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The Antibacterial Activity of Secondary Metabolites of Endophytic Bacterial Isolates of *Carica Papaya* against Some Clinical Isolates

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Abstract

Plants which are used in traditional medicines are of significant importance and therefore considerable research has been carried out on medicinal plants for bioactive compounds. However, limited research has been performed on the associated microorganisms, especially bacterial endophytes which could serve a good source of novel antibiotic agents. This study was aimed to determine the antibacterial activity of secondary metabolites of endophytic bacterial isolates of *Carica papaya* plant parts against some clinical isolates. Clinical isolates used in this study were *Escherichia coli, Klebsilla* species*, Staphylococcus aureus,* and *Salmonella* species. Endophytes were cultured, isolated and identified using morphological and biochemical tests and five endophytes were isolated and observed to be Gram negative rod. The endophytes were presumed to be *flavobacterium* species and *Pseudomonas* species. Endophtic bacterial isolates were further confirmed using molecular method. The confirmed bacteria strains were *Myroides odoratimimus* from *Carica papaya* leaf, and *Empedobacter brevis* from *Carica papaya* root and stem. Isolated endophytes were sub-cultured in nutrient broth and secondary metabolites were extracted from the medium using Ethyl acetate. Crude extracts of secondary metabolites were then subjected to Liquid Chromatography and Mass spectrometry (LCMS) where Lupinine, pyridine, Aniline, Polyketides and Cyanopyridine where the most prevalent metabolites detected in the three ethyl acetate secondary metabolite extracts of bacterial endophytes analysed. The crude extracts were tested against the test isolates for antibacterial activity and result shows that all the secondary metabolite had activity against at least two out of the four clinical isolates used in this study with zones of inhibition ranging from 9mm to 22mm. The zones of inhibition observed indicates that the secondary metabolites extracted from bacterial endophytes of *Carica papaya* leaf, stem and root have antibacterial activities and as such can be explored as good sources of antibacterial agents for the development of therapeutic drugs.

Key words: Antibacterial, Bacterial endophytes, Clinical isolates, Secondary metabolites

Introduction

Lots of information on the nutritive importance and antimicrobial properties of some medicinal plants are available. However, the endophytes associated with these plants are yet to be elucidated and functionally characterized. Endophytes are chemical synthesizers that grow within plant tissues without causing disease symptoms. They play a role as a selection system for microbes to produce pharmacologically active substances with low toxicity toward mammalians (Rahman *et al*., 2017;

Sharma and Mallubhotla, 2022). Endophytes are sometimes responsible for the medicinal properties of their hosts. Many endophytes synthesize bioactive compounds that can be used by plants for defense against pathogens and some of these compounds may be valuable as pharmaceutical drugs (Bariya *et al.,* 2022). The endophytic communities have been divided into different subgroups, such as 'obligate' or 'facultative,' which are associated with all types of plants. (Bariya *et al.,* 2022).

Valuable bioactive metabolites, such as alkaloids, steroids, terpenoids, lactones, quinines, lignans, and phenols, have been isolated from endophytic bacteria (Sharma and Mallubhotla, 2022). Endophytic bacteria from medicinal plants have also been considered for their antimicrobial activities (Xu *et al*., 2020).

The development of drug resistance among pathogens has reduced the therapeutic options for treatment and as such, new drugs and new sources of drugs are needed (Kushwaha *et al*., 2023).

Materials and Methods

Plants Collection and Identification

The leaf, stem and root of Pawpaw (*Carica papaya*) samples were collected in sample bags from Rafin guza area in Igabi Local Government Area of Kaduna State. The plant materials were then taxonomically identified at the Department of Biological Science, Kaduna State University, Kaduna, Nigeria.

Surface Sterilization of Plant Tissues

Fleshy collected leaves were washed under slow running water for 15 minutes. Samples were cut into about 1 cm pieces. Samples were sterilized by immersing the sample in 70% ethanol for 1 minute, sodium hypochloride for 3 - 4 minutes, again 70% ethanol for 1 minute and then rinsed with sterile water for 3 - 4 minutes. The sterilized leaf samples were then dried in the laminar air flow cabinet (Hlaing and Soe, 2018; Osuntokun *et al*., 2021). Confirmation of Surface sterilised plant tissues to be cultured for endophytic bacteria was done by culturing aliquots of water from the last rinsing of the plant tissues on nutrient agar and placing it in an incubator at 37ºC and then observing the media for growth after 24hours (Anjum and Chandra, 2015; Shukla and Wahla, 2019).

