



Isolation, Identification and Antibacterial Activity of Endophytic Bacteria from Leaves of *Acacia catechu*

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ABSTRACT

Drug resistance in bacteria is becoming an alarming problem in recent years. Post-antibiotic era is not so far. The need for novel antibiotics, chemotherapeutic agents and agrochemicals are utmost. Endophytes are microorganisms that reside in the tissues of living plants which are relatively unstudied and are potential sources of novel natural products for exploitation in medicine, agriculture and industry. The endophytes of medicinal plants participate in biochemical pathways and produce novel bioactive compounds. The aim of the study was to isolate, identify and to assess antibacterial activity of endophytic bacteria from leaves of *Acacia catechu*. 18 endophytic bacteria had been isolated and identified by sequencing 16S rRNA gene. The isolated endophytic bacteria were identified as *Bacillus humi*. Considerable *in vitro* antibacterial activities against a panel of human pathogenic microorganisms (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Salmonella Typhimurium* and *Escherichia coli*) were recorded. Among 18 isolates, 15 isolates had shown inhibitory activity against *Staphylococcus aureus* and *Salmonella Typhimurium* and 10 isolates had shown inhibitory activity against *Klebsiella pneumoniae*.

Keywords: *Acacia catechu*, endophytic bacteria, 16S rRNA gene, *Bacillus humi*

Antibiotic resistant bacteria have become increasing worldwide. Antibiotic resistant is defined as the reduction of effectiveness of an antibiotic during treatment of infectious diseases. Gram-negative bacteria are more resistant than Gram-positive bacteria due to presence of outer lipopolysaccharide membrane in their cell wall which limits access of antibiotic into their targets in the bacterial cells. Other mechanisms that contribute to antibiotic resistance in bacteria include modification of antibiotic target site (Aravamuthan *et al.*, 2010). However, the discovery of new antimicrobial compounds either from natural products has declined over the years and have come to a standstill compared to the Golden Antibiotic Age (1940-1960). Endophytic bacteria are ubiquitous in most plant species, either through their active colonization of plant tissues or their latent residence. Endophytes have

recently been defined as microorganisms that can be isolated from surface-disinfected plant tissues and that do not visibly harm the plant (Hallmann *et al.* 1997). Many endophytes have antimicrobial activity. Seo *et al.* (2010) found that some endophytic bacteria isolated from young radishes can be used as biocontrol agents against human and plant pathogens.

The importance of endophytes had been demonstrated over a long period as a source of pharmaceutical bioactive compounds, as many of endophytes were exposed to produce novel bioactive metabolites such as

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antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, immunosuppressive drugs and many related compounds. Endophytes are well known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity and are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans, etc.

Importantly, secondary metabolites produced by endophytes provide a variety of fitness enhancements and exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and induce resistance to drought, herbivorous, parasitism etc. and as sources of novel biologically active secondary metabolites. Endophytes enter the plant tissue chiefly through the root zone; on the other hand, above ground portions of plants, may also be used for entry, such as cotyledons, stems, and flowers (Rosales *et al.*, 2017).

Acacia catechu is a small or medium -sized thorny tree upto 15 m tall. It belongs to *Fabaceae* family and subfamily *Mimosoideae*. The plant has diverse pharmacological actions. The phytoconstituent isolated from various parts of plant include flavanoids (catechin, epicatechin, epigallocatechin, rocatechin, quercetin), alkaloids (kaempferol, taxifolin), tannins (gallic acid, phlobatannins) and sugars (d-galactose, d-rhamnose). *Acacia catechu* is one such plant showing multifarious medicinal properties viz. anti-bacterial, antioxidant, antipyretic, anti-diarrhoeal, anti-inflammatory and wound healing (Yadav *et al.*, 2014). Various investigators reported endophytic microbes from various plant exists in different ecosystems. Consequently, the opportunity to find new and interesting microorganism among myriads of plants in different ecosystems is great. Previously, numerous reports showed on diversity of endophytic bacteria, fungi in medicinal plants (Strobel *et al.*, 2004), but from available literature there is no report on endophytic bacteria from *Acacia catechu* particularly in India.

