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Research Article

ISOLATION, IDENTIFICATION OF MARINE FUNGI WITH ANTIBACTERIAL ACTIVITY

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A B S T R A C T

The marine environment is a tremendous source of natural products. Marine microorganisms have become an important source of pharmacologically active metabolites. Marine fungi are the potential and promising sources for biologically active secondary metabolite production. Secondary metabolites are the chemical compounds that are produced during the stationary phase of the organism. Many years of study revealed that fungi are excellent sources for novel bioactive secondary metabolites. In the present study fungi were isolated from marine soil samples and serial dilution method was performed to isolate single colonies. Later microscopic and macroscopic observations were done to identify the organism. Organisms are inoculated into medium for production of secondary metabolites. One week later ethyl acetate is added to the broth in 1:1 ratio and kept for incubation in orbital shaker at 170rpm. Solvent is separated by separating funnel and is extracted by rotavapour to collect the crude extract.

INTRODUCTION

New trends in drug discovery from natural sources emphasize on investigation of the marine ecosystem to explore numerous complex and novel secondary metabolites. These secondary metabolites are the sources of new lead treatment of many diseases such as cancer, AIDS, inflammatory condition, arthritis, malaria and viral, bacterial, fungal diseases (Nazar *et al.,* 2009). In marine environment, the fungi produce a variety of molecules with unique structures and exhibit various biological activities (Ravikuamr *et al.,* 2010)

Since the early 1990's the oceans have been recognized as one of the latest untapped environments of unknown microorganisms. Up to then, the occurrence of true marine strains of fungi has been only occasionally reported. With the advent of molecular biology tools, it has been possible to isolate and identify obligate marine fungi present in the oceans. Continuous investigations demonstrated that marine microorganisms are an unlimited source of novel biologically active secondary metabolites (Fenical, 1993; Gallagher *et al.,* 2010; Hughes & Fenical, 2010; Jensen & Fenical, 1994; Kelecom, 2002; Newman & Hill, 2006). Marine-derived fungi, in particular, have yielded an increasing number of biologically active secondary metabolites (Bugni & Ireland, 2004; Saleem *et al.,* 2007; Rateb & Ebel, 2011).

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Majority of compounds isolated from marine-derived fungal strains are polyketidederived, alkaloids, terpenes, peptides, phenolic compounds, steroids and glycosides.

Bioactive compounds from marine fungi have extensive use in the treatment of many diseases and these compounds act as the templates for synthetic modification. Several molecules isolated from various fungi are currently involved in the advanced stage of clinical trials. The biologically active molecules isolated from the fungus *Aspergillus* sp. showed strong antibiotic, analgesic and anti-inflammatory properties (Rajeev *et al.,* 2004). Some of the *Aspergillus* sp. which produced industrially important biologically active secondary metabolites are *Aspergillus niger* and *Aspergillus oryzae*. Many secondary metabolites produced by selected strains of these fungi are capable of eliciting toxicity in animals. The Secondary metabolites produced by *A. niger* are Naphtho-r-pyrones, Nigerazine B, Nigragillin, Ochratoxin A. and *A. oryzae* are Aspergillomarasmine, Cyclopiazonic Kojic acid, Maltoryzine, Naphtho-r-pyrones (Cynthia *et al.,* 2004**).** The present study describes the antimicrobial ability of the crude extracts against the Gram positive and Gram negative bacterial and fungal organisms.

MATERIALS AND METHODS

Collection of soil samples

Marine soil samples were collected from Bay of Bengal, Kakinada, East Godavari District of Andhra Pradesh, India. To avoid contamination, collected soil samples were stored in pre-sterilized polythene bags and used for the isolation of **fungi.**

Isolation of Fungi from Soil by serial dilution method

Soil samples were collected from Bay of Bengal, Kakinada. One gm of the soil sample was diluted in 9 ml of sterile saline. Ten-fold serial dilution was carried out, 0.1 ml of 10^{-1} to 10^{-9} dilutions were placed on Potato Dextrose Agar plates using a spread plate technique supplemented with chloramphenicol to inhibit the growth of bacteria, also the pH of the medium was adjusted to 5.6 to encourage the growth of the fungi. The plates were incubated at room temperature (28^{o}C) for 96 hours

