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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

against the reagent blank. BHA was used as standard for comparison. The decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

3. Results and discussion

Fungal strains were isolated from the rhizosphere of *R. annamalayana* in Pichavaram Mangrove forest. Five strains were found as predominant fungi and confirmed as *Trichoderma* species by Lacto phenol cotton blue staining method. Based on the morphological key characters and hyphae structures (Rifai, 1969), the strains were distinguished and designated as NPK1, NPK2, NPK3, NPK4 and NPK5 (Fig. 1).

3.1. FT-IR spectrum of crude extract of NPK2 strain

The crude extract was analyzed and the active five active peaks were obtained. Peak locations of FT- IR spectra were assigned for the crude extracts of *Trichoderma* sp., in the wavelength of 500–4000 cm^{-1} and are represented in Fig. 2. In the observed IR-frequency (νcm^{-1}) 2360.87 and 2106.27 νcm^{-1} was assigned to the primary amine. IR frequencies of 1653.00 corresponds to –C=O stretching of proteins. The frequencies of 1458.18 νcm^{-1} indicates N–H bending (CH_3-asymmetric bending of lipids), and 1097.5 represents the amide III (Po_2 symmetric stretching mainly nucleic acids). CH_3 bending absorption where IR-frequency 835.18 νcm^{-1} refers to CH out of plan bending occurs. Amide I of the crude extract was observed at a wave number of 1653 cm^{-1} against those of standard which is 1647 cm^{-1}. Amide II was represented at 1458.18 cm^{-1} against the standard (1461 cm^{-1}) which confirms CH_2 bend. The wave numbers of 1251.80 cm^{-1} correspond to the Amide III and can be compared with the standard of 1261 cm^{-1}. Peak at 835.18 cm^{-1} corresponds to the PO_2-asymmetric stretch DNA of (B-form) Adenine and it corresponds to the Amide III which was exhibited by a standard of 833 cm^{-1}. Peak at 648.08 cm^{-1} corresponds to the C≡C–II bending and it corresponds to the Amide III which is exhibited by a standard of 668 cm^{-1}.

3.2. Antibacterial activity of crude metabolites against human and fish pathogens

The crude ethyl acetate extract exhibited the wide spectrum activity against both human and fish pathogens. With increase in concentration the zone of inhibition was also found to increase (Table 1). In 200 µg/ml concentration, the crude extract showed the highest activity against *E. coli* and *S. aureus* at 19 mm and 18 mm, whereas the *B. cereus* and *V. cholerae* showed the moderate activity 17 mm and 16 mm respectively. The minimum zone of inhibition was observed in *B. subtilis* (13 mm). While against fish pathogen, *V. harveyi* showed maximum inhibition zone of 20 mm and *V. parahaemolyticus* showed 18 mm at 200 µg/ml. Comparing to both, the crude extract showed potential activities against human pathogens than fish pathogens (Table 2).

The growth of human pathogens was found considerably arrested with increased concentration. Minimum inhibitory concentration of the crude ethyl acetate extract against *B. subtilis* and *V. cholerae* was found at 200 µg/ml, similarly against *S. aureus* and *B. cereus* were recorded at 150 µg/ml whereas *E.coli* at 125 µg/ml. The MIC of crude extract against fish pathogens was found at 200 µg/ml and 150 µg/ml for *V. parahaemolyticus* and *V. harveyi* respectively. Leelavathi et al. (2014) have reported that the MIC of *T. harzianum* against bacterial pathogens ranged at lower concentration (50–100 µl/ml) which was found contradictory to the present results where the MIC was recorded in higher concentration. The present results are in accordance with Aytemir et al. (2003) who have reported that the MIC of crude extract of *Trichoderma* spp., was found at higher concentrations against human pathogens (150-256 µg/ml). This preliminary screening is an interesting evaluation of the potential antimicrobial activity of this crude extract. Our next approach will be focused on isolating and testing pure active compounds.

