

**EXPLORING MEDICINAL PLANTS AND THEIR SECONDARY
METABOLITES AS AN INHIBITOR OF α - GLUCOSIDASE TARGETING TYPE-2
DIABETES MELLITUS**

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INDIAN INSTITUTE OF TECHNOLOGY DELHI
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by

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Submitted

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Certificate

This is to certify that the thesis entitled " **Exploring medicinal plants and their secondary metabolites as an inhibitor of α - glucosidase targeting Type-2 Diabetes Mellitus**" being submitted by **Ms Monu Dinesh Ojha** 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.

Prof. Hariprasad P.

Centre for Rural Development and Technology

Indian Institute of Technology Delhi

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ABSTRACT

Diabetes mellitus (DM) is a multifactorial metabolic disorder characterised by abnormally elevated blood glucose levels (hyperglycemia). Diabetes Mellitus is caused by insufficient insulin synthesis or inappropriate insulin usage by the cells (American Diabetes Association). In 2017, more than 425 million people aged 20 to 79 had diabetes, including 72 million Indians; by 2045, this number is expected to rise to 629 million (IDF diabetes atlas - 2017). Hyperglycemia is associated with microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (cardiovascular, cerebrovascular, and peripheral artery disease) problems. In addition, hyperglycemic situations are reported to damage smaller and larger blood vessels. α -glucosidase inhibitors (α -GI) are used for treating Type 2-diabetes mellitus (T2-DM). They function by inhibiting the catalytic activity of α -glucosidase, slowing the glucose release rate from oligosaccharides. Thereby reducing the rate of glucose absorption in the small intestine. Thus, α -GIs are used primarily to treat postprandial hyperglycemia, are the first-line treatment for T2-DM and are recommended as first-line drugs for pre-diabetic conditions.

Natural products have been used since ancient times and are used by the current society as they are potential resources. With the future rise of the diabetic population and the limitations of the available drugs, it becomes a meaningful task to identify new phytochemicals with α -glucosidase inhibitory activity. Plants are a rich source of bioactive chemicals that display decisive pharmacological actions. α -GIs compounds are ubiquitously present in the plants. This study explored the potential of plants belonging to diverse families as α -GIs using a combinatorial approach.

Objective 1: The preliminary step was to establish a physical library of plant materials collected across different sites in India. In this objective, a high throughput screening of the plant extracts has been carried out to analyze their potential to inhibit α -glucosidase. This has

helped to validate the existing literature of various plants reported for α -glucosidase inhibition and find new plants with their potential to inhibit the enzyme. Among 420 crude extracts from 70 plants, 174 extracts had IC_{50} concentration of ≤ 3 mg/ml, which was dominated by the hexane extract. *Terminalia arjuna* – bark (Arjuna) ($IC_{50} \leq 0.001$ to 1.23 mg/ml), *Eucalyptus tereticornis* – leaves (forest red gum) (IC_{50} 0.001 to 2.18 mg/ml), *Artocarpus heterophyllus* – bark (jackfruit) (IC_{50} 0.005 to 1.15 mg/ml), *Curcuma longa* – rhizome bark (turmeric) ($IC_{50} \leq 0.001$ to 0.082 mg/ml), *Tribulus terrestris* – fruit (devil's weed) (IC_{50} 0.12 to 1.11 mg/ml), and *Cassia anugustifolia* - leaves (senna) (IC_{50} 0.045 to 3.06 mg/ml) were categorised based on the presence of α -glucosidase inhibitory plant secondary metabolite (PSM) in all six solvents. It suggested that these plants may have a broad range of PSM with different polarities that inhibit α -glucosidase at low concentrations in crude/purified form. For the plants like *Murraya koenigii*- leaves (kadi patta), *Swertia chirayita*- whole plant (chirayata), *Withania coagulans*- fruit (Punir dodi), *Dillenia indica*- fruit (elephant apple) only their hexane extracts showed IC_{50} values < 1 mg/ml. The others solvent extracts of these plants did not show any inhibition.

Objective 2: *In-silico* studies were conducted to understand the possible role of structurally diverse PSM in inhibiting α - glucosidase activity. A virtual PSM library of 2752 molecules from 50 shortlisted α -glucosidase inhibitor plants selected from the first objective was prepared. Further, they were subjected to molecular docking against α -glucosidase. The structural activity relationship studies revealed that among the top 100 molecules with the least binding energy, 34% belonged to sesquiterpenoids. Through ADMET and pkCSM studies, 539 molecules were found to have a drug-like properties. For the first time on in silico basis, this study reports the following molecules, Withanolide M, 17 β -hydroxyl withanolide K (17BHWK), Withanolide J, Coagulansins A and B, Withasomniferol C, Withacoagulin I, 1, 8-Dihydroxy-3-carboxy-9, 10-anthraquinone (DHCA), and Swerilactone C as a potential α -glucosidase inhibitors which can be studied further.

