Screening of *Catunaregam uliginosa* (Retz.) Sivar Leaf endophytes for its biological activity

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Abstract: *Catunaregam uliginosa (Retz.) Sivar with known therapeutic potential has immense value for its roots, leaves and flowers. The authors have attempted to study the residing endophytes for metabolites equivalent to the host products. The leaf endophytic fungi were morphotyped based on internal transcribed spacer–deoxyribonucleic acid (ITS–DNA) sequences and C. uliginosa endophytes identified by molecular typing include Penicillium sp, Fusarium sp., and Aspergillus sp. Molecular typing identified Fusarium sp. as Fusarium proliferatum with 94% similarity to the nearest genera and so its extract was screened for therapeutic capacities. Antioxidant activity of the extract exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity with IC_{50} of 51.25 ± 1.23 $\mu g.mL^{-1}$. Antimicrobial activity by disc diffusion in opposition to Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli and Bacillus subtilis significantly arrested bacterial growth. Bacterial biofilm inhibition capacity stained by acridine orange and ethidium bromide imaged by confocal laser scanning microscopy revealed bactericidal activity. Thus, studies on unexplored medicinal plant endophytes could pave path to identification of novel secondary metabolites as therapeutic agents and potential drug candidates.*

Keywords: *Anti-biofilm property, Biological activities, ITS, Leaf endophytes, Morphological typing*

1. Introduction

Medical practitioners and researchers are in continuous quest for natural medicine with no or less side effects. Ayurveda, a traditional Indian medicine system with about eighty percent population dependent on it utilizes plants as resource. Escalating world-wide research on the effectiveness of herbal medicine for chronic diseases has lead to putting forth scientific evidence for traditional beliefs. In our studies we report exploration of leaf endophytes of *Catunaregam uliginosa*, with its recent botanical nomenclature, *Tamilnadia uliginosa* (Retz.) Tirveng. & Sastre belonging to family Rubiaceae. It's been utilized in Ayurveda, Unani, and Siddha medicine systems to address a range of ailments with applications including treating ulcers, gastrointestinal, hepatic problems, wounds, and inflammation. The leaves are used to treat skin diseases as leprosy [1,2].

As an alternative to the host, phytotherapists searched for sources and reported endophytes with the capacity to generate related metabolites as the host plant [3]. Plants harbor microorganisms that could be bacteria and fungi collectively known as endophytes [4]. Studies have reported existence of more than one species of these microbes housed in a single host. These microbes could be symbiotic and bordering pathogenic living in plants tissues spending a part or their complete life cycle residing either intercellularly and/or intracellularly within the plant causing no overt negative effects. Several studies have indicated the ease with which they can be isolated from any part of the host using commonly available growth medium. They have reviewed on endophytes being pivotal provenance of secondary metabolites with specific biological task [5-7]. Usually, secondary metabolites synthesized by endophytes are highly regulated and modulated at the genetic level; however, when the external environment is tweaked, physiologically is affected resulting in further activation of these genes [8]. Metabolites from endophytes support growth of the host, aid with improvement to tolerate biotic/abiotic stress, increase resistance to pest/ insect attack or organic toxins [9]. The products of such interactions, the metabolites exhibit broad spectrum pharmaceutical property. These metabolites are pilots of pharmaceutical revolution leading to discoveries of drug with a range of biological activities are a boon. Secondary metabolites conferring biological activity comprise phenols, alkaloids, quinones, steroids, polyketides, terpenoids, and lignans [9]. These exhibit assorted biological properties such as antioxidant, antibacterial, antidiabetic, cytotoxicity, antimalarial, anti-tubercular and acetylcholinesterase hindrance [10-12].

The endophytes are todays` established expedient supply of natural ad-mixture with therapeutic values [6]. Exploring these as-yet untapped natural legacy increments the chance of unearthing novel entities with use in pharmaceutical sector leading to drug source. However, it remains important to constantly screen for novel entities from nature resources, endophytes and that too from seldom stumbled across medicinal plants [7].

With this scenario, we attempted to report on the endophytic flora from the leaves of medicinally important plant *C. uliginosa* as a lack of reports on endophytes residing in this pharmaceutically important tree was noted. Ethyl acetate extracts of the fungi were prepared, and they were tested for antioxidant and antimicrobial activities.