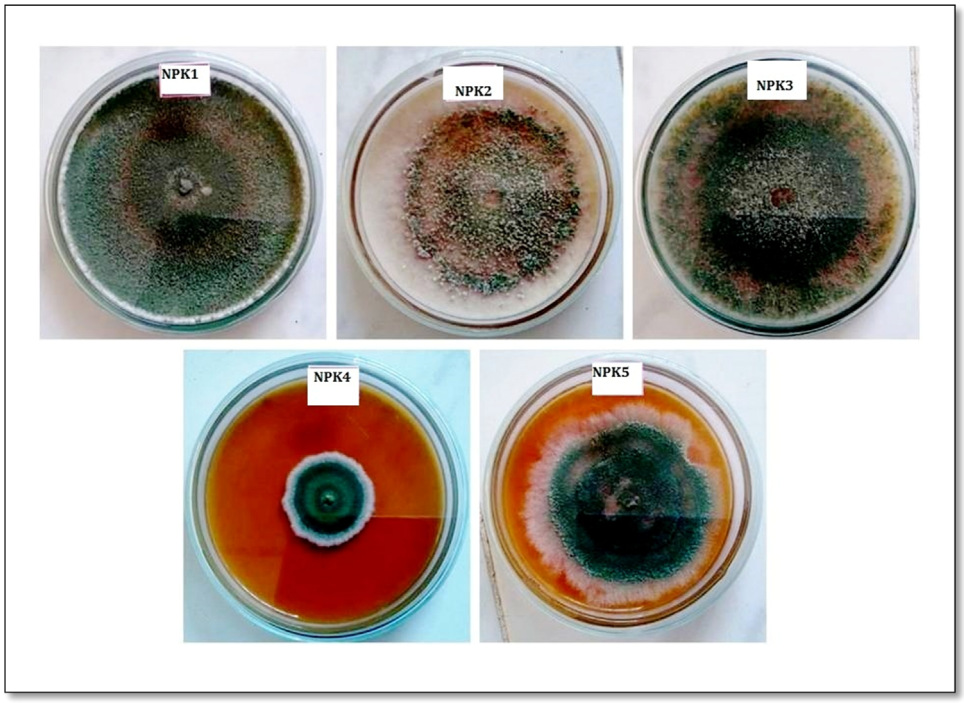

Fig. 1. Pure cultures of *Trichoderma* species.

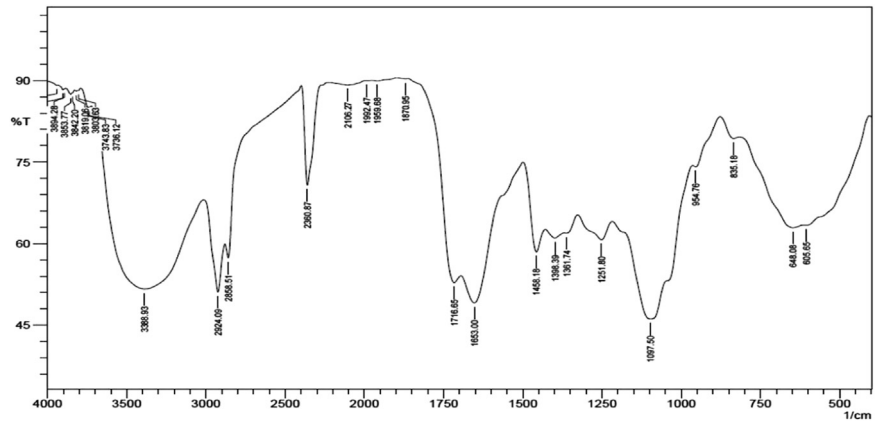

Fig. 2. FT-IR spectrum of crude extract of NPK2 strain.

Table 1
Antimicrobial activity of *Trichoderma* NPK2 crude extract against human and fish pathogens.

S. no.	Name of the strains	Zone of inhibition (mm)					
		50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	+*ve* (c)	-*ve* (c)
Human pathogens							
1	*E. coli*	14	16	17	19	24	–
2	*B. subtilis*	9	11	12	13	17	–
3	*S. aureus*	13	14	16	18	22	–
4	*V. cholerae*	12	13	13	16	19	–
5	*B. cereus*	13	14	15	17	21	–
Fish pathogens							
6	*V. parahaemolyticus*	11	13	14	15	18	–
7	*V. harveyi*	10	13	15	17	20	–

+++ Indicates highly turbid, ++ indicates turbid solution, + indicates cloudy solution, * indicates considerably arrest, – No growth, c-Control.

