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Antimicrobial activity of crude extracts from Mangrove-derived *Trichoderma* species against human and fish pathogens

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ABSTRACT

Mangrove environment holds a rich source for discovery of novel microbiota with potential applications in pharmaceutical science. Marine derived fungi are known to produce secondary metabolites with excellent biomedical applications when compared with that of terrestrial origin. The marine fungi produce bioactive compounds that could be used as a promising source to cure the human and fish diseases. Bearing this in mind, the rhizosphere fungi were isolated and tested for their antimicrobial properties. The potent fungal strain was chosen and subjected for mass scale cultivation followed by the extraction of secondary metabolites with ethyl acetate. The crude extract was examined for anti microbial and antioxidant properties. The results clearly states that the *Trichoderma* isolated from mangrove sediment are capable of suppressing the growth of human pathogens rather than fish pathogens and also hold significant antioxidant properties.

1. Introduction

Mangrove forests, the world's most productive ecosystems that enrich coastal waters, protect coastlines and enrich coastal waters with a yield of diversified commercial forest products, protect coastlines, and support coastal fisheries. However, the mangroves strives under extreme conditions such as highly fluctuating salinities, extreme tide actions, strong winds, high temperatures, muddy and anaerobic soils. There may be no other group of plants with such highly developed morphological, biological, ecological and physiological adaptations to extreme environmental conditions (Kathiresan et al., 2001). Mangrove environments hold a rich source for discovery of the new microbiota with extensive applications in pharmaceutical science (Gayathri et al., 2010; Boopathy and Kathiresan, 2010; Lin et al., 2001; Grant et al., 1996; Pointing and Hyde, 2000; Atri and Sharma, 2012). *Rhizophora annamalayana* is a natural mangrove, originated from the natural hybrid of *Rhizophora apiculata* and *Rhizophora mucronata*, and it is the only endemic species of Indian mangroves, confined to Pichavaram mangrove forest of Tamil Nadu (Kavitha and Kathiresan, 2011, 2012). Soil microorganisms associated with the rhizospheres of plants have been known to contribute in many processes in the soil which in turn may influence the plants growth and progression (Tilak et al., 2005; Shimoi et al., 2010). Some studies showed that roots of mangrove plants are a rich source of fungal endophytes (Manimegalai et al., 2013). Mostly the secondary metabolites are produced as a part of defense mechanisms against the predators or competitors (Vasant et al., 2013). Marine fungi are known to produce secondary metabolites with excellent biomedical applications when compared with that of terrestrial origin (Manimegalai et al., 2013). Endophytic fungi derived secondary metabolites possess high antimicrobial activity (Kaul et al., 2013). The marine fungi produce bioactive compounds with potent antibacterial, antifungal, anticancer, antiviral and anti-inflammatory properties that could be used as a promising source to cure the human diseases (Namikoshi et al., 2002; Jasti et al., 2005; Samuel et al., 2011). The present study attempted to test the antibacterial activity of trichoderma isolated from mangrove root soil against human and fish pathogens.

2. Material and methods

2.1. Sample collection

Mangrove rhizosphere samples were collected from Pichavaram (N11°25′26.7″, E079° 47′37.7″) Mangrove forest. Rhizosphere soil were collected from the rhizosphere of *Rhizophora annamalayana* at a depth of 5–10 cm by using corer. The samples were collected in sterile plastic bags and kept at 4 °C until transported to laboratory for further processing.

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2.2. Isolation of marine sediment fungi

The collected samples were serially diluted into 10^{-2}–10^{-5} (Askew et al., 1993). From the dilutions, the samples were placed on sterilized Trichoderma selective medium (TSM), using seawater and distilled water mixture in a ratio of 1:1 by pour plate technique, then the plates were incubated at 28 °C for 7 days with better fungal mat formation.

2.3. Identification of fungal strains

The isolated colonies were sub-cultured and maintained in TSM. The presumptive identification of the fungus was done on the basis of morphological examination of the lacto phenol cotton blue (Himedia) stained smear under microscope and stored at 4 °C for further use.

2.4. Extraction of secondary metabolites

Agar discs of 5 mm from isolated cultures was inoculated into 50 ml PDB (Potato dextrose broth) and kept in a shaker at 150 rpm for 5 days at room temperature. Inoculum was transferred to 1 L Erlenmeyer flask containing 500 ml PDB (Potato dextrose broth) broth and cultured for 28 days at room temperature. After 28 days of incubation, the medium was filtered using Whattman No.1 filter paper. The filtrates were poured in 1000 ml separating funnels and added equal volume of ethyl acetate and the residues (crude extracts) thus obtained were finally dried under rotary vacuum evaporator at 40 °C. These crude extracts were maintained at 4 °C for further studies.

