A Study on the Antimicrobial Potentials of an Endophytic Fungus Fusarium oxysporum NFX 06

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***Abstract*—Endophytes are fungi associated with plants without causing symptoms. They are quite diverse in nature and have enormous potential for production of important secondary metabolites for the pharmaceutical industry. Thus the aim of this work was to isolate an endophytic fungal strain possessing antimicrobial activity against the selected human pathogens. In this study we report for the first time microwave assisted extraction of secondary metabolites from an endophytic fungal strain NFX06 isolated from leaf of *Nothapodytes foetida* of Agumbe forest, Karnataka. The fungal strain was identified as *Fusarium oxysporum* NFX06 based on its macroscopical and microscopical characteristics. Further confirmation of the species was done by Internal Transcribed Spacer (ITS) sequencing and the nucleotide sequence was submitted to the GenBank with an accession number KC914432. The highest activity against all the four pathogenic strains [*Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 69548)] were exhibited by ethyl acetate extract with a Minimum Bactericidal Concentration (MBC) of about 30 μg/mL against *Staphylococcus aureus* and a Minimum Fungicidal Concentration (MFC) of about 50 μg/mL against *Candida albicans*.**

***Index Terms*—endophytic fungi, Fusarium oxysporum, ITS sequencing, microwave extraction, minimum inhibitory concentration, Nothapodytes foetida**

I. Introduction

Plants and fungi are the chief source of natural compounds used for medicine, in which medicinal plants and endophytes have attracted considerable interest for their wide variety of bioactive metabolites [1]-[3]. Demain and Sanchez [4] reported that production of bioactive secondary metabolites by medicinal plants and their endophytes have provided countless for therapeutic applications. The discovery of novel antimicrobial metabolites from endophytes is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens [5], [6].The antimicrobial compounds can be used as drugs and also as food preservatives in the control of food spoilage and food-borne diseases [7].

Bacon and White [8] described endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effect. It was estimated there may be as many as 1.5 million different fungal species on our planet [9]. Of these only about 10% have been discovered and nearly 1% is examined for their production of secondary metabolites. Endophytic fungi represent an important and quantifiable component of fungal diversity, with an estimate of at least 1 million species [10]-[12]. They are found in nearly all plant families and have been investigated to be a rich source of novel biological active secondary metabolites such as antibiotics, antimycotics, immunosuppressants, and anticancer compounds [13]. These compounds may have potential for use in modern medicine, agriculture, and industry. Thus endophytic fungi are considered to be rich and reliable source of genetic diversity with novel and undescribed species [14].

In this study an attempt has been made to isolate fungal endophytes of a medicinal plant *Nothapodytes foetida (Wights)* Sleumer, Syn. *N. nimmoniana*, *Mappia foetida* Miers, *Premna nimmoniana* Graham commonly known as "Amrutha' a medicinal plant of the family Icacinaceae [15]. It is a small tree distributed in the Western Ghats, a global bio diverse hot spot. As herb it was widely used in Indian traditional medicine for various types of cancers, HIV, malaria and few bacterial infections. Considerable efforts are in progress to map its populations in India since it has been classified as a 'vulnerable' species, as reported by Kumar and Ved [16]. Hence the aim of the present study was to screen endophytic fungal isolates possessing antimicrobial activity and also to determine the increase in efficiency of microwave assisted extract of NFX 06 strain.

II. Materials and Methods

A. Collection of Plant Materials

Figure 1. Nothapodytes foetida

Manuscript received August 8, 2013; revised October 9, 2013.

doi: 10.12720/jomb.3.3.162-166

The plant samples were collected from Agumbe forest of Western Ghats, South Karnataka, India "Fig. 1". The plant material was identified and authenticated by an experienced botanist. Fresh and healthy parts of the plant like leaves, stem, seed and fruits were cut with a sterile scalpel and stored at 4 ℃ in a sterile polythene bag prior to use.