Table 2
Minimum inhibitory concentrations (MIC) of *Trichoderma* NPK2 crude extract against human and fish pathogens.

S. no.	Name of the strains	50 μg/ml	75 μg/ml	100 μg/ml	125 μg/ml	150 μg/ml	200 μg/ml	+*ve* (c)	-*ve* (c)
Human pathogens									
1	*E. coli*	+++	++	+	*	–	–	–	+++
2	*B. subtilis*	+++	+++	+++	+++	+	*	–	+++
3	*S. aureus*	+++	+++	++	+	*	–	–	+++
4	*V. cholerae*	+++	+++	+++	++	+	*	–	+++
5	*B. cereus*	+++	+++	++	+	*	–	–	+++
Fish pathogens									
6	*V. parahaemolyticus*	+++	+++	+++	++	+	*	–	+++
7	*V. harveyi*	+++	+++	++	+	*	–	–	+++

+++ Indicates highly turbid, ++ indicates turbid solution, + indicates cloudy solution, * indicates considerably arrest, – No growth, c-Control.

3.3. Antioxidant capacity of the crude fungal extract

The crude extract of NPK2 strain which revealed broad spectrum of antimicrobial activities was selected for its antioxidant properties such as total antioxidant activity, scavenging ability on DPPH radicals, superoxide radical scavenging activity and hydroxyl radicals scavenging assay. Total antioxidant activity and DPPH radical scavenging activity varied among the concentrations of the crude extract. The total antioxidant activity was found higher (48.75%) whereas L-Ascorbic acid showed 67.85% at 250 μg/ml concentration. DPPH radical scavenging activity was record maximum (54.61%) while L-Ascorbic acid showed 80.05% at 160 μg/ml concentration (Figs. 3 and 4).

Superoxide radical scavenging activity and hydroxyl radical scavenging activity were found maximum at 71.45% and 67.43% at 500 μg/ml respectively. SOD of L-Ascorbic acid was found to be 84.28% at 500 μg/ml concentration (Fig. 5). The hydroxyl radical scavenging activity of the standard (BHA) was found maximum (83.11%) at 500 μg/ml concentration. DPPH free radical scavenging assay is widely used as the most accurate screening method used to evaluate the antioxidant activity (Kalidasan et al., 2015) and also it is not affected by metals and enzyme inhibition (Saravanakumar and Kathiresan, 2014) (Fig. 6).

Trichoderma extract displayed high free radicals scavenging activity whereas hydroxyl radicals are sensitive and bring the damage to the adjacent molecules (Zanwar et al., 2010; Chance

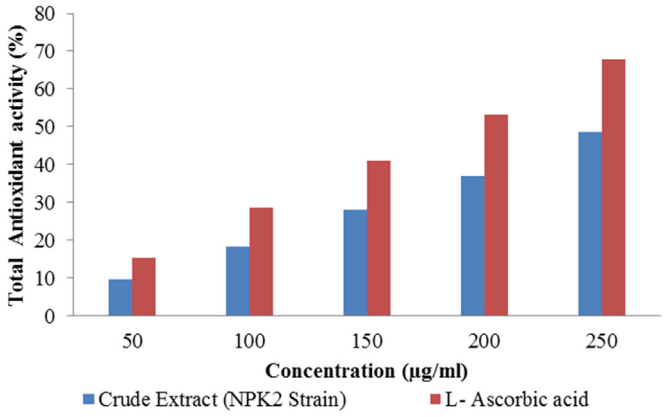

Fig. 3. Total antioxidant activity of Crude extract – NPK2 Strain.

