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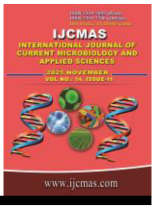

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Chromatographic Analysis of Ethyl Acetate Extract of Endophytic Bacteria Isolated from *Paederia foetida*

Sonali Priyadarshini and Chandi Charan Rath*

Department of Life Sciences, Rama Devi Women's University, Vidya Vihar, Bhubaneswar - 751022, Odisha, India

**Corresponding author*

ABSTRACT

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In the present study ten endophytic bacteria were isolated from *Paederia foetida* and were subjected to antibacterial activity test. The maximum zone of inhibition was observed by the isolate PFNP2 against *Staphylococcus aureu*s (17.6±0.5 mm) followed by *Salmonella sp.* (15.3±0.5 mm) and MRSA (14.6±0.5 mm). The bacterial isolate was identified as *Staphylococcus haemolyticus* by 16s rRNA gene sequencing with NCBI accession number PQ143273. Genes that are essential for the adaptation to the plants were mainly acquired by horizontal gene transfer which could explain the adaptation of such bacteria to plant environment. Presence of aromatic, aliphatic (C-H) and carboxylic acid (C=O) groups were detected in the ethyl acetate extract of the selected isolate by FTIR analysis. Bioactive compounds such as 2- Piperidinone, N- Hexadecanoic acid and Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- identified from the GC-MS mass spectra of the bacterial extract have been reported to have antimicrobial properties. This indicates that bacterial endophytes from *Paederia foetida* could be a source of antimicrobial compounds for use in biotechnological and pharmaceutical applications.

Introduction

One of the biggest issues plaguing the world healthcare today is resistant to antibiotics in microorganisms. Overuse of numerous antibiotics and chemotherapeutics have made many antimicrobial treatments ineffective due to the development of drug resistance which mounts pressure on the academicians and researchers to explore novel pharmaceuticals (Coque *et al.,* 2023). The resistance is mostly attributed to the lack of drug target, inactive enzymes and limitations to the entry of antimicrobial agents in the cells due to efflux pump (Zhang *et al.,* 2022). Since aromatic medicinal plants are reservoirs of huge number of secondary metabolites, they have been explored for new antimicrobial drugs to large extent.

However, the disadvantages regarding the use of large plant biomass cannot be overlooked as some of the medicinal plants are indigenous species (Alvin *et al.,* 2014). Therefore, to deal with such catastrophic condition, study of endophytes with antimicrobial properties is relevant and could result in the discovery of new antimicrobial substances. Most of the work has been based on fungal endophytes and their secondary metabolites. This prompted us to study the biomolecules generated by endophytic bacteria that were isolated from the therapeutic plant *Paederia foetida* with anti-bacterial properties. *Paederia foetida* also known as skunkvine, is a perennial herb with various medicinal properties, belongs to the family Rubiaceae. The plant has been reported to have antimicrobial, anti-oxidant, anti-inflammatory, anti-pyretic, anti-diabetic, anti-diarrhoea and anti-cancer properties (Roy *et al.,* 2021).

As the endophytic population and the plants, they reside which have ethnomedical qualities are closely related, they can be considered as a good prospect for the study of endophytes (Strobel, 2003). The present work is aimed to i) isolate endophytic bacteria from *Paederia foetida*, ii) to identify the selected isolate through 16s rRNA gene sequencing and iii) to identify the bioactive compounds through chromatographic techniques.

Materials and Methods

Sample collection

Fresh twigs of *Paederia foetida* were collected from healthy plants from puri district located at Lat 20.076436° Long 85.833441° in sealed polythene bags. The plant was identified by Dr. Kunja Bihari Satpathy, Emeritus professor of Department of Botany, School of applied sciences at CUTM, Bhubaneswar.

The plant parts were then surface sterilized for two minutes with 70% ethanol, washed twice with sterile distilled water, treated periodically with 4% sodium hypochlorite (NaClO) solution for four minutes, and then rinsed three times with sterilzed distilled water. The very last wash in the beaker was applied to a nutrient agar plate as a control to guarantee effective surface sterilization.

Isolation of endophytic bacteria

Nutrient agar and agar with 10% leaf extract were used to inoculate sterile plant sections on petri dishes. For two to four days, the plates were kept at 34±2° C. After examining, bacterial inoculum from the lining of plant parts were taken and were streaked onto nutrient agar surface and then re-streaked on fresh plates to get pure culture. Cultures were kept at 4°C for further use.