Media for Culturing Endophytic Bacteria Isolates

Nutrient agar being a universal media was used to culture endophytic bacteria. (Anjum and Chandra, 2015; Yan *et al*., 2018).

Isolation of Endophytic Bacteria

After proper drying of surface sterilized plant material, using aseptic procedure the surface of the leaf, root and stem was removed using a sterile scalpel in the laminar air flow cabinet and then plant tissues were cut into pieces and each piece was placed on nutrient agar medium supplemented with antifungal agents (niacin). Plates with plant tissues was sealed using parafilm tape and incubated at 28±2°C in order to recover the maximum possible colonies of bacterial endophytes. The observation was made for 48 hrs. After 48hrs of the bacterial cultures, morphologically different bacterial colonies were picked and repeatedly streaked in order to achieve pure bacterial isolates. All selected isolates were sub-cultured unto nutrient agar slants and finally, all the purified endophytes were maintained at 4°C till further use (Anjum and Chandra, 2015).

Identification of Endophytic Bacteria

Gram staining and biochemical reactions such as catalase, citrate utilization, indole, oxidase, methyl red and Voges-Proskauer test were used for preliminary identification of the endophytes (Mohammed *et al*., 2019).

Extraction of Secondary Metabolites

Endophytic bacterial isolates were inoculated in 800mL of nutrient broth and incubated for 2weeks in shaking incubator at 28 °C to 30°C at 120 rotations per minute (rpm). Broth containing the endophytes were then mixed with 800mL of Ethyl acetate and placed in a shaker for 30minutes. The mixtures were then transferred into separation funnel and allowed to sediment into layers. The supernatant containing the secondary metabolites were evaporated using rotary evaporator set at 40°C to get crude extract of secondary metabolites. The metabolites were then dissolved in methanol for subsequent separation and the crude extracts were analysed by Thin Layer chromatographic separation and Liquid Chromatography and Mass spectrometry (LCMS) (Rahman *et al*., 2017).

Antibacterial Activity of Secondary Metabolites of Endophytic Bacterial Isolates

Antibacterial activity of secondary metabolites of endophytic bacterial isolates of *Carica papaya* leaf, root and stem were analysed using Agar well diffusion method as described by Balouri (2018). The evaporated secondary metabolite of extracts of the endophytic bacterial isolates were dissolved in methanol to obtain concentrations of 10mg/mL and 20mg/mL while 10mg/mL of Ciprofloxacin dissolved with distilled water was used for the analysis. Zones of inhibition were observed, measured and recorded in millimeters (mm) after incubating the plates containing the clinical isolates and secondary metabolites at 37°C for 24hours. Endophytes that produced secondary metabolites that had best zones of inhibition against the clinical isolates according to plant parts were selected for further study.

Confirmation of endophytic bacterial isolates

Phenol-chloroform bacteria DNA extraction, 16S rRNA analysis, Gene Sequencing and BLAST analysis were carried out on the bacterial isolates to confirm their identity as prescribed by (Rahman *et al.,* 2017).

Identification of Secondary Metabolites using Liquid Chromatography and Mass Spectrometry Analysis (LCMS) Analysis

Liquid Chromatography and Mass Spectrometry Analysis was done using Waters e2695 separation module with W2998 PDA and couple to ACQ-QDA MS. About 50mg of each Sample was reconstituted in methanol and filtered using membrane filter (0.45 µm). The filtered samples were injected into LC system (Waters e2695), separations were done in C18 column (Sunfire C18 5.0µm 4.6mm x 150 mm). The analysis was conducted at a flow rate of 1.0 ml /min, sample and column temperature at 25°C. Solvent A (0.1% formic acid in water) and B (0.1% formic acid in Acetonitrile) were used as mobile phase with the following gradient: A ratio of A/B 95:5 was used and maintained for 1 min, then followed by A/B 5:95 for 13 min, to 15 min, A/B 95:5 to 17min, 19min and finally 20min. the PDA detector was set at 210-400nm with resolution of 1.2nm and sampling rate at 10 points/sec. The mass spectra were acquired with a scan range from m/z 100 – 1250 after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8kv (positive) and 0.8kv (negative); probe temperature 600°C; flow rate 10 mL/min; nebulizer gas, 45 psi. MS set in automatic mode applying fragmentation voltage of 125 V. The analysis and data were controlled and processed with Empower 3 (Garba *et al*., 2023).

