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Evaluation of antimicrobial, antioxidant and plant growth potential of endophytic bacteria isolated from *leucas aspera* and characterization of its bioactive metabolites

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Abstract
Exploration of bioactive compounds from plant endophytes increases the chance of finding novel compounds. Three endophytic bacteria having antimicrobial activity was isolated from the medicinal plant *Leucas aspera*. Due to superior antimicrobial activity, the strain isolated from roots of the plant was selected for further studies. It was characterized by morphological, cultural and biochemical studies. Scanning electron microscopic study supported the morphology of the cells. It has plant growth promoting attributes such as zinc solubilization, ammonia and organic acid production. Based on molecular and phylogenetic analyses, the strain presented 100% similarity to *Enterobacter ludwigii* strain Gol9. Bioactive compound was extracted from the isolate using a three solvent system and it showed zones of inhibition against both Gram negative and Gram positive bacteria. Thin layer chromatographic separation followed by FTIR and GC-MS analysis indicated a number of compounds. GC-MS analysis revealed six peaks of which the highest peak was shown by 1, 2-benzenedicarboxylic acid with a retention time of 40.761. The bioactive compound was found to possess anti-oxidant activities as well. These findings revealed that the endophytic bacterium isolated from *Leucas aspera* has the ability to produce potential bioactive compounds.

Keywords: endophytes, bioactive compounds, *Enterobacter ludwigii*, gc-ms, ftir, antioxidant activity

Introduction
The medicinal plant *Leucas aspera* is commonly known as “thumba” or “thumbai”, belonging to the family Lamiaceae. It is an annual, branched, herb or undershrub erecting to a height of 15-60 cm with stout and hispid acutely quadrangular stem and branches. It possess various pharmacological activities like antifungal, antioxidant, antimicrobial, antinociceptive and cytotoxic activity. It is known to produce numerous phytochemicals such as triterpenoids, oleanolic acid, ursolic acid, b-sitosterol, nicotine, sterols, glucoside, diterpenes and phenolic compounds [1]. The use of medicinal plants is growing rapidly as demand for herbal drugs, natural health products and secondary metabolites are increasing. However, medicinal plants are threatened with extinction due to overharvesting and habitat destruction [2]. Medicinal plants produce bioactive compounds at very low levels and there is difficulty in extraction and purification of such compounds [3]. Endophytes are known to produce the same or similar bioactive compounds as their host [4].

De Bary introduced the term endophyte and defined it as any organism that grows within plant tissues. One or more endophytes can be present in a single plant and they can colonize various structures of a plant such as the stem, roots, leaves, petioles, seeds and buds. The population of endophytes in a plant is variable. It depends on various factors such as host species, host developmental stage, climatic conditions and inoculum density. Endophytes can be ‘obligate’ or ‘facultative’. Obligate endophytes are those that depend on the metabolism of plants for their survival, being spread by different types of vectors or by vertical transmission. Facultative endophytes are those that can survive outside the body of the host during a certain stage of their lifecycle. Endophytes can be bacteria (actinomycetes or mycoplasma) or fungi [5].

Endophytic bacteria are a class of specialized rhizobacteria that have the ability to invade plant roots after the establishment of a rhizospheric population [6]. Epiphytic bacteria from the phyllosphere can also enter the host plants via natural openings such as stomata and hydathodes or wounds and cracks created by wind, insects, and pathogen Attacks [7].

Endophytes are known to produce a wide array of bioactive metabolites which can be used as drugs for the treatment of various diseases [5]. Bioactive compounds produced by endophytes are much more advantageous than those that are produced by plants alone. Some of the bioactive compounds include antibiotics, alkaloids, phenols, terpenoids, nanoparticles, phytohormones, various enzymes, anticancerous compounds, antioxidants and antiviral compounds. The antimicrobial agents are effective against multi-drug resistant pathogenic microbes. Amines and amides can be used as insecticidal agents. They also confer disease resistance to the host plants. Endophytes also enhance plant growth via production of phytohormones such as IAA, ADCC, siderophore production and phosphate solubilization enhances water and nutrient uptake [8].

In continuing the search for new endophytes from medicinal plants as a source of bioactive compounds, we isolated for the first time an endophytic bacteria belonging to *Enterobacter* sp. from the root of *Leucas aspera.* The bioactive compounds produced by the endophyte were purified and identified by GC-MS analysis.

Materials and Methods
Collection of host plant and surface sterilization
Healthy leaves, flowers and roots of *Leucas aspera*, were randomly collected from natural populations of Kothamangalam (10°03'27.2"N 76°36'57.4"E), Kerala, India and kept in sterilized polythene bags and brought to the

laboratory. The plant was identified by a botanist. The plant materials were cut into small pieces using a sterile blade. It was washed in distilled water and surface sterilized with 0.1% $HgCl_2$ for about 5 minutes and washed in sterile distilled water 3-4 times to remove the surface sterilizing agent. It was then placed in 70% ethyl alcohol for 5 minutes and again washed in sterile distilled water 3-4 times. Such sterilized samples were dried on blotting sheets. The final rinse water was streaked on Nutrient agar medium to ensure the efficiency of the surface sterilization procedure [9].

Isolation of bacterial endophytes

Three samples of each surface sterilized plant parts were aseptically placed on nutrient agar plates and incubated at 37ºC for 24- 36 hours. After incubation, the bacterial colony was subcultured on nutrient agar and each bacterial colony was checked for purity and subcultured again [9].

