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MOLECULAR PROFILING, *IN-VITRO* ANTIMICROBIAL AND ANTIOXIDANT ASSAYS OF ANTAGONISTIC FUNGUS *ASCOTRICHA SINUOSA* VJCH-18 FROM MANGROVES OF ANDHRA PRADESH, INDIA

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Abstract– The fungi from mangrove habitats are the immense source for biologically active secondary metabolites with therapeutic potential. The objective of the present study is to screen the potent fungi from mangrove sediments that could produce secondary metabolites active against pathogenic microbes. Soil samples collected from mangrove regions of Adavuladeevi, South coast of Andhra Pradesh, India were employed for isolation of fungi using serial dilution plate technique. To isolate fungi, three media viz. Czapek-Dox agar, potato-dextrose agar and Sabouraud-dextrose agar media supplemented with 2% sodium chloride were used. 18 fungal strains isolated from the samples were designated as VJCH-1 to VJCH-18 and evaluated for antimicrobial and antioxidant activities. Among them, VJCH-18 showed broad spectrum of antimicrobial and antioxidant activities. The strain was cultured in Sabouraud-dextrose broth to record growth phases. Ethyl acetate extract of culture broth was active against bacteria *(Escherichia coli, Proteus vulgaris)* and fungi (*Candida albicans*). The strain exhibited significant antioxidant activity against DPPH free radicals. Characterization of the strain was performed based on cultural, morphological and genomic characteristics using 18s r RNA sequencing and identified as *Ascotricha sinuosa* VJCH-18 (GenBank accession number: MK463994). The study suggested that fungi from unexplored mangrove habitats of South coast of Andhra Pradesh serve as a source for antimicrobial and antioxidant compounds.

INTRODUCTION

New trends in drug discovery from natural sources emphasize the need to explore mangrove ecosystem for novel chemical entities. Mangrove forests also referred to as mangrove swamps, tidal forests, tidal swamp forests etc. represent a transition zone between terrestrial and marine habitats (Kathiresan and Bingham, 2001; Khalil *et al.*, 2013). The mangrove habitat is an unexplored source for isolation of microbes that have the potential to produce bioactive secondary metabolites (Hyde, 1990). Recent reports revealed thatfungi of mangrove origin possess the capability to produce significant bioactive compounds (Zhou *et al.*, 2001). Extensive research has been done in recent years to unveil the bioactive potential of mangrove fungi. Many novel natural products from mangrove fungi inhibit or kill a wide range of harmful microbes that affect humans (Joel and Bhinba, 2013; Berby, 2005). Some of the bioactive secondary metabolites from fungi of mangrove origin include flavonoids, benzopyranones, phenolic acids, steroids, alkaloids, quinones, chinones, tetralones, terpenoids, xanthones etc. (Thatoi, *et al*., 2013) which are used in pharmaceutical and nutraceutical industries to produce anticancer, antioxidant, anti inflammatory, antidiabetic and other therapeutic agents (Firakova *et al*., 2007; Fernandes *et al*., 2015).

As part of our screening program to identify potential fungi from mangrove habitats, an attempt has been made to isolate fungi from mangrove

sediments of Adavuladeevi that possess antagonistic activity against microbes.

MATERIALS AND METHODS

Sample collection and isolation of fungi

Sediment samples were collected at a depth of 10 cm from mangrove habitats of Adavuladeevi (Lat, 15° 54'0 N; Long 80° 40'0 E), located at Guntur district of Andhra Pradesh, India. The samples were shade dried and used for isolation of fungi by serial dilution plate technique (Warcup, 1950) employing Czapek-Dox agar (CDA), Potato-dextrose agar (PDA) and Sabouraud-dextrose agar (SDA) media supplemented with 2% NaCl. Streptomycin (25µg/ml)was also added to retard bacterial growth. An aliquot of 0.1ml sample of each dilution (10^{-2} to 10^{-4}) prepared using serial dilution was spread evenly over the surface of media and incubated for 7-10 days at room temperature (35±2°C). The plates were examined for fungal colonies and representative colonies were picked up, preserved on SDA slants and stored at 4°C for further study.

Screening of fungi for the production of antimicrobial metabolites:

All the fungal strains were cultured in SD broth for twenty days. Culture broth obtained after filtration was extracted with ethyl acetate and tested for antimicrobial activity using agar-well diffusion assay against bacteria and fungi.

Test organisms

Bacteria: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (MTCC 3160), *Xanthomonas campestris* (MTCC 2286), *Bacillus megaterium* (NCIM 2187), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (ATCC 6380) and *Escherichia coli* (ATCC 9027).
Fungi: *Penicillium citrinum* (MTCC 6849) and *Candida albicans* (MTCC 183).

Identification of the potent fungal strain

Cultural, morphological characteristics of the strain VJCH-18

The potent fungal strain VJCH-18 was grown on different culture media such as SDA, CDA, NAM, MEA, YEA and PDA. After 7 days of incubation, colony characteristics were observed (Palacios, *et al.*, 2005). Initially, the fungal strain was identified basing on morphological features (Butler and Mann, 1959).

The conidial characteristic of strain was studied under the scanning electron microscope (SEM). The strain was fixed at 4°C for 4 h in 0.1 M phosphate buffer (pH 7.2) of 2.5% gluteraldehyde and post fixed for 4 h in 2% aqueous osmium tetroxide. Sample was dehydrated in different alcohol concentrations and dried to critical point drying with CPD (EMS 850) unit. The processed sample was mounted over stubs with double-sided carbon conductivity tape and a thin layer of gold coat over the samples was made using an automated sputter coater for 3 minutes and scanned under SEM (Model: JOEL-JSM 5600) at required magnifications (Lakshman, 2019).

Molecular profiling of the strain VJCH-18

The molecular analysis of fungal strain VJCH-18 was carried out using 18s r RNA sequencing. The strain was identified based on internal transcribed spacer (ITS) sequences. Genomic DNA extraction, amplification and 18S rRNA gene sequencing were performed as described in protocol (Chiranjeevi and Vijayalakshmi, 2020). Fungal genomic DNA was extracted from mycelia by using the cetytrimethyl ammonium bromide method. The rDNA ITS region was amplified using primers ITS1 and ITS4. The genomic analysis and calculation of pair wise 18S rRNA gene sequence similarities were obtained using BLAST. Phylogenetic tree was constructed with MEGA version 6.0 after multiple alignments of data (Lim *et al*., 2014).
GenBank Accession: The 18S rRNA gene sequences of the strain VJCH-18 were submitted in NCBI (National Center for Biotechnology Information).

Growth pattern of the strain VJCH-18

To determine the growth pattern, the culture was inoculated in to SD broth amended with 2% NaCl with pH 6.0 and incubated at 30±2°C at 180 rpm. The flasks were harvested at 4 day interval up to 28 days and growth was determined in terms of dry weight of biomass. The culture filtrate obtained after separating the biomass was extracted with ethyl acetateand evaporated to dryness in a water bath (Westley *et al*., 1997).

Extraction of secondary metabolites from broth and Bioactivity assay

The homogenous culture suspension prepared by suspending one week old culture in sterile saline was used to inoculate SD broth (seed medium) and incubated at 30±2°C for 7 days at 180 rpm. Seed

culture @ 10% was transferred to fermentation medium (SD broth)and incubated at 30±2°C for 28days under agitation at 180 rpm. By solvent extraction method, the compounds were recovered from filtrate. To extract the compounds, ethyl acetate was added to filtrate (1:1) and shaken vigorously. Solvent extract was evaporated to dryness on a water bath and the residue thus obtained was used to determine antimicrobial activity. Ethyl acetate itself served as negative control. About 80 µl of the crude extract and 80 µl of negative control were poured into separate wells. The standard antibiotic disc was placed on the agar surface as a positive control. Plates were incubated at 35±2°C for 24 - 48 h andactivity of bioactive metabolites was assessed by measuring the diameter of the inhibition zone through agar well diffusion method (Perez *et al.,* 1990).

Screening the strain for antioxidant activity using 1,1-*diphenyl-2-picrylhydrazyl (DPPH)*

The antioxidant property of crude extract was evaluated by DPPH free radical scavenging assay (Ankita and Chakrabortyet, 2021). 3 ml each of 0.1 mM DPPH solution and ethanolic extract (100 - 500 µg/mL) were mixed and incubated at darkness for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517 nm and DPPH radical scavenging activity (%) was calculated by the following equation:

% scavenging rate = (Control – Sample / Control) X 100

The half maximal inhibitory concentration (IC_{50}) was calculated using regression analysis. IC_{50} values signify the concentration of sample required to scavenging 50% of the DPPH free radicals.

RESULTS AND DISCUSSION

Isolation and screening of fungi from mangrove sediments

A total of eighteen fungal cultures were isolated from the sediments of Adavuladeevi mangrove habitats. All the strains were subjected to screening through antimicrobial assay and the strain VJCH-18 exhibited broad spectrum of antagonistic activity against bacteria and fungi tested.

Cultural and morphological characteristics of the strain VJCH-18

Cultural characteristics of the strain VJCH-18 were studied on six different media *viz.* NAM, SA, CDA, MEA, YEA and PDA and results are presented in table 1. The strain exhibited excellent growth and produced pale yellow pigment on SA and MEA media. There was no Pigment production was not found on CDA, YEA and PDA. NAM did not support the growth. The colonies are fluffy, white becoming black when mature and rigid. Branched filamentous mycelium produced conidiophores which are erect and straight, smooth and initially hyaline gradually turned to brown. Conidia are smooth, ellipsoidal/spherical (1.94 to 2.75 µm)with aslightly protruding basal hilum (Fig.2) (Stchigel and Guarro, 1998).

Molecular profiling of the strain VJCH-18

The Phylogenetic tree was constructed using MEGA software version 6 using maximum parsimony method. The strain VJCH-18 was identified as *Ascotricha sinuosa* (Fig.1) (Cheng *et al.,* 2015; Pavlicek *et al.,* 1999; Tamura *et al*., 2013). The partial 18S rRNA sequence of strain was submitted to the GenBank database with accession number MK463994.

Growth pattern and antimicrobial profile of *Ascotricha sinuosa* VJCH-18

The growth curve and antimicrobial profile of *Ascotricha sinuosa* VJCH-18 were studied at regular intervals up to 28 days in batch culture. The strain entered into log phase on 12th day and continued up to 20 days followed by stationary phase extended for 24 days (Fig.3). The antimicrobial spectrum of

Table 1. Cultural and morphological characteristics of the strain VJCH-18

S.No.	Media	Colony colour	Texture	Growth	Pigmentation
1	SA	White	Fluffy	Excellent	Pale yellow
2	CDA	Light white	Fluffy	Good	No Pigmentation
3	NAM	-	-	-	-
4	YEA	Light white	Fluffy	Good	No Pigmentation
5	MEA	White	Fluffy	Excellent	Pale yellow
6	PDA	White	Fluffy	Good	No Pigmentation

(SA-Sabouraud Dextrose agar, CDA- Czapek-Dox agar, NAM- Nutrient agar, YEA- Yeast extract agar, MEA- Malt extract agar, PDA- Potato dextrose agar)

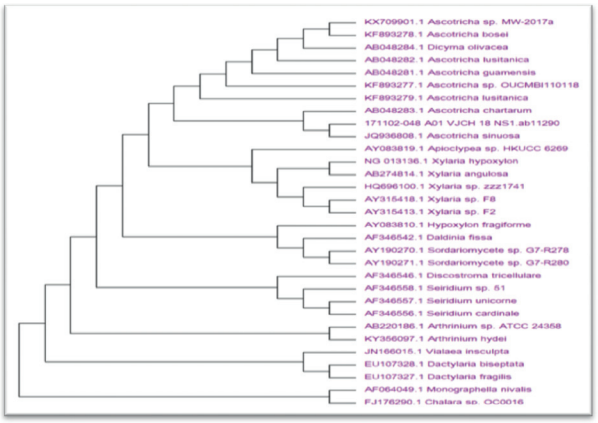
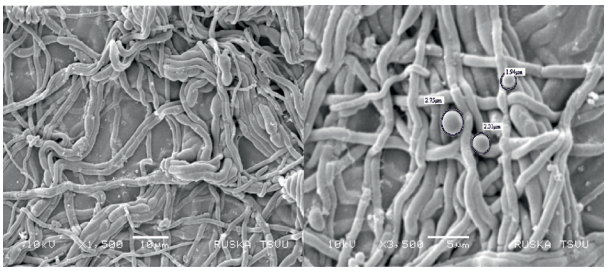


Fig. 1. Maximum parsimony tree based on partial 18S rRNA gene sequence showing relationship between strain VJCH-18 and related members of the genus *Ascotricha*



Mycelium Mycelium with conidia

Fig. 2. Scanning Electron Microscopic photographs of *Ascotricha sinuosa* VJCH-18

