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ORIGINAL RESEARCH ARTICLE

Antimicrobial Activity of Bacterial Endophytes from Medicinal Endemic Plant *Garcinia lancifolia* Roxb.

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Abstract: Endophytes are important source of bioactive metabolites having antibacterial, antifungal and antiviral activities. *Garcinia lancifolia* Roxb. is an endemic medicinal plant of Assam and is known for its antiscorbutic, astringent, cardiotonic, and emollient properties. The aim of this study was to isolate and identify the endophytic bacteria associated with *G. lancifolia* for potential antimicrobial activity. The five bacterial endophytes isolated from different parts of *G. lancifolia* were tentatively identified as *Bacillus cereus* (GlB_1), *B. megaterium* (GlB_2), *Staphylococcus* sp (GlB_3), *Corynebacterium xerosis* (GlB_4) and *C. kutscheri* (GlB_5) on the basis of their colony morphology and biochemical characteristics. The isolates were tested for antibiotic sensitivity, antibacterial and antifungal activities and plant growth promoting traits. All of them showed antibacterial and antifungal activities. Highest zone of inhibition (20 mm) was exhibited by GlB_1 against *K. pneumoniae* followed by GlB_3 and GlB_5 against *B. subtilis* and *E. coli* respectively. All the isolates showed positive result in production of I. A. A, NH_3 and solubilised phosphate.

Key Words: Bacterial endophytes; *Garcinia lancifolia;* Antimicrobial activity; Antifungal activity.

Introduction

Endophytes are microorganisms residing asymptomatically within the healthy plant tissues without causing any negative effects to their hosts (Bills 1996; Bacon and White 2000; Schulz and Boyle 2006). They are hypothesized to help their hosts by producing bioactive substances that confer resistance to the hosts. They are found in healthy tissues of almost all plants investigated so far, and have been accepted as a rich source of novel bioactive metabolites (Tan and Zou 2001) for use in agricultural and industry (Strobel and Daisy 2003) and therapeutic use in medicine (Tejesvi *et al.,* 2005). Plants growing in particular environmental setting having ethnobotanical uses, extreme age or interesting endemic locations generally harbour novel endophytes that may produce unique metabolites having diversified applications (Li *et al.,* 2005). Plants from a unique environmental setting and medicinally important are considered as a promising source of novel endophytes and their secondary metabolites (Strobel *et al.,* 2004). The isolation of endophytes from endemic medicinal plant is particularly important because both the habitat as well as the species is threatened because of destruction and loss of habitat owing to human activities (Tomita 2003). The endemic medicinal plants cannot be used for the treatment of diseases anymore because their population has decreased owing to their over exploitation as a source of medicine and therefore, the researchers are trying to isolate endophytes, which might produce similar bioactive compounds and can be used without destructing the plants (Li *et al.,* 2005). Bacterial endophytes play an important role in production of secondary metabolites such as antibiotics, anticancer drugs, volatile organic compounds, and fungicidal, insecticidal, and immunosuppressive agents. Endophytic bacteria are capable of reducing or preventing adverse effects of phytopathogens on their host plants (Chebotar *et al.,* 2015).

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G. lancifolia Roxb. of Clusiaceae is an evergreen tree which grows all over Assam. It has many medicinal properties and is extensively used in dysentery, diarrhoea, stomachache, fever, jaundice, diabetes and urinary problems (Chowdhury and Handique 2012; Baruah 2007). *G. lancifolia* is rich source of bioactive substances such as xanthones, biflavonoides, benzophenones with antimicrobial, cytotoxic and antioxidant acivities (Chowdhury and Handique 2012). It is believed that medicinal plants and their endophytic flora may produce similar metabolites. Some endophytes are capable of developing biochemical ability to produce compounds similar or identical to those produced by the host plant (Strobel 2003; Petrini *et al.,* 1992). Hence, it is important to study medicinal plants for their endophytic microflora for biodiversity and then to determine their medicinal properties. The present work, therefore, was taken up to study the endophytic bacterial population of endemic medicinal plant, *G. lancifolia* and its potent antimicrobial activity.

