Indian Journal of Chemistry
Vol. 64, June 2025, pp. 634-639
DOI: 10.56042/ijc.v64i6.16839

Bioactive compound from *Micrococcus luteus* associated with *Datura stramonium* L. seeds

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Received 2 February 2025; accepted (revised) 22 May 2025

Endophytic bacteria residing within plant tissues have garnered significant attention as prolific sources of bioactive compounds with diverse medicinal applications. Their economic and scientific importance lies in their ability to produce metabolites with antimicrobial, antitubercular, and other therapeutic properties, offering potential alternatives to synthetic drugs and contributing to sustainable biotechnological advancements. In this study, we explore *Micrococcus luteus*, an endophyte isolated from the seeds of *Datura stramonium L.*, for its capacity to produce bioactive metabolites. The bacterium has been identified using a combination of morphological, biochemical, and 16S rRNA sequencing methods. Fermentation and extraction processes facilitated the recovery of metabolites, which have been purified and characterized through chromatographic and spectroscopic techniques, including NMR and HRMS. The analysis reveals the presence of a diketopiperazine derivative. Biological evaluations demonstrate significant antimicrobial efficacy against *Staphylococcus aureus* and *Escherichia coli* and notable antitubercular activity against *Mycobacterium tuberculosis H37Rv* with a minimum inhibitory concentration (MIC) of 12.5 µg/mL. These results emphasise the dual economic and scientific value of endophytic bacteria such as *Micrococcus luteus*, not only as a sustainable source of bioactive compounds but also as a promising resource for developing novel treatments for infectious diseases, including tuberculosis.

Keywords: Endophytic microorganisms, Secondary metabolites, *Datura stramonium*, *Micrococcus luteus*, 16SrRNA

Endophytic bacteria, microorganisms that reside symbiotically within plant tissues without causing harm, have emerged as a prolific source of bioactive compounds with potential therapeutic applications[1]. These bacteria produce diverse secondary metabolites, many exhibiting potent biological activities, including antimicrobial, anticancer, and antioxidant properties[2]. Exploring endophytes offers a promising avenue for discovering novel bioactive molecules, especially in the fight against antibiotic-resistant pathogens and other pressing health challenges[3].

In this study, we focus on *Micrococcus luteus*, an endophytic bacterium isolated from the seeds of *Datura stramonium* L., a medicinal plant widely recognized for its pharmacological significance[4]. *Datura stramonium* has a rich history of use in traditional medicine, being valued for its alkaloid content and therapeutic potential in treating conditions like asthma, pain, and inflammation[5]. Among its parts, the capsules serve as a highly specialized microenvironment, rich in biochemical precursors, that can harbor unique endophytic communities with enhanced capabilities for producing bioactive metabolites[6]. As a reproductive structure, the capsule is metabolically active and serves as a protective niche for endophytes, offering a favorable habitat supporting microbial secondary metabolism[7]. This makes it an ideal candidate for isolating endophytes with pharmaceutical relevance. *Micrococcus luteus* was first described by Cohn in 1872 and has since garnered attention for its remarkable environmental resilience, including the ability to withstand high salinity, desiccation, and radiation[8]. These stress-tolerance mechanisms make it a model organism for studying bacterial survival under extreme conditions and a potential reservoir of

unique bioactive compounds. Furthermore, its fully sequenced genome has revealed insights into its versatile metabolic pathways and the biosynthesis of secondary metabolites[9]. As a Gram-positive, non-motile bacterium belonging to the family *Micrococcaceae*, *M. luteus* is widely distributed across diverse habitats such as soil, water, air, and human skin[10,11]. Its characteristic bright yellow pigmentation is attributed to carotenoid compounds, which have antioxidant and photoprotective properties[12]. Additionally, it produces pyridine metabolites in soil[13], salt-tolerant glutaminases in marine environments[14], alkaline esterases on human skin[15], exopolysaccharides in freshwater[16], and exhibits antimicrobial activity[17]; *Micrococcus luteus* mitigates heavy metal stress in tomato plants by reducing the accumulation of nickel and cadmium while fostering growth. Its ability to produce IAA and solubilise phosphate enhances stress resilience and nutrient uptake. *M. luteus* is thus a potentially valuable tool for augmenting plant resistance in polluted environments[18] making it invaluable for biotechnology and pharmaceutical advancements. Fig. 1 depicts bioactive compounds produced by *Micrococcus luteus* isolated from soil, freshwater, and marine environments.

