

Antimicrobial activity of Endophytes from aerial and non aerial parts of *Calotropis procera* against Pathogenic microbes

Preeti Juyal^{1*}, Vandana Shrivastava², Abhishek Mathur³

¹Himalayan University, Arunachal Pradesh, India;

²ITS Dental College, Ghaziabad (U.P), India.

³EBEC-NCFT, New Delhi, India

***Corresponding author:**

Preeti Juyal

Research Scholar, Dept. of Microbiology

Himalayan University, Arunachal Pradesh, India

E-mail: juyalpreeti@gmail.com; abhishekmthr@gmail.com

Abstract: Endophytes are the microbes which are invading in the host plant tissues and are believed to produce the similar metabolites as that of the plant tissue. The present study was performed for isolation, identification and determination of endophytes from leaves, stem and roots of *Calotropis procera* (Aak/Madar) antimicrobial potential against the dreadful pathogens and drug resistant strains. It is already reported that, *Calotropis procera* plays an important role in improving soil fertility and improved soil water holding capacity. The root bark is febrifuge, anti-helminthic, depurative, expectorant, and laxative. The powdered root promotes gastric secretions and useful in asthma, bronchitis, and dyspepsia. Flowers are useful in asthma, catarrh, anorexia, inflammations and tumours. In the present investigation, after the surface sterilization of leaves, stems and roots of the plant, different bacterial and fungal endophytes were isolated on Luria-Bertani (LB) and Potato dextrose agar (PDA) medium respectively. It was found that, 05, 06 and 03 fungal endophytic isolates were obtained from leaves, stems and roots respectively out of which 03 fungi were common in all the three. The dominant endophytic fungi were identified as *Aspergillus niger*, *Rhizopus stolonifer*, *Phoma hedericola*, *Curvularia pallaescens* and *Penicillium chrysogenum* and *Fusarium oxysporum*. Total of 10, 05 and 05 bacterial endophytes were isolated from leaves, stems and roots respectively. The dominant endophytic bacteria were identified as Gram positive bacilli (CPGPB), gram positive cocci (CPGPC) and gram negative bacilli (CPGNB). The antimicrobial activity of dominant endophyteic fraction viz. *Phoma hedericola* (PH), *Curvularia pallaescens* (CP), *Penicillium chrysogenum* (PC'), CPGPB, CPGPC and CPGNB were checked against test bacterial cultures viz. *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus* and *Pseudomonas aeruginosa*. The results were found to be very significant and surprising as all these endophytic solvent fractions showed potent antimicrobial activity against the test organisms.

Key words: *Calotropis procera*, Endophytes, antimicrobial activity, pathogens.

I. INTRODUCTION

Endophytes are those microorganisms that inhabit interior of plants especially leaves, stems, roots shows no apparent harm to host. These endophytes are diverse group of microbes which may be bacteria, fungi, actinomycetes etc [1-3]. It is meant that there is a great diversity of population of microbes residing in the tissues of most of the medicinal plants and thus are able to produce the similar kind of secondary metabolites as is produced by the specific plant tissue. Thus there is a need to explore the biodiversity and medical importance of such microbes residing in the plant tissues. To date, only a few plants have been broadly investigated for their endophytic biodiversity and their probable to produce bioactive secondary metabolites. Endophytic fungi are of biotechnological interest due to their potential as sources of secondary metabolites have proven useful for novel drug discovery [4]. Each plant has been reported to harbor one or more endophytes [5, 6]. Plant based natural constituents can be derived from any aerial or non aerial parts of the plant which may cause extinction of endangered plant species thus, there is a need to isolate and investigate the biodiversity of endophytes which can also produce similar kind of secondary metabolites as the part of the plant contains where the specific endophytic bacteria or fungus resides.

II. MATERIALS AND METHODS

Surface sterilization of plant tissues and Isolation of endophytes

Further the tissues of the plant were soaked in 70% alcohol for few seconds or in 0.5-3.5% sodium hypochlorite for 1-2 minutes followed by rinses in sterile double distilled water before placing it on a LB medium for isolation of endophytic bacteria [7]. For isolation of fungal endophytes surface sterilization of tissue requires 70% ethanol for 1-3 minutes, aqueous sodium hypochlorite (4% available chlorine) for 3-5 minutes again rinse with 70% ethanol 2-10 seconds and final rinse with double distilled water and drying in laminar air flow. Sterile knife blade was required to remove outer tissues from sample and to excise inner tissues. The PDA plates were kept for about 5-6 days for observation of growth of any fungal endophytes. All the plates were incubated at 28°C to promote the growth of endophytes and were regularly monitored for any microbial growth [8]. On observing the microbial growth, sub-culturing was done. Each endophytic culture were checked for purity and transferred to freshly prepared PDA plate. Appropriate controls will also be maintained in which no plant tissues were inoculated. The bacterial and fungal endophytes isolated were identified.

