Exploration of Selected *Streptomyces* Strains from Western Ghats for its Agriculture and Biomedical Application

by

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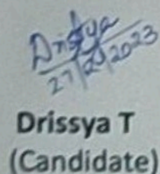

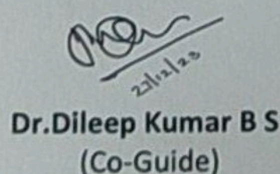

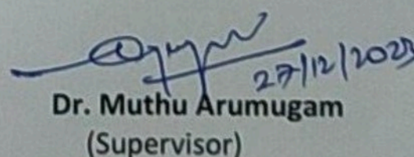

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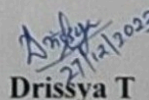

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ABBREVIATIONS

V

^{13}C	Carbon 13
^{1}H	Proton (Hydrogen -1)
ANOVA	Analysis of Variance
BGCs	Biosynthetic gene clusters
BLB	Bacterial Leaf Blight
BLS	Bacterial Leaf Streak
CAS	Chrome azurol S
CD	Circular dichroism
$CDCl_3$	Chloroform -d
CFU	Colony-forming units
COSY	Correlated Spectroscopy
COX-2	Cyclooxygenase -2
CSIR	Council for Scientific and Innovative Research
DFT	Density functional theory
DM	Differentiation medium
DMEM	Dulbecco’s modified Eagle’s medium
DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic acid
dw	dry weight,
ET	Ethylene
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FBS	Fetal bovine serum
Fe^{3+}	Ferric ion
$FeCl_3$	Ferric chloride
FT-IR	Fourier Transformation Infrared Spectroscopy
fw	fresh weight,
HBSS	Hanks balanced saline solution
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HMBC	Heteronuclear Multiple Bond Correlation
HR-ESI-MS	High-resolution electrospray ionisation mass spectrometry
HRP	Horseradish peroxidase
HSQC	Heteronuclear Single Quantum Coherence
HTDMA	Hexadecyl trimethyl ammonium
Hz	Hertz
IAA	Indole 3 acetic acid
IARI	Indian Agricultural Research Institute
IBMX	3- isobutyl-1-methylxanthine
ICAR	Indian Council of Agricultural Research
IMTECH	CSIR-Institute of Microbial Technology
ISR	Induced systemic resistance
ITCC	Indian Type Culture Collections
JA	Jasmonic acid
K_2O	Potassium Oxide

KBr	Potassium Bromide
LBB	Luria Bertani Broth
M	Molar
m/z	mass to charge ratio
Mb	Megabases
Mg	Magnesium
mg	Milli gram
MHA	Muller Hinton Agar
MHz	Megahertz
mL	Milli litre
mm	Milli metre
mM	Milli molar
MTCC	Microbial Type Culture Collection and Gene Bank
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)
NCCS	National Center for Cell Sciences
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
NUE	Nutrient use efficiency
P_2O_5	Phosphorus pentoxide
PAL	L-phenylalanine ammonia lyase
PBS	Phosphate buffer saline
PCM	Polarizable continuum model
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGP	Plant growth promotion
PGPB	Plant growth promoting bacteria
PGPR	Plant Growth Promoting Rhizobacteria
PGPR	Plant growth promoting Rhizobacteria bacteria
POD	Peroxidase
PPARγ	Peroxisome proliferator-activated receptor gamma
ppm	parts per million
PPO	Polyphenol oxidase
PSE	Phosphate Solubilizing Efficiency
PVDF	Polyvinylidene Fluoride
RB	Rice Blast
RIPA buffer	Radioimmunoprecipitation assay buffer
rl	root length,
rpm	Revolution per minute
rRNA	Ribosomal RNA
SA	Salicylic acid
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SB	Sheath Blight
SCRF	Self-Consistent Reaction Field.
SDS-PAGE	Sodium dodecyl- sulfate polyacrylamide gel electrophoresis
sdw	shoot dry weight
sh	shoot height
SPSS	Statistical Package For The Social Sciences
Sw	seed weight
TBE	Tris-Borate-EDTA

TLC	Thin layer chromatography
UNESCO	United Nations Educational Scientific and Cultural Organization
UV-Vis	Ultraviolet – Visible
V	Volts
v/v	Volume per volume
WHO	World Health Organization
YMA	Yeast Malt Agar
YMB	Yeast Malt Broth
δ	Chemical shifts
Δε	Molar circular dichroism
θ	Elipticity
µg	Micro gram
µl	Micro litre
µm	Micro metre
µM	Micro molar

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Chapter 1

Introduction

1.1. Introduction

Rice is a semi-aquatic plant belongs to the family Poaceae and genus *Oryza*. Almost 22 species of plants in this genus are known, of which 20 species are wild. Among them two species are most important and are used for human consumption, *O. glaberrima* and *O. sativa.* Rice (*O. sativa*) was first cultivated from 8000 to 15000 years ago in southeast Asia, such as India, Thailand, China, and North Vietnam. *O. glaberrima* is believed to have been domesticated about 3000 years ago, by African people who lived in the floodplains of river Niger. Nowadays rice is cultivated all over the world except Antarctica (Muthayya et al., 2014). More than 50% of the world's population relies on rice (*Oryza sativa*) which is a widely accessible staple food. It contributes, to the major sources of dietary energy, (20% of the global supply of dietary energy) in undernourished and poorest people in Africa and Asia as they cannot access or afford nutritious foods. Likewise, in some countries of Asia, rice provides 70% calorie supply. Thus, rice is regarded as one of the most strategically significant commodities worldwide. (Bin Rahman and Zhang, 2022). More than 80% of the rice consumed worldwide is produced by seven nations, they are China, India, Bangladesh, Myanmar, Indonesia, Vietnam, and Thailand. In recent decades rice production has increased due to the improvements in cultivation practices and the introduction of new varieties of rice such as hybrids. Average global share of rice production from 1994 to 2021 (**Figure 1.1**). The production of rice increased by more than three times between 1961 and 2019 (215 million tonnes in 1961 to 755 million tons in 2019) (Jiang et al., 2020).

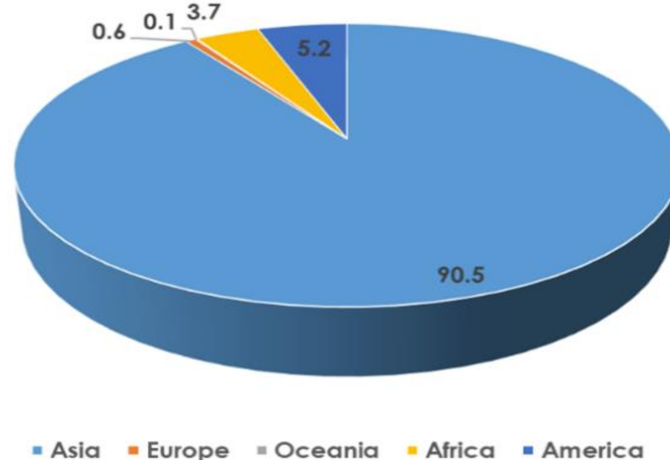


Figure 1.1. Average worldwide share of rice production in percentage between 1994 to 2021 (FAOSTAT, 2023).

But recently population growth has increased tremendously, rapid increase in the population is a key challenge to reduce hunger and malnutrition. Population growth from 1950 to 2019 was

2.5 billion to 7.7 billion and the global population is expected to increase up to 8.5 billion in 2030. Further growth will be expected in 2050 and 2100 (9.5 billion in 2050 and 10.9 billion in 2100) (Bin Rahman and Zhang, 2022). Nowadays there is a huge demand to produce rice, to feed the ever-expanding population. Generally, rice cultivation is passing through challenging situations like soil fertility, farmland availability, climate change, water, pests, and diseases (Jiang et al., 2020). Among them, rice diseases are the major problem in the agriculture sector, which brings down yield and quality. Another challenge includes the early identification and classification of diseases in the agricultural field. Rice is infected by various diseases caused by fungi, bacteria, and viruses (Jiang et al., 2020). This can decrease rice production by up to 10- 15%. Some of the rice disease that causes yield loss includes Rice Blast (RB), Bacterial Leaf Blight (BLB), Sheath Blight (SB), and Bacterial Leaf Streak (BLS). RB disease is caused by *Magnaporthe oryzae* and is the most devastating rice plant disease affecting the leaves of the plant. The causative agent of SB disease is *Rhizoctonia solani* which causes loss of rice production, quality degradation, etc (Shrivastava and Pradhan, 2020). Other two rice diseases BLS and BLB are caused by *Xanthomonas sps.* Both the diseases (BLS and BLB) are caused by Gram-negative bacteria such as *Xanthomonas oryzae pv. oryzicola* and *Xanthomonas oryzae pv. oryzae* respectively. BLS rice disease spreads quickly under favorable conditions and causes yield loss ranging from 8 to 32%. Tremendous loss in yield was noted especially in Asian and African countries.

BLB is one of the major devastating rice diseases throughout Asia. One of the most destructive rice diseases among others, this can cause a significant reduction in rice production. Depending on the geographic location, growth stage, rice variety, and environmental factors, BLB can cause severe destruction and yield loss of up to 50%. The loss caused by the kresek syndrome of BLB can reach up to 75%. Symptoms include the water-soaked region which appears at the tip and later spreads to the leaf base (Shrivastava and Pradhan, 2020). BLB disease was first identified in 1884 by farmers in the Fukuoka region of southern Japan. From the southern regions of Japan, it gradually spreads to various rice-growing regions. Damages of BLB disease significantly increased in the area where excess use of nitrogen fertilizers as well as the cultivation of hybrids and semi-dwarf rice varieties. As BLB has a widespread nature, it is observed in almost all rice-cultivating countries in the world (Jiang et al., 2020).To reduce the incidence of disease, improve yield, plant growth, and decrease environmental pollution, more activities are needed especially the chemical-free practice of agriculture. The use of agrochemicals can harm the environment as well as humans. Biological means of agriculture

improvement practices are well known and are efficient to increase yield, reduce disease development and environmental hazards. Microbes are utilized as biological agents (biocontrol agents or biofertilizers) to replace chemicals in managing crop productivity. Biocontrol is defined as the reduction of disease-causing properties or number of pathogenic organisms by the action of an individual or combination of microorganisms. Biocontrol agents inhibit pathogen growth by antagonistic activities like competition, parasitism, and antibiosis (Jacob et. al., 2018).

Beneficial microbes especially, rhizosphere microbes are essential for plant health. Microbes enhance the growth of plants by colonizing the rhizosphere (around the root system) are called as Plant Growth Promoting Rhizobacteria (PGPR). PGPR has two mechanisms one is direct mechanism and the other is indirect mechanism. In direct mechanism they stimulate the growth of the plant (biofertilization) and plant stress control (rhizoremediation) (Nozari et al., 2021). Direct mechanism includes the enhancement of nutrients in the soil by phosphate solubilization, iron sequestration, and nitrogen fixation, which helps the plant to grow in low-nutrient soils. PGPR can also produce necessary plant growth promoting hormones such as Indole 3 acetic acid (auxins), gibberellins, and cytokinin or compounds and enzymes that alter plant hormonal pathways (Viaene et al., 2016). Through the means of indirect mechanism, PGPR reduces the incidence of disease by controlling pathogenic organisms (biocontrol) possibly through competition of nutrients or niches and antibiosis (Nozari et al., 2021). These bacteria activate induced systemic resistance (ISR) that triggers immunity in a systemic manner and protects the plants by enhancing resistance against a wide range of pathogens and diseases (Viaene et al., 2016). Induced resistance is an alternative way to protect plants especially when various fungicides fail to control plant diseases. ISR by PGPR against pathogens was considered the most advisable method in crop protection (Anand et al., 2007). PGPR are widely studied for their important role in maintaining soil composition by providing necessary secondary metabolite, which helps in soil fertility and plant growth promotion. Hence, PGPR can be used as an alternative to agrochemicals such as pesticides and fertilizers (Viaene et al., 2016).

One of the major groups of beneficial microbes which act as PGPR is *Streptomyces*, they are Gram-positive microbes belonging to Actinobacteria, with the resemblance of both fungus and bacteria. They are a heterogeneous group of saprobic bacteria with mycelium, mostly free-living, some are endophytic and are found abundantly in the soil (Jacob et. al., 2018). They not only maintain the structure and integrity of the soil but are also involved in recycling of the

nutrients present in the soil. *Streptomyces* are widely studied genera of Actinobacteria for their ability to produce numerous economically important metabolites which have antimicrobial properties. Researchers focus on these organisms to isolate natural bioactive metabolites and utilize them in the medical field as well as in agriculture to control disease (Jacob et. al., 2018).

Another challenging field is the medical sector, mostly due to infectious as well as lifestyle diseases. With the discovery of "wonder drugs" (antibiotics) and various secondary metabolites derived from *Streptomyces* species and filamentous fungi, the medical field in twentieth century was transformed into a new era (Deshpande et al., 1988; Hiltner et al., 2015; Seipke et al., 2012). The discovery of the first antibiotic, penicillin from the fungus *Penicillium notatum* in 1928 by Alexander Fleming led to a shift in the isolation of bioactive natural products from plants to microorganisms for new drug generation (Arya et al., 2020). Antibiotics not only cure fatal diseases but also involved in life-saving surgeries like organ transplantation. However, the emergence of drug-resistant pathogens causes serious issue, which leads to the death of a huge number of people (about 17 million people every year) (Alam et al., 2022). Mortality increased, due to the lack of effective antibiotics for the treatment of infectious diseases caused by multi-drug resistance pathogens. The discovery of Streptomycin from the *Streptomyces griseus* in 1943 by soil microbiologist Selman Waksman and his student Albert Schatz, leads to a turning point to cure infectious disease. Streptomycin is the first effective antibiotic to treat *Mycobacterium tuberculosis* (Seipke et al., 2012). World Health Organization (WHO) classified this antibiotic as a critically important medicine for humans. In 1961 Bu'Lock used the term "Secondary metabolites" for some natural products of microbial origin (Běhal, 2000). These secondary metabolites are a significant source of valuable therapeutic agents and pharmaceuticals. Natural compounds derived from microorganisms play a major role in reducing various infections and life-threatening diseases such as cancer, diarrhea, atopic dermatitis, Crohn's disease anemia, and obesity (Arya et al., 2020).

Since 1929, approximately 50,000 natural products that have been isolated from microbial sources, of which more than 10,000 exhibited biological activity. Almost 1000 microbial products are used for various medicines and agrochemicals (Berdy, 2005; Subramani and Aalbersberg, 2012). *Bacillus, Actinomycetes, Pseudomonas, Cyanobacterial*, fungi, and *Myxobacteria* are the major sources of microbial metabolites or microbial natural products (Katz and Baltz, 2016). Natural products and derivative compounds contribute to more than 50% of available medications in modern medicine (Mahmoud et al., 2023). The majority of

microbial bioactive secondary metabolites are produced by the *Actinomycetes*, particularly the genus *Streptomyces*. They produce almost 70% of available antibiotics (Kim et al., 2021).

In recent years metabolic regulations, growth, and heredity of *Streptomyces* strains become research interest. As a result, they are the most frequently used bacteria for fermentation to develop effective pharmaceutical drugs (Mahmoud et al., 2023). To increase the chance of isolating new natural bioactive metabolites, researchers are interested in studying unexplored areas. One of the best sources of diverse microorganisms is the Western Ghat forest soil. In 2012 UNESCO considered the Himalayas and Western Ghats as two "mega diversity hotspots" in the world. Western Ghats consist of forests and mountain ranges extending from Central Maharashtra to the southern region of Kerala (Vasudevan et al., 2015). There are lots of investigations going on to isolate *Streptomyces* strains from this forest area. Even though, a vast area of this forest is still unexplored and thus such areas are effective resources for novel soil microbes including *Streptomyces*.

1.2. Objectives

With this background, the present work aimed to explore potential *Streptomyces* strains with antimicrobial activity from the soil of Malampuzha forest. Which is in the unexplored Western Ghat region of Palakkad District, Kerala. Soil *Streptomyces* were isolated in pure forms and selected strains were screened and evaluated for their ability to produce bioactive metabolites, Plant growth promotion, and resistance against rice disease with the following objectives.

1. Isolation, culturing, and purification of *Streptomyces* species from the soil of Malampuzha forest.
2. Functional *In vitro* screening against common plant and human pathogens.
3. Plant growth promoting activity of *Streptomyces* species in rice plant.
4. Elicitation of the defence-related enzyme by *Streptomyces* species in rice to induce systemic resistance against rice leaf blight disease.
5. Bioactive metabolites produced by *Streptomyces* species and its promising biomedical application.

1.3. References

Alam, K., Mazumder, A., Sikdar, S., Zhao, Y. M., Hao, J., Song, C., Wang, Y., Sarkar, R., Islam, S., Zhang, Y., & Li, A. (2022). *Streptomyces*: The biofactory of secondary metabolites. Frontiers in Microbiology, 13, 1–21.

Anand, T., Chandrasekaran, A., Kuttalam, S., Raguchander, T., Prakasam, V., & Samiyappan, R. (2007). Association of some plant defense enzyme activities with systemic resistance to early leaf blight and leaf spot induced in tomato plants by azoxystrobin and *Pseudomonas fluorescens*. Journal of Plant Interactions, 2(4), 233–244.

Arya, A., Nahar, L., Khan, H. U., & Sarker, S. D. (2020). Anti-obesity natural products.Annual Reports in Medicinal Chemistry, 55, 411-433.

Běhal, V. (2000). Bioactive products from *Streptomyces*. Advances in Applied Microbiology, 47, 113–156.

Berdy, J. (2005). Bioactive Microbial Metabolites. Journal of Antibiotics, 58(1), 1–26.

Bin Rahman, A. N. M. R., & Zhang, J. (2022). Trends in rice research : 2030 and beyond. Food and Energy Security. 12(390), 1–17.

Critically important antimicrobials for human medicine. (2019). 6th revision. Geneva: World Health Organization.

Deshpande, B. S., Ambedkar, S. S., & Shewale, J. G. (1988). Biologically active secondary metabolites from Streptomyces. Enzyme and Microbial Technology.10(6), 455–473.

FAOSTAT (www.fao.org/faostat/en/#data/QC/visualize accessed), 2023.

Hiltner, J. K., Hunter, I. S., & Hoskisson, P. A. (2015). Tailoring specialized metabolite production in *Streptomyces*. Advances in Applied Microbiology, 91, 237-255.

Jacob, S., Sajjalaguddam, R. R., & Sudini, H. K. (2018). Streptomyces sp. RP1A-12 mediated control of peanut stem rot caused by *Sclerotium rolfsii.* Journal of integrative agriculture, *17*(4), 892-900.

Jiang, N., Yan, J., Liang, Y., Shi, Y., He, Z., Wu, Y., Zeng, Q., & Liu, X. (2020). Resistance Genes and their Interactions with Bacterial Blight / Leaf Streak Pathogens (*Xanthomonas oryzae*) in Rice (*Oryza sativa* L.) an Updated Review. Rice, 13(3),1-12.

Katz, L., & Baltz, R. H. (2016). Natural product discovery: past, present, and future. Journal of Industrial Microbiology and Biotechnology. 43, 155–176.

Kim, J. H., Lee, N., Hwang, S., Kim, W., Lee, Y., Cho, S., Palsson, B. O., & Cho, B. K. (2021). Discovery of novel secondary metabolites encoded in actinomycete genomes through coculture. Journal of Industrial Microbiology and Biotechnology. 48, 1–16.

Mahmoud, M. G., Awady, M. E. E., Selim, M. S., Ibrahim, A. Y., Ibrahim, F. M., & Mohamed, S. S. (2023).Characterization of biologically active exopolysaccharide produced by *Streptomyces* sp. NRCG4 and its anti-Alzheimer efficacy: in-vitro targets. Journal of Genetic Engineering and Biotechnology. 21(1),76-93.

Muthayya, S., Sugimoto, J. D., Montgomery, S., & Maberly, G. F. (2014). An overview of global rice production supply trade and consumption. Annals of the New York Academy of Sciences. 1324(1),7–14.

Nozari, R. M., Ortolan, F., Astarita, L. V., & Santarém, E. R. (2021). *Streptomyces* spp. enhancevegetative growth of maize plants under saline stress. Brazilian Journal of Microbiology. 52(3), 1371–1383.

Seipke, R. F., Kaltenpoth, M., & Hutchings, M. I. (2012). *Streptomyces* as symbionts: An emerging and widespread theme? FEMS Microbiology Reviews. 36(4), 862–876.

Shrivastava, V. K., & Pradhan, M. K. (2020). Rice plant disease classification using color features : a machine learning paradigm. Journal of Plant Pathology.103,17-26.

Subramani, R., & Aalbersberg, W. (2012). Marine actinomycetes: An ongoing source of novel bioactive metabolites. Microbiological Research. 167(10), 571–580.

Vasudevan, G., Siddarthan, V., & Solai Ramatchandirane, P. (2015). Predominance of *Bacillus* sp. in soil samples of the southern regions of Western Ghats, India. Annals of Microbiology. 65(1), 431–441.

Viaene, T., Langendries, S., Beirinckx, S., Maes, M., & Goormachtig, S. (2016). *Streptomyces* as a plant's best friend?. FEMS Microbiology Ecology. 92(8), 1–10.

Chapter 2
Review of literature

2.1. Review of literature

2.1.1. Overview of Genus *Streptomyces*

The Genus *Streptomyces* was first characterized by Waksman and Henrici in 1943. Based on cell wall chemotype and morphology they are classified into Streptomycetaceae family (Anderson and Wellington, 2001; Pacios-michelena et al., 2021). *Streptomyces* are bacteria that belong to the class Actinomycetes. They are filamentous, spore-forming, and Gram-positive microbes residing in diverse environments, such as terrestrial, marine, unexplored habitats, extreme environments, symbionts, mangroves, and endophytes etc. Approximately 850 *Streptomyces* species have been studied in various environments. It is estimated that 1g of soil contains 10^9 CFU (colony-forming units) of bacteria, among them 10^7 CFU belong to actinobacteria. It has a linear and moderately large genome of about 8- 10Mb size with more than 70% of G+C % this feature differentiates them from other bacteria. This can form areal and substrate mycelium with extensive branching with complex morphology (Alam et al., 2022). During the vegetative phase, substrate mycelium lacks cross-walls with a diameter of approximately 0.5 to 1 µm (Anderson and Wellington, 2001). *Streptomyces* life cycle (**Figure 2.1**) begins from the germination of unigenomic spores which grow to form vegetative mycelium. Generally, aerial hyphae are produced in a nutrient-limiting environment and finally form septa with chain of mature uninucleate spores (Alam et al., 2022; Hiltner et al., 2015). Secondary metabolites in *Streptomyces* usually depend on the growth- phase. Metabolites are produced during the development of reproductive areal mycelium (Ngamcharungchit et al., 2023) Biosynthetic gene clusters (BGCs) in *Streptomyces* genome are responsible for the production of various bioactive compounds with agriculture and medical use (Alam et al., 2022). Similarly, the diverse secondary metabolism of this organism is also involved in the production of valuable bioactive molecules and antibiotics (Flärdh and Buttner, 2009).

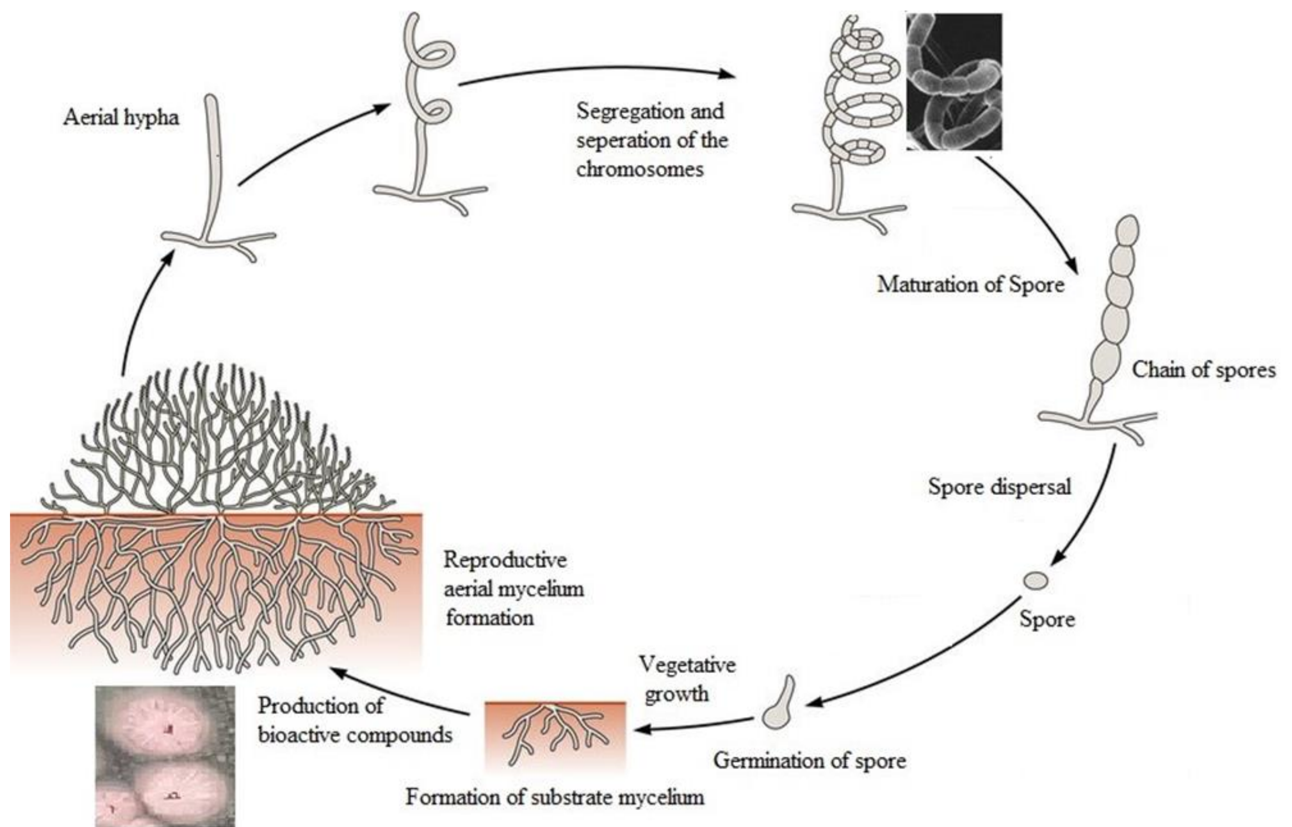


Figure 2.1. Life cycle of *Streptomyces* species (modified from Barka et al., 2016).

2.1.2. *Streptomyces*, A crucial producer of bioactive secondary metabolites

Streptomyces species are considered as the major source of natural products. They produce antibiotics which is approximately 70-80% of all natural products. Structurally diverse natural products are isolated from *Streptomyces* spp. such as tetracyclines, glycoproteins, macrolides, ansamycins, aminoglycosides, and terpenes. *Streptomyces hygroscopicus* is the best example, which can synthesize around 180 biologically active secondary metabolites (Alam et al., 2022). *Streptomyces*-derived secondary metabolites have active antiviral, antitumor, antimicrobial, cytotoxic, antihypertensive, antioxidative, Plant growth promoting, immunosuppressive, insecticide, herbicidal, (Alam et al., 2022) anti-obesity and anti-diabetes properties (Arya et al., 2020). Bioactive metabolites discovered from *Streptomyces* are classified into four different classes: (i) compounds that exhibit regulatory activities including siderophores, growth factors, and morphogenic agents (ii) antagonistic agents such as antibacterials, antivirals, anti-protozoans, and antifungals (iii) pesticides herbicides and insecticides as agrobiologicals (iv) enzyme inhibitors, antitumor agents, immunomodulators and neurological agents as pharmacological drugs. Antibiotics with diverse antibacterial activities are the most valuable

natural product of *Streptomyces* (Alam et al., 2022). Actinomycetes are primary antibiotic producers among all microorganisms and are estimated as 55% of existing antibiotics. Among all antibiotics, approximately 75% were isolated from *Streptomyces* species (Alam et al., 2022).

2.1.3. Antibiotics from *Streptomyces*

Nowadays *Streptomyces* are exploited for both commercial and medical antibiotics, they perform splendidly in these contexts. In some cases, same antibiotics have been produced by several species of *Streptomyces* and in other cases different (e.g., actinomycin and streptothricin) antibiotics are produced by one species (e.g., *S. hygroscopicus* and *S. griseus*). Antibiotics are classified into various types based on their chemical structure, mode of action, and spectrum of activity. Types of antibiotics from *Streptomyces* are glycopeptides, ansamycins (rifamycin), aminoglycosides (such as tobramycin, streptomycin, gentamicin kanamycin, and neomycin), anthracyclines (doxorubicin), β-lactams (carbapenems cephalosporin, and monobactams), macrolides (erythromycin, azithromycin, and clarithromycin), polyenes, nucleosides, peptides, tetracyclines, and polyesters (Alam et al., 2022). Almost 173 new compounds were discovered between 2015 to 2020 from terrestrial *Streptomyces*, majority of compounds exhibit antibiotic properties (Donald et al., 2022). The first antifungal antibiotic, polyene macrolide was discovered from *Streptomyces* species in the late 1950s. Amphotericin B is another polyene antifungal compound used as the standard medication for fungal infections. Antibiotics grouped under class morpholine and nucleosides analogue inhibit the synthesis of sterol and target the synthesis of DNA respectively (Ngamcharungchit et al., 2023). Cephamycin, tetracycline, monensin, kanamycin, chloramphenicol, mitomycin C, spectinomycin, fluorometabolites, polypeptide, avermectin, and streptomycin are some of the important antibiotics derived from *Streptomyces* species such as *S. clavuligerus, S. aureofaciens, S. cinnamonensis, S. kanamyceticus, S. venezuelae, S. lavendulae, S. spectablis, S. cattleya, S. avermitilis* and *S. griseus* respectively. Likewise a new soil *S. parvulus* strain MARS-17 isolated from Rajshahi, Bangladesh secretes actinomycin D (Alam et al., 2022).

2.1.4. Anti-obesity compounds from *Streptomyces*

Obesity is one of the major lifestyle diseases affected in both developed and developing countries. WHO explained obesity as one of the most neglected and blatantly visible health problems (Haslam and James, 2005). Natural products from microbial origin especially from *Streptomyces* species have a great effect in controlling the fat deposition and lipase inhibitory activity, thereby reducing the risk of obesity. In previous reports, *Streptomyces toxytricini,*

produces a potent pancreatic and gastric lipase inhibitor known as orlistat which diminishes the dietary fat absorption (Ibrahim, 2014; Lunagariya et al., 2014). Actinomycetes strain G7-GI (similar to *Streptomyces aburaviensis*) releases Ebelactone A and B a potent inhibitor of hog pancreatic lipase (Lunagariya et al., 2014). Panclicins and esterastin are another two anti-obesity compounds isolated from *Streptomyces sp.* NR0619 (Mohamed et al., 2014; Yun, 2010; Birari and Bhutani, 2007) and *Streptomyces lavendulae* respectively. Both valilactone and ebelactones derived from *Streptomyces albolongus* exhibit inhibitory activity against pancreatic lipase (Yun, 2010). Previous studies mentioned that *Streptomyces sp*. TK08330 produces borrelidin an 18-membered macrolide. In 3T3-L1 (fibroblast) cells, it inhibits the differentiation of adipocytes. Borrelidin reduced the PPARγ protein levels in adipocyte cells (Matsuo et al., 2015).

2.1.5. Other activities

Compounds produced from *Streptomyces* strains have various biological activities that are utilized to treat various diseases. Using natural products for controlling diseases is much more efficient, safe, and cost-effective. Compounds such as Coronamycins, Trioxacarcins, Munumbicins E-4 and E-5, Munumbicin D, Metacycloprodigiosin, Kakadumycin A, Gancidin-W, Antimycin A18, bafilomycin A1 from *Streptomyces* sp. MSU-2110, *Streptomyces ochraceus*, *Streptomyces* NRRL 3052, *Streptomyces* NRRL 30562, *Streptomyces spectabilis* BCC 4785, *Streptomyces* sp. NRRL 30566, *Streptomyces* sp. SUK 10, *Streptomyces albidoflavus* and *Streptomyces* sp. CS respectively. All these biological compounds from *Streptomyces* possess antimalarial activity (Ahmad et al., 2017). Exopolysaccharide produced by marine *Streptomyces* sp. NRCG4 have COX-2 inhibitory effect and promising antioxidant property. Exopolysaccharides serve as raw material for the treatment of Alzheimer's (Mahmoud et al., 2023).

Streptomyces peucetius is the best source of antitumor agent, Adriamycin. It inhibits the replication of DNA and is a potent anticancer drug. Bioactive compounds such as actinomycin D from *Streptomyces verticillus*, bleomycin from *Streptomyces peucetius*, anthracyclines (daunorubicin), and mitosanes (mitomycin C) isolated from *S. caespitosus* are effective cancer chemotherapeutics (Ngamcharungchit et al., 2023). Antiviral metabolites are also extracted from *Streptomyces* spp. one of the examples of such bioactive compound is Antimycin A derived from *Streptomyces kaviengensis.* Recent reports of a compound, Ivermectin shows effective inhibitor activity against SARS-CoV-2 (Ngamcharungchit et al., 2023).

2.1.6. Importance of *Streptomyces* in Agriculture: As Plant Growth Promoting Rhizobacteria (PGPR)

Term rhizobacteria were introduced in 1978 by Kloepper and Schroth to a bacterial community present in the soil that colonizes the plant root system, stimulates plant growth, and reduces the incidence of disease. In 1981 Kloepper and Schroth termed this group of beneficial bacteria as plant growth-promoting rhizobacteria (PGPR). PGPR is considered as a significant factor for the management of agriculture practices in an eco-friendly manner with innate genetic potential. PGPR are bacterial strains that fulfill at least two criteria of the following such as plant growth stimulation, rapid colonization, and biocontrol (Bhattacharyya & Jha, 2011).

For the past few years, researchers have been searching for PGPR and its mode of action to develop commercial biofertilizers using potential PGPR strains. PGPR strains promote growth of host plants by direct and indirect mechanism (**Figure 2.2**). The direct mechanism of PGPR includes producing phytohormones like IAA, gibberellins, and cytokinin, solubilizing minerals such as iron and phosphorus, siderophore production, and ammonia production. The indirect mechanism involves biocontrol including the synthesis of enzymes to hydrolyse cell wall of fungus, antibiotic production, and competition in rhizosphere for niches (Bhattacharyya & Jha, 2011). *Streptomyces* strains are efficient PGPR, they can stimulate the growth of the plants. Several studies revealed that the inoculation of *Streptomyces* strains in Arabidopsis plant resulted in increased biomass. It was also reported that *Streptomyces* strains inoculated crop plants like rice, sorghum, wheat, and tomato exhibited increased Biomass (Viaene et al., 2016).

Streptomyces spp. own special role in plant health, where they produce metabolites that promote plant growth, enhance uptake of nutrients, reduce stress, induce plant resistance, and prevent pests or pathogen attack through insecticidal or nematicidal activities (Dow et al., 2023). *Streptomyces* also produces compounds having herbicidal properties some of the structurally unrelated groups of herbicides are Herbimycins, herbicidins, bialaphos, oxetin, toyocamycin, and anisomycin (Deshpande et al., 1988)

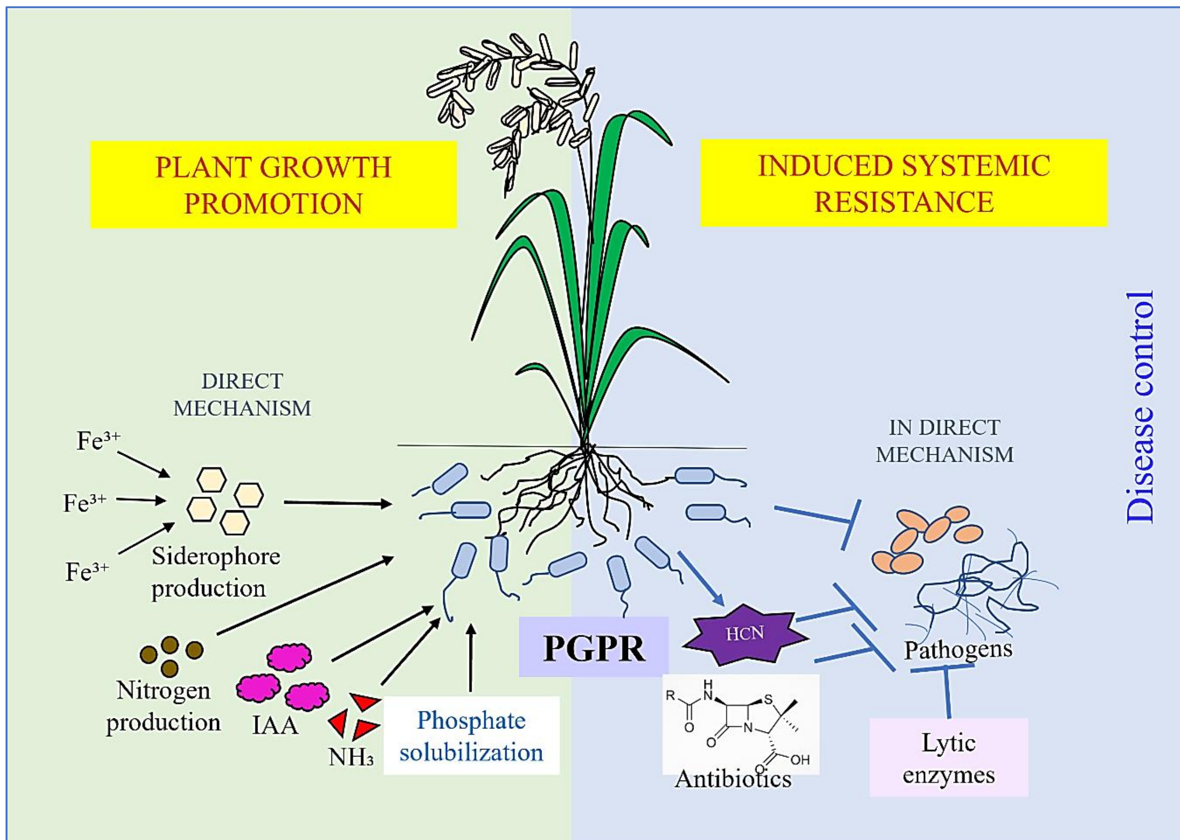


Figure 2.2. Mode of action of Plant Growth Promoting Rhizobacteria (PGPR) in controlling plant diseases and plant growth promotion of host plants by direct and indirect mechanism. (Direct mechanism includes plant growth promotion by producing phytohormones, solubilizing minerals, siderophore production, and ammonia production. Indirect mechanism involves biocontrol of pathogens by the production of enzymes and antibiotics).