Objective 3: Based on the literature and our experimental data *Withania coagulans* was selected to isolate bioactive compound for α - glucosidase inhibition. Only the hexane extract (2% yield) showed α - glucosidase inhibitory properties with IC₅₀ of 0.013 mg/ml and Ki of 0.012 mg/ml. The purification strategy of the bioactive fraction was designed, and at the final purification stage, the bioactive fraction S-IV-F3 showed IC₅₀ of 0.004 mg/ml and Ki of 0.0037 mg/ml. The characterization and structure elucidation studies showed that the bioactive fraction consisted of olefinic fatty acids and fatty acid methyl esters. The NMR analysis also supports the presence of fatty acid-like compounds in the S-IV-F3.

Objective 4: *Withania coagulans* fruit extract has shown a better α - glucosidase inhibiting activity than the standard drug acarbose. At various stages of its purification, it has shown increasing activity, thus inferring that it has the potential to be used as an α -glucosidase inhibitor. So, it becomes essential to understand the additional benefits it may provide to individuals consuming it to manage DM. *Withania coagulans* bioactive fractions were analysed for their antioxidants, anti-microbial, and cytotoxic activities. Enzyme kinetics studies revealed the non-competitive inhibition nature of bioactive fraction against α - glucosidase. This may be mainly due to the type of compounds present in the extracts and the molecules binding to the allosteric site of the enzyme, thus reducing the affinity of the enzyme towards the substrate from interacting with the active site of the enzyme. Among the five bacterial strains tested *S. aureus* was found to be highly sensitive to S-I with IC₅₀ of 4 mg/ml, followed by *B. cereus* (8 mg/ml), *K. pneumoniae* (25 mg/ml), *P. aeruginosa* (25 mg/ml), and *E. coli* (32 mg/ml). However, S-I showed lower antioxidant activity. Consumption of *W. coagulans* extract may provide additional benefits/ safety apart from diabetes management. The purification strategy and the reported molecules in this study have shown an immense potential to reduce cytotoxicity and may be represented as a potential venture for drug molecules.

Objective 5: As the bioactive fraction (S-I and S-IV-F3) of *W. coagulans* predicted non-competitive inhibition under *in-vitro* conditions, evaluating the PSMs for their interaction with the enzyme under in-silico conditions becomes interesting. In this objective, a library of known PSMs was prepared through GC-MS analysis of WC bioactive fractions. These PSMs were subjected to blind ensemble docking with the enzyme to evaluate the allosteric binding affinity to different allosteric pockets of the enzyme. The molecular dynamics studies used the Enzyme-PSM binary structure and Enzyme-PSMs-Isomaltose ternary structure. The molecular dynamic simulation (MDS) studies of selected molecules showed that Methyl 12,13-tetradecadienoate (MTDD) was consistently found to bind the allosteric site at pocket 3 throughout the simulation period. Further, binding to the allosteric site of the enzyme, MTDD reduced the affinity of the enzyme towards isomaltose, predicted its non-competitive nature of enzyme inhibition.

सार

मधुमेह मेलेटस (DM) एक बहु-कारक चयापचय संबंधी विकार है जिसकी विशेषता रक्त शर्करा के असामान्य रूप से उच्च स्तर (अतिशर्करा) है। मधुमेह मेलेटस अपर्याप्त इंसुलिन संश्लेषण या कोशिकाओं द्वारा अनुचित इंसुलिन उपयोग के कारण होता है (अमेरिकन डायबिटीज एसोसिएशन)। 2017 में, 20 से 79 वर्ष की आयु के 425 मिलियन से अधिक लोगों को मधुमेह था, जिसमें 72 मिलियन भारतीय शामिल थे; 2045 तक, इस संख्या के 629 मिलियन तक पहुंचने की उम्मीद है (IDF मधुमेह एटलस - 2017)। हाइपरग्लाइकेमिया माइक्रोवस्कुलर (रेटिनोपैथी, नेफ्रोपैथी और न्यूरोपैथी) और मैक्रोवस्कुलर (हृदयवाहिकीय, मस्तिष्कवाहिकीय और परिधीय धमनी रोग) समस्याओं से जुड़ा है। इसके अलावा, हाइपरग्लाइकेमिक स्थितियों को छोटी और बड़ी रक्त वाहिकाओं को नुकसान पहुंचाने की सूचना है।

