EXPLORING THE POTENTIAL OF PLANT SECONDARY METABOLITES FOR THEIR ANTIBIOTIC POTENTIATING ACTIVITY AGAINST MULTI-DRUG RESISTANT BACTERIA

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by

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CERTIFICATE

This is to certify that the thesis entitled, **"Exploring the potential of plant secondary metabolites for their antibiotic potentiating activity against multi-drug resistant bacteria"**, being submitted by **Ms. Bhani Kongkham** 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 requirement for the submission of this thesis. To the best of my knowledge, the results obtained in this thesis has 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

Since time immemorial plant extracts have been used for their antibacterial properties. In recent time, the active compounds have been isolated and their activities are established. However, considering the diversity of the plant kingdom and their secondary metabolites, further research needs to be done to identify potential metabolites having direct antibacterial and antibiotic potentiation ability. The identification of antibiotic potentiators is important considering the rapid development of bacterial resistance against the antibiotics thereby depleting the antibiotic pipeline. To address this issue of bacterial resistance and to find antibiotic potentiating plant metabolites, the present study was carried out under four objectives.

High throughput method was employed for screening of 180 extracts in different solvents obtained from 30 plants. These extracts were screened for β-lactamase inhibition and β-lactam potentiation ability against the MDR bacterial isolates (*Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Bacillus cereus*). Among the extracts, acetone extract of *Ficus religiosa* and hexane extract of *Acorus calamus* was found to be the most effective in terms of β-lactamase inhibition and ampicillin potentiation.

The metabolites present in F. religiosa were identified using HR-LCMS. Further, to understand the binding of the metabolites with Class A β-lactamases (TEM 1, SHV1, KPC-2 and 6D17) molecular docking and MD simulation studies were performed. This revealed that taxifolin and miquelianin showed the best binding affinity with all the four class A β-lactamases. Hydrogen bond occupancy of >200 % was seen with Glu166, Glu240, Asn132, etc. which are located in the enzyme active site. Additionally, the ADMET profile of the metabolites showed their drug likeliness and non-toxic nature.

Fractionation of bioactive compounds from *A. calamus* rhizome hexane (AC-R-H) extract was performed and its MIC, FIC index and mode of action was determined. AC-R-H bioactive fraction was found to reduce the MIC of ampicillin against *E. coli* (100 mg/mL to 25 mg/mL), *P. aeruginosa* (15 mg/mL to 3.25 mg/mL), *A. baumannii* (12.5 mg/mL to 1.56 mg/ml), and *B. cereus* (10 mg/mL to 1.25 mg/mL). Further it recorded synergistic activity with ampicillin against *B. cereus* (FICI = 0.365), *P. aeruginosa* (FICI = 0.456), and *A. baumannii* (FICI = 0.364). This activity can be attributed to its ability to alter the fatty acid composition of the bacterial cell membranes, hinder the membrane integrity, permeability, and cause cell membrane damage. It also showed good antibiofilm activity against *B. cereus* and a moderate β-lactamase inhibition (IC_{50} = 6.2 mg/mL). Characterization of AC-R-H bioactive fraction through UV-Vis, FT-IR, and GC-MS revealed Asarone as the major compound present in the bioactive fraction.

Finally, PLGA based formulation was prepared by encapsulating the clarified crude of *A. calamus*. The encapsulated particles exhibited size (300-500 nm), PDI (0.3-0.4) and zeta potential (-25 to -15 mV). The loading capacity and encapsulation efficiency of the particles were (40 to 60 %) and *in vitro* release profile showed burst release with ~70 % of cumulative release in 24 h. The encapsulated particles showed antibacterial efficacy and ampicillin potentiation against *A. baumannii*. Further, the cell toxicity studies of clarified crude and encapsulated particles (20 µg/ml) on Vero cells showed ~20 % inhibition. Overall, the study identifies two plants *F. religiosa* and *A. calamus* and their metabolites as potential candidates for countering multidrug resistance in bacteria. Also, the development of PLGA based formulation encapsulating *A. calamus* clarified crude showcases the feasibility to develop biocompatible formulation with multiple compounds.