Materials and Methods

Collection of Samples and Isolation of endophytes: Healthy barks, leaves and roots of *G. lancifolia* were collected aseptically in sterile plastic bags from different places of Assam. Plant materials were sealed immediately after collection and preserved at 4^0 C until they were processed. Endophytes were isolated from surface sterilized plant parts. Surface sterilization was performed using the modified method of Guo *et al.,* (2008) and Wang *et al.,* (2008). Plant materials were washed in running tap water and then sterile distilled water. This was followed by consecutive immersion in 75% ethyl alcohol for 1 min, 3.25% sodium hypochlorite for 5 min and again 75% ethyl alcohol for 30 secs and then dried with sterile tissue paper. Outer tissues of the collected samples were removed and inner tissues were cut into small pieces with sterile scalpel

and sets of four segments were evenly placed in each petridish containing nutrient agar (N. A) and potato dextrose agar (P. D. A). The plates were incubated in B. O. D incubator at 37 ± 1^0 C until bacterial growth appeared on the plates. After 2-3 days of incubation the endophytic bacteria were transferred with a sterile needle to a freshly prepared petriplates containing nutrient agar. The plates were sealed with parafilm and incubated for 48 h and were checked for its purity.

Identification of Bacterial Endophytes: Endophytic bacterial isolates were identified on the basis of colony morphology and biochemical characteristics based on Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

Antibiotic sensitivity test: An antibiotic sensitivity test was carried out using discs impregnated with antibiotics like streptomycin, tetracycline, and amoxicillin (30µg/disc) by following Kirby Baurer disc-diffusion method (Bauer *et al.,* 1996). The diameter of the zone of inhibition was recorded. The endophytic bacterial strains were categorized as resistant, intermediate and sensitive following the DIFCO Manual 10th edition. Antibacterial activity Cross-streak assay method (Williston *et al.,* 1947) was used for assaying the bacterial isolates for their antibacterial properties. The test organisms used for this assay were two-gram positive *Bacillus subtilis*, *Staphylococcus epidermidis* and two gram negative bacteria *Klebsiella pneumoniae* and *Escherichia coli*. Nutrient agar was inoculated with bacterial endophytes as a single streak at the centre of the petridish and incubated for seven days (30^0 C). The overnight grown cultures of the test organisms were streaked at a right angle to the endophytes and observed for growth or zone of inhibition after 24-48 hrs of incubation.

Antifungal activity: Endophytic bacteria were tested against four mould strains for their fungistatic activity using 24 h culture of each strain grown in nutrient broth at 37° C. Fungal test cultures were prepared using P. D. A media. They were inoculated with three separate 15 drops of each bacterial culture spotted in rows on the agar. The plates were incubated at room temperature for seven days and the fungal growth inhibition was scored (Owen and Hundley 2004).

Production of I. A. A. Bacteria were cultured (25±2 °C) for 48 h in the nutrient media supplemented with 100 and 200 µg/ml of L-Tryptophan and then centrifuged (8000 rpm, 10 min). Supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M, FeCl3 solution) and production of I. A. A was confirmed by the development of pink color (Brick *et al.,* 1991).

Phosphate solubilization: The bacterial isolates were inoculated at three to four places on the Pikovskaya media containing tricalcium phosphate in a plate and incubated (28±2 °C) for 2-3 days (Pikovskaya 1948). The development of a clear halo zone around bacterial isolates indicated positive phosphate solubilization activity.

Ammonia production: Freshly grown test bacterial isolates were inoculated in 10 ml peptone water in the tubes and incubated (28±2 °C) for 48 h. Development of a brown to yellow color on mixing Nessler's reagent (0.5 ml) indicated ammonia production (Cappuccino and Sherman 1992). Hydrogen cyanide (H. C. N) production. Bacterial isolates were streaked on petriplates of solidified King's B medium and a single disc of filter paper was placed in the lid of each petriplate. The plates were then sealed with parafilm and incubated (25±2 °C) for 72 h. The color change in the filter paper from deep yellow to dark brown was visually assessed for production of H. C. N (Bakker and Schippers 1987).

Results and Discussion

Biochemical characterization: A total of twenty-five bacterial isolates belonging to five species were recovered from different parts of *G. lancifolia* (Fig. 1).