Screening for antibacterial activities

Kirby-Bauer method

In this method broth cultures of the endophytic bacterial isolates were incubated for 24 hours at 34±2° C and centrifuged at 6000 rpm for 8 minutes. MH agar plates swabbed with test microbes were used to place sterilized paper discs dipped in bacterial broth supernatant (*Escherichia coli*, *Salmonella sp., Staphylococcus aureus*) and incubated at 34±2° C for 24 hours. The slightly visible clear rings around the discs observed, indicates zone of inhibition.

Bacteriocin like inhibitory substances production (BLIS)

Isolates from *Paederia foetida* were tested against *Salmonella sp.* (MTCC 1166), *Staphylococcus aureus* (MTCC 96) and *Escherichia coli* (MTCC 433) by the cross-streaking method on Muller-Hinton agar plates. The selected bacterial isolate was streaked in the middle of the agar plate, and incubated for 48 hours at 34±2° C. After completely removing the culture mass from the plates, it was subjected to 30 minutes of chloroform vapor and 30 minutes exposure to UV light. No organism growth on the line will determine whether the bacteriocin like substances secreted by the isolates are effective against test microorganisms.

Crude extract preparation of the isolates

Mueller-Hinton broth (300 ml) was inoculated with the selected endophytic bacterium culture and kept at 34±2° C for six days. For ten minutes, the culture was centrifuged at 8000 rpm. Using an equivalent volume of ethyl acetate (1:1), the supernatant was acidified and extracted three times with the solvent. To obtain the concentrated crude extract, the organic phase was first separated and dried. For further studies, it was kept at 4°C after dissolving it in 10% Dimethyl Sulfoxide (DMSO).

Agar well diffusion method

The ethyl acetate extract was tested against six test microorganisms *viz. Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella sp., Bacillus sp.*, *Staphylococcus aureus* and MRSA by agar well diffusion method. The culture broths of test microorganisms were swabbed on sterilized Muller-Hinton agar plates and wells were made on the plates using a cork borer. Three wells were filled with 20 µl of the ethyl acetate extract, whereas a control well with DMSO. Following an incubation period of 24 hours at 34±2° C, the zone of inhibition was evaluated.

Identification of the isolate

The chosen bacterial strain was identified utilizing high fidelity PCR polymerase for fragment amplification and the Sanger dideoxy sequence technique. The PCR sample underwent bidirectional sequencing. A single band was observed on agarose gel after gel electrophoresis.

Basic Local Alignment Search Tool (BLAST) was carried out to compare nucleotide sequences for the 16s rRNA gene on the National Centre for Biotechnology Information (NCBI) website. Highly similar sequences were selected from the BLAST result. Bootstrap analysis was performed using MEGA 12 genetic analysis tool, and the neighbor-joining technique was applied to create the phylogenetic tree.

FTIR

Using a Thermo Nicolet-6700 FT-IR, Fourier Transform Infrared Spectroscopy was done to identify the various functional groups present in the endophytic crude extract. For the analysis at the transmission mode (400-4000 cm-1), the extract was dried out.

To know the functional groups, present, different peaks obtained from the FTIR were noted which corresponds to the data provided in the chart for infrared absorption bands.

Gas Chromatography-Mass Spectrometry

For GC-MS analysis, a Perkin Elmer Model-Auto system XL turbomass with an Elite-5MS (30 meters x 0.250 mm x 0.250 µm) separating column was utilized. The oven temperature was set at 280°C and the mass range was established at 20–620 amu. The carrier helium gas flow rate was set at 1 milliliter per minute, and the EI source temperature was fixed at 220°C. A run lasting 50 minutes was performed, and for compound identification, the mass spectrum of the detected peaks was compared to the spectra of previously studied compounds in the NIST database.

Results and Discussion

Isolation of pure culture

In total ten endophytic bacterial isolates from stem (2), leaf (3), and (5) from the petiole were isolated. Slants were used to keep the pure cultivated isolates. The colonization frequency was observed to be higher in leaves (18.75%), then petiole (15.62%) and stem (6.25%).

Antibacterial activity of isolated endophytes against test microorganisms

Six out of ten isolates showed activity against *Escherichia coli*, *Salmonella sp.* and *Staphylococcus aureus* in the antibacterial screening and Two (PFNP1 and PFNP2) showed positive BLIS activity. Bacteriocins, which are produced by bacteria, are known to inhibit a number of microorganisms resistant to multiple drugs (Simons *et al.,* 2020). Both ribosomal and non-ribosomal enzymatic systems produce these antimicrobial compounds referred to as bacteriocin (Cotter *et al.,* 2013). The ethyl acetate extract of the isolates exhibited antimicrobial activity against six test microbes.

