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Isolation and Molecular Identification of Culturable Rhizosphere Bacteria Associated with the Medicinal Plant Justicia Adhatoda

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Abstract: ***The rhizosphere of medicinal plants represents a chemically rich and ecologically selective microhabitat that supports distinctive bacterial communities, yet the culturable diversity associated with many traditionally important species remains poorly documented. Justicia adhatoda (Malabar nut), a shrub widely used in Ayurvedic and Unani medicine for respiratory ailments, is one such plant whose rhizosphere microbiome has received little dedicated attention. In this study, rhizosphere soil was collected from healthy J. adhatoda plants and processed by serial dilution and spread-plating on nutrient agar. Twelve morphologically distinct colony types were recovered, of which six representative isolates were purified for identification. Preliminary screening by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) yielded only one high-confidence and one moderate-confidence species-level match, with the remaining four isolates falling below the reliable identification threshold. Three representative isolates spanning the confidence range were therefore subjected to 16S rRNA gene sequencing (Sanger platform, 27F/1492R primers) and BLASTn comparison against the NCBI database. This confirmed the isolates as Paenarthrobacter nitroguajacolicus (98.88% identity), Kocuria rosea (99.77% identity), and Micrococcus aloeverae (100% identity). The results demonstrate that combining rapid mass-spectrometric screening with confirmatory molecular sequencing provides a reliable, tiered workflow for characterising culturable rhizosphere bacteria of medicinal plants, and establish a taxonomically verified isolate collection suitable as a starting point for genome-level bioprospecting, pursued in a companion study.***

Keywords: Rhizosphere microbiology, Justicia adhatoda, MALDI-TOF mass spectrometry, 16S rRNA gene sequencing, bacterial isolation, medicinal plant microbiome.

I. INTRODUCTION

The rhizosphere the narrow zone of soil directly influenced by plant roots is among the most biologically active microenvironments in terrestrial ecosystems. Root exudates such as sugars, amino acids, organic acids, and secondary metabolites create a nutrient-rich zone that selectively shapes the surrounding microbial community [1]. This selective enrichment makes the rhizosphere a long-recognised "hotspot" for microorganisms with specialised metabolic capabilities, including many of the bacteria that have historically supplied antibiotics and other natural products to medicine [2].

Among rhizosphere inhabitants, plant growth-promoting rhizobacteria (PGPR) are particularly well studied for their roles in nitrogen fixation, phosphate solubilisation, and phytohormone production, while genera such as Streptomyces, Bacillus, Pseudomonas, and various Actinobacteria are frequently reported to produce antimicrobial, antifungal, and anti-inflammatory secondary metabolites [3]. The continuing rise of antimicrobial resistance has intensified interest in such under-exploited environmental reservoirs as sources of novel chemistry, since conventional screening of already well-studied taxa yields diminishing returns [2].

Medicinal plants add a further layer of selective pressure to this picture. Justicia adhatoda (Malabar nut; family Acanthaceae) is a dense evergreen shrub widely distributed across tropical and subtropical regions and long used in Ayurvedic and Unani medicine for asthma, bronchitis, and cough. Its therapeutic activity is attributed largely to quinazoline alkaloids such as vasicine and vasicinone, which possess bronchodilatory, antimicrobial, and anti-inflammatory properties [4], [5]. Root exudation of such phytochemicals is expected to create a distinctive chemical microenvironment in the rhizosphere, potentially selecting for bacteria with enhanced or specialised bioactivity. However, in contrast to well-studied medicinal and crop plants, the rhizosphere microbiome of J. adhatoda has not been systematically characterised.

Culture-dependent isolation remains the necessary first step for any bioprospecting pipeline that intends to progress from taxonomic survey to genome-level investigation, since it yields living isolates that can subsequently be sequenced, mined, and ultimately tested experimentally. Reliable species-level identification of such isolates, however, is not trivial: rapid proteomic fingerprinting methods such as MALDI-TOF MS are fast and inexpensive but depend on reference spectral libraries that are populated predominantly with clinical isolates, leaving many environmental and rhizosphere-associated taxa poorly represented.
Sequence-based identification via the 16S rRNA gene remains the most broadly reliable confirmatory method for bacteria at the species level and is treated here as the reference standard against which MALDI-TOF screening is compared.