Multiple studies highlight the significance of *Micrococcus luteus*, extracted from plant tissues in promoting growth and stress resistance. A strain from the soybean rhizosphere exhibited phosphate solubilization, IAA production, and biocontrol effectiveness[19]. The strain MlS14 enhanced plant growth by producing IAA and antifungal agents[20]. A desiccation-tolerant strain from the Cholistan Desert was discovered to promote maise growth[21]. *Cyperus conglomeratus* roots from Saudi Arabia produced *Micrococcus luteus* strain K39. Genomic research discovered salt and oxidative stress tolerance enzymes. It may increase plant growth under abiotic stress[22].

In this study, *M. luteus* was isolated from *Datura stramonium* capsules and identified using advanced morphological, biochemical, and molecular methods, including 16S rRNA sequencing[23]. Fermentation and extraction processes were employed to obtain bioactive metabolites purified and characterised using chromatographic and spectroscopic techniques[24]. Among the identified metabolites, diketopiperazine derivative exhibited significant antimicrobial and antitubercular activities, demonstrating the potential of *M. luteus* as a valuable resource for pharmaceutical innovation. This research highlights the importance of endophytic bacteria and their symbiotic association with medicinal plants in uncovering novel bioactive compounds to address global health challenges.

Material and methods

Endophyte isolation and identification

The capsule of *Datura stramonium*, collected from the premises of Andhra University, Visakhapatnam, Andhra Pradesh, India, was selected for isolation due to its rich reservoir of endophytes, which are often associated with reproductive structures providing a unique niche for microbial diversity. Capsules are

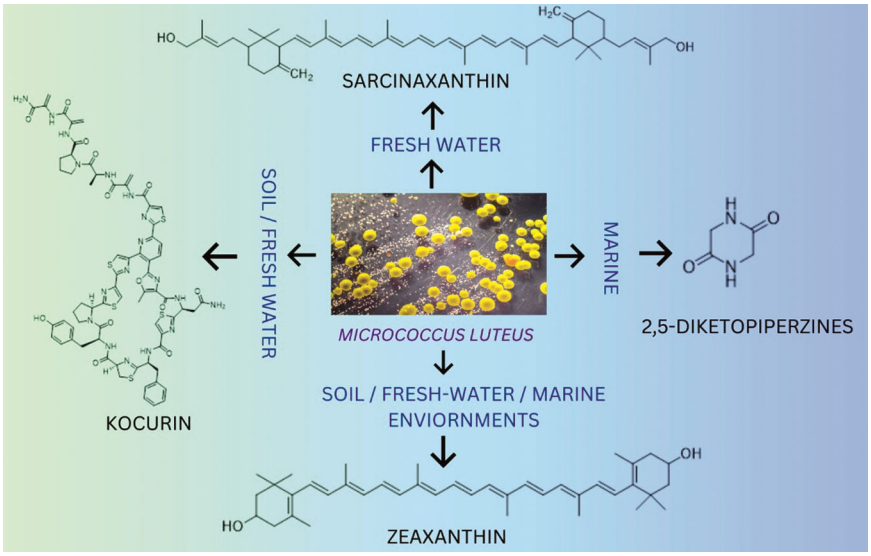


Fig. 1 — Bioactive compounds produced by *Micrococcus luteus* isolated from soil, freshwater, and marine environments