Primary screening of endophytic bacteria for antibacterial properties

Primary screening of isolates based on their inhibitory action against the test organisms, *Staphylococcus aureus*, *Pseudomonas* sp, *Escherichia coli* and *Klebsiella* sp. was conducted using cross-streak method. The test organisms were streaked at right angles to the line of growth of the endophytic bacteria which was previously inoculated and incubated at 37ºC for 24 hours. Such inoculated plates were re-incubated at 37ºC for 24 hours and the extent of growth inhibition against different test organisms were noted [10].

Secondary screening of endophytic bacteria

The endophytic bacteria (T) isolated from roots of Thumba (*Leucas aspera*) was selected for secondary screening on the basis of its inhibition against the test organisms in primary screening. A sample of the culture filtrate was assayed for antimicrobial activity. Using a sterile cotton swab, the test organism was spread over Mueller-Hinton agar plate surface and two holes with a diameter of 6-8mm were punched aseptically using a sterile tip on the agar plate. 50 µl culture filtrate was transferred to the well using a micropipette. The plate was incubated at 37ºC for 24 hours and observed for zone of growth inhibition. Peptone water as control along with a standard antibiotic disk of amikacin (AK) was placed in the plate [10].

Characterization of endophytic bacteria

Morphological, cultural and biochemical characterization

The size, shape, margin, elevation, consistency, opacity, pigmentation, Gram staining and motility of T was estimated along with various biochemical tests such as indole reduction test, methyl red test, Voges Proskauer test, citrate utilization test, nitrate reduction test, urease test, triple sugar iron test, catalase test and oxidase test [11].

SEM analysis

The isolate T was inoculated aseptically to 2ml peptone water in a sterile test tube and the tube was incubated at 37ºC for 24 hours**.** The suspension was centrifuged at 12, 000 rpm for 10 minutes and the supernatant was discarded. Using sterile tips, smears of the pellets were made on sterile glass slides (1mm * 1mm) and allowed to air dry. SEM analysis of the endophytic bacteria T was conducted to observe its morphology [12].

Plant growth promoting assays

Zinc solubilization

The organism was streaked on Pikovskaya's agar plate supplemented with zinc oxide as insoluble zinc source. The plate was incubated at 37ºC for 48- 72 hours. Clear halo around the streak line indicated zinc solubilization [13].

Ammonia production

The organism was inoculated into 10ml of peptone water and incubated at 37ºC for 48- 72 hours. After incubation 1ml of Nesseler's reagent was added to the tube. The development of brown to yellow color indicated positive ammonia production [14].

Phosphate solubilization

The organism was streaked on Pikovskaya's agar plate and was incubated at 37ºC for 48- 72 hours. Clear halo around the streak line indicated phosphate solubilization [24].

Organic acid production

The organism was inoculated into Pikovskaya's agar containing Methyl red indicator. It was incubated at 37ºC for 48- 72 hours. Color change from yellow to red shows organic acid production [13].

Indole acetic acid (IAA) production

The organism was inoculated into nutrient broth containing 0.1% Tryptophan and was incubated at 37ºC for 48- 72 hours. Presence of pink color indicated IAA production [15].

Total genomic DNA isolation, PCR amplification and sequencing

Genomic DNA of the isolate T was extracted using NucleoSpin® Microbial DNA isolation kit. The organism was inoculated into 3ml of Luria Bertanni Broth (LB) and incubated at 37ºC for 16 hours. The cells were harvested from the culture by centrifugation in a microcentrifuge tube and the supernatant was discarded. 100µl Elution Buffer BE was added and the cells were resuspended. The cell suspension was transferred into the NucleoSpin® Bead Tube Type B. 40µl Buffer MG and 10µl Liquid Proteinase K was added and the tube was closed. The NucleoSpin® Bead Tube was agitated and centrifuged 30s at 11,000 x g to clean the lid. 600µl Buffer MG was added, vortexed and centrifuged for 30s at 11,000 x g. The supernatant was centrifuged for 30s at 11, 000 x g. 500µl Buffer BW was added and centrifuged for 30s at 11,000 g. The NucleoSpin® Microbial DNA column was placed into a 1.5ml nuclease-free tube and 100 µl Buffer BE was added into the column. It was incubated at room temperature for 1 minute and centrifuged for 30s at 11,000 g to elute the DNA [16]. Agarose gel electrophoresis was used for the separation and visualization of the extracted DNA [17].

16s rRNA genes were amplified using forward primer A2: 5' AGAGTTTGATCCTGGCTCAG 3'and reverse primer S8: 5' TCTACGCATTTCACCGCTAC 3'. The PCR product was detected by agarose gel electrophoresis and was sent to AgriGenome Labs, Kakkanad, Kochi for sequencing [17].

Identification of endophytic bacteria and phylogenetic evaluation

The sequence was analyzed using NCBI Basic Local Alignment Search Tool (BLAST) and highly similar

sequences were found. The sequences were aligned using CLUSTAL W. A phylogenetic tree was constructed and the evolutionary history was inferred using the Neighbor-joining method. The reliability of the branching and clustering pattern was estimated from 1000 bootstrap replicates using MEGA-X. The sequence was deposited at GenBank and the accession number was obtained [17].

Determination of the effect of plant extracts on growth and antibacterial activity of endophytic bacteria

Roots of *Leucas aspera* were boiled in water to prepare the plant extract. 1 ml of bacterial suspension of T was mixed with 1 ml of peptone water and 1 ml of bacterial suspension of T was mixed with 1ml of plant extract. The tubes were incubated for a total of 120 hours. And readings were taken at a wavelength of 600 nm at 24 hours, 36 hours and 120 hours respectively.