सार

प्राचीन काल से वनस्पतियों के अर्क/सत्त का उपयोग उनके एंटीबैक्टीरियल गुणों के लिए किया जाता रहा है। हाल के समय में, उन वनस्पतियों के अर्क/सत्त से सक्रिय यौगिकों को अलग कर उनकी कार्यलाप को समझने की कोशिश की गई है। हालाँकि, सृष्टि जगत के वनस्पतियों और उनके द्वितीयक मेटाबोलाइट्स की विविधता को ध्यान में रखते हुए, प्रत्यक्ष एंटीबैक्टीरियल और एंटीबायोटिक सामर्थ्य वाले संभावित मेटाबोलाइट्स की पहचान करने के लिए और अधिक शोध किए जाने की आवश्यकता है। इसके अतिरिक्त, बैक्टीरिया के प्रतिरोध क्षमता में तेजी से विकास के कारण असरदार एंटीबायोटिक दवाओं की उपलब्धता में तेजी से कमी आने को ध्यान में रखते हुए नए एंटीबायोटिक सामर्थ्य वाले संभावित मेटाबोलाइट्स की खोज महत्वपूर्ण है। इसलिए, बैक्टीरिया के बढ़ते प्रतिरोध क्षमता से उत्पन्न परिस्थिति के समाधान करने और नए एंटीबायोटिक सामर्थ्य वाले संभावित मेटाबोलाइट्स को खोजने के लिए, वर्तमान अध्ययन को चार उद्देश्यों के तहत किया गया।

30 पौधों से प्राप्त 180 अर्क/सत्त की विभिन्न विलायकों में उच्च थ्रूपुट विधि के द्वारा जाँच किया गया। एमडीआर बैक्टीरियल आइसोलेट्स (*एस्चेरिचिया कोली, स्यूडोमोनस एरुगिनोसा, एसिनेटोबैक्टर बाउमन्नी* और *बैसिलस सेरिअस*) के प्रति β-लैक्टामेज़ नियंत्रण और β-लैक्टम सामर्थ्यता के लिए इन अर्कों/सत्तों की जांच की गई। इनमें से, *फाइकस रिलिजियोसा* के एसीटोन अर्क और *एकोरस कैलामस* के हेक्सेन अर्क को β-लैक्टामेज़ नियंत्रण और एम्पीसिलीन सामर्थ्यता के मामले में सबसे प्रभावी पाया गया।

फाइकस रिलिजियोसा में मौजूद मेटाबोलाइट्स की पहचान एचआर-एलसीएमएस का उपयोग करके किया गया। इसके अलावा, श्रेणी ए β-लैक्टामेस (टीईएम-1, एसएचवी-1, केपीसी-2 और 6डी17) के साथ मेटाबोलाइट्स के आबंधन को समझने के लिए आणविक डॉकिंग और एमडी सिमुलेशन अध्ययन किए गए। इससे पता चला कि टैक्सीफोलिन और मिकेलियानिन के चार श्रेणी ए β-लैक्टामेस के साथ सबसे अच्छा आबंधन होता है। एंजाइम सक्रिय साइट में अवस्थित ग्लू-166, ग्लू-240, एएसएन-132, इत्यादि में 200% से भी अधिक हाइड्रोजन बांड अधिभोग देख गया। इसके अतिरिक्त, मेटाबोलाइट्स के एडीएमईटी प्रोफाइल से उनकी गैर-विषाक्त प्रकृति और दवाई बनाने की संभावनाओं का भी पता चला।

एकोरस कैलामस राइजोम हेक्सेन (एसी-आर-एच) अर्क से बायोएक्टिव यौगिकों का अंशांकन करने के बाद इसके एमआईसी, एफआईसी सूचकांक और क्रियान्वयन के तरीके का निर्धारित किया गया। एसी-आर-एच बायोएक्टिव अंश *एस्चेरिचिया कोली* (100 मिलीग्राम/एमएल से 25 मिलीग्राम/एमएल), *स्यूडोमोनस एरुगिनोसा* (15 मिलीग्राम/एमएल से 3.25 मिलीग्राम/एमएल),

एसिनेटोबैक्टर बाउमन्नी (12.5 मिलीग्राम/एमएल से 1.56 मिलीग्राम/एमएल), और *बैसिलस सेरिअस* (10 मिलीग्राम/एमएल से 1.25 मिलीग्राम/एमएल) के एम्पीसिलीन के एमआईसी को कम करने के लिए उपयुक्त पाया गया। इसके अतिरिक्त, *बैसिलस सेरिअस* (एफआईसीआई = 0.365), *स्यूडोमोनस एरुगिनोसा* (एफआईसीआई = 0.456), और *एसिनेटोबैक्टर बाउमन्नी* (एफआईसीआई = 0.364) के प्रति एसी-आर-एच अर्क का एम्पीसिलीन के साथ सहक्रियात्मक गतिविधि का भी पता चला। इस गतिविधि को बैक्टीरिया कोशिका झिल्ली की फैटी एसिड संरचना को बदलने, झिल्ली की संपूर्णता और पारगम्यता में बाधा डालने और कोशिका झिल्ली को नुकसान पहुंचाने की क्षमता से सम्बद्ध किया जा सकता है। एसी-आर-एच अर्क ने *बैसिलस सेरिअस* के प्रति अच्छी एंटीबायोफिल्म गतिविधि और मध्यम β-लैक्टामेज़ नियंत्रण (IC_{50} = 6.2 मिलीग्राम/एमएल) भी दिखाया। एसी-आर-एच बायोएक्टिव अंश के यूवी-विज़, एफटी-आईआर और जीसी-एमएस निरूपण के माध्यम से बायोएक्टिव अंश में मौजूद प्रमुख यौगिक असारोन का पता चला।