more likely to harbour bioactive compound-producing endophytes, as these metabolites may play a role in seed protection and plant defence. Capsules of *Datura stramonium* were surface-sterilized using a standardised protocol to eliminate external contaminants and dissected aseptically to obtain seeds. The seeds were then plated on sterile nutrient agar (NA) (Table 1) to isolate endophytic bacteria, as NA provides a rich, non-selective medium supporting the growth of diverse bacterial taxa[25]. This approach was designed to ensure the successful recovery of cultivable endophytes from the internal tissues of the seeds. After incubation for 168 hours, bacterial colonies were selected based on distinct morphological characteristics and sub-cultured twice to ensure the purity of the isolates. To facilitate preliminary identification, standard microbiological and biochemical tests were performed. These included Gram staining, acid-fast staining, bacterial spore staining, and the hanging drop method to assess motility[26].

Of the nine isolated strains (DS-1 to DS-9), DS-5 was selected for advanced identification. This selection was based on its distinct morphological and biochemical traits and consistent and robust growth on the media chosen, indicating potential for bioactive metabolite production. The strain was sent to NCIM Pune for precise taxonomic identification using 16S rRNA sequencing, a gold-standard molecular technique for bacterial identification. Using the BLAST program, the 16S rRNA gene was amplified, sequenced, and compared against reference sequences in the GenBank database[27]. Phylogenetic analysis was conducted using MEGA software to construct a genetic relationship tree, confirming the classification of DS-5 as *Micrococcus luteus*[28]. The selection of DS-5 for 16S rRNA sequencing was a strategic decision, as it displayed unique phenotypic and biochemical features among the isolates, suggesting its potential as a producer of bioactive compounds. The comprehensive methodology ensured accurate taxonomic classification and validated the significance of selecting DS-5 as a promising candidate for further study on endophyte-derived bioactive metabolites.

Table 1 — Composition of nutrient agar medium

S. No.	Contents	Quantity for 100 (mL)
1	Beef extract	0.3 g
2	Peptone	0.3 g
3	NaCl	0.5 g
4	Distilled water	100 mL

Production of Bioactive Compounds

Bioactive compound production was performed using submerged fermentation, scaled to 10 litres, and incubating at 37°C and 135 rpm on a rotary shaker for 120 hours. The 120-hour incubation period was chosen based on preliminary studies demonstrating maximum metabolite yield. Ext extended incubation could lead to nutrient depletion and decreased production due to bacterial stress or autolysis. The culture was harvested, and the supernatant was separated from bacterial cells through centrifugation at 10,000 rpm. Equal volumes of ethyl acetate were used three times to extract bioactive compounds from the culture-free supernatant. The organic phases were pooled and concentrated using a rotary evaporator at 60°C to preserve the integrity of thermolabile compounds Fig. S2.

The crude extracts were screened for bioactive compounds using bioautography on thin-layer chromatography (TLC), with 10% EtOAc: Hexane solvent systems to identify spots indicative of bioactivity[29]. Column chromatography purified the identified compounds (Supplementary Table S1). Finally, structural characterisation of the bioactive compounds was performed using NMR and mass spectrometry[30]. This systematic and scalable methodology ensured consistent production and reliable analysis of bioactive metabolites.

Antimicrobial and Antitubercular Activities

The agar well diffusion technique was used to evaluate the bacterial susceptibility of the produced compounds at different doses. The antibacterial activity against *Staphylococcus aureus* (MTCC 3160) and *Escherichia coli* (MTCC 443) was evaluated using Rifampicin as the standard and DMSO as the control. Amikacin served as the standard, whereas DMSO was the control to assess antifungal efficacy against *Candida albicans* (MTCC 227) at a 100 µg/mL dose. The synthesized compounds were evaluated for their *in vitro* anti-tubercular efficacy against the H37Rv strain with the Microplate Alamar Blue test (MABA) on *Mycobacterium TB H37Rv (ATCC 27294)* with the activity reported as minimum inhibitory concentration (MIC) in µg/mL[31].