The maximum zone of inhibition was noted by PFNP2 against *Staphylococcus aureu*s (17.6±0.5 mm), *Salmonella sp.* (15.3±0.5 mm) and MRSA (14.6±0.5 mm).

Identification

The BLAST analysis showed maximum similarity with *Staphylococcus haemolyticus* strain XQYXY-B-1 16s rRNA gene. The genetic sequence was submitted to the gene bank with NCBI accession number PQ143273. The neighbor-joining method was used to create the phylogenetic tree for the identified gene sequence.

In a study on *Staphylococcus haemolyticus* from the rice seed, it was found that the core gene pool of about 1888 genes is conserved to all *S. haemolyticus* (Chaudhry *et al.,* 2020). In their study they found the genes encoding glycerophosphoryl diester phosphodiesterase (GDPD) with a GC content of 23.07% was present only in this particular strain. For the organism's adaptation and stress endurance, these genes induce increased accumulation of osmolytes such proline, glycine, and betaine inside the rice seeds.

Fig.1 Phylogenetic tree constructed with 16s rRNA gene sequence of Staphylococcus haemolyticus strain XQYXY-B-1 using neighbor-joining method (MEGA 12).

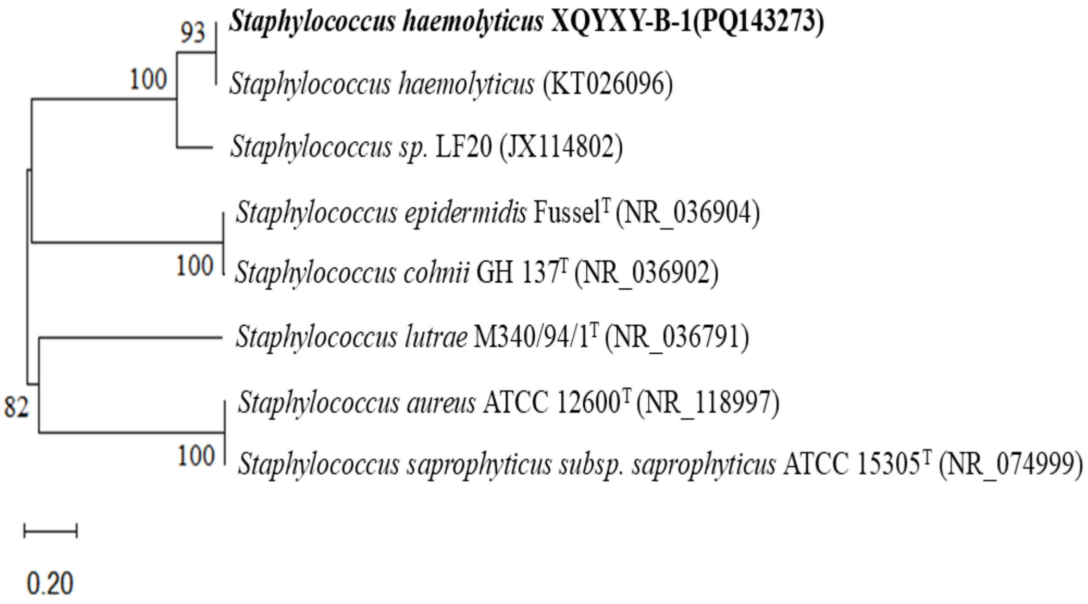


Fig.2 Fourier transform infrared spectroscopy (FTIR) spectrum of PFNP2 extract with different wave numbers within a range of 4000 cm^{-1} to 400 cm^{-1}