अंततः, *एकोरस कैलामस* के क्लारिफइड क्रूड को संपुटित करके पीएलजीए आधारित फॉर्मूलेशन तैयार किया गया। एनकैप्सुलेटेड कणों का आकार (300-500 एनएम), पीडीआई (0.3-0.4), जीटा क्षमता (-25 से -15 एमवी), लोडिंग क्षमता और एनकैप्सुलेशन दक्षता (40 से 60%) प्राप्त हुआ। इन-विट्रो रिलीज़ प्रोफ़ाइल से पता चला कि बर्स्ट रिलीज़ 24 घंटों में संचयी रिलीज़ का लगभग 70% होता है। एनकैप्सुलेटेड कणों में *एसिनेटोबैक्टर बाउमन्नी* के प्रति एंटीबैक्टीरियल गुण और एम्पीसिलीन सामर्थ्यता का पता चला। इसके अलावा, वेरो कोशिकाओं पर क्लारिफइड क्रूड और एनकैप्सुलेटेड कणों (20 माइक्रोग्राम/एमएल) के कोशिका विषाक्तता अध्ययन में लगभग 20% विनियंत्रण का पता चला। कुल मिलाकर, अध्ययन में बैक्टीरिया के मल्टीड्रग प्रतिरोध का मुकाबला करने के लिए संभावित दो पौधों *फाइकस रिलिजियोसा* और *एकोरस कैलामस* और उनके मेटाबोलाइट्स का पता चला। इसके अलावा, *एकोरस कैलामस* क्लारिफइड क्रूड को समाहित करने वाले पीएलजीए आधारित फॉर्मूलेशन का विकास कई यौगिकों के साथ जैव-संगत फॉर्मूलेशन विकसित करने की व्यवहार्यता को दर्शाता है।

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MRSA	Methicillin resistant *Staphylococcus aureus*
MIC	Minimum inhibitory concentration
FICI	Fractional inhibitory concentration index
MDR	Multi-drug resistant
PSM	Plant secondary metabolite
PBP	Penicillin Binding Protein
QS	Quorum Sensing
SAR	Structure Activity Relationship
EO	Essential Oil
WHO	World Health Organization
ADMET	Adsorption, Distribution, Metabolism, Excretion, Toxicity
ESBL	Extended Spectrum beta-lactamase
MD	Molecular dynamics
PDB	Protein Data Bank
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
SASA	Solvent Accessible Surface Area
Rg	Radius of Gyration
BE	Binding Energy
ABE	Average Binding Energy
HBO	Hydrogen Bond Occupancy
MMPBSA	Molecular Mechanics Poisson-Boltzmann Surface Area
AAR	Amino acid residue
BSA	Bioavailability score
TLC	Thin layer chromatography
AC-R-H	*Acorus calamus* rhizome hexane
OD	Optical Density
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
S-III-BAF	Stage three bioactive fraction
FTIR	Fourier Transform Infrared Spectroscopy
EtBr	Ethidium Bromide
SEM	Scanning Electron Microscopy
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
FAME	Fatty acid methyl ester
DMSO	Dimethyl sulfoxide
ANOVA	Analysis of variance
RFU	Relative Fluorescence Unit
PLGA	Poly (lactic-co-glycolic acid)
AC-H-CC	*Acorus calamus* hexane clarified crude
PVA	Poly vinyl alcohol
DCM	Dichloromethane
DI water	Deionized water
PDI	Polydispersity index
UV	Ultra-violet
HR-LCMS	High Resolution-Liquid Chromatography Mass Spectrometry

GC-MS	Gas Chromatography Mass Spectrometry
GC-FID	Gas Chromatography-Flame Ionization Detection
DLS	Dynamic Light Scattering