MATERIALS AND METHODS

The research was carried out in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, N.D.V.S.U., Jabalpur (M.P.).

Collection and sterilization of *Acacia catechu* leaves

Fresh leaves of *Acacia catechu* were procured from Tropical Forest Research Institute campus, Jabalpur, M.P. Mature healthy plant leaves were collected. Eighteen leaves samples were taken and separated for further isolation of endophytic bacteria. Samples were immediately brought to laboratory and were used within 24 hrs and finally processed for isolation of endophytic bacteria. The leaves were surface sterilized with 0.1% sodium hypochlorite for 5 minutes, 0.01% Bavistin, 0.05% streptomycin, 70% ethanol, followed by five times rinses in double distilled water for 5 minutes. Leaves were dried in laminar flow (Mahajan *et al.*, 2014).

Sterility check

To confirm that the surface of leaves were effectively sterilized, 1 ml of the sterile distilled water that was used in final rinse of surface sterilization procedures were plated on to nutrient agar media and incubated at 37°C for 24 hrs. Bacterial growths were observed after 24 hrs.

Preparation and sterilization of media

King's B (KB) media (HiMedia, India), Mueller Hinton media (HiMedia, INdia), Blood agar media (HiMedia, India) and BHI broth (HiMedia, INdia) were prepared by adding agar into the distilled water. Hot plate was used for the proper mixing of media and autoclaved at 121°C for 15-20 minutes at 15 lbs.

Inoculation of leaves and isolation of endophytic bacteria

The media were poured into different autoclaved Petri plates and leaves of the plants were embedded in Petri plates. These plates were then incubated at 37°C for 24 hrs. Characterization of the bacteria was done according to its morphology and by Gram's staining. After that a single colony was transferred into BHI broth and incubated at 37°C for 24 hours.

Purification of endophytic bacteria

For purification of endophytic bacteria, subculturing was mainly done by streaking a loop full of BHI broth on the

fresh pre solidified blood agar plates and then incubated at 37°C for 24 hrs. After incubation the colony was transferred into BHI broth and then incubated at 37°C for 24 hrs and purity was checked by Gram's staining and stored for further work.

Characterization of endophytic bacteria

The characterization was done on the basis of staining, morphological, biochemical and molecular methods.

Staining

Gram's staining was done where thin smears of obtained cultures were prepared on separate glass slides, air dried and heat fixed. Each smear was covered with crystal violet and allowed to stand for 1 minute followed by washing with distilled water. Further Gram's iodine was added and allowed to stand for 1 minute. Decolourized with 95% ethyl alcohol and the slide was rinsed with distilled water. Slide was flooded with safranin to counterstain and allowed to stand for 1 minute. Smear was rinsed with distilled water and dried with absorbent paper. Slides were then observed at 100X.

Morphological characterization

Form, elevation, margin, surface, opacity and chromogenesis of isolated endophytic bacterial colonies were noted for morphological characterization.

Biochemical characterization

Various biochemical tests like catalase (Slide method), coagulase (Tube method), Voges-Proskauer, ONPG, urease, arginine utilization and sugar fermentation tests (Hi media Identification test kit) were performed.

Molecular characterization

DNA isolation – An overnight grown isolated endophytic bacterial colony were picked and resuspended it in 1 ml of autoclaved water in a microfuge tube. Later centrifuged for one minute at 10,000-12,000 rpm. Supernatant was removed and 200 µl of Instagene matrix was added to the pellet and incubated at 56°C for 15-30 minutes. Centrifuged pellet was vortexed for 10 seconds and

then tube was placed in a 100°C boiling water bath for 8 minutes. Vortexed at high speed for 10 seconds. Spinned at 10,000-12,000 rpm for 2-3 minutes. Finally isolated DNA was stored at -20°C till further use.