Potato Dextrose agar

Ingredients	gm/L
Potato infusion	200
Dextrose	20
Agar	20
Final pH	5.6

Primary screening of antibacterial activity of marine fungi

All isolated fungal strains were preliminary screened for antimicrobial activity against human pathogens such as *Staphylococcus aureus* (MTCC-3160), *Streptococcus pyrogenes* (MTCC-442), *Ksebsiella pneumonia* (MTCC-452), *Escherichia coli* (MTCC-443), *Bacillus coagulans* (MTCC-5856), *Bacillus subtilis* (MTCC-441), *Corynibacterium glutamicum* (MTCC-2745), *Spinghomonas paucimobilis* (MTCC-6363) using plug agar method. These cultures were procured from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained in freshly prepared potato dextrose agar slants. The organisms were preserved at - 20 ºC in the presence of glycerol (15 %, v/v) for longer periods.

Discs (9mm) from 7 days old culture of the fungal isolates maintained in potato dextrose agar medium were picked up by using a sterile cork borer and then agar plug with mycelia was placed on surface of Nutrient agar medium seeded with test organisms separately. The plates were incubated for 2-5days at 37^{o}C. After incubation the diameter of the zone of inhibition was measured.

Production of antibacterial activity of marine fungi

Preparation of Potato Dextrose Broth Media

24 gm of potato dextrose broth (Hi-media) was weighted and dissolved in 1000 ml of distilled water. This prepared media was sterilized in an autoclave at 121°C, 15 lbs pressure for 15-20min.

Potato Dextrose Broth

Ingredients	gm/L
Potatoes infusion from	200.000
Dextrose	20.000
pH	5.1

Production of Metabolites

The pure culture of fungi was inoculated on to the sterilized Potato Dextrose Broth and the culture flasks were incubated at 27^{o}C for one week. After one week incubation ethyl acetate was added to the broth in 1:1 ratio and kept for incubation in orbital shaker at 170rpm for 5hr. Minimal shaking is required for dissolving the metabolites in to ethyl acetate solvent.

Separation of Metabolites

The metabolites were now dissolved in ethyl acetate solvent and separated by using separating funnels. In the separating funnel the media was added with ethyl acetate. To that some amount of ethyl acetate was added and allowed it to settle for few minutes. Later two layers were observed in the separating funnel. The bottom layer was broth layer which was discarded and the upper layer of ethyl acetate with metabolites was collected which is called the organic layer. The washes were repeated for three times to extract the complete metabolites. This separated extract was Rota vapored.

Antimicrobial activity of fungal extracts

The increase in prevalence of multiple drug resistance has slowed down the development of new synthetic antimicrobial drugs, and has necessitated the search for new antimicrobials from alternative sources. Natural compounds are a source of numerous therapeutic agents. Recent progress to discover drugs from natural sources has resulted in compounds that are being developed to treat cancer, resistant bacteria and viruses and immunosuppressive disorders.

Antibacterial assay

The antibacterial activity of various fungal extracts was assayed by Agar well diffusion method. The minimum concentration of the fungal extract to inhibit the growth of the microorganism was also determined by broth dilution method.

Test Microorganisms

The Bacterial cultures of *Bacillus coagulans* (MTCC-5856), *Staphylococcus aureus* (MTCC-3160), *Bacillus licheniformis* (MTCC-429), *Corynibacterium glutamicum* (MTCC-2745), *Escherichia coli(MTCC-443), Pseudomonas fluorescence*(MTCC-2453), *Klebsiell apneumoniae* (MTCC-452), *Sphingomonas paucimobilis* (MTCC-6363) grown overnight at 37°C temperature were used for testing the antibacterial activity.

Agar-well diffusion method

Reagents

Muller Hinton Agar Medium (1 L)

The medium was prepared by dissolving 33.9 gm of the commercially available Muller Hinton Agar Medium (HiMedia) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto petriplates (25-30ml/plate).

