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Research Article

Genotypic Characterization Based on 16S rDNA Sequencing and Antimicrobial Properties of Bacterial Endophytes from Garcinia pedunculata

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Abstract

Endophytic microorganisms residing within unique environmental niches of medicinal plants are increasingly recognized as significant producers of diverse secondary metabolites with pharmaceutical potential. *Garcinia pedunculata*, a medicinally important plant of Assam, is traditionally utilized in the tteatment of various ailments such as dysentery, diarrhoea, and jaundice; still its endophytic potential remains largely unexplored. This study framed to isolate and characterize endophytic bacteria associated with *G. pedunculata* and evaluate their antimicrobial properties. Antimicrobial screening revealed that isolates PDGB-6 and PDGB-7 possessed broad-spectrum activity, exhibiting the highest zones of inhibition (28 mm and 27 mm, respectively) against *Klebsiella pneumoniae*. Minimum inhibitory concentrations (MIC) were determined for the most potent strains, ranging from 125 µg/ml to 500 µg/ml. The potent isolates PDGB-6 and PDGB-7 were identified as *Klebsiella* sp. (Accession number KU710415) and *Bacillus pumilus* (Accession number KU710416), respectively based on molecular identification via 16S rDNA sequencing and phylogenetic analysis using Neighbor-Joining method. Optimization studies indicated distinct temperature and pH requirements for maximum metabolite production. These findings confirm that *G. pedunculata* harbours novel endophytic bacteria capable of producing potent antimicrobial agents, validating the ethnopharmacological importance of the host plant

Keywords: *Antimicrobial activity; Bacterial endophytes; Garcinia pedunculata; 16S rDNA sequencing.*

Introduction

Microorganisms are capable of colonizing asymptomatically the internal healthy tissues of plants. These microorganisms which are referred to as 'endophytes', often maintain a mutualistic and symbiotic relationship with the host, benefitting the host plant through various mechanisms, such as growth promotion and enhancing the defensive ability of the host (Afzal *et al.,* 2019). These microorganisms, which include both bacteria and fungi, often maintain a symbiotic or mutualistic relationship with the host plant, benefiting the plant through various mechanisms including the promotion of growth and protection against pests and pathogens (Morales-Cedeño *et al.,* 2021). The internal tissues of plants provide a unique protective niche, often characterized by a specific chemical environment. Consequently, endophytes have adapted to these conditions, and in many cases, have evolved the molecular machinery necessary to synthesize complex bioactive compounds, some of which may supplement the secondary metabolites produced by the host plant itself (Singh *et al.,* 2017).

The exploration of endophytes from medicinally important plants has obtained significant traction in recent years for the urgent need of new antimicrobial agents to combat rising drug resistance. Endophytes are considered an untapped reservoir of natural products, contributing to the "chemical synthesizers" theory inside plants (Ezeobiora *et al.,* 2021). *Garcinia pedunculata*, commonly known as "Bor Thekera" in Assamese, is an evergreen tree belonging to the family Clusiaceae. It holds a prominent place in traditional medicine, being used as an antiscorbutic, astringent, cardiotonic, and emollient. The fruit rinds are particularly rich in hy-

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droxycitric acid (HCA), ascorbic acid, and phenolics, and are traditionally prescribed for dysentery, diarrhoea, dyspepsia, and flatulence. Despite its rich ethnobotanical history and the known bioactive profile of the genus *Garcinia*, which include benzophenones, flavonoids, and garcinol. In spite of these, the endophytic biodiversity of *G. pedunculata* in the unique ecoclimatic zone of Assam remains largely uncharacterized.

Bacterial endophytes, including genera such as *Pseudomonas*, *Burkholderia*, and *Bacillus*, are well-documented producers of secondary metabolites with antibiotic, anticancer, and immunosuppressive properties (Hnamte *et al.,* 2024). New pathways in biochemical pharmacology are emerging from the theory that endophytes contribute to the curative abilities of their host plants (Semenzato *et al.,* 2024). While fungal endophytes have historically dominated natural product research, endophytic bacteria are increasingly recognized for producing novel compounds, which are active against various pathogens at low concentrations (Liu *et al.,* 2020).

This study emphases on the genotypic characterization of endophytic bacteria isolated from *G. pedunculata*. Utilizing 16S rDNA sequencing, a robust molecular tool for bacterial taxonomy (Liu *et al.,* 2017; Sun *et al.,* 2008), we aimed to identify potent antagonistic strains. Furthermore, we evaluated their antimicrobial potential against a broad spectrum of bacterial and fungal pathogens to validate the prospect of endophytes colonizing *G. pedunculata* as a source of novel bioactive compounds.

