

## Isolation of a Novel Antimicrobial Compounds Producing Fungus *Aspergillus niger* MTCC 12676 and Evaluation of its Antimicrobial Activity against Selected Pathogenic Microorganisms

Amrinder Singh<sup>1</sup>, Mukesh Kumar<sup>2</sup> and Raj Kumar Salar<sup>1\*</sup>

<sup>1</sup>Department of Biotechnology, Chaudhary Devi Lal University, Sirsa-125055, Haryana, India.

<sup>2</sup>Department of Biotechnology Engineering, Ambala College of Engineering and Applied Research, Devsthali-133101, Ambala, Haryana, India.

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A novel antimicrobial compounds producing strain of fungus was isolated from rhizospheric soil and identified as *Aspergillus niger* MTCC 12676 on the basis of morphological characters and molecular identification. Antimicrobial activity of this strain was evaluated by using different solvents against Gram positive bacteria *Streptococcus mutans* and *Staphylococcus aureus*, Gram-negative bacteria, *Pseudomonas fluorescense* and *Escherichia coli* and two yeast namely *Candida tropicalis* and *Candida glabrata*. Ethanol and ethyl acetate solvents exhibited significant antimicrobial activity against most of the tested microorganisms with more pronounced effect in case of ethyl acetate. MIC of ethanol extract ranged from 0.125-1.25 mg/ml whereas that of ethyl acetate was in the range of 0.312-0.0625 mg/ml. Antibiotic susceptibility pattern revealed that *Staphylococcus aureus* and *Candida glabrata* were resistant against the ciprofloxamine and fluconazole respectively. Combination of ciprofloxamine with different extracts (Ethanol and Ethyl acetate) exhibited synergistic effect in case of *E. coli*, *P. fluorescense* and *S. mutans*. Similarly, combination of the two extracts with antibiotic fluconazole had a positive synergistic effect in case of *C. albicans*.

**Keywords:** *Aspergillus niger*, Antimicrobial activity, MIC, Synergistic effect, rhizospheric soil.

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In the present world, there are many infectious diseases, re-emergent diseases and multidrug resistant bacterial diseases due to which there is continuous need of developing new antibiotics. Soil is an immense and practically unexploited source of potentially useful biological bioactive compounds. However, all biochemical chemical compounds are not well characterized. Interaction between compounds is a well-known phenomenon and substrate competition and antagonism are important factors in selection of

microbial communities in any given ecological niche (Lay, 1994). Rajalakshmi and Mahesh, 2014). Development of multiple drug-resistant microbes raised the need to search for new and novel antimicrobials for treatment of human diseases. One of the major problem in health care is that emergence of antibiotic resistance bacteria *Staphylococcus aureus* (MRSA). MRSA is now resistant to almost all commonly used antibiotics and even resistant to last choice of antibiotics vancomycin, has begun to appear. (Denyer *et al.*, 2004). Due to the rising incidences related to multidrug resistance amongst pathogenic microorganism, there is need to find out new antimicrobial sources (Gupta and Kumar, 2017).

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\* To whom all correspondence should be addressed.  
Tel.: +919896106467; Fax No. +91166624812;  
E-mail: rajsalar@rediffmail.com

Antibiotics are compounds produced by microorganisms that are able to inhibit the growth of other microorganisms (Rajalakshmi and Mahesh, 2014). In today's world, antibiotics are the compounds produced by a microorganism, or similar substance (produced wholly or partly by chemical synthesis), whose low concentrations can inhibit the growth of other microorganisms (Barrett *et al.*, 2005) and are considered as selective poison. Despite critical need for new antibiotics to treat drug-resistant infections and other infectious diseases, very few new antibiotics are being developed. Pathogens takes a huge variation in the time for emergence of resistance, which varies among organisms and antibiotics. When there is development of resistance in any pathogen, a new antibiotic is required, which is active against resistant one. In response to microbial resistance, the pharmaceutical industry has produced a remarkable range of antibiotics (Luzhetskyy *et al.*, 2007). Not only is there a problem in finding new antibiotics to fight old diseases (because resistant strains of bacteria have emerged), there is also a problem to find new antibiotics to fight new diseases. Over last few years, it has become clear that the emergence of acquired resistance to antibiotics in bacteria is still a serious threat to animals and humans. So, there is urgent need to search for antibiotics which differ in their modes of action (Salyers *et al.*, 2005).