Nutrient broth (1L)

One litre of nutrient broth was prepared by dissolving 13 gm of commercially available nutrient medium (HiMedia) in 1000ml distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

Chloramphenicol (standard antibacterial agent)

Muller Hintone Agar medium (High media) was dissolved in water in 100ml conical flask and was sterilized in an autoclave at 121^{o}C with 15lbp for 15min. After autoclaved the media was poured on to sterilized petriplates. Chloramphenicol was taken as positive control and DMSO was taken as negative

control for antibacterial activity. The antibacterial activity of fungal extract was evaluated by Agar well diffusion method (Perez *et al.,* 1990). Inocula were spread over the surface of agar plates with sterile glass spreader. To test the antibacterial activity of extracts final concentrations was made 60µg/ml and was poured on to the well and then plates were incubated for a period of 24h at 37^{o}C in incubater. After incubation diameter (mm) of the clear inhibitory zone formed around the well was measured.

Characterization of fungal isolates

Microscopic characterization

Identification of fungal isolates was done as per the manuals of Domsch *et al.,* (1980) and Barnett and Hunter (1972). After the isolation of fungal isolates they were subcultured on the PDA slants and plates. Later it was primarily subjected to the Lacto phenol cotton blue staining, and then analyzed the morphology by Scanning Electron Microscope (JSM-6610 instrument model, JEOL/EO format) to observe morphology of mycelium and spore structure.

Lactophenol cotton blue staining

A drop of the lactophenol cotton blue stain was placed in the center of clean glass slide. A fragment of the fungus colony was taken with a sterile needle and placed it on the drop of lacto phenol cotton blue stain. Gently the colony was teased and applied a cover slip. It should not be pushed down or tapped as this may dislodge conidia from the conidiophores. Then it was observed under required magnification.

Scanning electron microscopic (SEM) analysis

To observe the morphology of the selected fungal isolate under the SEM, the isolates were primarily fixed by 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 6) for 24hr at 4^{o}C and post fixed in 2% Osmium tetroxide for 4 hr in the phosphate buffer. Then these samples were washed and dehydrated with different concentrations of ethyl alcohol (30 to 80% for 20mimn). After completion of dehydration the samples were dried and mounted over the tube with double-sided carbon tape. A thin layer of gold coat was applied over the samples using an automated sputter coater by gold shadowing technique for 3 min, later the samples were scanned under SEM (JEOL-JSM-6610) at desired magnification.

Molecular Characterization of Fungal Isolates

ITS/5.8S rRNA gene sequence analysis

Molecular characterization of selected fungal isolate depending up on the diameter of zone of inhibition against selected human pathogens was done by intergenic transcribed spacer (ITS)/5.8S rRNA gene sequence analysis. The intergenic transcribed spacer (ITS) regions and the 5.8S rRNA gene sequence were used to analyze the interspecific relationships among the species. Taxonomic relationship among *Aspergillus* species is based on ITS sequence which explains the comparison with morphological and physiological features.

Phylogenetic tree

Construction of phylogenetic tree was based on evolutionary relationship of taxa based on ITS sequence data by Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree infereed from 100 replicates, which were taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). The evolutionary distances were computed using the Kimura2-parameter method (Kimura 1980) and evolutionary analyses were conducted in MEGA6 (Tamura 2011).

RESULTS AND DISCUSSION

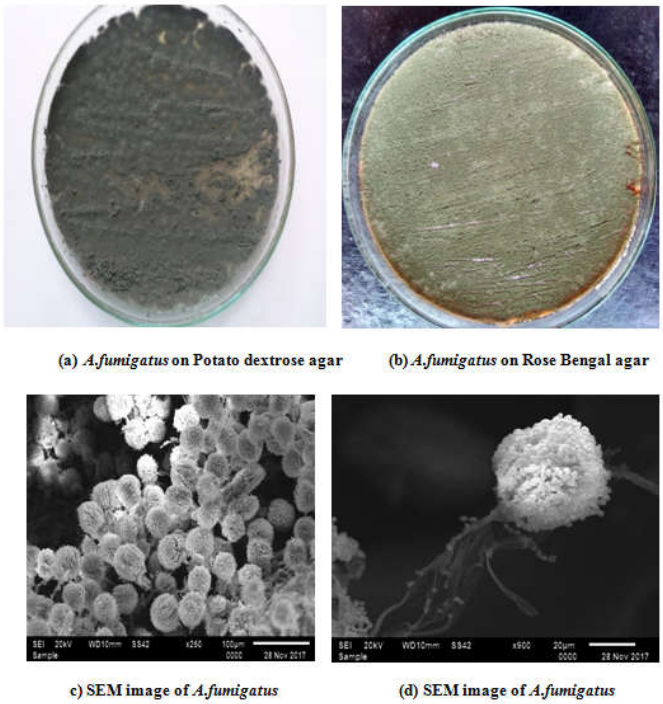
Isolation, Screening and Identification of fungal isolates