A. Related Work on Rhizosphere Bacterial Diversity

Several recent studies illustrate both the promise and the gaps in this field. Using shotgun metagenomics, Huang et al. [6] characterised the rhizosphere microbiome of ginseng and isolated a Streptomyces strain with strong antifungal activity, underscoring the value of combining community-level sequencing with targeted isolation. Mazumdar et al. [7] isolated a Streptomyces strain from Assam rhizosphere soil with activity against multidrug-resistant pathogens, while Sharma and Thakur [8] reported similarly potent actinomycete isolates from rhizosphere soils across Northeast India. Shami et al. [9] combined metagenomics with antibiotic-resistance-gene profiling in the Moringa oleifera rhizosphere, highlighting that bioprospecting efforts must also consider the resistome context of candidate producers. Tlou et al. [10] used next-generation sequencing to identify Bacillus and Streptomyces endophytes from medicinal plants with strong antimicrobial activity, reinforcing that plant-associated bacteria adapted to chemically rich hosts often show enhanced bioactivity. Comparable patterns have been reported outside the rhizosphere context as well for example in mangrove-associated bacteria [11] supporting the general hypothesis that ecologically distinctive niches favour distinctive microbial chemistry [12]–[15].
Despite this body of work, most studies continue to focus on a small number of well-characterised genera (chiefly Streptomyces and Bacillus), and dedicated surveys of medicinal-plant rhizospheres such as that of J. adhatoda remain scarce. There is also relatively little published comparison of rapid proteomic identification methods against molecular sequencing specifically for rhizosphere isolates, even though this comparison has direct practical value for laboratories designing bioprospecting workflows.

B. Aim and Objectives

This study aimed to isolate culturable bacteria from the rhizosphere of J. adhatoda and to establish their taxonomic identity using a tiered identification workflow. The specific objectives were: (i) to isolate and morphologically characterise bacterial colonies from J. adhatoda rhizosphere soil; (ii) to screen the isolates using MALDI-TOF MS and assess the reliability of this method for environmental isolates; and (iii) to confirm the identity of representative isolates using 16S rRNA gene sequencing and BLAST analysis against the NCBI database.

II. MATERIALS AND METHODS

A. Study Site and Sample Collection

Rhizosphere soil was collected from the root zone of healthy, mature J. adhatoda plants under aseptic conditions. Soil adhering tightly to the root surface was separated using sterile spatulas, transferred to sterile, labelled containers, and transported to the laboratory for immediate processing to preserve microbial viability.

B. Media, Reagents, and Instrumentation

Nutrient agar and nutrient broth (HiMedia) were used for isolation and maintenance of bacterial cultures, respectively, with distilled water used throughout for dilution and media preparation. Standard laboratory equipment was used for aseptic processing, including a laminar airflow cabinet, autoclave, hot-air oven, incubator, centrifuge, and micropipettes. Genomic DNA extraction reagents, PCR reagents (EmeraldAmp GT PCR Master Mix, Takara), and Sanger sequencing reagents (BigDye Terminator v3.1 Cycle Sequencing Kit, Thermo Fisher) were used for downstream molecular work as described below.

C. Isolation of Rhizosphere Bacteria

Soil samples were serially diluted in sterile distilled water and aliquots from each dilution were aseptically spread onto nutrient agar plates using the spread-plate technique. Plates were incubated at 28–37°C for 24–72 h. Morphologically distinct colonies distinguished by size, shape, colour, elevation, and margin were selected and purified by repeated streak-plating to obtain axenic cultures, which were maintained on slants for subsequent analysis.

D. Morphological and Biochemical Characterisation

Purified isolates were examined for colony morphology and Gram reaction, and screened using catalase and oxidase tests to obtain preliminary information on cell-wall type and enzymatic profile ahead of species-level identification.

E. MALDI-TOF MS Identification

Six purified isolates were analysed by MALDI-TOF MS on a Bruker Biotyper system. Spectra were matched against the BDAL and Filamentous Fungi reference libraries, and identifications were classified using the manufacturer's standard scoring scheme: scores of 2.00–3.00 were treated as high-confidence identifications (+++), 1.70–1.99 as low-confidence identifications (+), and scores below 1.70 as providing no reliable organism identification (−). A consistency category (A = high, B = low, C = none) was additionally assigned based on agreement between the top two spectral matches for each isolate.

