



ST. THOMAS' COLLEGE (AUTONOMOUS)

THRISSUR, KERALA-680001, INDIA

Phone: 0487 2420435, 2444486

E-mail: stethrissur@gmail.com

Visit us at stthomas.ac.in

Dr. Anto P V, B.Ed, Ph.D

Assistant professor

Research and P.G Department of Botany

Ph. no. 9446230315

E-mail: pvabotany71@gmail.com

CERTIFICATE

This is to certify that the thesis entitled "**Beneficial aspects of selective endophytic bacteria isolated from *Morinda L. species***" is an authentic record of research work carried out by **Mrs. Neenu A Santhosh** under my supervision in fulfilment of the requirement for the degree of Doctor of Philosophy, in Botany of University of Calicut. The results embodied in this thesis have not been included in any other thesis submitted previously for the award of any degree or diploma of any other university or institution. Also certified that the contents of the thesis have been checked using anti-plagiarism data base and no unacceptable similarity was found through the software check.

Thrissur

20 November 2021

Dr. Anto P V

(Research Guide)

Dr. ANTO P.V., Ph.D
Assistant Professor
Department of Botany
St. Thomas College
Thrissur - 680 001

DECLARATION

I, **Neenu A Santhosh**, hereby declare that the thesis entitled “**Beneficial aspects of selective endophytic bacteria isolated from *Morinda L. species***” submitted to the University of Calicut, for the award of the degree of Doctor of Philosophy in Botany is a bona fide record of the original research work carried out by me under the supervision and guidance of Dr. Anto P V, Assistant professor, Department of Botany, St. Thomas’ College (autonomous), Thrissur and that it has not been submitted earlier either in part or full for the award of any degree/diploma to any candidate of any University.

Thrissur

20.11.2021



Neenu A Santhosh

ACKNOWLEDGEMENT

First and foremost, I thank **Lord Almighty**, who showered his abundant blessings upon me which enriched my thoughts, deeds and gave me health, strength and confidence to complete my work successfully.

I wish to express my sincere gratitude to my Research guide **Dr. Anto P.V**, Assistant Professor, Department of Botany, St. Thomas' college, Thrissur, for his unceasing encouragement, strong support, excellent guidance, valuable suggestions, immense support as a result of which I could successfully complete this work.

I am grateful to our Principal, Rev. **Fr. Dr. Martin K.A** and former Principal, **Dr. K.L Joy**, Associate professor, Department of chemistry and **Dr. Ignatius Antony**, Associate professor, Department of Botany, St. Thomas, College (autonomous), Thrissur.

I express my gratitude to former Head and Associate Professor **Dr. C.D Varghese** and Assistant Professor **Dr. Vimala Jose**, Head of the Department of Botany, St. Thomas' college (Autonomous), Thrissur for all the facilities and help rendered for my research work.

I pay my deepest gratitude to **Dr. Sr. Marriette A Therattil**, former Principal and **Dr. Sr. Magie Jose**, Principal, St. Marys college, Thrissur for providing me lab facilities in their esteemed college during the initial stages of my research work.

I am grateful to **Dr. Sr. Meena K Cheruvathur**, Vice- Principal, Assistant Professor; **Dr. Regi Raphael K**, former HOD, Associate Professor; **Dr. Rekha K**, HOD, Assistant Professor; teaching and non-teaching staffs, Department of Botany, St. Marys College, Thrissur for their unconditional support.

I convey my sincere gratitude to Prof. **K.M James (Late)**, **Prof. Jacob Abraham Pulikal**, **Prof. Tony Jacob**, **Prof. N.V Joseph**, and all my beloved teachers for their blessings and constant encouragements.

I mention special thanks to **Dr. Geethu Elizabeth**, **Dr. Joby Paul**, **Dr. Thomas M.T** and **Dr. Sandhya Vincent**, Assistant professors, Department of Botany, St. Thomas College (autonomous), Thrissur for their support and encouragement throughout my research work.

I extend my sincere gratitude to **Dr. Ramdas Kuttan**, former Director, **Dr. Achuthan C Ragavamenon** and **Dr. Babu T D** Scientist, Amala Cancer Research Institute,

Thrissur for providing the facilities and permission for the consultation in their esteemed institution.

I am also thankful to **Mrs. Preetha**, lab Assistant, **Mr. Vaisakh**, Research associate and all research scholars, Amala Cancer Research Institute, Thrissur for their help and timely suggestions during my research work.

I am thankful to **CSIR**, New Delhi for providing me the fellowship to meet the financial assistance for the research.