Maintenance of Endophytes for Identification and Future Use

The purified endophytic isolates were transferred separately to LB/PDA slants and broths depending on the case for bacterial and fungal endophytes respectively and accessioned accordingly depending upon the plant parts from which they have been isolated. Finally all the purified endophytes were maintained at 4°C till further used. Different biochemical tests were done for identification of bacterial and fungal endophytes. The bacterial isolates were tested for their morphological and biochemical characteristics (catalase enzyme activity). Gram stains were performed to determine the characteristics of the cell wall, cell shape and the arrangement of cells. The morphology of the endophytic bacterial strains was observed on slides under a microscope. For staining, 15 µL of a bacterial culture that is grown in nutrient broth overnight at room temperature with shaking at 150 rpm were heat-fixed onto a slide and then stained. The fungal slides if isolated were stained with lactophenol. The structures were observed using a photomicroscope. The samples were then compared to other samples reported in the literature [9-11].

Production of Secondary metabolites

LB broth and Potato Dextrose broth were prepared and autoclaved. Endophytic bacterial and fungal cultures were inoculated in the medium separately within the flasks. Flasks were then incubated at 28°C for 10-14 days in shaker. After incubation, extraction was done with different solvents (Chloroform, Ethyl acetate). The organic phase were collected and kept for drying at 37°C. The dry weight of the extract was determined.

Determination of Antimicrobial activity

Culture Media

For antibacterial test, Soyabean Casein Digest agar/broth was used.

Inoculum

The bacteria viz. *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus* and *Pseudomonas aeruginosa* were inoculated into Soyabean Casein Digest broth and incubated at 37°C for 18 h and suspension were checked to provide approximately, 10⁵ CFU/ml.

Determination of diameter of zone of inhibition by well diffusion method

The agar well diffusion method was modified [12]. Soyabean Casein Digest agar medium (SCDM) was used for bacterial cultures. The culture medium was inoculated with the bacteria separately suspended in nutrient broth. A total of 8 mm diameter wells were punched into the agar and filled with secondary metabolites at 200 mg/ml. Standard antibiotic (Erythromycin, 1 mg/ml) was simultaneously used as the positive control. The plates were incubated at 37°C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The procedure for assaying antibacterial activity was performed in triplicates to confirm the readings of diameter of zone of inhibition observed for each of the test organism.

III. RESULTS AND DISCUSSION

In the present investigation, after the surface sterilization of leaves, stems and roots of the plant, different bacterial and fungal endophytes were isolated on Luria-Bertani (LB) and Potato dextrose agar (PDA) medium respectively. It was found that, 05, 06 and 03 fungal endophytic isolates were obtained from leaves, stems and roots respectively out of which 03 fungi were common in all the

three. The dominant endophytic fungi were identified as *Aspergillus niger*, *Rhizopus stolonifer*, *Phoma hedericola*, *Curvularia pallaescens* and *Penicillium chrysogenum* and *Fusarium oxysporum*. Total of 10, 05 and 05 bacterial endophytes were isolated from leaves, stems and roots respectively. The dominant endophytic bacteria were identified as Gram positive bacilli (CPGPB), gram positive cocci (CPGPC) and gram negative bacilli (CPGNB). The antimicrobial activity of dominant endophytes fractions viz. *Phoma hedericola* (PH), *Curvularia pallaescens* (CP), *Penicillium chrysogenum* (PC'), CPGPB, CPGPC and CPGNB were checked against test bacterial cultures viz. *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus* and *Pseudomonas aeruginosa*. The results were found to be very significant and surprising as all these endophytic solvent fractions showed potent antimicrobial activity against the test organisms. The results are shown in **Table 1** and **Figures 1-7**. Previous studies reported the endophytic fungi from *Calotropis procera* [13]. Antibacterial activity of twenty different endophytic fungi isolated from *Calotropis procera* were recorded [14].

IV. CONCLUSION

The present study suggests that endophytic isolates had a great biodiversity in *Calotropis procera*. These endophytes are potential source of antimicrobial metabolites. The study may thus lead to the isolation and identification of significant antimicrobial molecule (s) from such endophytes. Further identification of such molecules is required to formulate some novel/alternate antimicrobial agents against such dreadful pathogens.

