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Screening of Antagonistic Endophytic bacteria from *Phyllanthus niruri* leaves

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Abstract: Bacteria are common populace of surfaces and the internal tissues of most plants. These bacteria contribute to the health, growth and development of plants. In this study, 45 strains were isolated from *Phyllanthus niruri* plant leaves. *Phyllanthus niruri* is a medicinal plant used popularly to cure jaundice. Endophytic bacteria isolated were tested against human pathogens and the active strain was identified. Ethyl acetate extract of the strain 13 shows activity against *Candida albicans, Klebsiella pneumoniae* and *Escherichia coli*. High Performance Liquid Chromatography (HPLC) and Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) analysis were done. The data shows the presence of active compounds. Thus, the endophytic bacteria may yield a vast array of new compounds with novel activities that will provide new drugs in the fight against a number of pathogens currently resistant to conventional antibiotic therapies.
Key words: *Phyllanthus niruri*, Ethyl acetate, MALDI-TOF, Pathogens.

Introduction

Bacteria are frequent inhabitants of both the surfaces and the internal tissues of most plants and may have varied effects on host plant growth[1-3]. Plant coupled bacteria isolated from rhizoplane and phylloplane surfaces are known as epiphytes[4] whereas those isolated from the core of tissues, which they reside in without causing harm to the host, are called endophytes[5-6]. Bacterial endophytes can be isolated from surface-disinfected plant tissue or extracted from internal plant tissue[7]. Endophytes penetrate plant tissue primarily through the root zone; however, aerial portions of plants, such as flowers, stems, and cotyledons, may also be used for entry[8]. Endophytes inside a plant may either become contained at the point of entry or extend throughout the plant[7]. These microorganisms can live within cells [9], in the intercellular spaces [10] or in the vascular system [11].

The population density of endophytic bacteria can vary from 10^2 to 10^9 [12-13] and depends on many factors, including the plant being studied, the part under analysis, the developmental stage of the plant [14-15] the plant cultivar (genotype)[16] and the communication with other organisms, as well as other environmental-related factors[7]. *Phyllanthus niruri* plays a crucial role in curing wide range of diseases and they were used in unani, siddha and in ayurveda treatment[17]. *Phyllanthus niruri* is an herbaceous plant with an average height of 50 cm. Its fruits are found below the branches and it is structurally similar to the fruits of *E. officinalis.* It belongs to the Phyllanthaceae family. It is found in the tropical regions. It is widely used for the treatment of jaundice, syphilis, against constipation, gonorhoea and kidney disorders [18, 19].

Materials and Methods

Isolation of endophytic bacteria

Phyllanthus niruri leaves were collected in fields near veltech multitech campus, Chennai. The leaves were surface sterilized to get rid of surface bacteria. 5gm of surface sterilized leaves were added with 5ml of distilled water and crushed in a mortar. The resulted juice was plated on nutrient agar plates using cotton swab. The plates were incubated for 24 hours. After incubation, morphologically differential strains were streaked on nutrient agar plates. Upon re-streaking, individual strains were obtained and stored as slants.

Screening for antibiotic production (Agar overlay method)

Antibiotic production by endophytic bacteria was carried out by following the standard agar-overlay method. Initially the strain was spotted on Nutrient agar plates and allowed to grow for 24 hours. Test strains (MRSA, *E.coli, Klebsiella pneumoniae, Candida albicans, Bacillus subtilis* and *Pseudomonas aeruginosa*) were gently overlaid using soft agar over the strain. The soft agar was prepared by inoculating 1ml of test strain in 100 ml of soft agar (0.75% agar) and mixing thoroughly [21]. The overlaid plates were incubated at 37°C for 24h and the zones of inhibition (measured from the edge of the colony to the edge of the clear zone) were recorded.

Cold-ethanol precipitation for peptide antibiotics

The cold–ethanol precipitation of the culture broth was carried out following the slightly modified method of Schubert and Finn [20]. The potential strains were cultured in Nutrient Broth for 24 hrs and the cells were removed by centrifugation at 7000rpm for 30min in 4˚C. To the supernatant two volumes of ice-cold ethanol was added gradually while agitating with a magnetic stirrer. When the solvent addition was complete, the culture was agitated at 4˚C for at least 60min. The culture was then placed in an ice bucket and left overnight at 4˚C. The precipitate was separated from the supernatant by centrifugation at 5000rpm for 30min in 4˚C. The precipitate was dried in room temperature to remove the ethanol and then dissolved in 5ml of MilliQ water. The antimicrobial activity of the ethanol precipitate was carried out using agar-well diffusion method.

Agar well diffusion assay

The agar well diffusion assay was carried out using the modified method of Stein et al and Chellaram *et al*., [22-23]. Tryptic Soy Agarose (TSA) will be used as the assay medium. TSA was prepared by adding 3g Tryptic Soy broth powder (Hi-media, Mumbai, India) and1g of low electroendosmosis (EEO) Agarose in 100ml of double distilled water. Hundred micro liters of the extracts (ethanol precipitate/ crude biofilm) was poured into wells (6-mm diameter) of TSA plates previously seeded with the test strain. Plates were placed at 4˚C for 4 to 6h to allow diffusion of the substance into the agar, and their contents were subsequently incubated for 12 to 18 h at 37˚C. The presence or absence of inhibition zones around the wells was recorded. All well diffusion assays was carried out in triplicates.