2.1.7. Induction of systemic resistance in plants

Induction of plants defence pathway is another mechanism usually associated with disease control in plants by beneficial bacterial strains. This mechanism occurs due to the release of elicitors by the bacteria such as antibiotics, volatiles, and proteins. Elicitors can trigger the expression of defence-related genes and induce the jasmonic acid or ethylene (JA/ET), and salicylic acid (SA) pathways in plants. Induced systemic resistance (ISR) is a broad-spectrum resistance against a wide range of pathogens as well as abiotic stress. ISR is a primed state of resistance to abiotic or biotic stimulus which requires less energy when compared to full induction (O'Brien, 2017). Accordingly, ISR triggered by *Streptomyces* AcH 505 in oak (*Quercus sp.*) against powdery mildew also depends on JA/ET and SA pathways. In Arabidopsis, activation of either JA/ET or SA pathway was noticed when inoculated with endophytic *Streptomyces* strain (Viaene et al., 2016).

2.1.8. *Streptomyces* spp. control rice bacterial leaf blight

Bacterial leaf blight (BLB) is a serious rice disease caused by the bacterial pathogen *Xanthomonas oryzae pv. oryzae* (*X oryzae*). Pathogen enters through the hydathodes or wounds, actively multiplies inside the plant, and moves through xylem vessels, resulting in symptoms of rice blight disease (He et al., 2010). Epidemic potential of BLB can lead to severe loss in rice production. Generally, BLB disease management is through chemical control method, but the effectiveness is fully dependent on strain, due to resistance gained by the pathogen over chemicals. Microbes are much more effective in controlling disease incidence. Because they act via various mode of action to control plant pathogens, therefore nowadays microbes are commonly applied to crops to inhibit pathogens. Microbial biocontrol agents can inhibit pathogens through the production of secondary metabolites with antimicrobial properties (Shi et al., 2021).

Microbes and their metabolites gained researcher's attention as effective agents for plant protection because they reduce problems of environmental pollution caused by the use of chemical pesticides and fungicides (Prabavathy et al., 2006; Pacios-michelena et al., 2021). Microbial biocontrol is an effective 'green' alternative to agrochemicals for the management of weeds and plant pathogens (Dow et al., 2023). *Streptomyces* are effective biocontrol agents against rice disease BLB. Previous studies reported that soil *Streptomyces bottropensi*, isolated from Imsil-gun in South Korea which produce antibiotics Dunaimycin D3S and Bottromycin A2 which is effective against BLB. Another bioactive compound staurosporine produced from *Streptomyces sp*. MJM4426 protects the explant of rice (leaf) from *X oryzae* infection. Similarly, *Streptomyces toxytricini* VN08-A-12 can be able to reduce the lesion length of BLB infection and reduces yield loss (Shi et al., 2021)

2.2. References

Ahmad, S. J., Abdul Rahim, M. B. H., Baharum, S. N., Baba, M. S., & Zin, N. M. (2017). Discovery of antimalarial drugs from *Streptomycetes* metabolites using a metabolomic approach. Journal of tropical medicine, 2017.

Alam, K., Mazumder, A., Sikdar, S., Zhao, Y. M., Hao, J., Song, C., ... & Li, A. (2022). *Streptomyces*: The biofactory of secondary metabolites. Frontiers in Microbiology, 13, 968053.

Anderson, A. S., & Wellington, E. M. (2001). The taxonomy of *Streptomyces* and related genera. International journal of systematic and evolutionary microbiology, 51(3), 797-814.

Arya, A., Nahar, L., Khan, H. U., & Sarker, S. D. (2020). Anti-obesity natural products. In Annual Reports in Medicinal Chemistry 1(55). 411-433.

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., Clément, C., Ouhdouch, Y., & van Wezel, G. P. (2016). Taxonomy, Physiology, and Natural Products of Actinobacteria. Microbiology and Molecular Biology Reviews, 80(1), 1–43.

Bhattacharyya, P. N., & Jha, D. K. (2011). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. 28, 1327–1350.

Birari, R. B., & Bhutani, K. K. (2007). Pancreatic lipase inhibitors from natural sources : unexplored potential. Drug discovery today. 12 (19-20), 879–889.

O'Brien, P. A. (2017). Biological control of plant diseases. Australasian Plant Pathology, 46, 293-304.

Deshpande, B. S., Ambedkar, S. S., & Shewale, J. G. (1988). Biologically active secondary metabolites from *Streptomyces*. Enzyme and microbial technology, 10(8), 455-473.

Donald, L., Pipite, A., Subramani, R., Owen, J., Keyzers, R. A., & Taufa, T. (2022). *Streptomyces*: Still the biggest producer of new natural secondary metabolites, a current perspective. Microbiology Research, 13(3), 418-465.

Dow, L., Gallart, M., Ramarajan, M., Law, S. R., & Thatcher, L. F. (2023). *Streptomyces* and their specialised metabolites for phytopathogen control–comparative in vitro and in planta metabolic approaches. Frontiers in Plant Science, 14, 1151912.

Flärdh, K., & Buttner, M. J. (2009). *Streptomyces morphogenetics*: dissecting differentiation in a filamentous bacterium. Nature Reviews Microbiology, 7(1), 36-49.

Haslam, D. W., & James, W. P. T. (2005). Obesity. Lancet, 366, 1197–1209.

He, Y. W., Wu, J. E., Cha, J. S., & Zhang, L. H. (2010). Rice bacterial blight pathogen *Xanthomonas oryzae pv. oryzae* produces multiple DSF-family signals in regulation of virulence factor production. BMC microbiology, 10, 1-9.

Hiltner, J. K., Hunter, I. S., & Hoskisson, P. A. (2015). Tailoring specialized metabolite production in *Streptomyces.* Advances in applied microbiology, 91, 237-255.

Lunagariya, N. A., Patel, N. K., Jagtap, S. C., & Bhutani, K. K. (2014). Inhibitors of pancreatic lipase: state of the art and clinical perspectives. EXCLI journal, 13, 897.

Mahmoud, M. G., Awady, M. E. E., Selim, M. S., Ibrahim, A. Y., Ibrahim, F. M., & Mohamed, S. S. (2023). Characterization of biologically active exopolysaccharide produced by *Streptomyces sp.* NRCG4 and its anti-Alzheimer efficacy: in-vitro targets. Journal of Genetic Engineering and Biotechnology, 21(1), 76.

Mohamed, G. A., Ibrahim, S. R., Elkhayat, E. S., & El Dine, R. S. (2014). Natural anti-obesity agents. Bulletin of faculty of pharmacy, Cairo University, 52(2), 269-284.

Matsuo, H., Kondo, Y., Kawasaki, T., Tokuyama, S., & Imamura, N. (2015). Borrelidin isolated from *Streptomyces sp*. inhibited adipocyte differentiation in 3T3-L1 cells via several factors including GATA-binding protein 3. Biological and Pharmaceutical Bulletin, 38(10), 1504-1511.

Ibrahim, S. R., Elkhayat, E. S., & El Dine, R. S. (2014). Natural anti-obesity agents. Bulletin of Faculty of Pharmacy, Cairo University, 52(2), 269-284.

Ngamcharungchit, C., Chaimusik, N., Panbangred, W., Euanorasetr, J., & Intra, B. (2023). Bioactive metabolites from terrestrial and marine actinomycetes. Molecules, 28(15), 5915.

Pacios-Michelena, S., Aguilar Gonzalez, C. N., Alvarez-Perez, O. B., Rodriguez-Herrera, R., Chávez-González, M., Arredondo Valdes, R., & Ilyina, A. (2021). Application of *Streptomyces* antimicrobial compounds for the control of phytopathogens. Frontiers in Sustainable Food Systems, 5, 696518.

Prabavathy, V. R., Mathivanan, N., & Murugesan, K. (2006). Control of blast and sheath blight diseases of rice using antifungal metabolites produced by *Streptomyces sp*. PM5. Biological Control, 39(3), 313-319.

Shi, T., Guo, X., Zhu, J., Hu, L., He, Z., & Jiang, D. (2021). Inhibitory effects of carbazomycin B produced by *Streptomyces roseoverticillatus* 63 against *Xanthomonas oryzae pv. oryzae.* Frontiers in microbiology, 12, 616937.

Viaene, T., Langendries, S., Beirinckx, S., Maes, M., & Goormachtig, S. (2016). *Streptomyces* as a plant's best friend?. FEMS microbiology ecology, 92(8),119.

Yun, J. W. (2010). Possible anti-obesity therapeutics from nature–A review. Phytochemistry, 71(14-15), 1625-1641.

Chapter 3

Isolation, Purification, functional *in vitro* screening, and identification of *Streptomyces* species

3.1. Introduction

Soil harbors a wide range of microbial community, and based on its inhabitants and diversity of niches microbes produces a huge number of various metabolites with biological activities. Those metabolites from microbes including antimicrobial compounds possess significant medical and agricultural importance. In natural forests, soil microbes are essential biotic communities and are responsible for the functioning of the ecosystem. They actively participate in the transformation of nutrients in the ecosystem. Even though microbes are the primary source of diversity in life, still vast group of soil bacteria remain unknown because only a small portion of naturally existing microorganisms can be cultured. A fair amount of research has been done on the diversity of the flora and fauna of the Western Ghats, but fewer studies have been done on the diversity of fungal species and even less on the diversity of bacteria found in this region (Vasudevan et al., 2015). Among culturable soil microbes, *Streptomyces* have the highest ranking because of their ability to produce diverse metabolites that have significant importance to society (Salwan and Sharma, 2020).Thus they are described as microbial cell factories. During the past 80 years, thousands of metabolites with important biological activities, including antibacterial properties, have been discovered; many of these constitute a significant fraction of the antibiotic repertoire used today. Actinomycetes are responsible for producing around 25% (estimated as 80,000–100,000 metabolites) of all microbial bioactive metabolites (Ossai and Nybo, 2022). The discovery of nearly all major classes of antibacterial antibiotics during the "heroic" or "golden era" in the 1940s and early 1950s. It seemed that the primary issues with chemotherapy had been resolved in the 1950s and 1960s. Approximately 70 to 80 percent of all isolated compounds during this time were antibiotics that were found to be mostly obtained from *Streptomyces* species. Their main modes of action were against fungus and bacteria (Berdy, 2005).

This chapter aimed to isolate and investigate *Streptomyces* strains with antimicrobial potential from the soil of Malampuzha forest, Palakkad, Kerala. The study area (Malampuzha forest) is microbiologically underexplored area located in the Western Ghats. In this investigation, we evaluated the broad-spectrum antimicrobial effect of isolates against bacterial (plant and human) and fungal (Plant) pathogens. Furthermore, potential *Streptomyces* strains were identified by molecular analysis.

3.2. Materials and Methods

3.2.1. Materials

3.2.1.1. Chemicals

Different media used for the microbiological studies were procured from HiMedia Laboratories Pvt. Limited, Mumbai, India.

3.2.1.2. Test bacterial pathogen

The bacterial human pathogens for antibacterial studies were *Mycobacterium smegmatis* MTCC 99, *Bacillus cereus* MTCC 1305, *Staphylococcus aureus* MTCC 902, *S. simulans* MTCC 3610, *S. epidermidis* MTCC 435, (all Gram positive) and *Proteus mirabilis* MTCC 425, *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Salmonella typhi* MTCC 3216, *Pseudomonas aeruginosa* MTCC 2642, (all Gram negative). All these pathogens were procured from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India. Bacterial plant pathogens *Ralstonia solanacearum* BI-0011 and *Xanthomonas oryzae* were procured from Indian Type Culture Collections (ITCC), ICAR- Indian Agricultural Research (IARI), New Delhi and Regional Agricultural Research station, Pattambi, Kerala. Bacterial pathogens were grown and maintained on nutrient agar slants and kept for long-term storage at 4°C for further studies.

3.2.1.3. Test fungal pathogens

Fungal plant pathogens for antifungal studies were *Aspergillus nige*r MTCC 2756, *A. flavus* MTCC 183, *Fusarium oxysporum f.sp. pisi* ITCC 4814, *F. oxysporum f.sp. zingiberi* ITCC 2698 and *Rhizoctonia solani*. All these fungal strains were procured from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India, Indian Type Culture Collections (ITCC), New Delhi and Regional Agricultural Research station, Pattambi, Kerala. All the test fungal pathogens were grown maintained on PDA agar slants and kept for studies at 4°C.

3.2.2. Methods

3.2.2.1. Site, sample collection, and isolation of *Streptomyces* strains

Soil samples were collected from the Malampuzha forest (10° 83´78.19" N, 76° 66´19.96" E) of the Western Ghats region in Kerala, India. Soil samples were collected from 5cm - 10cm depth after removal of surface soil. Transferred the collected samples to sterile polythene bags and brought them to the laboratory for isolation. Collected soil was air dried for two to three days (to reduce Gram-negative bacterial population as well as to remove the moisture content) according to the procedure of Rotich et al (2017). 1g of soil was suspended in 9 ml of sterile

distilled water and was vortexed for a few minutes. From this, a wire loop of the suspension was streaked on Nutrient agar (NA), Potato Dextrose Agar (PDA), Yeast Malt Agar (YMA), Kings Medium B base (KMB), and Streptomycetes agar plates (in triplicates) were incubated at 28±2°C for 28 days. Emerging colonies with distinct morphological features were chosen and the purified *Streptomyces* strains were maintained on Streptomycetes agar. The viability of the isolated strains was checked in the above-mentioned media, where PDA was found best for the growth of *Streptomyces* strains, hence further maintenance and storage of the culture was done in PDA at 4°C. *Streptomyces* strains were kept in the presence of glycerol (50 % v/v) at -20°C for longer period of storage.

3.2.2.2. Screening of *Streptomyces* strains for antibacterial activities

3.2.2.2.1. Primary screening

Streptomyces isolates were screened for antibacterial activity by agar overlay technique (Alaadin et al., 2007). A loopful of inoculum (4-day old *Streptomyces* culture) was spot inoculated on the centre of the PDA plates and grown for 5 days at 28±2°C. After incubation (5 days) the PDA plates were covered with NA medium (0.6%) previously seeded separately with two test indicator human bacterial pathogens (*S. aureus* and *E. coli*) and incubated at 37°C to evaluate the antibacterial efficacy of isolates. The activity was noted after 24 h and expressed the zone of inhibition in mm.

3.2.2.2.2. Secondary screening

To test the broad-spectrum antibacterial properties of isolates. After confirming antibacterial activity against the indicator bacteria, potential *Streptomyces* strains were screened against ten human bacterial pathogens by agar overlay method. Isolates that exhibited the best zone of inhibition (Potential strains) were selected for antifungal studies.

3.2.2.3. Identification of potential isolates based on 16SrRNA gene sequencing

3.2.2.3.1. Genomic DNA Isolation

Isolation of the genomic DNA of bacteria was carried out using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. For isolation, 1g *Streptomyces* culture was taken in a microcentrifuge tube. 25 µl of proteinase K and 180 µl of TI buffer were added to the culture and incubated in a water bath at 56°C until cells were completely lysed. After that 5 µl of 100 mg/ml RNase A was added, and it was incubated at room temperature for 5 minutes. After adding 200 µl of B3 buffer, it was incubated at 70°C for 10 minutes. Then, 210 µl of absolute ethanol was added and thoroughly mixed by vortexing. Thereafter, the

mixture was pipetted into NucleoSpin® Tissue column which was then placed in a 2 ml collection tube. The mixture was then centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to 2 ml tube (new tube) and washed using 500 µl BW buffer. repeated washing using 600 µl of B5 buffer. Afterward, 50 µl BE buffer was used to elute the DNA from the NucleoSpin® Tissue column, which was placed in a clean 1.5 ml tube.

3.2.2.3.2. Agarose Gel Electrophoresis for DNA quality and quantity check

Isolated DNA quality was analyzed using agarose gel electrophoresis. To 5µl of DNA, 1 µl of gel-loading buffer (30% sucrose in TE buffer pH 8.0, 0.25% bromophenol blue) was added. agarose gel (0.8%) prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide was used to load samples. At 75 V, to perform electrophoresis, 0.5X TBE was used as the buffer until the front of the bromophenol dye moved to the bottom of the gel. A UV transilluminator (Genei) was used to visualize the gels and under UV light, the image was captured using a Gel documentation system (Bio-Rad).

3.2.2.3.3. PCR analysis

Polymerase Chain Reaction (PCR) contains 10 µl reaction volume composed of 1X PCR buffer (100mM Tris HCl, pH-8.3; 500mM KCl), 2.5mM MgCl2, 0.2mM each dNTPs (dATP, dGTP, dCTP, and dTTP), 1 unit of AmpliTaq Gold DNA polymerase enzyme, 4% DMSO, 0.1 mg/ml BSA, 5 pM of forward and reverse primers and DNA template. The following procedures were used to carry out the PCR amplification in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems): pre-denaturation for five minutes at 95°C, 35 cycles of denaturation for thirty seconds at 95°C, annealing for forty seconds at 60°C, extension for sixty seconds at 72°C, and a repair extension for seven minutes at 72°C.

Table 3.1. Primers used**.**

Target	Primer Name	Direction	Sequence (5’ → 3’)
16S rRNA	16S-RS-F	Forward	GGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

3.2.2.3.4. Agarose Gel electrophoresis of PCR product

PCR products were checked in agarose gels (1.2%) prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. To 5 µl of PCR products, 1 µl of 6X loading dye was added, mixed,

and loaded then at 75V power supply electrophoresis was performed for about 1-2 h with 0.5X TBE as electrophoresis buffer, until the bromophenol blue front had migrated to the bottom of the gel. A 2-log DNA ladder (NEB) was used as the molecular standard. After that, using UV transilluminator (Genei) visualized the gels, and the image was captured using a Gel documentation system (Bio-Rad) under UV light.

3.2.2.3.5. ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the unwanted dNTPs and primer removal from the mixture of PCR product with no interference in downstream applications. 2 µl of ExoSAP-IT added to 5 µl of PCR product, mixed well and incubated for 15 minutes at 37°C and is followed by enzyme inactivation for 15 minutes at 80°C.

3.2.2.3.6. Sequencing using BigDye Terminator v3.1

Using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) and the manufacturer's instructions, the sequencing reaction was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). PCR mix has the following components: 3.2 pM primer (either Forward or Reverse), 10-20 ng of ExoSAP treated PCR product, 0.28 µl Sequencing Mix, and 1.86 µl Reaction buffer. Using Sterile distilled water, the mix was made up to 10µl. Sequencing PCR temperatures consist of first cycle at 96°C for 2 minutes and is followed by 30 cycles for 30 sec at 96°C, 50°C for 40 sec, and 60°C for 4 minutes.

3.2.2.3.7. Post Sequencing PCR Clean up

Master mixes I and II were prepared using 10µl of milli Q and 2 µl of 125mM EDTA per reaction and 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol, respectively. Then, 12 µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed. To each reaction, 52 µl of master mix II was added. Mixed the content by inverting and incubated for 30 minutes at room temperature and spun for 30 minutes at 14,000 rpm. Decant the supernatant and 100 µl of 70% ethanol was added and spun for 20 minutes at 14,000 rpm. Decanted the supernatant and repeated (70%) ethanol wash. The supernatant was again decanted, and the obtained pellet was air-dried. Cleaned-up- air-dried product was then sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

3.2.2.3.8. Sequence Analysis

Quality of the sequence was analyzed by Sequence Scanner Software v1 (Applied Biosystems). Geneious Pro v5.6 were used for alignment of Sequence (Drummond et al., 2012).

3.2.2.3.9. Phylogenetic tree construction of selected strains

Under designated Accession numbers, the 16S rRNA sequence was submitted to the GenBank data library. Using MEGA version 11, the sequences were compared to other similar sequences that were obtained from the GenBank data source to create the phylogenetic tree (Tamura et al., 2021). The multiple sequence alignment tool ClustalW was used to align the sequences, and then the neighbour-joining method was used to examine the data and create bootstrap values based on 100 replicates.

3.3. Results

3.3.1. Site, sample collection

Sampling site is the Malampuzha forest in Kerala (**Figure. 3.1, Figure. 3.2**). From the forest area, 16 soil samples were collected. Soil samples were aseptically processed in various media for the isolation of *Streptomyces* strains.

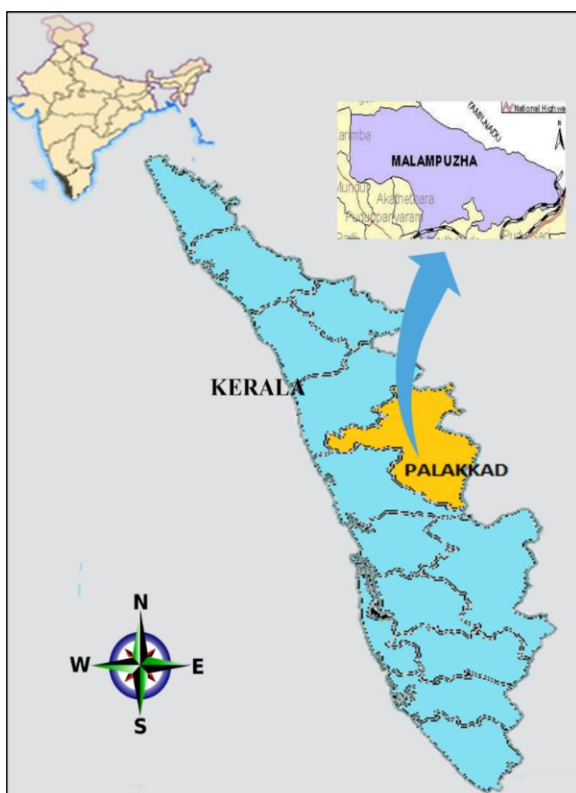


Figure 3.1. location of sample collection.

Figure 3.2. Image of Malampuzha forest.

3.3.2. Isolation of *Streptomyces* from soil samples

From collected soil samples 300 morphologically different *Streptomyces* strains were isolated in various media. Isolated strains were repeatedly cultured, and pure cultures thus obtained were maintained in PDA slants for further studies.

3.3.3. Screening of *Streptomyces* strains for their antibacterial activities

3.3.3.1. Primary screening

Primary screening of isolated *Streptomyces* strains with two pathogens as indicator microorganisms such as *E. coli*, Gram-negative, and *S. aureus*, Gram-positive. From isolated strains, 96 (32 %) *Streptomyces* strains showed antibacterial activity against *E. coli.* while 143 (47.33 %) exhibited activity against *S. aureus*. Of these, 22 (7.33 %) strains inhibit both the pathogens. The morphology of 22 antagonistic strains in PDA was illustrated in **Figure 3.3.**

3.3.3.2. Secondary screening

The twenty-two strains that exhibited activity against both (indicator pathogen) organisms in primary screening were taken for the secondary screening with ten bacterial human pathogens. Among them, NIIST D31 inhibits all test pathogenic bacteria (broad spectrum activity) *S. simulans* was the most inhibited (50.66±1.15 mm) followed by *E. coli* (46.00±1.00 mm) whereas *P. aeruginosa* (24.66±2.00 mm) (**Figure 3.4, Table 3.2**) was the least inhibited.

3.3.4. Antimicrobial activity against plant pathogens using live *Streptomyces* strains

In vitro, antagonism of isolated *Streptomyces* against fungal and bacterial pathogens with live *Streptomyces* strains inhibits various plant pathogens. *Streptomyces* strains NIIST D27 and NIIST D72 exhibit antifungal activity of more than 40 mm against test fungal pathogens. Compared to other isolates, NIIST D27 exhibited maximum zone of inhibition against *Aspergillus flavus* (43.66±0.57 mm), *Aspergillus niger* (54.66±0.57 mm). This strain also inhibits plant bacterial pathogens, *Xanthomonas oryzae* (74.33±0.57mm) *and Ralstonia solanacearum* (19.33±1.15mm). Similarly, another strain NIIST D72, inhibits all the plant pathogens tested including bacterial and fungal plant pathogens. NIIST D72 shows antagonistic activity by producing a zone of inhibition against *Aspergillus flavus* (26.66±0.57mm), *Aspergillus niger* (40.33±0.57mm), *Fusarium oxysporum f.sp. pisi* (59.66±0.57mm), *Fusarium oxysporum f.sp. zingiberi* (65.66±1.15mm), *Ralsotina solanacearum* (42.66±2.08mm), *Xanthomonas oryzae* (51.00±1.00mm), and *Rhizoctonia solani* (36.33±0.57mm) **(Figure. 3.5, Table. 3.3).** Moreover, these two strains having broad-spectrum antimicrobial activity against plant pathogens.

Figure 3.3. Antagonistic strains grown in potato dextrose agar (PDA) medium.

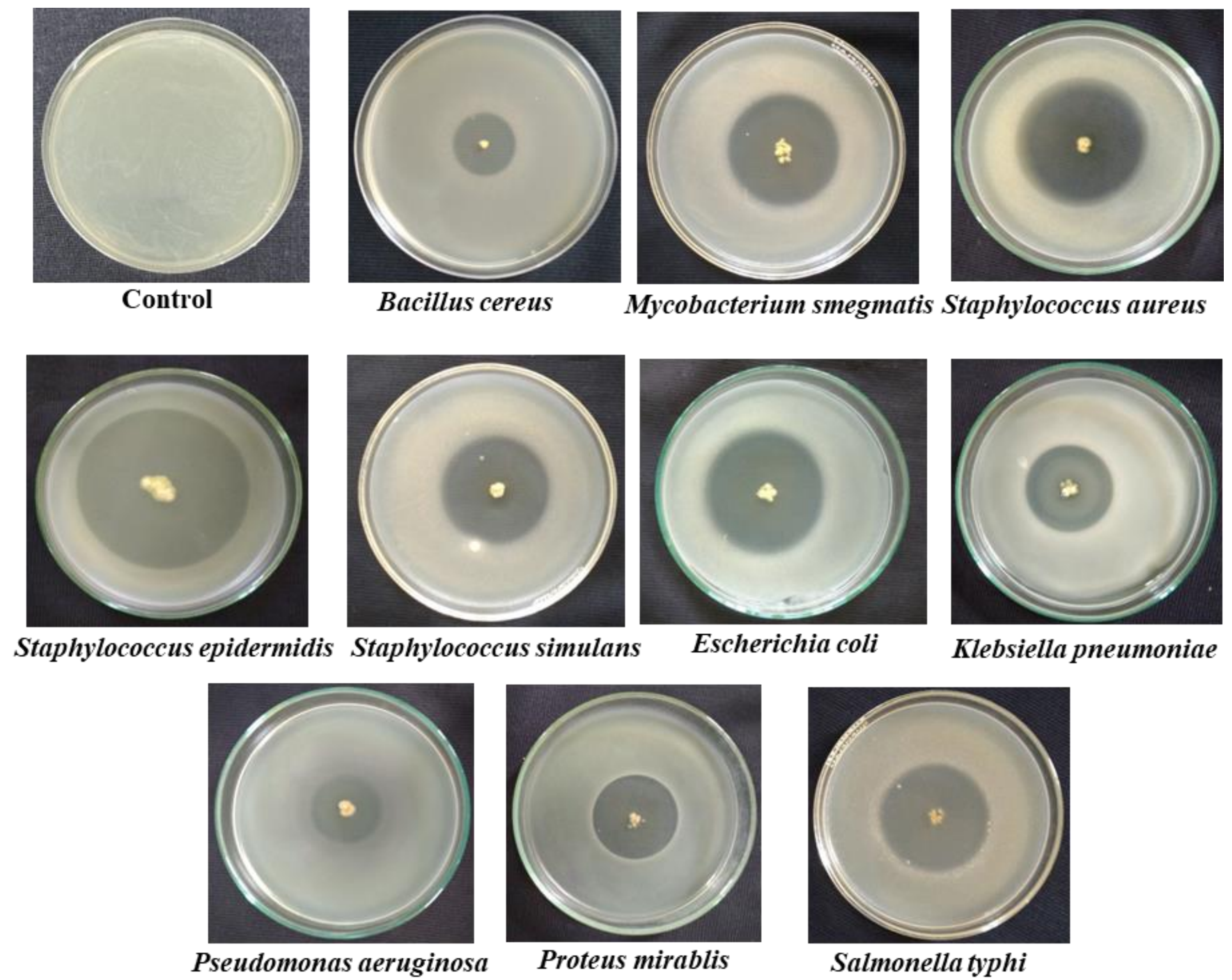


Figure 3.4. Broad spectrum antibacterial activity of NIIST D31 against human pathogens

Table 3.2. Antagonistic activity of Streptomyces strains through agar overlay method against human bacterial pathogen.

Strains	***Zone of inhibition in mm***									
	B. cereus	***M. smegmatis***	***S. aureus***	***S. epidermis***	***S. simulans***	***E. coli***	***K. pneumonia***	***P. mirablis***	***P. aeruginosa***	***S. typhi***
NIIST D6	45.66±2.30	21.66±1.15	16.66±0.57	13.66±1.15	9.33±0.57	29.00±1.00	36.00±1.00	14.66±0.57	0.00	0.00
NIIST D21	24.33±0.57	28.66±0.57	22.66±0.57	21.33±0.57	0.00	33.33±1.15	27.66±0.57	0.00	0.00	26.00±1.00
NIIST D25	24.66±0.57	23.00±2.64	39.33±0.57	38.00±1.73	26.6±0.57	20.33±0.57	36.33±0.57	11.33±0.57	0.00	50.00±1.00
NIIST D27	20.66±0.57	50.66±1.52	35.66±0.57	18.33±0.57	46.33±0.57	30.66±0.57	22.33±1.52	33.66±1.52	0.00	29.66±0.57
NIIST D31	**25.00±1.00**	**43.00±1.00**	**43.08±2.00**	**40.33±0.57**	**50.66±1.15**	**46.00±1.00**	**41.33±1.52**	**35.33±0.57**	**24.66±2.00**	**40.33±1.57**
NIIST D33	17.6±0.57	0.00	15.33±1.52	0.00	31.33±0.57	39.33±0.57	0.00	50.66±0.57	0.00	0.00
NIIST D34	35.00±1.00	0.00	22.66±1.52	22.33±0.57	24.66±2.51	39.00±1.00	0.00	22.66±0.57	0.00	34.33±0.57
NIIST D39	13.00±1.00	25.33±0.57	17.33±1.52	14.00±1.00	21.66±0.57	14.33±0.57	15.33±0.57	17.33±1.52	0.00	21.33±0.57
NIIST D41	31.33±1.15	30.66±0.57	16.33±0.57	9.66±0.57	21.66±0.57	53.66±1.52	25.00±1.00	14.33±0.57	0.00	24.66±1.10
NIIST D43	13.00±1.00	13.33±1.15	14.66±0.57	0.00	0.00	11.66±1.15	0.00	0.00	0.00	0.00
NIIST D47	15.33±0.57	0.00	12.66±0.57	0.00	0.00	11.66±0.57	14.33±0.57	17.66±0.57	10.66±0.57	17.00±1.00
NIIST D50	26.00±1.00	15.00±1.00	23.00±2.00	23.33±1.52	51.33±1.52	25.33±0.57	28.33±0.57	14.00±1.00	0.00	27.66±1.52
NIIST D52	12.00±1.00	11.33±0.57	11.33±0.57	12.66±0.57	0.00	11.66±0.57	13.00±1.00	14.00±1.00	0.00	11.66±0.57
NIIST D65	0.00	0.00	27.66±0.57	20.66±0.57	0.00	13.00±1.00	27.66±0.57	0.00	0.00	0.00
NIISTD70	20.66±0.57	14.33±1.15	12.66±0.57	27.33±0.57	33.66±1.15	27.33±0.57	21.00±1.00	0.00	0.00	20.33±0.57
NIIST D72	50.66±0.57	31.33±1.52	36.33±0.57	37.00±1.00	18.33±0.57	26.66±1.52	49.66±1.15	40.66±0.57	0.00	20.00±1.00
NIIST D75	48.33±0.57	41.00±1.00	8.33±0.57	0.00	36.33±0.57	39.66±0.57	11.33±0.57	44.00±1.00	9.00±0.57	36.33±0.57
NIIST D80	14.66±1.15	12.33±0.57	10.00±1.00	15.00±1.00	12.33±0.57	11.66±0.57	10.66±0.57	17.66±0.57	0.00	15.66±1.52
NIIST D82	16.66±0.57	33.00±1.73	12.33±0.57	26.66±0.57	30.66±0.57	30.66±0.57	11.66±1.15	0.00	0.00	21.66±0.57
NIIST D90	19.00±1.00	19.66±0.57	12.00±1.00	20.33±0.57	0.00	20.00±1.00	12.33±0.57	12.66±0.57	0.00	21.66±1.15
NIIST D110	12.66±1.15	0.00	22.66±0.57	0.00	0.00	13.33±1.52	0.00	0.00	0.00	0.00
NIIST D210	0.00	0.00	14.66±1.52	0.00	0.00	11.00±1.00	0.00	0.00	0.00	0.00

Values are readings of three independent experiments expressed as mean± Standard deviation in millimetre.

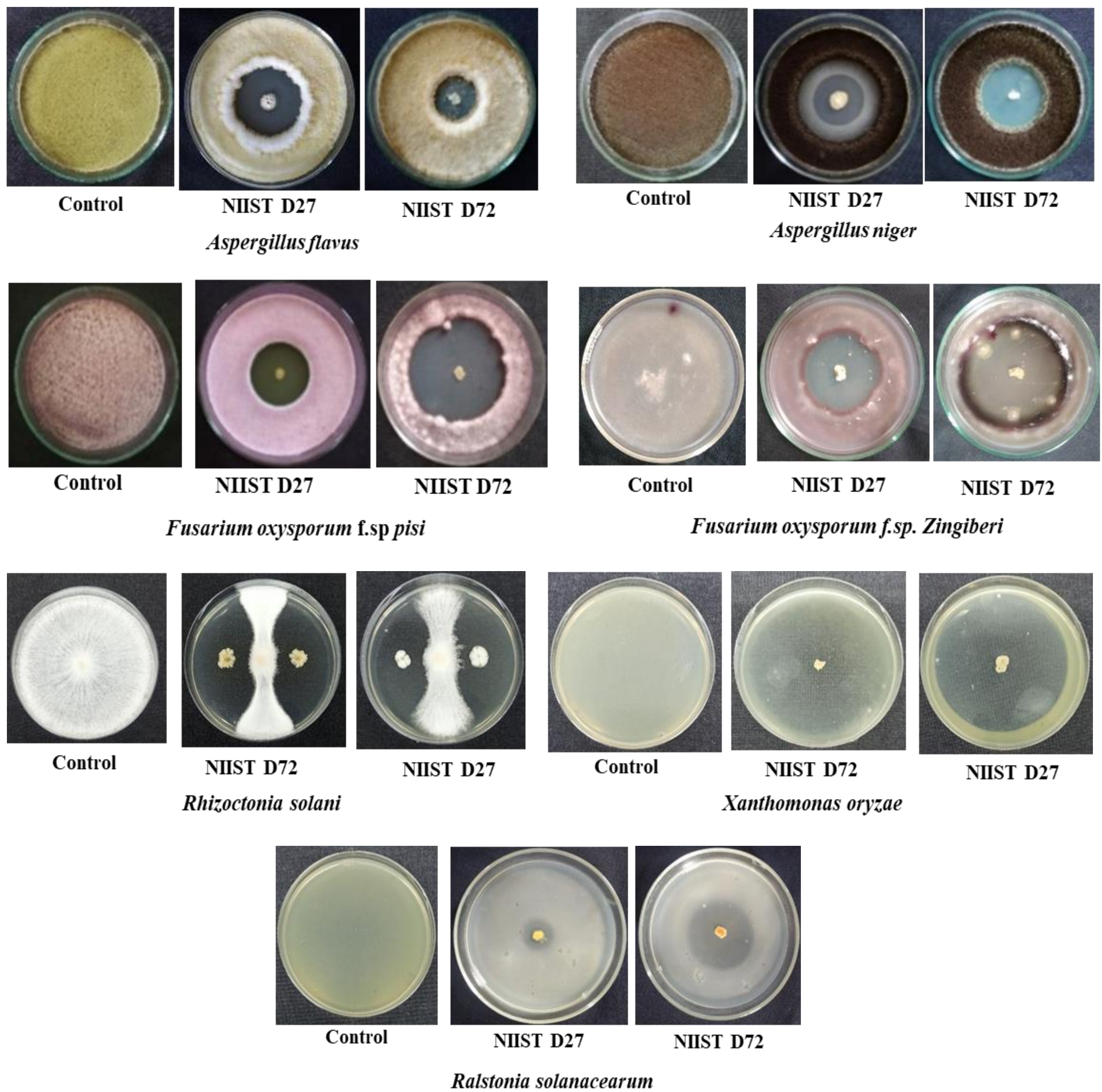


Figure 3.5. *In vitro* antagonism of NIIST D27 and NIIST D72 against plant pathogens

Table 3.3. Zone of inhibition recorded by the isolates against plant fungal and bacterial pathogen.

Isolates	Zone of inhibition in mm						
	Aspergillus flavus	***Aspergillus Niger***	***Fusarium oxysporum f.sp. pisi***	***Fusarium oxysporum f.sp. Zingiberi***	***Ralstonia solanacearum***	***Xanthomonas oryzae***	***Rhizoctonia solani***
NIIST D6	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NIIST D21	0.00	0.00	0.00	0.00	15.33±0.57	0.00	0.00
NIIST D25	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NIIST D27	43.66±0.57	54.66±0.57	35.00±1.00	51.33±1.15	19.33±1.15	74.33±0.57	34.66±0.57
NIIST D31	6.67±2.88	20.00±1.00	21.66±0.57	0.00	0.00	38.66±1.15	27.66±0.57
NIIST D33	0.00	0.00	0.00	0.00	13.66±2.30	0.00	0.00
NIIST D34	25.66±0.57	30.66±0.57	22.66±0.57	16.66±0.57	0.00	0.00	0.00
NIIST D39	0.00	15.66±0.57	15.33±0.57	0.00	0.00	0.00	0.00
NIIST D41	0.00	0.00	25.33±0.57	0.00	0.00	0.00	0.00
NIIST D43	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NIIST D47	15.00±1.00	16.66±2.08	25.33±0.57	20.66±0.57	0.00	20.66±1.52	21.66±0.57
NIIST D50	30.33±0.57	39.33±0.57	32.33±0.57	34.33±1.15	0.00	23.66±0.57	19.00±1.73
NIIST D52	0.00	19.00±1.00	20.00±1.00	0.00	0.00	14.33±0.57	0.00
NIIST D65	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NIIST D70	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NIIST D72	26.66±0.57	40.33±0.57	59.66±0.57	65.66±1.15	42.66±2.08	51.00±1.00	36.33±0.57
NIIST D75	0.00	29.66±0.57	33.66±0.57	0.00	0.00	33.66±1.52	26.66±1.52
NIIST D80	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NIIST D82	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NIIST D90	0.00	32.66±0.57	0.00	34.66±1.52	0.00	30.66±0.57	0.00
NIIST D110	32.66±0.57	39.33±1.15	31.33±0.57	28.00±2.64	0.00	0.00	0.00
NIIST D210	0.00	22.66±0.57	27.33±0.57	0.00	0.00	0.00	0.00

Values are readings of three independent experiments and expressed as mean± Standard deviation in millimetre.