Results

Morphological and Biochemical Characteristics of Endophytes Isolated from the Leafs, Roots and Stems of *Carica papaya* and *Mentha spicata* plants

From results on Table 1, the endophytes isolated from *Carica papaya* leaf, root and stem were designated as PL1 for *Carica papaya* leaf endophyte 1, PL2 for *Carica papaya* leaf endophyte 2, PR3 for *Carica papaya* root endophyte 3, PS4 for *Carica papaya* stem endophyte 4 and PS5 for *Carica papaya* stem endophyte 5, All the endophytes isolated were subjected to Gram staining and biochemical characterisation for their preliminary identification. As for their Gram reactions, PL1, PL2, PR3, PS4,

and PS5 all showed Gram negative rod colonies. Biochemical tests conducted were Catalase, Oxidase, Methyl Red, Citrate, Motility, Urease, Indole, and Vogues Proskauer test. All bacterial endophyte strains; PL1, PL2, PR3, PS4, PS5, were catalase positive. PL1, PR3 and PS5 had positive oxidase reaction while all others where oxidase negative. The strains PL1 and PS5 showed positive result for methyl red test while positive Voges Proskauer test was only exhibited by PS5. Bacterial endophyte strains PL2, PS4 and PS5 were citrate utilization positive while all others were negative. The overall morphological, gram reaction and biochemical results of the bacterial endophytes indicated that bacterial endophytes PL1, PR3 and PS5 from *Carica papaya* were all presumed to be *Flavobacterium* species while PL2 and PS4 were presumed to be *Pseudomonas* species.

Table 1: Morphological and Biochemical Characteristics of Endophytes Isolated from the Leafs, Roots and Stems of *Carica papaya*

Bacteria isolates	Morphology appearance	Gram reaction	CAT	COG	MR	VP	CIT	MOT	UR	IND	OXD	Probable Bacteria
PL1	Yellow and mucoid	Negative rod	+	NA	+	-	-	+	+	-	+	*Flavobacterium* spp.
PL2	Greenish yellow	Negative rod	+	NA	-	-	+	-	-	-	-	*Pseudomonas* spp.
PR3	Yellow and mucoid	Negative rod	+	NA	-	-	-	+	-	+	+	*Flavobacterium spp.*
PS4	Greenish yellow	Negative rod	+	NA	-	-	+	-	-	-	-	*Pseudomonas* spp.
PS5	Yellow and mucoid	Negative rod	+	NA	+	+	+	+	+	-	+	*Flavobacterium* spp.

Key: PL1= *Carica papaya* leaf endophyte 1, PL2: *Carica papaya* leaf endophyte 2, PR3= *Carica papaya* root endophyte 3, PS4= *Carica papaya* stem endophyte 4, PS5=*Carica papaya* stem endophyte 5, CAT= Catalase, OXD= Oxidase, MR= Methyl Red, CIT= Citrate, MOT= Motility, UR= Urease, IND= Indole, VP= Vogues Proskauer, += Positive reaction, - = Negative reaction, NA= not applicable.

Extraction Yields of Secondary Metabolites of Endophytic Bacterial Isolates of *Carica papaya* Leaf, Root and Stem

Results on Table 2 shows the mass of crude Ethyl acetate extracts of secondary metabolites of Endophytic Bacterial Isolates of *Carica papaya* Leaf (PL1), Root (PR3) and Stem (PS5) obtained from 800mL of Ethyl acetate supernatant after evaporation to obtain crude extracts. Endophytic bacteria PL1 yielded 1.30g of secondary metabolites, PR2 yielded 1.15g and PS5 yielded 1.24g.

Table 2: Extraction Yields of Secondary Metabolites of Endophytic Bacterial Isolates of *Carica papaya* Leaf, Root and Stem

Endophytic Bacteria	Secondary Metabolites of Endophytic Bacteria	Secondary Metabolites Yield (gram)
PL1	SPL1	1.30
PR3	SPR3	1.15
PS5	SPS5	1.24

Antibacterial Activity of the Extracted Secondary Metabolites against Clinical Isolates

From Table 3, secondary metabolites SPS5 and SSL1 extract of bactrerial endophytes *Empedobacter brevis* (PS5) had the highest antibacterial activity of 11mm zone of inhibition aganst *E. coli* while endophyte *Myroides odoratimimus*(PL1) had the highest zone of inhibition of 22mm against *S. aureus.* Similarly, while PR3 and PS5 endophytes both identified as *Empedobacter brevis* produced secondary metabolites that had the higest zones of inhibition of 13mm against *Klebsiella* species.