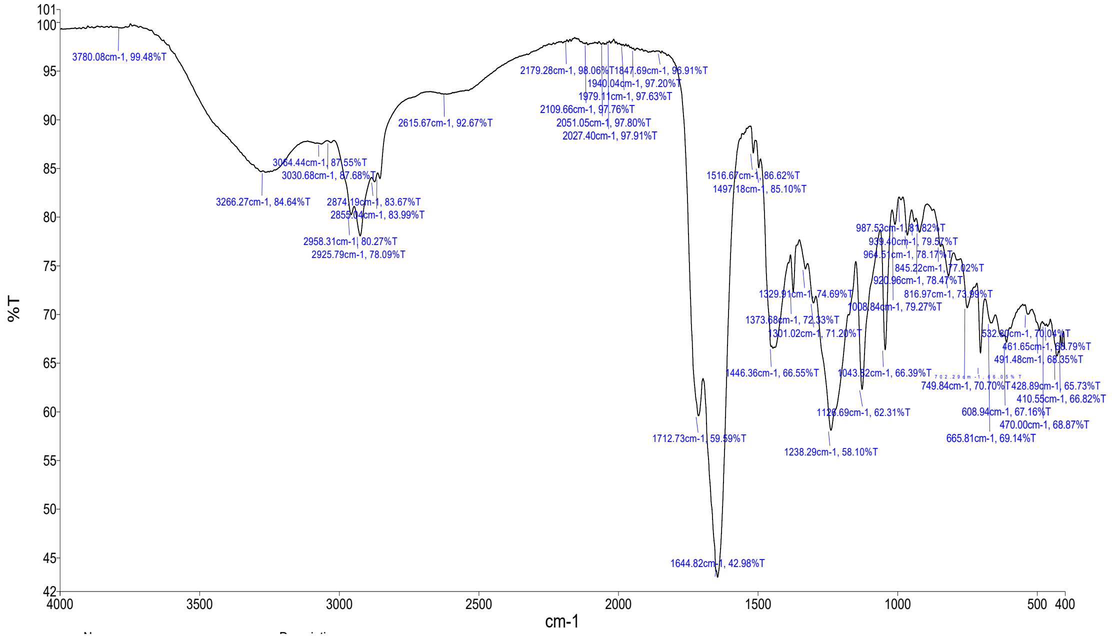


Fig.3 GC-MS chromatogram of PFNP2 bacterial extract showing different peak area

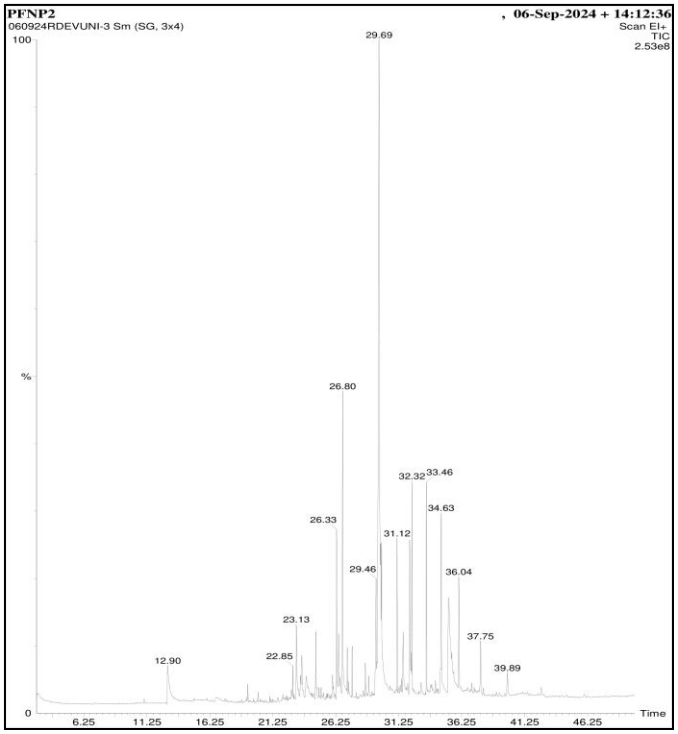


Table.1 Antibacterial Screening of endophytic isolates by Kirby-Bauer method

Endophytic isolates	*Salmonella sp.*	*E. coli*	*S. aureus*
PFNEP1	+	+	+
PFNEP2	+	+	+
PFNES	-	-	-
PFNEL1	+	+	+
PFNP1	+	+	+
PFNP2	+	+	+
PFNL	+	+	+
PFNS	+	-	+
PFWEP	+	+	-
PFNEL2	-	-	-

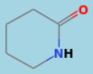
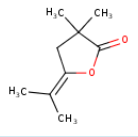
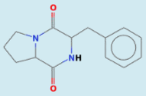
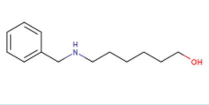
Table.2 Zone of Inhibition by PFNP2 extract against test microorganisms