3.3.5. Molecular identification of promising strains by 16srRNA sequencing

Potential strain NIIST D31 exhibits broad-spectrum antibacterial activity against all human pathogens tested and two strains (NIIST D27 and NIIST D72) which exhibited significant antimicrobial activity against all test plant pathogens were analysed phylogenetically. Using ClustalW software, the 16SrRNA sequence of potential isolates and related sequences that were obtained from GeneBank were aligned. Neighbour joining method was employed in MEGA 11 software to construct the phylogenetic tree. Phylogenetic analysis showed that the strain NIIST D27 is identified as *Streptoverticillium reticulum* (99.74% similarity), NIIST D72 as *Streptomyces rimosus* (similarity is 99.74%) **(Figure 3.6)** and NIIST D31 as *Streptomyces luteireticuli* **(Figure 3.7)** (with 99.5% similarity).

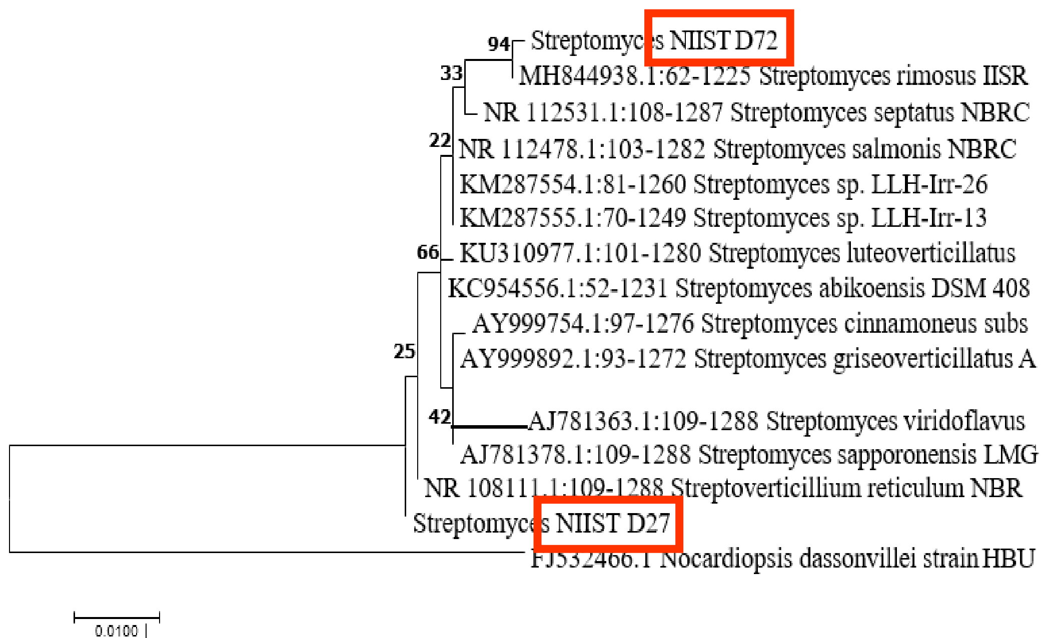


Figure 3.6. Phylogenetic tree of *Streptomyces* strain NIIST D27 and *Streptomyces* NIIST D72 strain.

Figure 3.7. Phylogenetic tree of *Streptomyces* NIIST D31 strain

3.3.5.1. 16SrRNA sequence of NIIST D27 and NIIST D72 with accession number

> ON054036. ***Streptoverticillium reticulum* strain NIIST D27**.16S ribosomal RNA gene, partialsequence**.**ACACGTGGGCAATCTGCCCTGCACTACTGGGACAAGCCCTGGAAA CGGGGTCTAATACCGGATACGACCTTCGAGCGCATGCTTGAGGGTGGAAAGCTCC GGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTAC CAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGA GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG AAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCT CTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTA ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATT GGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTT AACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGA ATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAG GCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACA GGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAGGTGTGGGCGAC ATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACG GCCGCAA

> ON013964. ***Streptomyces luteireticuli* strain NIIST D31**. 16S ribosomal RNA gene, partialsequence.GAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCT GGAAACGGGGTCTAATACCGGATACGACCGCTGACCGCATGGTTGGTGGTGGAA AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGAT GGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACT GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC ACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCG GGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAA

GAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCG
TTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGT
GAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTC
GGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTT
GGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTA
AGTGCCCCGCCTGGGGAGTACGGCCCGCAAAGGCTAAAACTCAAAGGAAATTG
ACGGGGGCC

> ON054060. ***Streptomyces rimosus*** **strain NIIST D72**. 16S ribosomal RNA gene, partial sequence.GGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACACACGACC
GCATGGTCTGTGTGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATC
AGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAG
AGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTG
AGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGT
GACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGG
CTTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATAC
GGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAAT
GCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACT
GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACGTCGTCCGTGC
CGCAGCTAACGCATTAAGTTCCCCGCCTGGGGGAGTACGGCCGCAAGGCTAAAA
CTCAAAGGAATTTGACGGGGGGG

3.4. Discussion

Actinomycetes are Gram-positive and filamentous bacteria that have similarities with both fungi and bacteria. High G+ C content is one of the peculiarities of their genome. They are widely distributed in nature and are also considered as saprophytic soil microbes. Majority of the soil actinomycetes belong to the *Streptomyces* genus (Khamna et al., 2008). They produce a large number of agriculturally and clinically important secondary metabolites, which include antitumor agents, more than half of naturally available antibiotics, and immunosuppressors commonly used in the medical field (Zhao et al., 2017). *Streptomyces* species are the largest producer of metabolites with antimicrobial nature (Manteca and Yagüe, 2019). Search for new antimicrobial metabolites from *Streptomyces* strains have been increased in recent years. Because there is an increased demand for potential strains (with antimicrobial activity) due to the widespread of resistant pathogenic microorganisms (both plant and human pathogens). Novel antimicrobial metabolites are needed to reduce the pathogen attack, and exploration of unexplored regions is good for isolating active *Streptomyces* strains (Almalki, 2020). Therefore, probability of isolating new and efficient metabolites with bioactive from actinobacteria can be increased.

In the present study, 16 soil samples were collected from the Malampuzha forest, (an unexplored region of Western Ghats) and 300 morphologically different *Streptomyces* strains were isolated from collected samples. These strains were screened for their antimicrobial activity against human and plant pathogens, among them 22 are potential strains having antagonistic properties. The phylogenetic position of potential strains suggested that the strains are belongs to *Streptomyces* genus. Screening of a huge number of isolates is required for the selection of potential strains and is supported by a study where 56 actinomycetes were isolated from the seashore of China from which only one potent strain (*Streptomyces chumphonensis* strain AM-4) was obtained (Hu et al. 2019). It was reported that from 172 isolated actinobacterial strains 96 exhibited antimicrobial activity among them 24 showed strong antimicrobial potential (Das et al., 2018). In a previous report, *Streptomyces sioyaensis exhibited antimicrobial activity against plant and human pathogens including Staphylococcus haemolyticus* MR-CoNS an antibiotic-resistant pathogen (Quinn et al., 2020).

Western Ghat soil is the best source of potential microbial community. In the present study, three potential *Streptomyces* strains active against human and plant pathogens (fungal and bacteria) were isolated from Western Ghat forest soil. Likewise, in an investigation conducted by Arasu et al. (2008) isolated potential *Streptomyces* spp. ERI-3 from the soil (forest rock soil) of the Western Ghats region in Tamil Nadu, India. This strain has potential antimicrobial activity against both fungi and bacteria. Antagonistic strain of the present study was identified using the 16SrRNA gene, which indicated that they were all members of the *Streptomyces* genus. This result is in line with prior research that found that *Streptomyces* sp. recovered more often than other actinobacterial genera with antibacterial activity (Das et al., 2018). Another study reported that from 60 isolates of actinobacteria only one potential strain named JJY4T, obtained which possess broad-spectrum antimicrobial activity. Phenotypic test and phylogenetic analysis of the strain confirmed that the strain is a novel species in the clade *Streptomyces violaceusniger* (Boudjeko et al., 2017). The strains in the present study were identified as *Streptoverticillium reticulum* NIIST D27 strain, *Streptomyces luteireticuli* NIIST D31 strain, and *Streptomyces rimosus* NIIST D72 strain by 16SrRNA gene sequencing. All these strains have antimicrobial properties. Various researchers mentioned about the potential nature of *Streptomyces* species isolated from the Western Ghats. For instance, *Streptomyces sp*. GOS1 from Agumbe, Western Ghats region in Karnataka, *Streptomyces sp*. RAMPP-065 isolated from Kudremukh, exhibited significant antimicrobial activity. Another stain from Kodachadri possess activity against fungal pathogens (Siddharth et al., 2020). From various

research reports, it was clear that *Streptomyces* species have huge potential for the production of novel natural metabolites (Xia et al., 2020).

3.5. Conclusion

In this chapter, 300 *Streptomyces* strains were isolated from the soil of Malampuzha forest. The antibacterial activity of each strain was assessed against two indicator pathogenic bacteria, *S. aureus* and *E. coli.* Out of the 300 strains examined, 22 had antibacterial capabilities against both pathogens and underwent secondary screening against ten human bacterial pathogens. One of these isolates, shows broad-spectrum antibacterial activity against the test bacterial human pathogens, and two strains that inhibit plant pathogens (both bacterial and fungal) were selected for further studies. All three potential strains were identified by 16SrRNA gene sequencing as *Streptomyces luteireticuli* NIIST D31 strain*, Streptoverticillium reticulum* NIIST D27 strain, and *Streptomyces rimosus* NIIST D72 strain respectively. *Streptomyces luteireticuli* NIIST D31 strain was selected for further studies (biomedical application) and the other two were selected for further studies in the agriculture field.

3.6. References

Alaadin, A. M., Al-Khateeb, E. H., & Jäger, A. K. (2007). Antibacterial activity of the Iraqi Rheum ribes root. Pharmaceutical Biology, 45(9), 688–690.

Almalki, M. A. (2020). Isolation and characterization of polyketide drug molecule from *Streptomyces* species with antimicrobial activity against clinical pathogens. Journal of Infection and Public Health, 13(1), 125–130.

Arasu, M. V., Duraipandiyan, V., Agastian, P., & Ignacimuthu, S. (2008). In vitro antimicrobial activity of Streptomyces spp . ERI-3 isolated from Western Ghats rock soil (India). Journal of Mycologie Medicale, 19, 22–28.

Berdy, J. (2005). Bioactive microbial metabolites. The Journal of antibiotics, 58(1), 1-26.

Boudjeko, T., Tchinda, M, A, R., Zitouni, M., Nana, T, V, A, J., & Lerat, S. (2017). *Streptomyces cameroonensis* sp .nov .,a Geldanamycin Producer That Promotes Theobroma cacao4 Growth. Microbes and Environments, 32(1), 24–31.

Das, R., Romi, W., Das, R., Sharma, H. K., & Thakur, D. (2018). Antimicrobial potentiality of actinobacteria isolated from two microbiologically unexplored forest ecosystems of Northeast India. BMC Microbiology, 18(1), 1–16.

Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular biology and evolution, 29(8), 1969-1973.

Hu, X., Cheng, B., Du, D., Huang, Z., Pu, Z., Chen, G., Peng, A., Lu, L., 2019. Isolation and identification of a marine actinomycete strain and its control efficacy against citrus green and blue moulds. Biotechnology & Biotechnological Equipment 33, 719-729.

Khamna, S., Yokota, A., & Lumyong, S. (2008). Actinomycetes isolated from medicinal plant rhizosphere soils: Diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. World Journal of Microbiology and Biotechnology, 25(4), 649–655.

Manteca, Á., & Yagüe, P. (2019). *Streptomyces* as a source of antimicrobials: novel approaches to activate cryptic secondary metabolite pathways. Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods, 119.

Ossai, J., Khatabi, B., Nybo, S. E., & Kharel, M. K. (2022). Renewed interests in the discovery

of bioactive actinomycete metabolites driven by emerging technologies. Journal of applied microbiology, 132(1), 59-77.

Quinn, G. A., Banat, A. M., Abdelhameed, A. M., & Banat, I. M. (2020). *Streptomyces* from traditional medicine: Sources of new innovations in antibiotic discovery. Journal of medical microbiology, 69(8), 1040.

Rotich, M. C. (2018). Bioprospecting for broad spectrum antibiotic producing actinomycetes isolated from virgin soils in Kericho county (Doctoral dissertation, JKUAT-COHES).

Salwan, R., & Sharma, V. (2020). Bioactive compounds of *Streptomyce*s: biosynthesis to applications. Studies in natural products chemistry, 64, 467-491.

Siddharth, S., Vittal, R. R., Wink, J., & Steinert, M. (2020). Diversity and bioactive potential of actinobacteria from unexplored regions of Western Ghats, India. Microorganisms, 8(2), 225.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. Molecular biology and evolution, 38(7), 3022-3027.

Vasudevan, G., Siddarthan, V., & Solai Ramatchandirane, P. (2015). Predominance of *Bacillus sp*. in soil samples of the southern regions of Western Ghats, India. Annals of microbiology, 65(1), 431-441.

Xia, H., Zhan, X., Mao, X. M., & Li, Y. Q. (2020). The regulatory cascades of antibiotic production in *Streptomyces*. World Journal of Microbiology and Biotechnology, 36(1), 13.

Zhao, Y., Liang, Y., Liu, L., Cheng, J., & Yuan, Y. (2017). Medium optimization for antifungal active substance production from *Streptomyces lydicus* using response surface methodology. Transactions of Tianjin University, 23, 78-86.

Chapter 4

Plant growth promotion studies of rice plants inoculated with *Streptoverticillium reticulum* and *Streptomyces rimosus*

4.1. Introduction

Oryza sativa, or Rice is one of the world's most significant agricultural commodities. It mostly grown in Asian nations such as China, India, Bangladesh, Indonesia, Thailand, Vietnam, Philippines, Myanmar, and Japan (Awla et al., 2017). The global population is expected to grow to 9.7 billion people by 2050 and 10.4 billion people by 2080. This creates the huge challenge of feeding the world within the limits of the environment (Shi et al., 2023). Globally, chemical fertilizers are used as primary input for improving rice production. The three nutrients that are most frequently administered for rice cultivation are nitrogen (N), phosphorus (P), and potassium (K). Minerals required for one ton of rice production are approximately calculated as follows: 15 to 20 kilograms of N, 30 kg K_2O, 11 kg P_2O_5, 7 kg Ca, 3 kg Mg, 3 kg S, 150 g Fe, 675 g Mn, 40 g Zn, 15 g B, 18 g Cu, 2 g Mo, and 52 kg Si (Naher et al., 2019). After green revolution, to increase rice production farmers have been using massive amounts of chemical fertilizers, which is one of the reasons why soil quality has declined and alteration of biodiversity and ecology. The physicochemical qualities of soil were seriously degraded as a result of extensive agricultural production with chemical fertilizers (Singh et al., 2014). To produce rice, chemical fertilizers have a 59 %–69 % role. The remaining fertilizers are left in crop residue, soil, water, and air, where they typically create environmental damage. Since we must feed the world's expanding population, we are unable to reduce the use of chemical fertilizers. In light of this situation, experts around the world are considering alternative strategies to decrease the usage of chemical fertilizers, such as boosting nutrient use efficiency (NUE), using balanced fertilization with higher fertilizer efficiency or recycling nutrients from organic sources (Naher et al., 2019). Enhancing NUE and meeting the need for chemical fertilizers to reach the targeted level of food production in the future can be accomplished through the use of biofertilizers (microbial) (Naher et al., 2019). Most chemical reactions that occur in soil are greatly influenced by soil microorganisms. They also participate in the synthesis and circulation of nutrients and actively contribute to soil fertility (Chinakwe et al., 2019). Microorganism colonies on the rhizosphere region of plant that improves plant growth are called Plant growth promoting rhizobacteria (PGPR). The synthesis of siderophores, fixation of nitrogen, solubilization of phosphates, and hormone biosynthesis, including indole-3-acetic acid (IAA), cytokinin are some of the most significant plant growth-promoting (PGP) activities of PGPR (Abbasi et al., 2019). *Streptomyces* are one of the group of microorganisms that can act as PGPR. They are considered as free-living soil microbes belongs to Actinomycetales (order) (Chen et al., 2018). *Streptomyces* are mostly found in soil, compost, freshwater, and marine environments. They are essential for plant protection, Plant growth promotion (PGP), organic material decomposition, and the production of secondary metabolites with commercial significance (Gopalakrishnan et al., 2014).

Using potential *Streptomyces* strains as PGPR to improve agriculture productivity is an alternative approach to reducing the harmful effects of agrochemicals.

4.2. Materials and Methods

4.2.1. Materials

4.2.1.1. Experimental site

The current study was conducted at the CSIR-NIIST Agro-Processing and Technology Division in the agro-climatic conditions of Thiruvananthapuram, Kerala, India. This experimental location was located at an elevation of 4.9 meters above the mean sea level, between 8.4725 °N and 76.9895 °E.

4.2.1.2. Soil and seeds for the study

The sandy loam soil used for the studies had a pH of 6.8, total nitrogen content of 0.896 percent, total content of phosphorus and total potassium of 0.215 percent and 0.474 percentage respectively. The soil had never been treated with synthetic agrochemicals or pesticides before. Rice seeds (Jyothi variety) purchased from Regional Agricultural Research Station, Pattambi, Kerala.

4.2.2. Methods

4.2.2.1. Evaluation of plant growth promotion traits

4.2.2.1.1. Phosphate solubilization

Pikovskaya's agar medium was used to evaluate phosphate solubilizing activity. For this, the *Streptomyces* were spot inoculated on the centre of the Pikovskaya's agar plates and kept for incubation at 28±2 °C for 8 to 10 days. Phosphate solubilization was detected by a clear zone produced around the inoculated *Streptomyces* and expressed as Phosphate Solubilizing Efficiency (% PSE) (Pikovskaya, 1948).

4.2.2.1.2. Production of ammonia

The isolates were screened to produce ammonia in peptone water (Cappuccino and Sherman, 2005). The production of NH3 was evaluated by inoculating *Streptomyces* strains in 5 ml of peptone water then incubated at 28±2°C for 10 days and 1 ml Nessler's reagent was added. The formation of yellow to brown precipitate indicated a positive reaction.

4.2.2.1.3. Production of Indole Acetic Acid (IAA)

Streptomyces were inoculated in yeast malt broth containing 0.5mg/ml L-tryptophan and incubated for 10 days at 28 ±2°C. After incubation centrifuged at 10,000 rpm for 20 minutes. To one milliliter

of supernatant, 2ml Salkowski's reagent and a drop of orthophosphoric acid were added and incubated for 20 minutes at room temperature (Sev et. al., 2016). Pink colour indicated the IAA production.

4.2.2.1.4. Siderophore production of isolates

Determination of siderophore production was done using blue indicator dye, chrome azurol S (CAS). Chrome azurol S (60.5 mg) was weighed and dissolved in distilled water (50 ml) and mixed with iron solution (10 ml) which contains 1mM $FeCl_3$, $6H_2O$, and 10Mm HCl (mixing of this solution turns blue). Weighed 72.9 mg hexadecyl trimethyl ammonium (HTDMA) dissolved in 40ml distilled water, HDTMA was slowly added with constant stirring to CAS, iron solution. This mixture is autoclaved and mixed with 900ml of autoclaved PDA (pH is adjusted to 6.8). To PDA plates with CAS, iron solution, and HDTMA, *Streptomyces* were inoculated and incubated for one week at 28±2°C. After incubation yellowish to orange halo was indicated as positive for the production of siderophore (Lakshmanan et. al., 2015).

4.2.2.2. Plant growth promotion studies

4.2.2.2.1. Seed sterilization

Rice seeds (Jyothi variety) were surface sterilized using 2.5 % sodium hypochlorite for 5 min followed by washing with sterile distilled water for three times and dried under a sterile condition.

4.2.2.2.2. PGPR studies in rice plant using promising strains under nursery conditions

For the experiment, pots were filled with soil and cow dung in a ratio of 3:1. To each pot 30 rice seeds were sown after surface sterilization. The experiment had five set replicates of each treatment. Experiment consists of four treatments such as NIIST D27, NIIST D72, NIIST D27+ NIIST D72, and control to perform the plant growth promotion study. For inoculum preparation, two strains were separately grown in Yeast Malt Broth (YMB) for seven days and centrifuged at 7000 rpm for 30 minutes. The pellets were homogenized and mixed with supernatant and optical density was adjusted approximately to get 10^8 CFU of bacteria for individual as well as for combination treatments. After that 150 ml of bacterial inoculum was added to soil (Morang et al., 2018) in the pots. After seven days from the day of germination, plants were uprooted and growth parameters like shoot height, root length, fresh and dry weight were measured up to 28 days with an interval of seven days. After one month, the plants were treated again with the same dosage of the corresponding bacterial strain. The yield parameters, such as dry weight of shoot (with and without seeds) and weight of 100 seeds were recorded on 120th day.

4.2.2.3. Statistical analysis

Using SPSS (version 25.0; IBM SPSS), one-way Analysis of Variance (ANOVA) was performed for statistical analysis of data. The data used in the plant growth study were shown as mean± standard deviation. Duncan's Multiple Range Test (DMRT) was used to assess statistical significance, and a p value < 0.05 was considered to denote a significant difference.

4.3. Results

4.3.1. Evaluation of plant growth promotion traits

In vitro, results suggest that *Streptoverticillium reticulum* NIIST D27 strain and *Streptomyces rimosus* NIIST D72 strain exhibited positive results for plant growth promoting traits such as phosphate solubilization, IAA, ammonia, and siderophores.

4.3.1.1. Phosphate solubilization

Both the *Streptomyces* strains, NIIST D27 (*Streptoverticillium reticulum*) and NIIST D72 (*Streptomyces rimosus*) solubilise insoluble phosphate to soluble form of phosphate. Phosphate solubilising efficiency of NIIST D27 and NIIST D72 (**Figure 4.1**) was calculated as 53.03 ±2.62 % and 38.78 ±2.09 % respectively.

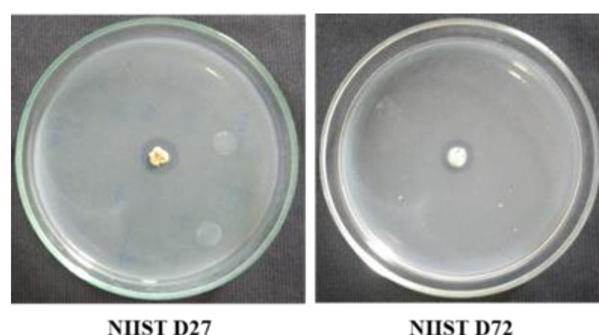


Figure 4.1. Phosphate solubilisation of isolates in Pikovskaya's agar plates

4.3.1.2. Production of ammonia

The strains, NIIST D27 and NIIST D72 produce ammonia in peptone water with the formation of characteristic yellow to brown precipitate (**Figure 4.2**).

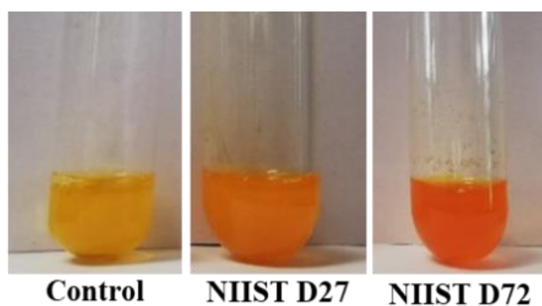


Figure 4.2. Ammonia production of isolates.

4.3.1.3. Production of IAA

IAA producing ability of the strains were analysed. Both strains are capable of producing IAA from the precursor tryptophan. IAA produced by the isolates NIIST D27 and NIIST D72 (**Figure 4.3**) were analysed by colorimetric method and estimated as 6.81 μg/ml and 4.01 μg/ml respectively.

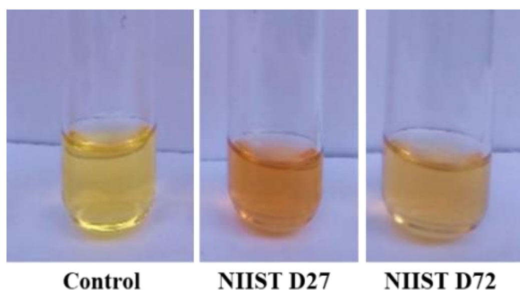


Figure 4.3. production of IAA by the isolates

4.3.1.4. Siderophore production of isolates

NIIST D27 and NIIST D72 produce siderophore by producing an orange zone around the colony **(Figure 4.4)** in plate assay (CAS agar plate). NIIST D27 produces 25.33±0.57 mm of zone and NIIST D72 produces slightly smaller zone of 15.66±0.57 mm.

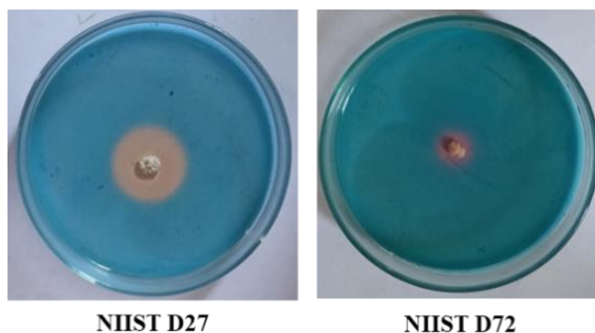


Figure 4.4. Siderophore production of isolates in CAS agar plate.

4.3.2. Effect of NIIST D27 and NIIST D72 on growth promotion and yield on rice plants.

4.3.2.1. Shoot height and root length

As on 28th Day, maximum shoot height was exhibited by NIIST D27+ NIIST D72 (35.98±2.53 cm) treatment followed by NIIST D27 (30.7±2.30 cm) which was 1.3-fold and 1.1-fold increase over the control. Likewise, on 28th day maximum root length was also recorded in combination treatment NIIST D27+ NIIST D72 (21.77±1.66 cm) followed by NIIST D27 alone (18.42±2.63 cm). Maximum increase of root length over the control was recorded by NIIST D27+ NIIST D72 (1.6-fold increase) followed by NIIST D27 (1.3-fold increase) treatment as depicted in (**Figure 4.5, Figure 4.6A, Table 4.1**).

4.3.2.2. Fresh and dry weight

On the 28th day, NIIST D27+ NIIST D27 showed the maximum fresh weight among the treatments (1.00±0.33 g), followed by NIIST D27 alone (0.80±0.09 g). NIIST D27+ NIIST D27 treatment exhibited a 2.2-fold increase in fresh weight followed by NIIST D27 alone which shows a 1.7-fold increase over control. Dry weight of each plants of various treatments was recorded on 7th to 28th day and found that on 28th day NIIST D27+ NIIST D27 had maximum dry weight of 0.21 g and was followed by NIIST D27 alone (0.18±0.01 g). Dry weight of combination treatment NIIST D27+NIIST D27 was a 1.7-fold increase over control and was followed by NIIST D27 (1.5-fold increase) (**Figure 4.5, Figure 4.6 B, Table 4.1**).

4.3.2.3. Yield studies in rice plant

Weight of the seeds was increased in combination treatment NIIST D27+ NIIST D72 compared to control and other treatments. Weight of 100 seeds were recorded as 2.29±0.10 g, weight of shoot with and without seeds were also recorded. It was noticed that NIIST D27+ NIIST D72 treatment has the highest dry weight. The dry weight was recorded as 1.46±0.30 g (shoot dry weight without seeds) and 14.37±1.00 g (shoot dry weight with seeds). Compared to the control NIIST D27+ NIIST D72 treatment exhibited 1.4-fold increase in seed weight, 1.3-fold increase in dry weight of shoot (with seeds), and 2.4-fold increase in shoot dry weight (without seed). (**Figure 4.6 C, Figure 4.7, Table 4.2**).

Table 4.1. Effect of various treatments on growth characteristics of rice plants from 7th to 28th day.

Treatments	Days	Shoot height (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
Control	7	9.61±1.33^{a}	6.47±1.44^{a}	0.09±0.02^{a}	0.02±0.01^{a}
	14	17.74±2.01^{a}	8.48±2.10^{a}	0.15±0.03^{a}	0.05±0.01^{a}
	21	22.26±2.04^{a}	10.24±0.95^{a}	0.21±0.03^{a}	0.04±0.01^{a}
	28	26.07±2.23^{a}	13.39±1.21^{a}	0.45±0.08^{a}	0.12±0.02^{a}
NIIST D72	7	16.09±1.32^{b}	8.49±1.06^{b}	0.24±0.07^{b}	0.03±0.01^{a}
	14	22.86±2.00^{b}	10.9±2.43^{a}	0.36±0.02^{b}	0.07 ±0.01^{b}
	21	26.44±2.48^{b}	12.84±1.51^{b}	0.41±0.09^{b}	0.09±0.01^{b}
	28	27.55±1.95^{a}	15.35±1.67^{a}	0.66±0.07^{b}	0.16±0.02^{b}
NIIST D27	7	18.16±1.91^{c}	14.1±1.91^{d}	0.35±0.06^{c}	0.05±0.01^{b}
	14	24.73±1.88^{c}	11.50±2.43^{b}	0.31±0.12^{bc}	0.08±0.01^{b}
	21	28.12±1.13^{bc}	17.88±1.71^{c}	0.50±0.15^{bc}	0.13±0.02^{c}
	28	30.7±2.30^{b}	18.42±2.63^{b}	0.80±0.09^{b}	0.18±0.01^{bc}
NIIST D27+ NIIST D72	7	20.94±0.77^{d}	10.13±1.17^{c}	0.34±0.05^{c}	0.07±0.01^{b}
	14	25.47±2.04^{c}	12.14±2.44^{b}	0.41±0.08^{c}	0.08±0.01^{b}
	21	29.73±2.03^{c}	18.97±1.51^{c}	0.55±0.12^{c}	0.16±0.01^{d}
	28	**35.98±2.53^{c}**	**21.77±1.66^{c}**	**1.00±0.33^{c}**	**0.21±0.04^{c}**

Values are the average of ten readings and expressed in mean±standard deviation. The same letters do not differ significantly at $p < 0.05$ in each column by DMRT (treatments compared to control).

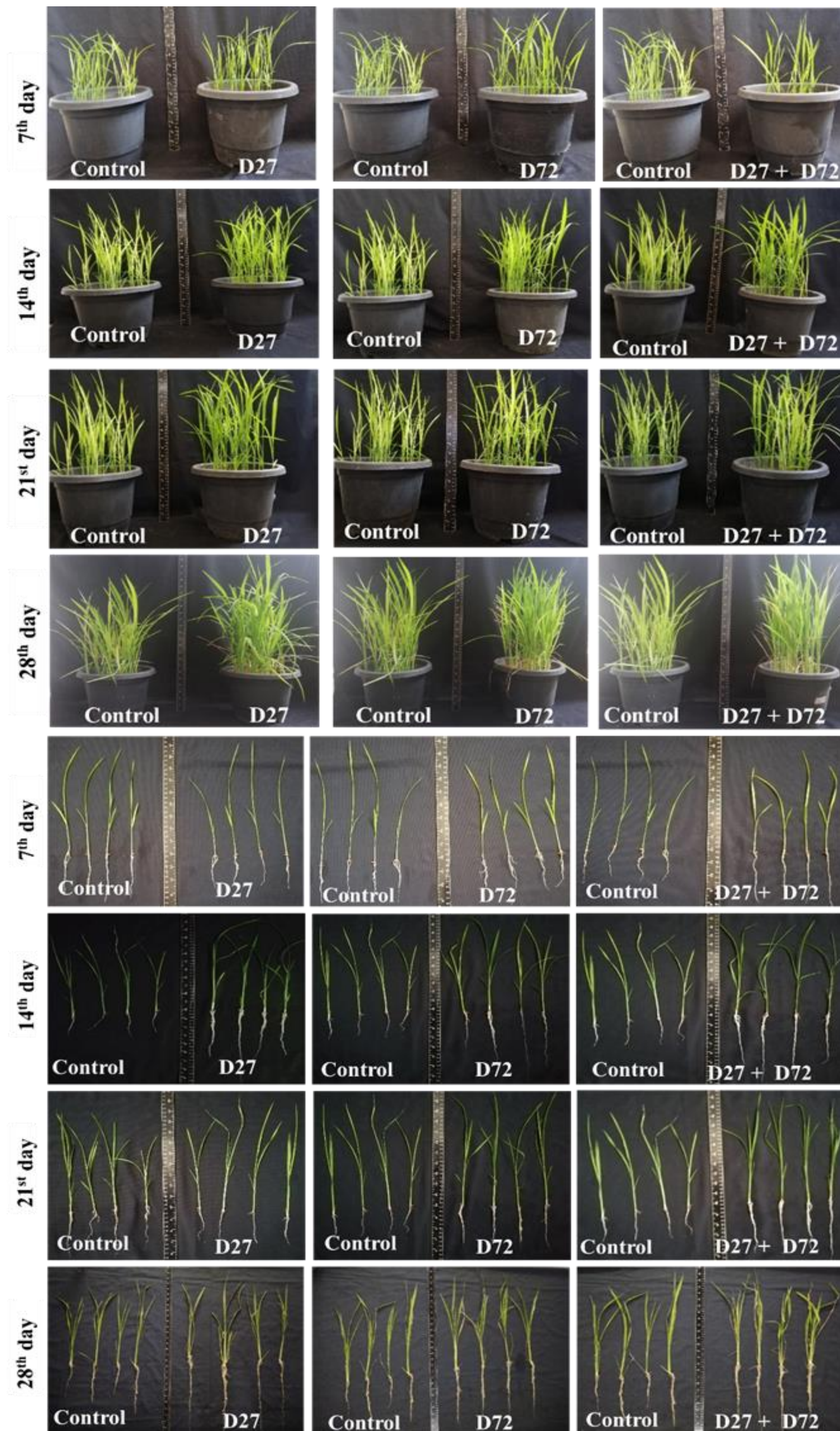


Figure 4.5. Effect of various treatments on plant growth promotion in rice plants from 7th to 28th day. (D27: NIIST D27, D72: NIIST D72).

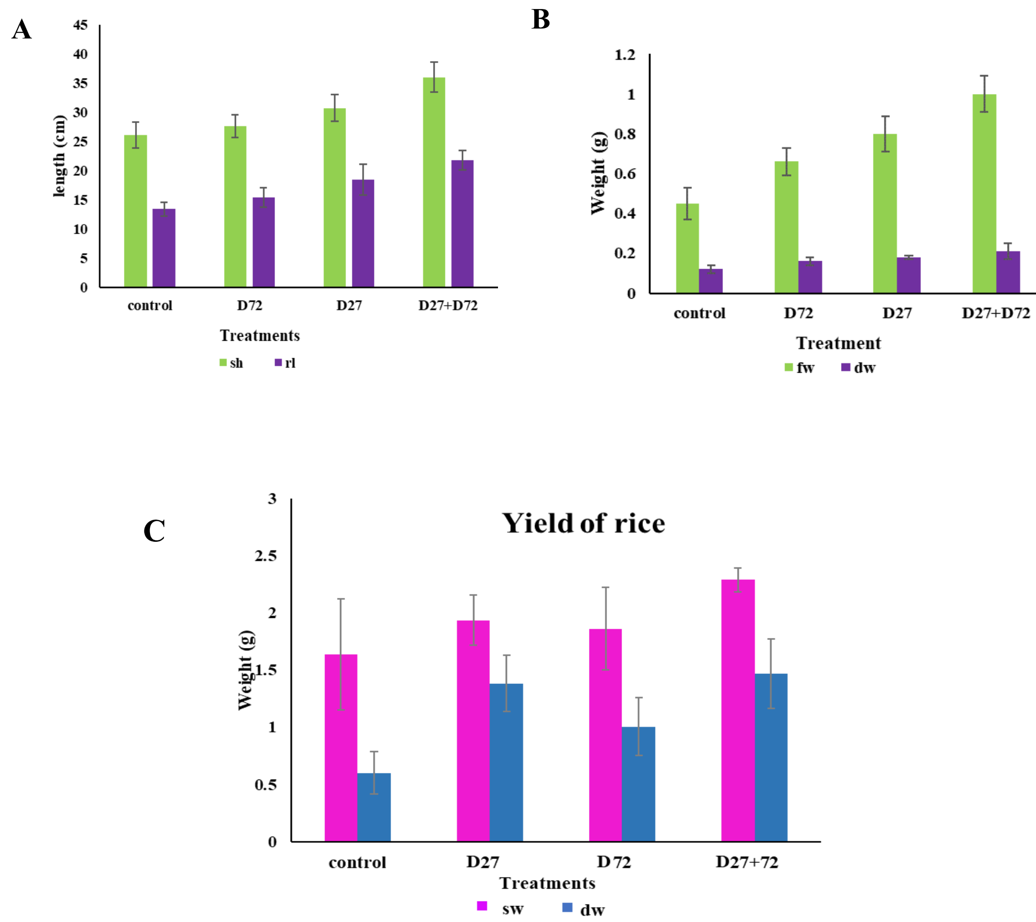


Figure 4.6. Effect of different treatments on Plant growth promotion in rice plants (A: Effect of treatments on shoot and root length on 28th day. B: Effect of treatments on fresh weight and dry weight on 28th day. C: Effect of treatments on yield parameters on 120th day). (sh: shoot height, rl: root length, fw: fresh weight, dw: dry weight, sw: seed weight, without seeds. The data was shown as mean± standard deviation. Statistical significance was performed by DMRT with p value < 0.05.

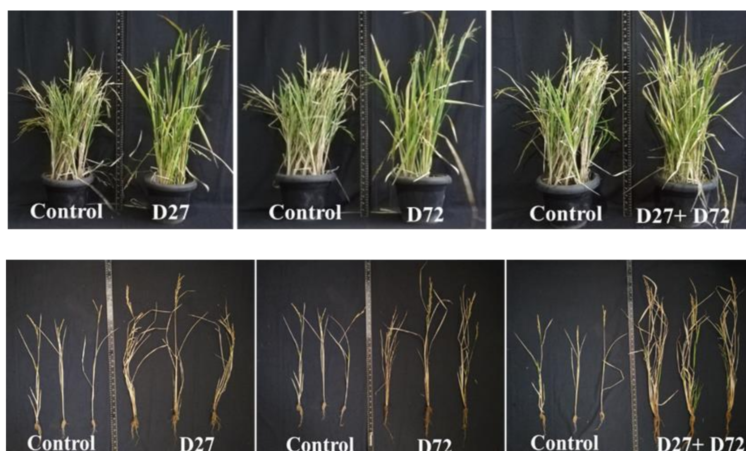


Figure 4.7. Effect of treatments on yield parameters in rice plant.

Table 4.2. Effect of various treatments on yield of rice.

Treatments	weight of 100 seeds (g)	sdw without seeds (g)	sdw with seeds (g)
Control	1.63 ± 0.48^{a}	0.60 ± 0.18^{a}	10.67 ± 0.75^{a}
NIIST D72	1.86 ± 0.36^{a}	1.00 ± 0.25^{b}	11.68 ± 0.69^{b}
NIIST D27	1.93 ± 0.21^{a}	1.38 ± 0.24^{c}	13.00 ± 0.70^{c}
NIIST D27+D72	2.29 ± 0.10^{b}	1.46 ± 0.30^{c}	14.37 ± 1.00^{d}

Values are average of 10 readings and expressed as mean±standard deviation. The same letters do not differ significantly at $p < 0.05$ in each column by DMRT (treatments compared to control, sdw: shoot dry weight).

