MKH 12 were inhibitory against *E. coli* and *S. aureus* (antibacterial) and MKH 16 was inhibitory against *C. albicans* (antifungal). Analysis of the crude metabolites of MKH 4, MKH 12 and MKH by thin layer chromatography (TLC) showed the presence of bioactive components with Rf value of 0.88, 0.80, 0.74, respectively. The potential isolates MKH 4, MKH 12 and MKH 16 were studied morphologically and microscopically and identified as *Aspergillus* sp. and could be studied further for enhancing its potential of antibiotic production.

Key words : Manikaran hot spring, fungi, test organism, TLC, *Aspergillus*

INTRODUCTION

Multidrug resistance amongst pathogenic microorganisms is a serious problem with which the whole world is grappling. Methicillin resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumonia* (PRSP) and vancomycin-resistant *Enterococcus faecium* (VRE) are some of the pathogenic organisms that have gained resistance and increased significantly in the last decade (Deshmukh *et al.,* 2015). Some key factors accountable for the development of multidrug resistance (MDR) among microorganisms include contamination at clinics, inadequate antibiotic utilization, self-prescription, and delay in diagnosis, which is responsible for 15.5% of hospital acquired infections (HAIs). This burning issue has led the researchers to investigate new drug scaffolds with broad range of antimicrobial activities or new modes of action essential to combat the battle against the ever-increasing multidrug resistance problem (Ventola, 2015). Antimicrobial metabolites have been found in a range of fungal species worldwide, and various fungal metabolites have been accepted as drugs, such as fusidic acid and cephalosporins. In the field of drug discovery, fungi have contributed a significant part by delivering new bioactive compounds such as anti-infection agents like penicillin, anti-cancer drugs like taxol, immune suppressant like cyclosporin, and cholesterol-lowering drugs like lovastatin (Zhang *et al.*, 2015; Deiske *et al.*, 2020). Different broad explorations, essentially on earth inferred and marine-determined fungi, have indicated that fungi are a prolific well spring of explicit bioactive compounds (Fakih and Almaqtri, 2019). It is now potentially vital to think about new methodologies and investigate all undiscovered common assets or natural sources that may furnish new classes of antimicrobial agents with novel instruments of activity to combat bacterial microbes or pathogens.

MATERIALS AND METHODS

Water samples were collected from Manikaran hot spring (Kullu), Himachal Pradesh, India. The samples were collected aseptically and

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were taken to the laboratory in sterile bags and stored in a refrigerator at 4°C for further use. The standard serial dilution method was used to isolate the fungal isolates from the collected samples. To make a stock solution, one ml of water sample was taken into test tubes containing 9 ml sterile water, respectively, and shaken well using a vortex mixer. Further, 1 ml stock solution and 9 ml sterile water were mixed to make dilutions up to 10^{-8}. Thereafter, 100 µl of inoculum from 10^{-4} to 10^{-8} dilutions were spread on to the surface of potato dextrose agar, malt extract agar, and sabouraud dextrose agar media plates (Dhanwal *et al.*, 2018) and were incubated at 30°C for 48-72 h. Distinct colonies were selected from the cultured plates and maintained on potato dextrose agar medium by streak plate method.

Test microbial strains were procured from MTCC (Microbial Type-culture Collection Center), IMTECH, Chandigarh, India. Table 1 shows the selected test microorganisms to study the antimicrobial activity. Test bacteria and test fungi were maintained on nutrient agar and potato dextrose agar, respectively, at 4°C with routine sub-culturing.

Table 1. Test organisms with their MTCC numbers

Strain	Type	MTCC No.
Escherichia coli	Gram-negative bacteria	1687
Staphylococcus aureus	Gram-positive bacteria	3160
Candida albicans	Fungus	183

Clinical Laboratory Standards Institute (CLSI, 2005) document M2-A8 guide lines were followed to prepare test microbial inocula for determining the antimicrobial assay. The loop culture of each bacterial strain was inoculated into 10 ml of fresh sterile nutrient broth and incubated overnight at 37°C. The turbidity was changed to 0.5 McFarland unit after incubation (Balouiri *et al.*, 2016). For *C. albicans*, the test strain was inoculated and incubated at 30 °C for 24 h in sterile yeast peptone dextrose (YPD) broth.

