# Isolation, Screening and Elucidation of Antimicrobial compound from *Aspergillus niger*

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**ABSTRACT**:

In recent years, there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat microbial resistance. Malformin is a metabolic product of *Aspergillus niger* and shows antibacterial activity against a variety of gram positive and gram negative organism. The present study aims to evaluating the In vitro antibacterial activity of *Aspergillus niger* extract at different concentrations were screened against *Bacillus subtilis* (MTCC 1133), *Enterobacter sp.,* (MTCC 11819), *Escherichia coli* (MTCC 584), *Klebsiella pneumoniae* (MTCC 9544), *Proteus vulgaris* (MTCC 744), *Pseudomonas aeruginosa* (MTCC 1036), *Salmonella typhi* (MTCC 3231), *Shigella sp.* (MTCC 1457), *Staphylococcus aureus* (MTCC 9886), *Streptococcus faecalis* (MTCC 389) and *Vibrio harveyi* (MTCC 7954) by disc diffusion and cup plate method. The antimicrobial activity of fungal extract were comparable with the standard antibacterial agent, ampicillin and were found to be active against all the organisms tested.



**Key words**: *Aspergillus niger*, Fungal extract, Antibacterial activity,

## 1. INTRODUCTION

Fungi are a biotic component of hydro ecosystems and an important link in the chain of trophic transformations in aquatic biosystem. Fungi are eukaryotic, heterotrophic organisms including both single celled yeasts and multi-cellular filamentous fungi [Hyde and Bussaban., 2007; Shearer *et al.,* 2007]. In freshwater ecosystem water fungi play a crucial role in nutrient cycling by breaking down, and have the ability to decompose organic materials [Kendrick, 1981].

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. Secondary metabolites are of intense interest to humans due to their pharmaceutical and toxic properties. Each species of Aspergillus can produce a range of secondary

metabolites associated with fungal growth and development [Hunter,1975; Devi *et al.,* 2009]. In some cases these metabolites have been implicated in disease, since they appear to be virulence factors. Interestingly, many of these secondary metabolites have been used in medicine for their antiviral, antibacterial, tumor suppressing, anti—hypercholesterolemic and immune suppressant activities [Jayanthi *et al., 2011;* Tan and Zou., 2001; Zhang and Song., 2006].

*Aspergillus niger* is a versalite filamentous fungus found in the environment all over the world in soil and decaying plant material, and it has been reported to grow on a large number of foods and feeds [Chandra and Arora, 2011]. Phytochemical studies have shown that fungi with antimicrobial activity contain bioactive constituents such as tannins, flavonoids, alkaloids, saponins [Darah *et al.,* 2014]. Antimicrobial activities of Aspergillus sp. against some clinically significant human pathogens have been reported [Fawzy *et al.,* 2014; Keating *et al.,* 2003*;* Martinez-Luis *et al.,* 2012].

The aim of the study was explore the antimicrobial activity of mycelial extracts of *A.niger* against bacterial human pathogens strain.Considering the vast potentiality of freshwater fungi as a source of drug for therapeutic application, an investigation was undertaken to screen various phytochemicals from the *Aspergillus* sp. and to analyse their properties.



## *2.* MATERIALS AND METHODS

### 2.1 Isolation of the fungal species

The water sample was aseptically collected from the selected pond and well, in pre-sterilized containers. Samples were collected thrice in a season for a period of one year (February 2011 to January 2012) to study the diversity and isolation of fungi. Samplings were carried out early in the morning. 0.1 ml of sample was transferred to potato dextrose agar (PDA) supplemented with ampicillin (150 mg/l) and streptomycin (100 mg/l) to inhibit the bacterial growth. The plates were incubated at room temperature (30 ± 2°C) for 7 days. Isolated colonies were subcultured in a PDA slant for further studies.

### 2.2 Identification of organisms

The technique was adopted for identification of the unknown isolated fungi using cotton blue in lacto phenol stain. The species encountered were identified in accordance with [Cheesbrough, 2000; Singh, 2015].

