

**PROFILING THE SECONDARY METABOLITES FROM
CHLORELLA MINUTISSIMA AND DEVELOPMENT OF
MILLET-BASED FUNCTIONAL FOOD**

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**CENTRE FOR RURAL DEVELOPMENT & TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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CHLORELLA MINUTISSIMA AND DEVELOPMENT OF
MILLET-BASED FUNCTIONAL FOOD**

by

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CERTIFICATE

This is to certify that the thesis entitled “**Profiling the secondary metabolites from *Chlorella minutissima* and development of millet-based functional food**” being submitted by **Ms. Koushalya S** 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 submission of this thesis. To the best of our 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

The present work investigated the therapeutic potential of the least explored *Chlorella minutissima* for nutraceutical application. The upscaling of the *C. minutissima* for biomass production was carried out using low-cost NPC media in different setup like 30 L photobioreactor and 150 L raceway. It is interesting to note that scaled-up cultivation by replacing BG11 with NPC media did not impact the biomass growth negatively which indicate that relatively expensive trace elements and other supplements are not required for this *C. minutissima* strain. *C. minutissima* was rich in abundant protein (26.11%) and essential elements (592.32 $\mu\text{g g}^{-1}$ of iron, and 182.11 $\mu\text{g g}^{-1}$ of zinc). Screening different solvent extracts (increasing polarity: hexane>chloroform>ethyl acetate>acetone>methanol>water) where acetone extract showed highest activities among other extracts. For the first time, this study revealed that acetone extract could protect macromolecules (DNA and Protein) from oxidative damage besides effectively reducing the oxidation of β -carotene and meat models by 79.75% at 200 ppm and 97.97% at 300 ppm concentrations, respectively.

Further to evaluate the therapeutic potential, this study employed bioassay-guided fractionation of *C. minutissima* to identify the potential AG inhibitors. The crude acetone extract of *C. minutissima* showed better inhibitory activity than acarbose standard against AG. Further, purification and spectral characterization of positive fractions of *C. minutissima* acetone extract led to the construction of virtual ASM library of 178 metabolites comprising major classes of terpenoids and alkaloids. Moreover, the computational studies (Ensemble Docking and Molecular Dynamic Simulation) of these metabolites against AG inhibition showed that Lucidine B obtained from *C. minutissima* was interacted with 3A4A with the least binding energy ($-318.089 \pm 26.202 \text{ KJ mol}^{-1}$) and hydrogen bond interaction with Glu408

(Hydrogen bond occupancy: 122.36 %) depicting non-competitive inhibition. Also, pharmacokinetics and toxicological profiling showed that the Lucidine B followed Lipinski 'Rule of five' and holds good drug-likeness properties. Also, AMES toxicity and oral rat acute toxicity suggest that Lucidine B is non-mutagenic (non-carcinogenic) and non-toxic.

In spite of high protein and bioactive metabolite content, *C. minutissima* contains low dietary fibre content which could be complemented by its incorporation into the millet for the development of functional food like cookies as a therapeutic solution for diabetic patients. Hence, the current study evidenced the possible use of nutritionally rich *C. minutissima* as the natural key ingredient for the development of Ready to use therapeutic food (RUTF) like cookie. The increased incorporation of 1, 2, and 6 % *C. minutissima* significantly enhanced the protein content, phenolics, and essential elements such as calcium, iron, and zinc of the cookies. The increased incorporation upto 6% *C. minutissima* significantly exhibited antioxidant potential, reduced the digestibility (69.60 %) and lowered the available sugar (low expected glycaemic index: 35.03) in the simulated digestive system. However, in the sensory analysis, the higher concentration (6 %) of *C. minutissima* scored less appreciation due to bitterness, despite better nutritional properties. Overall, this work provides a spotlight on the utilization of an under-explored *C. minutissima* as the source of protein and essential nutrients. *C. minutissima* was evidenced as a bioactive reserve of antioxidants and AG inhibitors associated with oxidative stress-related disorders like diabetes. This study delivered in-depth profiling and unveiled the mechanism of action of AG inhibition along with the development of *Chlorella*-incorporated millet cookies as a therapeutic functional food.

