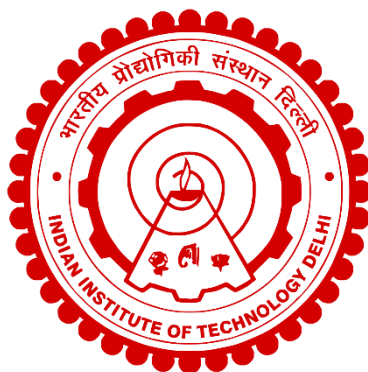


**EXPLORING THE POTENTIAL OF PHYTO-  
MOLECULES FROM INDIAN MEDICINAL PLANTS TO  
SUPPRESS THE MULTIDRUG RESISTANCE IN  
BACTERIA TARGETING THE B-LACTAMASE ENZYME**

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INDIAN INSTITUTE OF TECHNOLOGY DELHI  
MAY 2025**

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**Exploring the potential of phyto-molecules from Indian medicinal plants to suppress the multidrug resistance in bacteria targeting the  $\beta$ -lactamase enzyme**

**by**

**Leena**

**Centre for Rural Development and Technology**

**Submitted**

**In fulfilment of the requirements of the degree of Doctor of Philosophy**

**to the**



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**MAY 2025**



*Dedicated to my family*

## **Certificate**

This is to certify that the thesis entitled "**Exploring the potential of phyto-molecules from Indian medicinal plants to suppress the multidrug resistance in bacteria targeting the  $\beta$ -lactamase enzyme**" being submitted by **Ms. Leena** to the Indian Institute of Technology Delhi for the award of "**Doctor of Philosophy**" is a record of bonafide research work carried out by her. She has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis. To the best of my knowledge, the results contained in this thesis have not been submitted in part or full to any other university or institute for the award of any degree or diploma.

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## Abstract

From decades, antibiotics has been identified as powerful source for the treatment of bacterial disease. However, their effectiveness reduced over time due to the development of antibiotic resistance in bacteria. Further, their non-judicial usage impacted the environment negatively emphasizing the need of novel ecofriendly way of treating bacterial infections. Among the various mechanism through which bacteria resist antibiotics,  $\beta$ -lactamase is considered a key factor because of its wide distribution and high variability.  $\beta$ -lactamase production mainly responsible for drug failure against bacteria, it weakens  $\beta$ -lactam antibiotics which responsible for inhibiting bacterial cell wall synthesis. Centre for Disease Control and Prevention (CDC) and World Health Organization (WHO) also reported that humans are moving to post antibiotic era and unsafe in this condition. The need of the hour is to break this antibiotic resistance in bacteria to restore the activity of existing antibiotics or inventing newer generation of antibiotics. The aim of this study is to find a  $\beta$ -lactamase inhibitors from medicinal plants.

In Objective I, secondary metabolites from thirty plant samples were extracted in six different solvents, choose based on their increasing polarity. The extracts were concentrated, and  $\beta$ -lactamase inhibition assay was performed using chromogenic method, where nitrocefin used as substrate. Total seven extracts belong to four plants were showing >50% inhibition at 10-300  $\mu\text{g/ml}$ . The  $\beta$ -lactam potentiating activity of selected extracts were examined using checkerboard method against three multi drug resistance bacterial strains (*Bacillus cereus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*) which were showing synergistic and additive effects. Additionally, all seven extracts were showing good antioxidant activity, evaluated using the assay like Total Antioxidant Activity (TAA) and Ferric reducing antioxidant power (FRAP) Assay.

In Objective II, total seven extracts showing lowest  $\text{IC}_{50}$  values and effective antibiotic potentiation were selected for further studies. The inhibition kinetics studies revealed uncompetitive type of inhibition in all extracts. Metabolic profiling through High Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS) analysis identified 77 metabolites in all these extracts which dominated by polyphenolics and lipids compounds. Chemometric analysis correlating the all metabolites with  $\beta$ -lactamase inhibitory activity revealed that Partial Least Square Discriminant Analyses (PLS-DA) clearly segregated the extract according to the correlation between concentration and activity compared to Principal Component Analysis (PCA) analysis. Variable independent projection (VIP) score analysis revealed that metabolites responsible for segregation of