Morphological characterization of isolates

The isolation and identification of new bioactive compounds from marine microorganisms will be more effective in controlling diseases related to bacterial and fungal pathogens. Ability to produce large number of different secondary metabolites is associated mostly with the fungi. Isolation of fungi from soil samples and the rapid screening by plating on potato dextrose agar plates led to finding of fungal isolates capable of producing secondary metabolites. There are various methods for isolating the fungi but the simplest one is dilution plate method. In the present study 20 isolates from marine soil were identified. According to the colony characteristics, 20 isolates were screened from marine soil sample. The morphological features tabulated in Table 1. These 20 isolates were subjected for the primary screening of antibacterial activity of marine fungi. Pure cultures of common fungi were tentatively identified by comparing the characteristic features of fungi described in the manual of soil fungi (Gilman 1967). The isolated fungal strains were identified by at genus level on the basis of macroscopic characteristics like color, colony, morphology, shape, texture, diameter, and appearance of colony and by microscopic characteristics like mycelium, presence of specific reproductive structures, structure and shape of conidia and presence of mycelium.

Table 1 Characteristics of fungal isolates from marine soil

S.No	Isolate type	Diameter of colony (mm)	Color of colony	Reverse color
1	MF-1	25	Grey color	Cream
2	MF-2	62	Dark brown	Pale to yellow
3	MF-3	64	Black	Colorless
4	MF-4	60	Black powdery	Light green/Yellow
5	MF-5	5	Yellow	Yellowish green
6	MF-6	40	White	Yellowish white
7	MF-7	30	Brown	Light honey
8	MF-8	10	Black to grey with white layer	Cream
9	MF-9	20	Green to dark green	White to cream
10	MF-10	19	Black with white layer	Cream
11	MF-11	35	Black	Pale
12	MF-12	56	White with grey	Colorless to light yellow
13	MF-13	10	White with dusty yellow	White
14	MF-14	12	Dark green	Hyaline
15	MF-15	49	Light yellow	Yellow brown
16	MF-16	38	Green	Yellow brown
17	MF-17	20	Creamish white	Slightly cream
18	MF-18	19	White to pale yellow	Colorless to light orange
19	MF-19	67	White	Colorless
20	MF-20	43	Light greenish yellow	Light yellow



(a) *A.fumigatus* on Potato dextrose agar (b) *A.fumigatus* on Rose Bengal agar

c) SEM image of *A.fumigatus* (d) SEM image of *A.fumigatus*

Fig 1 Macroscopic (PDA and Rose Bengal agar) microscopic (Scanning electron micrograph SEM images)

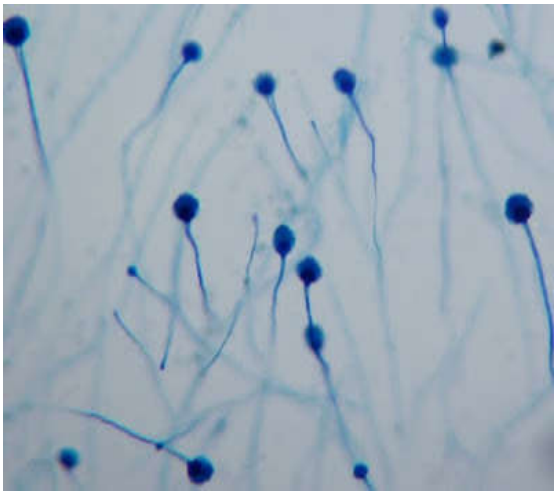


Fig 2 *Aspergillus fumigatus* (X250)