Table 3: Antibacterial Activity of the Extracted Secondary Metabolites against Clinical

Clinical Isolates	Secondary metabolites (10mg/mL)			Secondary metabolites (20mg/mL)			Control Ciprofloxacin 10mg/m
	SPL1	SPR3	SPS5	SPL1	SPR3	SPS5	
	Zones of inhibition						
E. coli	8	-	8	9	-	11	18
S. aureus	14	10	9	22	14	14	24
Salmonella spp.	-	10	6	9	-	14	16
Klebsiella spp.	-	9	10	6	13	13	18

Key: (-) = No zone of inhibition, SPL1=Secondary metabolites of *Carica papaya* leaf endophyte 1, SPL2= Secondary metabolites of *Carica papaya* leaf endophyte 2, SPR3= Secondary metabolites of *Carica papaya* root endophyte 3, SPS4= Secondary metabolites of *Carica papaya* stem endophyte 4, SPS5= Secondary metabolites of *Carica papaya* stem endophyte 5

Agarose gel image of amplified PCR products for identification of endophytic bacterial isolates of *Carica papaya* and *Mentha spicata* Leaf, root and stem.

Plate 1 shows the agarose gel image of the PCR products for identification of endophytic bacterial isolates of *Carica papaya* Leaf, root and stem where the Lane L on the image represents the Molecular marker with 1500basepairs amplification while Lane 1, 2 and 3 represents amplicons of endophytes PL1, PR3, PS5, respectively all having amplifications between 500-1500bp.

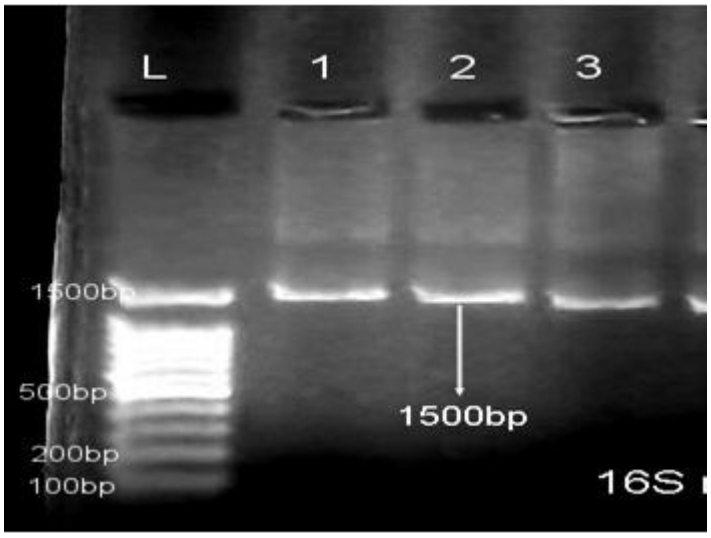

Plate 1: Agarose gel image of PCR products for identification of endophytic bacterial isolates of *Carica papaya* and *Mentha spicata* plant.

Lane L= Molecular marker

Lane 1, 2 and 3 represents amplicons of endophytes PL1, PR3 and PS5 respectively.

Nucleotide Sequences of the Polymerase Chain Reaction (PCR) products subjected to sequencing

Table 4 shows the PCR sequences of the six endophtes; PL1, PR3 and PS5 analysed using Sanger sequencing method.