extracts, among which 2-Hexylbenzothiazole, Luteolin 7-O-glucuronide, and Veranisatin C was found to be higher in *Syzygium cumini* indicate their positive involvement in separation of extracts. Person correlation analysis between metabolite abundance and IC<sub>50</sub> value revealed that compounds like (+)-Galocatechin, CDP-ethanolamine, Senampeline A, 3-Methylellagic acid 8-(2-acetyl rhamnoside), Cinnassiol A 19-glucoside, Gomisin B, Acutissimin A, Guajavin B, Veranisatin C, Luteolin 7-O-glucuronide, Nigakilactone B, and 2-Hexylbenzothiazole as a possible inhibitor of  $\beta$ -lactamase.

In Objective III, acetone extract of *Syzygium cumini* bark was selected for further purification and characterization due to its effective  $\beta$ -lactamase inhibition and antibiotic potentiating activity against multidrug-resistant bacteria. After extraction using a Soxhlet apparatus, the metabolites were separated using reverse-phase thin layer chromatography (RP-TLC) with a standardized mobile phase of water and acetonitrile (7:3). Combi-flash chromatography was then employed to fractionate the crude extract, and the active fractions were isolated. The separated fractions were analyzed for  $\beta$ -lactamase inhibitory activity, and further utilized using UV-VIS spectroscopy, high-performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS). LC-MS analysis identified major PSMs in the bioactive fraction, including 4-(2-Carboxyethyl)-2-methoxyphenyl  $\beta$ -D-glucopyranosiduronic acid, 4-( $\beta$ -D-Glucopyranosyloxy)-3,5-dimethoxybenzoic acid, 9,12-Octadecadienal, Erucamide, Eschweilenol C, Maraniol and Methyl ricinoleate.

Objective IV was aimed to identify the potential of  $\beta$ -lactamase inhibitors obtained from Objective II and III using computational tools. The study was conducted using 12 compounds from correlation analysis (Objective II) with  $\beta$ -lactamase inhibition, 7 compounds from *Syzygium cumini* bark extract (Objective III), and 206 compounds from literature. The study retrieved 3D coordinates of  $\beta$ -lactamase enzymes (SHV1, TEM1, CTX-M, and KPC-2) and performed molecular dynamics (MD) simulations using the GROMACS suite. Ensemble docking, consists multiple receptor conformations, were performed to shortlist the compounds for MD Simulation. Docking against enzyme catalytic residues revealed binding energies from 58.99 to -6.74 kcal/mol. Twelve compounds were shortlisted for simulation analysis, revealing structural and functional transitions during 100 ns simulations. MMPBSA analysis of the last 10 ns indicated that 1-Galloyl glucose, Dicoumarin,  $\alpha$ -Dichroine, Senampeline, and 2-Hexylbenzothiazole had the lowest binding energies and can be potential  $\beta$ -lactamase inhibitors.

Overall, the present study revealed the potential of plant secondary metabolites (PSMs) from *Syzigium cumini* bark as  $\beta$ -lactamase inhibitors and  $\beta$ -lactam potentiators. More in vitro and in vivo studies may bring these PSMs into the line of drug development.