2.5. Bacterial strains

Human bacterial pathogens such as *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Vibrio cholera* and *Bacillus cereus* were obtained from Microbial culture maintain Laboratory, Department of Medical Microbiology, Rajah Muthaiah Medical College, Annamalai University, Tamil Nadu, India. Fish pathogens such as *Vibrio parahaemolyticus* and *Vibrio harveyi* were obtained from Rajiv Gandhi Centre for Aquaculture, Nagapattinam District, Tamil Nadu, India.

2.6. Antibacterial assay for human and fish pathogens

The crude extracts were screened for the antibacterial activity using agar well diffusion method as described by Seedevi et al. (2013). The 24 h old cultures were swabbed in nutrient agar plates by using a sterile cotton swab aseptically. The wells were punched on swabbed plates using a sterile 5 mm well cutter. The stock solution was prepared at 10 mg/ml concentration in 10% DMSO. Four different concentrations such as 50, 100, 150 and 200 µg/ml were used. The standard tetracycline (1 mg/ml dissolved in 10% DMSO) and control 10% DMSO were loaded into the respectively labeled wells. The plates were incubated at 37 °C for 24 h. The results were obtained by measuring the diameter of inhibition zone for each well and expressed in millimeter.

2.7. Minimum inhibitory concentration (MIC)

The crude extract was determined for MIC (Seedevi et al., 2013). A stock solution of 1 mg/ml was prepared and was serially diluted to obtain various ranges of concentrations of (25, 75, 125, 150 and 200 µg/ml) and standard tetracycline (1 mg/ml), 0.5 ml of each of the dilutions contains 2.0 ml of nutrient broth. To the test tube, 0.5 ml of old bacterial culture was inoculated. A set of test tube containing broth alone was used as control. All test tubes and control were incubated at 37 °C for 24 h. After the period of incubation, the tube containing the least concentration of extract showing no visible sign of growth was taken as minimum inhibitory concentration.

2.8. FTIR analysis

The crude fungal extracts were analyzed using FT-IR Spectroscopy (Thermo Nicolet, USA). The diffuse reflectance technique was utilized in the mid-IR (500–4000 cm^{-1}) spectral region. The samples were mixed with KBr (about 200–400 mg) into a fine powder, placing the powder into the sampling cup, smoothing the powder, and compressing the powder bed into the holder using a compression gauge. The sample was placed in to light path and the spectrum was obtained by using ORIGIN (version 8.0).

2.9. Antioxidant activity

2.9.1. Total antioxidant activity

The total antioxidant activity was carried out according to the method described by Saravanakumar and Kathiresan (2014). Briefly, 2.0 ml of sample at various concentrations (10–160 µg/ml) was mixed with 1.0 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min under water bath. After the mixture had been cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The L-ascorbic acid was used as standards and the total antioxidant capacity is expressed as ascorbic acid equivalent.

2.9.2. Scavenging ability on DPPH radicals

The DPPH free radical scavenging activity of crude extract was determined. Briefly 0.1 mM solution of DPPH was prepared in 100% methanol, and 1 ml of this solution was added to 4 ml of sample in 40% methanol at various concentrations (10–160 g/ml). The mixture was shaken vigorously and incubated for 15 min at 30 °C in the dark. The reduction of the DPPH radical was measured by continuous monitoring of the decrease of absorption at 517 nm. The L-ascorbic acid was used as standards and the DPPH scavenging effect is calculated as follows:

$$\text{DPPH scavenging effect } (\%) = \frac{\Delta A \text{ sample} - \Delta A \text{ blank}}{\Delta A \text{ control}} \times 100$$

2.9.3. Superoxide radical scavenging activity (SOD)

The superoxide scavenging ability of crude extract was assessed by the method of Nishikimi et al. (1972). The reaction mixture, containing phosphorylated chitosan (0.05–0.5 mg/ml), PMS (30 mM), NADH (338 mM) and NBT (72 mM) in phosphate buffer (0.1 M, pH 7.4) was incubated at room temperature for 5 min and the absorbance was read at 560 nm against a blank. α-tocopherol were used as standard for comparison. The capability of scavenging the superoxide radical was calculated using the following equation:

$$\text{Scavenging ability } (\%) = \frac{\Delta A_{560} \text{ nm of control} - \Delta A_{560} \text{ nm of sample}}{\Delta A_{560} \text{ nmof control}} \text{x} 100$$

2.9.4. Hydroxyl radicals scavenging assay

The reaction mixture containing crude extract (50–3.2 mg/ml), was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 mM), EDTA (100 mM) and ascorbic acid (100 mM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C (Halliwell et al., 1987). The reaction was terminated by adding 1 ml of TBA (1%, w/v) and 1 ml of TCA (2% w/v) and then heating the tubes in a boiling water bath for 15 min. The contents were cooled and the absorbance of the mixture was measured at 535 nm