4.4. Discussion

Agrochemicals are one of the major causes of environmental pollution. Extensive use of chemical fertiliser for agriculture can reduce soil fertility and stability. An alternative ecofriendly approach to chemicals is needed to reduce harmful effects. Biological agents especially microbes are better to use as alternative for this because of their beneficial effect to improve growth of the plant by providing necessary components (PGPR). *Streptomyces* are one of the groups of microbes that can colonies the rhizosphere region of the plant and stimulate the growth. Although they are well-known as biocontrol agents, not as much is known about how they work as biofertilizers. Considering that they are among the most prevalent bacteria in the microflora and that they are highly successful at colonizing plant root systems, it is surprising that their potential to promote plant growth has not received enough attention (Olanrewaju and Babalola, 2019). Recently they have explored for their PGPR ability to improve crop yield. Stress alleviation and biostimulation activities of *Streptomyces* strains include the synthesis of phytohormones such as auxins (IAA), abscisic acid, cytokinins, and gibberellins which regulate the growth of plants (Rey & Dumas, 2017). Additionally, *Streptomyces* species have a beneficial impact on several rhizosphere activities, such as the promotion of arbuscular mycorrhizal and ectomycorrhizal symbiosis with host plants (which improves plant nutrition), fixation of nitrogen, solubilisation of insoluble phosphate, scavenging iron by the production of siderophore (Rey and Dumas, 2017).

In the present study, two potential *Streptomyces* strains NIIST D27 and NIIST D72 were isolated from Malampuzha forest, an unexplored area of Western Ghats. *In vitro* screening for the plant growth promoting ability of both strains were carried out. It was found that both have ability to produce necessary hormones, siderophore, ammonia, and solubilisation of phosphate. Based on the screening,

Plant growth promotion studies were carried out in rice plants (Jyothi variety) under nursery conditions.

Previous reports suggest that various PGPR studies conducted with *Streptomyces* strains in rice plants showed increased shoot length, root length, increased tillers, plant weight and also induces early flowering (Araujo et al., 2019). Similar results were observed in the present study, where combination of two potential *Streptomyces* strains (NIIST D27 + NIIST D72) enhances the growth in rice plants. It was noted that shoot height, root length, dry weight, and fresh weight of the plant increased by 1.3-fold, 1.6-fold, 1.7-fold, and 2.2-fold respectively. Likewise, Olanrewaju and Babalola (2019) mentioned that *Streptomyces griseus* not only has biocontrol activity but also has a huge effect on the dry weight of foliage, tiller number, grain yield, and early emergence of head in oats, wheat, and carrots when compared to control. In addition to that various investigations reported about the plant growth promotion potential of *Streptomyces* especially in wheat, tomato, rice pea, and beans (Gopalakrishnan et al., 2014). Certain strains of *Streptomyces* species can improve the fixation of nitrogen. For instance, nitrogen fixation in sugarcane was increased up to 9.16 % when inoculated with *Streptomyces chartreusis* (Al-tammar and Khalifa, 2023). Besides, several reports mentioned that some *Streptomyces* strains enhances the growth of rice plant in various ways such as *Streptomyces aurantiogriseus* solubilises phosphate, produces IAA and siderophore, (Vurukonda et al., 2021) *Streptomyces hygroscopicus* produce IAA and enhances the formation of adventitious roots (Fu et al., 2022). Similarly, rice plant inoculated with *Streptomyces palmae* PC 12 increases biomass by 50%, (Chaiharn et al., 2020) *Streptomyces* spp. JR9 enhances the shoot biomass by 18% over control, (Ntemafack et al., 2022) and *Streptomyces shenzhenesis* TKSC3 increases 33% of plant dry weight (Hata et al., 2021).

It was evident from previous research, that *Streptomyces* not only increases the plant growth of rice but also other crops such as *Solanum lycopersicum* (Tomato), *Cicer arietinum* (chickpea), *Glycine max* (soybean), banana plants, and *Gossypium herbaceum* (cotton), exhibited improved growth when inoculated with *Streptomyces hydrogenans* strain DH-16, (Barreales et al., 2020) *Streptomyces griseus* (CAI-24, CAI-121 and CAI-127), (Ankati et al., 2021) *Streptomyces* spp. CLV45, (Horstmann et al., 2020) *Streptomyces* sp. CB-75 growth- (Chen et al., 2018) and *Streptomyces globisporus* (Chen et al., 2021) respectively. From previous studies, it was perceived that *Streptomyces* strains have massive role in enhancing the growth of various crop plants which in turn increases the importance of such microbes in the agriculture near future. In the present study, we obtain two potential strains of *Streptomyces* from unexplored habitats which exhibit a significant increase in the growth of rice plants

which is in line with previous studies. Thus, the Western Ghat region of Kerala is the best source for novel as well as potential strains of *Streptomyces* with various agricultural applications.

4.5. Conclusion

Two *Streptomyces* strains*, Streptoverticillium reticulum* NIIST D27 strain and *Streptomyces rimosus* NIIST D72 strain isolated from Malampuzha forest show a positive result for PGPR traits such as phosphate solubilisation, production of ammonia, IAA and siderophore. Phosphate solubilising efficiency of NIIST D27 and NIIST D72 was 53.03 ±2.62 % and 38.78 ±2.09 % respectively. NIIST D27 produces 6.81 µg/ml and NIIST D72 produces 4.01 µg/ml of IAA from the precursor tryptophan. Siderophores produced by the isolates were confirmed by the production of an orange or yellow zone around the *Streptomyces* colony. NIIST D27 produces a larger zone of 25.33±0.57 mm compared to NIIST D72 (15.66±0.57 mm). Pot studies were carried out in rice plants with *Streptomyces* strain in single (NIIST D27, NIIST D72) and combination (NIIST D27+ NIIST D72) treatments along with control. Gradual increase in growth parameters was observed from 7th day to 28th day. Increased growth of rice plant was noticed in terms of shoot height (35.98±2.53 cm), root length (21.77±1.66 cm), fresh (1.00±0.33 g) and dry weights (0.21±0.04 g) in NIIST D27 + NIIST D72 (combination) treated plants on 28th day. Which is 1.3-fold, 1.6-fold, 2.2-fold, and 1.7-fold increase compared to the control respectively. Application of NIIST D27 +NIIST D72 improves the yield in terms of the weight of seeds and dry weight of shoot over control plants. It is concluded that strains from Malampuzha forest (an unexplored region of Western Ghats) have significant plant growth promoting activity.

4.6. References

Abbasi, S., Safaie, N., Sadeghi, A., & Shamsbakhsh, M. (2019). *Streptomyces* strains induce resistance to *Fusarium oxysporum f. sp. lycopersici* race 3 in tomato through different molecular mechanisms. Frontiers in Microbiology, 10, 1505.

Al-Tammar, F. K., & Khalifa, A. Y. (2023). An update about plant growth promoting *Streptomyces* species. Journal of Applied Biology and Biotechnology, 11(4), 34-43.

Ankati, S., Srinivas, V., Pratyusha, S., & Gopalakrishnan, S. (2021). *Streptomyces* consortia-mediated plant defense against Fusarium wilt and plant growth-promotion in chickpea. Microbial Pathogenesis, 157, 104961.

Araujo, R., Dunlap, C., Barnett, S., & Franco, C. M. (2019). Decoding wheat endosphere–rhizosphere microbiomes in *Rhizoctonia solani*–infested soils challenged by *Streptomyces* biocontrol agents. Frontiers in Plant Science, 10, 1038.

Awla, H. K., Kadir, J., Othman, R., Rashid, T. S., Hamid, S., & Wong, M. Y. (2017). Plant growth-promoting abilities and biocontrol efficacy of *Streptomyces* sp. UPMRS4 against *Pyricularia oryzae*. Biological Control, 112, 55-63.

Barreales, E. G., Payero, T. D., Jambrina, E., & Aparicio, J. F. (2020). The *Streptomyces filipinensis* gamma-butyrolactone system reveals novel clues for understanding the control of secondary metabolism. Applied and Environmental Microbiology, 86(18), e00443-20.

Cappucino, J. G., & Sherman, N. (2005). Microbiology a Laboratory Manual 7thEd. Aurora.

Chaiharn, M., Theantana, T., & Pathom-Aree, W. (2020). Evaluation of biocontrol activities of *Streptomyces* spp. against rice blast disease fungi. Pathogens, 9(2), 126.

Chen, Q., Bai, S., Zhang, T., Duan, C., Zhao, J., Xue, Q., & Li, Y. (2021). Effects of seed-coating preparations of living *Streptomyces globisporus* on plant growth promotion and disease control against verticillium wilt in cotton. Sustainability, 13(11), 6001.

Chen, Y., Zhou, D., Qi, D., Gao, Z., Xie, J., & Luo, Y. (2018). Growth promotion and disease suppression ability of a *Streptomyces sp*. CB-75 from banana rhizosphere soil. Frontiers in microbiology, 8, 2704.

Chinakwe, E. C., Nwogwugwu, N. U., Ibekwe, V. I., Chinakwe, P. O., Egbadon, E. O., & Adeleye, S. A. (2019). Isolation and evaluation of bacteria exhibiting multiple plant growth traits in the rhizosphere of yellow bell pepper (*Capsicum chinense*). Journal of Advances in Microbiology, 14(4), 1-6.

Fu, Z., Lei, P., Hu, Z., Yang, H., Cheng, W., Xiao, R., ... & Guo, Z. (2022). Characterization of endophytic *Streptomyces griseobrunneus* Ahn75 and its potential for biocontrol and plant growth-promotion in rice.

Gopalakrishnan, S., Vadlamudi, S., Bandikinda, P., Sathya, A., Vijayabharathi, R., Rupela, O., & Varshney, R. K. (2014). Evaluation of *Streptomyces* strains isolated from herbal vermicompost for their plant growth-promotion traits in rice. Microbiological Research, 169(1), 40-48.

Hata, E. M., Yusof, M. T., & Zulperi, D. (2021). Induction of systemic resistance against bacterial leaf streak disease and growth promotion in rice plant by *Streptomyces shenzhenesis* TKSC3 and *Streptomyces sp*. SS8. The plant pathology journal, 37(2), 173.

Horstmann, J. L., Dias, M. P., Ortolan, F., Medina-Silva, R., Astarita, L. V., & Santarém, E. R. (2020).

Streptomyces sp. CLV45 from Fabaceae rhizosphere benefits growth of soybean plants. Brazilian Journal of Microbiology, 51, 1861-1871.

Lakshmanan, V., Shantharaj, D., Li, G., Seyfferth, A. L., Janine Sherrier, D., & Bais, H. P. (2015). A natural rice rhizospheric bacterium abates arsenic accumulation in rice (Oryza sativa L.). *Planta*, *242*, 1037-1050.

Morang, P., Devi, S. P., Jha, D. K., Dutta, B. K., & Kumar, B. D. (2018). Tea root brown-rot fungus disease reduction and yield recovery with rhizobacteria inoculation in both nursery and field trials. Rhizosphere, 6, 89-97.

Naher, U. A., Ahmed, M. N., Sarkar, M. I. U., Biswas, J. C., & Panhwar, Q. A. (2019). Fertilizer management strategies for sustainable rice production. In Organic farming (pp. 251-267). Woodhead Publishing.

Ntemafack, A., Ahmed, S., Kumar, A., Chouhan, R., Kapoor, N., Bharate, S. B., ... & Gandhi, S. G. (2022). Plant growth promoting potential of butyl isobutyl phthalate and *Streptomyces* sp. from *Rumex dentatus* on rice. Applied Microbiology and Biotechnology, 106(7), 2603-2617.

Olanrewaju, O. S., & Babalola, O. O. (2019). *Streptomyces*: implications and interactions in plant growth promotion. Applied microbiology and biotechnology, 103, 1179-1188.

Pikovskaya, R. I., (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Microbiologiya, 17, 362-370.

Rey, T., & Dumas, B. (2017). Plenty is no plague: *Streptomyces* symbiosis with crops. Trends in Plant Science, 22(1), 30-37.

Sev, T. M., Khai, A. A., Aung, A., & Yu, S. S. (2016). Evaluation of endophytic bacteria from some rice varieties for plant growth promoting activities. *J Sci Innov Res*, *5*(4), 144-148

Shi, J., An, G., Weber, A. P., & Zhang, D. (2023). Prospects for rice in 2050. Plant, Cell & Environment, 46(4), 1037-1045.

Singh, H., Verma, A., Ansari, M. W., & Shukla, A. (2014). Physiological response of rice (*Oryza sativa* L.) genotypes to elevated nitrogen applied under field conditions. Plant Signaling & Behavior, 9(7), 29015.

Vurukonda, S. S. K. P., Giovanadri, D., & Stefani, E. (2021). Growth Promotion and Biocontrol Activity of Endophytic *Streptomyces* spp. Prime Archives in Molecular Sciences, 2nd Edition, 1, 1-55.

Chapter 5

Control of Rice Bacterial Leaf Blight (BLB) through Induced Systemic Resistance (ISR)

5.1. Introduction

In ecology, biodiversity refers to the variety of organisms that exist at the species, interspecies, and intraspecies levels. In the long run, microbes can be used to generate large amounts of money for both agriculture field and human health. In rhizospheric soil, microbial diversity exceeds that of eukaryotic species. More than 10 billion different species of microorganisms can be found in rhizosphere soil, they are included in archaea, eubacteria, and fungi (Kaari et al., 2023). The rhizosphere consists of both harmful as well as beneficial microorganisms. Every year, harmful microbes cause enormous reduction in agriculture all over the world. (Saeed et al., 2021). Major agricultural crops include rice, wheat, maize, etc. Over 3 billion people rely primarily on rice (*Oryza sativa*), one of the major cereal crops grown worldwide. The improvement in rice production has significantly improved the food security of the world (Yu et al., 2022). Rice is the primary food source in the world, and over 90% of it is produced in Asia. However, reductions in rice yields have been observed throughout the growing season, and these reductions are caused by various phytopathogens, including bacteria. The primary barrier to sustainable rice productivity is bacterial rice diseases (Ngalimat et al., 2021). In case of rice, global agriculture loss was estimated as 30 % due to the crop pathogens and pests. Among them, BLB is one of the diseases that cause higher crop loss (Savary et al., 2019). BLB in rice, caused by *Xanthomonas oryzae pv. oryzae* (*X. oryzae*), which lowers crop yield and the global economy. As of right now, there is no effective solution for this disease. While *X. oryzae* infection can be managed with antibacterial compounds such as kasugamycin, phenazin, and streptomycin. Farmers will still have difficulties because bactericides are costly, not practical to use these chemicals in paddy fields, and do not have a positive environmental impact, even though their application can reduce the severity of BLB in the field. In addition, bacterial strains resistant to antibiotics may arise because of the continuous use of such compounds. It is possible to develop new rice cultivars resistant to *X. oryzae*, such varieties eventually become susceptible to bacterial blight (Ham and Kim, 2018, Hastuti et al., 2012). A short-term increase in the productivity of agricultural crops was achieved by the use of agricultural chemicals like pesticides and fertilizers. Nevertheless, the application of these chemicals had a negative long-term effect on the soil's quality and texture. The use of chemical fertilizers can lead to the depletion of non-renewable energy sources because many of these products are produced using fossil fuels (Mosier et al., 2021). Microbial antagonists are being extensively studied for their potential to control plant diseases biologically. This approach is a green substitute for chemical pesticides in the management of crop diseases (Palaniyandi et al. 2013; Akanmu et al., 2021) especially, bacterial blight (Hastuti et al., 2012).

Biocontrol microorganisms are capable of reducing pathogen virulence factors either directly or indirectly through the production of biocontrol metabolites, detoxification, iron competition, and induction of systemic resistance (ISR) in host plants (Saeed et al., 2021). Utilizing microorganisms from the actinomycetes group for biological control is one aspect of integrated disease control (Hastuti et al., 2012). Actinomycetes are widely recognized for their ability to synthesis a wide range of extracellular enzymes and antibiotics. Numerous actinomycetes, particularly *Streptomyces*, have been identified and chosen to manage rice-related diseases, including rice blast, rice blight, and sheath blight. The vast majority of these antibiosis actinomycetes come from conventional sources like the soil and rhizosphere (Xu et al., 2017). PGPB (Plant growth promoting bacteria) applications on rice have demonstrated notable success. Numerous PGPB belonging to the genera *Bacillus*, *Enterobacter*, *Streptomyces,* and *Pseudomonas* have been proven to have beneficial effects on the growth and health of rice plants. According to studies, PGPB can function as bioinoculants by enhancing plant yield, health, and growth. As of right now, PGPB has also been proven to be effective against bacterial rice pathogens. Therefore, PGPB inoculation is becoming recognized as a successful strategy for reducing bacterial rice pathogens and increasing rice production via eco-friendly methods (Ngalimat et al., 2021).

5.2. Materials and Methods

5.2.1. Materials

5.2.1.1. Experimental site

Experiment was conducted in Thiruvananthapuram, Kerala, India at the Agro-Processing and Technology Division of CSIR-NIIST during a period of 2018 to 2022. This region is characterized by hot, tropical temperatures and monsoon rains.

5.2.1.2. Soil and Seeds

For the experiment each pot was filled with 3:1, soil (sandy loam soil): cow dung was done using and Rice seeds (Jyothi) were purchased from the Regional Agricultural Research Station, Pattambi, Kerala.

5.2.1.3. Pathogen

Rice pathogen, *Xanthomonas oryzae pv. oryzae* procured from the Regional Agricultural Research Station, Pattambi, Kerala.

5.2.2. Methods

5.2.2.1. Inoculum preparation

Streptomyces strains (NIIST D27 and NIIST D72) were grown in Yeast Malt Broth (YMB). After 7 days of growth optical density was adjusted to one (homogenised solution of 10^8 CFU/ml). Similarly, pathogens were cultured in nutrient broth for 18 to 24 hours at 37°C (diluted to get 10^8 CFU/ml solution).

5.2.2.2. Growth promotion studies with potential isolates and pathogen under nursery conditions

Plant growth promotion study comprises of five treatments: NIIST D27+P, NIIST D72+P, NIIST D27+NIIST D72+P, pathogen alone, and control. 2.5% sodium hypochlorite solution was used to surface sterilised rice seeds. After surface sterilisation, 30 seeds were sown per pot (soil and cow dung in a 3:1 ratio) and inoculated with 150 ml of 10^8 CFU of bacteria for individual as well as for combination treatments (Morang et al., 2018). Plants were uprooted on 7th, 14th, 21st, and 28th day after germination, and Plant growth parameters were measured such as shoot height, root length, fresh and dry weight. Yield parameters such as seed weight (100 seeds) and shoot dry (weight with or without seeds) plant were noted on the 120th day.

5.2.2.3. Induction of systemic resistance in rice plants with strains NIIST D27 and NIIST D72 under split root conditions

The strain's capacity to induce systemic resistance in rice plants was evaluated by split root experiments (Dutta et al., 2008) with some modifications. The rice seeds were surface sterilized with sodium hypochlorite solution (Dileep Kumar et al., 2001) and sown in 22×18 cm plastic pots and was filled with soil and cow dung in a ratio of 3:1. Five days old rice seedlings with similar sizes were selected for the study. Roots of each plant were split into two parts and planted in paper cups of 5×6 cm diameter filled with soil under split root setup (two paper cups with soil on the lower part and one cup above). Experiment consists of five treatments.

1. Pathogen (both halves of roots were treated with pathogen, *X. oryzae* (Pathogen: Pathogen).
2. NIIST D27+ Pathogen (half of roots were treated with NIIST D27 strain and the other half was treated with the pathogen to evaluate systemic resistance of NIIST D27 on the BLB (pathogen: NIIST D27).
3. NIIST D72+ Pathogen (half of roots were treated with NIIST D72 strain and the other half was treated with pathogen to evaluate systemic resistance of NIIST D72 on the BLB (pathogen: NIIST D72).

4. NIIST D27+ NIIST D72+ Pathogen (half of roots were treated with NIIST D27+ NIIST D72strain and the other half was treated with the pathogen to evaluate systemic resistance of NIIST D27+ NIIST D72 on the BLB (pathogen: NIIST D27+ NIIST D72).
5. Control (both halves of roots were untreated (control: control).

For this experiment, 10 ml of *Streptomyces* (NIIST D27 and NIIST D72) cell suspension (10^8 CFU/ml) inoculated (on 2nd day of after planting) to treatments 2 (10ml of NIIST D27), 3 (10ml of NIIST D72) and 4 (5ml of NIIST D27+ 5ml of NIIST D72). For pathogen treatments, cell suspension (10^8 CFU/ml) of *X. oryzae* was inoculated.

5.2.2.4. Determination of defence related compound and enzymes

Total phenol content in the leaf tissue of various treatments was determined by using the perocedure of Bray and Thorpe, (1954) and expressed as µg/gm tissue weight (fresh weight) using standard curve of catechol.

5.2.2.5. Determination of plant defence related enzymes

Defence related enzymes of the host plant was determined via Peroxidase (POD), L-phenylalanine ammonia lyase (PAL), and Polyphenol oxidase (PPO). Enzyme extract for the evaluation of POD, PAL, and PPO was prepared according to the procedure of Mahadevan and Sridhar (1986). POD activity was estimated according to Thimmaiah (1999) and expressed as units/min/g sample (fresh weight). Determination of PAL activity by the procedure of Sadasivam and Manickam (1991). PPO enzyme was estimated based on the protocol of Sadasivam and Manickam (1991).

5.2.2.5. Effect of *Streptomyces* strains on the incidence of BLB of rice under nursery conditions

Rice plants were evaluated for the appearance of BLB symptoms. On 28th day, the disease index in percentage was noted by counting the number of infected plants. For this, 15 plants from each treatment were evaluated (4 replicates) and disease index of BLB was recorded and estimated by the formula: Disease index (%) = Number of infected plants /Total number of plants observed × 100.(Udayashankar et al., 2011).

5.2.2.6. Statistical analysis

Using SPSS (version 25.0; IBM SPSS), ANOVA was performed for the statistical analysis of data. The data used in the plant growth study were shown as mean± standard deviation. DMRT was used to assess statistical significance, with a p value < 0.05 was considered to denote a significant difference.

5.3. Results

5.3.1. Effect of *Streptomyces* strains inoculated with pathogen on growth and yield of rice

5.3.1.1. Shoot height and root length

As of the 28th Day, maximum shoot height was exhibited by NIIST D27+ NIIST D72+P (36.36 ±2.89 cm) treated plants followed by NIIST D27+P (29.87±2.03 cm) which was1.73 -fold and 1.42-fold increase over the pathogen. Likewise, on 28th day maximum root length was also recorded in combination treatment, NIIST D27+ NIIST D72+ P (20.40±2.05 cm) followed by NIIST D27+P alone (17.51±1.14 cm). Maximum increase of root length over the control was recorded by NIIST D27+ NIIST D72+P (1.78- fold increase) followed by NIIST D27 (1.53- fold increase) treatment as depicted in (**Figure 5.1, Figure 5.2 A, Table 5.1).**

5.3.1.2. Fresh and dry weight

The treatment NIIST D27+NIIST D27+P (1.43±0.46 g) on the 28th day shows maximum fresh weight among the treatment and was followed by NIIST D27+P (0.64±0.11 g). NIIST D27+NIIST D27 +P treated plants exhibited 4.76- fold increase in fresh weight followed by NIIST D27+P which shows 2.13-fold increase over pathogen. Dry weight of various treatments was recorded on 7th to 28th day and found that on 28th day NIIST D27+ NIIST D27+ P having maximum dry weight of 0.45g and was followed by NIIST D27+P (0.16±0.02 g). Dry weight of combination treatment NIIST D27+NIIST D27+P was 6.42-fold increase over pathogen and was followed by NIIST D27+P (2.28-fold increase). When compared to control the growth of the plants (NIIST D72+ NIIST D72+ P) was increased in terms of fresh weight (3.32- fold) and dry weight (3.75- fold). **(Figure 5.1, Figure 5.2 B, Table 5.1).**

5.3.1.3. Yield studies in rice plant

Weight of the seeds was increased in combination treatment NIIST D27+ NIIST D72+ P compared to pathogen and other treatments. Weight of 100 seeds was recorded as 2.19±0.14 g, weight of shoot with and without seeds was also recorded. It was noticed that NIIST D27+ NIIST D72+P treated plants have highest dry weight. The dry weight was recorded as 1.55±0.29 g (shoot dry weight without seeds) and 14.27±1.74 g (shoot dry weight with seeds). Compared to pathogen NIIST D27+ NIIST D72+P treatment exhibited 2.02-fold increase in seed weight, 1.82-fold increase in dry weight of shoot (with seeds), and 4.42-fold increase in shoot dry weight (without seed). **(Figure 5.2 C, Figure 5.3, Table 5.2).**

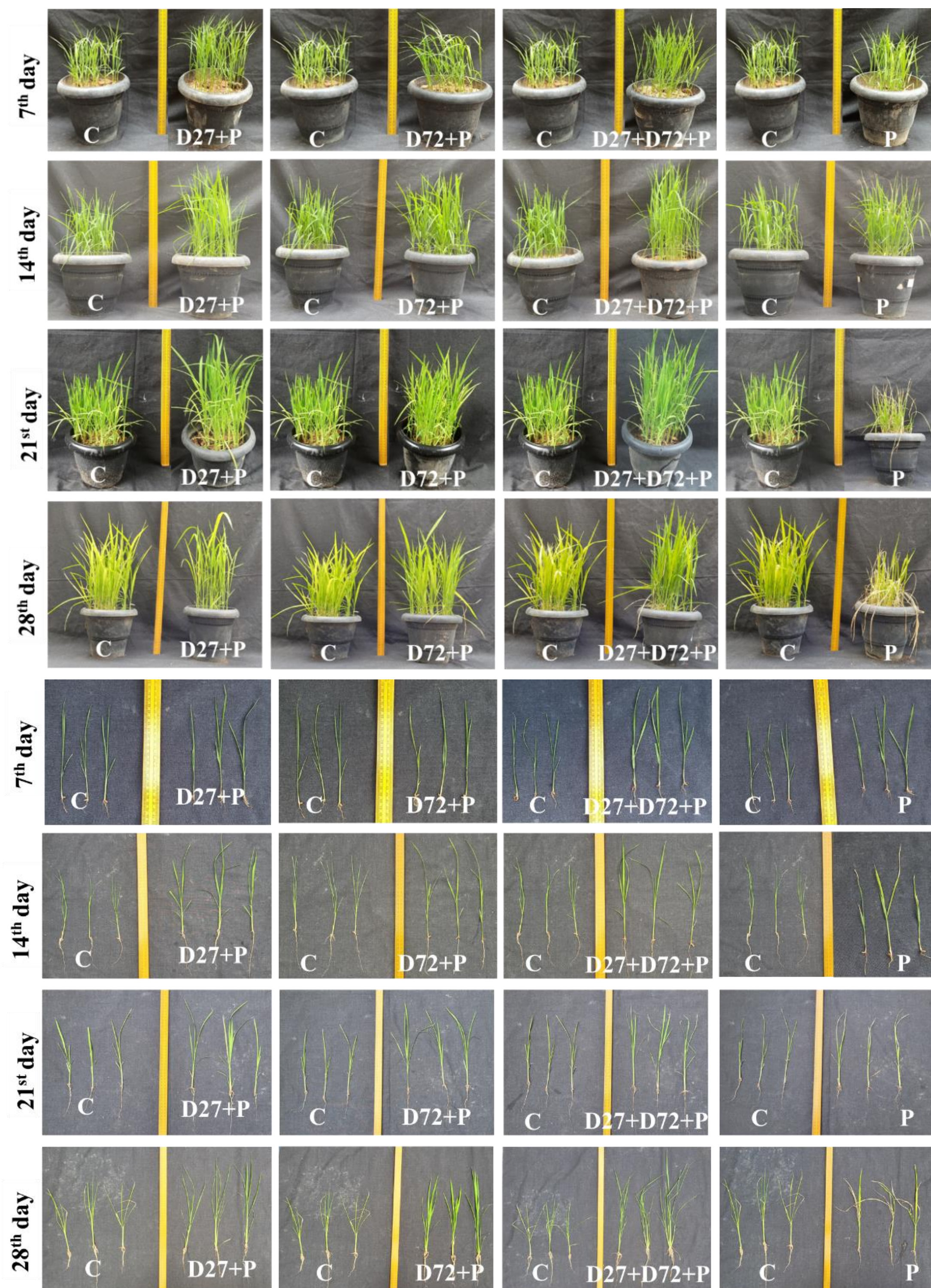


Figure 5.1. Effect of various treatments on plant growth promotion in rice plant from 7th to 28th day. (C: Control, P: Pathogen (*X. oryzae*), D27: NIIST D27, D72: NIIST D72).

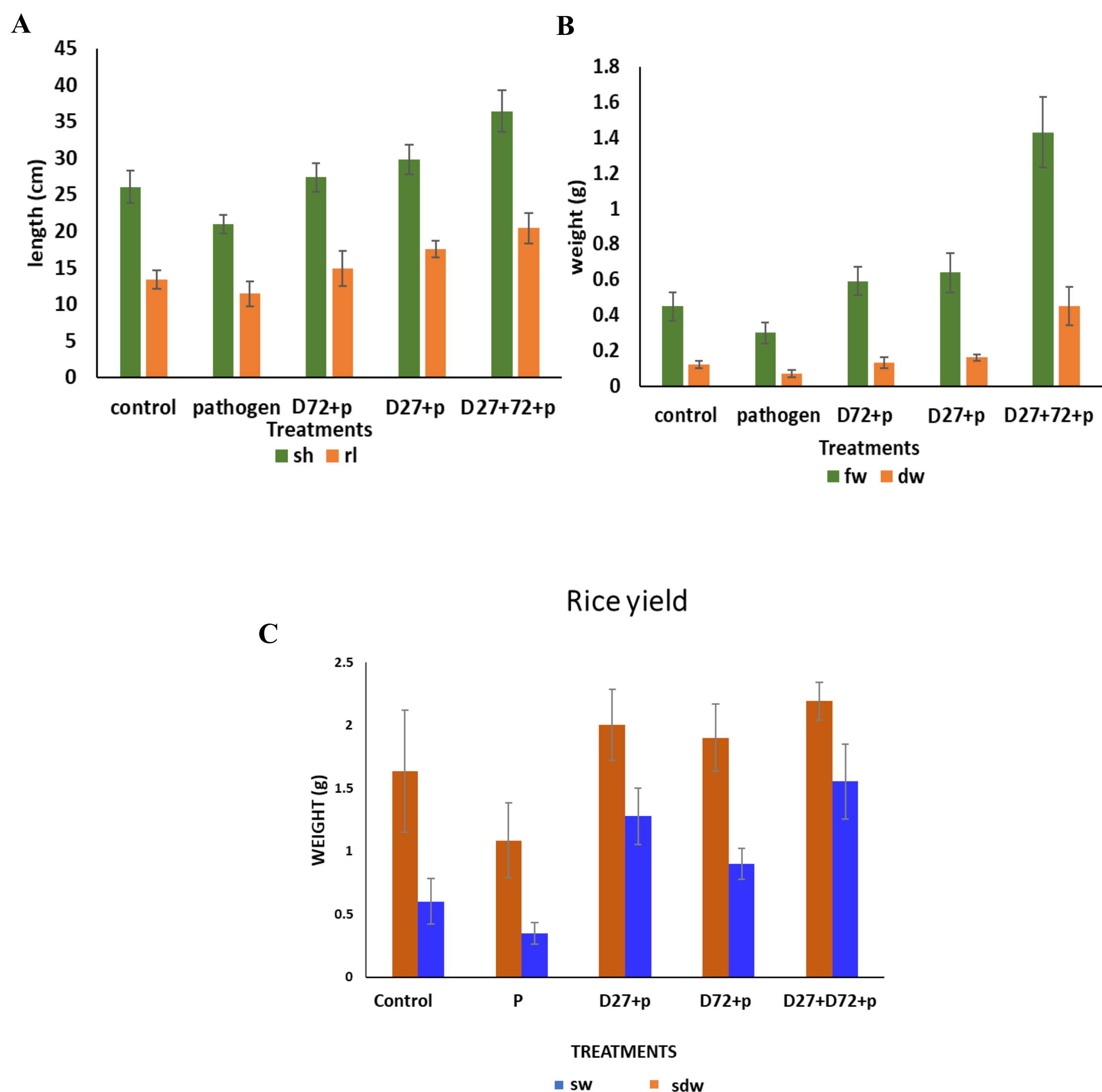


Figure 5.2. Effect of different treatments on Plant growth promotion in rice plants (A: Effect of treatments on shoot and root length on 28th day. B: Effect of treatments on fresh weight and dry weight on 28th day. C: Effect of treatments on yield parameters on 120th day). (sh: shoot height, rl: root length, fw: fresh weight, dw: dry weight, sw: seed weight, sdw: shoot dry weight (without seeds)). The data was shown as mean± standard deviation. Statistical significance was performed by DMRT with p value < 0.05.

Table 5.1. Effect various treatments on growth characteristics of rice plants from 7th to 28th day.

Treatments	Days	Shoot height (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
Pathogen	7	6.83±1.24^{a}	5.97±1.22^{a}	0.06 ±0.007^{a}	0.02±0.01^{a}
	14	16.65±1.94^{a}	6.04±2.43^{a}	0.14±0.03^{a}	0.04±0.02^{a}
	21	18.93±1.47^{a}	11.77±1.80^{a}	0.16 ±0.06^{a}	0.03±0.01^{a}
	28	20.96±1.24^{a}	11.43±1.74^{a}	0.30±0.06^{a}	0.07±0.02^{a}
Control	7	7.93±1.45^{a}	5.97±1.22^{a}	0.08±0.02^{a}	0.02±0.01^{a}
	14	18.01±1.78^{a}	8.95±1.87^{b}	0.14±0.03^{a}	0.04±0.01^{a}
	21	20.70±1.50^{a}	9.19±1.27^{a}	0.19±0.02^{a}	0.04±0.01^{a}
	28	25.76±1.79^{b}	12.98±0.98^{b}	0.43±0.07^{b}	0.12±0.03^{b}
NIIST D72 +P	7	15.95±2.75^{b}	7.98±1.28^{b}	0.28±0.04^{b}	0.05±0.01^{b}
	14	20.75±1.86^{a}	10.96±2.47^{c}	0.29±0.07^{b}	0.04±0.01^{a}
	21	27.16±1.03^{c}	11.72±1.40^{a}	0.32±0.03^{b}	0.08±0.01^{b}
	28	27.37±1.93^{b}	14.92±2.41^{b}	0.59±0.08^{b}	0.13±0.03^{b}
NIIST D27 +P	7	17.51±2.13^{b}	8.91±1.52^{b}	0.29±0.04^{b}	0.05±0.01^{b}
	14	20.85±1.93^{b}	11.53±2.52^{c}	0.32±0.16^{b}	0.06±0.04^{a}
	21	28.38±1.93^{c}	11.68 ±1.40^{a}	0.41±0.07^{c}	0.08±0.01^{b}
	28	29.87±2.03^{c}	17.51±1.14^{c}	0.64±0.11^{b}	0.16±0.02^{b}
NIIST D27+ NIIST D72+ P	7	18.41±0.92^{b}	9.31±1.06^{c}	0.35±0.14^{c}	0.07±0.03^{c}
	14	22.01±2.34^{b}	12.91±2.54^{c}	0.42±0.07^{c}	0.10±0.01^{b}
	21	29.62±2.84^{d}	15.91 ±2.66^{b}	0.53±0.05^{d}	0.13±0.02^{c}
	28	**36.36 ±2.89^{d}**	**20.40±2.05^{d}**	**1.43±0.46^{c}**	**0.45±0.11^{c}**

Values are average of ten readings and expressed as mean±standard deviation. The same letters do not differ significantly at $p < 0.05$ in each column by DMRT. (treatments compared to control).

Table 5.2. Effect of various treatments on yield of rice plant.

Treatment	weight of 100 seeds (sw) in g	sdw without seeds in g	sdw with seeds in g
Control	1.42±0.40^{b}	0.50±0.16^{b}	9.93±0.64^{b}
P (*X. oryzae*)	1.08±0.29^{a}	0.35±0.08^{a}	7.77±0.76^{a}
NIIST D72+P	1.90±0.26^{b}	0.90±0.12^{c}	11.34±1.66^{b}
NIIST D27+P	2.00±0.28^{c}	1.27±0.22^{d}	12.05±0.79^{c}
NIIST D27+ D72+P	**2.19±0.14^{c}**	**1.55±0.29^{e}**	**14.25±1.74^{d}**

Values are average of 10 readings and expressed as mean±standard deviation. The same letters do not differ significantly at $p < 0.05$ in each column by DMRT (treatments compared to pathogen, sdw: shoot dry weight).

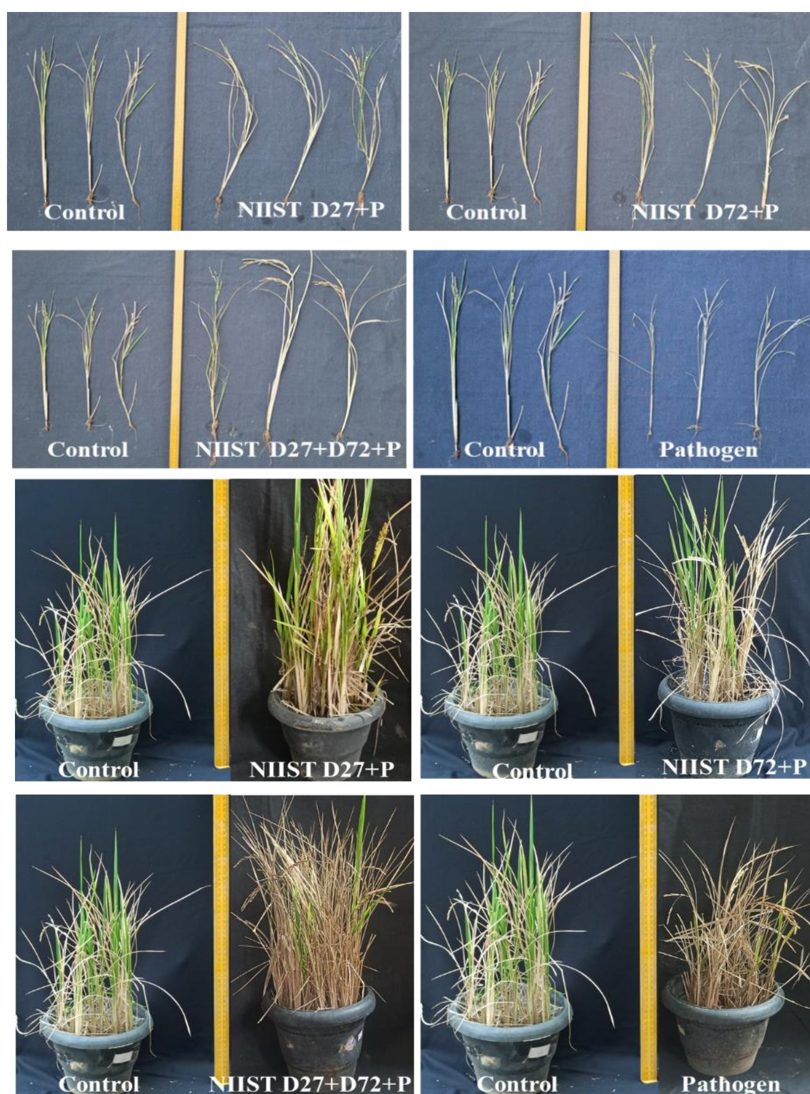


Figure 5.3. Effect of treatments on yield parameters in rice plant

5.3.2. Induction of systemic resistance in rice plant under spit-root conditions

Efficiency of *Streptomyces* strains (NIIST D27 and NIIST D72) to suppress bacterial leaf blight (BLB) in rice plants through the induction of systemic resistance (ISR) was also evaluated by split-root system (**Figure 5.4**). In rice seedlings inoculated with pathogen (only pathogen), exhibited symptoms of BLB on 14th Day and symptoms progresses. The typical symptoms of disease were observed such as yellowing leaves and wilting of seedling, as disease progresses leaves turns from yellow to straw coloured and whole plant wilt, dry up and die. While *Streptomyces* strain inoculated plant does not show any symptoms and remain healthy.