## शोध सारांश

दशकों से एंटीबायोटिक्स को बैक्टीरियल रोगों के इलाज के लिए एक शक्तिशाली साधन के रूप में पहचाना गया है। हालांकि, समय के साथ बैक्टीरिया में एंटीबायोटिक प्रतिरोध (रेज़िस्टेंस) विकसित हो जाने के कारण इनकी प्रभावशीलता कम हो गई है। इसके अलावा, इनका अनुचित उपयोग पर्यावरण पर भी नकारात्मक प्रभाव डालता है, जिससे बैक्टीरियल संक्रमण के उपचार के लिए एक नए, पर्यावरण-अनुकूल (इको-फ्रेंडली) तरीके की आवश्यकता महसूस की जा रही है। बैक्टीरिया द्वारा एंटीबायोटिक्स के प्रति प्रतिरोध विकसित करने के कई तरीकों में से,  $\beta$ -लैक्टामेज़ ( $\beta$ -lactamase) को एक प्रमुख कारक माना जाता है, क्योंकि यह व्यापक रूप से पाया जाता है और इसमें उच्च परिवर्तनशीलता होती है।  $\beta$ -लैक्टामेज़ का निर्माण मुख्य रूप से एंटीबायोटिक दवाओं की असफलता के लिए जिम्मेदार होता है, क्योंकि यह  $\beta$ -लैक्टाम एंटीबायोटिक्स को कमजोर कर देता है, जो बैक्टीरिया की सेल वॉल के निर्माण को रोकने के लिए जिम्मेदार होते हैं। सेंटर फॉर डिज़ीज़ कंट्रोल एंड प्रिवेंशन (CDC) और वर्ल्ड हेल्थ ऑर्गनाइज़ेशन (WHO) ने भी यह रिपोर्ट किया है कि मानवता एक "पोस्ट-एंटीबायोटिक युग" की ओर बढ़ रही है, जो अत्यंत असुरक्षित स्थिति है। इस स्थिति में सबसे ज़रूरी है कि हम बैक्टीरिया में विकसित हो चुके एंटीबायोटिक प्रतिरोध को तोड़ें, जिससे मौजूदा एंटीबायोटिक्स की कार्यक्षमता को फिर से बहाल किया जा सके या नई पीढ़ी की एंटीबायोटिक दवाएं विकसित की जा सकें।

उद्देश्य। के अंतर्गत, तीस पौधों के नमूनों से द्वितीयक चयापचयों (secondary metabolites) को छह विभिन्न घुलनशीलता वाले सॉल्वेंट्स में निकाला गया, जिन्हें उनकी बढ़ती ध्रुवीयता (polarity) के आधार पर चुना गया था। इन अर्कों को सघन (concentrate) किया गया और  $\beta$ -लैक्टामेज़ अवरोधन परीक्षण ( $\beta$ -lactamase inhibition assay) क्रोमोजेनिक विधि द्वारा किया गया, जिसमें नाइट्रोसेफिन (nitrocefin) को सब्सट्रेट के रूप में उपयोग किया गया। कुल सात अर्क, जो चार पौधों से संबंधित थे, ने 10–300  $\mu\text{g/ml}$  की सांद्रता पर 50% से अधिक अवरोधन दिखाया। चयनित अर्कों की  $\beta$ -लैक्टम को प्रभावी बनाने की क्रिया ( $\beta$ -lactam potentiating activity) को चेकरबोर्ड विधि द्वारा जांचा गया, जिसमें तीन मल्टी-ड्रग रेजिस्टेंट बैक्टीरियल स्ट्रेन्स — *Bacillus cereus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* — पर परीक्षण किया गया, जिसमें synergistic (सहक्रियात्मक) और additive (संयोजक) प्रभाव देखे गए। इसके अतिरिक्त, सभी सात अर्कों ने अच्छी एंटीऑक्सीडेंट गतिविधि भी प्रदर्शित की, जिसका मूल्यांकन कुल एंटीऑक्सीडेंट क्रियाशीलता (Total Antioxidant Activity - TAA) और फेरिक रिड्यूसिंग एंटीऑक्सीडेंट पावर (Ferric Reducing Antioxidant Power - FRAP) टेस्ट के माध्यम से किया गया।