B. Isolation of Endophytic Fungi

Isolation of endophytic fungi from plant samples was carried out as described by Wang *et al*. [17] with little modifications. Plant samples were washed under running tap water for 10 minutes followed by immersion in 75% ethanol for 1 minute and in sodium hypochlorite 2.5% for 10 minutes, drained and immersed in 75% ethanol again for 30 seconds. Finally, the samples were rinsed with sterile distilled water. Each plant sample was cut aseptically into 1 cm long segments and placed on petri-dishes containing potato dextrose agar (PDA) supplemented with chloramphenicol (50 µg/mL, Sigma) and streptomycin sulphate (250 µg/mL, Sigma). The plates were sealed using Parafilm^{TM} and incubated at 25 ℃±1 ℃ in a light chamber with 12 hours of light followed by 12 hours of dark cycles [18].

The petri dishes were monitored every day to check the growth of endophytic fungal colonies from the plant segments. As and when the hyphal tips emerged out from plant segments they were isolated and sub-cultured and brought to pure culture by serial sub-culturing. The same procedure without surface sterilization was used as negative control to check for contamination by fungi. Purity of the isolates was checked by preparing microscopic slides of every isolate by standard methods of microscopy.

C. Identification of Endophytic Fungi

Preliminary identification of fungal endophytes was carried out based on their microscopic and macroscopic characteristics. Lactophenol cotton blue staining method was used for staining the fungal cultures and visualized under microscope (MOTIC BA 400). Colonies were analyzed with respect to their average diameter, coloration of the mycelium, sporulation and production of acervuli, coloration of the medium, and the size and coloration of the conidia. Further identification was done based on molecular characterization. The genomic DNA was extracted from the mycelium and the ITS regions including the intervening 5.8s rDNA and flanking ITS1 and ITS2 were amplified using universal primers ITS 5 (5′GGAAGTAAAAGTAACAAGG3′) and ITS 4 (5′TCCTCCGCTTATTGATATGC3′) [19]. The nucleotide sequence data was submitted to Genbank.

D. Fermentation

The spore suspension was prepared by scraping out the fungal mycelium from the pure slant culture into 0.2% of Tween 80 and filtered using Whatmann No.1 filter paper. 100 µL of spore suspension was added to 250 mL of erlynmeyer flask containing Potato Dextrose Broth (PDB), Malt Extract Broth, (MEB), Czapek Dox Broth (CDB) and M4 medium containing (4% dextrose, 1% peptone, 0.06 % $ZnSO_4$, 0.035 % $MgSO_4.7H_2O$ and 0.04% KH_2PO_4) and incubated at 25 ℃ ± 2 ℃ and 120 RPM with normal light and dark condition for a period of 14 days.

E. Test Microorganisms

The test microorganisms used in this study included three bacterial strains [*Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853)], three fungal strains [*Aspergillus niger* (ATCC 16404), *A. flavus* (ATCC 11498), and *A. terreus* (MTCC 1281)] and one yeast, *Candida albicans* (ATCC 69548). All the cultures were procured from King's Institute Chennai. The bacterial cultures were sub-cultured every two weeks on fresh nutrient agar (NA) slants and incubated at 37 ℃ whereas the yeast and fungal cultures were sub-cultured every four weeks on the fresh PDA slants and incubated at 28 ℃ and 25 ℃ respectively.

F. Semipolar Extraction of Fungal Culture

After fermentation the fungal biomass was separated from the broth by filtration using cheese cloth and Whatmann No.1 filter paper. Liquid-liquid extraction was carried out by adding equal volume of ethylacetate with the broth. After overnight extraction the organic layer was transferred to a round bottom flask and concentrated to dryness using a rotary vacuum evaporator (Superfit, India) under reduced pressure. The resultant extract was then lyophilized and stored at 4 ℃ for further use.

G. Antimicrobial Activity of Biomass of NFX 06 Extracted Using Different Solvents by MAE

Fermentation was carried out in a 250 mL of Erlenmeyer flask containing M4 medium inoculated with 100 µL of spore suspension at 27 ℃ and at 120 rpm under normal light and dark condition for a period of 14 days. After fermentation broth and biomass was separated by filtration and the biomass was dried to constant weight in a hot air owen at 60 ℃ overnight. The dried biomass was ground to fine powder using pestle and mortar. 100 mg of powder was extracted using three different solvents like methanol, ethylacetate, hexane-acetone at constant temperature of 55 ℃ for 4 minutes with a power consumption of 65 W by Microwave (QAsh 8000 Microwave ashing system, Canada). The microwave assisted extract of fungal biomass obtained from M4 medium with three different solvents were tested by modified agar well diffusion assay (NCCLS), [20]. The magnitude of antimicrobial activity was assessed by the diameter (mm) of inhibition zones relative to those of positive and negative control. Streptomycin and Fluconazole were co-assayed as positive antimicrobial references with DMSO as negative control.