सार

वर्तमान कार्य में न्यूट्रास्युटिकल अनुप्रयोग के लिए सबसे कम खोजी गई क्लोरेला मिनुटिसिमा की चिकित्सीय क्षमता की जांच की गई। बायोमास उत्पादन के लिए सी. मिनुटिसिमा का अपस्केलिंग 30 एल फोटोबायोरिएक्टर और 150 एल रेसवे जैसे विभिन्न सेटअप में कम लागत वाले एनपीसी मीडिया का उपयोग करके किया गया था। यह ध्यान रखना दिलचस्प है कि एनपीसी मीडिया के साथ बीजी¹¹ को प्रतिस्थापित करके स्केल-अप खेती ने बायोमास विकास पर नकारात्मक प्रभाव नहीं डाला, जो दर्शाता है कि इस सी. मिनुटिसिमा स्ट्रेन के लिए अपेक्षाकृत महंगे ट्रेस तत्वों और अन्य पूरक की आवश्यकता नहीं है। सी. मिनुटिसिमा प्रचुर मात्रा में प्रोटीन (26.11%) और आवश्यक तत्वों (592.32 $\mu\text{g g}^{-1}$ आयरन, और 182.11 $\mu\text{g g}^{-1}$ जिंक) से समृद्ध था। विभिन्न विलायक अर्क (बढ़ती ध्रुवता: हेक्सेन>क्लोरोफॉर्म>एथिल एसीटेट>एसीटोन>मेथनॉल>पानी) की स्क्रीनिंग जहां एसीटोन अर्क ने अन्य अर्क के बीच उच्चतम गतिविधियां दिखाईं। पहली बार, इस अध्ययन से पता चला कि एसीटोन अर्क मैक्रोमोलेक्यूल्स (डीएनए और प्रोटीन) को ऑक्सीडेटिव क्षति से बचा सकता है, इसके अलावा β -कैरोटीन और मांस मॉडल के ऑक्सीकरण को क्रमशः 200 पीपीएम पर 79.75% और 300 पीपीएम सांद्रता पर 97.97% तक कम कर सकता है।

चिकित्सीय क्षमता का मूल्यांकन करने के लिए, इस अध्ययन ने संभावित एजी अवरोधकों की पहचान करने के लिए सी. मिनुटिसिमा के बायोएसे-निर्देशित अंशीकरण को नियोजित किया। सी. मिनुटिसिमा के कच्चे एसीटोन अर्क ने एजी के खिलाफ एकरबोस मानक की तुलना में बेहतर निरोधात्मक गतिविधि दिखाई। इसके अलावा, सी. मिनुटिसिमा एसीटोन अर्क के सकारात्मक अंशों के शुद्धिकरण और वर्णक्रमीय लक्षण वर्णन के कारण 178 मेटाबोलाइट्स की वर्चुअल एएसएम लाइब्रेरी का निर्माण हुआ, जिसमें टेरपेनोइड्स और एल्कलॉइड्स के प्रमुख वर्ग शामिल थे। इसके अलावा, एजी निषेध के खिलाफ इन मेटाबोलाइट्स के कम्प्यूटेशनल अध्ययन (एन्सेबल डॉकिंग और आणविक गतिशील सिमुलेशन) से पता चला है कि सी. मिनुटिसिमा से प्राप्त ल्यूसिडाइन बी को सबसे कम बाध्यकारी ऊर्जा (-318.089 ± 26.202 केजे मोल⁻¹) और हाइड्रोजन बंधन के साथ 3 ए 4 ए के साथ इंटरैक्ट किया गया था। ग्लू408 के साथ सहभागिता (हाइड्रोजन बांड अधिभोग: 122.36%) गैर-प्रतिस्पर्धी अवरोध को दर्शाता है। इसके अलावा, फार्माकोकाइनेटिक्स और टॉक्सिकोलॉजिकल प्रोफाइलिंग से पता चला है कि ल्यूसिडाइन बी ने लिपिंस्की के 'पांच के नियम' का पालन किया है और इसमें दवा जैसे अच्छे गुण हैं।

इसके अलावा, एमईएस विषाक्तता और मौखिक चूहे की तीव्र विषाक्तता से पता चलता है कि ल्यूसिडाइन बी गैर-उत्परिवर्तजन (गैर-कार्सिनोजेनिक) और गैर-विषाक्त है।