Results and Discussion

Phylogenetic identification of isolated bacterial endophytes

Capsules of *Datura stramonium* were surface sterilised, cut open with sterile scalpel seeds, and

aseptically plated on nutrient agar (NA) medium to isolate endophytic bacteria. After a 168-hour incubation period, nine distinct bacterial strains were successfully isolated, as shown in Fig.S1 (supplementary information); among these, strain DS-5 was selected for further study based on its significant antibiotic activity demonstrated *via* submerged fermentation, extraction, and subsequent bioautography of the organic extract. Morphological and biochemical characterisation (VITEK 2) revealed that the endophytic bacterial strains were Gram-positive, non-motile, and negative for oxidase and catalase activities Table S2. DS-5 was identified as *Micrococcus luteus* using 16S rRNA sequencing (NC111223A) Table S3. The 16S rRNA gene was amplified, sequenced, and compared with reference sequences in the GenBank database using the BLAST program. The genomic characterisation of the isolate DS-5 is shown in Fig S3. Phylogenetic analysis using MEGA software confirmed its taxonomic classification and genetic relationship within the *Micrococcus luteus,* as shown in Fig. 2. This comprehensive approach ensured accurate identification. It validated DS-5 as a promising candidate for bioactive compound production.

The fermentation of *Micrococcus luteus* (DS-5) at *p*H 7.0 and 37°C for 120 hours. This incubation period was ideal for maximum metabolite production, as it allowed sufficient time for the bacterium to reach the stationary phase, where secondary metabolite biosynthesis typically peaks. The scaled-up fermentation was conducted with a total production volume of 10 litres, yielding 0.225 grams of crude extract per litre (a total of 2.25 grams). The crude extract was obtained by extracting the culture-free supernatant with ethyl acetate three times and concentrating the organic layer using a rotary evaporator. Purification of the crude extract through column chromatography led to the isolation of the major bioactive compound DSM-05, identified as a diketopiperazine derivative, a well-known class of secondary metabolites with reported antimicrobial properties, supporting the therapeutic potential of *Micrococcus luteus*. The findings highlight the significance of *Micrococcus luteus* from *Datura stramonium* as a source of bioactive compounds with potential applications in combating infectious diseases (Fig. 3).