III. Results

The endophytic fungal strain NFX 06 isolated from the leaf of the medicinal plant *Nothapodytes foetida* was found to be *Fusarium oxysporum* "Fig. 2" based on its morphological and molecular characteristics. The

nucleotide sequence obtained by ITS sequencing was deposited in the GenBank with an accession number *KC914432.*

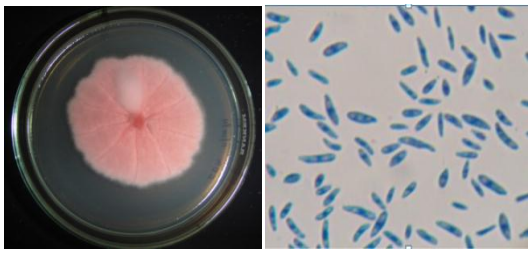

Figure 2. Colony morphology and Spore image of *Fusarium oxysporum* NFX06

A. *Antimicrobial Activity of Fusarium Oxysporum NFX 06 Grown in Different Medium Extracted with Solvent*

The effect of different media (PDB, MEB, CDB and M4) on the production of secondary metabolites was determined from the broth obtained after fermentation. The broth was extracted with ethyl acetate and analyzed for its antimicrobial activity.

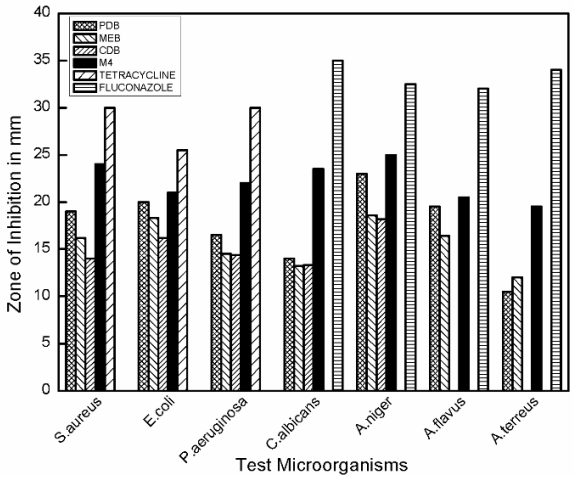

Figure 3. Antimicrobial activity of NFX 06 grown in different medium

It was found that the isolate NFX 06 had good promising antimicrobial activity against human pathogenic bacteria (*S. aureus, E. coli and P. aeruginosa*) yeast (*C. albicans*) and fungi (*A. niger, A. flavus* and *A. terreus*). As shown in "Fig. 3", the antimicrobial activity against the selected human pathogens was exhibited more by the secondary metabolites of medium M4.Maximum activity of about 21.5, 22.4, 18.2, 19.2, 18.5, 20.5 and 13.5 mm zone of inhibition was found against *S. aureus, E. coli, P. aeruginosa, C. albicans, A. niger, A. flavus* and *A. terreus respectively*. The activity was found to be in the order of *A. terreus < P. aeruginosa <A. niger <C. albicans <A. flavus <S. aureus <E. coli.*

B. *Antimicrobial Activity of Fusarium Oxysporum NFX 06 Biomass Extracted Using Different Solvents by MAE*

The antimicrobial activity of the isolate NFX06 tested from biomass extracted using three different solvents such as methanol, ethyl acetate and acetone–hexane (1:1) has also exhibited quite good activity against the selected human pathogens indicating the efficiency of extraction of compounds having biological activities by MAE. The activity in terms of inhibition zone is given in the Table. I.