F. Molecular Identification by 16S rRNA Gene Sequencing

Representative isolates spanning the range of MALDI-TOF confidence levels were selected for confirmatory molecular identification. Genomic DNA was extracted using Prepman Ultra lysis reagent (Thermo Fisher) and the 16S rRNA gene was amplified by PCR using the universal bacterial primers 27F and 1492R with EmeraldAmp GT PCR Master Mix (Takara). Amplicons were verified by agarose gel electrophoresis, purified using ExoSAP-IT Express reagent, and sequenced by Sanger cycle sequencing (BigDye Terminator v3.1, forward primer 27F) on a 3500XL Genetic Analyzer (Applied Biosystems, 24-capillary configuration). Electropherograms were assembled and edited in CodonCode Aligner, and consensus sequences were compared against the NCBI 16S ribosomal RNA (Bacteria and Archaea) database using BLASTn [16] to determine the closest phylogenetic match, percentage identity, and query coverage for each isolate.

G. Data Analysis

Identification outcomes from MALDI-TOF MS and 16S rRNA sequencing were compared to assess concordance and to evaluate the reliability of proteomic screening relative to molecular confirmation for rhizosphere-derived isolates.

III. RESULTS

A. Colony Morphology and Isolation Outcome

Spread-plating of serial soil dilutions produced visible colonies on all inoculated plates after 72 h of incubation, confirming a diverse culturable microbial population in the J. adhatoda rhizosphere soil. Based on differences in colour, size, shape, and texture, twelve morphologically distinct colony types were recognised. Following purification by repeated subculturing, six isolates showing the clearest morphological distinctions were retained for identification.

B. MALDI-TOF MS Screening

The six purified isolates were screened by MALDI-TOF MS. Identification confidence varied considerably: one isolate (I17) returned a high-confidence match (score 2.09; consistency category A) to Deinococcus wulumuqiensis, and one isolate (I16) returned a moderate/low-confidence match (score 1.89; consistency category B) to Micrococcus luteus. The remaining four isolates (I14, I15, I18, I19) either matched below the reliable identification threshold or, in the case of I18, produced no detectable spectral peaks (Table I). Overall, only one of six isolates (17%) could be identified with high confidence by MALDI-TOF MS alone, underscoring the limited coverage of rhizosphere-associated taxa in the reference spectral library used.

TABLE I. SUMMARY OF MALDI-TOF MS IDENTIFICATION RESULTS FOR SIX RHIZOSPHERE BACTERIAL ISOLATES

Isolate ID	Best-Match Organism	Score	Confidence	Consistency
I17	Deinococcus wulumuqiensis	2.09	High (+++)	A (high)
I16	Micrococcus luteus	1.89	Low (+)	B (low)
I15	No reliable identification*	1.69	None (−)	C (none)
I14	No reliable identification	1.55	None (−)	C (none)
I19	No reliable identification	1.39	None (−)	C (none)
I18	No peaks detected	0.00	None (−)	C (none)

**Best available spectral match for isolate I15 was Paenarthrobacter nicotinovorans (score 1.69), below the reliable identification threshold; this isolate was carried forward to molecular sequencing as the highest-scoring representative of the low-confidence group.*

Range	Interpretation	Symbols	Color
2.00 - 3.00	High-confidence identification	(+++)	green
1.70 - 1.99	Low-confidence identification	(+)	yellow
0.00 - 1.69	No Organism Identification Possible	(-)	red

Fig. 1 MALDI-TOF MS score interpretation criteria used to classify identification confidence (Bruker Biotyper system).

Category	Interpretation
(A)	**High consistency:** The best match is a high-confidence identification. The second-best match is (1) a high-confidence identification in which the species is identical to the best match, (2) a low-confidence identification in which the species or genus is identical to the best match, or (3) a non-identification.
(B)	**Low consistency:** The requirements for high consistency are not met. The best match is a high- or low-confidence identification. The second-best match is (1) a high- or low-confidence identification in which the genus is identical to the best match or (2) a non-identification.
(C)	**No consistency:** The requirements for high or low consistency are not met.