उच्च प्रोटीन और बायोएक्टिव मेटाबोलाइट सामग्री के बावजूद, सी. मिनुटिसिमा में आहार फाइबर की मात्रा कम होती है जिसे मधुमेह के रोगियों के लिए चिकित्सीय समाधान के रूप में कुकीज़ जैसे कार्यात्मक भोजन के विकास के लिए बाजरा में शामिल करके पूरक किया जा सकता है। इसलिए, वर्तमान अध्ययन ने कुकी जैसे उपयोग के लिए तैयार चिकित्सीय भोजन (आरयूटीएफ) के विकास के लिए प्राकृतिक प्रमुख घटक के रूप में पोषण से भरपूर सी. मिनुटिसिमा के संभावित उपयोग का प्रमाण दिया है। 1, 2, और 6% सी. मिनुटिसिमा के बढ़े हुए समावेश ने कुकीज़ की प्रोटीन सामग्री, फिनोलिक्स और कैल्शियम, आयरन और जिंक जैसे आवश्यक तत्वों को काफी बढ़ा दिया। 6% सी. मिनुटिसिमा तक बढ़े हुए समावेशन ने एंटीऑक्सीडेंट क्षमता को महत्वपूर्ण रूप से प्रदर्शित किया, पाचनशक्ति को कम किया (69.60%) और सिम्युलेटेड पाचन तंत्र में उपलब्ध चीनी (कम अपेक्षित ग्लाइसेमिक इंडेक्स: 35.03) को कम कर दिया। हालाँकि, संवेदी विश्लेषण में, बेहतर पोषण गुणों के बावजूद, सी. मिनुटिसिमा की उच्च सांद्रता (6%) को कड़वाहट के कारण कम सराहना मिली। कुल मिलाकर, यह कार्य प्रोटीन और आवश्यक पोषक तत्वों के स्रोत के रूप में कम खोजे गए सी. मिनुटिसिमा के उपयोग पर प्रकाश डालता है। सी. मिनुटिसिमा को मधुमेह जैसे ऑक्सीडेटिव तनाव-संबंधी विकारों से जुड़े एंटीऑक्सीडेंट और एजी अवरोधकों के बायोएक्टिव रिजर्व के रूप में प्रमाणित किया गया था। इस अध्ययन ने गहन प्रोफाइलिंग प्रदान की और एक चिकित्सीय कार्यात्मक भोजन के रूप में क्लोरेला-निगमित बाजरा कुकीज़ के विकास के साथ-साथ एजी निषेध की कार्रवाई के तंत्र का अनावरण किया।

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Abbreviations

CM	: <i>Chlorella minutissima</i>
ROS	: Reactive oxygen species
DM	: Diabetes Mellitus
AD	: Alzheimer's disease
AChE	: Acetylcholinesterase
PBR	: Photobioreactor
FL	: Flask study
RC	: Raceway
NPC	: Nitrate, phosphate, and carbonate
DPPH	: 2,2-Diphenyl-1-picryl-hydrazyl
ABTS	: 2,2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt
FRAP	: Ferric Reducing Antioxidant Power
DNA	: Deoxyribonucleic acid
BSA	: Bovine serum albumin
SDS-PAGE	: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
AAPH	: 2,2-azobis (2-amidinopropane) dihydrochloride
BHA	: Butylated hydroxyanisole
BHT	: Butylated hydroxytoluene
TBA	: Thiobarbituric acid
CHN	: Carbon Hydrogen Nitrogen
ICPMS	: Inductively coupled plasma mass spectrometry
GA	: Gallic acid
TBARS	: Thiobarbituric acid reactive substances
MDA	: Malondialdehyde
PNPG	: p- nitrophenol-a-D-glucopyranoside
GAE	: Gallic acid equivalents
QCTE	: Quercetin equivalents
PUFA	: Polyunsaturated fatty acids
FAs	: Fatty acids
IC ₅₀	: Half-maximal inhibitory concentration
ASM	: Algal secondary metabolite

TLC	: Thin layer chromatography
PNPG	: 4-Nitrophenyl- β -D- glucopyranoside
ED	: Ensemble Docking
MD	: Molecular dynamic
PDB	: Protein Data Bank
BE	: Binding Energy
HR-LCMS	: High Resolution Liquid Chromatograph Mass Spectrometer
Q-TOF	: Quadrupole-Time of Flight
RMSD	: Root mean square deviation
RMSF	: Root mean square fluctuation
Rg	: Radius of gyration
SASA	: Solvent-accessible surface area
HBN	: Hydrogen bond number
HBO	: Hydrogen bond occupancy
MM-PBSA	: Molecular Mechanic Poisson-Boltzmann Surface Area
PCA	: Principal component analysis
pkCSM	: Small-molecule pharmacokinetics prediction
GRAS	: Generally Recognized As Safe
FDA	: Food and Drug Administration
EFSA	: European Food Safety Authority
RUTF	: Ready to use therapeutic food
CF	: Cookie formulations
LVR	: Linear viscoelasticity region
TDF	: Total dietary fibre
IVD	: <i>In vitro</i> digestibility
HI	: Hydrolysis index
eGI	: Expected glycaemic index