### 2.3 Production of secondary metabolites from the fungal isolates

The fungal isolates, namely *Aspergillus niger*, were inoculated in Potato dextrose broth. The fermentation was carried out by the submerged fermentation. The spore suspension was inoculated in 250 ml Erlenmeyer flask containing 100 ml of the fermentation medium and incubated on a rotary shaker at 175 rpm and 28 °C for 7 days.

### 2.4 Extraction of secondary metabolites

The extracellular metabolites in the media were obtained by means of liquid—liquid extraction technique with ethyl acetate. The organic fractions were combined and the solvent removed under reduced pressure at 35 ºC to yield crude extracellular extracts. The fungal isolates were exhaustively extracted with methanol and the solvent removed under reduced pressure at 35 ºC to yield crude intracellular extracts [Zain *et al*., 2008]. The extracts were subjected to preliminary phytochemical and antimicrobial.

### 2.5 Preliminary phytochemical screening of fungal metabolites

The ethyl acetate extract of the selected fungi were analyzed for the presence of the secondary metabolites such as alkaloids, saponins, terpenoids, flavonoids, steroids, phenols, tannins, carbohydrates and proteins by standard methods given by [Harborne, 1998].



### 2.6 Antimicrobial activity

**Test microorganisms**

The bacteria such as *Bacillus subtilis* (MTCC 1133), *Enterobacter* sp., (MTCC 11819), *Escherichia coli* (MTCC 584), *Klebsiella pneumoniae* (MTCC 9544), *Proteus vulgaris* (MTCC 744), *Pseudomonas aeruginosa* (MTCC 1036), *Salmonella typhi* (MTCC 3231), *Shigella* sp. (MTCC 1457), *Staphylococcus aureus* (MTCC 9886), *Streptococcus faecalis* (MTCC 389) and *Vibrio harveyi* (MTCC 7954) were used. Their sensitivity to the reference antibiotics was checked using ampicillin as a positive control.

### 2.7 Agar disc diffusion method

The antibacterial substance impregnated on the paper disc diffusion through the agar and prevents growth of bacteria in areas where active concentration is reached. The Muller Hinton Agar medium is poured into the Petri plate. After the medium was solidified, the inoculums were spread on the solid plates with sterile swab moistered with the bacterial

suspension. The disc was placed in MHA plate at different concentrations (250 µg, 500 µg, 750 µg and 1000 µg) with antibiotic Ampicillin (1 mg/disc) as reference standard.

### 2.8 Cup plate method

Antimicrobial susceptibility testing of the crude intracellular and extracellular methanolic extracts of *Aspergillus* species were determined by the cup plate method.The antibacterial activity is expressed as the zone of inhibition in millimeters, which is measured with a zone reader [Seeley and Van Denmark, 1975].

## 3. RESULTS

### 3.1 Isolation and Identification

The summary of fungal species isolated in the present study is presented in Table 1. The study reveals that the occurrence of species from the samples of the pond and well water in the Muthalakurichy panchayat mostly contained more than one species. It has been observed that *Aspergillus sp*., dominated among all the sampling stations tested, a total of seven fungi were isolated and identified. The species *A. niger, A. flavus, A. fumigatus, A. granulosus, A. versicolor* belongs to family Trichocomaceae. *Tricoderma* sp., belong to Hypocreaceae, *Candida* sp., come under the family Saccharomycetae.



**Table 1.** Morphology and microscopic observation of fungal species.

| Morphology | LPCB stain | Microscopic observation |
|---|---|---|
| Formed white and quickly becoming black with conidial production. Reverse is pale yellow and growth may produce radial fissures in the agar. | *A. niger* | Hyphae are septate and hyaline. Conidial heads are radiate initially, splitting into columns at maturity. |
| Formed olive to lime green colonies with a cream reverse. Texture is woolly to cottony to somewhat granular. | *A. flavus* | Hyphae are septate and hyaline. Conidial heads are radiated to loosely columnar with age. Conidiophores are coarsely roughened, uncoloured, up to 800µm long×15-20µm wide. |