Materials and Methods

Isolation of endophytes

Healthy bark of *G. pedunculata* were collected aseptically in sterile polythene bags, sealed immediately, and preserved at 4°C until processing. The plant materials were surface sterilized following a modified protocol (Kandasamy & Kathirvel, 2023) to eliminate epiphytic microorganisms. After removing the outer layers, the inner tissues of the surface-sterilized plant parts were cut into small segments using a sterile knife. These segments were then placed onto the Nutrient Agar (NA) plates and media amended with the bark extracts of the host plant to enhance isolation efficiency (da Silva Pinto *et al.,* 2025). Plates were incubated in a BOD incubator at 37±1°C. Emergent endophytic growth was transferred to fresh Nutrient Agar plates to maintain the purity of the cultures, which were subsequently preserved at 4°C.

Identification of Bacterial Endophytes

Bacterial isolates were initially characterized based on physiological and biochemical properties, including Gram staining. Definitive identification of potent strains was achieved through molecular analysis targeting the 16S rDNA region (Younas *et al.,* 2023). Genomic DNA was extracted, and the 16S rDNA region was amplified using universal primers (Forward: 5′ GTGTAGCGGTGAAATGCG 3′; Reverse: 5′ ACGGGGGCGTGTGTACAA 3′). The resulting amplicons (~1200 bp) were sequenced. Identification was confirmed based on nucleotide homology using NCBI GenBank and RDP databases. Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA4/6 software to determine evolutionary relationships (Hnini *et al.,* 2022).

Antimicrobial Screening

The antimicrobial activity of the isolated strains was evaluated using the agar cup diffusion method (Sebola *et al.,* 2019). Bacterial endophytes were cultured in Nutrient Broth (NB) at 37±1°C for 48 hours with shaking at 150 rpm. Following incubation, the cultures were filtered and centrifuged at 10,000 rpm for 15 minutes to prepare the crude extracts which were then dissolved in dimethyl sulphoxide (DSMO, 1mg/1mL). For antimicrobial assay, two Gram-positive bacteria viz. *Bacillus subtilis* (Bs) and *Staphylococcus epidermidis* (Se), two Gram-negative bacteria *Klebsiella pneumoniae* (Kp) and *Escherichia coli* (Ec), and one fungal pathogen *Candida albicans* (Ca) were used. Nutrient Agar (for bacteria) and Potato Dextrose Agar (for fungi) plates were inoculated with 0.2 ml of the respective test pathogen suspensions. Wells (6 mm diameter) were bored into the agar and loaded with 100 µL of the crude extract. Tetracycline and fluconazole served as positive antibacterial and antifungal controls, respectively, while DMSO served as the negative control. After incubation at 37°C for 24 hours, the zone of inhibition (mm) was measured. Bacterial strains exhibiting broad-spectrum antagonistic activity were designated as potent strains.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the ethyl acetate extracts was determined via broth microdilution (Semenzato *et al.,* 2024). Stock solutions prepared in DMSO were serially diluted in 96-well plates using RPMI 1640 medium to achieve con-

centrations ranging from 1000 to 35 µg/ml. Each well was inoculated with approximately 2.5 x10^4CFU/ml of the test organism. Plates were incubated at 28±2°C. The MIC was defined as the lowest concentration resulting in no visible growth.

Results

A total of 22 endophytic isolates recovered from *G. pedunculata* exhibited antimicrobial activity. Among the 22 bacterial isolates, 82% inhibited at least one test organism of both Gram-positive and Gram-negative bacterial test organism. A subset of 36% showed dual activity against both bacterial and fungal test organism.

Detailed screening revealed that isolates PDGB-6 and PDGB-7 possessed the most significant broad-spectrum antagonistic potential. The antimicrobial profiles of these strains are summarized in **Table 1**. Isolate PDGB-6 exhibited the highest zone of inhibition against *K. pneumoniae* (28 mm) and significant activity against *B. subtilis* (20 mm) and *S. epidermidis* (18 mm). Isolate PDGB-7 showed maximal inhibition against *K. pneumoniae* (27 mm) and *B. subtilis* (21 mm) **(Figure 1).**

Identification of the potent strains PDGB-6 and PDGB-7 were done through molecular characterization using 16S rDNA sequence analysis. PCR amplification of the 16S rDNA region yielded a product of approximately 1200 bp. Sequence alignment using NCBI GenBank and the RDP database confirmed the identities of the isolates. Strain PDGB-6 was identified as *Klebsiella* sp. (Accession No. KU710415). Strain PDGB-7 was identified as *Bacillus pumilus* (Accession No. KU710416). The phylogenetic relationships were reconstructed using the Neighbor-Joining method (**Figure 2** & **3**).

