ANTIBACTERIAL ACTIVITY OF ISOLATED ENDOPHYTIC FUNGI FROM RAUVOLFIA SERPENTINA (L.) BENTH. EX KURZ

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INTRODUCTION

Endophytic fungi are the most important part of biodiversity and beneficial for the survival of other organisms. Endophytes play an essential role in ecological processes that includes mutualism, parasitism and commensalism [1-3]. These are symbiotic microorganisms, which live internal tissue of the plant body without causing any negative effects in the host plants [4]. The reproductions in endophytic fungi take place by spores and vegetative growth showing the formation of conidia and hyphae [5]. These are found in different parts of host plant like leaves, petioles, stem, twigs, bark, root, fruit, flower and seeds [1]. Generally, endophytic fungi are classified into different classes based on their host range, colonisation in plants and type of tissue colonised, diversity of plants, transmission and fitness benefits [6]. Endophytic fungi are produced a number of active novel bioactive compounds like alkaloids, peptides, steroids, terpenoids, phenols, quinones and flavonoids which are beneficial in agriculture, industries and in pharmaceutical industries for the production of medicine, drugs and natural biochemicals that provide protection against pathogenic organisms [7, 8].

Sandhu et al. [9] isolated the endophytic fungi from Calotropis procera (Linn.). R. Br. plant of Jabalpur region and tested their antibacterial activity against Escherichia coli, Klebsiella pneumoniae, Streptococcus pyogenes, Salmonella typhimurium and Enterococcus sp. The endophytic fungi Trametes hirsute produce podophyllotoxin and other related aryl tetralin lignans bioactive compounds which used against cancer [10]. In other research work Sandhu et al. [11] also isolated the endophytic fungi Aspergillus fumigatus, Aspergillus niger, Fusarium solani, Aspergillus repens, Alternaria alternata, Alternaria sp., Phoma hederaeola and Fusarium oxysporum from Menthe viridis and observed their antibacterial activity against the test bacterial strain and also optimized various parameters for maximum production of antibacterial bioactive compounds.

In the present work endophytic fungi were isolated from the medicinal plant Raufolvia serpentina also known as Sarpa gandhara. It is perennial woody rootstock plant commonly found all over the Indian subcontinent and South East Asian countries. More than 80 types of alkaloids are isolated from Raufolvia species like reserpine [12]. The bioactive compounds isolated from Raufolvia serpentina used in a number of treatments like insomnia, hypnotic, sedative, sexual aggression, anti-hypertensive and in cardiovascular diseases. Therefore, the present study was to isolated, identified and screening of antibacterial activity of endophytic fungi isolated from different parts of Raufolvia serpentina.

MATERIALS AND METHODS

Collection of plant sample

Different parts of medicinal plant Raufolvia serpentina (L.) Benth. ex Kurz (root, stem and leaves) were choosen for isolation of endophytic fungi. The sample was collected from plant nursery of Jawaharlal Nehru Krishi Vishwa Vidyalaya (JNKVV) Jabalpur (M. P.). The research work was conducted during February to March 2015.

Isolation of endophytic fungi

Isolation of endophytic fungi was carried out by methods described by Petri [13] with some modification. The plant material was rinsed gently in tap water to remove dust and debris. Then the plant samples were cut into small fragments. Each sample was surface disinfection with 70% ethanol for 1 min and immersed in sodium hypochlorite (NaOCl) for 30 s and then rinsed in sterile distilled...
Calculation of colonising frequency

Colonization frequency (CF %) was calculated as described by Suryanarayanan et al. [14] and Pothita et al. [15]. Colonization frequency (%) of an endophyte species was equal to the number of segments colonised by a single endophyte divided by the total number of segments observed x 100.

Colonizing Frequency % = Number of segment colonized by fungi / Total number of segment x 100

Morphological and molecular identification of isolated fungal strain

Morphological identification

Endophytic fungi isolated from *R. serpentina* were identified by using slide culture technique [16]. Morphological identification of endophytic fungi was done on the basis of their macroscopic and microscopic features such as colour, shape and growth of cultured colonies and the generated data was compared with available literature.