अल्फा-ग्लूकोसिडेज इनहिबिटर (α -GI) का उपयोग टाइप 2-मधुमेह मेलेटस (T2-DM) के इलाज के लिए किया जाता है। वे अल्फा-ग्लूकोसिडेज की उत्प्रेरक गतिविधि को रोककर काम करते हैं, जिससे ओलिगोसेकेराइड से ग्लूकोज रिलीज दर धीमी हो जाती है। इस प्रकार छोटी आंत में ग्लूकोज अवशोषण की दर को कम करता है। इस प्रकार, α -GIs मुख्य रूप से पोस्टप्रीडियल हाइपरग्लाइकेमिया के इलाज के लिए उपयोग किए जाते हैं, T2-DM के लिए प्रथम-पंक्ति उपचार हैं और प्री-डायबिटिक स्थितियों के लिए प्रथम-लाइन दवाओं के रूप में अनुशंसित हैं।

प्राकृतिक उत्पादों का उपयोग प्राचीन काल से किया जाता रहा है और वर्तमान समाज द्वारा किया जाता है क्योंकि वे संभावित संसाधन हैं। मधुमेह रोगियों की भविष्य की वृद्धि और उपलब्ध दवाओं की सीमाओं के साथ, अल्फा-ग्लूकोसिडेज निरोधक गतिविधि वाले नए फाइटोमोलेक्यूल्स की पहचान करना एक सार्थक कार्य बन जाता है। पौधे जैव सक्रिय रसायनों का एक समृद्ध स्रोत हैं जो निर्णायक औषधीय क्रिया प्रदर्शित करते हैं। α -GI यौगिक सर्वव्यापी रूप से पौधों में मौजूद होते हैं। इस अध्ययन में विभिन्न परिवारों से संबंधित पौधों की क्षमता को अल्फा-जीआई के रूप में एक संयुक्त दृष्टिकोण का उपयोग करके खोजा गया।

उद्देश्य 1 : पौधों से अल्फा-ग्लूकोसिडेज निरोधक की पहचान पहला चरण भारत के विभिन्न स्थानों से एकत्र किए गए पौधों की सामग्री का एक भौतिक पुस्तकालय स्थापित करना था। इस उद्देश्य में, अल्फा-ग्लूकोसिडेज को रोकने की उनकी क्षमता का विश्लेषण करने के लिए पौधों के अर्क की उच्च थ्रूपुट जांच की गई। इससे विभिन्न पौधों के बारे में मौजूदा शोध को मान्य करने में मदद मिली जिन्हें पहले से ही अल्फा-ग्लूकोसिडेज निषेध के लिए रिपोर्ट किया गया है और एंजाइम को बाधित करने की उनकी क्षमता वाले नए पौधों को खोजने में मदद मिली। 70 पौधों से लिए गए 420 कच्चे अर्क में से, 174 अर्क में IC_{50} सांद्रता ≤ 3 मिलीग्राम/एमएल थी, जो मुख्य रूप से हेक्सेन निकालने पर हावी थी। टर्मिनलिया अर्जुन (छाल) ($IC_{50} \leq 0.001$ से 1.23 मिलीग्राम/एमएल), यूकेलिप्टस टेरेंटिकोर्निस (पत्तियां) (IC_{50} 0.001 से

2.18 मिलीग्राम/एमएल), अर्तोकार्पस हेटेरोफिलस (छाल) (IC_{50} 0.005 से 1.15 मिलीग्राम/एमएल), कर्क्यूमा लोंगा (प्रकंद छाल) (हल्दी) ($IC_{50} \leq 0.001$ से 0.082 मिलीग्राम/एमएल), ट्रिब्युलस टेरेस्ट्रिस (फल) (IC_{50} 0.12 से 1.11 मिलीग्राम/एमएल), और कैसिया अनुगुस्तिफोलिया (पत्तियां) (IC_{50} 0.045 से 3.06 मिलीग्राम/एमएल) को सभी छह विलायकों में अल्फा-ग्लूकोसिडेज़ निरोधक पौधों के द्वितीयक चयापचयों की उपस्थिति के आधार पर वर्गीकृत किया गया था। इससे यह सुझाव दिया गया कि इन पौधों में विभिन्न ध्रुवीयताओं के साथ PSM की एक विस्तृत श्रृंखला हो सकती है जो कच्चे/शुद्ध रूप में कम सांद्रता में अल्फा-ग्लूकोसिडेज़ को रोकती है। मुरैया कोएनिगी (पत्तियां), स्वर्टिया चिरयता (पूरा पौधा), विथानिया कोगुलन्स (फल), डिलीनिया इंडिका (फल) जैसे पौधों के केवल उनके हेक्सेन अर्क में IC_{50} मान < 1 मिलीग्राम/एमएल था। इन पौधों के अन्य विलायक अर्क ने कोई निषेध नहीं दिखाया।