Compounds produced from microorganisms are divided into two types: primary and secondary metabolites. Primary metabolism is governed by primary metabolites produced by microorganisms. Primary metabolism is step by step enzyme-catalyzed reactions which provide the cell with energy, synthetic intermediate products and macromolecules such as protein and nucleic acids. Products of primary metabolism are associated with cell growth and their maximum production occurs in the logarithmic phase of growth in a batch culture (Martin and Demain, 1980). In contrast to primary metabolism, secondary metabolism has no apparent function in the organism. The organism continues to exist if secondary metabolism is blocked. Many type of secondary metabolites are produced which inhibits the growth of the surrounding strains. These secondary metabolites, acts as antibiotics to maintain their predominance in the surrounding microbial population.

Several compounds with antibiotic activity have been isolated from microorganisms till date, but only a few of them are clinically useful (Morgan *et al.*, 2005). The reason for this is because only compounds with selective toxicity can be used clinically they must be highly effective against a microorganism but have minimal toxicity to human cells. The discovery and development of new effective antimicrobial compounds with new mechanisms of action is still required for infectious disease research programs (Thomashow *et al.*, 2008). Rhizosphere soil is mostly viewed from the perspective of how beneficial microorganisms can inhibit the growth and activity of various pathogenic microorganisms (Richardson *et al.*, 2009). In view of the above facts, the present study reports an attempt for the isolation of potent soil fungal strain possessing antimicrobial activity against the selected human pathogenic microorganisms.

## MATERIALS AND METHODS

### Sample collection

Soil samples for the present study were collected from different regions of Delhi, Haryana, Punjab, Himachal Pradesh and Jammu and Kashmir. During this process, wild and cultivated plants were dug out carefully and soil adhering to roots was collected removing 1-2 inches of top soil with sterilized spatula. In addition to the rhizosphere soil, sample was also collected from various other sites like bank of river, crop fields, farmhouse backyards, household wastes. A total of 150 soil samples were collected in sterile polyethylene bags and were labeled with date and site of collection. The soil samples were processed immediately for determining moisture content and pH.

### Isolation of microorganisms from soil

Serial dilution agar plate method was used for the isolation of fungi from the soil samples (Cappucino and Sherman, 1996 and Aneja, 2003) through use of streptopenicillin supplemented potato dextrose agar. One gram of dry soil was suspended in 9 ml sterilized distilled water blank No. 1(stock suspension) and vortex for 10-15 minutes to obtain uniform suspension of microorganisms. 1.0 ml of suspension was transferred, while in motion, from the stock

suspension (No.1), into sterile water blank number 2 with sterile pipette under aseptic conditions to make 1: 10 ( $10^{-2}$ ) dilution and vortex it well for five minutes. Further dilutions from  $10^{-3}$  to  $10^{-7}$  were made by pipetting 1.0 ml suspension into sterile water blanks numbered 3, 4, 5, 6 and 7 from water blanks labeled 2, 3, 4, 5 and 6 respectively. Finally 1.0 ml aliquots of the suspension of final five dilutions i.e.  $10^{-3}$  to  $10^{-7}$  were added to labeled and sterilized Petri plates. Approximately, 25 ml cooled ( $45^{\circ}\text{C}$ ) molten PDA (with streptopenicillin) was added to each Petri plate and mixed gently by rotation. After solidification of the agar media, the inoculated PDA plates were incubated in an inverted position at  $25^{\circ}\text{C}$  for 6 days. The fungi exhibiting antagonistic activity in plate with others was taken as promising isolate and cultured for further studies.

#### Test microorganisms

The test microorganisms used in the present study were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India, which included Gram positive bacteria, *Streptococcus mutans* (MTCC Nos.497) *Staphylococcus aureus* (MTCC No.7443), Gram-negative bacteria, *Pseudomonas fluorescence* (MTCC No.1748) *Pseudomonas aeruginosa* (MTCC NO.1688) and *Escherichia coli* (MTCC No.40) and yeast namely *Candida tropicalis* (MTCC No. 3421), *Candida glabrata* (MTCC NO.3814) and *Candida albicans* (MTCC NO.227). The slants of modified nutrient agar were used for maintaining *S. mutans* strains, nutrient agar for *P. fluorescence*, *S. aureus* and *E. coli* and malt extract agar for *C. albicans* strains and *C. glabrata*. All the slants were kept at  $4^{\circ}\text{C}$  in the refrigerator for further studies.