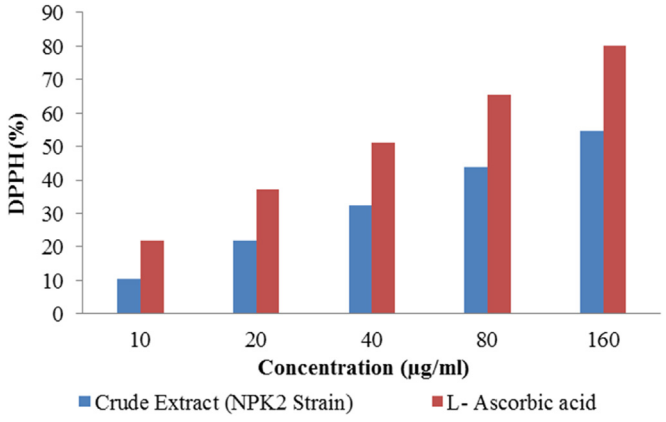

Fig. 4. DPPH antioxidant activity of Crude extract – NPK2 Strain.

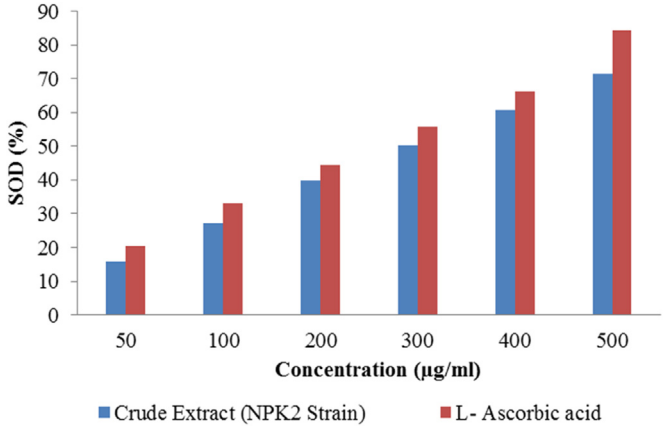

Fig. 5. Superoxide scavenging activity of Crude extract – NPK2 Strain.

et al., 1979). *Trichoderma* showed increasing hydroxyl scavenging activity with increasing concentration of the extract. Thus hydroxyl scavenging activity is highly dependent on concentrations of the crude extract (Saravanakumar and Kathiresan 2014).

The present results are in accordance with previous studies such as bacteria, fungi and plants (Lu and Foo 2000; Kim et al., 2002; Gomathi et al., 2013a; Saravanakumar and Kathiresan, 2014). Antioxidant activity has also been reported in endophytic fungal extract of *Trichoderma* (EMFCAS8) (Saravanakumar and Kathiresan, 2014). The present study reveals that mangrove derived *Trichoderma* sp., are rich source of natural antioxidants.

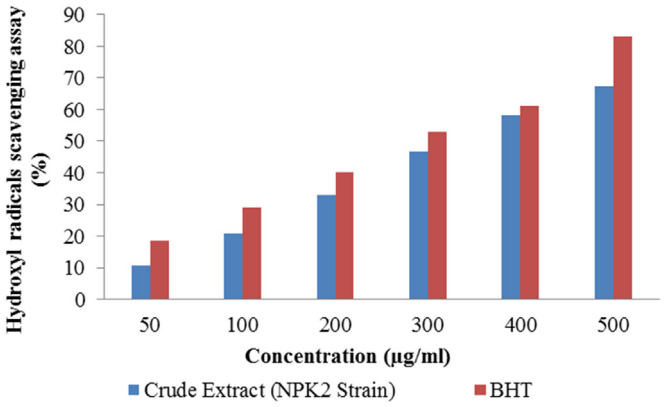

Fig. 6. Hydroxyl radical scavenging activity of Crude extract – NPK2 Strain.

4. Conclusion

Totally, 13 strains were isolated from the rhizosphere of *R. annamalayana* in Pichavaram mangrove forest. The crude extract of the potent fungi was characterized by FTIR analysis and subjected for antimicrobial and antioxidant properties. It is evident from the present study that the marine environment harbors a potential source of bioactive metabolites. The crude ethyl acetate extracts of marine derived *Trichoderma* sp., exhibited a promising anti microbial activity against both the human and fish pathogens in a dose dependent manner. Comparing to both, the crude extract showed potential activities against human pathogens rather than fish pathogens. Owing to its increased free radical scavenging activity, further investigation on the purification of this metabolite will provide novel and efficient secondary metabolites towards developing potent antimicrobials.

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