Fig. 2 Consistency categories used to evaluate agreement between the top two MALDI-TOF spectral matches for each isolate.

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
I16 (+) (B)	A10 -5 10/y (Standard)	Micrococcus luteus	1.89	Micrococcus luteus	1.81
I17 (+++) (A)	A10 -4 9/r (Standard)	Deinococcus wulumuqiensis	2.09	Deinococcus wulumuqiensis	2.08
I18 (-) (C)	A10 -2 6/y (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
I19 (-) (C)	A10 -3 3/w (Standard)	**No Organism Identification Possible**	1.39	**No Organism Identification Possible**	1.34

Fig. 3 Representative MALDI-TOF MS output summarising best- and second-best-match organisms and score values for the six screened isolates.

C. Detailed Spectral Identification of Selected Isolates

Isolates spanning the confidence range I17 (high confidence), I16 (low/moderate confidence), and I15 (no reliable identification) were carried forward for confirmatory molecular analysis.

1) *Isolate I17:* MALDI-TOF MS matched isolate I17 to Deinococcus wulumuqiensis with high confidence (score 2.09; consistency category A), as detailed in Fig. 4.

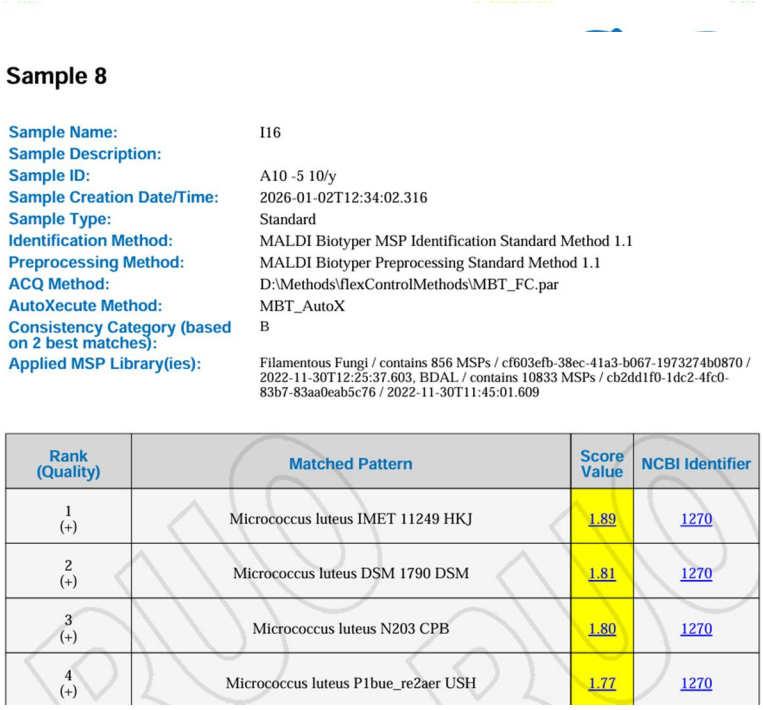
Sample 9

Sample Name:	I17
Sample Description:	
Sample ID:	A10 -4 9/r
Sample Creation Date/Time:	2026-01-02T12:34:02.316
Sample Type:	Standard
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
AutoXecute Method:	MBT_AutoX
Consistency Category (based on 2 best matches):	A
Applied MSP Library(ies):	BDAL / contains 10833 MSPs / cb2dd1f0-1dc2-4fc0-83b7-83aa0eab5c76 / 2022-11-30T11:45:01.609, Filamentous Fungi / contains 856 MSPs / cf603efb-38ec-41a3-b067-1973274b0870 / 2022-11-30T12:25:37.603

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+++)	Deinococcus wulumuqiensis CIP 110212T CIP	2.09	1298
2 (+++)	Deinococcus wulumuqiensis DSM 28106 DSM	2.08	1298
3 (+++)	Deinococcus wulumuqiensis DSM 28115T DSM	2.06	1298
4 (+)	Deinococcus wulumuqiensis 150821_5 GPSA	1.99	1298

Fig. 4 MALDI-TOF MS report for isolate I17, matched with high confidence to Deinococcus wulumuqiensis.

