STUDIES ON β-LACTAMASES AND THEIR INHIBITION USING BIOACTIVE PHYTOCHEMICALS TO OVERCOME "ANTIBIOTIC RESISTANCE"

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by

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DEPARTMENT OF CHEMISTRY

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

to the

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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Dedicated to my parents

CERTIFICATE

This is to certify that the thesis entitled ***"Studies on β-lactamases and their inhibition using Bioactive Phytochemicals to overcome "Antibiotic Resistance"*** being submitted by **Miss Pooja** to the Indian Institute of Technology Delhi for the award of the degree of ***Doctor of Philosophy*** in Chemistry is a record of Bonafide research work carried out by her. **Miss Pooja** has worked under my guidance and supervision and has fulfilled the requirements for the thesis submission, which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

Date: 21st July 2025

Place: New Delhi

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Pooja

New Delhi

Abstract

The rapid emergence and global dissemination of β-lactamase producing bacterial strains have significantly undermined the clinical utility of β-lactam antibiotics, which constitute one of the most widely used classes of antimicrobial agents. These enzymes hydrolyze the β-lactam ring, rendering antibiotics ineffective and leading to increased morbidity, mortality, and healthcare costs. The growing resistance crisis underscores the urgent need for alternative therapeutic strategies, including the identification of novel β-lactamase inhibitors that can restore antibiotic efficacy. In this context, plant-derived bioactive metabolites offer a valuable and underexplored reservoir of chemical diversity with potential antimicrobial and enzyme inhibitory properties. Combining traditional ethnobotanical knowledge with modern scientific tools allows for the systematic exploration of phytochemicals as prospective β-lactamase inhibitors. Furthermore, integrating *in vitro* screening with *in silico* approaches such as molecular docking, pharmacokinetic modeling, and molecular dynamics simulations enhances the rational identification and characterization of plant-based compounds with drug-like potential. This multidisciplinary approach is crucial for advancing the development of novel, plant-derived β-lactamase inhibitors as complementary or synergistic agents to existing antibiotic therapies.

The rising resistance to β-lactam antibiotics due to β-lactamase production by pathogenic bacteria necessitates the search for novel inhibitors. This study investigates the β-lactamase inhibitory potential of seven traditionally used medicinal plants, *Berberis aristata*, *Solanum nigrum*, *Curcuma amoda Roxb*., *Momordica charantia*, *Azadirachta indica*, *Punica granatum*, and *Camellia sinensis*. Different plant parts were collected (seeds, fruit, leaves and bark), dried, and extracted sequentially using solvents of increasing polarity (hexane < chloroform < ethyl acetate < acetone < methanol < water). A total of 60 extracts were screened for inhibitory activity against purified β-lactamase using nitrocefin-based spectrophotometric

assays. Among the screened extracts, the methanolic and acetone extracts of *Azadirachta indica* inner and outer bark and *Punica granatum* peel exhibited significant β-lactamase inhibition. The outer bark extracts of *A. indica* showed the highest inhibition of 98% and 94% with IC_{50} values of 2.1 μg/ml and 4.6 μg/ml for methanol and acetone extracts, respectively. In contrast, *P. granatum* peel extracts demonstrated moderate inhibition of 72% and 67% with higher IC_{50} values of 1140 μg/ml and 1200 μg/ml. Enzyme kinetic studies revealed an uncompetitive inhibition mode for the outer bark extracts of *A. indica*, while inner bark and *P. granatum* peel extracts exhibited non-competitive inhibition. These findings underscore the potential of phytochemicals from *Azadirachta indica* and *Punica granatum* as promising β-lactamase inhibitors, offering a natural alternative for combating antibiotic resistance.

The emergence of β-lactamase producing pathogens poses a critical challenge to β-lactam antibiotics. The present study evaluates the synergistic potential of selected medicinal plant extracts in combination with ampicillin to enhance antibacterial efficacy against β-lactamase producing bacteria. Six previously identified plant extracts exhibiting β-lactamase inhibitory activity were evaluated for synergistic interactions with ampicillin against *Bacillus tropicus* and *Bacillus subtilis*. A checkerboard assay was employed in 96-well microtiter plates, and bacterial growth inhibition was monitored in presence of different combinations of selected plant extracts and ampicillin via OD600 measurements over 24 hours, followed by resazurin based viability assessment. The fractional inhibitory concentration (FIC) was calculated to quantify synergy. Synergistic mapping and dose-response profiles were generated using the Highest Single Agent (HSA) model via the Combenefit tool. Methanolic and acetone extracts of *Azadirachta indica* inner and outer bark, as well as *Punica granatum* peel, demonstrated significant synergy with ampicillin. Among these, acetone and methanolic extracts of *A. indica* outer bark exhibited the strongest synergistic effects showing FIC < 0.5, and additive effects were observed with its acetone extract. This study underscores the promise of plant-derived