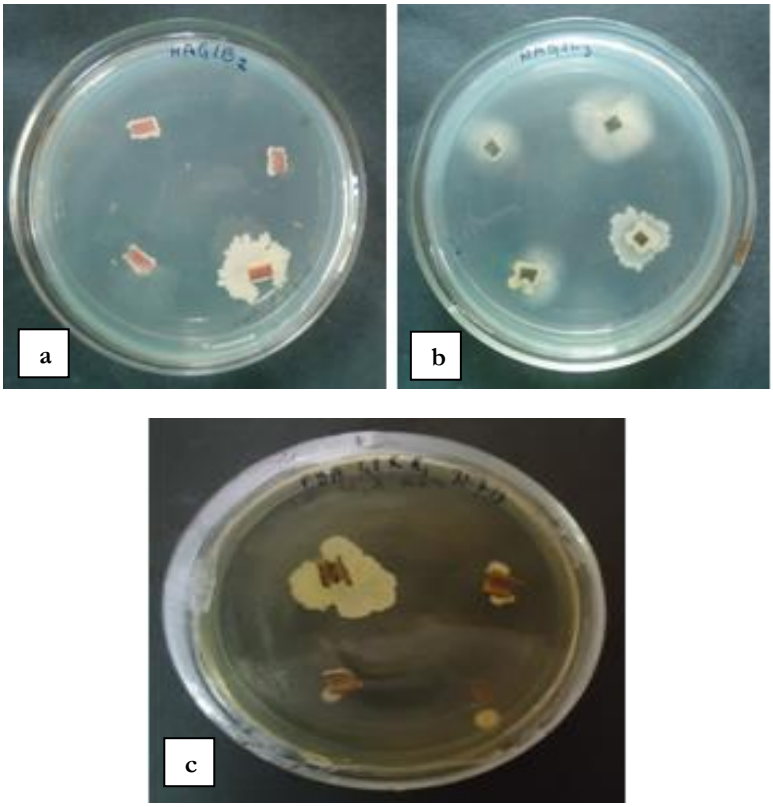

Figure 1: Isolation of bacterial endophytes from (a) bark, (b) leaf and (c) root of *G. lancifolia*

All the bacterial isolates were gram positive and rod shaped except strain GlB_3 which was coccus in shape. Eight strains (GlB_1) were identified as *Bacillus cereus*, six (GlB_2) as *B. megaterium*, four (GlB_3) *Staphylococcus* sp, three (GlB_4) *Corynebacterim xerosis* and four (GlB_5) *C. kutscheri*. Strains GlB_3, GlB_4 and GlB_5 showed positive for catalase activity whereas only GlB_4 reduced nitrate. Isolates GlB_1 and GlB_2 were able to form spore. Mannitol was fermented by isolates GlB_2 and GlB_3 (Table 1).

Table 1: Biochemical characteristics of bacterial strains isolated from different parts of *G. lancifolia*

Characteristics	GlB_1	GlB_2	GlB_3	GlB_4	GlB_5
Gram staining	+	+	+	+	+
Shape	Rod	Rod	Coccus	Rod	Rod
Spore formation	+	+	-	-	-
Catalase	-	-	+	+	+
Nitrate reduction	-	-	-	+	-
Mannitol	-	+	+	-	-
Voges-Proskaur test	-	-	-	-	-

Endophytic bacteria have been isolated from diverse categories of plants. Endophytic bacteria like *Bacillus* sp., and *Staphylococcus* sp were isolated from citrus and maize plants. *B. megaterium* was isolated from carrot, maize and citrus plants (Araujo *et al.,* 2001; Surette *et al.,* 2003). In the present study too *B. cereus*, *B. megaterium* and *Staphylococcus* sp were isolated frequently from different parts of *G. lancifolia*.

Antibiotic sensitivity: Antibiotic sensitivity assay was performed against three different antibiotics by disc diffusion method. The three antibiotics (30µg/disc) were streptomycin, tetracycline and amoxicillin. The results showed that bacterial strains GlB_2 and GlB_4 were most sensitive to antibiotic tetracycline and streptomycin respectively whereas GlB_5 appeared highly resistant to streptomycin as well as amoxicillin (Table 2).

Table 2: Antibiotic sensitivity of isolated endophytes

Bacterial strains	Zone of inhibition (mm) Antibiotics		
	Streptomycin	Tetracycline	Amoxicillin
GlB_1	22(S)	21(S)	10(I)
GlB_2	23(S)	28(S)	18(S)
GlB_3	17(S)	10(I)	22(S)
GlB_4	35(S)	20(S)	18(S)
GlB_5	6(R)	23(S)	8(R)

(S)-Susceptible; (R)-Resistant; (I)-Intermediate

Antibacterial activity: All the bacterial isolates tested showed antibacterial activity. Highest zone of inhibition (20 mm) was by GlB_1 against *K. pneumoniae* followed by GlB_3 and GlB_5 against *B. subtilis* and *E. coli* respectively (Table 3).