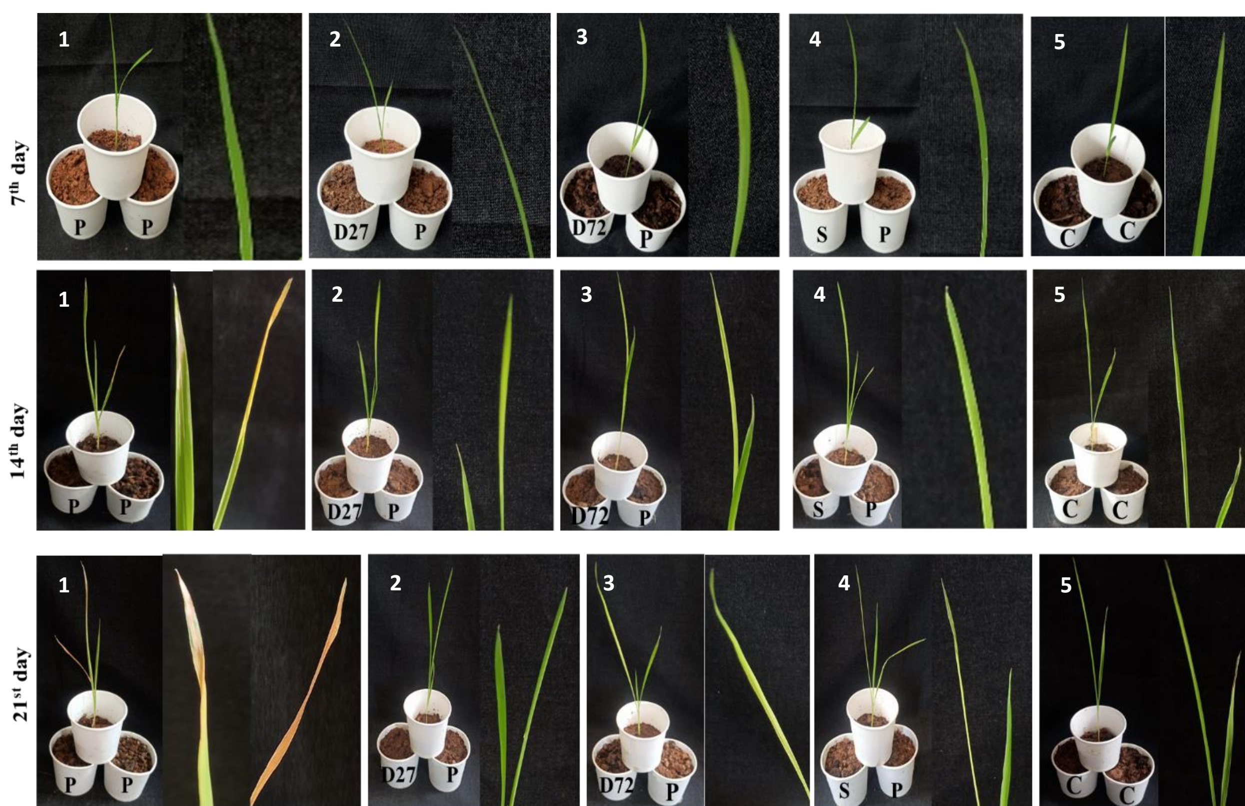


Figure 5.4. Split root experiment of rice plant using *Streptomyces* isolates and *Xanthomonas oryzae*. Treatments, 1: (Pathogen (P): Pathogen(P)), 2: (NIIST D27 (D27): Pathogen (P)), 3: (NIIST D72 (D72): Pathogen (P)), 3: (NIIST D27 + NIIST D72 (S): Pathogen (P)), 5: (Control (C): Control (C)

5.3.3. Effect of various treatment on incidence of bacterial leaf blight in rice under nursery condition

PGPR provides protection against the BLB disease in rice. Various treatment shows varying degree of protection and incidence of symptoms (Disease index). When the plants treated with single PGPR strain (NIIST D27 or NIIST D72) incidence of disease symptoms decrease. Lowest disease symptom and higher protection against BLB was noted in plants treated with the combination of both strains. Pathogen alone treated plants show symptoms of BLB (**Figure 5.5**) and shows higher disease incidence of 76.66%. When the plant treated with NIIST D27, NIIST D72 and Combination (both strains together) disease incidence reduces to 40.33%, 53.33% and 30% respectively (**Table 5.3**). NIIST D27+NIIST D72+P exhibited highest protection (60.8%) against BLB in rice plant. Plants treated with *Streptomyces* remain heathy compared to pathogen alone treated plants.

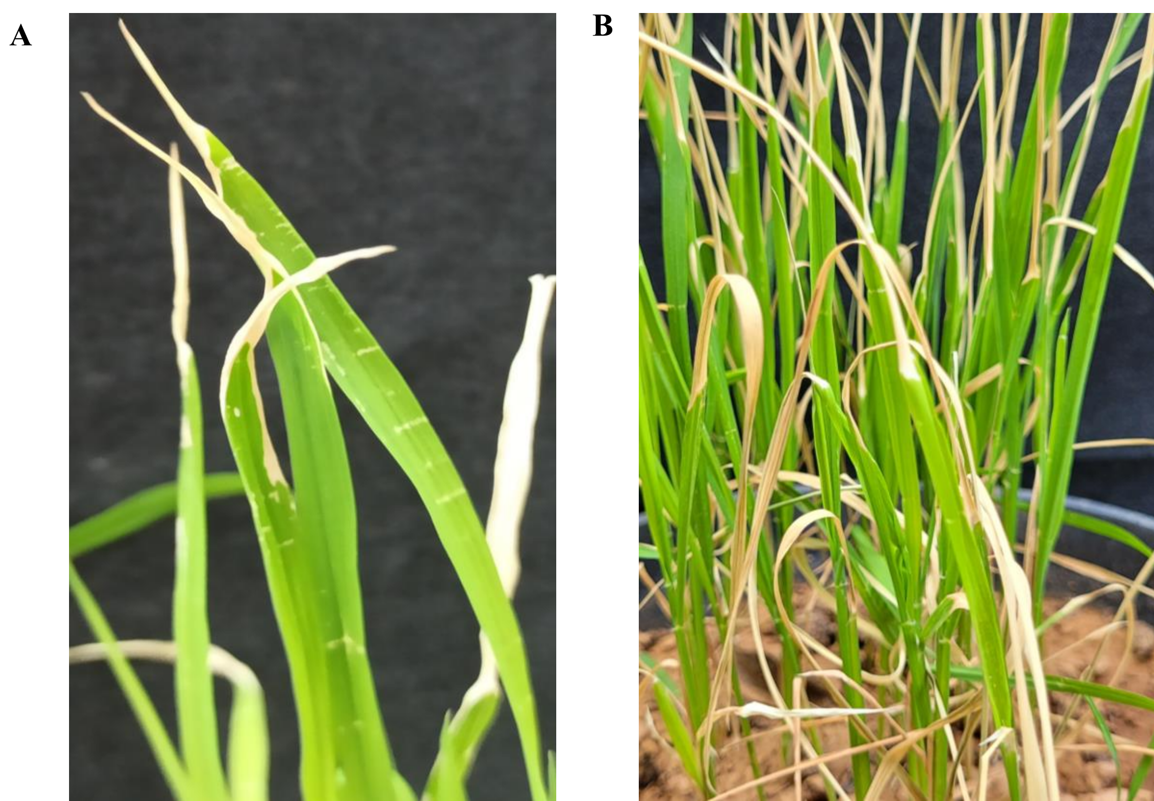


Figure 5.5. Symptoms of BLB in Pathogen (*X oryzae*) alone treated plants. A: on 14th day, B: on 28th day.

Table 5.3. Effect of different treatments on incidence of bacterial leaf blight of rice, grown under nursery conditions.

Treatments	Disease index of BLB in percentage (%)	Percentage protection from BLB (%)
Pathogen (*X. oryzae*)	76.66^c	0.00
NIIST D27 + P	48.33^b	36.95
NIIST D72 + P	53.33^b	31.4
NIIST D27+ NIIST D72 +P	30^a	60.8
Control	0.00	0.00

Disease index is the mean of four replicates in percentage. Means followed by same letters do not differ significantly at $p < 0.05$ in each column by DMRT.

5.3.4. Pathogenesis-related compound and enzymes

5.3.4.1. Total phenolic content

The treatments NIIST D27+P, NIIST D72+P, and NIIST D27+NIIST D72+P exhibited gradual increase in the total phenol content from 7th day to 28th day and higher phenolic content was noticed at 28th day in NIIST D27+NIIST D72+P treated plants (188.13 µg) and is followed by NIIST D27+P (179.4 µg) and NIIST D72+P (157 µg). Lower levels of phenol content were exhibited by the control (69.10 µg) and Pathogen alone treatment (54.75 µg). In the case of pathogen, phenol content increases up to the 14th day and then reduces on 28th day **(Figure 5.6 D).**

5.3.4.2. Determination of defence related enzymes

5.3.4.2.1. PAL enzyme activity

PAL activity in rice plants with different treatments on various days up to the 28th day is given in **Figure: 5.6 C**. From the figure, it is evident that the lowest and highest PAL activity was noted in pathogen (0.062 unit/g) and NIIST D27+NIIST D72+P (0.276 unit) treated plants respectively on the 28th day. NIIST D27+P treated plant shows slightly higher (0.260 unit) PAL activity than plants treated with NIIST D72+P (0.227 unit). PAL activity of the pathogen treated plant increases up to the 14th day and gradually decreases up to the 28th day.

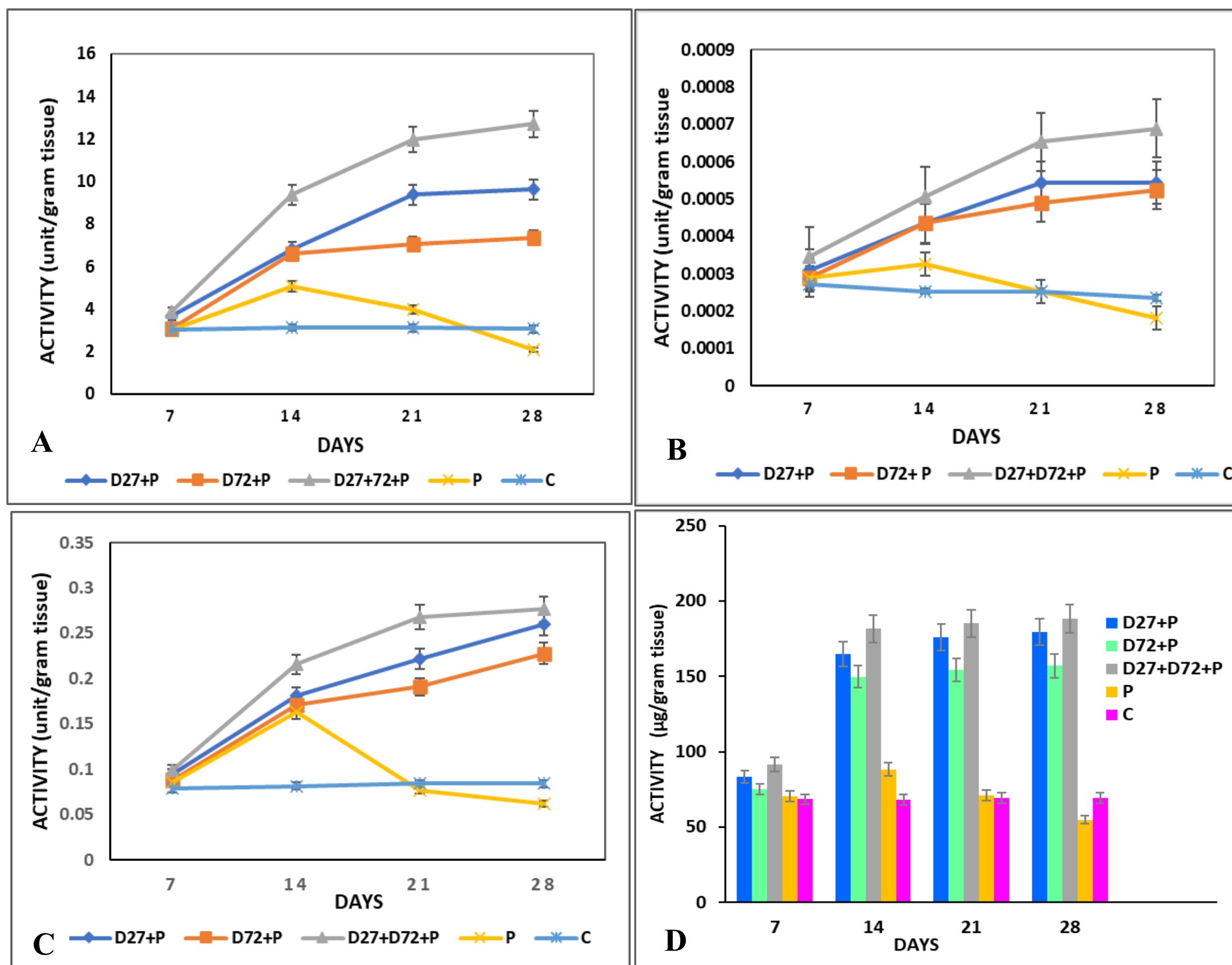


Figure 5.6. Effect of treatments on induction of defence related enzymes and defence related compound (A: Peroxidase (POD); B: Polyphenol oxidase (PPO), C: Phenylalanine ammonia lyase (PAL), D: Total phenol content). The data was shown as mean± standard deviation. Statistical significance was performed by DMRT with p value < 0.05.

5.3.4.2.2. POD enzyme activity

Rice plant treated with combination of streptomyces strain (NIIST D27+NIIST D72+P) enhances PAL activity (12.7 unit) compared to other treatments. Here also the pathogen treated plants exhibited the lowest PAL activity (2.08 unit) on the 28th day. Slightly higher PAL activity was noticed on the 14th day. The control plant does not show much increase in enzyme activity and was found to be similar on all days observed as 3.01 unit, 3.12 units,3.1 units, and 3.05 units on the 7th, 14th, 21st, and 28th day respectively (**Figure 5.6 A)**.

5.3.4.2.3. PPO activity

Like other enzymes, here also, maximum activity of enzyme was recorded in rice plants treated with combination of strains, NIIST D27+ NIIST D72+ P followed by NIIST D27+P. Similarly, here also

lowest activity was observed in pathogen alone (without *Streptomyces*) treated plants after a gradual increase in PPO enzyme up to the 14th day. PPO activity is represented in **Figure 5.6 B**.

5.4. Discussion

BLB management is a challenge nowadays because of the epidemic nature of this disease. Researchers are trying to find a better way to control BLB and secure rice productivity for the future. The best cost-effective approach to manage disease is by BLB management techniques, like using resistant cultivars. However, because of the vast diversity of the pathogens, it has only been partially successful. Conversely, certain substances, like agrochemicals, can control BLB but are hazardous to the environment. Increased use of chemical fungicides reduces the plant's resistance to diseases as well as influencing the emergence of resistant pathogens (Chung et al 2015). Eco-friendly disease management practices can be adopted for sustainable agriculture. Shifting from chemicals to an alternative green method for agriculture is needed nowadays, because of the negative impact caused by the chemical to the environment. Potential microbes from unexplored environments contribute to society in various ways, especially in the field of agriculture. Various microbes, specifically *Streptomyces,* are potential PGPR and biocontrol agents. Hence, researchers show special interest in potential *Streptomyces* strains nowadays to improve future agriculture. For instance, a study reported on the biocontrol ability of an endophytic *Streptomyces* species against rice BLB by inhibiting the growth of *X. oryzae* (Cheng et al., 2016). Similar results were obtained in the present study, where *Streptomyces* strains isolated from the Malampuzha forest have a potential effect in controlling the rice pathogen *X. oryzae* and improves the growth of plants. Combination treatment of NIIST D27 and NIIST D72 along with pathogen (NIIST D27+NIIST D72+P) shows increased plant growth such as increase in root length (1.78- fold increase), shoot length (1.73- fold), fresh (4.76- fold) and dry weight (6.42- fold) compared to pathogen alone treated plants. Combination of *Streptomyces* strains not only improves the growth of the plants but also increases the yield (seed weight of 100 seeds: 2.19±0.14 g, shoot dry weight with seeds: 14.25±1.74 g). This study revealed that *Streptomyces* strains can improve plant growth along with enhancing resistance to pathogen thereby controlling the incidence of various diseases. In such a way, *Streptomyces toxytricini* VN08-A-12 was chosen from 2690 Actinomycetes strains due to its potent growth-inhibitory properties against various *X. oryzae* races. In addition to reducing *X. oryzae* lesion lengths in field trails, it also enhanced rice yield in two distinct rice cultivars (Van Hop et al., 2014). Similarly, through the production of staurosporine, *Streptomyces sp*. MJM4426 can protect leaf explants of rice from *X. oryzae* (Cheng et al., 2016). In agriculture, *Streptomyces sp* are successfully used as PGPR strains and biocontrol agents. Owing to the fact that it can reduce environmental problems caused by chemicals to an extent along with the reduction of plant diseases

as well as improving the growth and yield of plants. According to the present study, *Streptomyces* strains NIIST D27 and NIIST D72, increased plant growth and suppressed bacterial leaf blight (BLB) in rice plants by reducing the disease incidence. Biocontrol agents control the pathogen attack by inducing systemic resistance in plants, this enhances the defence related enzymes like PAL, POD, and PPO. These are the key enzymes that provide resistance in host plants against pathogen. In the study, it was clear that the amount of these enzymes increased in *Streptomyces* treated rice plants compared to pathogen alone treatment and untreated control. As the inoculated *Streptomyces* strains induce systemic resistance in rice plants that leads to the reduction in the incidence of disease, it was evaluated and confirmed by split-root experiments and enzyme assay (defence relate enzymes). The combination treatment (NIIST D27+ NIIST D72+ P) remains healthier than all other treatments because defense related enzymes were found to increase in this treatment than others (Both strains together contribute to the inhibition of pathogen in pot experiments). Single treatments also reduce the incidence of diseases but much more effective in controlling BLB is achieved by the inoculation of both strains together (30% disease incidence and 62.55% reduction in disease over pathogen alone treated plant). But pathogen alone treated (disease incidence 76.66%) plants produce low levels of defense related enzymes. Hence it shows typical symptoms of BLB on the 14th day and the symptoms gradually increases the subsequent day. So, the *Streptomyces* strains have potential to control BLB. These results resemble the prior studies, in particular, the research report of Xu et al., (2017) mentioned that endophytic *Streptomyces endus* OsiSh-2 can control rice blast in agriculture fields. Additionally, 2,4-ditert-butylphenol isolated from *Streptomyces* strain UT4A49 enhances the growth of tomato plant and inhibits the pathogen *R. solanacearum*. Other than that, *Streptomyces* are explored for various natural products for instance Blasticidin-S, Mildiomycin, Kasugamycin, Polyoxins, and Oxytetracycline. All these compounds are effective against various bacterial and fungal plant diseases including rice blast. (Kaari et al., 2023).

Moreover, *Streptomyces globisporus* JK-1 fermented culture filtrate was more effective than the frequently used chemical fungicide tricyclazole in reducing rice blast by the inhibition of *Magnaporthe oryzae* (Newitt et al., 2019). Due to the potential ability of the *Streptomyces* strains**,** three thousand pesticides and herbicides, with properties such as phytotoxicity, nematicide, insecticide, and plant growth-regulating, have been developed. Actinomycete metabolite is much more effective than metabolite of fungal origin, which decreases phytotoxicity (Pacios-Michelena et al., 2021). Also, various biocontrol products derived from *Streptomyces* species are found in the markets including ArzentTM, Mycostop Actinovate, Micro108 R Actino-Iron and YAN TEN *S. saraceticus* derived from *Streptomyces* species *Streptomyces griseoviridis* K61, *Streptomyces lydicus* WYEC 108, *Streptomyces*

hygroscopicus, and *S. saraceticus* KH400 (Olanrewaju and Babalola, 2019, Law et al., 2017). Mycostop R was proven to control *Ceratocystis radicicola* which causes black scorch disease of date palm. It also inhibits the soil pathogen *Fusarium oxysporum f.sp. lycopersici* of tomato and *Verticillium dahlia* (Law et al., 2017). Thus, *Streptomyces* are microorganisms that are widely used as bio-controls, bio-remediators, bio-fertilizers and bio-stimulators. (Al-tammar and Khalifa, 2023).

5.5. Conclusion

Streptomyces strains (NIIST D27 and NIIST D72) were evaluated for their plant growth promoting and disease control ability in rice plants under nursery conditions (pot experiments). It was found that combination of these strains shows better results compared to single treatment, untreated control, and pathogen alone treated plants. Combination treatment (NIIST D27+NIIST D72+P) exhibited improved growth and enhanced yield compared to other treatments. Similarly, it induces systemic resistance (ISR) in plants thereby reducing the disease incidence, which is evident from the enzyme assay, where the host plant produces high levels of defense related enzymes (PAL, POD, and PPO) and compound (total phenol content). Split root experiment was conducted to conform the ISR and was observed that plants treated with *Streptomyces* remained healthy compared to pathogen-treated plants. Pathogen-alone treated plants show typical BLB symptoms from the 14th day and the disease symptoms increase by each day. The *Streptomyces* have a potential effect in controlling BLB and improving the growth of the plants, so this can be used as an alternative to chemicals. Thereby reducing the environmental pollution caused by agrochemicals.

5.6. References

Akanmu, A. O., Babalola, O. O., Venturi, V., Ayilara, M. S., Adeleke, B. S., Amoo, A. E., ... & Glick, B. R. (2021). Plant disease management: leveraging on the plant-microbe-soil interface in the biorational use of organic amendments. Frontiers in Plant Science, 12, 700507.

Al-Tammar, F. K., & Khalifa, A. Y. (2023). An update about plant growth promoting *Streptomyces* species. Journal of Applied Biology and Biotechnology, 11(4), 34-43.

Bray, H. G., & Thorpe, W. (1954). Analysis of phenolic compounds of interest in metabolism. Methods of biochemical analysis, 27-52.

Cheng, J., Park, S. B., Kim, S. H., Yang, S. H., Suh, J. W., Lee, C. H., & Kim, J. G. (2016). Suppressing activity of staurosporine from *Streptomyces* sp. MJM4426 against rice bacterial blight disease. Journal of Applied Microbiology, 120(4), 975-985.

Chung, E. J., Hossain, M. T., Khan, A., Kim, K. H., Jeon, C. O., & Chung, Y. R. (2015). *Bacillus oryzicola sp. nov*., an endophytic bacterium isolated from the roots of rice with antimicrobial, plant growth promoting, and systemic resistance inducing activities in rice. The plant pathology journal, 31(2), 152.

Dileep Kumar, B. S., Berggren, I., & Mårtensson, A. M. (2001). Potential for improving pea production by co-inoculation with fluorescent Pseudomonas and Rhizobium. Plant and soil, 229, 25-34.

Dutta, S., Mishra, A. K., & Dileep Kumar, B. S. (2008). Induction of systemic resistance against fusarial wilt in pigeon pea through interaction of plant growth promoting rhizobacteria and rhizobia. Soil Biology and Biochemistry, 40(2), 452–461.

Ham, Y., & Kim, T. J. (2018). Anthranilamide from *Streptomyces spp*. inhibited *Xanthomonas oryzae* biofilm formation without affecting cell growth. Applied Biological Chemistry, 61(6), 673–680.

Hastuti, R. D., Lestari, Y., Saraswati, R., Suwanto, A., & Chaerani. (2012). Endophytic *Streptomyces* spp. as Biocontrol Agents of Rice Bacterial Leaf Blight Pathogen (*Xanthomonas oryzae pv. oryzae*). HAYATI Journal of Biosciences, 19(4), 155–162.

Kaari, M., Manikkam, R., Annamalai, K. K., & Joseph, J. (2023). Actinobacteria as a source of biofertilizer/biocontrol agents for bio-organic agriculture. Journal of Applied Microbiology, 134(2), lxac047.

Law, J. W. F., Ser, H. L., Khan, T. M., Chuah, L. H., Pusparajah, P., Chan, K. G., ... & Lee, L. H. (2017). The potential of Streptomyces as biocontrol agents against the rice blast fungus, *Magnaporthe oryzae* (*Pyricularia oryzae*). Frontiers in microbiology, 8, 3.

Mahadevan, A., & Sridhar, R. (1982). Methods in physiological plant pathology.

Morang, P., Devi, S. P., Jha, D. K., Dutta, B. K., & Kumar, B. D. (2018). Tea root brown-rot fungus disease reduction and yield recovery with rhizobacteria inoculation in both nursery and field trials. Rhizosphere, 6, 89-97.

Mosier, S., Cordova, S., & Robertson, G. P. (2021). Restoring soil fertility on degraded lands to meet food, fuel, and climate security needs via perennialization. Frontiers in Sustainable Food Systems, 5, 706142.

Newitt, J. T., Prudence, S. M., Hutchings, M. I., & Worsley, S. F. (2019). Biocontrol of cereal crop diseases using *Streptomycetes*. Pathogens, 8(2), 78.

Ngalimat, M. S., Mohd Hata, E., Zulperi, D., Ismail, S. I., Ismail, M. R., Mohd Zainudin, N. A. I., ...

& Yusof, M. T. (2021). Plant growth-promoting bacteria as an emerging tool to manage bacterial rice pathogens. Microorganisms, 9(4), 682.

Olanrewaju, O. S., & Babalola, O. O. (2019). *Streptomyces*: implications and interactions in plant growth promotion. Applied microbiology and biotechnology, 103, 1179-1188.

Pacios-Michelena, S., Aguilar Gonzalez, C. N., Alvarez-Perez, O. B., Rodriguez-Herrera, R., Chávez-González, M., Arredondo Valdes, R., ... & Ilyina, A. (2021). Application of *Streptomyces* antimicrobial compounds for the control of phytopathogens. Frontiers in Sustainable Food Systems, 5, 696518.

Palaniyandi, S. A., Yang, S. H., Zhang, L., & Suh, J. W. (2013). Effects of actinobacteria on plant disease suppression and growth promotion. Applied microbiology and biotechnology, 97, 9621-9636.

Saeed, Q., Xiukang, W., Haider, F. U., Kučerik, J., Mumtaz, M. Z., Holatko, J., ... & Mustafa, A. (2021). Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: A comprehensive review of effects and mechanisms. International Journal of Molecular Sciences, 22(19), 10529.

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., & Nelson, A. (2019). The global burden of pathogens and pests on major food crops. Nature ecology & evolution, 3(3), 430-439.

Sadasivam, S., &Manickam, A. (1991). Biochemical methods. 2nd edn, New age International (P) Limited Publishers, Chennai, India, pp. 256

Thimmaiah, S.K. (1999). Standard methods of biochemical analysis. Kalyani Publishers, New Delhi, India, pp. 545.

Udayashankar, A. C., Nayaka, S. C., Reddy, M. S., & Srinivas, C. (2011). Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae pv. oryzae*. Biological Control, 59(2), 114-122.

Van Hop, D., Hoa, P. T. P., Quang, N. D., Ton, P. H., Ha, T. H., Van Hung, N., ... & Thom, V. T. (2014). Biological control of Xanthomonas oryzae pv. oryzae causing rice bacterial blight disease by Streptomyces toxytricini VN08-A-12, isolated from soil and leaf-litter samples in Vietnam. *Biocontrol Science*, *19*(3), 103-111.

Xu, T., Li, Y., Zeng, X., Yang, X., Yang, Y., Yuan, S., ... & Zhu, Y. (2017). Isolation and evaluation of endophytic *Streptomyces endus* OsiSh-2 with potential application for biocontrol of rice blast disease. Journal of the Science of Food and Agriculture, 97(4), 1149-1157.

Yu, S., Ali, J., Zhou, S., Ren, G., Xie, H., Xu, J., ... & Zhang, Q. (2022). From Green Super Rice to green agriculture: Reaping the promise of functional genomics research. Molecular Plant, 15(1), 9-26.

Chapter 6

Extraction of crude metabolite, Isolation, and characterization of pure compounds from *Streptomyces luteireticuli* (NIIST D31)

6.1. Introduction

In both developed and developing nations, obesity is a leading lifestyle disease. According to the WHO, obesity is one of the most ignored and apparent health issues (Haslam and James, 2005). Obesity is one of the most serious risks to global health in this millennium, with over 1 billion adults being overweight and at least 300 million of them considered clinically obese (Arbeeny, et al 2004; Harrold, et al 2003). Today's most rapidly evolving areas of biomedical research include the regulation of energy homeostasis for metabolic diseases (Birari & Bhutani, 2007). Species of *Streptomyces* are believed to be the main source of natural products. Which includes antimicrobial, anticancer, antifungal, antimalarial, and anti-obesity. Microbially derived natural products, particularly those derived from *Streptomyces* species, have a significant impact on regulating fat deposition and lipase inhibitory activity, thereby mitigating the risk of obesity. According to earlier studies, *Streptomyces toxytricini* produces orlistat, a strong pancreatic and gastric lipase inhibitor that reduces the absorption of dietary fat (Mohamed et al., 2014 ; Lunagariya et al., 2014). Similarly, Ebelactone B obtained from *Streptomyces aburaviensis*, shows a potential effect in the inhibition of pancreatic lipase. Likewise, *Streptomyces* sp. NR 061 produces a group of compounds panciclins A, B, C, D, and E having pancreatic lipase inhibitory activity (Tucci, 2010; Mohamed et al., 2014). From all these, it was evident that the importance of *Streptomyces* species in controlling obesity. The production of novel therapeutics is constantly aided by the use of natural products. Even in this case, the only medication used clinically is orlistat. As a result, there is a great need for newer discoveries from natural sources that should be developed as novel anti-obesity therapeutics. Natural compounds have the advantage of chemo-diversity and biological friendliness (Lunagariya et al., 2014). In the present study, a novel compound (compound 1) and five other compounds were isolated from *the Streptomyces luteireticuli* NIIST D31 strain. In addition, compound 1 was evaluated for its effect on PPAR-γ expression in 3T3L-1 cells.

6.2. Materials and methods

6.2.1. Materials

Solvents for the compound extraction were purchased from Merck Life Sciences, Mumbai, India. The antibiotics disc was used as standard (positive control) and media for culturing bacteria were procured from Hi-Media.

6.2.1.1. Chemicals and cell culture materials

Penicillin-streptomycin antibiotics, Fetal Bovine Serum (FBS), bovine calf serum trypsin - ethylenediaminetetraacetate (EDTA), phosphate buffer saline (PBS), and Hanks balanced saline solution (HBSS) were purchased from Gibco, USA. Dulbecco's modified Eagle's medium (DMEM) was purchased from Hi Media (Mumbai, India). Oil Red O stain, dexamethasone, powder 3- isobutyl-1-methylxanthine (IBMX), skimmed milk and insulin were purchased from Sigma-Aldrich Co. USA. 3T3-L1 pre-adipocytes were obtained from National Center for Cell Sciences (NCCS, Pune, India). We bought all of the primary and secondary antibodies for western blotting from Santa Cruz Biotechnology in the United States. The kit for chemiluminescence detection was bought from Advansta in the United States.

6.2.1.2. Test bacterial pathogens

Bacillus cereus MTCC 1305, *Staphylococcus aureus* MTCC 902, *Mycobacterium smegmatis* MTCC 993, *Proteus mirabilis* MTCC 425, *Escherichia coli* MTCC 2622, and *Pseudomonas aeruginosa* MTCC 2642 were the test bacterial pathogens used for antibacterial activity These strains procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India). They were then kept at 4 °C on nutrient agar slants.

6.2.2. Methods

6.2.2.1. Culturing of NIIST D31 and extraction of crude metabolite

The *Streptomyces* sp. NIIST-D31 strain was cultured in yeast malt broth and maintained at 28 ± 2 °C in a shaking incubator (Lab Companion IS97IR, Jeio Tech, Korea). Once the culture had grown for ten days, it was centrifuged for 20 minutes at 10,000 rpm. To the supernatant, an equal volume of solvent (Ethyl acetate: methanol 95:5) was added and the extraction was repeated twice to get maximum metabolite. The organic layer obtained was concentrated by using a rotary evaporator. Repeated the culturing of *Streptomyces* strains to obtain more metabolite.

6.2.2.2. Antimicrobial activity of crude metabolite against human pathogens

Disc diffusion method was used to assess the antibacterial activity of crude extract (Jacob et al. 2017) method against six test bacterial human pathogens. Antibacterial activity of the crude organic extract was carried out by inoculating test bacteria in nutrient agar, which was incubated at 37 °C for 18 h and adjusted to 1 x 10^6 CFU/mL and agar overlay to MHA agar plates. A sterile disc of 6 mm impregnated with 30 µL of the crude organic extract (10 mg/mL concentation) dissolved in 100% methanol and

placed in the center of each Petri plate. Then incubated, for 24 hours at 37 °C and Inhibition Zone (measured in millimetres) was recorded. Disc with only methanol was kept as negative control and antibiotic disc (chloramphenicol or ampicillin) as positive control.

6.2.2.3. Isolation of compounds from *Streptomyces* NIIST D31 strain crude extract

Crude metabolite (890 mg) obtained from the first batch was purified using silica gel column chromatography (100–200 mesh). To obtain fractions, hexane/ethyl acetate combination (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 100:0) of solvents was used by gradient elution. Thin layer chromatography of all fractions was monitored under short and long-wavelength UV. Compound isolation was achieved by additional purification of the resultant fractions using column chromatography (100–200 mesh). For some fractions, preparative TLC was used for the purification of compounds. $CDCl_3$ or CD_3OD were used to dissolve the isolated compounds and recorded ^{1}H (proton) and ^{13}C (Carbon) NMR (Nuclear Magnetic Resonance) on a Bruker AscendTM 500 MHz spectrometer at 500 and 125 MHz, respectively. The chemical shifts (δ) were given in parts per million (ppm), coupling constants in Hz, and multiplicity as s for singlet, t for triplet, d for doublet, and dd for double doublet.

6.2.2.4. Structure elucidation of compounds

With the help of the 2D NMR experiments COSY (Correlated Spectroscopy), HSQC (Heteronuclear Single Quantum Coherence), NOESY (Nuclear Overhauser Effect Spectroscopy), and HMBC (Heteronuclear Multiple Bond Correlation), the structure of compound 1 was successfully determined and the HR-ESI-MS data were acquired from a Thermo Scientific Exactive mass spectrometer equipped with an Orbitrap analyzer and ions are expressed in m/z. JASCO 810 spectrometer equipped with peltier thermostatic cell holders was used for Circular dichroism (CD) measurements. The CD spectra were recorded as θ in millidegrees, and they were converted to Δε using the formula $\Delta\varepsilon = \theta/(33982cl)$, where c is the concentration in M, l is the path length in cm, and Δε is the difference in the molar absorptivity for oppositely polarized light in M^{-1} cm^{-1}. To measure UV-Vis absorbance, a Shimadzu UV2600 UV-Vis spectrophotometer was used. A JASCO P-2000 digital polarimeter was used to record a specific optical rotation. Using a PerkinElmer Series FT-IR spectrum-2, the infrared spectrum was recorded over the wavenumber range of 4000–400 cm^{-1} at a resolution of 2 cm^{-1}. The compound was carefully inserted between the KBr pellets in order to do FT-IR measurements of the compound.

6.2.2.5. Computational methods

Stereochemical analysis of compound 1 in the singlet state was carried out by density functional theory (DFT) method B3LYP/6-311G(d,p), (Becke, 1993) using Gaussian09 suite of programs. For SCRF, the polarizable continuum model (PCM) (Miertuš et al., 1981) is selected for the solvent methanol as implemented in Gaussian09.

6.2.2.6. Evaluation of isolated compound for biological activities

6.2.2.6.1. cell culture and treatments

The 3T3-L1 pre-adipocytes were cultured to the confluence under a humidified 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) at 37 °C. Which contains 1% penicillin-streptomycin and 10% bovine calf serum. The cells had reached confluence after two days (day 0). Then the 3T3-L1 pre-adipocyte cells were cultured in differentiation medium (DM) containing 10 µg ml−1 insulin, 10% fetal bovine serum, 1 µM dexamethasone, and 0.5 mM IBMX, Two days after stimulation with differentiation media, including 0.5 mM IBMX, 10 µg ml−1 insulin and 1 µM dexamethasone (day 2), then the medium was changed to a 10% FBS/DMEM medium which contains 10 µg ml−1 insulin. After two days (4th day), the medium was changed to 10% FBS/DMEM medium and cultured in 10% FBS/DMEM medium repeated, every 2 days. By the 8th day, cells achieve full differentiation.

6.2.2.6.2. Cell viability assay on 3T3L-1 cells with compound 1

For cell viability assay, an MTT assay of the compound was carried out to find the toxic effects of compound 1 on 3T3L-1. Various concentrations of compound 1 were used in cell viability assay (5 µM, 10 µM, 20 µM and 30 µM).

6.2.2.6.3. Effect of compound 1 in the differentiation of adipocytes

During the adipocyte differentiation, 3T3-L1 cells (cells between 0 to 4 days) (Kim, et al., 2020) were treated with compound 1 of two concentrations (5 µM and 10 µM). The experiment consists of four groups as follows.

(a) C1 group: 3T3-L1 pre-adipocyte cells without differentiation.
(b) C2 group: Differentiated 3T3-L1 cells.
(c) 1 (5 µM) group: 3T3-L1 cells treated with 5 µM of compound 1.
(d) 1 (10 µM) group: 3T3-L1 cells treated with 10 µM of compound 1.

6.2.2.6.4. Oil Red-O-staining

To assess lipid accumulation in the adipocyte cells, Oil Red O staining was performed. For this, on the 8th day of differentiation, cultured 3T3-L1 cells were washed with cold phosphate-buffered saline (PBS) and fixed the cells with 4% formaldehyde at 25 °C. Thereafter, adipocyte cells were washed with PBS and permeabilized with 0.1% Triton X 100 followed by Oil Red O staining at room temperature for 20 min. After that, cells were visualized under a light microscope and absorbance was spectrophotometrically measured at 490 nm (Poornima, et al., 2022).