#### Preparation of microbial inoculum

McFarland standard has been used to standardize the approximate number of microorganisms (bacteria and yeasts) in a liquid suspension by comparing turbidity of test organism suspension with McFarland standard. It is a mixture of two chemical constituents {9.95 ml barium chloride (1.175%) + 0.05 ml sulphuric acid (1.0%)}. The reaction between the two chemicals results in production of a fine precipitate of barium sulphate. When shaken well, the turbidity of 0.5 McFarland standard is visually comparable to a bacterial suspension of approximately  $1.5 \times 10^8$

cells/ml. The inoculum of different test pathogens was adjusted according to above prepared 0.5 McFarland standard. The McFarland tube was stored at  $4-5^{\circ}\text{C}$  and was prepared afresh after every 3 to 4 months (Andrews, 2001).

#### Preparation of crude extract

The fungal crude extract was obtained by inoculating a disc of fungal isolate in potato dextrose broth and incubating for 10-11 days at  $27-29^{\circ}\text{C}$  in a BOD incubator. After the incubation, the biomass was filtered through Whatman filter paper No. 1. Bioactive compounds present in the filtrate were extracted by solvent extraction using ethyl acetate as a solvent. Equal volume (1:1) of culture filtrate and ethyl acetate were taken in separating funnel of 1000 ml and agitated for an hour. The mixture was allowed to stand on tripod stand till organic and aqueous layer separated out as upper and lower phase. The solvent layer was evaporated to dryness at  $40^{\circ}\text{C}$  to get concentrated crude extract. The dried crude extract was scratched with the help of spatula and dissolved in DMSO and was used for testing of antimicrobial activity against various test pathogens.

#### Evaluation of antimicrobial activity

The antimicrobial activity of purified fungal isolates was determined by using agar well plate method. 100  $\mu\text{l}$  of each of the test bacterial culture standardized at 10 cell/ml from 24 hour old incubated broth culture was aseptically transferred and spread over solidified Muller Hinton agar plates. Wells were dug on the seeded plate with the help of sterile cork borer (4 mm). Each well was filled with 100  $\mu\text{l}$  of crude fungal extracts. The plates were incubated at  $37^{\circ}\text{C}$  for 24 to 48 hours. Antimicrobial activity of fungal isolates against test pathogens was determined by measuring the zone of growth inhibition around the fungal colonies (Corrado and Rodrigues, 2004).

#### Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). MICs were determined following the method as described by Hirasawa et.al. (1999) with slight modifications. Different concentrations were prepared using DMSO (64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625mg/

ml) and agar well diffusion method was carried out. The test were carried out in triplicate and the mean were recorded.

#### **Determination of antimicrobial susceptibility and synergistic activity of promising fungal isolate with antibiotics**

Antibacterial activity was measured using agar well diffusion method according to the National Committee for Clinical Laboratory Standard. Petri plates containing approximately 25-30 ml of sterilized Mueller Hinton agar medium were inoculated using a cotton swab with a 4-6 h old culture of the bacterial strains. Wells (6 mm diameter) were punched in the agar with sterilized cork and filled with 30  $\mu$ l antibiotics and in case of synergistic effect, 30  $\mu$ l of each fungal isolate was also added into the well. The plates were incubated at 37°C for 18-24 hours. The antibacterial activity was assessed by measuring the inhibition zone diameter (mm) around the well. The average of three replicates for each crude fungal metabolite, antibiotic and combination was calculated. Synergism effect was considered when combinations exhibited enlargement of combined inhibition zone size by 0.5 mm.

#### **Identification of potential fungal isolate**

The fungal strain that exhibited highest antimicrobial activity was selected for subsequent studies and was identified on the basis of its morphological, physiological, biochemical characteristics and rDNA gene sequence of ITS region. Genomic DNA was isolated and rDNA of ITS region was amplified and the PCR product was bidirectionally sequenced using forward (ITS4) and reverse (ITS5) primers. The rDNA gene sequence of ITS region was used to carry out BLAST alignment search tool of NCBI Genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

## **RESULTS AND DISCUSSION**

#### **Selection of Promising fungal isolates**

Eight fungal isolates: A21 (MTCC 12676), A23, A63, A72, A129 (*Aspergillus* spp.), A56 (*Penicillium* spp.) and A103, A113 (*Cladosporium* sp.) were found to be effective

against test pathogens. On the basis of maximum diameter of zone of growth inhibition against test pathogens using agar well diffusion assay obtained from screening studies, the fungal isolate A21 proved most promising and was selected for further study.