2) *Isolate I16:* Isolate I16 was matched to Micrococcus luteus with low/moderate confidence (score 1.89; consistency category B), as detailed in Fig. 5.



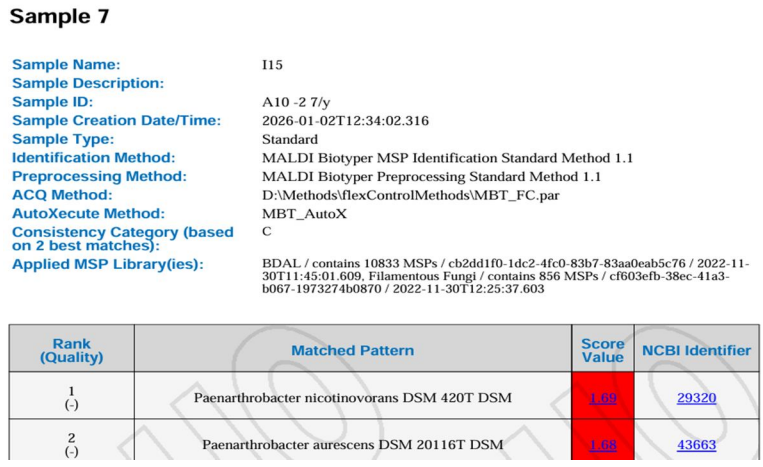
Sample 8

Sample Name:	I16
Sample Description:	
Sample ID:	A10 -5 10/y
Sample Creation Date/Time:	2026-01-02T12:34:02.316
Sample Type:	Standard
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
AutoXecute Method:	MBT_AutoX
Consistency Category (based on 2 best matches):	B
Applied MSP Library(ies):	Filamentous Fungi / contains 856 MSPs / cf603efb-38ec-41a3-b067-1973274b0870 / 2022-11-30T12:25:37.603, BDAL / contains 10833 MSPs / cb2dd1f0-1dc2-4fc0-83b7-83aa0eab5c76 / 2022-11-30T11:45:01.609

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (+)	Micrococcus luteus IMET 11249 HKJ	1.89	1270
2 (+)	Micrococcus luteus DSM 1790 DSM	1.81	1270
3 (+)	Micrococcus luteus N203 CPB	1.80	1270
4 (+)	Micrococcus luteus P1bue_re2aer USH	1.77	1270

Fig. 5 MALDI-TOF MS report for isolate I16, matched with low/moderate confidence to Micrococcus luteus.

3) *Isolate I15:* Isolate I15 could not be reliably identified by MALDI-TOF MS (best match Paenarthrobacter nicotinovorans, score 1.69, below the reliable identification threshold), as detailed in Fig. 6.



Sample 7

Sample Name:	I15
Sample Description:	
Sample ID:	A10 -2 7/y
Sample Creation Date/Time:	2026-01-02T12:34:02.316
Sample Type:	Standard
Identification Method:	MALDI Biotyper MSP Identification Standard Method 1.1
Preprocessing Method:	MALDI Biotyper Preprocessing Standard Method 1.1
ACQ Method:	D:\Methods\flexControlMethods\MBT_FC.par
AutoXecute Method:	MBT_AutoX
Consistency Category (based on 2 best matches):	C
Applied MSP Library(ies):	BDAL / contains 10833 MSPs / cb2dd1f0-1dc2-4fc0-83b7-83aa0eab5c76 / 2022-11-30T11:45:01.609, Filamentous Fungi / contains 856 MSPs / cf603efb-38ec-41a3-b067-1973274b0870 / 2022-11-30T12:25:37.603

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (-)	Paenarthrobacter nicotinovorans DSM 420T DSM	1.69	29320
2 (-)	Paenarthrobacter aurescens DSM 20116T DSM	1.68	43663

Fig. 6 MALDI-TOF MS report for isolate I15, selected from the low-confidence group as the highest-scoring remaining isolate.