उद्देश्य 2: इन-सिलिको अध्ययन अल्फा-ग्लूकोसिडेज़ गतिविधि को रोकने में संरचनात्मक रूप से विविध प्लांट सेकेंडरी मेटाबोलाइट्स (PSM) की संभावित भूमिका को समझने के लिए इन-सिलिको अध्ययन किए गए। पहले चरण से चुने गए 50 अल्फा-ग्लूकोसिडेज़ निरोधक पौधों से 2752 अणुओं का एक आभासी पुस्तकालय बनाया गया था। इसके बाद, इन अणुओं को अल्फा-ग्लूकोसिडेज़ एंजाइम के विरुद्ध आणविक डॉकिंग प्रक्रिया के अधीन किया गया। विश्लेषण से पता चला कि कम से कम बंधन ऊर्जा वाले शीर्ष 100 अणुओं में से 34% सेस्क्वीटेरपेनोइड्स (Sesquiterpenoids) वर्ग से संबंधित थे। ADMET और PKCSM अध्ययनों के माध्यम से, 539 अणुओं में दवा जैसी गुण पाए गए।

यह अध्ययन पहली बार इन-सिलिको आधार पर निम्नलिखित अणुओं को संभावित अल्फा-ग्लूकोसिडेज़ निरोधक के रूप में रिपोर्ट करता है, जिन्हें आगे के अध्ययन की आवश्यकता है: विथानोलाइड एम (Withanolide M), 17 β -हाइड्रॉक्सिल विथानोलाइड के (17BHWK), विथानोलाइड जे (Withanolide J), कोएगुलान्सिन ए और बी (Coagulansins A and B), विथसोमनिफेरोल सी (Withasomniferol C), विथाकोएगुलिन आई (Withacoagulin I), 1, 8-डायहाइड्रॉक्सी-3-कार्बोक्सी-9, 10-एंथ्राक्विनोन (DHCA), स्वेरिलैक्टोन सी (Swerylactone C)। ये खोजें भविष्य में मधुमेह उपचार के लिए नई दवाओं के विकास में मददगार हो सकती हैं।

उद्देश्य 3: विथानिया कोगुलन्स से अल्फा-ग्लूकोसिडेज़ निरोधक का पृथक्करण अध्ययन और हमारे प्रायोगिक आंकड़ों के आधार पर, अल्फा-ग्लूकोसिडेज़ निषेध के लिए जैव सक्रिय यौगिक को अलग करने के लिए विथानिया कोगुलन्स को चुना गया था। केवल हेक्सेन अर्क (2% उपज) ने 0.013 मिलीग्राम/एमएल के IC_{50} और 0.012 मिलीग्राम/एमएल के K_i के साथ अल्फा-ग्लूकोसिडेज़ निरोधक गुणों को दिखाया। जैव सक्रिय अंश के शुद्धिकरण की रणनीति तैयार की गई थी, और अंतिम शुद्धिकरण चरण में, जैव सक्रिय अंश S-IV-F3 ने 0.004 मिलीग्राम/एमएल का IC_{50} और 0.0037 मिलीग्राम/एमएल का K_i दिखाया। लक्षण वर्णन और संरचना स्पष्टीकरण अध्ययनों से पता चला कि जैव सक्रिय अंश में

ओलेफिनिक फैटी एसिड और फैटी एसिड मिथाइल एस्टर शामिल थे। NMR विश्लेषण भी S-IV-F3 में फैटी एसिड जैसे यौगिकों की उपस्थिति का समर्थन करता है।