#### **Identification of fungal isolate**

Identification of the fungal isolate A21 was done on the basis of colony characteristics (colony growth, colour and production of exudate) and sporulating structures (conidiogenous cells, vesicle, conidial head and conidia) by following manual of Kirk et al. (2001). Morphological characterization suggests that it is a fungal strain belonging to *Aspergillus* genera. For molecular analysis, the rDNA of ITS region was amplified and the PCR product was bidirectionally sequenced using forward (ITS4) and reverse (ITS5) primers. Based on BLAST search analysis the fungus isolate A21 was identified as *Aspergillus niger*. Based on maximum identity score first 90 sequences were selected and aligned using multiple sequence alignment software program clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using muscle (Fig. 1). A living culture of the isolate is deposited in Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India (Accession No.12676).

#### **Determination of antimicrobial activity**

In the present study, crude extracts of *Aspergillus niger* MTCC 12676 were prepared in different solvents (Methanol, petroleum ether, ethanol, n-butanol and ethyl acetate) and analyzed for their antimicrobial activity determined against the test microbial pathogens. The results presented in Table 1 shows that antimicrobial activity of the fungal strain *A. niger* MTCC 12676 was obtained only in case of ethanol and ethyl acetate solvents. These extracts exhibited significant antimicrobial activity against most of the tested microorganisms. In ethanolic extracts, the zone of growth inhibition ranged from 14-24 mm whereas the ethyl acetate extracts resulted in zones of 16-36 mm. Among the tested pathogens, *Candida albicans* was found to be most sensitive yielding 24 mm and 36 mm diameter zones against ethanol and ethyl acetate extracts respectively. However, none of the solvent was able to exhibit the antimicrobial activity against *Pseudomonas aeruginosa* and *Candida tropicalis*.

There are also previous reports suggesting the antimicrobial activity of *Aspergillus sp.* against the pathogenic microorganisms (Furtado *et al.*, 2002 and Sunkar and Nachiyar, 2011).

**Determination of minimum inhibitory concentration (MIC)**

The minimum inhibitory concentration of ethanolic extract of *A. niger* MTCC 12676 and of ethyl acetate extracts are shown in Table 2. MIC of ethanol extract was 0.125 mg/ml for *Escherichia coli*, *Staphylococcus aureus* and 1.25mg/ml for

*Pseudomonas florescence*, *Streptococcus mutans* and *Candida albicans*. The lowest MIC was observed in case of *Escherichia coli* (.125mg/ml). On the other hand, MIC of ethyl acetate extract was 0.312mg/ml for *Escherichia coli*, *Pseudomonas florescence*, *Candida albicans* and 0.0625mg/ml for *Staphylococcus aureus* and *Streptococcus mutans*. *Candida glabrata* had highest MIC of 2.5mg/ml among all the tested microorganisms in both the solvents. The present results are in accordance to Geetanjali et al. (2016) who reported