I convey my heartfelt thanks to **Mr. Sanjo**, Librarian of St. Thomas' college (Autonomous), Thrissur and Librarian of Central Library, KAU, Vellanikkara.

I extend my thanks to the lab assistant **Mr. Joy** and **Mr. Paulson**, Botany Department, St. Thomas' College (autonomous), Thrissur for their co-operation and encouragement during this work.

I mention special thanks to **Dr. Girija**, Head of Department of Microbiology, Vellanikkara, and all project assistants of that department for giving an opportunity to doing a part of my work in their department.

I convey my sincere gratitude to Agrigenome Pvt. Ltd, Kakkanad; STIC CUSAT, Cochin; KVASU, mannuthy and **Mrs. Beena** Omicsgen, Kakkanad, for providing lab facilities.

I extend my deep sense of gratitude to **Dr. Shahina P.M**, **Dr. Surekha** and **Dr. Anusree Dharman** for their immense help, assistance and suggestions during the final stages of my research.

My profound sense of gratitude is extended to the research colleagues in my lab, **Mrs. Seena**, **Mrs. Alina**, **Ms. Nimmy**, **Mrs. Keerthana**, **Mrs. Afsana**, **Mrs. Dhanya**, **Mrs. Reshma**, **Mrs. Smitha**, **Ms. Aiswariya**, **Mrs. Rameena**, **Mrs. Hridhya**, **Mrs. Lakshmi**, **Mrs. Sreeshma**, **Mrs. Aparna**, **Mrs. Blessy**, **Ms. Reshma Rajan**, **Mr. Shaibu** and **Mr. Jithin** for rendering their valuable suggestions and endless support and motivation.

I also thank all teachers and research colleagues of various departments for the support and help.

I am much indebted for the encouragement and immense support of all my family members, especially my mother **Jancy**, father **Santhosh**, brother **Nikhil**, mother-in-law **Thankamma**, my husband **George** and two lovely sons, **Gan** and **Gene**.

Neenu A Santhosh

Dedicated to my lovely family

PREFACE

In nature, no eukaryotes exist without prokaryotes. All the living organisms carry prokaryotes in them for their survival and existence. Endophytic bacteria residing inside the living tissue causing no harm to the host plays a major role in controlling the host's physiology and metabolism. They show symbiotic association with the plant they reside and consume the metabolites from the host for their growth. They also secrete such consumed compounds as secondary metabolites, characteristic of the host's active compounds. Therefore, if the host plants have good medicinal property, the endophytes in it may possess the similar character of the host.

Endophytic microorganisms have an inexhaustible source of chemical compounds and can biosynthesize a wide variety of beneficial secondary metabolites. They are the depot of novel drugs with various biological activities of pharmaceutical importance. Research on the endophytes reports that they utilize the chemical compound of the host for their metabolism and secrete it as extracellular metabolites. In vice versa, these endophytes are symbiotically related to the growth and development of the host plant. The search of these potent endophytic bacteria from the medicinally important plant led to the present study of isolation of such bacteria from the plant *Morinda L.* species. Also, the work has showcased the pharmacological activities of the endophytic bacteria.

The study suggest that the isolated endophytic bacterial species have important antimicrobial, antioxidant, anti-inflammatory, and anticancer activities. Also, the study focused on the use of the bacterial sample in nanoparticle production. The differences in the biological activities among the isolates can be due to the varied bioactive compounds present in it.

LIST OF TABLES

2.1	Some common endophytic bacterial species from the agronomic plant.	10
2.2	Commonly used primers targeting bacterial 16S rRNA gene sequence.	17
2.3	Some selected bioactive compounds reported from bacteria.	22
4.1	Phenotypic characterization of bacteria from <i>M. citrifolia</i> .	73
4.2	Phenotypic characterization of bacteria from <i>M. pubescens</i> .	74
4.3	Physiological characterization of bacteria from <i>Morinda</i> species.	75
4.4	Biochemical screening of bacteria from <i>M. citrifolia</i> .	76
4.5	Biochemical screening of bacteria from <i>M. pubescens</i> .	77
4.6	Identity percentage of the 16S rRNA gene sequences of the isolates with the NCBI database.	78
4.7	Hierarchical classification of isolated bacterial strains.	80
4.8	Summary of OTUs clustered from pre-processed sequences of sample NMT.	89
4.9	Diversity indices among different major phyla.	91
4.10	GC-MS analysis of ethyl alcohol extract of sample NMC01.	94
4.11	GC-MS analysis of ethyl alcohol extract of sample NMC15.	95
4.12	GC-MS analysis of ethyl alcohol extract of sample NMT03.	96
4.13	GC-MS analysis of ethyl alcohol extract of sample NMT04.	97
4.14	GC-MS analysis of ethyl alcohol extract of sample NMT06.	98
4.15	GC-MS analysis of ethyl alcohol extract of sample NMT07.	99
4.16	GC-MS analysis of ethyl alcohol extract of sample NMT08.	100
4.17	GC-MS analysis of ethyl alcohol extract of sample NMT09.	101
4.18	Total phenolic content and percentage yield of sample extract.	102
4.19	X-ray diffraction data (2θ and d-spacing of nanocrystal peak).	106
4.20	Heavy metals removal by synthesized γ -Fe ₂ O ₃ nanoparticle.	107
4.21	Degradation of different dye by synthesized γ -Fe ₂ O ₃	108