Antimicrobial activity of fungal isolates was tested against three test organisms viz., *S. aureus* (gram-positive), *E. coli* (gram-negative) and *C. albicans* (fungi) using the perpendicular cross-streak method (Dudeja *et al.*, 2020). Fungal isolates were streaked in the middle of muellerhinton (MH) agar plates as a straight line and incubated at 30°C for 48-72 h. The test organisms were prepared as described earlier and the density was adjusted to 0.5 McFarland. Thereafter, the test organisms were streaked perpendicular to the fungal isolate on the MH agar medium. Three plates of muellerhinton agar streaked with the test organisms without the fungal isolate were used as a negative control. All the plates were incubated at 37°C and observed for the zone of inhibition for 24-48 h. The fungal isolates that showed inhibition of test bacteria by the absence of growth of the test organism near the isolates was isolated and maintained at -20°C in 60% of sterile glycerol stock.

Fungal isolates with antimicrobial activity were characterized phenotypically by colonial observation on PDA media. Fungal colony characteristics like elevation, form, colony morphology, surface texture, surface colour, margin, mycelium colour, conidia colour and characteristics of spores were recorded. The microscopic observations were carried out using a compound microscope and the slides of selected fungal isolates were prepared using water and glycerol as mountant, whereas lactophenol blue was used for staining. Microscopic characteristics such as spore structures, seriation, special hyphal characteristics, shape of vesicle and growth patterns were observed.

Detection of bioactive compounds from the solvent extracts of selected fungal isolates was performed by analytical TLC (thin-layer chromatography) (Astuti *et al.*, 2014). Thin-layer chromatography was performed on silica-based gel slides with a solvent system (ethyl acetate : methanol in the ratio of 4 : 6) of 25 ml in a glass beaker. The concentrated ethyl acetate extract of selected fungal crude extract was prepared by adding 0.1 mg of crude extract and 100 ml of ethyl acetate. Thereafter, 10 ml of 1.0 mg/ml ethyl acetate extract was dropped on each spot on the TLC silica plate (TLC silica gel 60G Merck; Darmstadt, Germany) and was run until the solvent reached the top. The TLC plate was air-dried and the solvent traces were removed completely; the chromatograms thus developed were visualized at 254 nm under UV light and exposed to iodine vapors.

RESULTS AND DISCUSSION

A total of eight fungal isolates were isolated

from the samples on PDA (potato dextrose agar), CDA (czapekdox agar) and SDA (sabouraud dextrose agar) medium by using spread plate method as shown in Fig. 1. The isolated fungal colonies were purified and maintained on PDA medium containing chloramphenicol (antibacterial) by streaking as shown in Fig. 2. The plates were incubated at 30°C for 48-72 h. The fungal isolates thus developed were then screened for their antimicrobial activity and maintained at 4°C till further use.

During primary screening, out of eight fungal isolates, streaked perpendicular to three test organisms, three fungal isolates showed growth inhibition against one of the three test organisms (*E. coli, S. aureus* and *C. albicans*) and six fungal isolates showed no activity as shown in Fig. 3. Fungal isolates MKH 4 and MKH 12 showed inhibition of test strain *E. coli* and *S. aureus*; MKH 16 showed inhibition of test strain *C. albicans* and were further studied for thin layer chromatography (TLC). The primary screening of eight fungal isolates for the antimicrobial activity against three clinical test organisms is summarized in Table 2.