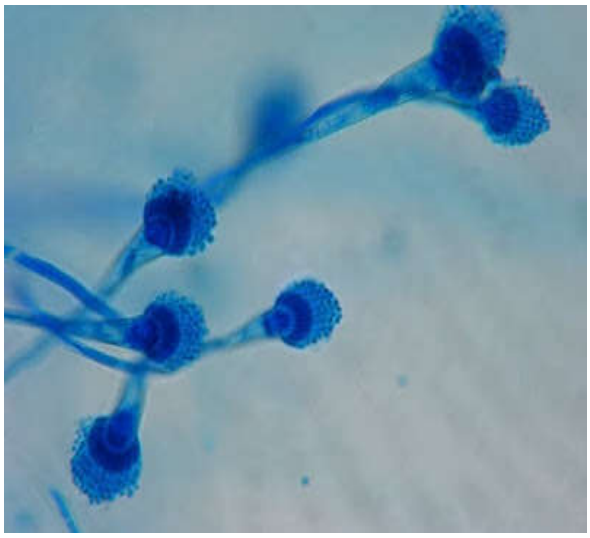


Fig 3 *Aspergillus fumigatus* (X400)

Primary screening of antibacterial activity of marine fungi

Primary screening for antimicrobial activity was done by the cross-streak method (Iwai *et al.,* 1982), on potato dextrose agar (PDA). Marine associated microorganisms are the potential sources for biotechnologically valuable compounds. The isolated fungal strains were screened by plug agar method against human pathogens *Bacillus coagulans* (MTCC-5856), *Staphylococcus aureus* (MTCC-3160), *Bacillus licheniformis* (MTCC-429), *Corynibacterium glutamicum* (MTCC-2745), *Escherichia coli* (MTCC-443), *Pseudomonas fluorescence* (MTCC-2453), *Klebsiell apneumoniae* (MTCC-452), *Sphingomonas paucimobilis* (MTCC-6363). Twenty isolated fungal strains exhibited antimicrobial activity against human pathogens. Among the 20 isolates, 7 isolates such as MF-1, MF-4, MF-6, MF-7, MF-10, MF-13 and MF-17 showed significant antimicrobial activity (Table 2) against human pathogens. Among these isolates MF-1 showed excellent antimicrobial activity against *Sphingomonas pacucimobilis* (16mm); *Bacillus subtilis* (19mm); *S.aureus* (17mm); *Streptococcus pyrogenes* (10mm); *E coli* (15mm); *B.coagulans* (17mm); *Corynibacterium glutamicum* (25mm); *Klebsiella pneumonia* (20mm) (Table1). The isolate MF-1 showed maximum zone of inhibition against *Corynibacterium glutamicum* (20mm).The fungal strains MF-1, MF-4, MF-6, MF-7, MF-10, MF-13 and MF-17 were selected for the secondary screening.

Table 2 Antibacterial activity of marine fungi (Zone of inhibition in mm)

Isolate	*Sphingo monas paucimobilis*	*Bacillus subtilis*	*Staphyl ococcus sureus*	*Strepto coccus pyrogenes*	*Esche richia coli*	*Bacillus coagulans*	*Coryni bacterium glutamicum*	*Klebsiella pneumoniae*
MF-1	16	19	17	10	15	17	25	20
MF-2	10	-	9	8	11	13	-	9
MF-3	-	11	-	-	9	15	13	8
MF-4	15	12	16	-	18	-	19	14
MF-5	12	-	14	10	9	-	-	-
MF-6	-	15	17	14	-	14	19	16
MF-7	18	17	10	19	-	12	20	14
MF-8	11	9	16	18	12	-	11	9
MF-9	10	-	13	11	9	-	12	15
MF-10	16	17	-	15	19	12	15	16
MF-11	9	10	11	-	16	15	12	-
MF-12	11	-	-	15	12	11	-	14
MF-13	-	14	15	12	10	-	17	19
MF-14	12	12	-	11	9	9	-	11
MF-15	16	-	17	11	12	10	16	11
MF-16	11	11	-	15	14	-	-	14
MF-17	13	-	10	14	-	17	15	19
MF-18	-	-	15	16	12	11	9	7
MF-19	8	11	-	12	10	9	13	14
MF-20	14	11	10	-	17	-	10	11