2. Material and Methods

2.1. Collection of Leaf Samples

The Kigga region in Western Ghats of Chikmagalore District in Karnataka State was surveyed and *C. uliginosa*. leaves were collected and placed in plastic zip lock covers. The sample specimens (*C. uliginosa* # IOE LP0015) were filed at the department. The leaf samples were processed within 48 hours of collection for fungal endophyte isolation.

2.2. Isolation of Fungal Endophytes from Leaves

The leaves were washed in running tap water to remove the stuck dust and grime particles. The surface was cleaned under aseptic conditions to avoid contamination by sequential rinse with ethanol (70%; v/v) for 1 minute, followed by 4.5% (v/v) sodium hypochlorite (3% available chlorine) wash for 3 min and finally washed with sterile water (1 minute) to remove traces of surface sterilant [13]. The leaf bits were then dried tapping with a sterile blotting paper to remove excess of moisture. The leaf bits (10–15 bits; 0.5 to 1-cm) were arranged in sterile water agar media (15 g/L) supplemented with 250 ppm chloramphenicol in a sterile petri dish. Incubation was followed for 15 days, at 28ºC with alternate dark and light cycles for 16 hours for endophyte growth. The emerging fungal endophytes were carefully harvested and placed in sterile potato dextrose agar (PDA) media to induce sporulation. The fungal endophyte isolated were preserved in cryovials on broth (PDB) containing glycerol (-80ºC; 15%, v/v).

2.3. Isolation of Genomic DNA of Endophytes, its Amplification by PCR and their Identification by Sequencing of ITS and nBLAST

Fungal endophytes were identified based on their colony characters (**Figure 1C),** spore morphology or fruiting bodies (**Figure 1E**) visualized by Research Stereo Zoom Microscope (Stereo Discovery V20; Carl Zeiss, Germany) using standard identification manuals [14,15]. Endophytic fungal cultures on PDA media were harvested, their genomic DNA was obtained by CTAB (cetyltrimethyl ammonium bromide) methodand was stored in Tris EDTA buffer (100 mL) [16]. Quantification and purity of the genomic DNA was checked in Nanospectrophotometer (Thermo 200°C; Thermo Fisher Scientific, USA). Pure DNA (ratio 260–280 nm) with the values (1.6/1.8) devoid of contamination with protein/phenol or RNA was subjected to agarose gel electrophoresis (1%) containing ethidium bromide and was visualized in gel documentation system (Geldoc XRT (BioRad, USA). Amplification by PCR was by using Universal ITS (internal transcribed spacer) primers for fungi, [ITS1:5`-TCC GTAGGTGAACCTGCG G-3`; ITS4: 5`-TCCTCCGCTTATTGATATGC-3`][17] in PCR tubes (0.2 mL) containing reaction mixture (25 mL) with genomic DNA (1-mL; 50 ng/mL) in a cycler (thermal; Master Cycler gradient; Eppendorf, Germany). The programincluded initial denaturation (95ºC for 5 min, 94ºC for 3 min), annealing of primer (55ºC for 1 min) and extension (2 min and 10 min, 72ºC) repeated for 35 cycles [16]. The amplified products (5 mL) were loaded on agarose gel (1%) and DNA bands were visualized along with standard ladder (100–10,000 bp) for molecular weight determination. For sequencing, the PCR amplified products were sent to Chromous biotech, Bangalore and they were analyzed by National Center for Biotechnology Information (NCBI) nucleotide basic local alignment search tool (nBLAST).

2.4. Preparation of Endophyte Extracts

Agar pieces (0.5 cm^2) with actively growing fungal endophytic colony were inoculated onto sterile potato dextrose broth (PDB; 1L) and the flasks were incubated with alternate light (8 hours) and dark cycles (16 hours) in stationary phase (25 ± 2°C) for 15 days. The mycelial mat in the broth was sonicated and the whole mass was filtered through double layer muslin cloth. For extraction of metabolites, the filtrate was mixed with equal volumes of ethyl acetate in a separating funnel and the blend was strongly agitated. The upper solvent layer was separated, concentrated to solid sticky mass (10 mg) in Heidolph rotary evaporator set at 42°C[18] and was stored in a colored glass container (4°C) for analysis.