Table 2. Primary screening of fungal isolates for antimicrobial activity against *E. coli, S. aureus* and *C. albicans* by using perpendicular streak plate method

S. No.	Isolate	*E. coli*	*S. aureus*	*C. albicans*
1.	MKH 2	-	-	-
2.	MKH 13	-	-	-
3.	MKH 1	-	-	-
4.	MKH 4	+	+	-
5.	MKH 12	+	+	-
6.	MKH 16	-	-	+
7.	MKH 9	-	-	-
8.	MKH 8	-	-	-

Morphologically the fungal isolates differed in growth pattern and colony appearance on PDA media (Table 3). The fungal isolates showed similar features when observed microscopically by staining with lactophenol blue and the microscopical characteristics observed (Table 4). Based on the morphological and microscopical features the fungal isolates MKH 4, MKH 12 and MKH 16 were identified as *Aspergillus* sp.

Ethyl acetate extracts of fungal isolates were screened for the detection of antimicrobial

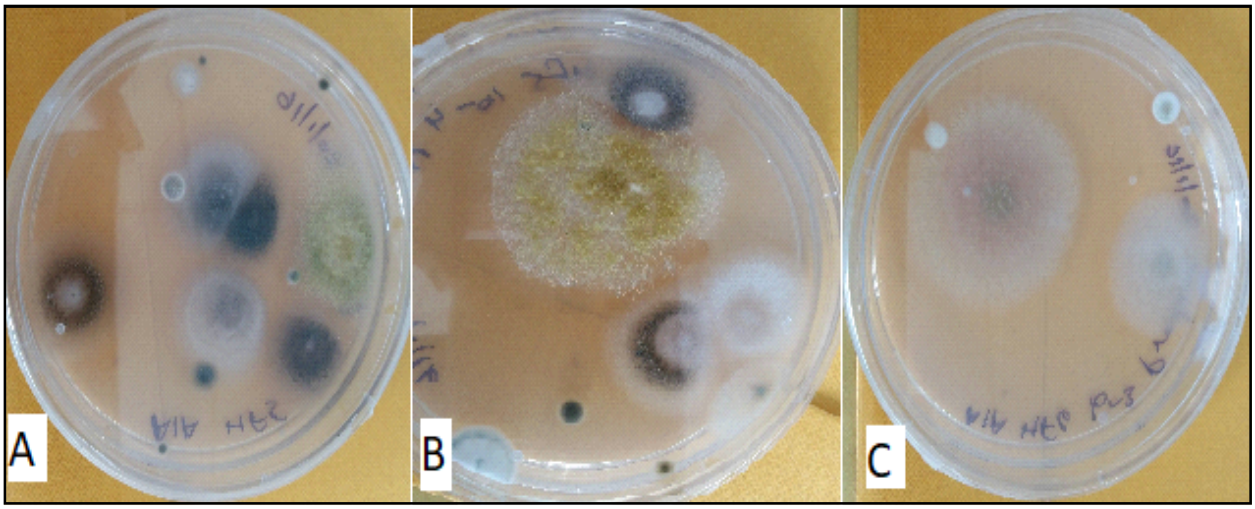

Fig. 1. Isolation of fungal isolates from different samples by spread plate technique (A, B and C).

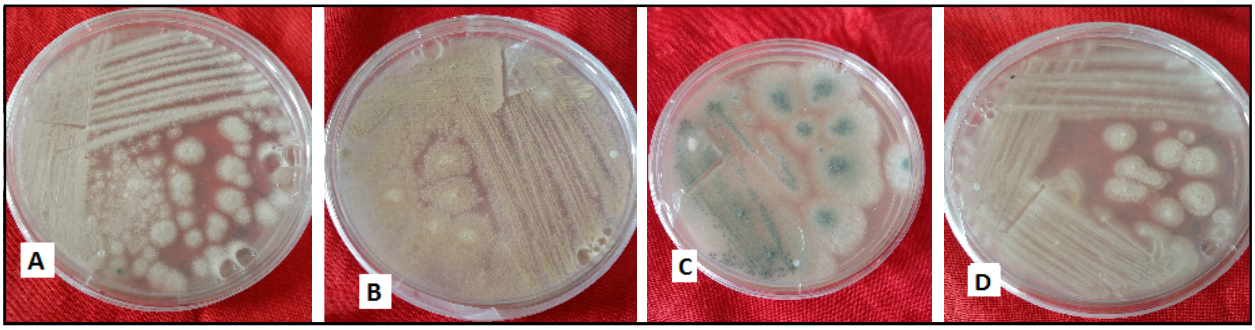

Fig. 2. Pure fungal isolates in PDA media plates by streak plate method (A-D).