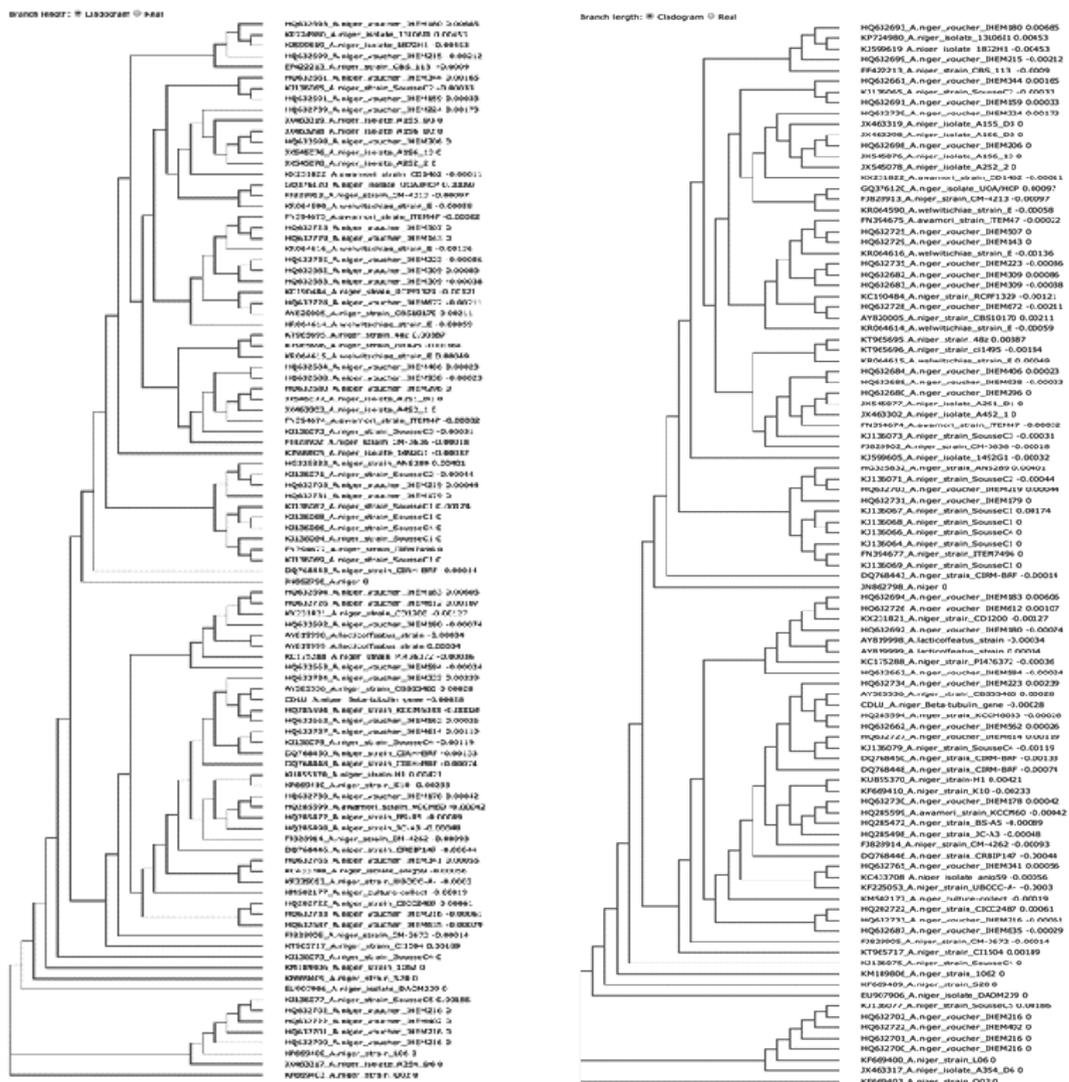


Fig.1. Phylogenetic tree of *Aspergillus niger* MTCC 12676

fungal antimicrobial activity against most of the microorganisms used in the present study with a closely matched MIC values.

#### Determination of antimicrobial susceptible pattern of tested microorganisms

Table 3 reveals the antibiotic susceptibility pattern of test pathogens. In case of gram-positive bacteria, *Pseudomonas aeruginosa* showed the maximum susceptibility to antibiotic ciprofloxacin producing inhibition zones of 32 mm, whereas *Streptococcus mutans* had zone of 28 mm. On the other hand, Gram-negative bacteria, exhibited zone of 30 mm and 26 mm for *Escherichia coli* and *Pseudomonas fluorescens* respectively against the same antibiotic. However, *Staphylococcus aureus* was found to be resistant against ciprofloxacin. Similarly, the fungi *Candida tropicalis* (26 mm) and *Candida albicans* (29 mm) also exhibited

susceptibility pattern against antibiotic fluconazole except *Candida glabrata*.

#### Synergistic activity of promising fungal isolate with antibiotics

The synergistic activities of ethanol and ethyl acetate extracts of *A. niger* MTCC 12676 with commercial antibiotics were determined to observe changes in their antimicrobial efficacy (Table 4). When both the extracts were combined with ciprofloxacin, no synergistic effect was observed in case of Gram-negative bacterium *P. aeruginosa* and Gram-positive *S. aureus*. However, this combination of ciprofloxacin with different extracts (Ethanol and Ethyl acetate) exhibited synergistic effect through significant inhibition of the growth of Gram-negative bacteria *E. coli*, *P. fluorescens* and Gram-positive bacterium *S. mutans* as indicated by significant increase in the

**Table 1.** Antimicrobial activity of different crude extracts of *A. niger* MTCC 12676 against various pathogenic microorganisms

Solvent	Diameter of zone of inhibition (mm)							
	Gram-negative			Gram-positive			Yeast	
	Ec	Pa	Pf	Sa	Sm	Ct	Ca	Cg
Methanol	-	-	-	-	-	-	-	-
Petroleum Ether	-	-	-	-	-	-	-	-
Ethanol	21±0.23	-	18±0.1	19±0.23	20±0.42	-	24±0.13	14±0.21
n-Butanol	-	-	-	-	-	-	-	-
Ethyl Acetate	29±0.47	-	24±0.31	21±0.21	26±0.21	-	36±0.37	16±0.23