To perform molecular identification of bacteria, marker gene for 16S rRNA was used. The PCR used universal primers 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3') to amplify approximately 1500 bp of 16S rRNA gene (Edward *et al.*, 1989).

PCR reaction mixture

Readymix Taq Master mix with MgCl₂ (Himedia) was used to prepare PCR reaction mixture. In a PCR TUBE, 25 µl reaction mixture was prepared as follows:

Components	Quantity
Taq Master mix	12 µl
Forward primer	1.5 µl
Reverse primer	1.5 µl
DNA	5 µl
Deionized water	5 µl
Total	25 µl

Mixed gently and briefly centrifuged to collect all components to the bottom of the tube. The PCR tubes with all the components were then transferred to thermal cycler (Bio-Rad laboratories Inc. USA). The PCR protocol designed for 25 cycles is as follows and was kept same for all the primers used.

PCR amplification conditions

Initial denaturation step at 95°C for 2 min., 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, followed by final extension step at 72°C for 10 min.

Agarose Gel Electrophoresis of PCR products

The PCR products were tested for amplification of specific gene by agarose gel electrophoresis using 2.0 per cent agarose gel in 1x Tris Borate EDTA Buffer (Sigma-Aldrich, U.S.A.). A total volume of 35 ml of 2.0 per cent agarose (Sigma-Aldrich, U.S.A.) was prepared in 1x Tris Borate EDTA Buffer and placed in microwave oven until melted.

Molten agarose was allowed to cool to about 55°C and ethidium bromide was added to give a final concentration of 0.5µg/ml. The gel was poured on to electrophoresis trough fitted with comb. The gel was allowed to set on a flat surface for about 15 minutes. Electrophoresis trough was placed in an electrophoresis tank filled with 1x Tris Borate EDTA Buffer. Samples were prepared on a parafilm by mixing 1 µl of Gel Loading Buffer (Sigma- Aldrich, U.S.A.) and 5 µl of PCR products. Prepared samples were then loaded in parallel with 100 bp ladder (Direct load PCR 100 bp low ladder, Sigma- Aldrich, U.S.A.). Electrophoresis was done at 70 volts for 10 minutes, then at 50 volts for 2 hour. Gel was viewed under a UV Trans-illuminator and photographed with gel documentation system (*BIO RAD* Gel Doc EZ Images) for future analysis. PCR products were purified using Montage PCR clean up kit (Millipore).

Data sequencing was done at Triyat Genomics, Nagpur, Maharashtra. Sequencing results were compared with existing sequences using Basic Local Alignment Search Tool program on National Centre for Biotechnology Information site (www.ncbi.nlm.nih.gov) to obtain the homology. The program MUSCLE 3.7 was used for multiple alignments of sequences. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model.

Antibacterial assay

In vitro antibacterial activity of endophytic bacteria isolated from *Acacia nilotica* were studied. Various known pathogenic bacterial cultures viz. *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella* Typhimurium (ATCC 13311), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 6538) and *Streptococcus pyogenes* (ATCC 12386) were procured from Hi media. Mc-Farlands Nephelometer was used for the determination of concentration of known culture as described by Henric *et al.* (1956). 1 ml of known culture containing 3.0×10^9 cfu/ml as determined by Mc-Farlands Nephelometer was used for antibacterial activity of endophytic bacteria. For determination of antibacterial activity of endophytic bacteria, broths were centrifuged at 4°C at 12000 rpm for 30 minutes. Supernatant of each of these broths were taken, sterile disc were soaked in these broths in a sterile test tubes for 24 hrs and dried in laminar

flow. After drying the discs were used immediately for disc impregnation in the inoculated plates as described by Kirubaharan *et al.* (1999) with slight modifications. Ciprofloxacin discs were used as control drug to compare the effect of treatment during *in vitro* study. The prepared bacterial inoculums were evenly spread on a sterile Mueller Hinton agar plate as per method described by Bauer *et al.* (1969). The known antibiotic Ciprofloxacin (Ci) disc was simultaneously placed as a control for antibiotic sensitivity. The dried disc was incubated at 37°C for 24 hrs. Results were recorded as positive (growth) or negative (no growth) and zone of inhibition of growth exerted by these impregnated discs.