D. Molecular Identification by 16S rRNA Gene Sequencing

16S rRNA gene sequencing of the three selected isolates (samples A1, A2, A3) produced high-quality partial sequences (708–863 bp) that showed strong similarity to known bacterial species in the NCBI database, with 100% query coverage and E-values of 0 in all cases (Table II). Isolate A3 was identified as Paenarthrobacter nitroguajacolicus (98.88% identity), isolate A2 as Kocuria rosea (99.77% identity), and isolate A1 as Micrococcus aloeverae (100% identity). Notably, isolate A3 corresponds to the isolate that MALDI-TOF MS had matched, with low confidence, to a congeneric species, while the MALDI-TOF top hits for the other isolates were not confirmed at the species level by sequencing, illustrating the value of molecular confirmation even where proteomic screening returns an apparently confident match.

TABLE II. 16S RRNA GENE SEQUENCING AND BLASTN IDENTIFICATION OF REPRESENTATIVE RHIZOSPHERE ISOLATES

Sample	Identified Species	Query (bp)	% Identity	E-value	Accession	Acc. Length
A3	Paenarthrobacter nitroguajacolicus	708	98.88	0	NR_027199.1	1488
A2	Kocuria rosea	863	99.77	0	NR_028924.1	1480
A1	Micrococcus aloeverae	732	100.00	0	NR_134088.1	1411

IV. DISCUSSION

Twelve morphologically distinct colony types recovered from a single rhizosphere soil sample confirm that the rhizosphere of J. adhatoda supports a culturable bacterial community of appreciable diversity, consistent with the broader literature on rhizosphere microbiology [1]. The subsequent identification results, however, highlight an important methodological point for researchers working with environmental and rhizosphere-derived bacteria: MALDI-TOF MS, despite its speed and low per-sample cost, correctly identified only one of six isolates (17%) with high confidence. This is consistent with previous observations that commercial MALDI-TOF reference databases are weighted toward clinically relevant organisms, leaving many environmental and soil-associated taxa under-represented or entirely absent (see also the broader diversity gaps noted for rhizosphere metagenomes generally [13], [14]). Relying on MALDI-TOF MS alone in such contexts risks both missed identifications and, more concerningly, confident-looking but incorrect calls when a close congener happens to be present in the database.

16S rRNA gene sequencing, by contrast, produced unambiguous species-level identifications (98.9–100% identity) for all three isolates carried forward, confirming its continued value as the reference standard for bacterial identification, particularly for organisms poorly represented in proteomic spectral libraries. The three identified taxa Paenarthrobacter nitroguajacolicus, Kocuria rosea, and Micrococcus aloeverae all belong to genera (Micrococcaceae) that include species previously reported elsewhere as producers of antimicrobial and other bioactive secondary metabolites, although, to our knowledge, none of the three species has been specifically reported from the rhizosphere of a medicinal Justicia species before. This supports the broader hypothesis that medicinal-plant rhizospheres, shaped by root-exuded phytochemicals such as vasicine and vasicinone, may select for microbial taxa with enhanced or distinctive biosynthetic potential [4], [5].

Minor variation in percentage identity across the three isolates (98.88–100%) is consistent with the presence of strain-level diversity, which is commonly observed in rhizosphere populations subject to localised selective pressure from root exudates [1]. Taken together, these results support a practical recommendation for rhizosphere bioprospecting workflows: MALDI-TOF MS remains useful as a rapid first-pass triage tool to prioritise isolates for further work, but molecular sequencing should be treated as obligatory for definitive identification, especially where an isolate is intended for downstream genomic or biosynthetic characterisation. A limitation of the present study is that only three of the six purified isolates were carried through to molecular confirmation; the remaining three (including the two additional low-confidence MALDI-TOF isolates not sequenced here) remain of unconfirmed identity and represent a natural target for future work. The taxonomically confirmed isolates reported here form the basis for a genome-level investigation of their biosynthetic gene cluster repertoire, which is reported separately in a companion study.

V. CONCLUSION

This study establishes a taxonomically verified collection of culturable rhizosphere bacteria from Justicia adhatoda, obtained through a tiered identification workflow combining rapid MALDI-TOF MS screening with confirmatory 16S rRNA gene sequencing. Of six morphologically distinct isolates purified from rhizosphere soil, three were confirmed by sequencing as Paenarthrobacter nitroguajacolicus, Kocuria rosea, and Micrococcus aloeverae, each with high sequence identity (>98%) to reference database entries. The comparatively poor performance of MALDI-TOF MS for these environmental isolates (one high-confidence match out of six) reinforces the need for molecular confirmation when characterising rhizosphere and other non-clinical bacterial communities. As genera within Micrococcaceae are known elsewhere to harbour biosynthetic potential for antimicrobial and anti-inflammatory compounds, the isolates identified here represent a promising, taxonomically secure starting point for genome mining and natural product discovery, pursued in a companion genome-based study of the same three isolates.