Secondary screening of fungal extracts for antimicrobial assay

The present study reveals that among 20 isolates, seven fungal isolates were found to be having more promising antibacterial activity against human pathogens and hence were selected for the production and extraction of secondary metabolites. Cephalosporin C is the first classical example of β-lactum antibiotic isolated from marine derived fungi *Cephalosporium acremoninum* (Blunden, 2001). The ethyl acetate extracts of the selected fungal isolates (MF-1, MF-4, MF-6, MF-7, MF-10, MF-13, MF-17) was yielded more production in potato dextrose broth. All the isolates showed promising growth during the production of bioactive secondary metabolites. In this study the culture filtrates of selected 7 isolates were used for bioassay against human pathogens by agar well diffusion assay. Among the 7 isolates MF-1 fungal extract showed

excellent antimicrobial activity against *Sphingomonas paucimobilis* (32mm); *Bacillus subtilis* (29mm); *S.aureus* (30mm); *Streptococcus pyrogenes* (34mm); *E.coli* (36mm); *B.coagulans* (30mm); *Corynibacterium glutamicum* (35mm); *Klebsiella pneumonia* (20mm) (Table 3 and Fig.4-11). The fungal isolate MF-1 showed maximum zone of inhibition against *E.coli* (36mm) and *Sphingomonas paucimobilis* (32mm), *Bacillus subtilis* (29mm), *Streptococcus pyrogenes* (34mm), *Bacillus coagulans* (30mm), *Corynibacterium glutamicum* (35mm), *Klebsiella pneumonia* (24mm), *Staphylococcus aureus* (30mm). The isolate MF-6 also showed good antimicrobial activity against *Sphingomonas paucimobilis* (24mm); *Bacillus subtilis* (27mm); *Streptococcus pyrogenes* (34mm); *E.coli* (26mm); *B.coagulans* (26mm); *Corynibacterium glutamicum* (29mm); *Klebsiella pneumonia* (27mm) (Table 3). Maximum zone of inhibition showed against *Corynibacterium glutamicum* (29mm).

Table 3 Antibacterial activity of fungal extract

Organism	Zone of inhibition (mm)						
	MF-1	MF-4	MF-6	MF-7	MF-10	MF-13	MF-17
Sphingomonas paucimobilis	32	-	24	21	-	19	10
Bacillus subtilis	29	-	27	15	10	20	-
Streptococcus pyrogenes	34	30	34	29	21	20	28
Escherichia coli	36	19	26	20	17	16	27
Bacillus coagulans	30	27	26	24	28	22	25
Coryni bacterium glutamicum	35	25	29	28	29	30	24
Klebsiella pneumonia	24	26	27	29	22	29	21
Staphylococcusaureus	30	18	15	22	32	19	20