TABLE I. ANTIMICROBIAL ACTIVITY OF *FUSARIUM OXYSPORUM* NFX06 EXTRACTED USING DIFFERENT SOLVENTS BY MAE

Test Micro-organism	Methanol extract	Ethyl Acetate extract	Acetone-Hexane extract	Strepto mycin	Flucona zole
Zone of inhibition in mm					
S. aureus	21.5	23.0	18.2	30.0	NI[a]
P. aeriginosa	19.4	21.1	17.2	25.5	NI[a]
E. coli	20.2	22.3	16.7	30.0	NI[a]
C. albicans	18.6	21.6	19.1	NI[a]	35
A. niger	18.4	22.8	17.5	NI[a]	32.5
A. flavus	19.7	22.4	17.9	NI[a]	32.0
A. terreus	18.2	21.4	18.4	NI[a]	34.0

[a] No inhibition

The highest antimicrobial activity was found in the ethyl acetate extract which was in the order of *S. aureus* > *A. niger* > *A. flavus* > *E. coli* > *C. albicans* > *P. aeruginosa* > *A. terreus.* Comparing to the other two solvents ethyl extract has shown good activity complementary to the positive controls.

C. *Determination of Minimum Inhibitory Concentration*

Different concentrations of crude ethyl acetate extract ranging from 10 – 100 µg were assessed against the test microorganisms. The crude extract of *Fusarium oxysporum* NFX 06 was most effective against the gram positive bacteria *S. aureus* with a minimum bactericidal concentration (MBC) of 30 µg/mL. Whereas MBC for other test microorganism was found to be ≤ 80 µg/mL. For *C. albicans*, minimum fungicidal concentration (MFC) was about 50 µg/mL and MFC for other test ≤ 100 µg/mL.

IV. DISCUSSION

Endophytic fungi have been recognized as a repository of novel secondary metabolites, possessing beneficial biological activities [21], [22]. This is the first report on the antimicrobial activity of endophytic fungus *Fusarium oxysporum* NFX06 isolated from leaf of the medicinal plant *N. foetida* collected from Agumbe forest Karnataka. The antimicrobial activity by dual culture method of *Nodulisporium sp*. isolated from *N. foetida* collected from Joida forest Karnataka was reported by [23].

From the results it was evident that this fungus has the potential for extraction of novel metabolites possessing antimicrobial activity against a broad range of pathogens not only from the broth as well as from the biomass. Generally utilization of fermentation broths has been reported for extraction of antimicrobial compounds. This is the first report showing the extraction of metabolites possessing antimicrobial activity using MAE.

The MIC for the selected pathogenic bacterial strains was 30 to ≤ 80 µg/mL, whereas MIC of ≤ 100 µg/mL for fungal pathogen and 50 µg/mL for yeast was obtained. For the same *S. aureus* ATCC 25923 the MIC 125 µg/mL from the crude extract of fungal endophyte of Sesbania grandiflora (L.) Pers was reported by Powthong et al [24].

According to Rios and Recio [25], in the search for substances of natural origin with antimicrobial activity, those that present concentrations higher than 1 mg/mL for extract and 0.1 mg/mL for isolated compounds should be avoided. However, the evaluation of activity is very interesting in case of concentration below 100 µg/mL for extracts and 1 µg/mL for isolated compound. Our observation reveals that the endophytic fungi of *N. foetida* possess a potential for isolation of antimicrobial agents against broad-spectrum of pathogens which are prone to develop resistance against the commercially available antibiotics [26].

As reported in the literature [27]-[29], medicinal plants provide a unique environment for endophytes where it receives nutrition and protection from the host plant, while in turn the endophytes protects the host plant from various biotic and abiotic stress.

In this regard, we speculate that medicinal properties of different parts of *N. foetida* may be fully or partially dependent on the endophytic fungi as they are the chemical synthesizers inside the plants [30] and vice versa. Because of the increasing demand and widespread of multi drug resistant pathogens there is a need to search for new antimicrobial agents [31]-[33]. Thus this work would provide a scope to study the various biological activities of the endophytic fungal isolates.

ACKNOWLEDGEMENT

The authors are thankful to Manipal Life Science Centre for providing the facilities for sequencing. And we are grateful to Retired. Prof. G. K. Bhat, Poornaprajna College Mangalore help us in finding the plant.

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