Molecular identification of potential fungi

Isolation of DNA

According to Sandhu, [17] total genomic DNA from the fungi was isolated by LETS buffer (Lithium chloride-EDTA-Tris HCl and sodium dodecyl sulphate). A conical flask with 50 ml PDB media was inoculated with a loop full of conidia and incubated for 6 to 7 d. Then the mycelia was harvested, washed with distilled water and hand pressed for removal of excess water from the mycelia, dried with filter papers and treated with liquid nitrogen, further crushed in a pestle-mortar by adding 0.7 ml LETS (LETS (0.1 M LiCl, 10 Mm EDTA, 10 Mm HCl and pH 8) and 0.5% SDS) extraction buffer. The crushed mycelia were poured in the centrifuge tubes and vortexed for few min and centrifuged at 5000 rpm for 10 min. After centrifugation, the supernatant was transferred into another sterilised tube, and the pellet was discarded. One ml of phenol: chloroform: isomyl alcohol (25:24:1) was administered in the tubes and vortexed for 1 min at medium speed. It was centrifuged at 10000 rpm in a centrifuge for 10 min. Two distinct layers were formed in the centrifuge tube one aqueous layer and another organic phase. The aqueous layer was transferred in other sterilised tube, and 70% chilled absolute ethanol was added to the sample and kept on ice for 15 min. After this, spin for 15 min in a microcentrifuge at 4 °C at 10000 rpm for 10 min. The supernatant was removed, and the pellet was dried by inverting the tubes gently and washed with 70% ethanol. The pellet was preserved in nuclease free water for future use.

Quantification and PCR amplification of isolated DNA [18, 19]

The quantity of the isolated DNA was checked in UV-VIS spectrophotometer. From the stock 1 μl DNA was mixed with 49 μl sterilised distilled water to get 50 times dilution. The A260/280 was recorded to check the purity of DNA. PCR amplification of ITS region was done in 20 μl of reaction mixture containing PCR buffer, 1X (Kapapa, SA); MgCl₂, 3 mmol; dNTP mix, 0.25 mmol; Taq DNA polymerase, 0.05 μl; primer, 1 picomol and template DNA, 50 ng. Sterilised nuclease free water was used as negative control. The oligonucleotide primers used for PCR amplification was ITS4 and ITS6 as depicted in table 1. Thirty-five cycles of PCR were performed by using denaturation at 94 °C for 10 s annealing at 48 °C for 30 s, followed by extension 72 °C for 6 min.

| Oligonucleotide | Sequences (5’-3’) | GC % | Tm Value | Length | Product size |
|-----------------|------------------|------|----------|--------|-------------|
| ITS 4           | TCC TCC GCT TAT TGA TAT G | 50   | 51.0 °C  | 19     | 700 bp      |
| ITS 6           | GAA GGT GAA GTC GTA ACA AGG | 60   | 56.0 °C  | 21     |             |

Source of bacteria strain

The six pathogenic bacteria strain *Bacillus subtilis*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Enterococcus sp.* was provided by the Fungal Biotechnology and Invertebrate Pathology Laboratory (FBPIL), RDV, Jabalpur (M. P.). Bacterial cultures were maintained on nutrient agar medium slant (NAM) and incubated at 37 °C for 24 h in the bacterial incubator and stored at 4 °C for preservation.

Mass production of secondary metabolites [20]

For mass production of a secondary metabolite from endophytic fungi, 250 ml of Potato dextrose broth (PDB) was prepared in 500 ml flasks. The medium was autoclaved at 121 °C and after that inoculated with various fungal cultures and incubated at 26±1 °C in the fungal incubator for 7th 14th and 21st days. After respective days of inoculation (7, 14 and 21 d) the crude extract of the culture broth was filtered by using Whatman filter paper no.1 and observed their antibacterial activity against test bacteria.

Screening of endophytic fungi

The antibacterial activity of endophytic fungi was observed by using Agar well diffusion method [21] against six pathogenic bacteria. In this method, 25 μl of bacterial suspension was spread over the nutrient agar medium plates and allowed to diffuse. The well was made aseptically in the plates with the help of cork borer (8 mm in diameter), and 80 μl of metabolites was introduced into the well. Then the plates were incubated in the bacterial incubator at 37 °C for 24 h. Finally, antibacterial activity was resolute by measuring the diameter of zone of inhibition by using Hi-Media Antibiotic zone scale.