6.2.2.6.5. Western blot analysis

After the respective treatments, adipocyte cells were lysed with RIPA buffer and a protease inhibitor cocktail (Sigma Aldrich, USA). The cell suspensions were then centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatant was used for immunoblot analysis and the concentration of protein was analysed by using the bicinchoninic acid kit (Pierce, Rockford, IL, USA). Then equal amount of proteins was separated by 10% SDS-PAGE and transferred to PVDF. TBS-Tween 0.1% was used for blocking, which contain 5% non-fat skimmed milk (Bio-Rad, Hercules, CA, USA) immediately after blocking membranes were incubated overnight at 4 °C with primary antibodies. On the other day, membranes were washed with TBS-Tween 0.1% and washed membrane were incubated with HRP-conjugated respective secondary antibodies for 2–4 h at room temperature. Repeated washing procedure three times with TBS-Tween 0.1% and the membranes were developed using Western Blot Hyper HRP Substrate (Takara Bio Inc. USA) and analysed the protein bands (Bio-Rad ChemiDoc MP imaging systems, USA).

6.3. Results

6.3.1. Metabolite extraction from NIIST D31 strain

From the 7.8 L of fermented culture broth 890 mg of crude metabolite was obtained as the first batch. Second batch fermentation leads to the extraction of 1.31 g of metabolite obtained from 7.8 L.

6.3.2. Antibacterial activity of crude metabolite against human pathogen

Antibacterial activity of crude metabolite against six human bacterial pathogens. Crude metabolite of NIIST D31 inhibits all the pathogens tested (**Figure 6.1**). Maximum zone of inhibition was produced against *Mycobacterium smegmatis* 29.00±1.00 mm and is followed by *Pseudomonas aeruginosa* 23.66±1.15 mm and *Bacillus cereus* 22.66±0.57 mm (**Table 6.1**). Least antibacterial activity of the extract was noted against *Escherichia coli* 14.00±1.00 mm followed by *Proteus mirablis.* When

compared with positive control (antibiotics), crude extract efficiently inhibits the three bacteria such as *Bacillus cereus, Mycobacterium smegmatis,* and *Staphylococcus* aureus by producing a larger zone of inhibition than the antibiotics (Chloramphenicol) used. In the case of other three bacteria, *Escherichia coli, Pseudomonas aeruginosa,* and *Proteus mirablis,* antibiotics produce a larger inhibition zone than the crude metabolite.

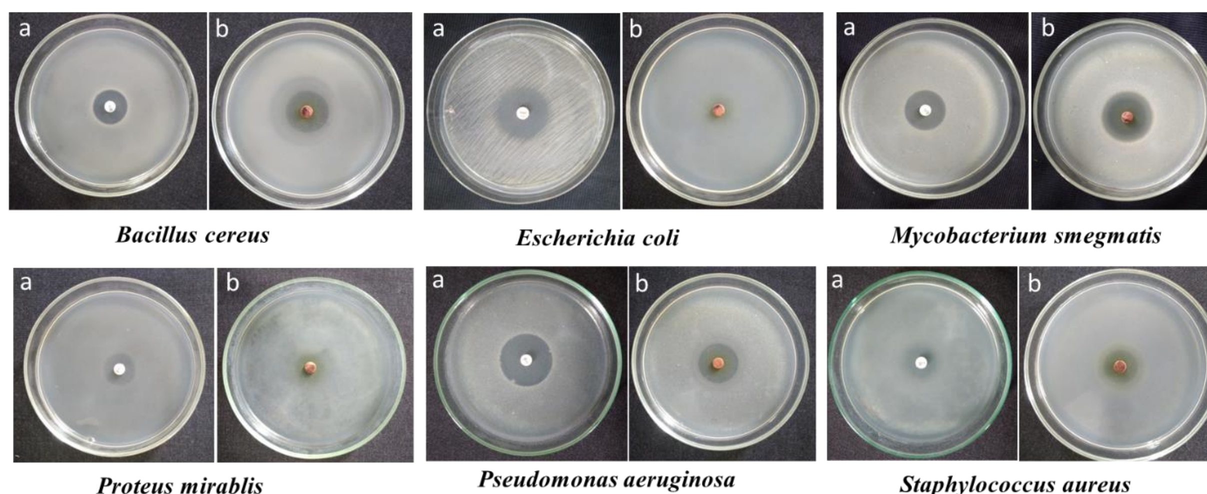


Figure 6.1. Antimicrobial activity of NIIST D31 crude extract against human bacterial pathogens. (a: control (chloramphenicol (against *B. cereus*, *M. smegmatis*, *P. mirablis*, *P. aeruginosa*, and *S. aureus*) and Ampicillin (against *E. coli*)), b: NIIST D31 crude metabolite).

Table 6.1. Antimicrobial activity of crude metabolite of NIIST D31.

	Zone of inhibition in mm	
Bacteria	**Control (Antibiotics)**	**NIIST D31 crude metabolite**
Bacillus cereus	18.33±0.57	22.66±0.57
Escherichia coli	21.00±1.00	14.00±1.00
Mycobacterium smegmatis	24.00±1.00	29.00±1.00
Proteus mirablis	18.66±0.57	16.33±0.57
Pseudomonas aeruginosa	28.66±0.57	23.66±1.15
Staphylococcus aureus	15.33±0.57	21.00±1.00

Values are triplicates of independent experiments expressed in mean±standard deviation in mm. (control/ antibiotics: chloramphenicol for all except *E. coli* (Ampicillin)).

6.3.3. Compound isolation from crude metabolite

Compound 1 (17 mg, eluted at 60% ethyl acetate), Compound 2, (4 mg eluted at 1:1 hexane: ethyl acetate), Compound 3, (2.5 mg, eluted at 30% ethyl acetate), Compound 4, (6.4 mg, eluted at 60% ethyl acetate), and Compound 6 (9.4 mg, eluted at 60% ethyl acetate) were isolated after the purification of metabolites (obtained from the first batch) using column chromatography (100–200 mesh). Using silica gel (230–400 mesh) column chromatography to purify the second batch of the

organic extract (1.31 g, extracted from 7.8 L fermented broth), the following compounds were obtained: compound 1 (20.9 mg), compound 2 (5 mg), compound 3 (4.9 mg), compound 4 (3.9 mg), compound 5 (7.6 mg eluted at 100% ethyl acetate), and compound 6 (1.7 mg). NMR clearly shows that compound 2 was obtained as a mixture from both batches; subsequent purifications by column chromatography resulted in the loss of compound 2, yielding 1.6 mg.

6.3.4. Structural characterization of compounds

Compound 1 is a colourless amorphous solid with molecular formula $C_{22}H_{23}NO_8$, mass was calculated as 430.1502 $[M + H]^+$ and was observed as 430.1497, from HR-ESI-MS along with ^{1}H and ^{13}C NMR data, implying 12 degrees of unsaturation. The IR spectra of compound 1 displayed characteristic absorption (ν_{max}) of the carbonyl group at 1669 $cm^{-1}$and nitro groups at 1510 and 1342 cm^{-1}. From the ^{1}H and ^{13}C NMR spectra, it was confirmed that the occurrence of a methoxy, four aromatic protons, three methyl singlets, three sp3 methine protons, two sp3 methylene protons, six quaternary sp^2 carbons, and a carbonyl C3 (δ_C 180.6 ppm). The presence of aromatic protons with ortho-coupling constant of 8.4 Hz and downfield signals specify the presence of a para-nitro substituted phenyl moiety. Structure elucidation of isolated compounds was carried out via ^{1}H-^{13}C HMBC data (**Figure 6.2).** Presence of HMBC correlations of methyl H-17 with C10, C11, C12; H-12 (s, δ_H 4.08 ppm) with C11, C13 (quaternary aromatic carbon): aromatic proton H-14 with methine C12 (δ_C 59.6 ppm) and based on chemical shifts, conformed the present of an oxygenated backbone C10-C11-C12. And was found that the C12 is attached to the phenyl moiety and observed a methyl C17 on 4° carbon C11. Presence of HMBC correlations of methylene proton H-7 with C5, C6, C8, C10; H-10 (s, δ_H 3.48 ppm) with C7, C8 (4°), C11; methylene proton H-9 with C6, C7, C8, C10 indicated the presence of oxygenated backbone C9-C8-C10-C11-C12. The presence of a tetrahydrofuran ring was evident based on the chemical shift of methine H-6, δ_H 5.16 ppm, COSY correlation with H-7, and HMBC correlations. The downfield shift for H-6 is explained by the attachment of its carbon to the 4° sp^2 carbon C-5 (δ_C 153.1 ppm).H-6 shows HMBC correlations with C4, C5, C7, C8, and C4 is confirmed as a 4° sp^2 carbon of δ_C 121.1 ppm, and observed a difference of >30 ppm with C5 suggest that C4 and C5 are part of an unsaturated ketone, and further confirmed by the presence of HMBC correlation of methyl H-20 with C3, C4, C5. The presence of HMBC correlation of methyl H-19 with C1, C2, C3, methyl H-18 with C1 and since the chemical shift difference between C1 and C2, >60 ppm, the presence of another unsaturated carbonyl to the same ketone (C3) is evident. According to the chemical shift differences, mentioned above, double bonds of both C1, C2, and C4, C5 are bound to the same C3 carbonyl and both C1, C5 are attached to the same oxygen atom, where C1 is attached to methoxy, evocative to an α-methoxy-γ-pyrone, found as a head group in polyketides. Ultimately, according to the molecular

formula with two oxygen atoms and remaining two degrees of unsaturation, the carbons (oxygenated carbons) C8, C10, C11, and C12 are involved as two oxygenated rings in a vicinal diepoxide. This indicates the presence of an aureothin backbone in compound 1 with an exceptional epoxidized double bonds (**Figure 6.2**).

NOESY correlations with respect to the conserved "R" configuration of aureothin's C6 were taken into consideration for the stereochemical determination. The configuration of stereocenters C8-C10 oxirane in relation to C6 can be determined with the help of formational restrictions imposed by the presence of spiro [2.4] heptyl moiety with C8 spiro carbon.

NOESY correlations for H-9a with H-7a, H-6 with H-9b and H-10 with H-7a, H-7b (Figure 1). The NOESY correlation for H-10 with both H-7a and H-7b as a result of equatorial orientations and pseudo axial, which places oxirane ring above the plane (**Figure 6.2**). The NOESY correlation between H-10 and H-9a, or between H-9a and H-9b, will occur if the oxirane ring is discovered below the plane. The C8-C10 configuration was thus determined by the NOESY correlation. According to a significant additional NOESY correlation between H-12 and H-17, the C11-C12 oxirane originated from the corresponding Z-double bond, indicating that compound1 is a vicinal diepoxide of alloaureothin (novel compound).

Other NOESY correlations were not considered when determining the relative configuration of the stereocenters of the C11-C12 oxirane because of the free-rotating C10-C11 single bond. Therefore, a computational method was used to compare experimental and quantum mechanically calculated ECD spectra (**Figure 6.3**). This helped to determine the structure of compound 1 by revealing the configuration of the C11–C12 oxirane ring above the plane. The configuration of compound 1 obtained at the B3LYP/6-311G(d,p) level revealed a dipole moment of 14.45 D and an energy of −1508.81055491 a.u.

Compound 2 was identified as 2,3-dimethoxybenzamide (**Figure 6.2**) by the presence of dimethoxy functionalities and three different aromatic proton signals in its ^{1} H NMR, which also showed characteristic broad singlets corresponding to NH_2 protons of a primary amide. similarly, compound 3 was identified as 2,3-dihydroxybenzamide (**Figure 6.2**) from its ^{1} H NMR. Proton (^{1}H) NMR of compound 4 showed distinctive aromatic signals in the range of 6-7 ppm, and its ^{13}C NMR demonstrated the presence of a primary amide functionality, identifying compound 4 as 1H-pyrrole 2-carboxamide (**Figure 6.2**) based on HR-ESI-MS analysis, compound 5 showed m/z 120.0449 that corresponds to [M-OH]+, both HR-ESI-MS analysis and NMR, helped to identify compound 5 as 4-

aminobenzoic acid (**Figure 6.2**). From HR-ESI-MS and NMR data, Compound 6 was easily identified as 1,6-dimethoxyphenazine (**Figure 6.2**).

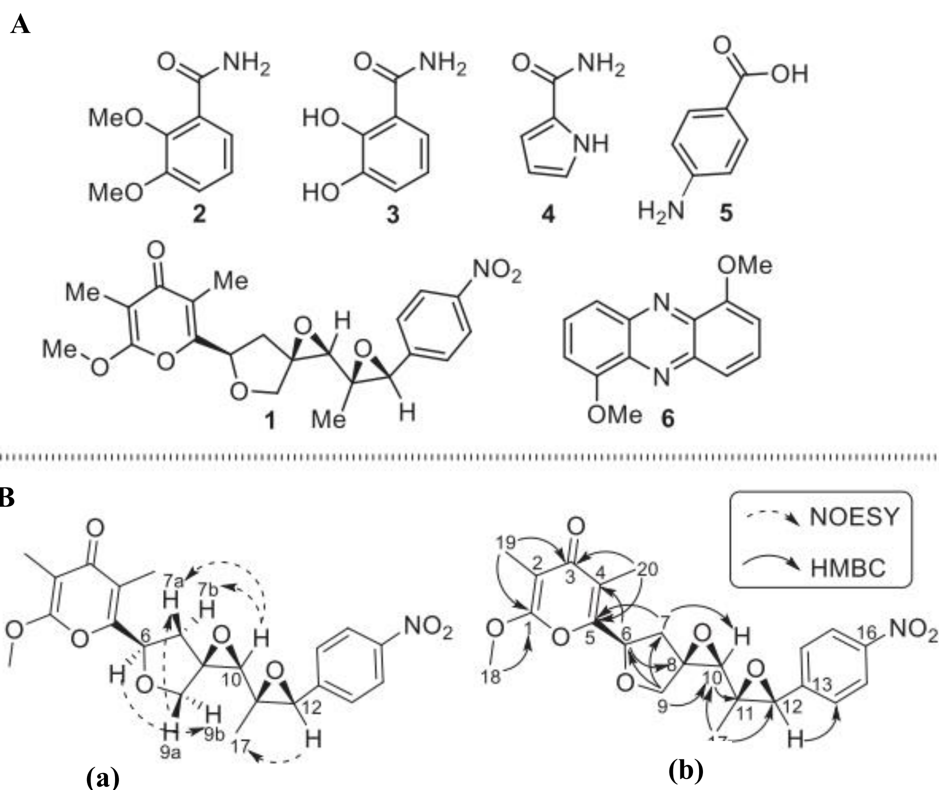


Figure 6.2. A: Structures of compounds Isolated (**1-6**) from *Streptomyces* sp. NIIST-D31 strain; **B**: key ^{1}H-^{1}H NOESY **(a)** and ^{1}H-^{13}C HMBC (**b**) correlations of compound 1.

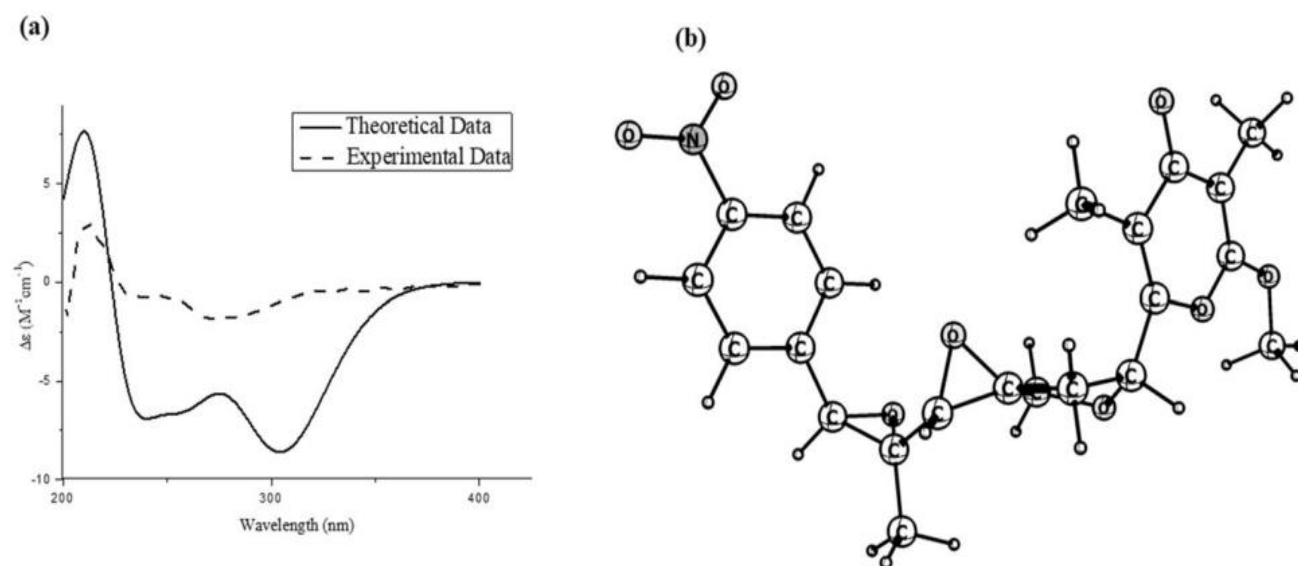


Figure 6.3. Stereochemistry and Conformation of compound 1. **(a)** Experimental and calculated ECD spectrum for compound 1; **(b)** Optimized structure of compound 1.

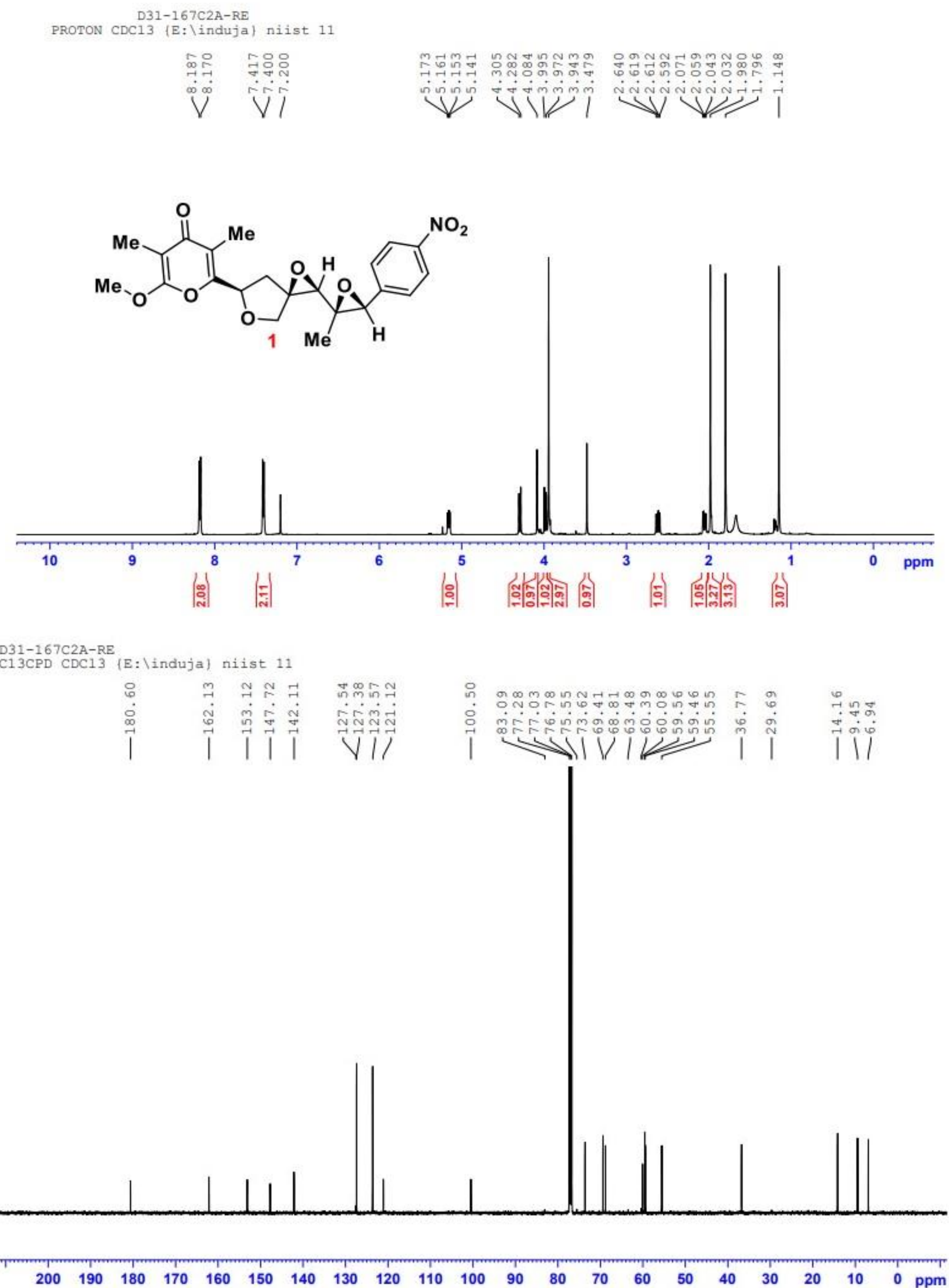
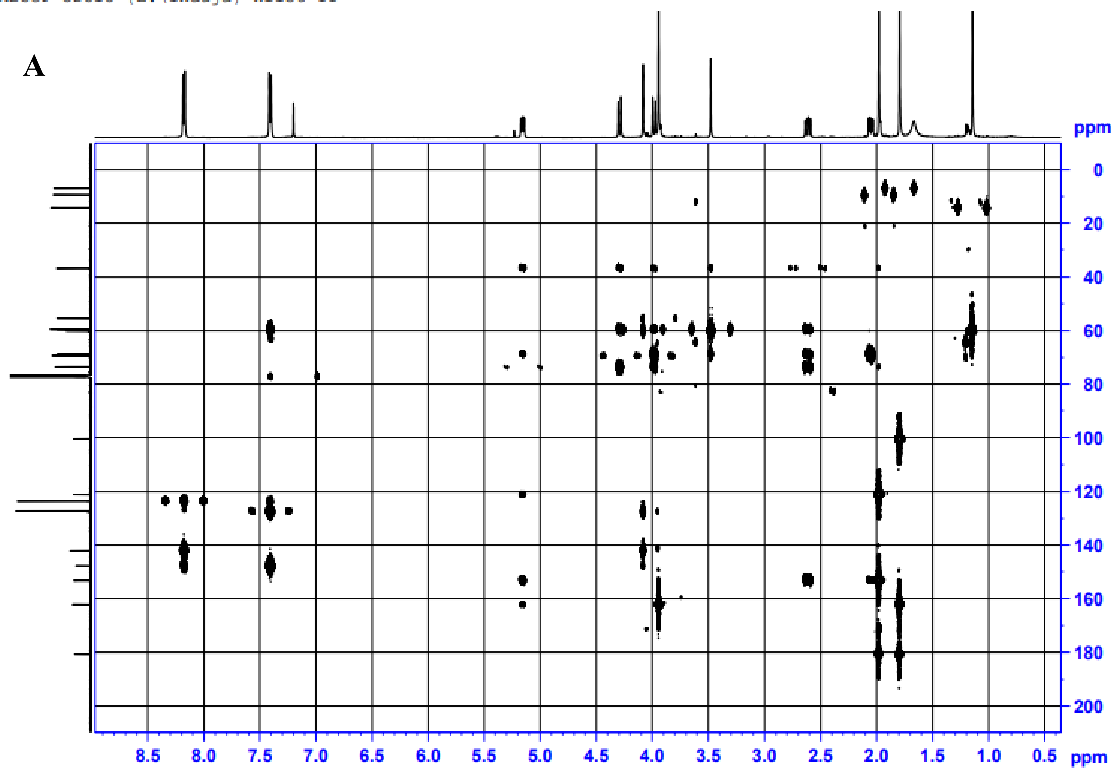


Figure:6.4. ^{1}H and ^{13}C NMR of compound 1.



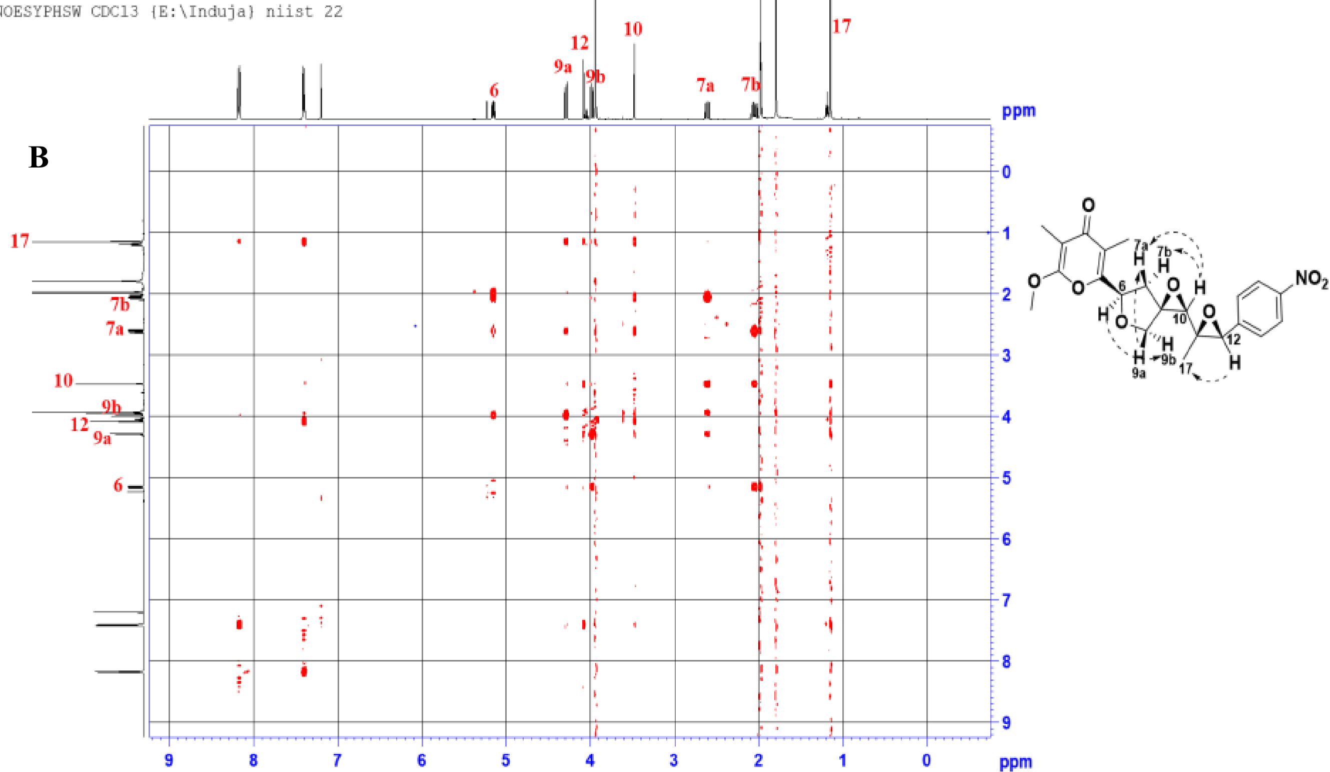


Figure:6.5. A: HMBC of compound 1; **B** :NOESY (delay D8: 1.5") of compound 1 with annotation.

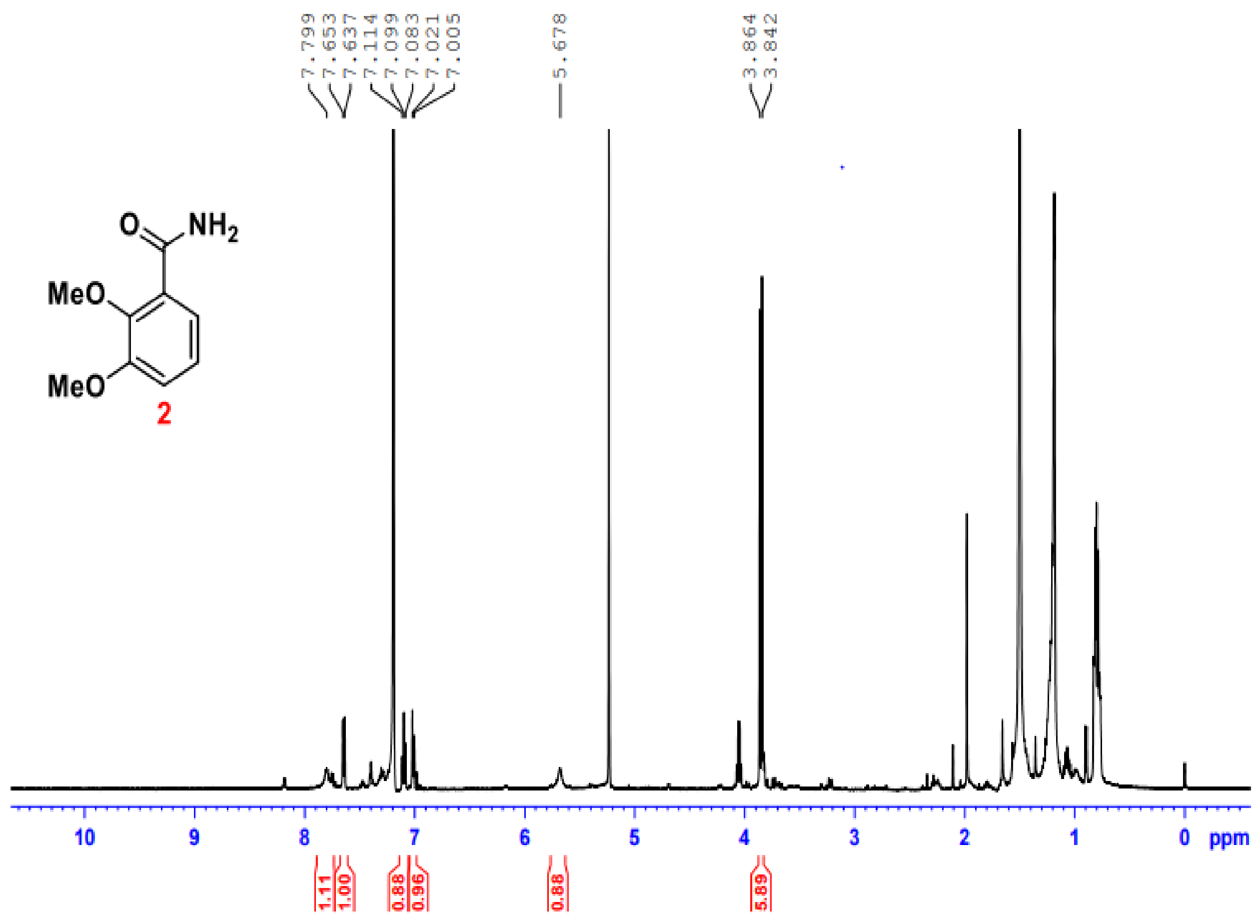


Figure 6.6. ^{1}H and HRMS of compound 2

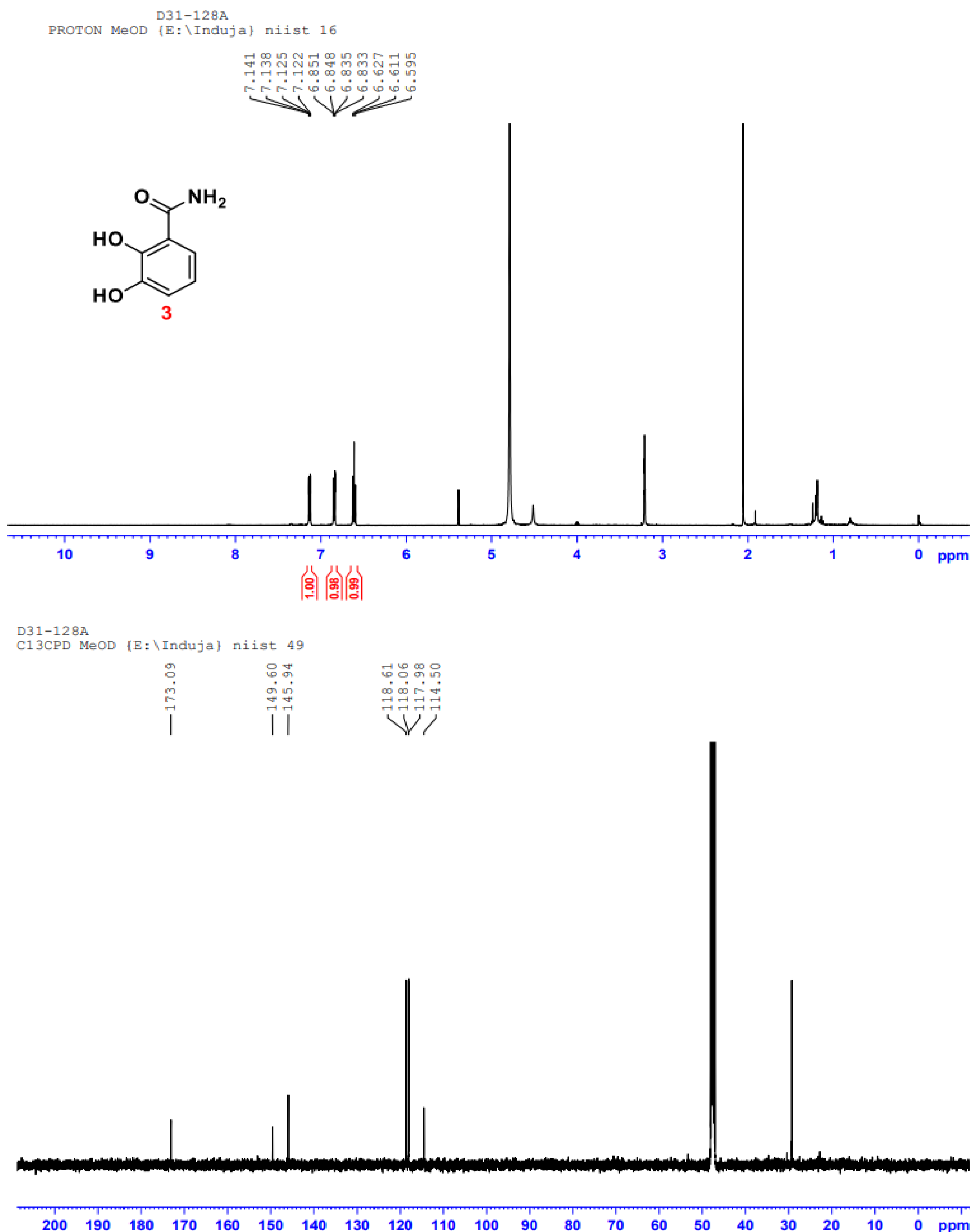
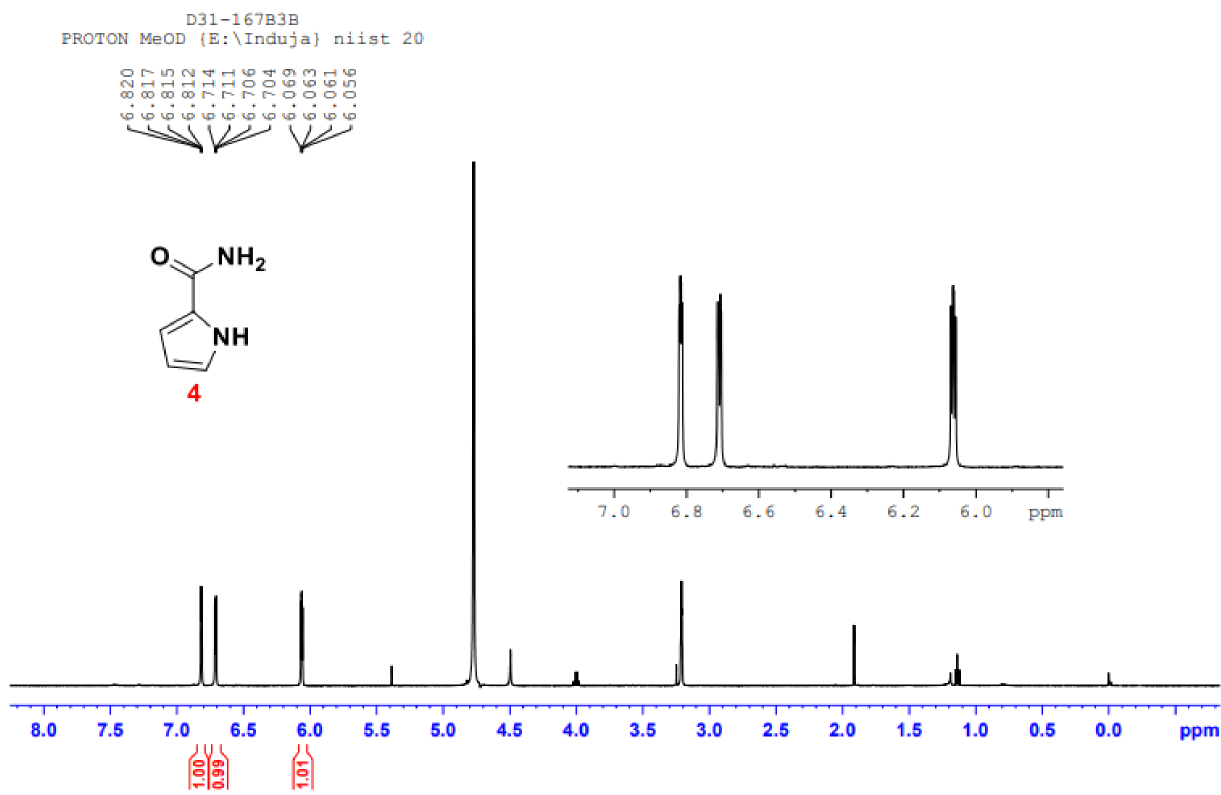


Figure 6.7. ^{1}H and ^{13}C NMR of compound 3.