Table 1: Antimicrobial activity of different bacterial endophytes isolated from *Garcinia pedunculata*

Endophytic Bacterial strains	Test Organisms				
	Bs	Se	Kp	Ca	Ec
PDGB-1	+	++	++	+	-
PDGB-2	++	++	++	-	-
PDGB-3	-	+	-	+	+
PDGB-4	-	-	-	-	-
PDGB-5	+	++	+	++	-
PDGB-6	++	++	+++	++	++
PDGB-7	++	++	+++	++	++
PDGB-8	++	-	-	+	-
PDGB-9	-	-	-	-	-
PDGB-10	+	++	-	-	+
PDGB-11	+	-	++	+	-
PDGB-12	-	+	++	-	-
PDGB-13	+	+	-	-	+
PDGB-14	+	-	++	-	+
PDGB-15	++	++	-	-	++
PDGB-16	++	++	++	-	-
PDGB-17	-	-	+	-	-
PDGB-18	-	-	-	-	-
PDGB-19	-	++	++	++	-
PDGB-20	++	+	-	-	+
PDGB-21	+	+	++	+	-
PDGB-22	+	+	-	+	-
Flucanozole	-	-	-	-	++
Tetracycline	+++	+++	+++	++	-
Negative control	-	-	-	-	-

'+'<10 mm, '++'=10mm to 20mm, '+++'>20mm, '-'no zone of inhibition; **Bs**- *Bacillus subtilis*, **Se**- *Staphylococcus epidermidis*, **Kp**- *Klebsiella pneumoniae*, **Ec**- *Escherichia coli* and **Ca**- *Candida albicans*.

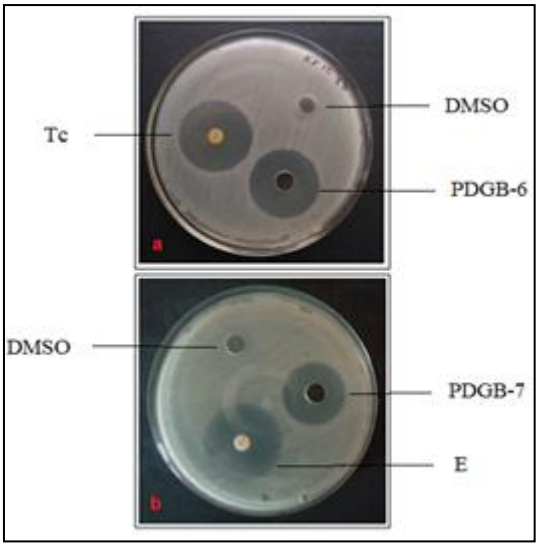

Figure 1: Antimicrobial activity of the crude extract of (a) *Klebsiella* sp. strain PDGB-6 (b) *B. pumilus* strain PDGB-7 against *K. pneumoniae*, co-assayed with tetracycline (Tc-30mcg/disc), erythromycin (E-15mcg/disc) and DMSO.