उद्देश्य II के अंतर्गत, कुल सात ऐसे अर्कों का चयन किया गया जिन्होंने सबसे कम IC<sub>50</sub> मान और प्रभावशाली एंटीबायोटिक सहयोग (potentiation) दिखाया था, ताकि इन पर आगे के अध्ययन किए जा सकें। इनहिबिशन किनेटिक्स (Inhibition kinetics) अध्ययन से पता चला कि सभी अर्कों में "अनकम्पेटिटिव अवरोधन" (uncompetitive inhibition) प्रकार पाया गया। हाई रिज़ोल्यूशन लिक्विड क्रोमैटोग्राफी मास स्पेक्ट्रोमेट्री (HR-LCMS) के माध्यम से मेटाबोलिक प्रोफाइलिंग की गई, जिसमें कुल 77 मेटाबोलाइट्स की पहचान हुई। इन मेटाबोलाइट्स में पॉलीफिनोलिक यौगिक और लिपिड्स की प्रमुखता पाई गई। केमोमेट्रिक विश्लेषण (Chemometric analysis), जिसमें सभी मेटाबोलाइट्स को  $\beta$ -लैक्टामेज़ अवरोधन गतिविधि से जोड़ा गया, ने दिखाया कि Partial Least Square Discriminant Analysis (PLS-DA) ने अर्कों को उनके एकाग्रता और क्रियाशीलता के बीच संबंध के अनुसार स्पष्ट रूप से अलग किया, जो कि Principal Component Analysis (PCA) की तुलना में अधिक प्रभावी था। Variable Importance in Projection (VIP) स्कोर विश्लेषण ने उन मेटाबोलाइट्स की पहचान की जो अर्कों के विभाजन के लिए जिम्मेदार थे। इनमें 2-Hexylbenzothiazole, Luteolin 7-O-glucuronide, और Veranisatin C को *Syzygium cumini* में अधिक मात्रा में पाया गया, जो कि अर्कों के पृथक्करण में उनकी सकारात्मक भूमिका का संकेत देता है। पियरसन सहसंबंध विश्लेषण (Pearson correlation analysis), जो कि मेटाबोलाइट की प्रचुरता और IC<sub>50</sub> मान के बीच किया गया, से पता चला कि निम्नलिखित यौगिक संभवतः  $\beta$ -लैक्टामेज़ के अवरोधक (inhibitor) हो सकते हैं: (+)-Galocatechin, CDP-ethanolamine, Senampeline A, 3-Methylelagic acid 8-(2-acetyl rhamnoside), Cinnassiol A 19-glucoside, Gomisins B, Acutissimin A, Guajavin B, Veranisatin C, Luteolin 7-O-glucuronide, Nigakilactone B, and 2-Hexylbenzothiazole. इन सभी यौगिकों को  $\beta$ -लैक्टामेज़ अवरोधन में संभावित रूप से सक्रिय माना गया है।

उद्देश्य III के अंतर्गत, *Syzygium cumini* की छाल के एसीटोन अर्क को आगे की शुद्धिकरण और लक्षण निर्धारण (characterization) के लिए चुना गया, क्योंकि इसने मल्टी-ड्रग रेसिस्टेंट बैक्टीरिया के विरुद्ध प्रभावी  $\beta$ -लैक्टामेज़ अवरोधन और एंटीबायोटिक सहयोगी (potentiating) गतिविधि प्रदर्शित की थी। सॉक्सलेट उपकरण (Soxhlet apparatus) की सहायता से अर्क निकाले जाने के बाद, मेटाबोलाइट्स को रिवर्स फेज़ थिन लेयर क्रोमैटोग्राफी (RP-TLC) द्वारा अलग किया गया, जिसमें जल और एसीटोनीट्राइल (7:3) का मानकीकृत मोबाइल फेज़ उपयोग किया गया। इसके पश्चात कॉम्बी-प्लैश क्रोमैटोग्राफी के माध्यम से कच्चे अर्क को विभिन्न भागों में विभाजित किया गया, और सक्रिय अंशों को पृथक किया गया। पृथक किए गए अंशों की  $\beta$ -लैक्टामेज़ अवरोधन गतिविधि का परीक्षण किया गया, और इनका आगे विश्लेषण UV-VIS स्पेक्ट्रोस्कोपी,

हाई परफॉर्मेंस लिक्विड क्रोमैटोग्राफी (HPLC) और लिक्विड क्रोमैटोग्राफी-मास स्पेक्ट्रोमेट्री (LC-MS) द्वारा किया गया। LC-MS विश्लेषण में जैवसक्रिय अंश (bioactive fraction) में प्रमुख प्लांट स्पेशिफिक मेटाबोलाइट्स (PSMs) की पहचान की गई, जिनमें निम्नलिखित यौगिक शामिल थे: 4-(2-Carboxyethyl)-2-methoxyphenyl  $\beta$ -D-glucopyranosiduronic acid, 4-( $\beta$ -D-Glucopyranosyloxy)-3,5-dimethoxybenzoic acid, 9,12-Octadecadienal, Erucamide, Eschweilenol C, Maraniol and Methyl ricinoleate. इन यौगिकों की उपस्थिति से यह संकेत मिलता है कि वे  $\beta$ -लैक्टामेज़ अवरोधन और एंटीबायोटिक सहक्रियाशीलता में सक्रिय भूमिका निभा सकते हैं।