Agar well diffusion method was used to check inhibition against the test organism *Staphylococcus aureus*. Mueller-Hinton agar plate surface is inoculated by spreading the test organism over the entire agar surface using a cotton swab. Three holes with a diameter of 6-8 mm was punched aseptically using a sterile tip or Cork borer on the agar plates. 50 µl of the bacterial suspension, plant extract, mixture of bacteria and plant extract along with peptone water as control were transferred to the wells using a micropipette. The plate was incubated at 37ºC for 24 hours and observed for zone of growth inhibition. This was performed at 24 hours, 36 hours and 120 hours [9, 10].

Extraction of Bioactive Compound from endophytic bacteria

Solvent selection

Growth from a mature slant culture of T was inoculated into 5ml of Nutrient broth aseptically and incubated for 37ºC for 24 hours. The solvents ethyl acetate, chloroform, acetone and hexane were selected. 1ml of each solvent was transferred into 4 sterile test tubes and 1ml of the suspension of T was added to each of the test tubes aseptically and vortexed. Aqueous and solvent layers were separated into sterile test tubes and dried in a waterbath. 500µl dimethyl sulfoxide (DMSO) was added to each. The zone of inhibition for each suspension against the test organism *Staphylococcus aureus* was determined using Agar well diffusion method and the solvent which exhibited the largest zone was selected [10].

Fermentation

Growth from a mature slant culture of the strain T was inoculated aseptically into four 1000ml conical flasks containing 500ml of Nutrient broth and incubated at 37ºC for 14 days with shaking. After the incubation period, extraction of bioactive compound was carried out using two methods [18].

Single solvent system

1000ml of the cultured media was added to equal amounts of ethyl acetate (1:1 ratio) and mixed thoroughly and left overnight to settle in a separating funnel. The aqueous and the solvent layers were separated, transferred to clean flasks and dried. 25µl of each extract in ethanol were used to determine the antibiotic activity against *Staphylococcus aureus* using agar well diffusion method [19].

Three solvent system

A three solvent system entailing petroleum ether, ethyl acetate and acetone was used for the recovery of bioactive compound from 1000ml of cultured media. Equal amount of petroleum ether was added to the cultured media (1:1 ratio) and mixed thoroughly and left overnight to settle in a separating funnel. The aqueous and the solvent layers were separated and tested for antimicrobial activity used Agar well diffusion method. The second solvent acetone was added to the aqueous extract and centrifuged at 12,000rpm for 15 minutes and the resultant precipitate was discarded after testing for its antimicrobial activity by redissolving it in 10ml methanol. The remaining solution was concentrated using a waterbath and equal volumes of ethyl acetate was added (1:1 ratio). It was mixed thoroughly and the aqueous and solvent layers were separated using a separating funnel. The antimicrobial activity of the solvent and the aqueous extract were checked using Agar well diffusion method against *Staphylococcus aureus*. The aqueous extract was discarded after establishing the presence of bioactive compound in the solvent extract [18].

Phytochemical screening

Phytochemical Analysis of aqueous and solvent extracts of T

The extracted solvent and aqueous extract from T was analyzed for the presence of phytochemicals such as carbohydrates, alkaloids, proteins, amino acids, saponins and flavonoids [20].

Thin layer chromatography (TLC)

TLCs were performed on silica gel plates. The chromatograms were developed using ascending method at room temperature. Using a capillary tube, a spot of the extract was applied 1.5cm above the bottom of the TLC plates and the spots were allowed to air dry. Different solvent systems were used to optimize the eluting process: methanol: chloroform (9:1, 1:9, 5:5, 3:7; v/v), ethanol: chloroform (9:1, 1:9, 5:5; v/v), chloroform: acetone (9:1, 1:9, 5:5; v/v) and butanol: glacial acetic acid: Water (4:1:5; v/v). Once the solvent moved upto 80% of the plates, it was taken out and allowed to air-dry. The TLC plates were visualized under ultraviolet light for checking fluorescence and the chromatograms were observed under iodine vapors. To detect phenols, terpenes, sugars and steroids, Anisaldehyde-sulphuric acid reagent can be sprayed over the TLC plate and heated at 100-105ºC. It yields violet, blue, red, grey or green products depending on the compound. The pink colored background obtained can be bleached by exposure to steam [21, 22, 23].

R_f values are calculated using the formula:

R_f = Distance moved by solute/ Distance moved by solvent

FTIR analysis

FTIR analysis was used to characterize the function groups present in the bioactive compound. The chemical structure of the compound can be proposed from the results obtained [24].

Gas chromatography mass spectroscopy (GC-MS) analysis

The GC-MS analysis of the bioactive compound was performed using Shimadzu GC-MS (model number: QP2010S) with ELITE-5MS column was used. A library

search was carried out using NIST 11 and WILEY 8 to identify the compounds [24].

Biological assays

Antifungal activity

The antifungal activity of the aqueous extracts obtained by the two methods were assessed using Agar disk-diffusion method. Sterile paper disks were spotted with 50µl of the extracts and allowed to dry before placing on the surface of solid Rose Bengal medium in petri dishes freshly seeded with the test organisms *Penicillium* and *Aspergillus species.* The plates were incubated at 30ºC in a humidified environment for one week and inspected daily. After the incubation period, the antifungal activity was assessed [25].

Immersion or agar overlay bioautography

The chromatogram was covered with a molten Mueller-Hinton agar medium seeded with *Staphylococcus aureus*. It was allowed to solidify and incubated at 37ºC for 24hours. After incubation it was stained with a tetrazolium dye. 2-(4-iodophenyl)-3(4-nitrophenyl)-5-phenyl-2H-tetrazolizum chloride (INT) was used as the visualizing reagent. The zones of microbial growth inhibition are indicated by the tetrazolium salt which is a dehydrogenase activity-detecting reagent. The metabolically active microorganisms convert the terazolium salt into its corresponding deeply colored formazan [26].