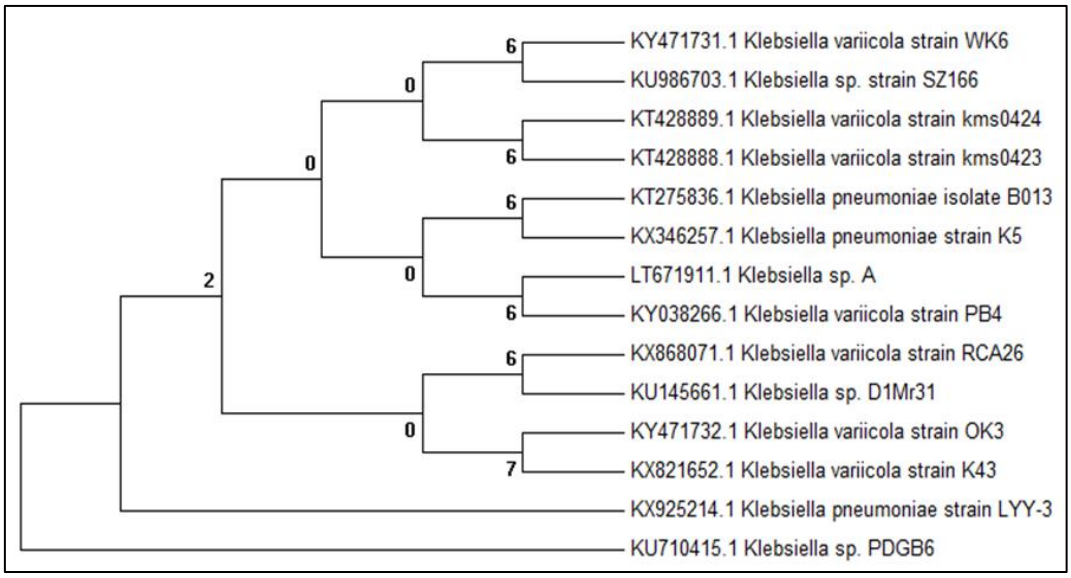

Figure 2: Phylogenetic analysis based on 16S rDNA sequence indicating the position of the strain PDGB-6 in MEGA 4 package using Neighbor-Joining method.

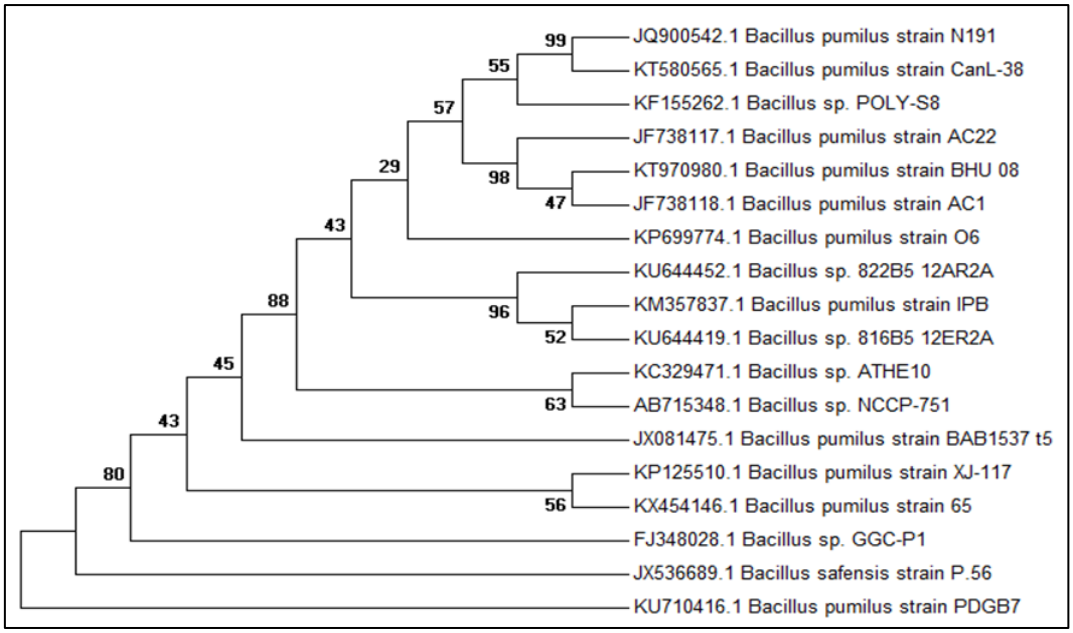

Figure 3: Phylogenetic analysis based on 16S rDNA sequence indicating the position of the strain PDGB-7 in MEGA6 package using Neighbor-Joining method

For PDGB-6, the MIC values were 125 µg/ml against *B. subtilis* and *S. epidermidis*, 110 µg/ml against *K. pneumoniae*, and 250 µg/ml against *E. coli*. PDGB-7 showed MIC values of 125 µg/ml against *B. subtilis* and *K. pneumoniae*, and 250 µg/ml against *S. epidermidis*. The highest MIC (lowest efficacy) was observed against *E. coli* (500 µg/ml) for both strains (Table 2 and 3).

Table 2: Zone of inhibition and minimum inhibitory concentration (MIC) of crude extract of isolated bacterial endophyte *Klebsiella* sp. strain PDGB-6 against different test organisms

Test organisms	Zone of inhibition (mm)	MIC
Bacillus subtilis	20	125±1
Staphylococcus epidermidis	18	125±1
Klebsiella pneumoniae	28	110±1
Escherichia coli	15	250±1
Candida albicans	13	500±1

Table 3: Zone of inhibition and minimum inhibitory concentration (MIC) of crude extract of isolated bacterial endophyte *B. pumilus* strain PDGB-7 against different test organisms

Test organisms	Zone of inhibition (mm)	MIC
Bacillus subtilis	21	125±1
Staphylococcus epidermidis	20	250±1
Klebsiella pneumoniae	27	125±1
Escherichia coli	10	500±1
Candida albicans	14	250±1

Growth parameters were optimized for maximum metabolite production. For *Klebsiella* sp. (PDGB-6), maximum growth (OD 0.35) and antagonistic activity (28 mm zone) were recorded at 30°C, pH 6.0, and an incubation period of 60 hours. For *B. pumilus* PDGB-7, optimum production required 35°C, pH 6.5, and 48 hours of incubation.