	nanoparticle.	
4.22	Percentage of fungal growth inhibition by isolated bacteria.	110
4.23	The IC ₅₀ value of samples and standard drug of different antioxidant assay.	117
4.24	The IC ₅₀ value of sample extract on cytotoxicity towards DLA cell lines.	118
4.25	Inhibition of carrageenan-induced acute inflammation by NMC15 extract.	122
4.26	Inhibition of carrageenan-induced acute inflammation by NMT07 extract.	123
4.27	Inhibition of formalin-induced acute inflammation by NMC15 extract.	124
4.28	Inhibition of formalin-induced acute inflammation by NMT07 extract.	125
4.29	Showing the number of EAC-bearing mice that survived in each group.	126
4.30	The mean survival days and percent increase in lifespan of EAC- bearing mice.	127
4.31	Antitumor effect of NMT07 on DLA solid tumor.	128
4.32	Hematological parameters of DLA tumor-bearing mice on the 30 th day.	130

LIST OF FIGURES

1.1	Schematic representation with an overview of the study design.	8
2.1	Schematic representation of bioactive compounds produced by <i>Bacillus</i> sp.	26
3.1	Habit of <i>M. citrifolia</i> .	40
3.2	Habit of <i>M. pubescens</i> .	41
4.1	Schematic representation of code names given to endophytic isolates.	71
4.2	Photograph showing endophytic bacterial culture from <i>Morinda</i> species.	72
4.3	Photograph showing gel electrophoresis of 16S rRNA gene of the isolates.	79
4.4	Phylogenetic tree of endophytic isolates from <i>Morinda</i> species.	81
4.5	Phylogenetic tree of isolated strains of <i>Exiguobacterium</i> sp.	82
4.6	Phylogenetic tree of isolated strains of <i>Bacillus</i> sp.	83
4.7	Phylogenetic tree of isolated strains of <i>Micrococcus</i> sp.	84
4.8	Phylogenetic tree of isolated strains of <i>Brevundimonas</i> sp.	85
4.9	Phylogenetic tree of isolated strains of <i>Microbacterium</i> sp.	85
4.10	Phylogenetic tree of isolated strains of <i>Brevibacterium</i> sp.	86
4.11	Gel profile of bacterial PCR amplicon.	86
4.12	Graphical representation of base quality distribution of Read 1 and Read 2 of sample NMT.	87
4.13	Sequencing chromatograms of Read 1 and Read 2 of sample NMT.	88
4.14	Contig length distribution of the sequence of sample NMT.	89
4.15	Graphical representation of OTUs classification at the phylum level of sample NMT.	90
4.16	Graphical representation of OTUs classification at the genus level of sample NMT.	91
4.17	Plates showing endophytic bacterial isolates selected for the study.	92
4.18	Photograph of lyophilized ethyl alcohol extracts of selected	93