extracts, especially from *Azadirachta indica*, as adjuvants to conventional antibiotics, offering a novel strategy to combat β-lactamase mediated resistance.

This research focuses on the in-silico characterization of bioactive compounds from *Azadirachta indica* outer bark extracts to identify potential β-lactamase inhibitors. Methanolic extract of *Azadirachta indica* outer bark was subjected to GC-MS analyses, leading to the identification of 62 metabolites. These compounds were further docked against Class A PDB ID: 6SP6, Class C PDB ID: 4WYY, and Class D PDB ID: 5L2F, β-lactamase enzymes using AutoDock Vina. Twelve metabolites exhibited strong binding affinities across all β-lactamase classes, showed binding energy < –7.5 kcal/mol. Nimbiol, nimbinone, nimbionone, and sugiol showed the highest inhibitory potential against all three different β-lactamase enzymes. The pharmacokinetic profiling of shortlisted four compounds was done using SwissADME,

The top metabolites were further analyzed through Density Functional Theory (DFT) using Gaussian 09 (B3LYP/6-31+G(d,p)) to investigate their electronic properties. HOMO–LUMO energy gaps ranged between 4.5–6.2 eV, indicating chemical stability and favorable reactivity. Electrostatic potential (ESP) mapping revealed the donor and acceptor regions essential for protein–ligand interaction. Molecular Dynamics (MD) simulations were performed using GROMACS 2019 to validate docking results and analyse the dynamic stability of ligand–enzyme complexes over 100 ns. The complexes demonstrated consistent RMSD, low RMSF, and stable radius of gyration (Rg) values, indicating robust binding and minimal conformational fluctuations. Sustained hydrogen bonding throughout the simulations further confirmed strong and stable interactions.

These findings highlight the potential of *Azadirachta indica* derived phytochemicals as novel inhibitors targeting diverse classes of β-lactamase enzymes, offering promising leads for the development of next-generation antibacterial agents.

सार

बीटा-लैक्टामेज उत्पादक जीवाणु उपभेदों के तेजी से उभरने और वैश्विक प्रसार ने बीटा-लैक्टम एंटीबायोटिक दवाओं की नैदानिक उपयोगिता को काफी हद तक कम कर दिया है, जो रोगाणुरोधी एजेंटों के सबसे व्यापक रूप से इस्तेमाल किए जाने वाले वर्गों में से एक है। ये एंजाइम बीटा-लैक्टम रिंग को हाइड्रोलाइज करते हैं, जिससे एंटीबायोटिक अप्रभावी हो जाते हैं और रुग्णता, मृत्यु दर और स्वास्थ्य सेवा लागत में वृद्धि होती है। बढ़ते प्रतिरोध संकट वैकल्पिक चिकित्सीय रणनीतियों की तत्काल आवश्यकता को रेखांकित करता है, जिसमें एंटीबायोटिक प्रभावकारिता को बहाल करने वाले नए बीटा-लैक्टामेज अवरोधकों की पहचान शामिल है। इस संदर्भ में, पौधे से प्राप्त बायोएक्टिव मेटाबोलाइट्स संभावित रोगाणुरोधी और एंजाइम अवरोधक गुणों के साथ रासायनिक विविधता का एक मूल्यवान और कम खोजा गया भंडार प्रदान करते हैं। पारंपरिक नृवंशविज्ञान संबंधी ज्ञान को आधुनिक वैज्ञानिक उपकरणों के साथ मिलाने से संभावित बीटा-लैक्टामेज अवरोधकों के रूप में फाइटोकेमिकल्स की व्यवस्थित खोज की अनुमति मिलती है। इसके अलावा, आणविक डॉकिंग, फार्माकोकाइनेटिक मॉडलिंग और आणविक गतिशीलता सिमुलेशन जैसे इन सिलिको तरीकों के साथ इन विट्रो स्क्रीनिंग को एकीकृत करने से दवा जैसी क्षमता वाले पौधे-आधारित यौगिकों की तर्कसंगत पहचान और लक्षण वर्णन में वृद्धि होती है। यह बहु-विषयक दृष्टिकोण मौजूदा एंटीबायोटिक उपचारों के पूरक या सहक्रियात्मक एजेंट के रूप में उपन्यास, पौधे-व्युत्पन्न β-लैक्टामेज अवरोधकों के विकास को आगे बढ़ाने के लिए महत्वपूर्ण है। रोगजनक बैक्टीरिया द्वारा β-लैक्टामेज उत्पादन के कारण β-लैक्टम एंटीबायोटिक दवाओं के लिए बढ़ते प्रतिरोध ने नए अवरोधकों की खोज को आवश्यक बना दिया है। यह अध्ययन सात पारंपरिक रूप से उपयोग किए जाने वाले औषधीय पौधों, बर्बेरिस एरिस्टाटा, सोलनम निग्रम, करकुमा अमोडा रोक्सब, मोमोर्डिका चारेंटिया, एज़ाडिरेक्टा इंडिका, पुनिका ग्रेनेटम और कैमेलिया साइनेंसिस की β-लैक्टामेज निरोधक क्षमता