Discussion

The present study establishes *G. pedunculata* as a reservoir for diverse and bioactive bacterial endophytes. While the genus *Garcinia* is well-known for its phytochemical constituents such as benzophenones and flavonoids, the exploration of its associated microbial community in the North Eastern region of India has been limited. Our results align with the hypothesis that medicinal plants growing in unique environmental settings harbour novel endophytes capable of producing unique metabolites (Singh *et al.,* 2021).

The isolation of *Bacillus* and *Klebsiella* species as dominant antagonists is consistent with global literature on endophytic diversity. *Bacillus* species are among the most ubiquitous bacterial endophytes and are renowned for their capacity to produce a wide array of antimicrobial peptides and lipopeptides (Mahlangu & Tai, 2022). The identification of *Bacillus pumilus* PDGB-7 in this study corroborates findings by other researchers who have isolated this species from mangrove plants and medicinal roots, noting its broad-spectrum activity against plant and human pathogens (Singh *et al.,* 2017). The high inhibition zone (28 mm) against *K. pneumoniae* suggests the production of potent antibacterial compounds by this strain.

The detection of *Klebsiella* sp. PDGB-6 as a potent endophyte is noteworthy. While often associated with clinical pathogenicity, *Klebsiella* sp. are frequently recovered as endophytes from diverse plant hosts, including rice and maize (Sun *et al.,* 2008). In this study, *Klebsiella* sp.PDGB-6 exhibited significant activity against *S. epidermidis*, suggesting that the endophytic adaptation might drive the production of specific secondary metabolites involved in host defense or competition within the plant tissue. The molecular identification based on 16S rDNA sequencing provided robust confirmation of these taxonomies, a method widely accepted for differentiating closely related bacterial species (Romero *et al.,* 2014; Yu *et al.,* 2013).

Our study also noted the influence of environmental factors on the recovery of endophytes. Higher isolation rates were observed in leaf tissues compared to bark and roots, and frequency was higher during the summer season. This supports the observation that tissue type and seasonal climatic factors, such as rainfall and temperature, significantly influence endophytic colonization and diversity (Liu *et al.,* 2017; Mahlangu & Tai, 2022). The presence of growth-promoting factors in leaf extracts, as evidenced by improved isolation when media was amended with plant extracts, highlights the intimate biochemical relationship between the host and its microbiome (Afzal *et al.,* 2019).

Furthermore, while this paper focuses on bacterial endophytes, the broader study context revealed the presence of bioactive fungal endophytes, such as *Penicillium citrinum*. This co-occurrence suggests a complex microbial community within *G. pedunculata* where bacteria and fungi may coexist or compete, potentially driving the evolution of potent antimicrobial defense mechanisms (Hnamte *et al.,* 2024).

The optimization data suggests that the production of bioactive metabolites by these endophytes is tightly regulated by environmental parameters. The specific requirements for pH and temperature (e.g., 30°C and pH 6.0 for *Klebsiella* sp.) indicate that industrial upscaling of these metabolites would require precise fermentation controls. The findings contribute to the growing body of evidence that endophytic bacteria from ethnobotanical sources are promising candidates for drug discovery (Ezeobiora *et al.,* 2021; Semenzato *et al.,* 2024).

Conclusion

This study represents the first report on the isolation and genotypic characterization of bioactive bacterial endophytes from *Garcinia pedunculata* in Assam. We successfully identified potent strains *Klebsiella* sp. PDGB-6 and *Bacillus pumilus* PDGB-7using 16S rDNA sequencing. These isolates demonstrated significant broad-spectrum antimicrobial activity, particularly against *Klebsiella pneumoniae* and *Staphylococcus epidermidis*. The results validate the traditional medicinal use of *G. pedunculata* and suggest that its therapeutic properties may be partially modulated by its endophytic constituents. The optimization of growth conditions established here provides a foundation for future biotechnological applications. Further research into the structural elucidation of the specific metabolites produced by these bacteria is warranted to develop novel antimicrobial agents from this under-exploited natural resource.

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