Detection of antioxidant agents by bioautography

The developed chromatogram is sprayed with 0.2% 2,2-diphyenyl-1-picrylhydrazyl radical (DPPH) in methanol/ethanol and the plate is examined in daylight. The maximum absorption of DPPH is at 217nm which decreases upon reduction during a reaction with a radical scavenger. The color change following the reaction can thus be observed. The free-radical scavengers appear as yellow/cream spots against a purple background [26].

Comparison of antibacterial activities of bioactive compound alone and in combination with standard antibiotics

Antibacterial Activity of Bioactive Compound

The antibacterial activity of the obtained bioactive compound was assessed by Kirby- Bauer method against the test organisms *Staphylococcus aureus*, *Pseudomonas* sp., *Escherichia coli* and *Klebsiella* sp. [10].

Determination of synergism or antagonism pattern of bioactive compound with standard antibiotics

The antibacterial activity of the bioactive compound along with standard antibiotics (Bacitracin, penicillin, amikacin and vancomycin) were assessed by Kirby- Bauer method to analyze synergism and antagonism against the test organisms *Staphylococcus aureus*, *Pseudomonas* sp., *Escherichia coli* and *Klebsiella* sp. [10, 27].

Results and Discussion

In the present endeavor, the well-known medicinal plant *Leucas aspera* was utilized for the isolation of endophytic bacteria. From healthy root, leaf and flower tissues, three bacterial strains were isolated, namely T, Tl and Tf respectively (Fig 1). The effectiveness of the surface sterilization method was verified as no microbial growth was observed in the control plates. It ensured that the isolates were endophytes. These were streaked on Nutrient agar plates and maintained.

The antibacterial potential of the isolated strains were tested using primary and secondary screening methods. The primary screening of isolates revealed that the isolate T obtained from root tissues of Thumba (*Leucas aspera*) presented inhibitory action against the test organisms. The largest inhibition was observed against *Staphylococcus aureus* (Table 1). Based on primary screening, T was selected for secondary screening of the isolate to confirm its inhibitory activity. The largest inhibition was observed against *Staphylococcus aureus* (Table 2). Various endophytes that possess antibacterial properties against pathogens have been previously isolated from medicinal plants. Such endophytes had nearly 60% bioactive activities [28]. On the basis of the antibacterial potential among the three strains, the strain T was considered for further studies.

Colonies of the endophytic bacteria T on Nutrient agar was observed to be small, circular, entire, flat, translucent and mucoid. It produced a green pigment. The isolated was revealed to be Gram negative, motile and rod shaped. It was VP positive but indole and MR negative. It was catalase and oxidase positive and able to utilize citrate and hydrolyze urea. SEM analysis also revealed rod shaped bacteria (Fig 2).

Bacterial endophytes are known to enhance the growth of their host plants. The endophyte T exhibited growth promoting attributes such as ammonia and organic acid production as well as zinc solubilization. These attributes have been previously reported in several endophytic strains like *Actinobacter*, *Pseudomonas* and *Bacillus* sp [29].

The DNA of the isolate T was extracted and 16s rRNA genes were amplified by applying PCR, followed by sequencing and a BLAST- based approach. The obtained sequence revealed a 634bp nucleotide sequence which showed hits against the databases with high identities (99%-100%). The sequence was deposited in NCBI GenBank (accession number MW245831) and the best hits were selected to construct a phylogenetic tree using MEGA-X (Fig 4). Based on the phylogenetic and BLAST analyses, the bacterial endophyte named T was assigned as *Enterobacter* sp., since the closest strains in the NCBI database were belonging to *Enterobacter* sp, and the most similar one is *Enterobacter ludwigii* strain Gol9 with 100% sequence identity.

Enterobacter sp. are known to be present as endophytes in various other plant species such as *Musa* sp. [30], *Ocimum sanctum* [31], *Brassica chinensis* L [32], *Vochysia divergens* [33], *Panicum virgatum* [34] and *Ophiopogon japonicas* [35].

Readings were taken at a wavelength of 600 nm at 24 hours, 36 hours and 120 hours respectively to assess the growth of bacteria and it was seen that the plant extracts did not enhance the growth of the endophytic bacteria (Table 3, Fig 5). Zone of growth inhibition was measured and it was observed that the plant extract of *Leucas aspera* did not enhance the inhibitory activity of T (Table 4).

The ethyl acetate fraction exhibited the largest zone of growth inhibition (16 mm) against the test organisms and it was selected for extraction of bioactive compound from the isolate T. This result is consistent with various other literature reports, stating that ethyl acetate is a hydrogen bond acceptor molecule and is better suited to extract electron donor solutes more than other solvents such as

chloroform. This was further confirmed in the studies by Kavitha *et al* and Mtunzi *et al*, where ethyl acetate was observed to have been the finest extractant for antimicrobial compounds [36, 37].

The bioactive compound was extracted using two methods and it was revealed that the second method was more efficient. It was observed that the bioactive compound extracted using the second method yielded a wider zone of inhibition (20 mm) than the bioactive compound extracted using the first method (10 mm) against *Staphylococcus aureus* (Fig 3).

In phytochemical screening, a positive biuret test was observed which indicated the presence of proteins and amino acids in the aqueous extract of T.

TLCs were performed on silica gel plates and the chromatograms developed with the solvent system methanol: chloroform (9:1, v/v) yielded spot with an Rf value of 0.730. It was visualized using ultraviolet light and iodine vapors. The spot indicated a presence of steriods upon spraying with anisaldehyde-sulphuric acid reagent.