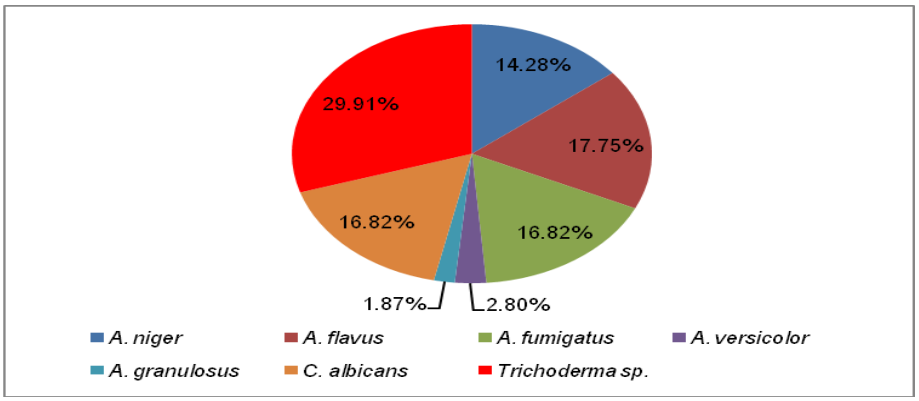
| Morphology | LPCB stain | Microscopic observation |
|---|---|---|
| White colony | *Tricoderma* sp. | Septate,hyaline,hyphae,conidiophores phalides and conidia are observed. |
| Gray-green with a slight yellow reverse. | *A. fumigatus* | Hyphae are septate and hyaline. Conidial heads are strongly columnar in an undisturbed culture. Conidiophores are smooth-walled uncoloured up to 300µm long and terminate in a dome shape vesicle. |
| Dull brown, plane or irregularly furrowed, mostly floccose, uneven in texture. | *A. granulosus* | Hyphae are septate and hyaline. Conidial heads radiate. Conidiophores are thin walled, smooth pale brown up to 350-500 long and 3.5-5.5 wide. |
| Variously coloured and may range from very pale green to greenish blue, pinkish green, salmon green or dark green. Reverse is reddish to uncoloured. | *A. versicolor* | Hyphae are septate and hyaline. Conidial heads are biseriate and loosely radiate. Conidiophores measure 120-700µm in length are hyaline to pale brown. |
| White to cream, soft and smooth to wrinkled | *Candida albicans* | Abundant branched pseudohyphae and true hyphae with blastoconidia are present. The blastoconidiaare formed in grape-like clusters along the length of the hyphae. |



### 3.2 Fungal diversity in freshwater ponds and wells

In monsoon season all ponds and wells showed maximum diversity of fungi such as *Aspergillus* sp. (60%), *Trichoderma* sp. (27.27%), *Candida* sp. (15.90%) except the pond 5 ($P_5$). The fungal species such as *A. versicolor* and *A. granulosus* are present only in monsoon season. In post-monsoon season *Aspergillus* sp. (52.77%), *Trichoderma* sp. (30.55%) and *Candida* sp. (16.66%) were observed [Fig. 1]. The diversity of fungi is less in pre-monsoon season is restricted to *Aspergillus* sp. (36.6%), *Trichoderma* sp. (40.9%), *Candida* sp. (22.7%).

**Figure 1.** Percentage of fungal diversity in water samples




### 3.3 Phytochemical analysis

The preliminary phytochemical screening of all the isolates showed the presence of secondary metabolite secretions such as flavonoids, tannins, phenols, steroids, alkaloides, terpenoids carbohydrates, proteins and saponins. The result of qualitative analysis of the phytochemicals were summarised in Table 2. The chemical composition of fungi varied depending on the species; however, *A. versicolor* extracts lack alkaloids and saponins.