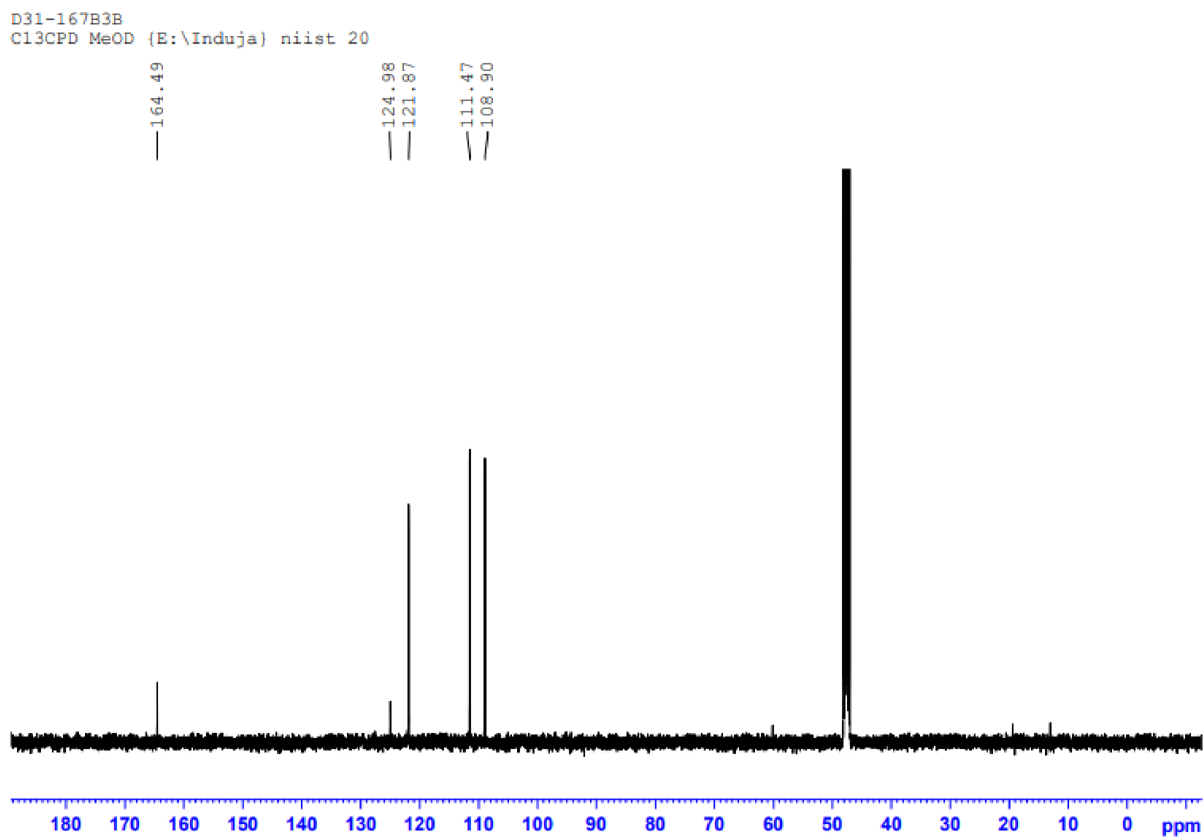
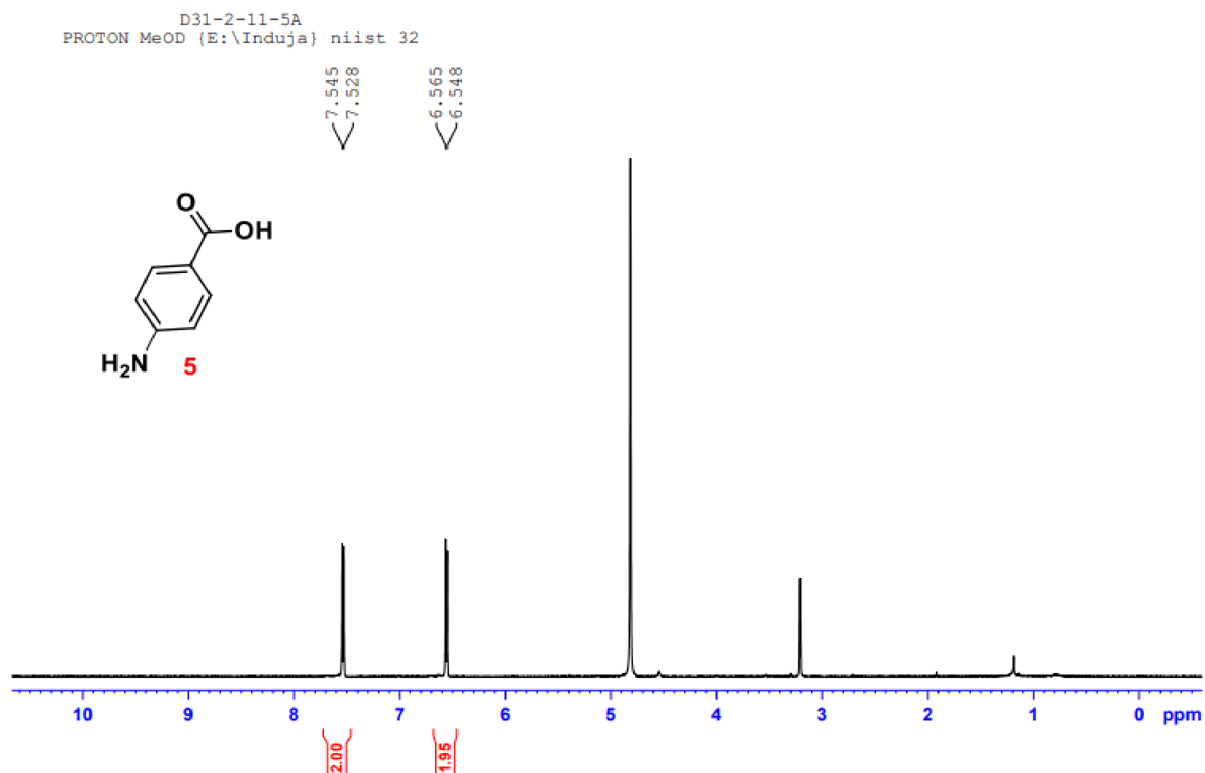


Figure 6.8. ^{1}H and ^{13}C NMR of compound 4.



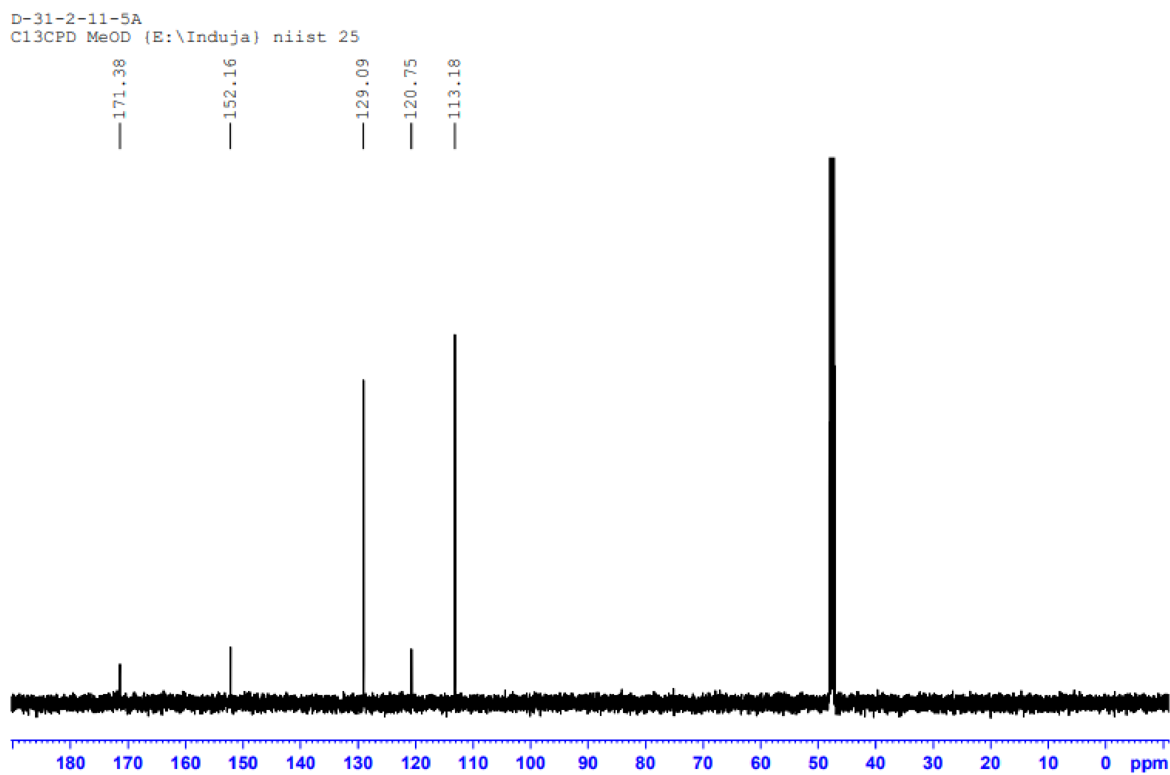


Figure 6.9. ^{1}H NMR, ^{13}C NMR and HRMS of compound 5.

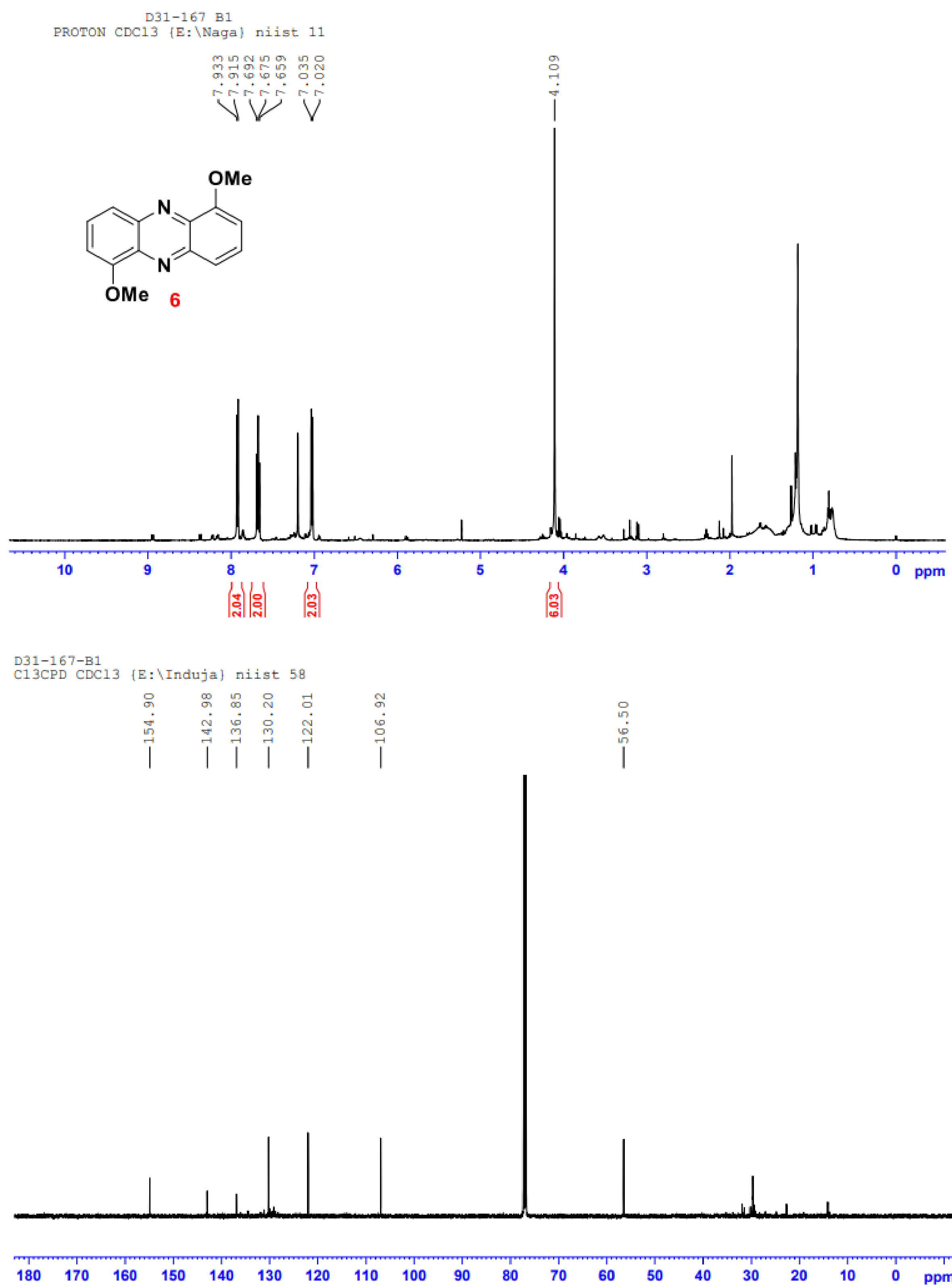


Figure 6.10. ^{1}H and ^{13}C NMR of compound 6.

6.3.5. Biological activity of compounds

6.3.5.1. Cell viability assay

Compound 1 was found to be non-toxic to 3T3L-1 cells up to 30 µM. maximum viability was observed at 5 µM and 10 µM these two concentrations of compound 1 was selected for further studies.

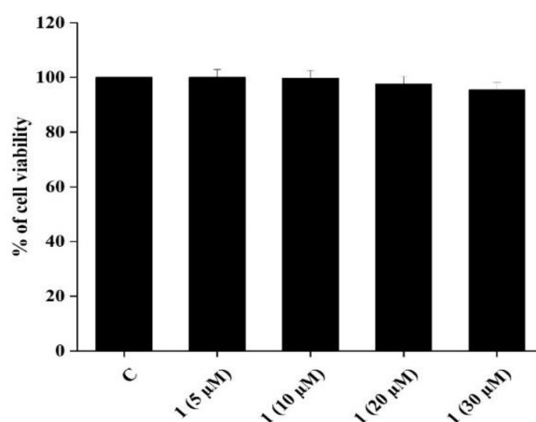


Figure 6.11. Effect of various concentration of Compound 1(5, 10, 20 and 30µM) on viability of 3T3L-1cells analyzed by MTT assay. Data are expressed in mean ± SEM where n = 3.

6.3.5.2. Effect of compound 1 on expression of PPAR-γ in 3T3-L1 cells

In the C2 group, the expression of PPAR-γ increased by 26.88% ($p \leq 0.05$) when compared to C1 group (**Figure 6.12**). when compared to C2, there was observed a decreased expression of PPAR-γ when treated with compound **1** (decreased by 38.45% with 5 µM and 48.84% with 10 µM).

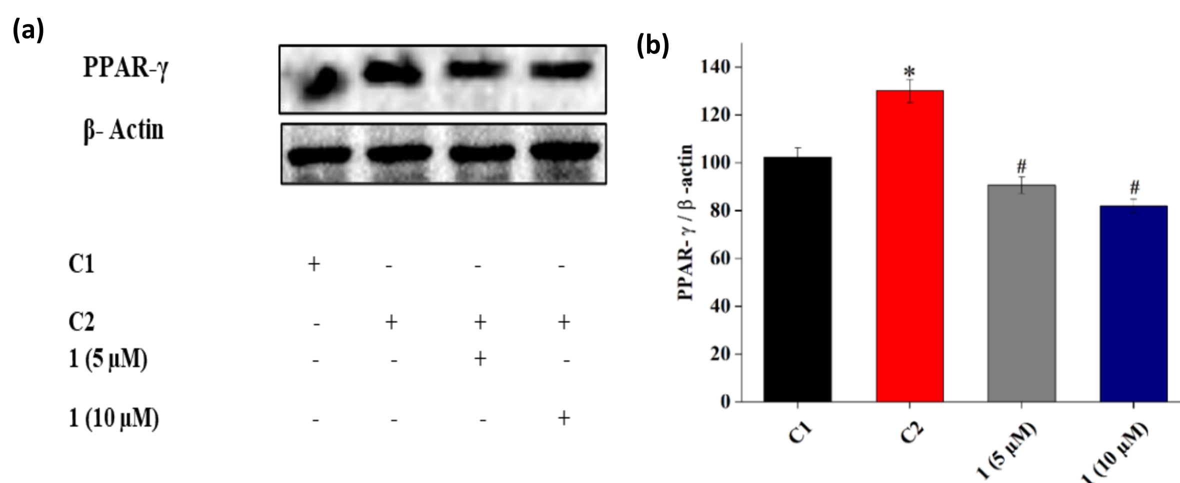


Figure 6.12. Effect of compound **1 (Alloaureothin vicinal diepoxide)** on expression of PPAR-γ: **(a)** protein expression in various experimental groups**. (b)** The relative intensity of bands quantified with β-actin. Pre-adipocyte cells (C1); Differentiated 3T3L-1 cells (C2); Differentiated 3T3L-1 + 5 µM compound **1**; Differentiated 3T3L-1 +10 µM compound **1**). Data expressed in mean ± SEM; where n = 3. * Represents the significant difference from the group C1 ($p \leq 0.05$) and # Represents the significant difference from the group C2 ($p \leq 0.05$).

6.3.5.3. Effect compound 1 (Alloaureothin vicinal diepoxide) in lipid accumulation

Both the concentration (5 µM and 10 µM) of compound **1** exhibited a significant reduction of lipid accumulation (17.55% when treated with 5 µM and 23% with 10 µM) in a dose-dependent manner. When compared to the compound (10 µM.), C2 group (Differentiated 3T3L-1) a significant increase in lipid content. (31.99%) (**Figure 6.13**).

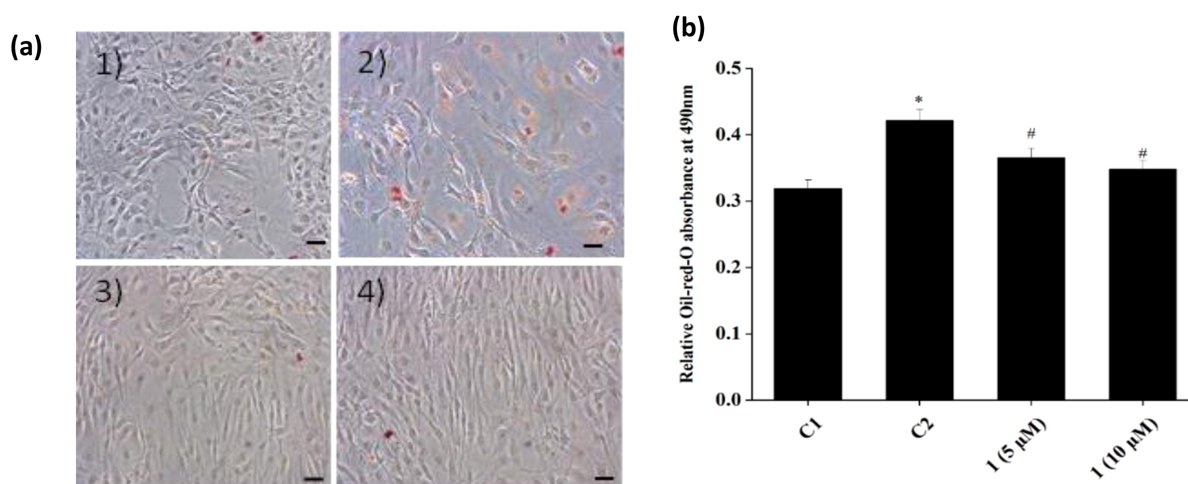


Figure 6.13. Effect of **Alloaureothin vicinal diepoxide (Compound 1)** on lipid accumulation in 3T3-L1 cells (Adipocyte cells): **(a)** Oil Red O staining of 3T3-L1 cells showing lipid accumulation (phase-contrast microscopic image, scale bar 50 µm.). 1) C1: Pre-adipocyte; 2) C2: Differentiated 3T3L-1 cells (3) Differentiated 3T3L-1 cells + compound **1** (5 µM); 4) Differentiated 3T3L-1 cells + compound **1** (10 µM). **(b)** Absorbance recorded at 490 nm after Oil-Red-O staining. Data expressed in mean ± SEM; where n = 3. # Represents the significant difference from the group C2 and * Represents the significant difference from the group C1 ($p \leq 0.05$).

6.3.5.4. Antimicrobial, anticancer, and anti-inflammatory activity

Compound 1 was explored for their biological activity (antimicrobial activity against human pathogenic bacteria and fungus), anticancer and anti-inflammatory activity). But the compound does not show positive result. Other compounds (compound 2, 3, 4, 5 and 6) were tested for their antibacterial activity against human pathogens (*B. cereus*, *M. smegmatis*, *P. mirablis*, *P. aeruginosa S. aureus* and *E. coli*) but compounds show negative result.

6.4. Discussion

More than half of the medications available in modern medicine are made from natural products or their derivatives (Mahmoud et al., 2023). Most microbial bioactive metabolites are produced by actinomycetes, specifically *Streptomyces* genus (Kim et al., 2021). Various compounds from *Streptomyces* strains have significant role in controlling obesity. Some of them includes, a strong

pancreatic and gastric lipase inhibitor called orlistat, which is produced by *Streptomyces toxytricini,* reduces the absorption of dietary fat. Another compound is a powerful inhibitor of hog pancreatic lipase, Ebelactone A and B, is released by the Actinomycetes strain G7-GI, which is related to *Streptomyces aburaviensis* (Lunagariya et al., 2014). Moreover, in present study a novel compound identified as a vicinal diepoxide of alloaureothin was isolated along with other five compounds from Streptomyces sp. NIIST D31 strain. Alloaureothin, a polyketide metabolite produced by *Streptomyces sp*. MM23 was reported in a study condected by Ueda et al (2007). Similarly, Compound 2 was isoalted and identified as 2,3-dimethoxybenzamide and compound 3 as 2,3-dihydroxybenzamide. Previous investigations reported that *Streptomyces* species are well known to produce benzamides (Chen, et al., 2011; Yang et al., 2015; Zhang et al 2019; Shaaban et al., 2012). Compound 4 is identified as 1H-pyrrole 2-carboxamide, this compound is also reported from *Streptomyces sp*. RM-14-6 (Shaaban et al 2017). Compound 5 and compound 6 were identified as 4-aminobenzoic acid and 1,6-dimethoxyphenazine respectively. Initially, compound 1 was evaluated for antimicrobial, anti-inflammatory activity and cytotoxicity against colon and breast cancer cells but there was no activity when compared to preciously reported parent compounds alloaureothin or aureothin. Later noticed about the compound plakdiepoxide with a vicinal diepoxide shown significant activity as a potent modulator of PPAR-γ (Chinese, et al., 2016). PPAR-γ is important gene and the most investigated transcriptional factor for adipogenesis (Hamm, et al., 1999). PPAR-γ expression significantly increased during the adipocyte differentiation. They directly affect the accumulation of fat in the cells (Li, et al.,2016) precious research reported that natural products possess anti-adipogenic properties through the inhibition of PPAR-γ (Choi, et al., 2018; Shyni, et al., 2021). For instance, the 18-membered macrolide borrelidin is produced by *Streptomyces sp.* TK08330. In 3T3-L1 cells, it inhibits the differentiation of adipocytes. In adipocyte cells, the amount of PPAR-γ protein was decreased by bororelidin (Matsuo et al., 2015). Therefore, we studied and compared the PPAR-γ expression with compound 1 in preadipocytes (C1) and differentiated adipocytes (C2). In the current investigation, PPAR-γ expression was higher in the C2 group (26.88%) than in the C1 group. Comparing PPAR-γ with compound 1 to C2, there was a reduction in expression (38.45% with 5 μM and 48.84% with 10 μM). Both the concentrations (5 μM and 10 μM) of compound 1 exhibited a significant reduction of lipid content in adipocyte cell in a dose-dependent manner (17.55% reduction with 5 μM and 23% with 10 μM). It was noted that C2 group showed a significant increase in lipid content of 31.99%, when compared with compound 1. Lipid accumulation in 3T3-L1 cells were checked with Oil-red-O staining and confirmed the potential nature of compound 1 to inhibit adipogenesis. Form this study it was found that compound 1(alloaureothin vicinal diepoxide) is a novel compound isolated from the

Streptomyces sp. NIIST D31, having potential activity to inhibit adipogenesis. So, this compound could be explored for lipolytic property in future to check the ability of the compound against anti-obesity.

6.5. Conclusion

Crude extract of *Streptomyces luteireticuli* NIIST D31 strain exhibited abroad range of antibacterial activity. Crude organic extract was subjected to repeated purification through column chromatography and preparative TLC. Six pure compounds were isolated, structural elucidation of the compounds were carried out using the data of 2D NMR experiments COSY, HSQC, NOESY and HMBC. Compound 1 is identified as a novel derivative compound of alloaureothin called alloaureothin vicinal diepoxide, Similarly, Compound 2, 3, 4, 5 and 6 were identified as 2,3-dimethoxybenzamide, 2,3-dihydroxybenzamide, 1H-pyrrole 2-carboxamide, 4-aminobenzoic acid and 1,6-dimethoxyphenazine respectively. all the compounds were tested for their antibacterial activity, but compounds do not show significant activity. Compound 1 were tested for anticancer and anti-inflammatory activity, but compound show negative results in both studies. Finally, the compound 1 was explored for the PPAR-γ expression in adipocyte cells as well as the lipid accumulation of compound treated cells. It was found that the compound has anti adipogenic potential and it also reduces lipid accumulation in 3T3-L1 cells (adipocyte cells). So, isolated novel compound (alloaureothin vicinal diepoxide) is a potential inhibitor of adipogenesis and having an anti-obesity property.

6.6. References

Arbeeny, C.M., (2004) Addressing the unmet medical need for safe and effective weight loss therapies. Obesity Research & Clinical Practice, 12, 1191–1196.

Becke, A.D. (1993). Density-functional thermochemistry. III. The role of exact exchange. Journal of Chemical Physics, 98, 5648–52.

Birari, R. B., & Bhutani, K. K. (2007). Pancreatic lipase inhibitors from natural sources: unexplored potential. *Drug discovery today*. *12*(19-20), 879-889.

Chen, G., Gao, H., Tang, J., Huang, Y., Chen, Y., Wang, Y., et al. (2011). Benzamides and quinazolines from a mangrove actinomycetes *Streptomyces sp*. (No. 061316) and their inhibiting Caspase-3 catalytic activity in vitro. Chemical and Pharmaceutical Bulletin, 59, 447–51.

Chianese, G., Yu, H.B, Yang, F., Sirignano, C., Luciano, P., Han, B. N., et al. (2016). PPAR modulating polyketides from a Chinese Plakortis simplex and clues on the origin of their chemodiversity. Journal of Organic Chemistry. 81, 5135–43.

Choi, R.Y., Lee, H.I., Ham, J.R., Yee, S.T., Kang, K.Y., Lee, M.K., Heshouwu. (2018) (Polygonum multiflorum Thunb.) ethanol extract suppresses pre-adipocytes differentiation in 3T3-L1 cells and adiposity in obese mice. Biomedicine & Pharmacotherapy. 106, 355–62.

Hamm, J.K., el Jack, A.K., Pilch, P.F., & Farmer, S.R. (1999). Role of PPAR gamma in regulating adipocyte differentiation and insulin-responsive glucose uptake. Annals of the New York Academy of Sciences. 892, 134–4.

Harrold, J., et al. (2003). Neuroendocrine targets for the treatment of obesity: physiological roles and unrealized opportunities. Central Nervous System Agents in Medicinal Chemistry. 3, 141–155.

Haslam, D. W., & James, W. P. T. (2005). Obesity. Lancet. 366, 1197–1209.

Jacob, J., Reshma, U.R., Syama, H. P., Jayamurthy, P., & Dileep Kumar, B.S. (2017). Enhanced Antibacterial Metabolite Production through the Application of Statistical Methodologies by a *Streptomyces Nogalater* NIIST A30 Isolated from Western Ghats Forest Soil. PLoS ONE. 12 (4), 1–21.

Kim, H., Kim, J.H., Zuo, G., Lim, S.S. (2020). Anti-obesity effect of Melandrium firmum Rohrbach extract in 3T3-L1 cells and high-fat diet-induced obese C57BL/6N mice. Food Science & Nutrition, 8, 2251–61.

Kim, J. H., Lee, N., Hwang, S., Kim, W., Lee, Y., Cho, S., Palsson, B. O., & Cho, B. K. (2021). Discovery of novel secondary metabolites encoded in actinomycete genomes through coculture. Journal of Industrial Microbiology and Biotechnology, 48(3–4), 1–16.

Li, K.K., Liu, C.L., Shiu, H.T., Wong, H.L., Siu, W.S., Zhang, C., et al. (2016). Cocoa tea (C*amellia ptilophylla*) water extract inhibits adipocyte differentiation in mouse 3T3-L1 preadipocytes, Scientific Reports, 6, 20172.

Lunagariya, N. A., Patel, N. K., Jagtap, S. C., & Bhutani, K. K. (2014). Inhibitors of pancreatic lipase: state of the art and clinical perspectives. *EXCLI journal*, *13*, 897.

Mahmoud, M. G., Awady, M. E. E., Selim, M. S., Ibrahim, A. Y., Ibrahim, F. M., & Mohamed, S. S. (2023). Characterization of biologically active exopolysaccharide produced by *Streptomyces sp.* NRCG4 and its anti-Alzheimer efficacy: in-vitro targets. *Journal of Genetic Engineering and Biotechnology*, *21*(1), 76.

Matsuo, H., Kondo, Y., Kawasaki, T., Tokuyama, S., & Imamura, N. (2015). Borrelidin isolated from Streptomyces sp. inhibited adipocyte differentiation in 3T3-L1 cells via several factors including GATA-binding protein 3. *Biological and Pharmaceutical Bulletin*, *38*(10), 1504-1511.

Miertuš, S., Scrocco, E., & Tomasi, J. (1981). Electrostatic interaction of a solute with a continuum. A direct utilizaion of AB initio molecular potentials for the prevision of solvent effects. *Chemical Physics*, *55*(1), 117-129.

Mohamed, G. A., Ibrahim, S. R., Elkhayat, E. S., & El Dine, R. S. (2014). Natural anti-obesity agents. *Bulletin of faculty of pharmacy, Cairo University*, *52*(2), 269-284.

Padula, D., & Pescitelli, G. (2018). How and how much molecular conformation affects electronic circular dichroism: the case of 1,1- Diarylcarbinols. Molecules, 23, 128.

Poornima, M.S., Sindhu, G., Billu, A., Sruthi, C.R., Nisha, P., Gogoi, P, et al. (2022). Pretreatment of hydroethanolic extract of Dillenia indica L. attenuates oleic acid induced NAFLD in HepG2 cells via modulating SIRT-1/p-LKB-1/AMPK, HMGCR & PPAR-α signaling pathways. Journal of Ethnopharmacology, 292, 115237.

Shaaban, K.A., Saunders, M.A., Zhang, Y., Tran, T., Elshahawi, S.I, & Ponomareva, L. et al. (2017). Spoxazomicin D and Oxachelin C, potent neuroprotective carboxamides from the appalachian coal fire-associated isolate *Streptomyces sp.* RM-14-6 J. Journal of Natural Products, 80, 2–11.

Shaaban, K.A., Shepherd, M.D., Ahmed, T.A., Nybo, S.E., Leggas, M., Rohr, J. (2012). Pyramidamycins A-D and 3-hydroxyquinoline-2-carboxamide; Cytotoxic benzamides from *Streptomyces sp*. DGC1. Journal of Antibiotics. 65, 615–22.

Shyni, G.L., Sajin, K.F., Mangalam, S.N., & Raghu, K.G. (2021). An in vitro study reveals the anti-obesity effects of 7- methoxy-3-methyl-5-((E)- prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran from Myristica fragrans. European Journal of Pharmacology, 891, 173686.

Tucci, S. (2010). The role of lipid and carbohydrate digestive enzyme inhibitors in the management of obesity: a review of current and emerging therapeutic agents. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy, 3, 125–143.

Ueda, J., Hashimoto, J., Nagai, A., Nakashima, T., Komaki, H., Anzai, K, et al. (2007). New aureothin derivative, alloaureothin, from *Streptomyces* sp. MM23. Journal of Antibiotics, 60, 321–4.

Yang, X., Peng, T., Yang, Y., Li, W., Xiong, J., Zhao, L., et al. (2015). Antimicrobial and antioxidant activities of a new benzamide from endophytic *Streptomyces* sp. YIM 67086 Natural Product Research. 29, 331–520.

Zhang, X., Shu, C., Li, Q., Lian, X.Y., & Zhang, Z., (2019). Novel cyclohexene and benzamide derivatives from marine-associated *Streptomyces* sp. ZZ502. Natural Product Research. 33, 2151–921.

Chapter 7

Summary, conclusion, and future prospects

7.1. Summary, conclusion, and future prospects

Agriculture and biomedical fields require enough attention, because of the emergence of diverse pathogens with high resistance. In the case of agriculture, this can reduce the productivity of crops and in the medical field this can threaten human life. To overcome these, efficient and ecofriendly/ harmless medications or controlling measures are needed. Natural products/biological methods are the best ways to solve this problem and have a positive impact on human and the environment. Natural products derived from microbes, especially from *Streptomyces* are much more efficient because *Streptomyces* are the major source of natural products. Many researchers successfully explored *Streptomyces* species for agriculture and medicine. Hence the present study was carried out. The study focused on the isolation of *Streptomyces* strains from the unexplored Malampuzha forest in Western Ghats, Kerala. From 16 soil samples collected 300 morphologically distinct *Streptomyces* strains were isolated. Isolated strains were subjected to antibacterial activity against two indicator pathogenic bacteria, (human pathogens) *S. aureus* and *E. coli.* From, 300 strains examined, 22 strains were obtained with antibacterial potential against both the test pathogens. A secondary screening of the 22 strains was carried out against 10 bacterial human pathogens, and plant pathogens. An isolate, NIIST D31 exhibited broad-spectrum activity (antibacterial) against all test human pathogens, and two strains, NIIST D72 and NIIST D27 inhibit both bacterial and fungal plant pathogens especially rice pathogen (Against *Xanthomonas oryzae*; zone of inhibition produced by NIIST D27 was 74.33±0.57mm and NIIST D72 was 51.00±1.00mm) tested. All three strains were chosen for further studies. Using 16SrRNA sequencing three strains were identified as *Streptomyces luteireticuli* NIIST D31 strain*, Streptoverticillium reticulum* NIIST D27 strain, and *Streptomyces rimosus* NIIST D72 strain respectively. For biomedical applications, the *Streptomyces luteireticuli* NIIST D31 strain was selected because of its potential ability to inhibit pathogenic human bacteria whereas the other two were chosen for agriculture application.

The *Streptoverticillium reticulum* NIIST D27 strain and *Streptomyces rimosus* NIIST D72 strain were evaluated for their Plant Growth Promotion ability. Both strains produce PGPR traits (*In vitro* conditions) such as siderophore production, phosphate solubilisation, IAA production, and production of ammonia. NIIST D27 produces more IAA, (6.81 µg/ml) than NIIST D72 (4.01 µg/ml). So, the strains are capable of producing IAA from tryptophan. In the case of phosphate solubilization, NIIST D27, and NIIST D72 are efficiently solubilising phosphate and phosphate solubilising efficiency was estimated as 53.03 ±2.62% and 38.78 ±2.09% respectively. Siderophore production of the isolates was analyzed and confirmed by the production of an orange or yellow halo (zone) around *the Streptomyces* colony in the CAS agar plate. NIIST D27 produces an orange zone of 25.33±0.57mm and NIIST D72

produces a zone of 15.66±0.57mm. For the validation of PGPR traits, pot studies were conducted in rice plants (Jyothi variety). The experiment consists of different treatments such as rice plants inoculated with *Streptomyces* strain in a single treatment (NIIST D27, NIIST D72) as well as the combination (NIIST D27+NIIST D72) of strains along with control (untreated plants). Growth parameters such as root length, shoot height, fresh and dry weight were observed and measured from 7th day to 28th day. An increase in the growth of rice seedlings was noticed in NIIST D27 +NIIST D72 treated rice plants on 28th day, growth parameters were recorded in terms of root length (21.77±1.66 cm), shoot height (35.98±2.53 cm), fresh (1.00±0.33 g) and dry weights (0.21±0.04 g). Which was found to be 1.6-fold,1.3-fold, 2.2-fold, and 1.7-fold increase compared to control (untreated plants) respectively. in addition, the application of both the strains together (NIIST D27 +NIIST D72) enhances the yield. Compared to the control combination treated plants exhibited 1.3-fold increase in shoot dry weight (14.37±1.00 g, with seeds), 1.4-fold increase in seed weight (2.29±0.10 g), and 2.4-fold increase in shoot dry weight (1.46±0.30 g, without seed). Both strains have a potential role in improving the growth of rice plant. Further study continued to evaluate both disease control ability and plant growth promoting ability of the strains with pathogen (*Xanthomonas oryzae*) under pot experiments (nursery conditions). It was noted that a combination of these strains (NIIST D27+NIIST D72+P) shows better results compared to single treatments (NIIST D27+P and NIIST D72+P), control (untreated plants), and pathogen-alone treated plants. Combination treatment (NIIST D27+NIIST D72+P) exhibited improved growth (1.73-fold increase in shoot height, 1.78 – fold increase in root length, fresh weight increased by 4.76-fold and dry weight increased by 6.42-fold) and enhanced yield (2.02-fold increase in seed weight (100 seeds) and 4.42-fold in shoot dry weight with seeds) compared to pathogen alone treated plants. Similarly, inoculated *Streptomyces* strains induce systemic resistance (ISR) in plants, and this reduces the incidence of BLB disease, which is evident from the enzyme assay (defense relate enzyme). Where rice plants inoculated with a combination of *Streptomyces* strains (NIIST D27+NIIST D72+P) produce higher levels of defense related compound (total phenol content) and enzymes (PAL, POD, and PPO) followed by NIIST D27+P and NIIST D72+P compared to pathogen alone treated rice seedling. Split root experiment was carried out to evaluate and confirm the ISR in plants produced due to the inoculation of *Streptomyces* strains. From these experiments, it was clear that plants treated with *Streptomyces* strains remain healthy (Disease incidence of NIIST D27+72+P: 30%; NIIST D27+P: 48.33%; NIIST D72+P:53.33%; Pathogen alone treated plant: 76.66%) compared to pathogen alone treated plants. Pathogen-alone treated rice plants exhibited typical symptoms of BLB from the 14th day and the disease progresses day by day. Both the *Streptomyces* strains are potent plant growth promoting agent and potent biocontrol agent against rice disease BLB.

For the biomedical application, NIIST D31 was selected based on the antibacterial activity of live organism against human pathogens. To isolate compounds having biomedical applications, crude metabolites were extracted from the fermented culture of this strain in ethyl acetate: methanol (95: 5%). The antibacterial activity of the crude metabolite was evaluated against six test bacterial human pathogens, and it was found that crude extract inhibits the test bacterial pathogens. The crude extract of *Streptomyces luteireticuli* NIIST D31 strain shows a broad range of antibacterial activity against test pathogens. To explore the antibacterial activity of the metabolites, compound isolation was carried out. For this crude organic extract was subjected to repeated purification by using column chromatography and preparative TLC. Six compounds were isolated and structural elucidation of the compounds was done with the data of 2D NMR experiments COSY, HSQC, NOESY, and HMBC. Compound 1 is a novel derivative compound of alloaureothin and is identified as alloaureothin vicinal diepoxide. Likewise, other compounds (Compound 2, 3,4,5 and 6) were identified as 2,3-dimethoxybenzamide, 2,3-dihydroxybenzamide, 1H-pyrrole 2-carboxamide, 4-aminobenzoic acid and 1,6-dimethoxyphenazine. Isolated compounds were evaluated for their antibacterial activity against human pathogens unfortunately, the result is not like the crude extract, pure compounds do not show any significant antibacterial activity. Furthermore, novel compound (Compound 1) was explored for anti-inflammatory and anticancer activity but no significant activity (negative results) for either study. Then, compound 1 was explored for the expression of PPAR-γ in adipocyte cells and lipid accumulation, according to the previous research results, where similar compounds show positive results. Finally, it was found that the compound 1 has anti-adipogenic potential and it also reduces lipid accumulation in adipocyte cells (3T3-L1 cells). From this, it was clear that alloaureothin vicinal diepoxide (novel compound) is a potential inhibitor of adipogenesis and has anti-obesity properties.