Table 4: Nucleotide Sequences of the PCR products subjected to sequencing

Sample code	Nucleotide sequences
PL1	CGGGCGGGGAAGCTTATCGCTTTCGCTGAGCCACTCAGGCGGAAAACCGAACAGCTAGTATCCATCGTT TACGGCGTGGACTACCAGGGGGTCTAATCCTGTTCGCTCCCCCCGCTTTCTTTCATCAGCGTCAATAAAT ACGTACTAACCTGCCTTCTCAATTGGGATTCCATGTAATATCTAAGCATTTCACCGCTACACTACATATTC TACTTACTTCCATATTATTCAAGCTCTGCAGTATCAATGGCAGTGTCCTAGGTAAGCTAGGAAATTTCAC CACTGACTTACAAAGCCGCCTACGAACCCTTTAAACCCAATAATTACGGATAACGCTCGGATCCTCCGTA TTACCGCGGATGCTGGSACGGAGTTAGCCGATCCTTATTCTTACAGGACCGCCAAGTCCCTACTCGGAGG GAGGTATCTTCCTGTACAAAAGCAGTTTACAATCCATAGGACCGTCATGCTGCACGCGGGATGGCTGGTT CAGAGATGCCTCCATTGACCAATATTCCTCACTGCTGCCTCCCGTACGAGTCTGGTGCGTGTGTCAGTAC CATCGTGGGGGATCTCCCTCTCAGGACCCCTAAGCATCTTTGCCTTGGTATGCCGTTACCACACCAACTA CCTAATGATACGAATGCCCATCTTATACCGATAAATCTTTATTATCAATACGATGCCATATCGATAAACC ATGGAGCATTAATCCTAATTTCTTCGGGCTATTCCCCTGTATAAGGTAGGTTGCATACACCGTTACTCAC CCGTGCGCCGGTCTCAAAAAAGCAAGCTTCCTTCTACCCCTCGACTTGCATGTGATTAGGCCTGCCCGCT AGCGTTCATCCTGAGCCCAGGATCAACTCTGCGG
PR3	CGGGGGGGATACTTATAACTTTCGCTTAGCCACTGAAGCCGAAACCCCAACAGCTAGTATCCATCGTTTA CGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGTCCATCAGCGTCAGTTGATAC TTAGTGACCTGCCTTCGCAATTGGTGTTCTGCGTAATATCTAAGCATTTCACCGCTACACTACACATTCCA GCCACTTCAACATCACTCAAGACTAACAGTATCAATGGCAGTTCCACAGTTAAGCTGCGGGATTTCACCA CTGACTGATTAATCCGCCTACGGACCCTTTAAACCCAATAAATCCGGATAACGCTTGCACCCTCCGTATT ACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTATTCTTCTGGTACCTTCAGCTACTCACACGTGAGTA GGTTTATCCCCATATAAAAGTAGTTTACAACCCATAAGGCAGTCGTCCTACACGCGGGATGGCTGGATC AGGGTTCCACCCATTGTCCAATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACC AGTGTGGGGGATCACCCTCTCAGGCCCCCTAAACATCATCGTCTTGGTGAGCCGTTACCTCTCCAACTAA CTAATCTTACGCATGCCTATCCTACTGCGATAAATCTTTAAAATTAAATGATGCCATTCAATCTGTTATAA AGAATTAATCCTCCTTTCGAAGGGCTATCCTTTTCTGTAGGGCAAGTTGCATACGCGTGTACACACCCCG CGCGCCGGTCTCAGATTCCCGAAAGAATCGTACCCCCTCGGCTTGCATGTGTTAGGGCTCCCCGCTAGCG KTCATCCCTGAGACCAGGGATCAAAACTCCTCAACGAGGG
PS5	GGGGGGGGGGATACTTATAACTTTCGCTTAGCCACTGAAGCCGAAACCCCAACAGCAAGTATYCATCGT TTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGTCCATCAGCGTCAGTTGA TACTTAGTGACCTGCCTTCGCAATTGGTGTTCTGCGTAATATCTAAGCATTTCACCGCTACACTACACATT CCAGCCACTTCAACATCACTCAAGACTAACAGTATCAATGGCAGTTCCACAGTTAAGCTGCGGGATTTCA CCACTGACTGATTAATCCGCCTACGGACCCTTTAAACCCAATAAATCCGGATAACGCTTGCACCCTCCGT ATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTATTCTTCTGGTACCTTCAGCTACTCACACGTGA GTAGGTTTATCCCCAGATAAAAGTAGTTTACAACCCATAAGGCAGTCGTCCTACACGCGGGATGGCTGG ATCAGGCTTCCACCCATTGTCCAATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTA CCAGTGTGGGGGTTCACCCTCTCAGGCCCCCTAAAGATCATCGTCTTGGTGAGCCGTTACCTCTCCAACT AACTAATCTTACGCATGCCTATCCTACTGCGATAAATCTTTCAAAATTAAATGATGCCATTCAATCTGTT ATAAAGTATTAATCCTCCTTTCGAAGGGCTATCCTTTTCAGTAGGGCAAGTTGCATACGCGTTACGCACC CGTGCGCCGGTCTCAGATTCCCGAAAGAATCGTACCCCTCGGCTTGCATGTGTTAGGCCTCCCGCTAGCG TTCATCCTGAGCCAGGATTCAAACTCATACGGTGG