2.5. Estimation of Total Phenol Content in the Extract

The phenol content in endophyte ethyl acetate extracts was determined by Folin-Ciocalteu calorimetric method. Test sample (100 µL) was reacted with Folin-Ciocalteu reagent (0.75 mL; diluted 10 fold) and allowed to stand for 5 minutes. After neutralization with saturated sodium carbonate (60 g/L) it was incubated in the dark for 1.5 hours at 22°C and the absorbance was measured at 725 nm using UV/visible spectrophotometer (U-3900;Hitachi). Gallic

acid standard (25–250 µg/mL) was prepared under similar conditions. Total phenol content was quantified using this gallic acid standard graph and phenol content was expressed as gallic acid equivalence (GAE; µg/mg of extract).

2.6. Free Radical Scavenging Ability of the Extracts by DPPH Radical Scavenging Assay

DPPH (1, 1-Diphenyl-2-picryl hydrazyl) method for determining the antioxidant potential was followed as described by Brand-Williams *et al* [19] at 37°C in dark for 30 min and the absorbance was recorded at 517 nm (Spectra max 340, multimode plate reader, Molecular devices). The reaction mixture included extract (5 µL; 1-mg/mL) mixed with DPPH solution (95 µL; 300 µM). Scavenging activity of DPPH radical was estimated in comparison with a methanol (negative control). Ascorbic acid and quercetin (25–250 $\mu g.mL^{-1}$; Sigma-Aldrich, St. Louis, MO, USA) were used as positive control. The values (IC_{50}) representing the concentration of endophyte extract required to scavenge DPPH (50%) radicals was reported.

2.7. Anti-microbial Activity

2.7.1 Agar Disc Diffusion Method: Gram (−) bacteria *Pseudomonas aeruginosa* (ATCC 27853); *Escherichia coli* (MTCC 724) and Gram (+) bacteria, *Bacillus subtilis* (MTCC 441) and *Staphylococcus aureus* (MTCC 96) were procured from the Institute of Microbial Technology, Chandigarh, India. The extract (1:10 diluted; 1, 2.5, 5.0, and 10.0 µg) was loaded on two 10 mm discs placed on these plates and incubated (15 to 18 hours at 37oC). The diameter of inhibition zones was reported comparing against chloramphenicol (positive standard) under similar experimental conditions [20].

2.7.2. Assay of Loss of Bacterial Biofilm Analyzed by Confocal Laser Scanning Microscopy

To observe the biofilm structure, CLSM (LSM 710 Carl Zeiss, Germany) was used. The samples were prepared from the overnight cultures of Gram (-) bacteria, *P. aeruginosa* nd *E. coli* with the absorbance set at 1.5 ± 2.0 at 600 nm [21]. The assay was conducted in a 6-well plate containing cover slips and growth media (2 mL) in all the wells. To the test wells, overnight bacterial suspensions (750 µL) were introduced and after 24 hours incubation at 37°C the extracts (200 µL, 750 µg) were added. The biofilm formed on the cover slips in wells were removed carefully and were washed with PBS. Ethidium bromide and acridine orange (20 µL; 1 µg in 400 µL; HiMedia, India) were used to stain the biofilm (5 minutes) at room temperature. After three washes with PBS, the cover slips with stained biofilm on them were placed on glass slide in an inverted position and the CLSM images of the samples were imaged. The results presented are images of three independent experiments.

3.0 Statistical Analysis

All experiments and measurements were made in triplicate and the values are reported as the mean ± standard deviation (SD). The results were subjected to variance analysis followed by Tukeys` test to analyse differences between the endophyte ethyl acetate extract and controls. Statistically significant differences ($p < 0.001$) were reported.

4. Results

The present study gives an insight into the endophytic fungal community associated with the leaves of *C. uliginosa* as this could be the first report on the endophytes from their leaves. The steps involved in the study are detailed in **Figure 1**.