RESULTS

Isolation of endophytic fungi

In the present study, seven endophytic fungi were isolated from the different parts viz. leaf, stem and root of the *R. serpentina* collected from plant nursery of Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV) Jabalpur M. P. (India) as depicted in table 2. A total 32 segments (leaf 16, stem 8 and root 8) of *R. serpentina* were processed for the isolation of endophytic fungi.

| Plant part | Name of endophytic fungi | Class |
|------------|--------------------------|-------|
| Leaf       | Aspergillus niger        | Eurotiomycetes |
| Leaf       | Penicillium citrinum     | Eurotiomycetes |
| Leaf       | Cladosporium sp.         | Dothideomycetes |
| Leaf       | Curvularia lunata        | Eurotiomycetes |
| Stem       | Aspergillus sp.          | Eurotiomycetes |
| Stem       | Alternaria sp.           | Dothideomycetes |
| Root       | Aspergillus fumigatus    | Eurotiomycetes |
Identification of isolated fungi

Morphological identification of endophytic fungi

The fungal isolates were purified on PDA plates and identified on the basis of their characteristic morphology that includes: colonies on plates, the morphology of spore, hyphae and color. Based on a morphological feature they were identified as Aspergillus niger, Penicillium citrinum, Cladosporium sp., Alternaria sp. and Aspergillus fumigatus as shown in Table 3.

Molecular identification of potent fungi

For the molecular identification both the designed oligonucleotide primers ITS4 (TCC TCC GCT TAT TGA TAT G) and ITS6 (GAA GGT ATTATCTCAAGTTGACCTCAGATCAGGTAGGAATAACCGCC) show strong specificity for fungal DNA sequences. Using BLAST (NCBI), the percentages of identical matches of ITS4 and ITS6 DNA sequences in the Gene Bank database (NCBI) were determined to 98%. This result was obtained by giving denaturation 94 °C for 30 s, annealing 48 °C for 30 s and final extension for processing of DNA amplification was given 72 °C for 6 min. The molecular sequence of Penicillium citrinum is:

Table 3: Colonization frequency (%) of isolated endophytic fungi

| Plant parts | Name of endophytic fungi | % frequency of colonization | No. of isolates |
|-------------|--------------------------|----------------------------|----------------|
| Leaf        | Aspergillus niger        | 12.25 %                    | 2              |
| Leaf        | Penicillium citrinum     | 6.25 %                     | 1              |
| Leaf        | Cladosporium sp.         | 12.50 %                    | 2              |
| Leaf        | Curvularia lunata        | 18.75 %                    | 3              |
| Stem        | Aspergillus sp.          | 12.50 %                    | 1              |
| Stem        | Alternaria sp.           | 25.00 %                    | 2              |
| Root        | Aspergillus fumigatus    | 37.50 %                    | 3              |

Detection of antibacterial activity

To detect the antibacterial activity of metabolites extracted from the endophytic fungi were examined after 7th, 14th and 21st days by Agar well diffusion method against six pathogenic bacteria. The antibacterial assay was carried out in triplicates. From the isolated endophytic fungal strain Penicillium citrinum was showed maximum zone of inhibition against B. subtilis (23.0±0.12 mm), E. coli (19.9±0.16 mm), S. pyogenes (19.2±0.59 mm), Enterococcus sp. (17.2±0.08 mm), K. pneumoniae (18.9±0.16 mm) and S. typhimurium (15.0±0.16 mm) as well as the positive control (Streptomycin) showed the zone of bacterial inhibition ranging from 15.46±0.15 mm to 21.20±0.59 mm shown in table 4.

Table 4: Antibacterial activity of isolated fungal strain

| Name of fungal isolates | Zone of inhibition (in mm) |
|-------------------------|----------------------------|
|                         | E. coli | B. subtilis | Enterococcus sp. | S. pyogenes | K. pneumoniae | S. typhimurium |
| F. sp.                  | -       | -           | -                | 12.0±0.10   | 13.1±0.30    | -              |
| A. sp.                  | 05.0±0.12 | -        | -                | 18.0±0.2    | 15.1±0.25   | 17.3±0.36     | 16.0±0.20       |
| C. sp.                  | 14.0±0.21 | 10.9±0.20 | -                | -           | 14.0±0.12   | -              |
| Lunata                 | -       | 14.4±0.46  | -                | -           | 14.9±0.16   | 16.1±0.16     |
| Asp. sp.                | -       | -           | -                | -           | -           | -              |
| P. citrinum             | 19.9±0.16 | 23.0±0.12 | 17.2±0.08        | 17.2±0.15   | 18.9±0.16   | 15.1±0.16     |
| N. sp.                  | -       | -           | -                | 15.4±0.15   | 21.2±0.59   | 17.4±0.10     | 18.4±0.58       |
| Streptomycin (1 mg/ml)  | 16.1±0.11 | 19.2±0.10 | 15.4±0.15        | 21.2±0.59   | 17.4±0.10   | 18.4±0.58     |

*Each value in the table is represented as mean±SD (n = 3). F. sp.: Fusarium sp., A. sp.: Alternaria sp., C. sp.: Cladosporium sp., C. lunata: Curvularia lunata; Asp. sp.: Aspergillus sp., P. citrinum: Penicillium citrinum and N. sp.: Nigrospora sp.