की जांच करता है। पौधों के विभिन्न भागों (छाल, बीज, फल, पत्ते और छाल) को एकत्र किया गया, सुखाया गया, और बढ़ते ध्रुवता (हेक्सेन < क्लोरोफॉर्म < एथिल एसीटेट < एसीटोन < मेथनॉल < पानी) के विलायकों का उपयोग करके क्रमिक रूप से निकाला गया। नाइट्रोसेफ़िन-आधारित स्पेक्ट्रोफोटोमेट्रिक परख का उपयोग करके शुद्ध β-लैक्टामेस के विरुद्ध निरोधात्मक गतिविधि के लिए कुल 60 अर्क की जांच की गई। जांचे गए अर्क में, एज़ाडिराच्टा इंडिका की आंतरिक और बाहरी छाल और पुनिका ग्रैनेटम के छिलके के मेथनॉलिक और एसीटोन अर्क ने महत्वपूर्ण β-लैक्टामेस अवरोध प्रदर्शित किया। ए. इंडिका की बाहरी छाल के अर्क ने क्रमशः 2.1 μg/ml और मेथनॉल और एसीटोन अर्क के लिए 4.6 μg/ml के IC_{50} मानों के साथ 98% और 94% का उच्चतम अवरोध दिखाया। इसके विपरीत, पी. ग्रेनेटम छिलके के अर्क ने 1140 μg/ml और 1200 μg/ml के उच्च IC_{50} मानों के साथ 72% और 67% का मध्यम अवरोध प्रदर्शित किया। एंजाइम गतिज अध्ययनों ने ए. इंडिका की बाहरी छाल के अर्क के लिए एक गैर-प्रतिस्पर्धी अवरोध मोड का खुलासा किया, जबकि आंतरिक छाल और पी. ग्रेनेटम छिलके के अर्क ने गैर-प्रतिस्पर्धी अवरोध प्रदर्शित किया। ये निष्कर्ष एज़ाडिरेक्टा इंडिका और पुनिका ग्रेनेटम के फाइटोकेमिकल्स की क्षमता को आशाजनक β-लैक्टामेज अवरोधकों के रूप में रेखांकित करते हैं, जो एंटीबायोटिक प्रतिरोध का मुकाबला करने के लिए एक प्राकृतिक विकल्प प्रदान करते हैं। β-लैक्टामेज उत्पादक रोगजनकों का उद्भव β-लैक्टम एंटीबायोटिक दवाओं के लिए एक गंभीर चुनौती है। छह पहले से पहचाने गए पौधों के अर्क जो β-लैक्टामेज निरोधात्मक गतिविधि प्रदर्शित करते हैं, का बैसिलस ट्रोपिकस और बैसिलस सबटिलिस के खिलाफ एम्पीसिलीन के साथ सहक्रियात्मक अंतःक्रियाओं के लिए मूल्यांकन किया गया। 96-वेल माइक्रोटिटर प्लेटों में एक चेकरबोर्ड परख का उपयोग किया गया था, और 24 घंटे से अधिक समय तक OD600 माप के माध्यम से चयनित पौधों के अर्क और एम्पीसिलीन के विभिन्न संयोजनों की उपस्थिति में जीवाणु वृद्धि अवरोध की निगरानी की गई थी, इसके बाद रेसाज़ुरिन आधारित व्यवहार्यता मूल्यांकन किया गया था। सहक्रिया को मापने के लिए आंशिक निरोधात्मक सांद्रता (FIC) की गणना की गई थी। कॉम्बिनिफ़िट टूल के माध्यम से उच्चतम एकल एजेंट (HSA) मॉडल का उपयोग करके सहक्रियात्मक