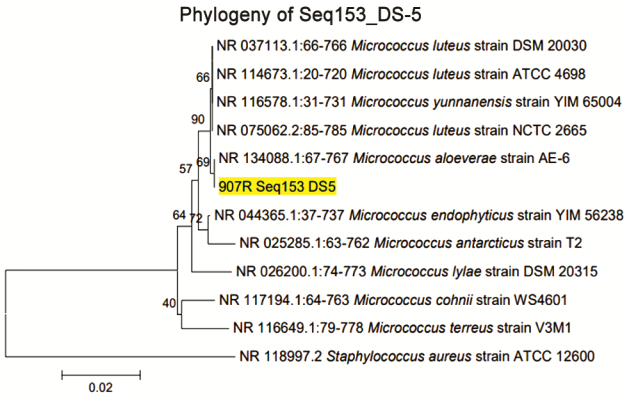


Fig. 2 — Evolutionary relationships of taxa

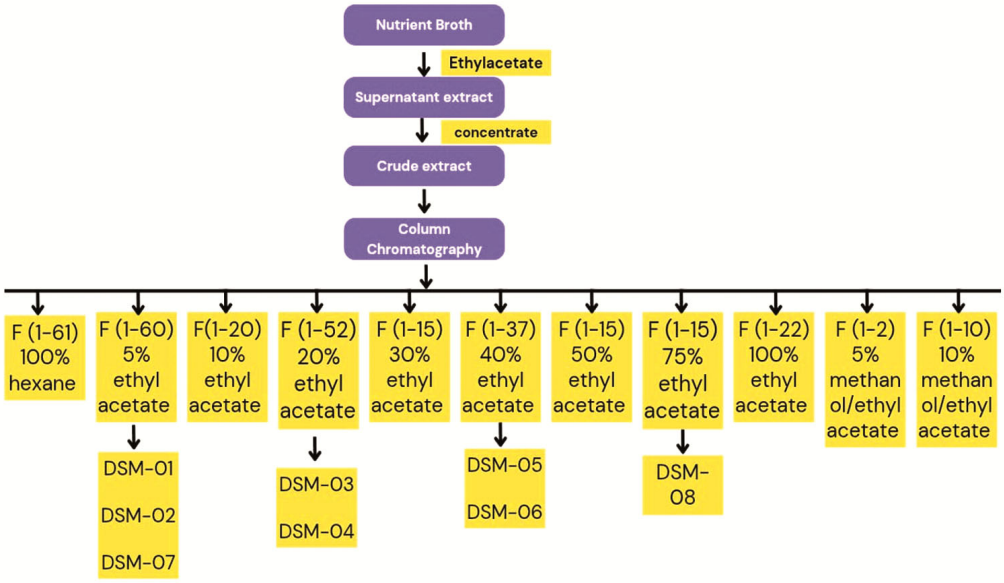


Fig. 3 — Workflow for bioactive compound extraction and fractionation

MABA Assay Results

For standard rifampicin, complete inhibition of bacterial growth is observed at 25, 12.5, 6.25, and 3.125 µg/mL (dark wells). Growth appears at 1.56 and 0.78 µg/mL (pink wells), indicating the MIC is 3.125 µg/mL. DSM-05: Complete inhibition occurs at 25 and 12.5 µg/mL (dark wells), while bacterial growth is evident from 6.25 µg/mL and lower (pink wells). The MIC for DSM-05 is 12.5 µg/mL (Fig. 4).

Structural characterisation of the isolated compound DSM-05

Compound DSM-05 is obtained as a white crystalline solid from fraction 11-14(40% ethyl acetate in hexane). Its molecular formula was found to be C11H18N2O3 using HRMS, Fig.S4. The compound's IR spectrum has shown characteristic signals for the presence of aliphatic CH3 at 3048-3032 cm^{-1}, CH2 at 2977-2953 cm^{-1}, amide -NH at 3468 cm^{-1}, and hydroxy (-OH) at 3554 cm^{-1} functional groups[32]. The ^{1}H NMR (400MHz, $CDCl_3$, Fig. S5, and ^{13}C NMR (100MHz, $CDCl_3$, Fig. S6 spectrum of DSM-05d has shown diagnostic signals for diketopiperazine structure at δ 4.54 (1Hdd, 6.4) and 4.10 (1Hdd, 2.8) for methine protons on alpha carbon of peptide unit and at δ166.15 and 170.23 for the dipeptide carbonyls indicating the presence of a diketopiperazine ring in DSM-05 (Ref. 33). Further analysis of the proton NMR, ^{1}H-^{1}H COSY Fig. S7, and NOESY Fig. S11 confirmed that the compound DSM-05 is a diketopiperazine formed from hydroxyproline and leucine (Fig. 5 and Fig. 6), a compound earlier reported from *Bacillus licheniformis* LB 8CT (Ref. 34) and *Nocardiopsis* sp. HT88 (Ref. 33). Figs S4-S11 show Spectral data is in complete agreement with the literature confirming the structure of DSM-05 as 7-Hydroxy-3-isobutylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione)), reported first for the time from *Micrococcus luteus*.

Antimicrobial activity and anti-tubercular activity

The antibacterial activity of compound DSM-05 was evident against the tested bacterial strains. At a 100 µg/mL concentration, none of the isolated compounds exhibited appreciable antifungal activity. The isolated compounds demonstrated significant antimicrobial activity, particularly against *Staphylococcus aureus* and *Escherichia coli*, with minimum inhibitory concentration (MIC) values ranging from 20 µg/mL to 1.56 µg/mL. Exhibited antitubercular activity, achieving a MIC µg/mL against *Mycobacterium tuberculosis* H37Rv. This activity is comparable to standard antitubercular drugs such as Rifampicin, highlighting its therapeutic potential.