VI. ACKNOWLEDGMENT

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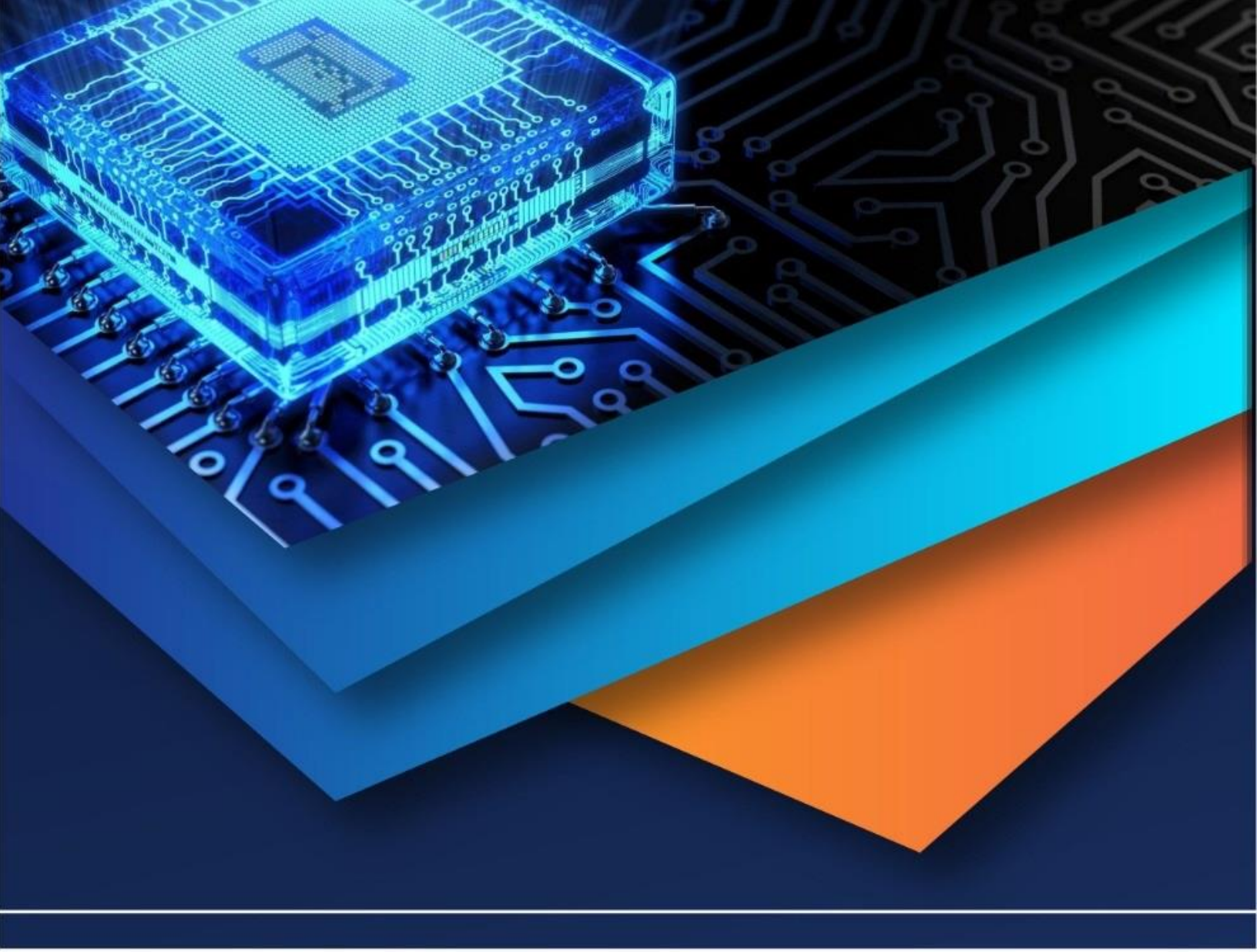

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