Test microorganisms	ZOI (mm)
E. coli	14.3±0.5
Salmonella sp.	15.3±0.5
Bacillus sp.	14.3±0.5
S. aureus	17.6±0.5
P. aeruginosa	14.3±0.5
MRSA	14.6±0.5

Table.3 Functional groups detected by FTIR

Wave number (cm^{-1})	Assignments	Interpretation of the band
3266	Broad band of-OH stretch	Presence of hydroxyl group
3030	C-H stretch	Aromatic group
2958 **2925**	Asymmetric stretch of CH_3 and CH_2 group	
2874 **2855**	Symmetric stretch of CH_3 and CH_2 group	
1712	C=O stretch	Carboxylic acid group
1644	C=N stretch	
1516-1446	C=C stretch	Aromatic group
1373 **1329**	C-H bend	Aliphatic and aromatic
1301	O-H bend	Carboxylic acid group
1238 **1126** **1043** **1008**	C-O C-N (single bond stretch) C-C	
987-920	N-H bend	
845-702	C-H bend	Aromatic and aliphatic group

Table.4 Compounds identified in PFNP2 extract by GC-MS

Sl no.	Compound name	Molecular formula	MW	RT (min)	Area %	Molecular structure	Chemical nature
1	2- Piperidinone	C_5H_9ON	99	12.903	4.246		Delta-lactam
2	5-Isopropylidene-3,3-Dimethyl-dihydrofuran-2-one	$C_9H_{14}O_2$	154	26.328	3.653		Heterocyclic aliphatic
3	N- Hexadecanoic acid	$C_{16}H_{32}O_2$	256	26.808	7.182		Saturated long-chain fatty acid
4	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	244	32.130	3.730		Heterocyclic organic compound
5	1-Hexanol, 6-[(phenylmethyl)amino]-	$C_{13}H_{21}NO$	207	35.227	9.24		A substituted benzylamine and an amino alcohol
6	Hexadecane	$C_{16}H_{34}$	226	32.315	3.664		Alkane hydrocarbon

They also discovered the genes encoding lantibiotics, its transporter genes and two alcohol dehydrogenase (ADH) in the microorganism. Most new genes for the adaptation to the plants were mainly acquired by horizontal gene transfer e.g. localization of type II lanthipeptide gene cluster by HGT. Novel evolutionary characteristics associated with *S. haemolyticus* adapatation to the plants from being a human associated bacterium may become a contributing factor towards the study of resistance to the antibiotics.

FTIR

The bacterial extract's infrared spectra, which had several peaks between 4000 cm^{-1} to 400 cm^{-1} was analysed. The presence of compounds such as aromatic groups, aliphatic and, carboxylic acids, C=N stretch contributing to piperidinone (from GC-MS) and aromatic C-H bend were detected.

GC-MS analysis

Peaks in the chromatogram were identified by comparing the mass spectra in NIST database (Table 4). Six compounds were identified. The compound 5-Isopropylidene-3,3-Dimethyl-dihydrofuran-2-one has previously been isolated from the extracts of *Streptomyces misionensis* V16R3Y1 as a minor compound (Saadouli *et al.,* 2020). Not much data on its activity is available in the literature. 2- Piperidinone (4.24%) identified from the GC-MS analysis has antimicrobial (Saadouli *et al.,* 2020) and anti-asthmatic (Mamillapalli, 2020) properties. 1-Hexanol, 6-[(phenylmethyl) amino] - with highest area of 9.24% is mostly used as reagent in the manufacture of pharmaceuticals especially drugs targeting neurological disorders. N- Hexadecanoic acid (7.18%), has antibacterial and antioxidant properties (Ganesan *et al.,* 2024). The heterocyclic compound Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- identified from the mass spectra has demonstrated antimicrobial effectiveness against multidrug resistant *Staphylococcus aureus* (Kiran *et al.,* 2018).

In conclusion, endophytic bacteria isolated from *Paederia foetida,* exhibiting most effective antibacterial activity was identified and the bacterial extract was analyzed for bioactive compounds *via.* FT-IR and GC-MS. Further analysis to characterize the bioactive principles for future pharmaceutical prospect is of significance and could immensely contribute to novel drug development. Though primary, study such as this is a prerequisite to tap the biotechnological potential of these microorganisms for use in pharmaceutical industries towards the development of newer drugs.

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Author Contributions

Sonali Priyadarshini: The computational framework, analyzed the data and wrote the manuscript. Chandi Charan Rath: Conceived the original idea and designed the model.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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