The bioactive compound obtained was subjected to IR analysis to detect functional groups which may lead to propose a possible chemical structure for them. Fig 6 shows the IR results of the bioactive compound. The different peaks corresponding to different functional groups are given in Table 5. Six compounds were detected by GC-MS analysis (Table 6). The compound 1, 2-benzenedicarboxylic acid exhibited the highest peak with a retention time of 40.761. This compound is known to possess antimicrobial and anticancer properties. It has been previously isolated from endophytes of *Verticillium dahliae*, a marine *Streptomyces* sp. [38]. The mass spectral analysis of each component was illustrated in Fig 7. The extracted bioactive compound did not inhibit the growth of the test fungi, *Penicillium* and *Aspergillus species*. Bioautography being a rapid, inexpensive and effective technique was used for the detection of antibacterial and antioxidant activities of the bioactive compound [39]. The chromatogram sprayed with the visualizing reagent INT showed zone of microbial growth inhibition surrounded by pink colored metabolically active cells. The bioactive compound showed antioxidant activity. The free-radical scavengers appeared as cream colored spots against a purple background when the developed chromatogram was sprayed with DPPH in methanol. The bioactive compound exhibited significant anti-bacterial activity against Gram negative and Gram positive test organisms. The largest zone of growth inhibition was observed against *Staphylococcus aureus* (Table 7). Endophytes have established resistance mechanisms to combat pathogenic intrusion by the production of secondary metabolites [39]. Several secondary metabolites produced by endophytes possess antimicrobial properties [40]. Bioactive compounds are known to enhance the activity of standard antibiotics [27]. In this study there was no significant synergistic or antagonistic activity of the bioactive compound with standard antibiotics against test organisms. The antibacterial activity of the bioactive compound in combination with a standard antibiotic penicillin had a synergistic effect against *Pseudomonas* sp. While the compound had an antagonistic effect against *Pseudomonas* sp., when in combination with the standard antibiotic amikacin.

When tested against *Staphylococcus aureus* the bioactive compound acted synergistically in combination with penicillin and antagonistically with vancomycin. The bioactive compound in combination with amikacin exhibited a synergistic effect and in combination with vancomycin, it exhibited an antagonistic effect against *Klebsiella* sp.

When tested against *E.coli*, the bioactive compound in combination with vancomycin exhibited an antagonistic effect (Table 8).

Tables and Figures

Table 1: Primary screening of endophytic bacteria for antibacterial properties

Endophytic bacteria	Zone of inhibition against test organisms (mm)			
	Staphylococcus aureus	*Pseudomonas* sp.	*Escherichia coli*	*Klebsiella* sp.
T	7	2	1	1
Tl	0	0	0	0
Tf	0	0	0	0

Table 2: Secondary screening

Test organisms	Zone of growth inhibition (mm)		
	T	Amikacin (AK)	Control (C)
Staphylococcus aureus	20	23	0
Pseudomonas sp	0	25	0
Escherichia coli	0	18	0
Klebsiella sp.	15	20	0

Table 3: Effect of plant extract on growth of endophytic bacteria

Time	Growth of Bacteria (600 nm)		
	Blank	Plant Extract + Bacteria	Bacteria
24 hours	0	0.9605	1.0145
36 hours	0	0.9141	1.569
120 hours	0	0.9706	1.2589

Table 4: Effect of plant extract on antibacterial activity of endophytic bacteria

Time	Zone of Growth Inhibition (mm)		
	T	Plant Extract + Bacteria	Plant
24 hours	25 mm	20 mm	0 mm
36 hours	30 mm	25 mm	0 mm
120 hours	50 mm	35 mm	0 mm

Table 5: Absorption peaks of functional groups obtained in the FTIR analysis of bioactive compound

Peak number	Group frequency (cm^{-1})	Functional group
1	3430.04	Heterocyclic amine
2	2997.95	Alkanes
3	2913.91	Methyne
4	1659.42	Amide
5	1435.88	Carbonate ion
6	1406.18	Carboxylate (Carboxylic acid salt)
7	1311.75	Dialkyl sulfones
8	1016.67	Phosphate ion
9	951.55	Aromatic phosphates
10	699.48	Aryl thioester
11	668.71	Thioester
12	526.67	Aryl disulfides

Table 6: Compounds identified from T by GC-MS

Peak	Retention time	Chemical name	Chemical structure	Molecular weight ($g.mol^{-1}$)
1	7.246	1-Hexanol, 2-ethyl	$C_8H_{18}O$	130.231
2	10.207	2-ethylhexyl acetate	$C_{10}H_{20}O_2$	172.26
3	18.048	Quinoline,1,2-dihydro-2,2,4-trimethyl	$C_{12}H_{15}N$	173.25
4	38.689	Decanoic acid, 2-ethylhexyl ester	$C_{18}H_{36}O_2$	284.5
5	40.761	1,2-benzenedicarboxylic acid	$C_8H_6O_4$	166.1308
6	41.908	2-ethylhexyl decanoate	$C_{18}H_{36}O_2$	284.5

Table 7: Antibacterial activity of bioactive compound

Test organism	Zone of growth inhibition(mm)
Pseudomonas sp	8 mm
Staphylococcus aureus	10 mm
Klebsiella sp	16 mm
E.coli	11 mm