उद्देश्य IV का उद्देश्य था कि उद्देश्य II और III में प्राप्त  $\beta$ -लैक्टामेज़ इनहिबिटर्स की कम्प्यूटेशनल टूल्स के माध्यम से संभावनाओं की पहचान की जाए। यह अध्ययन कुल 12 यौगिकों (जो उद्देश्य II के सहसंबंध विश्लेषण से प्राप्त हुए थे), *Syzygium cumini* की छाल के अर्क से प्राप्त 7 यौगिकों (उद्देश्य III), और साहित्य से लिए गए 206 यौगिकों पर आधारित था। अध्ययन के लिए  $\beta$ -लैक्टामेज़ एंजाइम्स — SHV1, TEM1, CTX-M, और KPC-2 — के 3D समन्वय (coordinates) को प्राप्त किया गया और GROMACS सॉफ्टवेयर सूट का उपयोग करते हुए मॉलिक्यूलर डायनामिक्स (MD) सिमुलेशन किया गया। एन्सेम्बल डॉकिंग (ensemble docking), जिसमें एंजाइम की कई संरचनाओं (receptor conformations) को शामिल किया गया, को लागू करके यौगिकों को MD सिमुलेशन के लिए शॉर्टलिस्ट किया गया। एंजाइम की catalytic residues के विरुद्ध डॉकिंग करने पर बाइंडिंग एनर्जी 58.99 से  $-6.74$  kcal/mol के बीच पाई गई। बारह यौगिकों को सिमुलेशन विश्लेषण के लिए चुना गया, जिससे 100 ns सिमुलेशन के दौरान उनकी संरचनात्मक और कार्यात्मक गतिशीलता (transitions) का खुलासा हुआ। अंतिम 10 ns के MMPBSA विश्लेषण से यह निष्कर्ष निकला कि निम्नलिखित यौगिकों की बाइंडिंग एनर्जी सबसे कम थी और ये संभावित  $\beta$ -लैक्टामेज़ अवरोधक (inhibitors) हो सकते हैं: 1-गैलॉयल ग्लूकोज, डिकौमरिन,  $\alpha$ -डाईक्रोइन, सेनाम्पेलीन और 2-हेक्साइलबेंज़ोथियाज़ोल।

यह अध्ययन *Syzygium cumini* की छाल के पौधों के द्वितीयक मेटाबोलाइट्स (PSMs) को  $\beta$ -लैक्टामेज़ अवरोधक और एंटीबायोटिक सहयोगी के रूप में सामने लाता है। इन विट्रो और इन विवो अध्ययन इन PSMs को औषधि विकास की दिशा में ले जा सकते हैं।

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## Abbreviations

PSMs	Plant secondary metabolites
IC <sub>50</sub>	Half-maximal inhibitory concentration
MIC	Minimum inhibitory concentration
FIC	Fractional inhibitory concentration
K <sub>m</sub>	Michaelis-Menten constant
V <sub>max</sub>	Maximum velocity
μg	Microgram
ml	Microliter
Kcal	Kilocalorie
KJ	Kilo joule
PCA	Principal Component Analysis
PLS-DA	Partial Least Square Discriminant Analyses
VIP	Variable importance in the projection
HPLC	High performance liquid chromatography
HR-LCMS	High resolution liquid chromatography-mass spectrometry
RP-TLC	Reverse phase-thin layer chromatography
BE	Binding energy
MD	Molecular dynamic
SDF	Structure Data File
PDB	Protein databank file
2D	2 dimension
3D	3 dimension
ns	nano second
nm	nano meter
NVT	Constant Number of atoms, Volume, and Temperature
NPT	Constant Number of atoms, Pressure, and Temperature
VWE	Van der Waals energy
EE	Electrostatic energy
PSE	Polar solvation energy
RMSD	Root mean square deviation

RMSF	Root mean square fluctuation
Rg	Radius of gyration
SASA	Solvent accessible surface area
HBO	Hydrogen bond occupancy
MMPBSA	Molecular mechanics Poisson–Boltzmann surface area
ADME/T	Absorption Distribution Metabolism Excretion/Toxicity