Mass determination

Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrum of the crude extracts were acquired on an Ultraflex Bruker mass spectrometer, equipped with a nitrogen laser of wavelength 337nm. Samples were prepared by mixing equal amounts of samples with the matrix solution (α-cyano-4-hydroxy cinnamic acid) saturated with 0.1% TFA and acetonitrile (1:1). Measured masses have an error of ~ ±3Da.

Results and Discussion

There are 45 individual strains were isolated from plant leaves. All the strains were tested against pathogens using agar overlay method. The active strains were selected and ethanol precipitation was done to obtain the active fraction. These active fractions were tested for its activity against test organisms using well diffusion technique. The strain 13 showed activity against *Candida albicans, Klebsiella pneumoniae* and *Escherichia coli*. Highest activity of 9mm was noted against *C. albicans,* 8mm against *Klebsiella pneumoniae* and 6mm against *E.coli*.

HPLC purification of the peptides of the strain 13 (Fig.1) was carried out. A HPLC fraction was found to be active (Fig.2), the MALDI-TOF spectra showed two prominent peptides (1148 Da & 1729 Da) (Fig.3). Peak found at 14.5 mins (HPLC) shows the presence of active compound. The presence of active compound was confirmed by the peak formation at 1148 Da and 1729 Da of MALDI-TOF.

Interaction between epbiotic bacteria and their host are knows to play a important role in environmental ecosystem but this association has received little attention. Presence of antagonistic bacteria on the surface of the soft coral and some medicinal plants have reported that control the some potent pathogens and observed than prominent activity[21,23,24]. Present finding suggests that significant epibiotic bacterial strain was isolated and active compound has been characterized.

Fig 1: Endophytic bacteria isolated from *Phyllanthus niruri* leaves

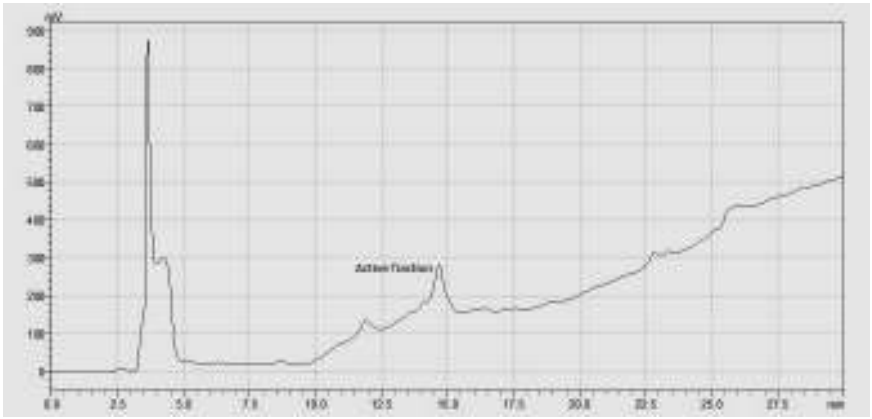

Fig 2: HPLC trace of the crude extract (ethanol ppt.) of 13

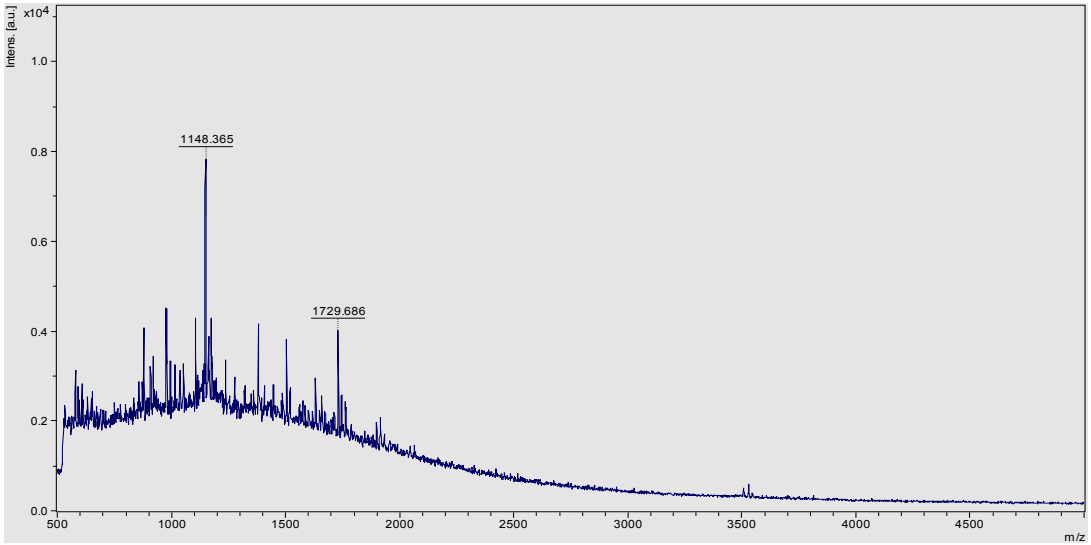

Fig 3: MALDI-TOF spectra of the HPLC active fraction of strain 13

Conclusion

The endophytic bacteria isolated from *Phyllanthus niruri* leaves shows excellent activity against *Candida albicans*. HPLC and MALDI-TOF results confirmed the presence of active compounds. Hence, along with further studies the compound purified from strain 13 can be used as medicine. However, 16s rRNA sequencing has to be done to identify the active strain. Further purification may result in the extraction of active compounds that are novel and efficient.

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