Table 8: Synergism and antagonism pattern of bioactive compound

Test Organisms	Zone of inhibition (mm)							
	Antibiotics				Antibiotics And Bioactive Compound			
	Penicillin	Amikacin	Bacitracin	Vancomycin	Penicillin	Amikacin	Bacitracin	Vancomycin
Pseudomonas sp.	7mm	21mm	7 mm	7 mm	8 mm	20 mm	7 mm	7 mm
S. aureus	7 mm	27 mm	7 mm	16 mm	11 mm	27 mm	11 mm	12 mm
Klebsiella sp.	50 mm	25 mm	13 mm	29 mm	50 mm	26 mm	13 mm	28 mm
E.coli	27 mm	28 mm	11 mm	21 mm	27 mm	28 mm	11 mm	20 mm

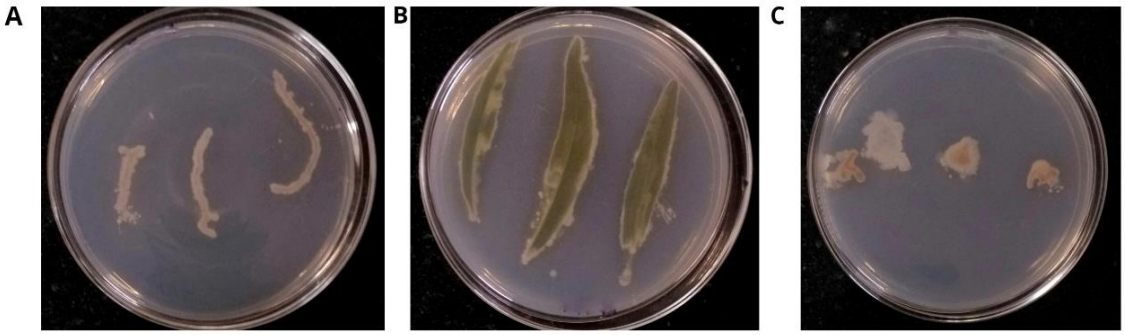

Fig 1: Endophytic Bacteria from Roots, leaves and flowers of Leucas aspera

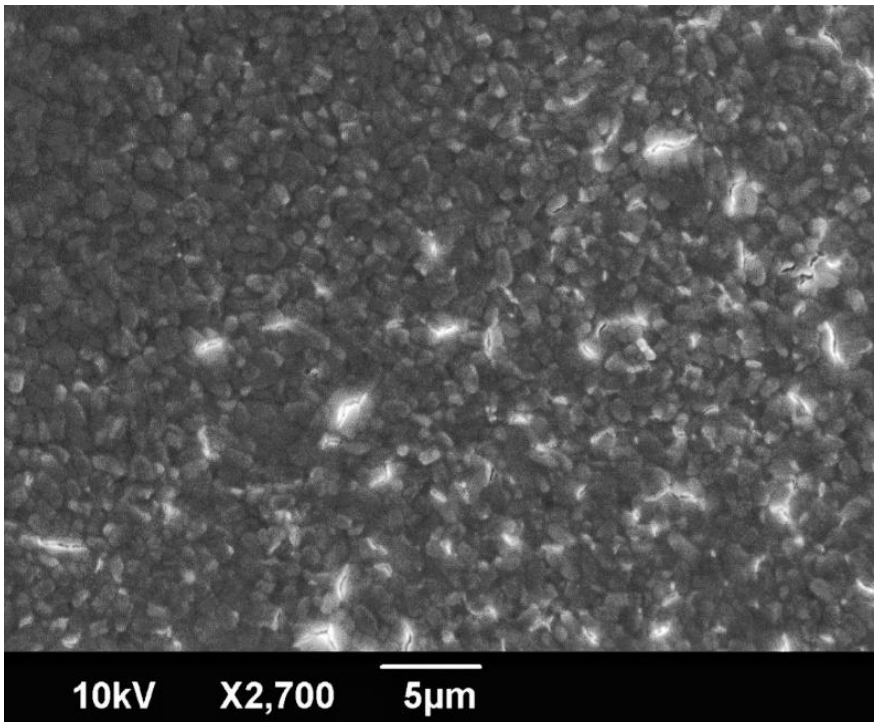

Fig 2: SEM image of endophytic bacteria (T)

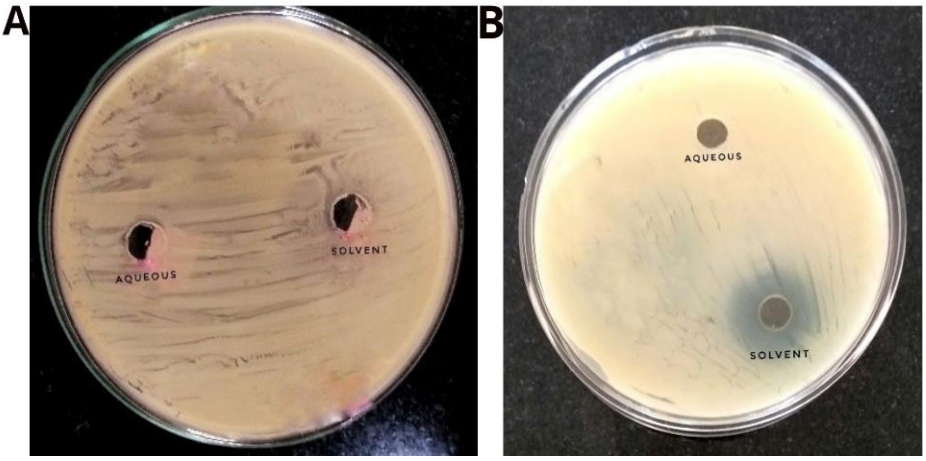

Fig 3: Antibacterial activity of method 1 (A) and method 2 (B)