Table 3: Antibacterial activities of endophytic bacterial isolates

Bacterial strains	Test Organisms Zone of inhibition (mm)			
	Bs	Se	Kp	Ec
GlB_1	6	15	20	12
GlB_2	8	-	16	-
GlB_3	15	10	5	10
GlB_4	-	10	8	-
GlB_5	-	-	10	15

Bs- *Bacillus subtilis*; Se- *Staphylococcus epidermidis*;
Kp- *Klebsiella pneumoniae*; Ec- *Escherichia coli*;
'-'absence of zone of inhibition

Strain GlB_1 and GlB_3 were considered as most active strains as it was active against all the test bacteria. Endophytes like *Bacillus, Pseudomonas and Burkholderia* are well known for their diverse range of secondary metabolites which show antimicrobial activities. *Pseudomonas* sp isolated from many grass sp produce novel antimicrobial compound called ecomycins (Miller *et al.,* 1998). It was also found that these compounds were able to inhibit the human pathogen *Cryptococcus neoformans* and *Candida albicans*. Present investigation revealed that all the isolates with antimicrobial activity can be helpful in the inhibition of the plant pathogens and also be in their defence mechanisms. Antifungal activity. To examine the fungistatic properties of the endophytes, four fungal species were used as test organisms. These were *Candida albicans, Aspergillus niger, Alternaria alternata* and *Trichoderma viridae*. Fungistatic activity was indicated by zone of inhibition in the area where the endophytic bacterial strains were applied on the agar plates. All the endophytic isolates except GlB_2 showed activity against all the test organisms. Only strain GlB_1 showed activity against *Aspergillus niger* (Table 4).

Table 4: Fungistatic activities of endophytic bacterial isolates

Bacterial strains	Test Organisms			
	Ca	An	Aa	Tv
GlB_1	+	+	-	+
GlB_2	-	-	-	-
GlB_3	-	-	+	+
GlB_4	+	-	+	+
GlB_5	+	-	-	-

Ca-*Candida albicans*; An-*Aspergillus niger*;
Aa- *Alternaria alternata*; Tv-*Trichoderma viridae*;
'+' Presence of fungistatic activity;
'-' Absence of fungistatic activity

GlB_1, GlB_4 and GlB_5 showed activity against *C. albicans*, GlB_3 and GlB_4 against *A. alternata* and GlB_1, GlB_3 and GlB_4 inhibited the growth of *T. viridae*. Kumar *et al.,* (2015) isolated a number of endophytes from *Cassia tora*. Their isolates also showed antifungal activity which may be helpful to the host to resist the virulent plant pathogenic fungi (Kumar *et al.,* 2015).

P. G. P traits analysis. All the bacterial isolates produced indole acetic acid (I. A. A) as well as ammonium. All the isolates were capable of solubilizing phosphate (Table 5).

Table 5: Plant growth promoting traits of bacterial isolates of *G. lancifolia*

Bacterial strains	IAA Production	Phosphate Solubilization	NH_3 Production	HCN Production
GlB_1	+	+	+	-
GlB_2	+	+	+	-
GlB_3	+	+	+	-
GlB_4	+	+	+	-
GlB_5	+	+	+	-

None of them, however, produced H. C. N. Endophytic bacteria possess the capacity to solubilize phosphate (Verma *et al.,* 2001). They also produce I. A.A (Lee *et al.,* 2004) and promote plant growth. The nitrogen fixing endophytes alleviate N deficiencies in N-poor soils (Reiter et al. 2003). In this study also all the isolates produced I. A. A, NH_3 and carried out phosphate solubilisation and consequently helped their hosts to grow better.

Conclusion

From the results it is concluded that *G. lancifolia* is a perfect habitat for bacterial endophytes, which might act as a rich source of bioactive compounds with good antimicrobial activity. The ability of endophytic isolates to produce I. A. A, NH_3 and to carry out phosphate solubilisation indicates that these endodophytes can be used for the improvement of growth of their hosts. The biological activity of the bacterial endophytes owing to their secondary metabolite

producing ability indicated their medicinal properties and necessitates further detail investigation.

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