मानचित्रण और खुराक-प्रतिक्रिया प्रोफ़ाइल तैयार की गई थी। एज़ाडिरेक्टा इंडिका की आंतरिक और बाहरी छाल के मेथनॉलिक और एसीटोन अर्क, साथ ही पुनिका ग्रैनेटम छिलके ने एम्पीसिलीन के साथ महत्वपूर्ण सहक्रिया का प्रदर्शन किया। इनमें से, ए. इंडिका बाहरी छाल के एसीटोन और मेथनॉलिक अर्क ने सबसे मजबूत सहक्रियात्मक प्रभाव प्रदर्शित किया, जिसमें FIC < 0.5 दिखाया गया, और इसके एसीटोन अर्क के साथ योगात्मक प्रभाव देखे गए। यह अध्ययन पादप-व्युत्पन्न अर्क, विशेष रूप से अज़ादिराच्टा इंडिका से, पारंपरिक एंटीबायोटिक दवाओं के सहायक के रूप में, β-लैक्टामेज मध्यस्थ प्रतिरोध का मुकाबला करने के लिए एक नई रणनीति पेश करने की संभावना को रेखांकित करता है। यह अध्ययन संभावित β-लैक्टामेज अवरोधकों की पहचान करने के लिए अज़ादिराच्टा इंडिका बाहरी छाल के अर्क से जैव सक्रिय यौगिकों के इन-सिलिको लक्षण वर्णन पर केंद्रित है। अज़ादिराच्टा इंडिका बाहरी छाल के मेथनॉलिक अर्क का जीसी-एमएस विश्लेषण किया गया, जिससे 62 मेटाबोलाइट्स की पहचान हुई

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LIST OF ABBREVIATIONS

AMR	Antimicrobial Resistance
PBPs	Penicillin Binding Proteins
MBLs,	Metalloβ-lactamases
BLIs	β-lactamase inhibitors
MRSA	Methicillin resistance *Staphylococcus aureus*
ESBL	Extended-spectrum β-lactamase
CRE	Carbapenem-resistant Enterobacteriaceae
WHO	World Health Organization
CDC	Centres for Disease Control and Prevention
VRE	Vancomycin-resistant Enterococci
CRE	Carbapenem-resistant Enterobacteriaceae
D-alanyl-D-alanine	D-alanyl-D-alanine
KPC	*Klebsiella pneumoniae* carbapenemase
EGCG	Epigallocatechin Gallate
HTS	High-throughput screening
VS	Virtual Screening
MD	Molecular dynamics
QM	Quantum mechanics
SBDD	Structure based drug design

LBDD	Ligand-based drug design
QSAR	Quantitative structure-activity relationship
ADMET	Absorption, distribution, metabolism, excretion, and toxicity
FDA	Food and Drug Administration
MDR	Multi Drug Resistant
MIC	Minimum Inhibitory Concentration
FIC	Fractional inhibitory concentration
OD	Optical Density
GC-MS	Gas Chromatography-Mass Spectrometry
PDB	Protein data Bank
DFT	Density Functional Theory
PSMs	Plant Secondary Metabolites
NIST17	National Institute of Standards and Technology 2017 Mass Spectral Library
HOMO	Highest Occupied Molecular Orbital
LUMO	Lowest Unoccupied Molecular Orbital
GROMACS	GROningen MAchine for Chemical Simulations
ACPYPE	AnteChamber PYthon Parser interfacE

LINCS	Lennard-Jones and Coulomb interactions
PME	Particle Mesh Ewald
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
SASA	Solvent Accessible Surface Area
Rg	Radius of gyraion
TPSA	Topological polar surface area
GMP	Good Manufacturing Practice