Results obtained from the present investigation concluded that *Streptomyces* strains from Malampuzha forest, an unexplored region of Western Ghats in Palakkad district, Kerala, are potent strains with significant antimicrobial activity and Plant Growth Promoting activity. They are ideal biocontrol agents in controlling BLB and PGPR strains. Such strains can replace agrochemicals. Additionally, they reduce the harmful effects caused by agrochemicals. So, *Streptomyces* from that area can be used to improve both agriculture and biomedical field.

The present study under nursery conditions shows *Streptoverticillium reticulum* NIIST D27 strain and *Streptomyces rimosus* NIIST D72 strain have potential PGPR (improving the rice productivity) and biocontrol properties against BLB. So, this reveals biofertilizer application of these *Streptomyces* strains in the future in agriculture after necessary field trials. Furthermore, validation of antiobesity

properties of the alloaureothin vicinal diepoxide through the appropriate animal model and subsequent clinical trials towards biomedical applications.

ANNEXURE I- Media composition

Potato Dextrose Broth (PDB) g / L

Potato infusion	200
Dextrose	20

Final pH 5.1 ± 0.2 at 25ºC

Potato dextrose agar (PDA)– PDB supplemented with 20g Agar.
pH is 5.6 ± 0.2.

Luria Bertani Broth (LBB) g / L

Tryptone	10
Yeast Extract	5
Sodium chloride	10

Final pH 7.5 ± 0.2 at 25ºC

Nutrient broth (NB) g / L

Peptone	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0

pH is 7.3 ± 0.1

Nutrient agar (NA) –NB supplemented with 20g Agar.
pH is 7.3 ± 0.1

Muller Hinton Agar (MHA) g / L

Beef infusion	300
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17

Final pH 7.3 ± 0.1 at 25ºC

Streptomyces Agar g / L

Malt extract	10
Yeast extract	4
Dextrose	4
Calcium carbonate	2
Agar	12

Yeast Malt Broth (YMB) g / L

Dextrose	10
Peptone	5

Malt extract	3
Yeast extract	3

Final pH 6.2 ± 0.2 at 25ºC

Pikovskaya's Agar g / L

Yeast extract	0.50
Dextrose	10
Calcium phosphate	5
Ammonium sulphate	0.50
Potassium chloride	0.20
Magnesium sulphate	0.10
Manganese sulphate	0.0001
Ferrous sulphate	0.0001
Agar	15

ANNEXURE-II AcSIR Course work

Code	Course Work	Credits	Status
BIO-NIIST-1-0001	Biostatistics	1	Completed
BIO-NIIST-1-0004	Research Methodology/Communication/ethics	1	Completed
BIO-NIIST-1-0003	Basic Chemistry	1	Completed
BIO-NIIST-2-4101	Biotechniques and Instrumentation	1	Completed
BIO-NIIST-2-006	Protein science and proteomics	2	Completed
BIO-NIIST-2-4103	Basics and Applied Microbiology	2	Completed
BIO-NIIST-3-382	Enzymology and Enzyme technology	2	Completed
BIO-NIIST-1-0002	Computation/bioinformatics	1	Completed
BIO-NIIST-3-4103	Microbial Diversity and Ecology	2	Completed
BIO-NIIST-3-4101	Seminar course	1	Completed
BIO- NIIST -4-0001	Project Proposal	2	Completed
BIO-NIIST-4-0002	Review Writing	2	Completed
BIO-NIIST-4-0003	CSIR 800	4	Completed

ABSTRACT OF THE THESIS

<table>
<tr><td>Name of the student : Drissya T
Faculty of study : Biological Sciences</td><td>Registration No : 10BB17A39022
Year of Submission : 2023</td></tr>
<tr><td colspan="2">AcSIR Academic Centre/CSIR Lab : CSIR NIIST, Thiruvananthapuram
Name of the Supervisor (s) : Dr. Muthu Arumugam (Supervisor),
Dr. Dileep Kumar B S (Co supervisor)</td></tr>
<tr><td colspan="2">Title of the thesis: “Exploration of Selected Streptomyces strains from Western Ghats for its Agriculture and Biomedical Application”</td></tr>
</table>

Streptomyces were isolated from Malampuzha forest in Palakkad district, a part of Western Ghats in Kerala. From 300 streptomyces strains isolated 3 were selected based on the antimicrobial activities against plant and fungal pathogens. NIIST D27 and NIIST D72 were selected for agriculture purpose based on the *in vitro* antibiosis results against plant especially rice pathogen (Against *Xanthomonas oryzae*; zone of inhibition produced by NIIST D27 was 74.33±0.57mm and NIIST D72 was 51.00±1.00mm). These strains were inoculated to rice plants and evaluate the plant growth promoting ability and the emergence of BLB disease with the presence of *X. oryzae*. These two strains have the ability to produce plant growth promotion train in *in vitro* conditions such as phosphate solubilisation, IAA production, siderophore production and ammonia production. PGP traits were evaluated by pot experiments and was found that the combination of these strains (NIIST D27+ NIISTD72) have the ability to improve plant growth by increasing the plant growth parameters as well as yield parameters compared to control. Similarly, these strains in combination (NIIST D27+ NIIST D72+ P) can reduces BLB incidence in Rice plant under pot studies by the production of defence related enzymes and compound such as PAL, POD, PPO, and total phenol content. Similarly, the strain NIIST D31 were selected for biomedical application because of the broad-spectrum antimicrobial activity against pathogenic human bacteria. Crude metabolites were extracted from the fermented culture of NIIST D31 and metabolites were subjected to column chromatography and preparative TLC which leads to the purification and isolation of 6 pure compounds. All the six compounds were identified as as alloaureothin vicinal diepoxide, 2,3-dimethoxybenzamide, 2,3-dihydroxybenzamide, 1H-pyrrole 2-carboxamide, 4-aminobenzoic acid and 1,6-dimethoxyphenazine. Alloaureothin vicinal diepoxide is a novel compound and is a derivative aureothin. All compounds were subjected to antimicrobial activity and result obtained was negative. The novel compound was explored for the expression of PPAR-γ in adipocyte cells and lipid accumulation. It was found that the compound has antiadipogenic potential and having anti-obesity property.

List of publications

List of Publications from the thesis work

- Drissya, T., Induja, D.K., Poornima, M.S., Jesmina, A.R.S., Prabha, B., Saumini, M., Suresh, C.H., Raghu, K.G., Kumar, B.D. and Lankalapalli, R.S. 2022. A novel aureothin diepoxide derivative from Streptomyces sp. NIIST-D31 strain. The Journal of Antibiotics, 75(9), pp.491-497

List of Publications not directly linked to the thesis

- R. S. Jesmina, Dehannath K. Induja, Thankappan Drissya, C. R. Sruthi, K. G. Raghu, Shijulal Nelson-Sathi, B. S. Dileep Kumar and Ravi S. Lankalapalli. In vitro antibacterial effects of combination of ciprofloxacin with compounds isolated from Streptomyces luteireticuli NIIST-D75. The Journal of Antibiotics 2023. DOI: 10.1038/s41429-023-00600-7.
- Jubi Jacob, Gopika Vijayakumari Krishnan, Drissya Thankappan, Dileep Kumar Bhaskaran Nair Saraswathy Amma.2020. Endophytic bacterial strains induced systemic resistance in agriculturally important crop plants. Microbial Endophytes. Elsevier. http://dx.doi.org/10.1016/B978-0-12-819654-0.00004-1

Conference and seminar attended

Conferences proceedings

- 29th APSI Scientist Meet & International Conference on "Drug Discovery and Agribiotechnology and pharmaceutical sciences" held at Ahmedabad, Gujarat, on November 23-25, 2019. (Second prize for oral presentation)

Seminar Attended

- International Seminar on "Life Sciences for Sustainable Development- Issues and Challenges" held at University College, Thiruvananthapuram, on October 3-5,2019.

Abstract of conferences proceedings

- 29th APSI Scientist Meet & International Conference on "Drug Discovery and Agribiotechnology and pharmaceutical sciences" held at Ahmedabad, Gujarat, on November 23-25, 2019. (Second prize for oral presentation)

PLANT GROWTH PROMOTION STUDIES AND METABOLITE EXTRACTION OF *STREPTOMYCES* STRAINS ISOLATED FROM MALAMPUZHA FOREST SOILS

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ABSTRACT

Soil samples were collected from Malampuzha forest in Western Ghats region of Palakkad district at Kerala, South India. Microorganisms were isolated from the soil samples employing different media such as Potato Dextrose Agar, Nutrient Agar, King's Base B, Yeast Malt agar and Streptomyces Agar. From 16 soil samples 300 organisms (all are *Streptomyces*) were isolated and screened for *in vitro* antibiosis against ten bacterial pathogens. After preliminary screening, 25 organisms exhibited activities against the pathogens. Among them, seven potential strains exhibiting an inhibition zone more than 50 mm (including the diameter of inoculated organism) were selected for further studies. The strains were tested for *in vitro* antibiosis against common fungal pathogens such as *Aspergillus niger, Aspergillus flavus,* and *Fusarium oxysporum* 4814, *F. udum* by dual culture method. Furthermore, the plant growth promotion traits such as production of ammonia, IAA and the ability of phosphate solubilisation were checked. Plant growth promotion studies, both individual as well as in combinations in rice plant was done by measuring the shoot height, root length, fresh and dry weight and chlorophyll a, chlorophyll b and total chlorophyll up to 28th day of germination. The treatment with a combination of NIIST D72 + NIIST D27 exhibited increased shoot height, root length, fresh weight and dry weight compared to control. Further experiments regarding the strains, bioactive metabolite production is in progress.

Abstract of seminar Attended

- International Seminar on "Life Sciences for Sustainable Development- Issues and Challenges" held at University College, Thiruvananthapuram, on October 3-5,2019.

IN VITRO ANTIBIOSIS AND PLANT GROWTH PROMOTION STUDIES OF *STREPTOMYCES* STRAINS ISOLATED FROM MALAMPUZHA FOREST SOILS

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Theme: Microbial technology and Nanotechnology

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ABSTRACT

Soil samples were collected from Malampuzha forest, an unexplored area in Western Ghats region of Palakkad district at Kerala, South India. Microorganisms were isolated from the soil samples employing different media such as Potato Dextrose Agar, Nutrient Agar, King's Base B, Yeast Malt agar and Streptomyces Agar. From 16 soil samples, 300 organisms were isolated and screened for *in vitro* antibiosis against ten bacterial pathogens. After preliminary screening, 25 organisms exhibited activities against the pathogens. Among them, seven potential strains exhibiting an inhibition zone more than 50 mm (including the diameter of inoculated organism) were selected for further studies. The strains were tested for *in vitro* antibiosis against common fungal pathogens such as *Aspergillus niger, Aspergillus flavus,* and *Fusarium oxysporum* 4814 by dual culture method. Furthermore, the plant growth promotion traits such as production of ammonia, IAA and the ability of phosphate solubilisation were analysed. Plant growth promotion studies, both individual as well as in combinations in rice plant was done by measuring the shoot height, root length, fresh and dry weight and chlorophyll a, chlorophyll b and total chlorophyll up to 28th day of germination. The treatment with a combination of NIIST D72 + NIIST D27 exhibited increased shoot height (22.89% increase), root length (39.60%), fresh weight (62.56%) and dry weight (89.47%) compared to control.

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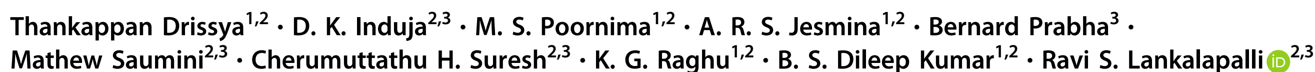
ARTICLE

A novel aureothin diepoxide derivative from *Streptomyces* sp. NIIST-D31 strain



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Abstract

A novel vicinal diepoxide of alloaureothin was isolated from *Streptomyces* sp. NIIST-D31 strain along with three carboxamides, *p*-aminobenzoic acid and 1,6-dimethoxyphenazine. Exhaustive 2D NMR analysis and analysis of experimental, theoretical CD spectra aided in establishing the structure of compound **1**. Compound **1** inhibits adipogenesis and accumulation of lipid droplets during the differentiation of 3T3-L1 cells.

Introduction

Vicinal diepoxide is a distinct functionality in natural products, reported from plant, animal and microbial kingdom. Examples of vicinal diepoxides on an acyclic chain include gummiferol from the leaves of *Adenia gummifera* [1], spatol from the brown seaweed *Spatoglossum schmittii* [2], diepoxy-3β-hydroxy-7-tirucallene isolated from *Azadirachta indica* [3], and plakdiepoxide from Chinese sponge *Plakortis simplex* [4]. Elysiapyrone A contain a vicinal diepoxide in cyclic system, an endoperoxide derivative, isolated from sea slug *Elysia diomedea* [5]. Hedamycin derived from *Streptomyces griseoruber* possess an anthraquinone backbone with a vicinal diepoxide in the side-chain [6]. Some of the other structural analogs of hedamycin with a vicinal diepoxide side-chain include DC92-B and DC92-D from *Actinomadura* sp. [7], ankinomycin from *Streptomyces* sp. SF2587 [8], and chromoxymycin from *Streptomyces rubropurpureus* [9]. Herein, we report a novel alloaureothin vicinal diepoxide **1** isolated from *Streptomyces* sp. NIIST-D31 strain, along with benzamides **2-3**, 1*H*-pyrrole-2-carboxamide **4**, 4-aminobenzoic acid **5**, and 1,6-dimethoxyphenazine **6** (Fig. 1), in addition, compound **1** has been evaluated for its effect on PPAR-γ expression.

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Materials and methods

Microorganism

The *Streptomyces* sp. NIIST-D31 strain was isolated from soil samples of Western Ghats Forest of Malampuzha (10°83´78.19“N, 76°66´19.96“E) of Palakkad district, Kerala (Figure S1, see, Supplementary Material). Molecular identification of the strain was carried out based on the 16S rRNA sequencing. The sequence was aligned with the nucleotide sequence of *Streptomyces* genera in Gene bank database using BLAST. *Streptomyces* sp. NIIST-D31 strain exhibited 99.5% homology with *Streptomyces luteireticuli* using NCBI BLAST (Figure S2, see, Supplementary Material). The GenBank accession number for the partial 16S rRNA gene sequence of *Streptomyces* sp. NIIST-D31 strain is ON013964.

Fermentation, extraction, isolation and structural characterization

Streptomyces sp. NIIST-D31 strain was inoculated in Yeast Malt Broth and kept in shaking incubator at 28 ± 2 °C. After

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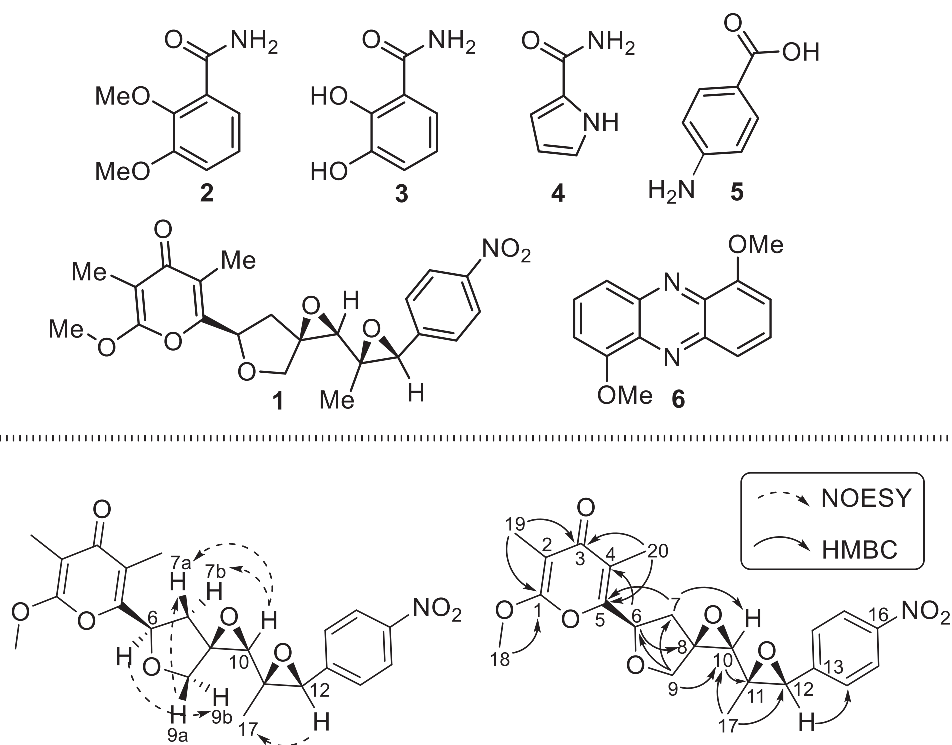

Fig. 1 Compounds isolated from *Streptomyces* sp. NIIST-D31 strain. Structures of compounds **1-6**; key ^{1}H-^{1}H NOESY and ^{1}H-^{13}C HMBC correlations of compound **1**

ten days of growth, the fermented culture was centrifuged at 10,000 rpm for 20 min. The supernatant was collected and added equal volume of solvent (Ethyl acetate:methanol 95:5), and repeated the extraction twice in order to obtain maximum metabolites from the supernatant. The organic layer was concentrated using rotary evaporator. The crude organic extract (890 mg, obtained from 7.8 L fermented broth), as the first batch, was purified by column chromatography using silica gel (100–200 mesh) and hexane/ethyl acetate combination of solvents by gradient elution to obtain fractions. Thin layer chromatography of the resulting fractions was monitored by short and long-wavelength UV. Further purification of the resulting fractions by column chromatography (100–200 mesh) led to isolation of compound **1** (17 mg), compound **2** (4 mg), compound **3** (2.5 mg), compound **4** (6.4 mg), and compound **6** (9.4 mg). Purification of the second batch of the organic extract (1.31 g, obtained from 7.8 L fermented broth) using silica gel (230–400 mesh) afforded compound **1** (20.9 mg), compound **2** (5 mg), compound **3** (4.9 mg), compound **4** (3.9 mg), compound **5** (7.6 mg), and compound **6** (1.7 mg). Compound **2** was obtained as a mixture from both the batches, as evident from NMR, repeated purifications by column chromatography led to a loss of compound **2**, yielding 1.6 mg. The isolated compounds were dissolved in $CDCl_3$ or CD_3OD and recorded ^{1}H and ^{13}C NMR on a Bruker Ascend^{TM} 500 MHz spectrometer at 500 and 125 MHz, respectively. The chemical shifts (δ) were given in parts per million (ppm), coupling constants in Hz, and multiplicity as s for singlet, t for triplet, d for doublet, dd for double doublet, etc. Structure elucidation of **1** was carried out with the aid of 2D NMR experiments COSY, HSQC, HMBC, and NOESY. The HR-ESI-MS data were obtained from a Thermo Scientific Exactive mass spectrometer with Orbitrap analyser and the ions are given in m/z. Circular dichroism (CD) measurements were performed on JASCO 810 spectrometer equipped with peltier thermostatic cell holders. CD spectra recorded as θ in millidegrees, and converted to Δε using the equation $\Delta\varepsilon = \theta/(33982cl)$ where, Δε is the difference in the molar absorptivity for oppositely polarized light in $M^{-1}\,cm^{-1}$, c is the concentration in M and l is the path length in cm. Melting point was determined on a Fisher-Johns melting point apparatus and is uncorrected. UV-Vis absorbance was carried out on a Shimadzu UV-2600 UV-Vis spectrophotometer. Specific optical rotation was recorded using JASCO P-2000 digital polarimeter. Infrared spectrum was recorded using a PerkinElmer Series FT-IR spectrum-2 at a resolution of $2\,cm^{-1}$ over the wavenumber range of 4000–400 cm^{-1}. FTIR measurements of the compound was carried out by carefully placing the compound in between the KBr pellets.

Computational methods

Stereochemical analysis of **1** in the singlet state was performed by using the density functional theory (DFT) method B3LYP/6-311G(d,p) [10], using Gaussian09 suite of programs. The energies, oscillator strengths, rotational

strengths of first 24 electronic excitations and ECD spectra were calculated with the time-dependent DFT (TD-DFT) approach [11], using the B3LYP/6-311G (d,p) level of theory in conjunction with self-consistent reaction field (SCRF) method to include solvent effect of methanol. For SCRF, the polarizable continuum model (PCM) [12] is selected for the solvent methanol as implemented in Gaussian09.

Chemicals, cell culture, and treatments

Dulbecco's modified Eagle's medium (DMEM) was purchased from HiMedia (Mumbai, India). Fetal bovine serum (FBS), bovine calf serum, penicillin-streptomycin antibiotics, trypsin - ethylenediaminetetraacetate (EDTA), Hanks balanced saline solution (HBSS), and phosphate buffer saline (PBS) were purchased from Gibco, USA. Skimmed milk powder, and Oil Red O stain, insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone were purchased from Sigma-Aldrich Co. USA. Chemiluminescence detection kit was purchased from Advansta, USA. All primary and secondary antibodies used in western blotting were purchased from Santa Cruz Biotechnology, USA.

3T3-L1 pre-adipocytes were obtained from National Center for Cell Sciences (NCCS, Pune, India) and cultured to the confluence at 37 °C under a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), including 10% bovine calf serum and 1% penicillin-streptomycin. Two days after the cells had reached confluence (day 0), 3T3-L1 pre-adipocyte cells were cultured in differentiation medium (DM) containing 10% fetal bovine serum, 10 μg ml^{-1} insulin, 0.5 mM IBMX, and 1 μM dexamethasone. Two days after stimulation with differentiation media, including 0.5 mM IBMX, 1 μM dexamethasone, and 10 μg ml^{-1} insulin (day 2), the medium was changed to a 10% FBS/DMEM medium containing 10 μg ml^{-1} insulin. After two days (day 4), the medium was changed to 10% FBS/DMEM medium and cultured in 10% FBS/DMEM medium every 2 days. Full differentiation was achieved by day 8. The cell viability assay was carried out to find the toxic effect of **1** on 3T3L-1, and **1** was found to be non-toxic up to 30 μM in 3T3L-1 cells (Figure S15, see, Supplementary Material). During differentiation, **1** was used at concentrations of 5 μM and 10 μM to study the differentiation inhibition of adipocytes in the 3T3-L1 culture between days 0 and 4 [13]. The experimental group consists of: (a) C1 group: 3T3-L1 preadipocyte cells without differentiation and any treatment are designated as C1. (b) C2 group: Differentiated 3T3-L1 cells are indicated as C2. (c) **1** (5 μM) group: 3T3-L1 cells treated with 5 μM of **1**. (d) **1** (10 μM) group: 3T3-L1 cells treated with 10 μM of **1**.

Oil Red-O-staining

Oil Red O staining was done to check lipid accumulation in the cells. The cultured 3T3-L1 cells were washed with cold phosphate-buffered saline (PBS) on 8th day of differentiation and the cells were fixed with 4% formaldehyde at 25 °C. After fixation, the cells were washed with PBS and permeabilized with 0.1% Triton X 100 followed by Oil Red O staining for 20 min at room temperature. The cells were then visualized under a light microscope. Absorbance was spectrophotometrically measured at 490 nm [14].

Western blot analysis

After respective treatments, cells were lysed with RIPA buffer and protease inhibitor cocktail (Sigma Aldrich, USA). Cell suspensions were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was collected and used for immunoblot analysis and the protein concentration was analysed by using the bicinchoninic acid kit (Pierce, Rockford, IL, USA). Then an equal amount of proteins was separated by 10% SDS-PAGE and transferred to PVDF. After blocking with TBS-Tween 0.1% containing 5% non-fat skimmed milk (Bio-Rad, Hercules, CA, USA), membranes were incubated with primary antibodies overnight at 4 °C. After washing with TBS-Tween 0.1%, the membranes were incubated with HRP-conjugated respective secondary antibodies for 2–4 h at room temperature. Further washed thrice with TBS-Tween 0.1%, then the membranes were developed using Western Blot Hyper HRP Substrate (Takara Bio Inc. USA), and the protein bands were analyzed (Bio-Rad ChemiDoc MP imaging systems, USA) [15].

Results and discussion

Compound **1** was obtained as a colourless amorphous solid and its molecular formula was determined as $C_{22}H_{23}NO_8$, calculated as 430.1502 $[M + H]^+$ and observed 430.1497, from HR-ESI-MS along with ^{1}H and ^{13}C NMR data, implying 12 degrees of unsaturation. The IR spectra displayed characteristic absorption (ν_{max}) of nitro groups at 1510 and 1342 cm^{-1} and carbonyl group at 1669 cm^{-1}. The ^{1}H and ^{13}C NMR spectra showed signals of a methoxy, three methyl singlets, four aromatic protons, two sp^3 methylene protons, three sp^3 methine protons, a carbonyl C3 (δ_C 180.6 ppm), and six quaternary sp^2 carbons (Table 1). Aromatic protons with ortho-coupling constant (8.4 Hz) and downfield signals indicate the presence of a para-nitro substituted phenyl moiety. Structure elucidation was carried out with the aid of ^{1}H-^{13}C HMBC data (Fig. 1). Presence of HMBC correlations of aromatic proton H-14

Table 1 ^{1}H and ^{13}C NMR data of compound **1** ($CDCl_3$)

Carbon	^{13}C (δ_C)	^{1}H NMR (δ_H)
1	162.1	–
2	100.5	–
3	180.6	–
4	121.1	–
5	153.1	–
6	73.6	5.16 (dd, J = 5.85, 10.00 Hz)
7a	36.8	2.62 (dd, J = 10.20, 13.66 Hz)
7b		2.05 (dd, J = 5.83, 13.73 Hz)
8	68.8	–
9a	69.4	4.29 (d, J = 11.56 Hz)
9b		3.98 (d, J = 11.56 Hz)
10	59.5	3.48 s
11	60.4	–
12	59.6	4.08 s
13	142.1	–
14	127.4	7.41 (d, J = 8.40 Hz)
15	123.6	8.18 (d, J = 8.45 Hz)
16	147.7	–
17	14.2	1.15 s
18	55.5	3.94 s
19	6.9	1.8 s
20	9.5	1.98 s

with methine C12 (δ_C 59.6 ppm); methyl H-17 with C10, C11, C12; H-12 (s, δ_H 4.08 ppm) with C11, C13 (quaternary aromatic carbon), C14; and based on chemical shifts, an oxygenated backbone C10-C11-C12 is present, wherein C12 is attached to the phenyl moiety, and the presence of methyl C17 on 4° carbon C11. Presence of HMBC correlations of H-10 (s, δ_H 3.48 ppm) with C7, C8 (4°), C11; methylene proton H-7 with C5, C6, C8, C10; methylene proton H-9 with C6, C7, C8, C10 suggest the presence of oxygenated backbone C9-C8-C10-C11-C12. Based on chemical shift of methine H-6, δ_H 5.16 ppm, COSY correlation with H-7, and based on HMBC correlations, *vide supra*, presence of a tetrahydrofuran ring was evident, and the reason for downfield shift for H-6 is attributed to the attachment of its carbon to 4° sp^2 carbon C-5 (δ_C 153.1 ppm). H-6 exhibited HMBC correlations with C4, C5, C7, C8, and C4 is a 4° sp^2 carbon (δ_C 121.1 ppm), and difference of greater than 30 ppm with C5 suggest C4 and C5 are part of an unsaturated ketone, further confirmed by presence of HMBC correlation of methyl H-20 with C3, C4, C5. Presence of HMBC correlation of methyl H-18 with C1 and methyl H-19 with C1, C2, C3 and based on chemical shift difference between C1 and C2, greater than 60 ppm, presence of another unsaturated carbonyl to the same ketone (C3) is apparent. Based on the chemical shift differences, stated above, both C1, C2 and C4, C5 double bonds are bound to the same C3 carbonyl, and C1, C5 are attached to the same oxygen atom, where C1 is attached to methoxy, reminiscent to an α-methoxy-γ-pyrone, present as a head group in polyketides. Finally, based on the molecular formula with two further oxygen atoms and remaining two degrees of unsaturation, the oxygenated carbons C8, C10, C11 and C12 must be involved as two oxygenated rings as a vicinal diepoxide, which revealed the presence of an aureothin backbone in **1** with an unprecedented epoxidized double bonds (Fig. 1). For stereochemical determination, NOESY correlations relative to the conserved '*R*' configuration of C6 of aureothin was considered. The presence of spiro[2.4]heptyl moiety with C8 spiro carbon impose conformational restriction that aid in determination of configuration of stereocenters C8-C10 oxirane relative to C6. There were NOESY correlations for H-6 with H-9b; H-9a with H-7a; and H-10 with H-7a, H-7b (Fig. 1, Figure S5, see, Supplementary Material). NOESY correlation for H-10 exist with both H-7a and H-7b due to pseudo axial and equatorial orientations, which places the oxirane ring above the plane as shown in Fig. 1, if the oxirane is below the plane then H-10 will have NOESY correlation with H-9a or with both H-9a, H-9b instead. Thus, the NOESY correlation served as a determining factor in C8-C10 configuration. Another important NOESY correlation of H-12 with H-17 suggest that the C11-C12 oxirane emanated from the corresponding Z-double bond, thus, suggesting **1** as a vicinal diepoxide of alloaureothin. Alloaureothin is a polyketide metabolite that was reported from *Streptomyces* sp. MM23 [16]. Owing to the free-rotating C10-C11 single bond, other NOESY correlations were not considered in determination of relative configuration of stereocenters of C11-C12 oxirane. Hence, we adopted a computational approach based on the comparison between experimental and quantum-mechanically calculated ECD spectra (Fig. 2), which revealed the configuration of C11-C12 oxirane ring above the plane, enabling structure elucidation of **1**. This configuration of **1** obtained at B3LYP/6-311G(d,p) level showed energy −1508.81055491 a.u. and dipole moment 14.45 D. The impact of conformational factors with the change of configuration of C11-C12 oxirane ring provides different chiroptical response in ECD [17], which is evident from Figures S12–14 (Supplementary Material). The vicinal diepoxide formation of **1** can be postulated as epoxidation of 1,3-diene of alloaureothin by monooxygenases. If there is an involvement of an endoperoxide intermediate formed from alloaureothin by endoperoxide synthetase then 1,3-diene must take s-*cis* conformation, which will lead to undesired stereochemistry based on the proposed formation in elysiapyrone A and plakdiepoxide [4, 5].

Compound **2** exhibited characteristic broad singlets corresponding to NH_2 protons of a primary amide in its ^{1}H

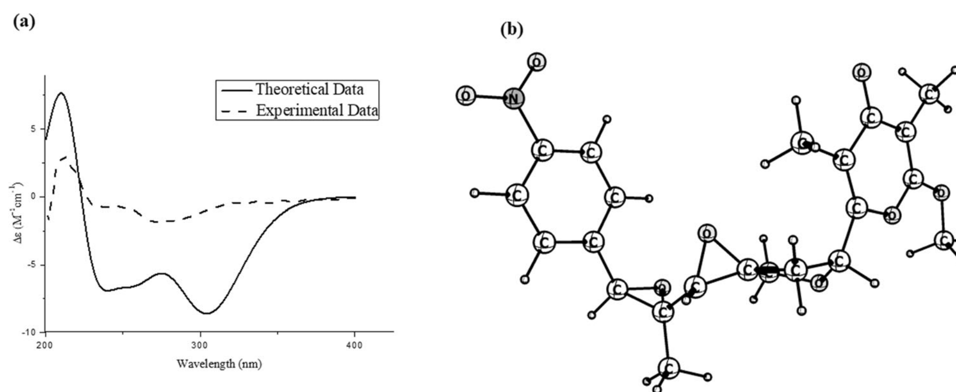

Fig. 2 Stereochemistry and Conformation of **1**. **a** Experimental and calculated ECD spectrum for compound **1**; (**b**) Optimized structure of compound **1**

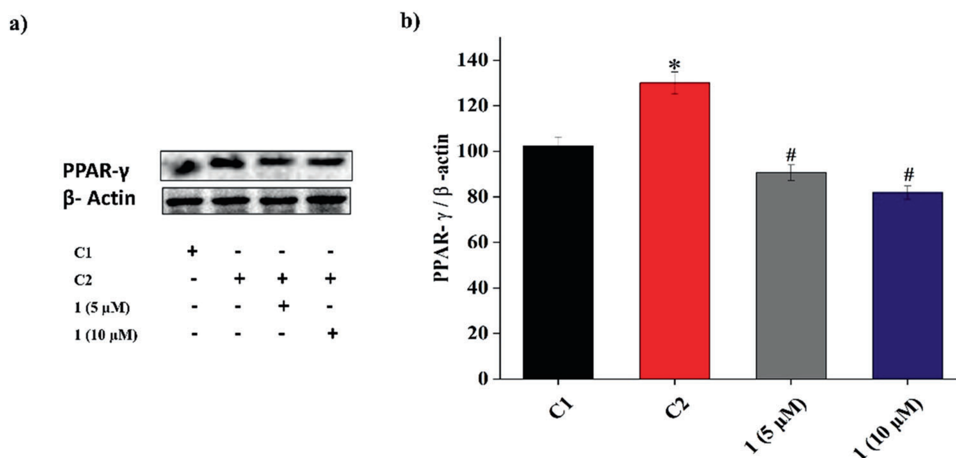

Fig. 3 Effect of compound **1** on PPAR-γ. **a** The protein expression in different experimental groups. **b** The relative intensity of each band was quantified with β-actin. Pre-adipocyte (C1); Differentiated 3T3L-1 (C2); Differentiated 3T3L-1 + compound **1** (5 μM); Differentiated 3T3L-1 + compound **1** (10 μM). Data are expressed as mean ± SEM; where $n = 3$. *denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$)

NMR. In addition, the three distinct aromatic proton signals and the presence of dimethoxy functionalities confirmed compound **2** as 2,3-dimethoxybenzamide (Fig. 1), similarly, compound **3** was identified from its ^{1}H NMR as 2,3-dihydroxybenzamide (Fig. 1), benzamides are indeed known from *Streptomyces* species [18–21]. Compound **4** exhibited characteristic aromatic signals in the region of 6–7 ppm in its ^{1}H NMR of a heterocycle, presence of a primary amide functionality was evident from ^{13}C NMR, which revealed the identity of compound **4** as 1*H*-pyrrole-2-carboxamide (Fig. 1). Compound **4** has been reported from *Streptomyces* sp. RM-14-6 and from marine sponges [22–24]. Based on HR-ESI-MS analysis, compound **5** exhibited m/z 120.0449 that correspond to $[M\text{-}OH]^+$, which along with NMR, enabled identification of compound **5** as 4-aminobenzoic acid (Fig. 1). Compound **6** was readily identified by NMR and HR-ESI-MS data as 1,6-dimethoxyphenazine (Fig. 1).

Initially, **1** was evaluated for cytotoxicity against cancer cells but there was no significant activity compared to reported parent compounds aureothin or alloaureothin. However, plakdiepoxide with a vicinal diepoxide was shown as a potent modulator of PPAR-γ [4]. PPAR-γ is the most studied transcriptional factor and important gene for adipogenesis [25]. The expression of PPAR-γ increased significantly during differentiation. They have a direct impact on the development of fat accumulation in cells [26]. Studies have shown that natural products have anti-adipogenic property by inhibiting PPAR-γ [27, 28], hence, we analyzed and compared the expression of PPAR-γ with **1** with differentiated adipocyte (C2) and pre-adipocyte (C1). In the present study, the expression of PPAR-γ was increased in the C2 group (26.88%; $p \leq 0.05$) when compared to C1 (Fig. 3). There was a decrease in the expression of PPAR-γ with **1** (38.45% with 5 μM and 48.84% with 10 μM) when compared to C2. Both the concentrations of **1** caused a significant reduction of lipid content in a dose-dependent manner (17.55% with 5 μM and 23% with 10 μM; $p \leq 0.05$; Fig. 4) when compared to the C2 group which showed a significant increase (31.99%). Oil-red-O staining also confirmed the inhibition of adipogenesis with **1**. Antiadipogenic compounds are expected to have anti-obesity property, based on these preliminary observations, **1** could be explored for its lipolytic property to check its potential against anti-obesity.

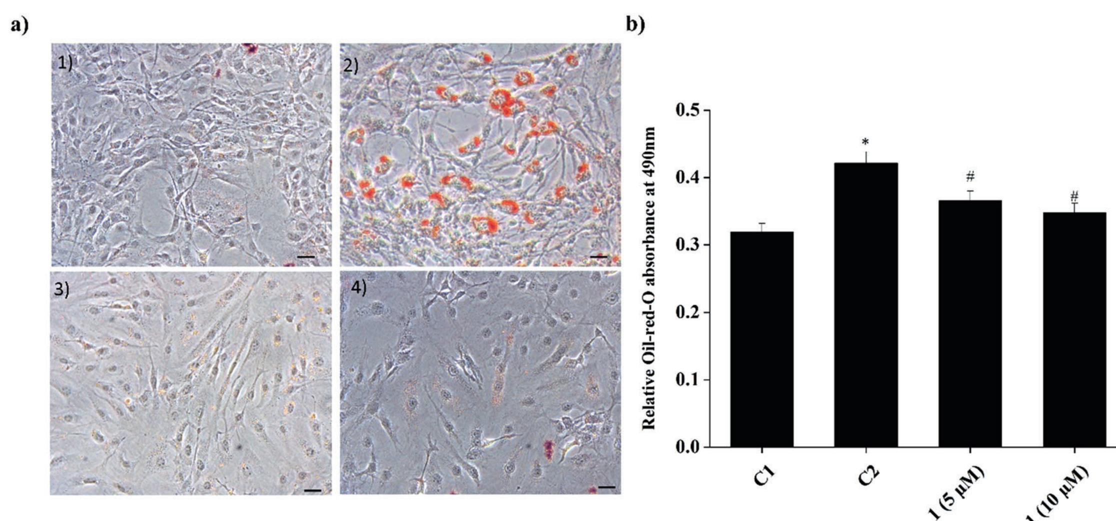

Fig. 4 Effect of compound **1** on lipid accumulation in 3T3-L1 cells. **a** The lipid accumulation was assessed by Oil Red O staining. Representative phase-contrast microscopic images of Oil Red O stained 3T3-L1 were presented, scale bar 50 μm. (1) Pre-adipocyte(C1) (2) Differentiated 3T3L-1 (C2) (3) Differentiated 3T3L-1 + compound **1** (5 μM) (4) Differentiated 3T3L-1 + compound **1** (10 μM). **b** Absorbance was read at 490 nm after Oil-Red-O staining. Data are expressed as mean ± SEM; where $n = 3$. *denotes significant difference from the C1 group ($p \leq 0.05$) and # denotes significant difference from the C2 group ($p \leq 0.05$)

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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References

1. Fullas F, Brown DM, Wani MC, Wall MR, Chagwedera TE, Farnsworth NR, et al. Gummiferol, a cytotoxic polyacetylene from the leaves of Adenia gummifera. J Nat Products. 1995;58:1625–8.
2. Gerwick WH, Fenical W, van Engen D, Clardy J. Isolation and structure of spatol, a potent inhibitor of cell replication from the brown seaweed Spatoglossum schmittii. J Am Chem Soc. 1980; 102:7991–3.
3. