



## Biological activity of secondary metabolites isolated from mangrove fungi *Neurospora crassa*

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### Publication Info

Paper received:  
17 December 2011

Revised received:  
12 April 2012

Re-revised received:  
30 June 2012

Accepted:  
08 August 2012

### Abstract

The screening of antimicrobial compounds from mangrove-associated fungi is a promising way to meet the increasing threat of drug-resistant strains of human pathogens. In the present study, a novel foliar fungus was isolated from the leaves of *Rhizophora mucronata*. Crude fungal extracts were obtained through solvent extraction. The fungal extracts exhibited an effective antimicrobial activity against bacterial strains. This study reports the cytotoxic activity of the secondary metabolite produced by the fungus against Hep 2 (HeLa derivative) cell line by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay. GC-MS investigation of the secondary metabolites revealed the active components of the fungal metabolite. The DNA of the fungus was sequenced by amplifying the ITS region of the 5.8s rRNA to be submitted in the Genbank and an accession number was assigned confirming it to be a new strain.

### Key words

Antibacterial, Anticancer, Hep2

### Introduction

Infectious diseases are the leading cause of death worldwide and the consequences of antibiotic resistance has become a worldwide concern (Westh *et al.*, 2004). Hence there has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, mainly because of the indiscriminate use of commercial antimicrobial drugs commonly employed to treat infectious diseases. One well-established fact is the presence of antibacterial substances in the higher plants. (Srinivasan, *et al.*, 2001). So the search for new antimicrobial substances from various sources including medicinal plants species which have been serving as the best natural source of drugs and medicines since the beginning of civilization is still on by the scientific community. According to the recent research evidences, Indian mangroves contained antibacterial (Chandrasekaran *et al.*, 2009) and antifungal (Bose and Bose, 2008) properties. Mangroves and mangrove associates are widely used throughout the world

(Ravikumar *et al.*, 2010) in folklore medicines to combat diseases.

Mangroves are proved as the best biodiversity "hotspots" for fungi (Shearer *et al.*, 2007). These constitute the second largest ecological group of marine fungi (Sridhar, 2004). Endophytes of mangroves are an important group of marine fungi. Generally, they consist of those fungi with the ability to cycle nutrients that also involves degradation of mangrove detritus matter. In order to retain their predominance, they adopt chemical means as part of their competitive mechanisms. The metabolites they produce therefore possess various bioactivities

While mycotoxins are a sulking reality, a significant number of fungal metabolites have found utility as important pharmaceuticals and agrochemicals, or as lead compounds in the development of new drugs. More than 200 bioactive metabolites isolated from mangroves of tropical and sub-tropical populations that belong to steroids, triterpenes,

saponins, flavonoids, alkaloids, tannins and phenolic are found to have a wide range of therapeutic possibilities (Bandaranayake, 1998).

There is now a continuous and urgent need to discover new antimicrobials with diverse chemical structures and novel mechanism of action for new and reemerging infectious diseases (Rojas *et al.*, 2003). The main objective of this study was to screen the secondary metabolites from fungus, *Neurospora crassa* isolated from the leaves of mangrove plant *Rhizophora mucronata* for antimicrobial and cytotoxic activity.

### Materials and Methods

**Isolation and extraction of fungi :** Fresh elder leaves from mangrove species *R. mucronata* from Pichavaram mangrove forest southeast coast of India were collected, thoroughly washed and grinded using distilled water and seawater in 1:1 ratio under aseptic conditions. 1ml of the above was mixed with 10 ml of sterile water (distilled water: seawater; 1:1) to get dilution  $10^{-1}$ . The serial dilution was repeated till  $10^{-6}$ . From each dilution plating was done in Sabouraud's agar by spread plate technique and incubated at 27°C for 5 days and the pure culture was isolated on agar plate.

The pure culture was grown in Sabouraud's dextrose broth culture medium at 28°C for 5 days. Then a preinoculum was prepared by introducing small fragments (1cm square) of the growth culture into 250 ml Erlenmeyer flasks containing Sabouraud's dextrose broth and cultivated on a rotary shaker at 200rpm, 28°C for 5 days. Then the mycelium and the filtrate were separately subjected to solvent extraction using ethyl acetate, butanol and chloroform.

The fresh mycelium was washed thrice (distilled water: sea water 1:1), plotted between folds of Whatman filter paper no 1, crushed using mortar and pestle with ethyl acetate and methanol and subjected to sonication (Sartorius Labsonic) for 3-4 hrs for intracellular metabolites, further centrifuged at 2000-2500 rpm for 5 mins and the supernatant collected for studies. The filtrate was extracted several times with ethyl acetate, butanol and chloroform (v/v) in a separating funnel. The extracts from mycelia and filtrate were evaporated under vacuum at 50°C till dryness. The solid material was dissolved in ethyl acetate for bioassays.

**Antibacterial assay:** Antibacterial activity was carried out against a panel of laboratory standard pathogenic strains such as *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) by agar well diffusion method. Antibacterial activity was determined using the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003). Pre-warmed Mueller-Hinton agar (MHA) plates were seeded

with  $10^7$ - $10^8$  cfu suspension of test bacteria. Extracts (Biomass and filtrate) were pipetted (10 µl) onto sterile paper discs (6 mm diameter) and placed onto the surface of inoculated agar plates. Gentamicin sulphate (10 µg) was used as the positive control. Plates were incubated at 37°C for 48 hr. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts. Then different concentrations were performed for one solvent, which showed maximum activity against the bacterial pathogens.

**MTT assay for cytotoxicity :** Cytotoxicity of extracts at various concentrations (15- 1000 µg ml<sup>-1</sup>) was assessed for Hep2 and MCF7 using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) but with minor modification, following 72 hr of incubation. Assay plates were read using a spectrophotometer at 520 nm. Data generated were used to plot a dose-response curve of which the concentration of extract required to kill 50% of cell population (IC<sub>50</sub>) was determined.

$$\text{Cell viability (\%)} = \text{Mean OD/control OD} \times 100$$

**GC Mass analysis for quantification :** The crude extract was quantified using gas chromatograph (Shimadzu QP2010) equipped with a VF-5 ms column (diameter 0.25 mm, length 30.0 m, film thickness 0.25µm) mass spectrometer (ion source 200°C; EI -70 eV), programmed at temperature 40-650°C with a rate of 4°C min<sup>-1</sup>. Injector flow rate was 200°C; carrier gas was He of 99.9995% purity, column flow rate 1.51 ml min<sup>-1</sup>, injection mode -split.

**Fungal isolation and identification :** The total DNA of mangrove fungi was extracted using the EZNA kit (Omega). The internal transcribed spacers (ITS) of ribosomal DNA (rDNA) were amplified employing the combination of a conserved forward primer ITS1 (50- TCCGTAGGTGAA CCTGCGG-30) and reverse primer ITS4 (50- TCCTCCGCTTA TTGATATGC-30). The polymerase chain reaction product was about 0.7 kb. The purified ITS rDNA was sequenced. The sequence data have been submitted to GenBank with an accession number. The sequences were aligned manually using CLUSTAL X version 1.8 with sequences of representative strains retrieved from the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases. Phylogenetic trees were produced using the neighbor-joining algorithms from the PHYLIP package version 3.5c.

### Results and Discussion

Plants derived medicines have made significant contribution towards human health. Microorganisms found ubiquitous in all plant species in the world, contribute to their host plants by producing plenty of substances that provide protection and ultimately survival value to the plant.

**Table 1** : Antibacterial activity of the crude metabolite of *Neurospora crassa* extracted with different solvents

| Bacterial pathogens  | Z one of inhibition (mm) |          |         |          |            |          |
|----------------------|--------------------------|----------|---------|----------|------------|----------|
|                      | Ethyl acetate            | Standard | Butanol | Standard | Chloroform | Standard |
| <i>E.coli</i>        | 12                       | 13       | 12      | 10       | -          | -        |
| <i>P. aerogenosa</i> | 14                       | -        | -       | -        | -          | -        |
| <i>B. subtilis</i>   | 15                       | 12       | 15      | -        | 12         | -        |
| <i>P. vulgaris</i>   | 15                       | -        | -       | -        | -          | -        |
| <i>K. pneumonia</i>  | 13                       | 10       | -       | -        | -          | -        |

Many researches have proven that mangrove associated fungi are a new and potential source of novel natural products for exploitation in modern medicine, agriculture and industry (Jensen and Fenical, 2000).

Surface sterilization of the leaves of *R. mucronata* followed by cultivation using Sabouraud's agar media was the best way to isolate pure strains of fungi. Thus the isolate was identified as *Neurospora crassa* VB2 using ITS sequencing.

Crude extracts isolated from fungi grown in Sabouraud's broth were tested for their ability to inhibit growth of human pathogenic bacteria. Table 1 shows the antibacterial activity of *Neurospora crassa* (filtrate) extracted with butanol, ethylacetate and chloroform. In sharp contrast to the ethyl acetate extracts, butanol and chloroform extracts showed no appreciable activity. Successive isolation of secondary metabolites from fungi is also largely dependent on the type of solvent used in the extraction procedure (Rani Juneius and Selvin, 2012). It was found in this study that only the fungal extract of ethyl acetate provided more consistent antimicrobial and cytotoxic activity.

Ethyl acetate extracts from filtrate exhibited growth-arresting activity at different concentrations against the test organisms with an inhibition zone between 15 mm- 20 mm as shown in Table 2. Except *B. subtilis* the standard proved ineffective to inhibit the growth of the other four test organisms. The growth of *P. vulgaris* was not inhibited by lower concentrations (50ml and 100ml) of the metabolite but the inhibition zone at 125ml and 150ml was measured as 18mm and 20 mm. Inhibition zones were absent in the case of *E.coli* also except for one zone that measured 15mm at 125ml concentration. The zones of inhibition seemed to increase proportionately with the concentration of the extract.

As far the cytotoxic analysis against Hep2 cell lines were concerned IC50 values were found to be 125  $\mu\text{g ml}^{-1}$  with a cell viability of 46.15 at an absorbance of 0.24 (Table 3). However, these values represented in Table 3 shows that the values were within the cutoff point of the National Cancer Institute criteria for cytotoxicity ( $\text{IC}_{50} < 20 \mu\text{g ml}^{-1}$ ) in the screening of crude plant extracts. The GC MS analysis

**Table 2** : Antibacterial activity of the ethyl acetate extracts of *Neurospora crassa* at different concentrations

| Bacterial pathogens  | Zone of inhibition in mm for different concentration |                  |                   |                   |                   |
|----------------------|--|------------------|-------------------|-------------------|-------------------|
|                      | Standard   | 50 $\mu\text{l}$ | 100 $\mu\text{l}$ | 125 $\mu\text{l}$ | 150 $\mu\text{l}$ |
| <i>P. vulgaris</i>   | -  | -                | -                 | 18                | 20                |
| <i>B. subtilis</i>   | 12   | 15               | 17                | 15                | 20                |
| <i>P. aerogenosa</i> | -  | 17               | 15                | 15                | 18                |
| <i>E.coli</i>        | -  | -                | -                 | 15                | -                 |
| <i>K. pneumonia</i>  | -  | 12               | 17                | 18                | 20                |

**Table 3** : Cytotoxicity of *Neurospora crassa* on Hep2 cell line

| Concentrations ( $\mu\text{g ml}^{-1}$ ) | Dilutions | Absorbance | Cell viability |
|--|-----------|------------|----------------|
| 1000                                     | Neat      | 0.07       | 13.46          |
| 500                                      | 1:1       | 0.15       | 28.84          |
| 250                                      | 1:2       | 0.20       | 38.46          |
| 125                                      | 1:4       | 0.24       | 46.15          |
| 62.5                                     | 1:8       | 0.29       | 55.76          |
| 31.25                                    | 1:16      | 0.32       | 61.53          |
| 15.625                                   | 1:32      | 0.40       | 76.92          |
| Cell control                             | -         | 0.52       | 100            |

revealed that the active principals might be 9-Octadecenoic acid with the RT 34.012. Two new oxidized sterols obtained from the active fraction of a mangrove fungus *Aspergillus awamori* from the soils around the mangrove plant *Acrostichum speciosum* showed cytotoxic activity against A549 cell line and their structures were elucidated using spectroscopic methods as 22E-7 $\alpha$ -methoxy-5 $\alpha$ , 6 $\alpha$ -epoxyergosta-8(14), 22-dien-3 $\beta$ -ol and 22E-3 $\beta$ -hydroxy-5 $\alpha$ , 6 $\alpha$ , 8 $\alpha$ , 14 $\alpha$ -diepoxyergosta-22-en-7-one. (Hao *et al.*, 2010). Zheng *et al.* 2003, Deng *et al.*, 2007, Tariq *et al.* 2006 and Chen *et al.* 2006 reported the isolation of fungi from *A. marina*, and found that most of them showed antibacterial or antitumor activities. 3-chlordeoxylapachol, a secondary metabolite obtained from the chloroform extract of mangrove tree was active against KB human cancer cells in the murine hallow fibre antitumour model (Jones *et al.*, 2005). Also the antibacterial activity of aqueous and methanol extracts of leaves/shoots of five salt marsh halophytes and six mangroves was studied against methicillin resistant clinical

isolates of *Staphylococcus aureus* (Chandrasekran *et al.*, 2009).

The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). The percentage of similarity between the fungi and database suggests it as novel strain. Thus, the novel strain was named as *Neurospora crassa* strain VB2 and made publically available in GenBank with an assigned accession number HQ271350

Biological activity was examined in the crude extract of the secondary metabolite of the mangrove foliar fungus *Neurospora crassa*. The results indicated selective antibacterial activity of the extract isolated with ethyl acetate against five test organisms and cytotoxic activity against Hep 2 cell line thus showing that Pichavaram mangrove ecosystem is an important source of fungi possessing biologically active secondary metabolites.

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