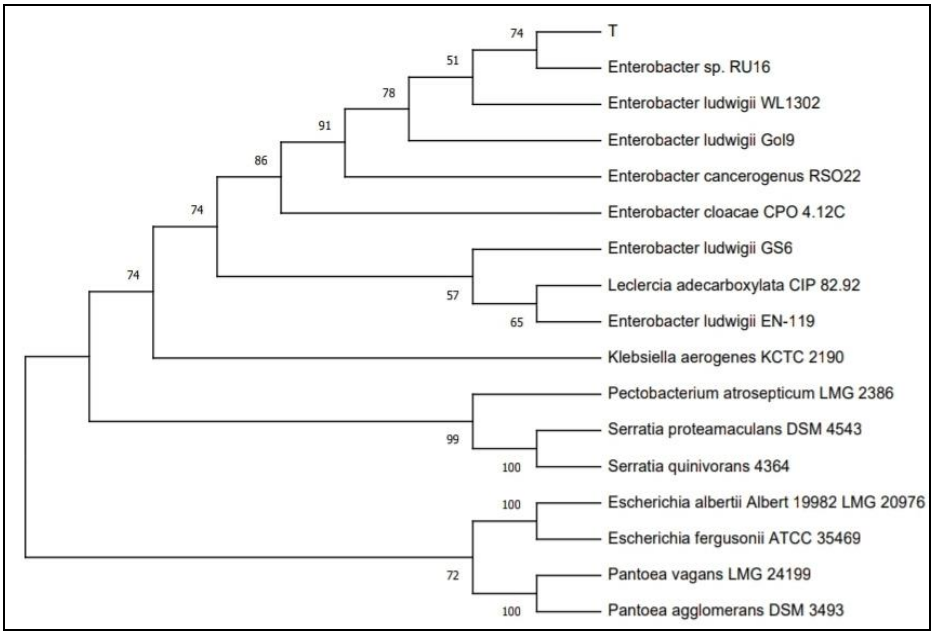

Fig 4: Neighbor joining phylogenetic tree of endophytic bacteria strain T

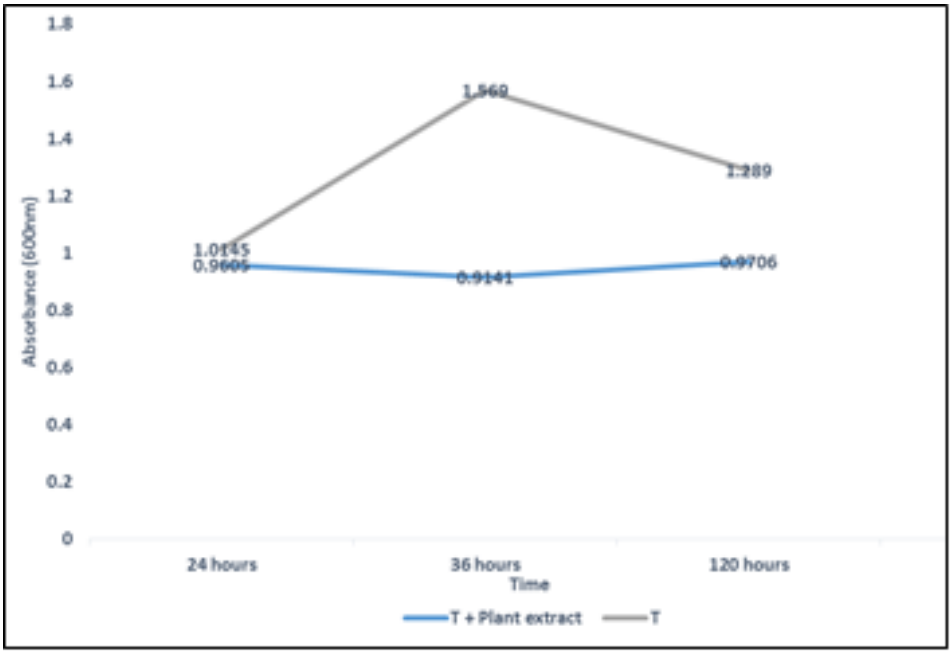

Fig 5: Effect of plant extract on growth of endophytic bacteria

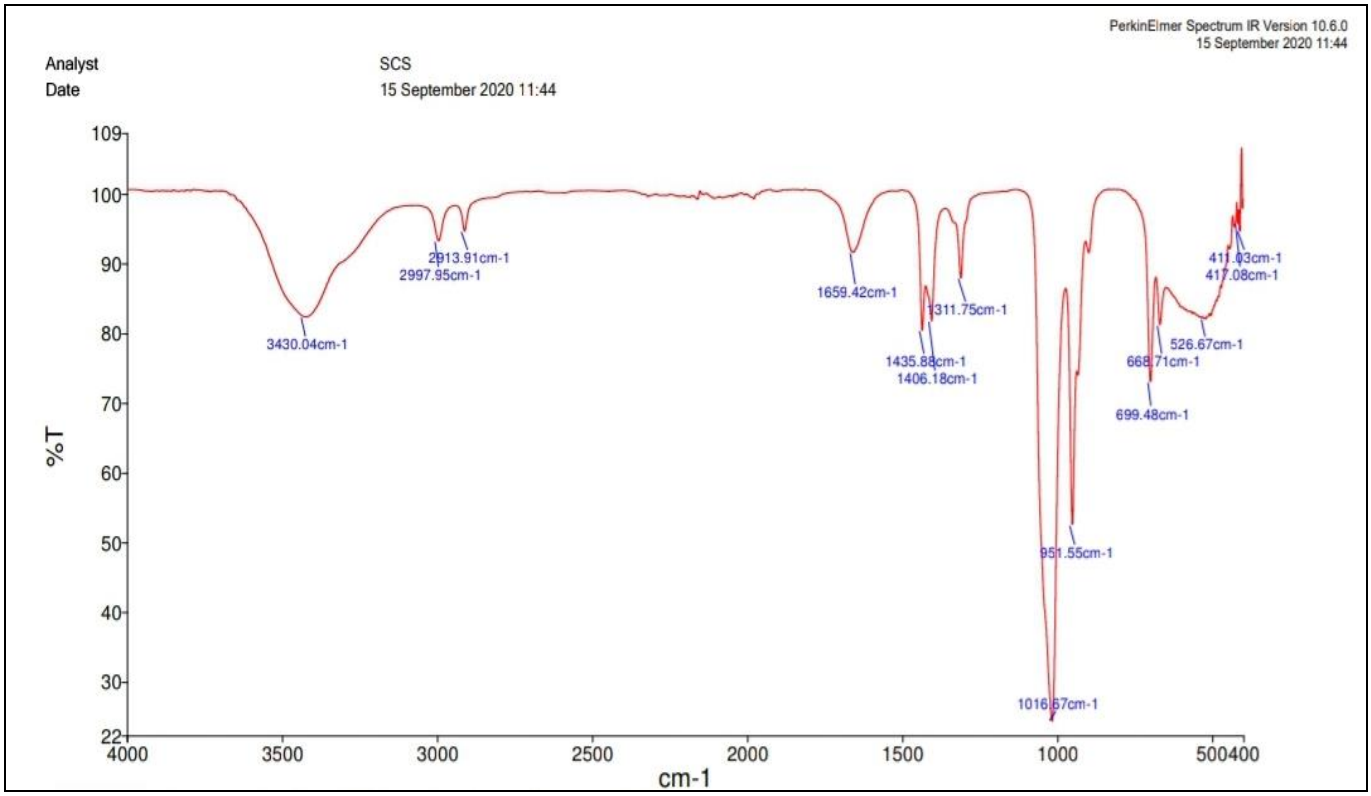

Fig 6: Infrared spectrum of bioactive compound by endophytic bacteria

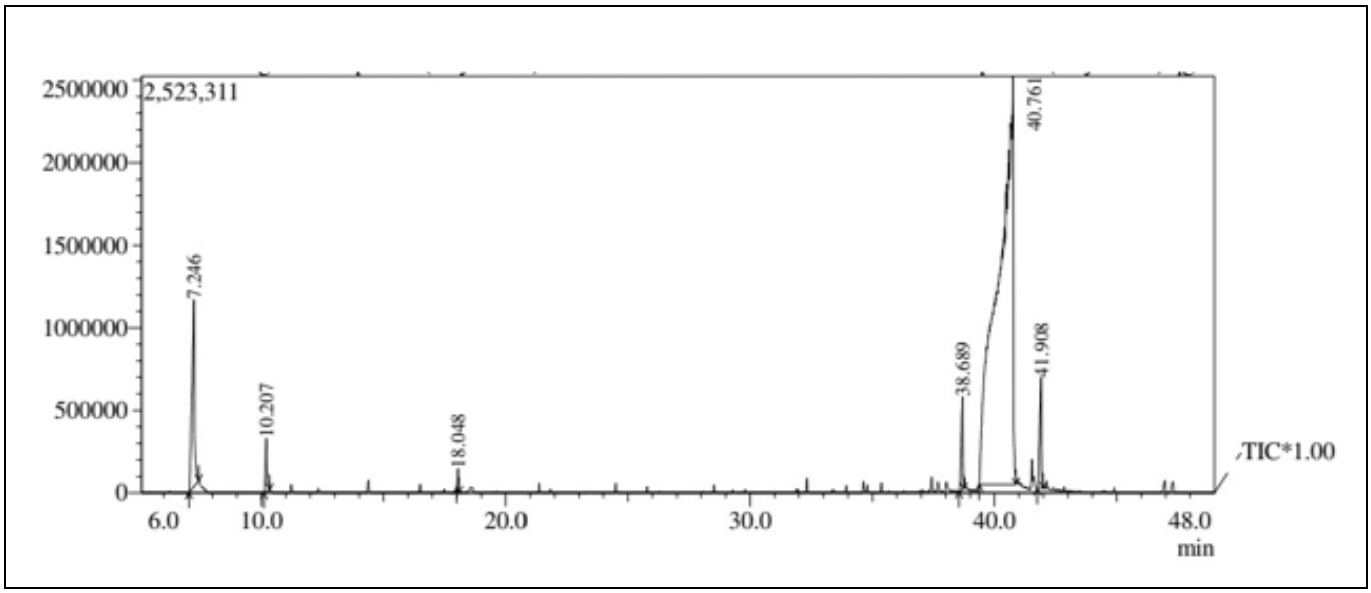

Fig 7: GC-MS chromatogram of bioactive compound

Conclusion

This is the first study to report the presence of bacterial endophytes in *Leucas aspera*. The most promising strain isolated from roots of the plant showed 100% similarity with *Enterobacter ludwigii* strain Gol9. The endophyte exhibited significant antimicrobial activity against Gram positive as well as Gram negative bacteria. It was found to possess plant growth promoting properties such as zinc solubilization, organic acid and ammonia production. The endophyte was able to produce a number of antimicrobial compounds which have been identified by GC-MS analysis. The highest peak was shown by 1, 2-benzenedicarboxylic acid with a retention time of 40.761. The bioactive compound was found to possess anti-oxidant activities as well. These findings highlight the importance of endophytic bacteria as a source of bioactive compounds for the development of new drugs.

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