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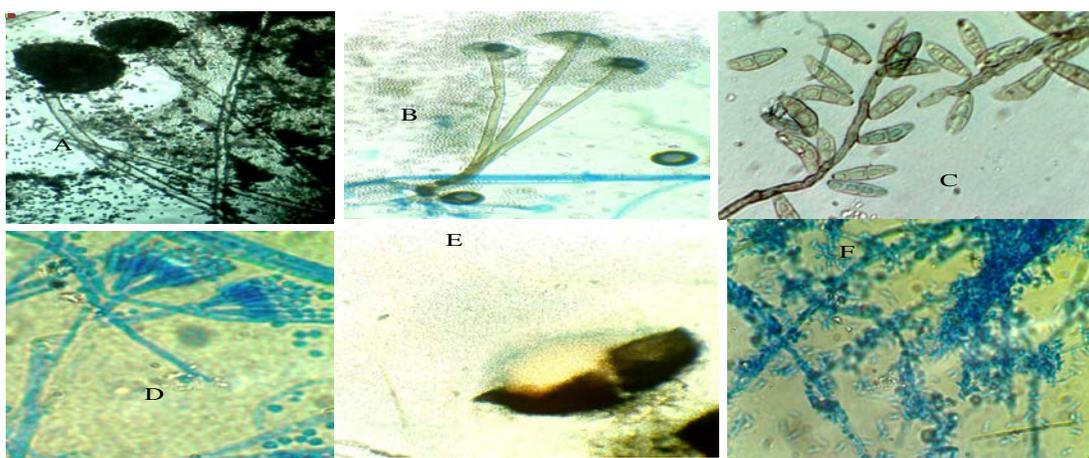
Figure 1: Inoculation of surface sterilized parts of *Calotropis procera* on LB and PDA



Figure 2: Growth of bacterial and fungal endophytes in leaves, stems and roots of *Calotropis procera*



Figure 3: Isolation of pure cultures of bacterial and fungal endophytes on LB and PDA media



Microscopic identification of fungal endophytes (A) *Aspergillus niger*; (B) *Rhizopus stolonifer*; (C) *Curvularia pallaescens*; (D) *Penicillium chrysogenum*; (E) *Phoma hedericola*; (F) *Fusarium oxysporum*

Figure 4: Microscopic taxonomical features of the fungal endophytes isolated

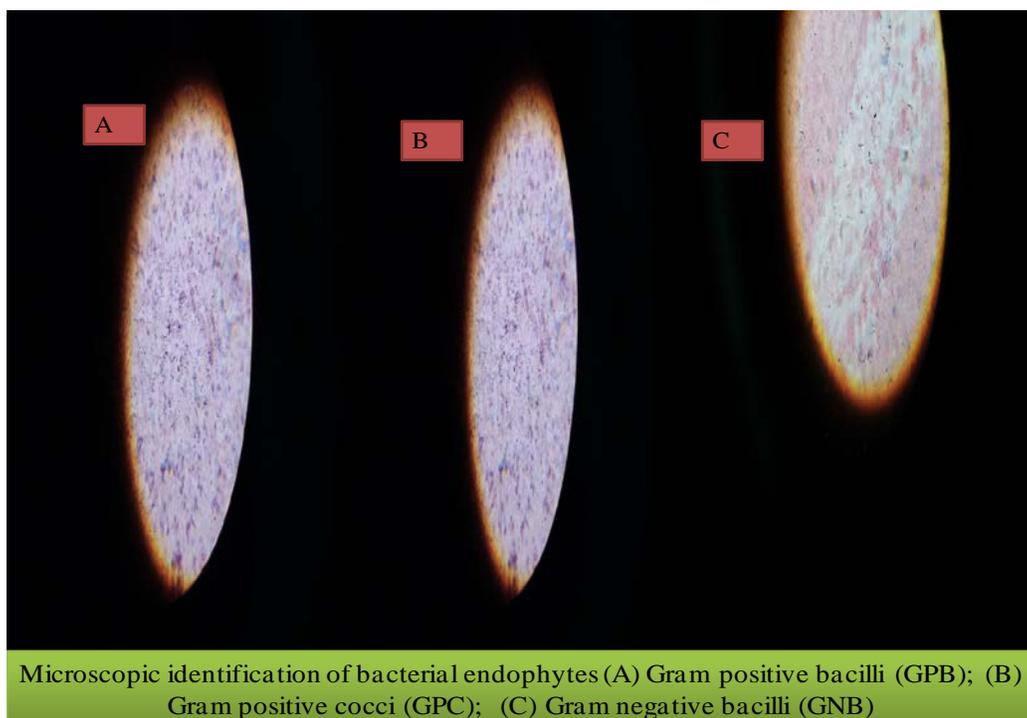
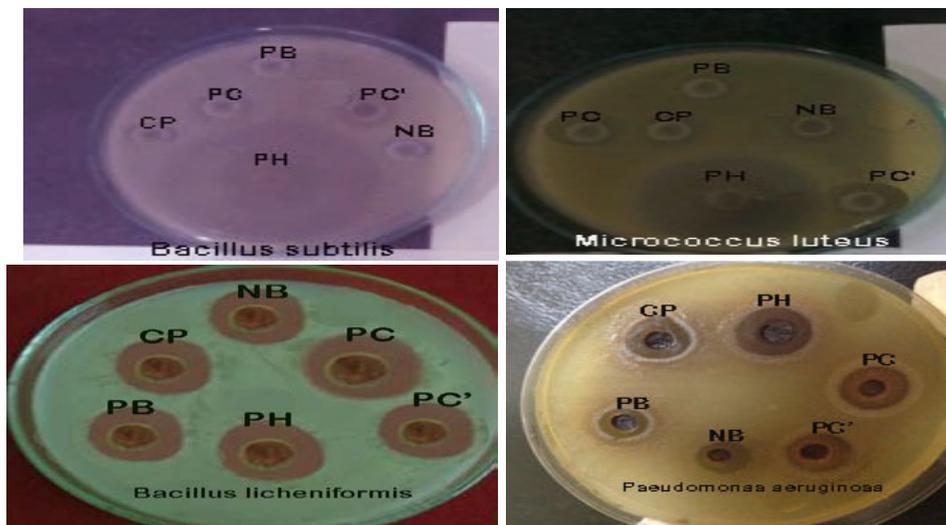