	samples.	
4.19	GC-MS chromatogram of ethyl alcohol extract of sample NMC01.	94
4.20	GC-MS chromatogram of ethyl alcohol extract of sample NMC15.	95
4.21	GC-MS chromatogram of ethyl alcohol extract of sample NMT03.	96
4.22	GC-MS chromatogram of ethyl alcohol extract of sample NMT04.	97
4.23	GC-MS chromatogram of ethyl alcohol extract of sample NMT06.	98
4.24	GC-MS chromatogram of ethyl alcohol extract of sample NMT07.	99
4.25	GC-MS chromatogram of ethyl alcohol extract of sample NMT08.	100
4.26	GC-MS chromatogram of ethyl alcohol extract of sample NMT09.	101
4.27	TPC of sample extract (a) and calibration curve of standard gallic acid (b).	102
4.28	Photograph of visual observation of synthesized iron oxide nanoparticles.	103
4.29	UV-Vis absorption spectrum of iron oxide nanoparticles of NMC01.	104
4.30	FTIR analysis of iron oxide nanoparticles of NMC01.	104
4.31	EDAX image of iron oxide nanoparticles of NMC01.	105
4.32	SEM image of iron oxide nanoparticles of NMC01.	105
4.33	X-Ray Diffraction curves of iron oxide nanoparticles of NMC01.	106
4.34	TEM micrograph (A) with SAED pattern (B) of iron oxide nanoparticles of NMC01.	107
4.35	Graph showing heavy metal removal from treated water using AAS.	108
4.36	Photograph of dye decoloration by synthesized γ -Fe ₂ O ₃	109

	nanoparticle.	
4.37	Plates showing antibacterial activity of <i>Exiguobacterium aurantiacum</i> NMC1 by cross streak method.	109
4.38	Plates showing antifungal activity of isolates against phytopathogen <i>Sclerotium rolfsii</i> by dual culture method.	111
4.39	Plates showing antifungal activity of isolates against phytopathogen <i>Aspergillus</i> sp. by dual culture method.	112
4.40	DPPH radical scavenging activity of ethyl alcohol extract of samples.	113
4.41	IC ₅₀ value of the sample extract in DPPH assay.	113
4.42	SRSA activity of ethyl alcohol extract of samples.	114
4.43	IC ₅₀ value of sample extract in SRSA assay.	114
4.44	LPI activity of ethyl alcohol extract of samples.	115
4.45	IC ₅₀ value of the sample extract in LPI assay.	115
4.46	FRAP assay of ethyl alcohol extract of samples.	116
4.47	IC ₅₀ value of the sample extract in FRAP assay.	116
4.48	In vitro cytotoxicity analysis of 8 samples towards DLA cell lines.	118
4.49	Cell viability profile of NMC15 extract against MCF-7 breast cancer cell lines.	119
4.50	Cell viability profile of NMT07 extract against MCF-7 breast cancer cell lines.	119
4.51	Inflammation- induced in mice [A] before induction and [B] after induction.	120
4.52	Effect of sample NMC15 on carrageenan-induced acute inflammation in reducing the paw edema in Swiss albino mice.	121
4.53	Effect of sample NMT07 on carrageenan-induced acute inflammation in reducing the paw edema in Swiss albino mice.	122
4.54	In vivo anti-inflammatory effect of sample NMC15 on formalin-induced chronic inflammation edema of Swiss albino mice.	124
4.55	In vivo anti-inflammatory effect of sample NMT07 on formalin-induced chronic inflammation edema of Swiss albino mice.	125
4.56	EAC-induced ascites tumor in mice.	126

4.57	Effect of sample NMT07 on survival pattern of ascites tumor-bearing mice.	127
4.58	Effect of NMT07 extract on DLA-induced solid tumor development in mice.	129
4.59	Macroscopic view of variation in tumor size of mice in different groups.	131
4.60	DLA-induced tumor macroscopy and microphotography of control mice.	131
4.61	DLA-induced tumor macroscopy and microphotography of Cyclophosphamide-treated mice.	132
4.62	DLA-induced tumor macroscopy and microphotography of lower dose of NMT07 extract-treated mice.	132
4.63	DLA-induced tumor macroscopy and microphotography of higher dose of NMT07 extract-treated mice.	133

ABBREVIATIONS

° C	Degree Celsius
μL	Microliter
μg	Microgram
mL	Milliliter
mg	Milligram
mM	Millimolar
M	Molar
Kg	Kilogram
g	Gram
v/v	Volume per volume
w/v	Weight per volume
b.wt.	Body weight
bp	Base pair
cm	Centimeter
nm	Nanometer
Å	Angstrom
min	Minute
sec	Second
h	Hour
%	Percentage
~	Approximately
rpm	Revolutions per minute
kV	Kilovolt
mA	Milliampere
pH	Potential of Hydrogen
IC	Inhibitory concentration
θ	Theta
λ	Lambda
α	Alpha
β	Beta
γ	Gamma
OD	Optical density
Cu	Copper
W	Watt
Cos	Cosine
Sin	Sine
CO₂	Carbon dioxide
MCF-7	Michigan Cancer Foundation-7
RPMI	Roswell Park Memorial Institute
SOD	Superoxide dismutase