Source: NCBI Online Database

NCBI Nucleotide BLAST Analysis of Endophytic Bacterial Isolates of *Carica papaya* and Leaf, Root and Stem

Table 5 shows the result of the nucleotide BLAST of the PCR sequences of the bacterial endophytes analysed and results obtained indicated that PL1 had 95.34% similarity with *Myroides odoratimimus* strain 3J2MO5 16S ribosomal RNA gene, partial sequence, PR3 had 97.89% similarity with *Empedobacter brevis* strain LMG 4011 16S ribosomal RNA, partial sequence while PS5 had 99.7% similarity with *Empedobacter brevis* strain Emp_bre 16S ribosomal RNA gene, partial sequence.

Table 5: NCBI Nucleotide BLAST Analysis of Endophytic Bacterial Isolates of *Carica papaya* and *Mentha spicata* Leafs, Roots and Stems

S/No	Isolate DNA code	Max score	Total Score	Query cover	e-value	% ID	Accession number	Description of identified isolate
1	PL1	1362	1362	98%	0.0	95.34%	PQ656455.1	*Myroides odoratimimus* strain 3J2MO5 16S ribosomal RNA gene, partial sequence
2	PR3	1489	1489	98%	0.0	97.89	NR_042471.2	*Empedobacter brevis* strain LMG 4011 16S ribosomal RNA, partial sequence
3	PS5	1576	1576	98%	0.0	99.77	PQ722375.1	*Empedobacter brevis* strain Emp_bre 16S ribosomal RNA gene, partial sequence

Liquid Chromatography and Mass Spectroscopic (LCMS) Analysis of Ethyl Acetate Extract of Secondary metabolites of Endophytic Bacterial Isolates of *Carica papaya* Leafs, Roots and Stems with Molecular Weight (m/z) at Positive ionization mode

Results on Table 6 shows the LCMS analysis of Ethyl acetate extract of secondary metabolites of endophytic bacterial isolates of *Carica papaya* leaf, root and stem with molecular weight (m/z) at Positive mode. Secondary metabolites with higher peaks identified in SPL1 were Lupinine, Pyridine, Benzeneacetonitrite and Norspermidine while those identifies from SPR3 were Lupinine and Aniline. Those secondary metabolites identified in SPS5 were 6-Methoxytaxifolin, Lupinine, N-Methyltyramine, Phenylethylamine, Aminoacetone and Aniline.

Table 6: Liquid Chromatography and Mass Spectrometry (LC Waters e2695 separation module with W2998 PDA and couple to ACQ-QDA MS) Analysis of Ethyl Acetate Extract of Secondary metabolites of Endophytic Bacterial Isolates of *Carica papaya* Leafs, Roots and Stems with Molecular Weight (m/z) at Positive ionization mode

Sample	Retention time (min)	Molecular formula	Base peak (m/z)	Exact mass (m/z)	Metabolite class	Metabolite name
SPL1	1.490	$C_{10}H_{19}NO$	170.223	169.1467	Alkaloids	Lupinine
	17.545	C_5H_5N	80.205	79.0422	Alkaloids	Pyridine
	1.621	C_8H_7N	118.158	117.0578	Tryptophan alkaloids	Indole
	2.336	$C_8H_{17}N_3$	132.177	131.1422	Alkaloids	Norspermidine
SPR3	1.395	$C_{10}H_{19}NO$	170.235	169.1467	Alkaloids	Lupinine
	17.536	C_8H_7N	94.098	93.0578	Benzenoids	Aniline
SPS5	4.926	$C_{10}H_{14}O_8$	335.214	334.0689	Polyketides	6-Methoxytaxifolin
	1.493	$C_{10}H_{19}NO$	170.095	169.1467	Alkaloids	Lupinine
	2.575	$C_9H_{13}NO$	152.151	151.0997	Alkaloids	N-Methyltyramine
	6.484	$C_8H_{11}N$	122.114	121.0891	Alkaloids	Phenylethylamine
	10.216	C_3H_7NO	73.894	73.0528	Organic oxygen compounds	Aminoacetone
	17.531	C_8H_7N	94.330	93.0578	Benzenoids	Aniline

Sources: Metabolomics workbench (Online database), Mass Bank (Online database).