Compound DSM-05: IR (KBr): 3400, 1685, 1630, 1450, 1250 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ cyclo(*cis*-Hyp-Leu) NH group at 5.82 (1H, s,4-NH), 4.62 (1H, s, H-4), 4.10 (1H, dd, $J = 2.8$, H-2'), 4.54(1H,m, H-2),3.77(1H, dd, $J = 4.4$, H-5), 2.44(1H, t, H-3), 1.79 and 1.58 (1H, m, H-3'), 1.63 (2H, t, $J = 6.9$ Hz, H-4'), 0.97 (3H, d, $J = 6.4$ Hz, H-5') and

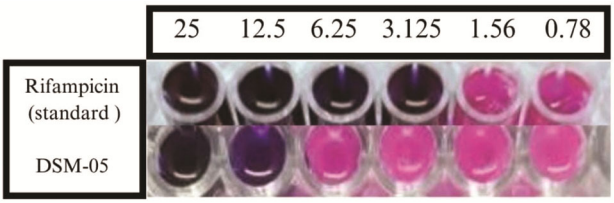


Fig. 4 — Anti-TB activity of strains

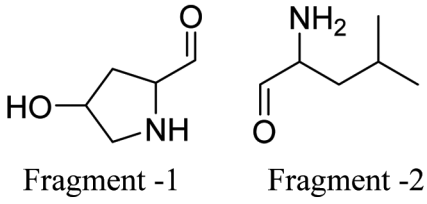
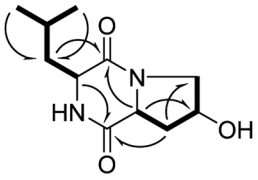


Fig. 5 — Fragment-1 and Fragment-2



(3*S*,7*S*,8a*S*)-7-hydroxy-3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione

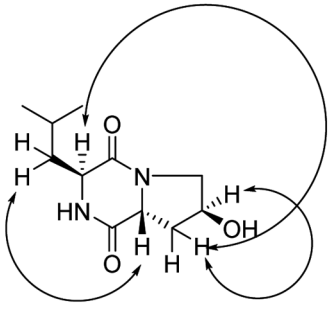


Fig. 6 — Key 1H-1H COSY (bold), NOESY (double-headed arrow)

1.04 (3H, d, J = 6.4, Hz, H-6'); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.23 (C-1), 166.15(C-2'), 68.57 (C-4), 57.32, 54.37 (C-5), 53.41(C-2'), 38.63(C-3'), 37.53 (C-3), 24.77 (C-4'), 23.26(C-6') and 21.21 (C-5'); HREIMS: *m/z* 227.1396 (M+1). Calcd for $C_{11}H_{18}N_{20}$: 226.1318.

Conclusion

This is the first report of *Micrococcus luteus's isolation from the Datura stramonium L* seeds. Bioactivity-guided screening resulted in the isolation of an antitubercular diketopiperazine (7-hydroxy-3-isobutylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione) from the fermented media of *Micrococcus luteus*. This study further affirmed endophytes' potential as a source of bioactive secondary metabolites.

Acknowledgments

The authors acknowledge the National Collection of Industrial Microorganisms, Pune, India, for supporting molecular characterisation efforts. The authors also thank the Director of the NMR Research Center at Andhra University in Visakhapatnam for providing the spectral data.

Supplementary Information

Supplementary information is available in the website http://nopr.niscpr.res.in/handle/123456789/58776. The supplementary information includes data on the isolation of endophytic bacterial colonies, purification processes, biochemical tests, 16S rRNA sequencing, HRMS, and NMR spectra.

Disclosure statement

The authors report no potential conflict of interest.

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