उद्देश्य 4: अल्फा-ग्लूकोसिडेज निरोधक क्षमता में विथानिया कोगुलन्स फल का अर्क मानक दवा अकार्बोज से भी बेहतर पाया गया। शुद्धिकरण के विभिन्न चरणों में इसकी गतिविधि बढ़ती गई है, जिससे यह संकेत मिलता है कि इसे अल्फा-ग्लूकोसिडेज अवरोधक के रूप में उपयोग करने की अपार संभावना है। इसलिए, यह समझना महत्वपूर्ण हो जाता है कि मधुमेह प्रबंधन के लिए इसका सेवन करने वाले व्यक्तियों को यह अतिरिक्त लाभ कैसे प्रदान कर सकता है। विथानिया कोगुलन्स से प्राप्त जैव सक्रिय अंशों का उनके एंटीऑक्सिडेंट, रोगाणु-रोधी और कोशिकाविषाक्त गतिविधियों के लिए विश्लेषण किया गया। एंजाइम कीनेटिक्स अध्ययनों से पता चला है कि जैव सक्रिय अंश अल्फा-ग्लूकोसिडेज के प्रति गैर-प्रतिस्पर्धी निषेध का स्वरूप प्रदर्शित करता है। यह मुख्य रूप से अर्क में मौजूद यौगिकों और एंजाइम के एलोस्टेरिक स्थान से जुड़ने वाले अणुओं के कारण होता है, जो सबस्ट्रेट के एंजाइम के सक्रिय स्थान से जुड़ने के प्रति एंजाइम की समानता को कम करता है। परीक्षण किए गए पांच जीवाणु उपभेदों में से *S. aureus* को S-I के प्रति अत्यधिक संवेदनशील पाया गया, जिसका IC₅₀ 4 मिलीग्राम/एमएल था, इसके बाद *B. cereus* (8 मिलीग्राम/एमएल), *K. pneumoniae* (25 मिलीग्राम/एमएल), *P. aeruginosa* (25 मिलीग्राम/एमएल), और *E. coli* (32 मिलीग्राम/एमएल) का स्थान रहा। हालांकि, S-I ने कम एंटीऑक्सिडेंट गतिविधि दिखाई। विथानिया कोगुलन्स अर्क का सेवन मधुमेह प्रबंधन के अलावा अतिरिक्त लाभ प्रदान कर सकता है। इस अध्ययन में प्रस्तुत शुद्धिकरण रणनीति और रिपोर्ट किए गए अणुओं ने कोशिकाविषाक्तता को कम करने की अपार संभावना दिखाई है और दवा अणुओं के लिए एक संभावित पहल का प्रतिनिधित्व कर सकते हैं।

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Abbreviations

%	Percentage
°C	Degree Celsius
μ	Micron
μg	Microgram
2D	2 dimension
3D	3 dimension
A1C/ HbA1C	Glycated hemoglobin
ADMET	Absorption Distribution Metabolism Excretion Toxicity
ATCC	American Type Culture Collection
CRDT	Centre for Rural Development and Technology
BAS	Bioavailability score
CRF	Central Research Facility
DM	Diabetes Mellitus
DMSO	Dimethyl sulfoxide
EE	Electrostatic energy
FDA	Food and Drug Administration
FDA	Food and Drug Administration
g	Gram
GC-MS	Gas chromatography–mass spectrometry
HBO	Hydrogen bond occupancy
HP-TLC	High Performance-Thin Layer Chromatography
IC	Inhibitory concentration
IC ₅₀	Inhibitory concentration at 50 %
IDF	International Diabetes Federation

IITD	Indian Institute of Technology Delhi
Kcal	Kilocalorie
kg	Kilogram
KJ	Kilojoule
MDS	Molecular dynamics simulation
min	Minute
ml	microliter
ml	Milliliter
mM	Millimolar
MMPBSA	Molecular mechanics Poisson–Boltzmann surface area
MTDD	Methyl 12,13-tetradecadienoate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
N	Normal
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
nm	Nanometer
NPT	Constant Number of atoms, Pressure, and Temperature
ns	Nanosecond
NVT	Constant Number of atoms, Volume, and Temperature
OD	Optical Density
PDB	Protein databank file
PME	Particle Mesh Ewald
PPG	Post-prandial glucose
PSE	Polar solvation energy

PSM	Plant secondary metabolite
R _g	Radius of gyration
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
SASA	Solvent accessible surface area
SDF	Structure Data File
T1-DM	Type 1 Diabetes mellitus
T2-DM	Type 2 Diabetes mellitus
VWE	Van der Waals energy
WC	<i>Withania coagulans</i>
WCf	<i>Withania coagulans</i> fruit
α -GI	α -glucosidase inhibitor