Discussion

All the endophytes isolated were subjected to gram staining and biochemical reactions for their preliminary identification and were observed to be Gram negative rod. In case of the biochemical characterization of the endophytic bacteria, catalase test was indicated by bubble formation after addition of bacterial culture on the drop of hydrogen peroxide and all strains; PL1, PL2, PR3, PS4 and PS5, were catalase positive. The presence of cytochrome C oxidase enzyme in oxidase test was showed by PL1 and PL5. The strains PL1 and PS5 showed strong positive result after adding methyl red reagent comparable with the control while Voges-proskauer test used to examine acetone production was only exhibited by PS5 and showed strong positive result indicated by the red coloured ring formation on the broth surface. The blue colour of media indicated the use of citrate as sole source of carbon and PL2, PS4 and PS5 strains were citrate positive (Barrow and Feltham, 2003; Cheesebrough, 2006).

Endophytes PL1, PR3 and PS5 from *Carica papaya* were all were presumed to be *Flavobacterium* species due to their Gram negative rod shape and being catalase and oxidase positive (Strepparava *et al.,* 2014; Enisoglu-Atalay*et al.,* 2018). Endophytes PL2 and PS4 in this study were presumed to be *Pseudomonas* species due to their light green colony appearance on nutrient agar and Gram negative rod appearance when viewed with microscope. This finding is in line with the description of Olutiola *et al*., (2000) and the findings of Sarjono *et al.,* (2020), who isolated *Pseudomonas* specie in closest correlation with *Pseudomonas deceptionensis*, *Pseudomonas endophytic*, *Pseudomonas psychrophile, Pseudomonas fragi* ATCC 4973, *Pseudomonas fragi* NBRC 3458 from *Pseudomonas* genus.

In this study as shown on Table 3, the secondary metabolites analysed had antibacterial activity against the clinical isolates analysed. Secondary metabolites from *Empedobacter brevis* (PS5) had the highest antibacterial activity of 11mm zone of inhibition against *E. coli* while secondary metabolite from endophyte *Myroides odoratimimus* (PL1) had the highest zone of inhibition of 22mm against *S. aureus* while PR3 and PS5 endophytes both identified as *Empedobacter brevis* produced secondary metabolites that had the higest zones of inhibition of 13mm against *Klebsiella* species. According to Sarjono *et al*., (2019), the inhibitory zone with a diameter of 20 mm or more has very strong antibacterial potential, the inhibitory zone with a diameter of 10-20 mm has strong antibacterial potential, the inhibitory zone with a diameter of 5-10 mm has moderate antibacterial potential, and the inhibitory zone with a diameter of less than 5 mm has weak antibacterial potential. Overall, the secondary metabolites tested against the clinical bacterial isolates *E. coli, S. aureus*, *Salmonella* spp and *Klebsiella* species in this study had inhibitory zones of between 6mm to 22mm and as such are regarded to have moderate to strong antibacterial potentials.

The result of the nucleotide BLAST of the PCR sequences of the bacterial endophytes analyzed indicated that PL1 endophyte was *Myroides odoratimimus*, PR3 was *Empedobacter brevis,* PS5 was *Empedobacter brevis,* SL1 was *Bacillus tropicus*, SR2 was *Myroides odoratimimus* and SS4 was *Empedobacter brevis* (NCBI, 2025).

In this study, Liquid chromatographic and Spectral Analisyis (LCMS) of crude extracts of secondary metabolites produced by endophytic bacterial isolates of *Carica papaya* and *Mentha spicata* Leaf, root and stem revealed the presence of various secondary metabolites including Lupinine, Pyridine, Indole, Norspermidine, Aniline, 6-Methoxytaxifolin, N-Methyltyramine, Phenylethylamine, Aminoacetone and 2-Acetylpyrrolidine as shown on Table 4.7.

For example, Lupinine which is one of the dominant secondary metabolite discovered in all the crude extracts analysed in this study, is an elementary representative of a large quinolizidine alkaloid group (*Nurkenov et al.,* 2022). Referring to a pharmacological action, lupinin has the bactericidal and low sedative effects. It also possesses the short-term anthelmintic and hypotensive properties (*Nurkenov et al.,* 2022).