Broad-spectrum activity of endophytic fungi

For the broad-spectrum activity of endophytic fungi against 6 pathogenic bacterial strains are shown in table 5 and fig. 2. Penicillium citrinum showed a maximum zone of inhibition against E. coli (19.2±0.30 mm), B. subtilis (23.0±0.10 mm), Enterococcus sp. (17.3±0.20 mm), S. pyogenes (19.3±0.15 mm), K. pneumoniae (18.4±0.32 mm) and S. typhimurium (15.6±0.45 mm). Similarly, Alternaria sp. was also showed a zone of inhibition against Enterococcus sp. (18.2±0.25 mm), S. pyogenes (15.0±0.25 mm), K. pneumoniae (17.6±0.58 mm), S. typhimurium and (16.8±0.80 mm), E. coli (05.7±0.43 mm). In the case of Curvularia lunata it showed antibacterial activity against B. subtilis (13.5±0.30 mm), K. pneumoniae (15.7±0.77 mm) and S. typhimurium (16.7±0.32 mm).
### Table 5: Broad-spectrum activity of selected endophytic fungi

| Endophytic fungi          | E. coli (in mm) | B. subtilis (in mm) | Enterococcus sp. (in mm) | S. pyogenes (in mm) | K. pneumoniae (in mm) | S. typhimurium (in mm) |
|---------------------------|----------------|--------------------|--------------------------|--------------------|-----------------------|------------------------|
| Alternaria sp.            | 05.7±0.43      | -                  | 18.2±0.25                | 15.1±0.40          | 17.6±0.58             | 16.8±0.80              |
| Curvularia lunata         | -              | 13.5±0.30          | -                        | -                  | 15.7±1.07             | 16.7±0.32              |
| Penicillium citrinum      | 19.02±0.30     | 23.0±0.10          | 17.3±0.20                | 19.3±0.15          | 18.4±0.32             | 15.6±0.45              |

*Each value in the table is represented as mean±SD (n=3).*

### DISCUSSION

The seclusion of endophytic fungi from medicinal plant produces a number of bioactive compounds, which have a therapeutic value. Therefore, in the present work endophytic fungi were isolated from the medicinal plant *R. serpentina* and observed their antibacterial activity against six pathogenic bacteria. The endophytic isolated *Penicillium citrinum* was displayed the best result against all the test strains. Similar, result was observed by Sandhu *et al.* [20] isolated the endophytic fungi from *Saraca indica* and detect their antibacterial activity against pathogenic bacteria and pointed to these endophytic fungi have the capability to produce a number of antibacterial bioactive compounds of multiple uses. Shekhawat *et al.* [22] were also isolated 35 endophytic fungi from leaves, the root of *Melia azedaricha* L. and observed their antibacterial activity against test strains. The endophytic fungi *Aspergillus fumigatus*, *Aspergillus japonicas*, *Aspergillus niger*, *Fusarium semitectum*, *Curvularia pallescens*, *Phoma hedericola*, *Alternaria tenuissima*, *Fusarium solani*, *Drechslera australien* and *Aspergillus repens* isolated from the medicinal plant *Ricinus communis* (L.) plant and observe their antibacterial activity against pathogenic bacteria [23]. Previous research worker had examined the production of the anti-leukemic and anti-tumor drug taxol from the endophytic fungi like *Taxomyces andreanae* and *Pestalotiopsis microsora* of *Taxus* sp. [24, 25].

The molecular identification of *Penicillium citrinum* was also done by using oligonucleotide primer for easy identification and comparison of molecular characteristics of the fungi with other organisms. Silva *et al.* [26] also identified the different strain of *Aspergillus* species on the basis of their morphological as well as molecular characteristics. Nyongesa *et al.* [27] isolated the *Aspergillus* species from the maize kernel and soil of maize field of Nandi County and identified on the basis of macroscopic and microscopic morphological characteristics.

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### CONFLICT OF INTERESTS

The authors have no potential conflict of interest regarding publication of the said manuscript.

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