**Table 2.** Phytochemical analysis of fungal sp

| **Compounds** | ***A. niger*** | ***A. fumigatus*** | ***A. versicolor*** | ***Trichoderma* sp.** |
|---|---|---|---|---|
| Alkaloids | + | + | - | + |
| Saponins | + | + | - | + |
| Terpenoids | + | - | - | + |
| Flavonoids | + | + | + | + |
| Steroids | + | _ | _ | - |

| | | | | |
|---|---|---|---|---|
| Phenols | + | + | - | + |
| Tannins | + | + | + | + |
| Carbohydrates | + | + | + | + |
| Proteins | + | + | + | + |

+ = presence, - = Absence

### 3.4 Agar disc diffusion method

**Antibacterial activity of crude extract from *A. niger***

*A. niger* had broad spectrum activity against tested bacterial isolates. It possess more potent activity having the zone of inhibition ranging from 14.63 ± 0.189 mm to 7.3 ± 0.216 mm. The present investigation showed the efficiency of the extracts against the selected pathogenic bacteria [Table:3] The highest activity observed was14.63 ± 0.189 mm against *E.coli* and lowest activity in *K. pneumoniae* 7.3 ± 0.216 mm.

**Table 3.** Antibacterial acitivity of the extracts of *A. niger*.

| **Organism** | **Zone of inhibition in mm** | | | | **Std-Ampicillin (1 mg/ml)** |
|---|---|---|---|---|---|
| | **250 µg/ml** | **500 µg/ml** | **750 µg/ml** | **1000 µg/ml** | |
| *Bacillus subtilis* | 10.87 ± 0.094 | 11.9 ± 0.163 | 13.87 ± 0.094 | 14.1 ± 0.082 | 16 ± 0 |
| *Enterobacter* sp. | 6.93 ± 0.125 | 8.87 ± 0.047 | 8.03 ± 0.33 | 9.07 ± 0.047 | 11 ± 0 |
| *Escherichia coli* | 11.3 ± 0.141 | 12.77 ± 0.17 | 13.87 ± 0.125 | 14.63 ± 0.189 | 15 ± 0 |
| *Klebsiella pneumoniae* | 5.07 ± 0.047 | 5.93 ± 0.205 | 7.03 ± 0.047 | 7.5 ± 0.082 | 9 ± 0 |
| *Proteus vulgaris* | 7.00 ± 0.216 | 8.23 ± 0.189 | 9.03 ± 0.189 | 9.37 ± 0.094 | 10 ± 0 |
| *Pseudomonas aeruginosa* | 9.03 ± 0.047 | 9.7 ± 0.141 | 10.97 ± 0.17 | 11.83 ± 0.125 | 13 ± 0 |

| | | | | | |
|---|---|---|---|---|---|
| *Salmonella typhi* | 4.80 ± 0.082 | 5.93 ± 0.047 | 6.1 ± 0.082 | 8.07 ± 0.047 | 10.5 ± 0 |
| *Shigella* sp. | 8.00 ± 0.00 | 8.73 ± 0.205 | 9.67 ± 0.094 | 9.47 ± 0.047 | 10 ± 0 |
| *Staphylococcus aureus* | 9.9 ± 0.082 | 10.93 ± 0.094 | 11.27 ± 0.094 | 11.93 ± 0.125 | 13 ± 0 |
| *Streptococcus faecalis* | 5.87 ± 0.094 | 6.47 ± 0.047 | 7.07 ± 0.047 | 7.3 ± 0.216 | 9 ± 0 |
| *Vibrio harveyi* | 6.93 ± 0.17 | 7.93 ± 0.047 | 9.00 ± 0.141 | 8.57 ± 0.047 | 10 ± 0 |

### 3.5 Cup Plate Method

**Antibacterial activity of extra and intracellular extract of *A. niger***

The results revealed that both intracellular and extracellular extracts showed antimicrobial activity against the selected human pathogens. The extracellular extracts of *A. niger* showed highest inhibition activity against *E. coli* (17.13 ± 0.094 mm), *B. subtilis* (14.77 ± 0.094 mm), *P. aeruginosa* (13.8 ± 0.141 mm), and *S. aureus* (13.8 ± 0.141 mm) and it showed lowest inhibition zone against *S. typhi*, and *K. pneumoniae* (6.87 ± 0.094 and 6.07 ± 0.047 mm).



The intracellular extract of *A. niger* showed maximum inhibitory activity against *P. aeruginosa* (14.6 ± 0.141 mm), *E. coli* (13.97 ± 0.17 mm), *B. subtilis* (13.03 ± 0.047 mm) followed by *S. aureus* (12.93 ± 0.047 mm) and the lowest inhibitory activity was against *P. vulgaris* (6.8 ± 0 mm).