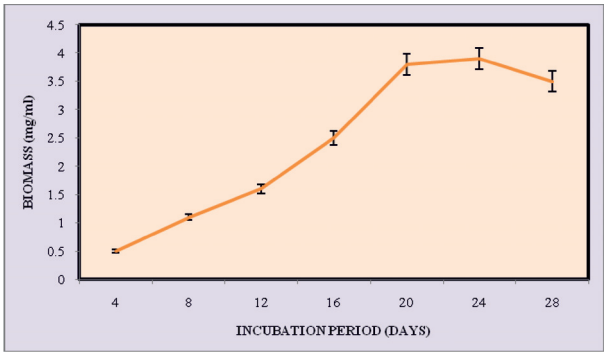


Fig. 3. Growth pattern of *Ascotricha sinuosa* VJCH-18

strain cultured on SD broth for 28 days was tabulated (Table 2). The secondary metabolites produced by 20 day-old culture showed high antimicrobial activity against *E. coli*, *P. vulgaris* and *C. albicans*. Production of antimicrobial metabolites by 20-day old *Simplicillium lanosoniveum* VJCH-19 of mangrove origin was reported (Chiranjeevi and Vijayalakshmi, 2020). while15-day old culture extracts of *Stachybotrys chlorohalonata* (Rajesh, *et al.*,

Table 2. Antimicrobial activity of *Ascotricha sinuosa* VJCH-18

Test Strains	Zone of inhibition (mm)
Bacteria	VJCH-18
Escherichia coli	30.18±0.20
Bacillus megaterium	27.08±0.06
Bacillus subtilis	28.10±0.10
Staphylococcus aureus	26.19±0.09
Proteus vulgaris	29.19±0.21
Pseudomonas aeruginosa	25.19±0.20
Xanthomonas campestris	26.32±0.36
Fungi	
Candida albicans	19.26±0.16
Penicillium citrinum	17.23±0.15

2014). and *Cladosporium cladosporioides* VJLB-37 (Bhavani and Muvva, 2020). showed high antimicrobial activity.

Antioxidant assay using *diphenylpicrylhydrazyl (DPPH)*

The crude extract of *Ascotricha sinuosa* VJCH-18 was evaluated for its antioxidant activity using DPPH. The DPPH produces violet/ purple color in ethanol and fades to shades of yellow color due to the presence of antioxidants. The optical density was measured at 517nm using UV-Vis spectrophotometer. A strong yellow color indicates high capability of extracts to scavenge free DPPH radicals and strong antioxidant potential. The antioxidant potential of ethyl acetate extract of *Ascotricha sinuosa* was explored in a dose dependent (100 – 500 µg/ml) manner as shown in Fig. 4. An increase in DPPH scavenging ability was observed with increase in concentration of extract. The results showed dose dependent scavenging activity and it was expressed as IC_{50} (µg/ml). The radical scavenging activity (%) was found to be 46.16±0.16, 55.67±0.09, 66.51±0.18, 72.84±0.21 and 78.52±0.14 at concentrations of 100,200,300,400 and 500 mg/ml (fig. 4).

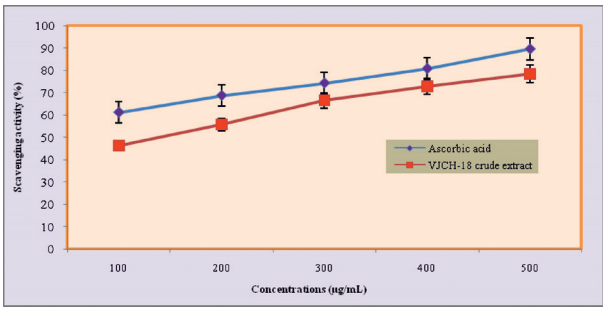


Fig. 4. Antioxidant assay of *Ascotricha sinuosa* VJCH-18

Statistical analysis: Values are the means of three replicates ± SD. The results are statistically analyzed and found to be significant at 5% level.

CONCLUSION

A potent fungal strain possessing antimicrobial and antioxidant activities was isolated from mangrove sediments and identified as *Ascotricha sinuosa* VJCH-18 based on cultural, and morphological features as well as genomic analysis. Further studies on purification and characterization of bioactive secondary metabolites of *Ascotricha sinuosa* VJCH-18 are in progress.

To the best of our knowledge, this is the first report on molecular profiling, *In-vitro* antimicrobial and antioxidant properties of potent fungus *Ascotricha sinuosa* VJCH-18 isolated from mangrove of Andhra Pradesh, India.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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