Figure 5: Microscopic images of the bacterial colonies observed after gram staining

Table 1: Antimicrobial activity of the endophytic fractions/secondary metabolites against the pathogens

Endophytic fractions/Secondary metabolites	Diameter of zone of inhibition (mm)			
	<i>Bacillus subtilis</i>	<i>Micrococcus luteus</i>	<i>Bacillus licheniformis</i>	<i>Pseudomonas aeruginosa</i>
<i>Phoma hedericola</i> (PH)	58.0	45.0	28.0	18.0
<i>Curvularia pallaescens</i> (CP)	12.0	10.0	18.0	14.0
<i>Penicillium chrysogenum</i> (PC')	10.0	15.0	20.0	15.0
Gram positive bacilli (PB)	10.0	8.0	28.0	12.0
Gram positive cocci (PC)	13.0	14.0	30.0	18.0
Gram negative bacilli (NB)	8.0	12.0	15.0	16.0



*Gram positive bacilli (PB); gram positive cocci (PC); gram negative bacilli (NB); *Phoma hedericola* (PH), *Curvularia pallaescens* (CP), *Penicillium chrysogenum* (PC')

Figure 6: Antimicrobial activity of the endophytic fractions

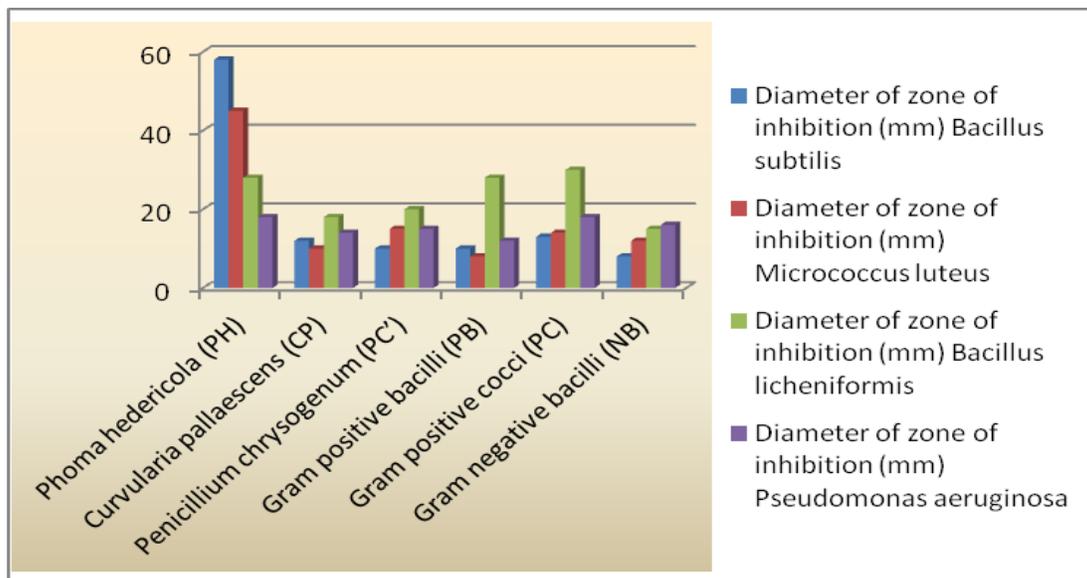


Figure 7: Graphical representation of the antimicrobial activity of the endophytic fractions/secondary metabolites