RESULTS AND DISCUSSION

Isolation of endophytic bacteria

A total 18 isolates of endophytic bacteria were obtained and were characterized using gram's staining (Fig. 1).



Fig. 1: Growth of endophytic bacteria from leaves of *Acacia catechu* on King's B media

Gram's staining

Endophytic bacteria isolated from leaves of *Acacia catechu* showed that 100 per cent isolates were gram positive and were found to be rods (Fig. 2).

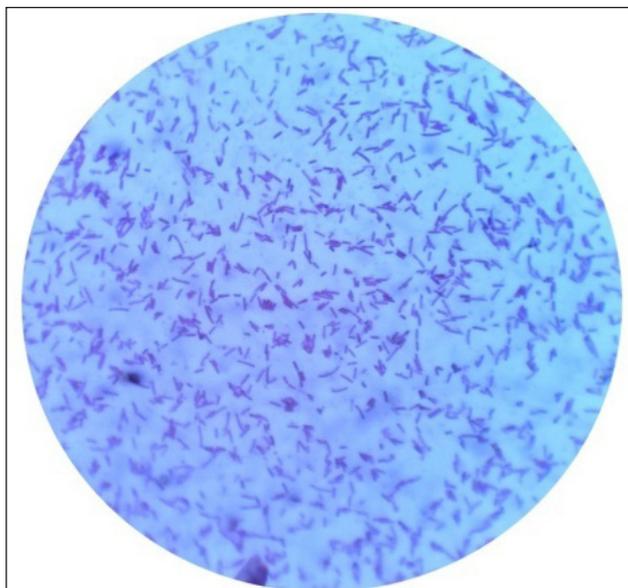


Fig. 2: Light microscopic view of endophytic bacteria isolated from *Acacia catechu* after Gram's staining

Morphological characterization

Growth characteristics of endophytic bacteria isolated from *Acacia catechu* from T.F.R.I campus showed that 84 per cent were irregular in shape while 16 per cent circular in shape, 89 per cent were flat elevation on petriplate while

11 per cent were raised elevation, margin of 67 per cent colonies were entire while 33 per cent were undulated, the surface of the growth was smooth for the entire colonies and 96 per cent growth were opaque and white in colour (Table 1).

Biochemical characterization

The endophytic bacteria isolated from *Acacia catechu* had shown positive reaction to catalase and sugar fermentation test (maltose) and negative reaction to coagulase, VP, ONPG, urease and arginine utilization tests. The phenetic characterization and biochemical profiling indicated the presence of endophytic bacteria in the leaves of *Acacia catechu*.

Molecular characterization

Endophytic bacteria were identified by comparing 16S rRNA gene sequences obtained from bacterial isolates and those deposited in (NCBI) GenBank database using BLAST tool to obtain the sequences that displayed maximum similarity. Results of this analysis showed that one given sequence was actually similar to those sequences of more than one species. The amplified PCR products were run on agarose gel electrophoresis using 2.0 per cent agarose gel in 1x Tris Borate EDTA Buffer, where the

Table 1: Growth of endophytic bacteria isolated from leaves of *Acacia catechu* on King's B media