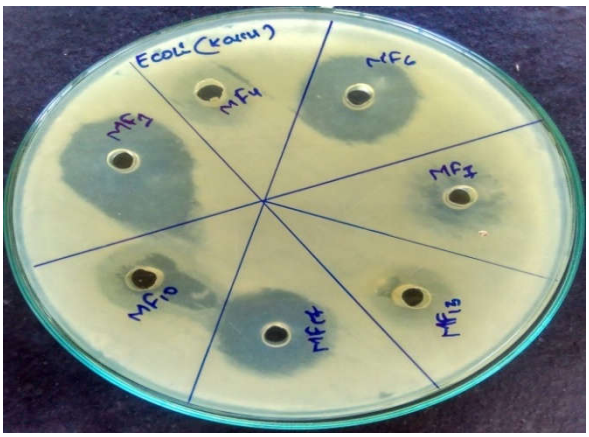


Fig 4 Antibacterial activity against *Escherichia coli*

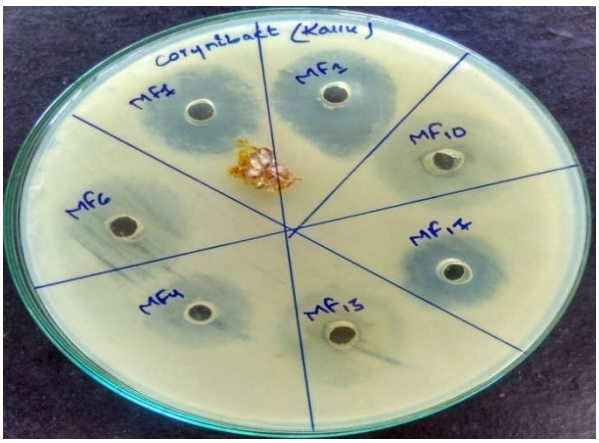


Fig 5 Antibacterial activity against *Corynibacterium glutamicum*

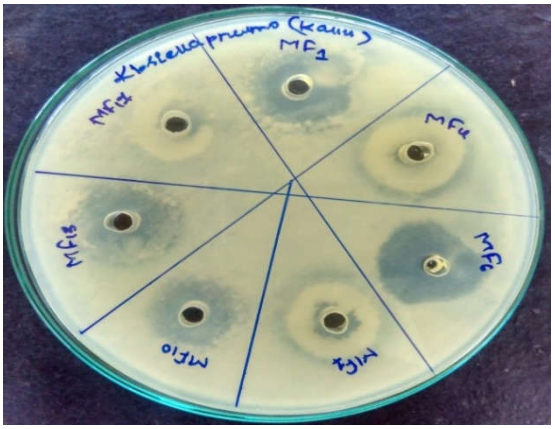


Fig 6 Antibacterial activity against *Klebsiella pneumoniae*

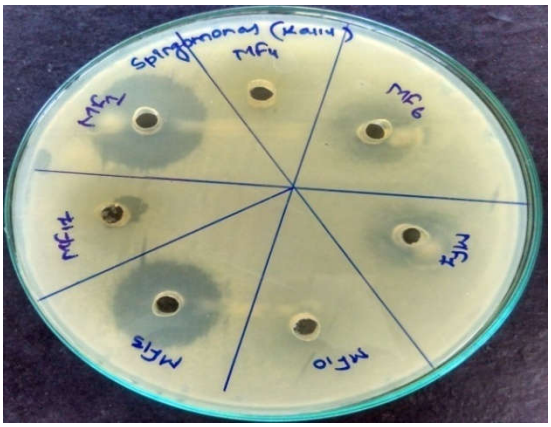


Fig 7 Antibacterial activity against *Sphingomonas paucimobilis*

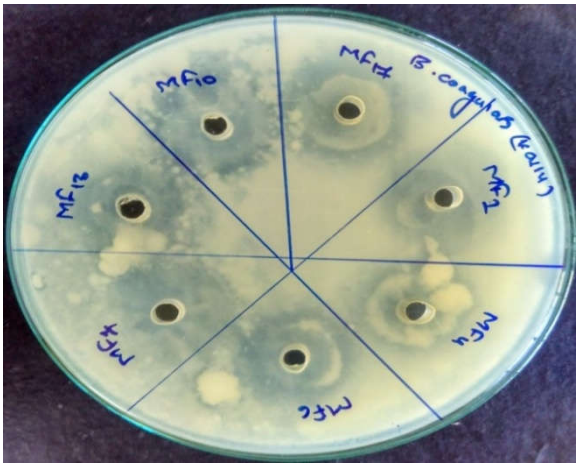


Fig 8 Antibacterial activity against *Bacillus coagulans*

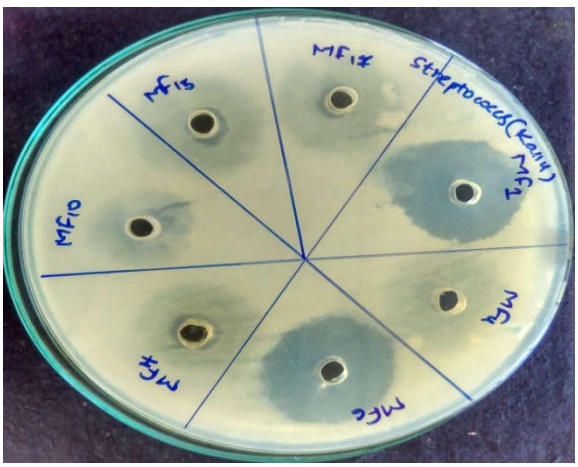


Fig 9 Antibacterial activity against *Streptococcus pyrogenes*

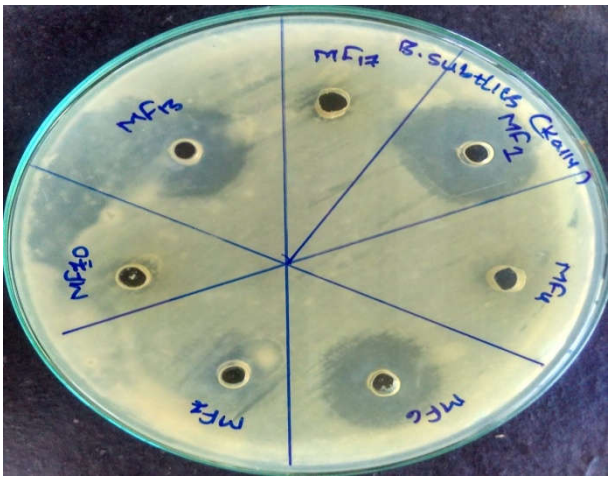


Fig 10 Antibacterial activity against *Bacillus subtilis*

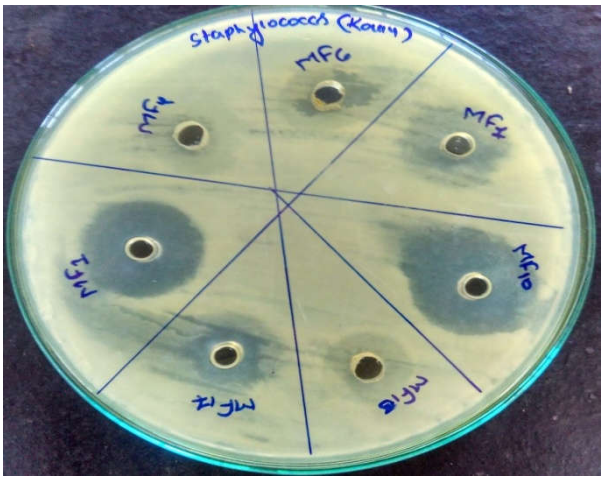


Fig 11 Antibacterial activity against *Staphylococcusaureus*

Molecular characterization of Aspergillus fumigatus

Among the 7 isolates, MF-1 showed maximum zone of inhibition against human pathogens and then the MF-1 isolate was subjected to molecular characterization. Molecular characterization of the isolate (MF-1) was performed by ITS/5.8 rRNA gene sequence analysis and the isolate confirmed as *Aspergillus fumigatus*. ITS/5.8 rRNA gene sequence and phylogenetic Evolutionary relationship of taxa based on ITS sequence were shown in Figure 2 and 3. Park *et al.,* (2007) and Litaker *et al.,* (2007) reported that intergenic