4.1. Isolation and Identification of Fungal Endophytes by Morphotyping and Molecular Typing

A total of 19 isolates were recovered from a total of 91 leaf segments. Endophytic DNA was extracted as reported in materials and methods and the amplified ITS-DNA presented a clear band on agarose gel (1%) of molecular weight 500 bp. *Fusarium* sp. exhibited 98% similarity to its nearest genera was identified as *Fusarium proliferatum* by its colony, spore morphology (**Figure 1 E**).

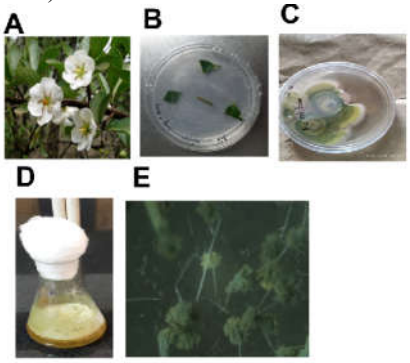

Figure 1: *C. uliginosa* floweres (A); its leaves plated (B); isolated endophyte *Fusarium* fungus (C); growth of the endophyte in broth (D); *Fusarium* sp. Visualized in stereozoom microscope (E)

The analysis of ITS regions of the DNA identified the endophyte (**Table 1**).

Table 1: The DNA sample and endophyte identification by ITS sequences

Endophyte code	Endophyte identified	DNA (ng/100 mL)	Total DNA	Percentage similarity to the nearest genera	GenBank accession number
CUL-06	*Penicillium* sp	123	1.86	*Penicillium gravinicasei* (98%)	MG600581.1
CUL-23	*Fusarium* sp	131	1.89	*Fusarium proliferatum* (94%)	MH109276
CUL-51	*Fusarium* sp	120	1.79	*Fusarium solani* (92%)	MH109274
CUL-82	*Aspergillus* sp	119	1.72	*Aspergillus* sp (91%)	MH594213

CUL- *Catunaregam uliginosa*

Ethyl acetate extract of this microbe was prepared and it was subjected to evaluation of biological activities in the present studies.

4.2. Total Phenol Content and DPPH Radical Scavenging Assay of *F. proliferatum* Extract

The total phenol content was expressed as equivalents of Gallic acid in microgram/mg of the sample. The extract exhibited a phenol concentration of 41.20 ± 0.38 mg GAE/mg for the extract. The endophyte extract exhibited scavenging of DPPH with the IC_{50} value of 51.25 ± 1.23 µg/mL^{-1}. The ascorbic acid exhibited IC_{50} values of 28 ± 3.12 µg.mL-1.

4.3. Antibacterial Activity

4.3.1. Agar Disc Diffusion Assay

Inhibition zone studies by agar-disc diffusion method exhibited a clear antimicrobial effect of the extract. A statistically significant inhibition of both gram negative and gram positive bacteria were recorded (**Figure 2**). The endophyte extract were screened using four microbes, Gram (−) bacteria *E. coli* (MTCC 724), *P. aeruginosa* (ATCC 27853) and Gram (+) bacteria, *S. aureus* (MTCC 96) and *B. subtilis* (MTCC 441) by disc diffusion. Extract (ethyl acetate; 10 mg) significantly arrested *P. aeruginosa* (1.1 cm) and *S. aureus* (0.5 cm) followed by bacteria *E. coli* and *B. subtilis* (0.9 cm).

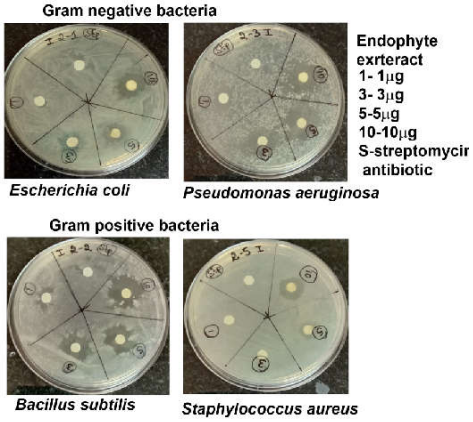

Figure 2: Antibacterial assay of *F. perforetum* ethyl acetate extract

4.3.2. Assay of Loss of Bacterial Biofilm Visualized by Confocal Laser Scanning Microscopy

Gram (-) strains of *E. coli* (MTCC 724) and *P. aeruginosa* (ATCC 27853) reported as biofilm forming strains were included in the present study. From sample observations, biofilm formation on the cover slip support was observed for the controls, *P. aeruginosa* (**Figure 3a**) and *E. coli* (**Figure 3c**). They were live cells visualized green stained by