**Table 4.** Antibacterial activity of extract from *A. niger* by cup plate method.

| **Organism** | **Zone of inhibition in mm** | | **Std-Ampicillin (1 mg/ml)** |
|---|---|---|---|
| | **Extracellular (1 mg/ml)** | **Intracellular (1 mg/ml)** | |
| *Bacillus subtilis* | 14.77 ± 0.094 | 13.03 ± 0.047 | 14 ± 0 |

| | | | |
|---|---|---|---|
| *Enterobacter* sp. | 10.1 ± 0.082 | 9.53 ± 0.249 | 10.5 ± 0 |
| *Escherichia coli* | 17.13 ± 0.094 | 13.97 ± 0.17 | 18 ± 0 |
| *Klebsiella pneumoniae* | 6.07 ± 0.047 | 4.93 ± 0.047 | 6.5 ± 0 |
| *Proteus vulgaris* | 7.9 ± 0.082 | 6.8 ± 0 | 8.4 ± 0 |
| *Pseudomonas aeruginosa* | 13.8 ± 0.141 | 14.6 ± 0.141 | 16 ± 0 |
| *Salmonella typhi* | 6.87 ± 0.094 | 8.77 ± 0.047 | 9 ± 0 |
| *Shigella* sp. | 7.6 ± 0.141 | 7.97 ± 0.094 | 8 ± 0 |
| *Staphylococcus aureus* | 13.8 ± 0.141 | 12.93 ± 0.047 | 15 ± 0 |
| *Streptococcus faecalis* | 7.1 ± 0.141 | 8.07 ± 0.125 | 10 ± 0 |
| *Vibrio harveyi* | 9.63 ± 0.125 | 9.03 ± 0.094 | 10 0 |



## 4. DISCUSSION

Fungi are diverse in nature and water molds consequently exhibit seasonally in aquatic system. Results revealed that pond and wells possessed vast diversity of watermolds such as *Aspergillus* sp., *Tricoderma* sp., and *Candida* sp.

The fungal species isolated in the present study showed the presence of various phytochemicals like phenols, tannins, alkaloids, terpenoids, sterols, carbohydrates, proteins and flavonoids. Phenols, tannins, and flavonoids amount were found high in *A. niger*. These phenolic compounds are antioxidant constituents and are beneficial in terms of nutritional value [Ayala-Zavala *et al*., 2012]. The major natural products of secondary metabolism in fungi are phenolic compounds. The flavonoids and phenols have antioxidant activity [Chandra, P, Arora 2012; Govindappa *et al., 2011;* Yadav *et al.,*

2014] .These compounds possess a diverse range of beneficial biological activities, which contribute to their potent effects on inhibiting carcinogenesis.

The antimicrobial activity of intracellular and extracellular extracts of *A. niger* was active against tested bacterial strains comprising both Gram negative and Gram positive organisms. Recently [Radji *et al*., 2010] used the crude ethyl acetate extract of fungi, and found to have antibacterial activity against various bacteria such as, *S. aureus, B. substilis, E. coli, P. aeroginosa, S. typhi* and *V. harveyi.* The antimicrobial activity of the crude extract from *A. niger* was found to be higher than the *A. fumigatus*. The results obtained were similar to that of previous reports. It was previously reported the varying effect of antimicrobial and anticancer activity of some soil fungi extracts depend upon the type of isolated fungi [Ning *et al*., 2003, Johannes *et al.,* 2009].The intracellular extracts of *A. niger* showed highest activity against Gram positive bacteria than the Gram negative bacteria. These differences may be reasoned to the fact that the cell wall in the Gram positive bacteria is composed of a single layer whereas the Gram negative cell wall is multilayered structure [Yao and Moellering, 1995].

## CONCLUSION

The present investigation clearly shows that *A.niger* possess novel metabolites with antimicrobial activity against microbial pathogens. Antimicrobial compound isolation from fungi can meet the ever growing need of antimicrobial novel compounds. Hence the *A.niger* might be promising source for novel antimicrobial and anticancer agents.

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