Ec: *Escherichia coli*, Pa: *Pseudomonas aeruginosa*, Pf: *Pseudomonas fluorescens*, Sa: *Staphylococcus aureus*, Sm: *Streptococcus mutans*, Ct: *Candida tropicalis*, Ca: *Candida albicans*, Cg: *Candida glabrata*

\*Mean diameter± Standard deviation

**Table 2.** Minimum inhibitory concentration (MIC) of crude extracts of *A. niger* MTCC 12676 prepared in different solvents against the test pathogens

Solvent	Diameter of zone of inhibition (mm)							
	Gram-negative			Gram-positive			Yeast	
	Ec	Pa	Pf	Sa	Sm	Ct	Ca	Cg
Methanol	-	-	-	-	-	-	-	-
Petroleum Ether	-	-	-	-	-	-	-	-
Ethanol	0.125	-	1.25	0.125	1.25	-	1.25	2.5
n-Butanol	-	-	-	-	-	-	-	-
Ethyl Acetate	0.312	-	0.312	0.0625	0.0625	-	0.312	2.5

Ec: *Escherichia coli*, Pa: *Pseudomonas aeruginosa*, Pf: *Pseudomonas fluorescens*, Sa: *Staphylococcus aureus*, Sm: *Streptococcus mutans*, Ct: *Candida tropicalis*, Ca: *Candida albicans*, Cg: *Candida glabrata*

\*Mean diameter± Standard deviation

**Table 3.** The antibiotic susceptibility pattern of test microorganisms

Strains Used	Ciprofloxacin	Fluconazole	Diameter Of Zone Of Inhibition (mm)***
<i>Escherichia coli</i>	+	-	30±0.33
<i>Pseudomonas aeruginosa</i>	+	-	32±0.67
<i>Pseudomonas fluorescense</i>	+	-	26±0.37
<i>Staphylococcus aureus</i> *	+	-	NA
<i>Streptococcus mutans</i>	+	-	28±0.45
<i>Candida tropicalis</i>		+	26±0.23
<i>Candida albicans</i>		+	29±0.13
<i>Candida glabrata</i> **		+	NA

\**Staphylococcus aureus*: resistant to Ciprofloxacin

\*\**Candida glabrata*: resistant to Fluconazole

\*\*\*Mean Diameter ± Standard Deviation

NA: No activity

**Table 4.** Synergistic effect of different solvent extracts of *A. niger* MTCC 12676 with selected antibiotics

Strains Used	Ciprofloxacin	Fluconazole	Crude Extract	Diameter of Zone of Inhibition (Mm)***	
				ET	EA
<i>Escherichia coli</i>	+	-	+	32±0.21	34±1.09
<i>Pseudomonas aeruginosa</i>	+	-	+	32±0.21	32±0.21
<i>Pseudomonas fluorescense</i>	+	-	+	27±0.67	28±0.76
<i>Staphylococcus aureus</i> *	+	-	+	18±0.78	22±0.33
<i>Streptococcus mutans</i>	+	-	+	30±0.23	32±0.21
<i>Candida tropicalis</i>	-	+	+	26±.43	26±.43
<i>Candida albicans</i>	-	-	+	38±1.09	38±1.09
<i>Candida glabrata</i> **	-	+	+	13±0.65	16±0.76

Abbreviations: ET: Ethanol, EA: Ethyl acetate

\*Mean Diameter ± Standard Deviation

diameter of the zone of inhibition. Similarly, a positive synergistic effect was observed in case of *C. albicans*, where zone of inhibition increased significantly in response to the combination of the two extracts with antibiotic fluconazole. However, no effect was observed in case of *C. tropicalis* and *C. glabrata*.

### CONCLUSION

A number of *Aspergillus species* have been studied to be the potent source of antimicrobial compounds, but a lot more still remained to be explored. In the present study, a novel strain of *Aspergillus niger* MTCC 12676 was isolated from the soil and investigated for the

antimicrobial activity against some disease causing microorganisms. The results obtained indicates that the crude extract of this fungi was very effective against the growth of these microorganism and could represent a possible source of antimicrobial compounds against the tested microorganisms. It was also revealed that fungi also inhibited the growth of *Staphylococcus aureus* and *Candida glabrata* both of which exhibited resistance against the commercial antibiotics ciprofloxacin and fluconazole, respectively. Further studies are required for the isolation and characterization of the antimicrobial compounds which could be used for pharmaceutical applications for treatment and control of infectious disease causing bacteria and fungus.

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