acridine orange. The addition of the extract (200 µL, 750 µg) resulted in a loss in the biofilm and this observation was visualized by CLSM studies. Patches and broken down biofilm architecture and with decreased micro-colonies containing dead cells of *P. aeruginosa* (**Figure 3b**) and *E. coli* (**Figure 3d**) was observed red stained by ethidium bromide.

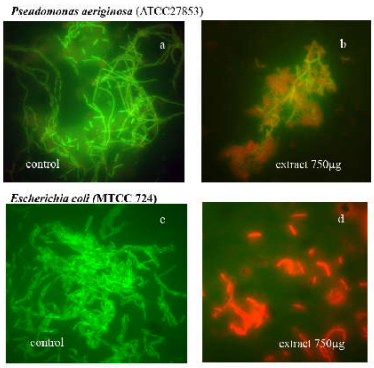

Figure 3: Biofilm inhibition assay of ethyl acetate extract. Controls, *P. aeruginosa* (a); *E. coli* (c). *F. proliferatum* extract (ethyl acetate) + *P. aeruginosa (b); F. proliferatum* extract (ethyl acetate) + *E. coli* (d).

5. Discussion

Treatment of bacterial induced infections involves antibiotics, and its prolonged application has resulted in antibiotic resistance exhibited by them leading to hindering the ability to treat even commonly reported bacterial infections. These infections could be pneumonia, gonorrhea, tuberculosis, or blood poisoning resulting it impossible to cure with antibiotics becoming less useful. A rise in research interest related to endophytic microbes in medicinal plants for their ability to generate broad spectrum metabolites with therapeutic potential is observed [3-13]. Bioprospecting has been applied as a tool to commercialize medicinal plants and the diverse endophytes residing in its tissues. Endophytes are reported paragon of metabolites like flavonoids, phenols, chinones, alkaloids etc., exhibiting several biological activities, including antioxidant [22], antibacterial [23] and anti-cancer [24] revolutionizing pharmaceutical industry. It is reported that of the 35% microbes isolated from medicinal plants several of them (about 80%) are reported to provide molecular entities with biological activity contributing 82% of the natural drugs to the commercial establishments. This has led to discoveries of several unique secondary metabolites that have been used as drug [25,26].

This edged the authors in the present study to initiate scientific screening of endophytes from a valuable medicinal tree, *C. uliginosa.* It is reported that plant-based drug is generally not produced to the required quantities as its production is dependent on specific developmental stage, environmental conditions, stress and/or availability of specific nutrient. Further, it was visualized that microbes (endophytes) residing in their tissues could be screened for potential sources of novel natural production that could find applications in agriculture, medicine, and drug industries.

In this direction, our work could be the first study of fungal endophytes from *C. uliginosa*. The fungal endophytes were cultivated, and four fungal isolates were morphotyped with their molecular identity revealed by ITS–DNA sequences analysis (**Table 1**). One of the isolates, screened for therapeutic capacities was the *Fusarium* sp. identified as *F. proliferatum* by molecular typing exhibiting 94% similarity to the nearest genera. Antioxidant capacity of ethyl acetate extract exhibited DPPH scavenging capacity.

Further, we provide here the anti-bacterial capacity of the extract screened against four microbes (**Figure 2**) which significantly arrested the growth of *P. aeruginosa* and *S. aureus* followed by *E. coli* and *B. subtilis*. Bacterial biofilm inhibition capacity stained by acridine orange and ethidium bromide imaged by confocal laser scanning microscopy revealed the loss of microcolonies (**Figure 3**).

This scientific activity could lead to identification of many microbes with potential in identification of novel metabolites leading to future drug development. Our laboratory is continuing to pursue this research. Data collection is a valuable exercise for understanding the extent of the potential of endophytic *Fusarium* species reported to be a true fungal factory for exploitation directed towards wide variety of applications.

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