According to Wang *et* al., (2022). Structurally diverse nitrogen-containing heterocycles, such as pyrroles, imidazoles, oxazoles, pyridines, and quinolones, are naturally occurring secondary metabolites that often exhibit significant pharmacological activities, including antibacterial, antifungal, antiparasitic, and anticancer activities. Indole also known as benzopyyroles is a secondary metabolite produced in response to specific environmental conditions found in plants and microorganisms.

Many naturally occurring compounds are reported to possess a pyridine nucleus. These are considered alkaloids. Pyridine scaffold compounds and materials are valued for their biological, medicinal, optical, chemical, and physical properties among nitrogen-based heterocycles (Islam *et al*., 2023). According to Marinescu and Popa, (2022), Pyridine is a privileged nucleus among heterocycles and its compounds have been noted for their therapeutic properties, such as antimicrobial, antiviral, antitumor, analgesic, anticonvulsant, anti-inflammatory, antioxidant, anti-Alzheimer's and anti-ulcer or antidiabetic. 2-Acetylpyrrolidine is a pyrrolidine derivative. Pyrrolidines are part of a broader class of heterocycles that can exhibit antimicrobial properties (Seipp *et al*., 2021; Wang *et al*., 2022).

6-Methoxytaxifolin is a flavonoid derivative. Flavonoids are known for their broad spectrum of biological activities, including antimicrobial properties (Kumar & Pandey, 2013).

Norspermidine is a polyamine involved in various biological processes. Polyamines have been shown to have antimicrobial effects by interacting with bacterial membranes (Childs *et al*., 2018). Aniline is an aromatic amine with limited antimicrobial activity. It is more commonly associated with industrial applications than biological activities. N-Methyltyramine is a phenethylamine derivative with potential biological activities. Phenethylamines can exhibit antimicrobial effects, but detailed study on N-methyltyramine's efficacy against bacterial pathogens has not been established. Phenylethylamine is a naturally occurring amine with various biological activities. Aminoacetone is a compound involved in metabolic pathways but lacks documented antimicrobial activity against clinical isolates used in this study.

The antibacterial activities portrayed by the secondary metabolites used against the clinical isolates in this study could be attributed to the presence of the bioactive secondary metabolites such as lupinine, pyrimidine and pyrrolidine identified in them which are known for their antibacterial activities (*Nurkenov et al.,* 2022; Islam *et al*., 2023). Similarly, Keskin *et* al., 2017, El Gaamouch *et* al., 2021 and *Nurkenov et al.,* (2022) reported in their study that lupinine compounds have high antibiotic activity against plague and cholera microbes. Also, antibacterial activity of pyridine derivatives were observed against series of bacteria like *A. baumannii, A. iwoffii, Enterobacter sp., Klebsiella planticola*, *E. coli, S. aureus, P. aeruginosa, S. typhi, S. dysenteriae* and *S. maltophilia* (Eldeab, 2019; Karunanidhi *et al*., 2019; Özgeri; 2021; Ragab *et al*., 2021; Marinescu and Popa, 2022).

Therefore, the presence of these phytochemical constituents in the secondary metabolites by LCMS could be the reason for the antibacterial activities on the clinical isolates.

Conclusion

Preliminary isolation and identification of endophytic bacterial isolates from leaf, root and stem of *Carica papaya* using Gram staining and biochemical characterization revealed that PL1, PR3 and PS5 from *Carica papaya* were all presumed to be *Flavobacterium* species while PL2 and PS4 were presumed to be *Pseudomonas* species.

Three endophytes were selected for further analysis and their identities were confirmed using their PCR sequences where PL1 endophyte was confirmed to be *Myroides odoratimimus* while PR3 and PS5 were *Empedobacter brevis.*

Antibacterial activities of the secondary metabolites tested against the clinical bacterial isolates *E. coli, S. aureus*, *Salmonella* spp and *Klebsiella* species in this study had inhibitory zones of between 6mm to 22mm and as such were regarded to have moderate to strong antibacterial potentials.

The LCMS analysis of the secondary metabolites indicated the presence of many metabolites such as Lupinine, Pyridine, Benzeneacetonitrite, Norspermidine, Aniline, 6-Methoxytaxifolin, N-Methyltyramine, Phenylethylamine, Aminoacetone and 2-Acetylpyrrolidine out of which Lupinine and Pyridine are alkaloid derivatives and known to have antibacterial activities.

The antibacterial activities portrayed by the secondary metabolites used against the clinical isolates in this study could be attributed to the presence of the bioactive secondary metabolites identified in them which are known for their antibacterial activities.

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