transcribed spacer (ITS) region was found to be most promising for identification of the *Aspergillus* species. Witiak *et al.,* (2007) reported that the Bootstrap support was generally used for relationship among species with in sections and subgenera.

TCTATCGTACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACG
GCCGCCGGGGAGGCCTTGCGCCCCCGGGCCCGCGCCCGCC
GAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCT
GAGTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGAT
CTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCG
ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTC
TTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATG
CCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGT
GTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAA
GGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGG
CTTTGTCACCTGCTCTGTAGGCCCGGCCGGCGC

Fig 12 *Aspergillus fumigatus*, Strain "(MF-1)", ITS/5.8S rRNA gene sequence data

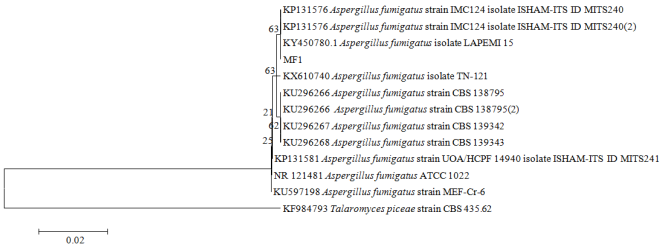


Fig 13 Evolutionary relationship of taxa based on ITS Sequence data

The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.,* 1987). The optimal tree with the sum of branch length = 0.16284750 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein *et al.,* 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 424 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

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