Sl. No.	Isolate No.	Form	Elevation	Margin	Surface	Opacity	Chromo-genesis
1	TF1-1a	Irregular	Raised	Entire	Smooth	Opaque	Absent
2	TF1-1b	Irregular	Flat	Entire	Smooth	Opaque	Absent
3	TF1-1c	Irregular	Raised	Entire	Smooth	Opaque	Absent
4	TF1-1d	Irregular	Flat	Undulated	Smooth	Opaque	Absent
5	TF1-1e	Irregular	Flat	Undulated	Smooth	Opaque	Absent
6	TF1-1f	Circular	Flat	Entire	Smooth	Opaque	Absent
7	TF1-2a	Irregular	Raised	Entire	Smooth	Opaque	Absent
8	TF1-2b	Irregular	Flat	Entire	Smooth	Glistening	Absent
9	TF1-2c	Irregular	Raised	Entire	Smooth	Opaque	Absent
10	TF1-2d	Irregular	Flat	Undulated	Smooth	Opaque	Absent
11	TF1-2e	Irregular	Flat	Undulated	Smooth	Opaque	Absent
12	TF1-2f	Circular	Flat	Entire	Smooth	Opaque	Absent
13	TF1-3a	Irregular	Raised	Entire	Smooth	Opaque	Absent
14	TF1-3b	Irregular	Flat	Entire	Smooth	Opaque	Absent
15	TF1-3c	Irregular	Raised	Entire	Smooth	Opaque	Absent
16	TF1-3d	Irregular	Flat	Undulated	Smooth	Opaque	Absent
17	TF1-3e	Irregular	Flat	Undulated	Smooth	Glistening	Absent
18	TF1-3f	Circular	Flat	Entire	Smooth	Opaque	Absent

fragments with 1500 bp were observed (Fig. 3). Based on 16S rRNA sequence analysis, endophytic bacteria isolated from *Acacia catechu* were identified as *Bacillus humi*. Krishnan *et al.* (2012) had isolated endophytic bacteria from different papaya varieties and 16S rRNA sequence revealed that *Bacillus* species were the predominant bacterial endophytes (Fig. 4). The result in the study indicates that distribution of endophytic bacteria mainly depends on environmental conditions such as temperature, humidity, UV irradiation and nutrients in the apoplast and not only on host (Sun *et al.*, 2013). The higher frequency of *Bacillus* species in *Acacia catechu* leaves may indicate that they have formed a beneficial association with the plant.

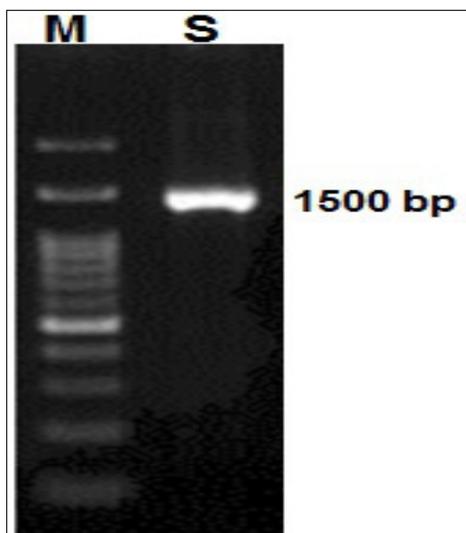


Fig. 3: Agarose gel electrophoresis of 16S rRNA gene Polymerase Chain Reaction product of endophytic bacteria isolated from leaves of *Acacia catechu*. Lane M: 100 bp ladder; Lane S: PCR product

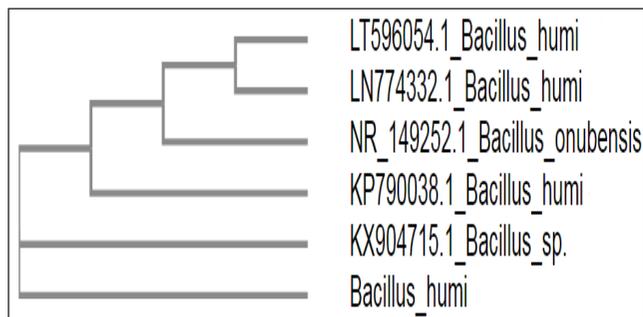


Fig. 4: Construction of Phylogenetic tree

In vitro antibacterial activity of isolated endophytic bacteria

The endophytic bacteria isolated from *Acacia catechu* shown antibacterial activity as 80 per cent isolates inhibited growth of *Staphylococcus aureus* (Fig. 5), 85 per cent of isolates inhibited growth of *Salmonella Typhimurium* (Fig. 6) and 60 per cent of isolates inhibited growth of *Klebsiella pneumoniae* (Fig. 7) and no isolates had inhibited growth of *Streptococcus pyogenes*, *Bacillus cereus* and *Escherichia coli* (Fig. 5).