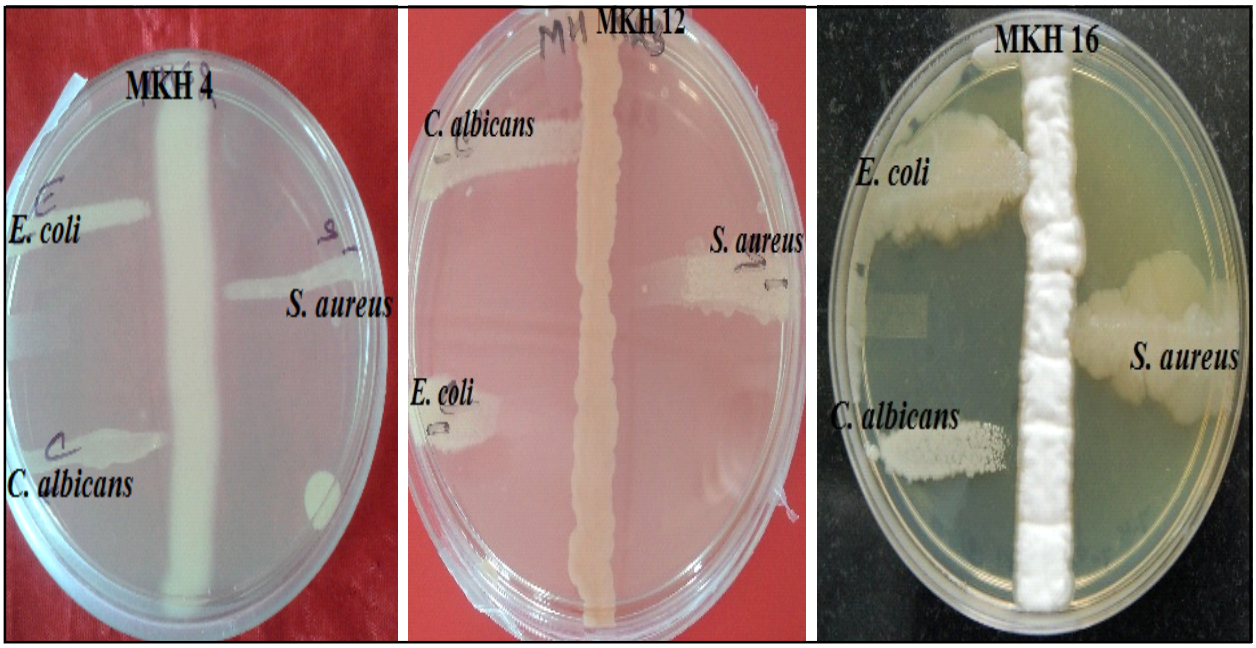

Fig. 3. Primary screening of fungal isolates against three test organisms (*E. coli, S. aureus* and *C. albicans*).

Table 3. Morphological characteristics of fungal isolates on PDA growth media

Features	MKH 4	MKH 12	MKH 16
Form	Irregular	Irregular	Filamentous
Elevation	Raised	Raised	Flat
Margin	Filiform	Filiform	Entire
Surface colour	Dark green	Brown	Light green
Reverse colour	Cremish	Cremish	White
Texture	Velvety	Velvety	Powdery
Sporulation	Heavy	Heavy	Moderate
Conidia colour	Dark olive green	Brown	Light green to brown
Mycelium	White	White	Cremish
Colony diameter after five days (mm)	62±2.5	58±2.5	65±1.0

Table 4. Microscopical characteristics of fungal isolates MKH 4, MKH 12 and MKH 16

Seriation	Uniseriate
Type of hyphae	Septate
Type of spore	Conidiospores
Shape of vesicle	Globose
Conidial head	Columnar
Conidiophore	Short and smooth

compound(s) using TLC bioautography. TLC metabolic profiling of the *Aspergillus* sp. extracts of ethyl acetate showed the presence of intense band under UV transilluminator at 254 nm. Retention factor (*Rf*) value as a ratio of the distance travelled by the solute/distance travelled by the solvent provided a quantitative measure of specific components and their properties in a mixture.

The *Rf* value for ethyl acetate extract of *Aspergillus* sp. MKH 4, MKH 12 and MKH 16 were observed to be 0.88, 0.80 and 0.74, respectively (Fig. 4). Padhi and Tayung (2015) reported two UV-visible components obtained from crude extracts of *Aspergillus* sp. The main active component showed a strong peak at 240-260 nm with a maximum absorbance of 1508 nm with an *Rf* value of 0.93.

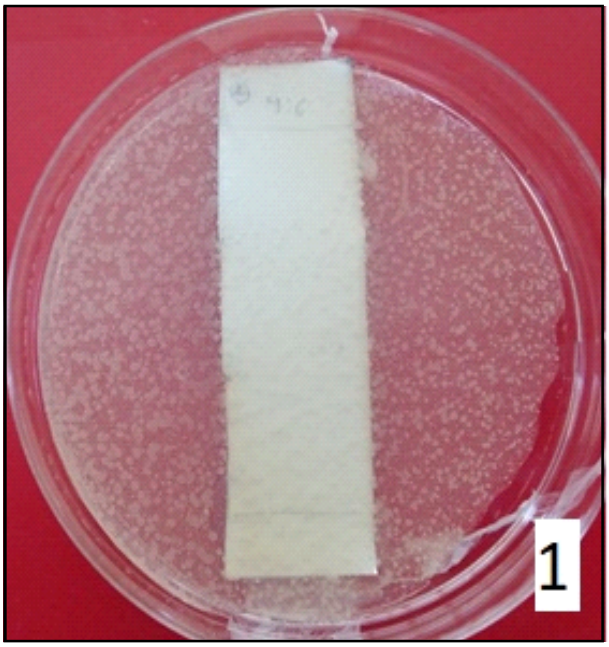

Fig. 4. TLC plate showing separation of components from the crude extract of fungal isolate MKH 4 with solvent system ethyl acetate : methanol (4 : 6).

Several fundamental drugs are expensive and have adverse side effects on the host, and some microorganisms do not offer effective antibiotics and others create multidrug resistance. To address this problem, many researchers are working into finding new antimicrobial agents and expanding the viability of these agents. Although the research for new microorganisms from the unexplored territories will prompt new organically dynamic secondary metabolites, just only a tiny portion (0.1-1%) of all the microorganisms has been exploited so far. In this context, there has been a considerable focus on antibiotic producing microbes from different unexplored habitats to discover novel and potent antibiotics (Davies and Behroozian, 2020). In the present study, Manikaran hot spring area was explored and eight fungi were isolated. The isolated fungi screened for antimicrobial activity against three test organisms showed significant antimicrobial activity. This will lead to large-scale fermentation of selected fungal isolates and characterization of bioactive compounds for further studies. The fungal isolates exhibiting antimicrobial activity were studied for morphological and microscopical features and identified as *Aspergillus* sp. The MKH 4 and MKH 12 isolates were showing inhibition against *E. coli* and *S. aureus* (antibacterial) and isolate MKH 16 was found to be active against *C. albicans* (antifungal). The results obtained in TLC studies suggested the presence of bioactive compounds, which could be used for further investigation of bioactive compounds. The study validates that the isolated fungi from Manikaran hot spring could be useful as antibacterial and antifungal agents.

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