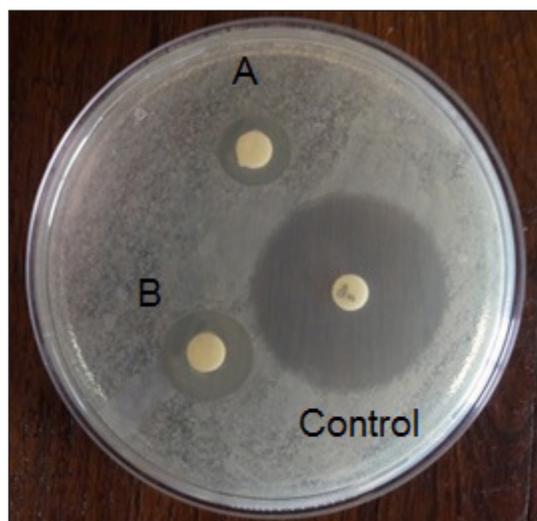


Fig. 5: *In vitro* antibacterial activity of endophytic bacteria isolated from leaves of *Acacia catechu* (A, B) against *Staphylococcus aureus*

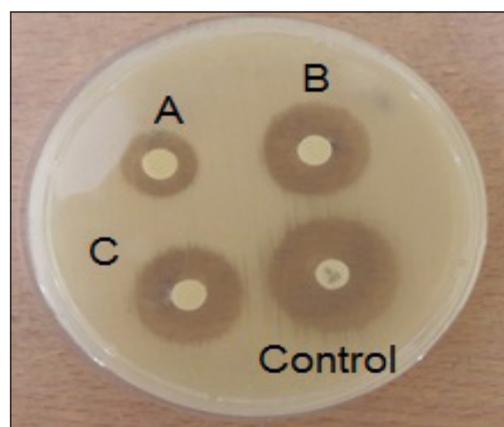


Fig. 6: *In vitro* antibacterial activity of endophytic bacteria isolated from leaves of *Acacia catechu* (A,B,C) against *Salmonella Typhimurium*

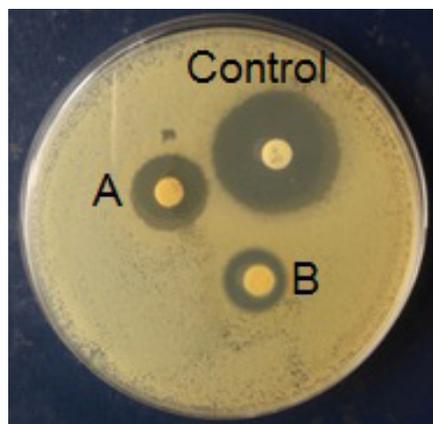


Fig. 7: *In vitro* antibacterial activity of endophytic bacteria isolated from leaves of *Acacia catechu* (A, B) against *Klebsiella pneumoniae*

This antibacterial effect which I have seen in my experimentation may be due to presence of metabolites which suggests that these metabolites of endophytic bacteria might had diffused in the culture medium and suppressed the growth of pathogenic bacteria. The findings of my studies are very similar with the reports of following researchers viz. Roy and Banerjee (2010) had isolated endophytic bacteria from *Vinca rosea* and one of the isolated endophytes produced potential antimicrobial activity against some selected pathogenic bacteria like *Bacillus cereus*, *Klebsiella pneumoniae*, *Vibrio cholera*, *Escherichia coli*. Sunkar and Nachiyar (2013) had found antibacterial activity of endophytic bacteria isolated from *Brassica oleracea* and the isolated endophyte was showing significant antimicrobial activity against *Klebsiella pneumoniae* NCIM 2883 (20 mm), *Staphylococcus aureus* ATCC 25923 (25 mm), *Escherichia coli* ATCC 25922 (13 mm) and *Salmonella typhi* ATCC 6539 (12 mm).

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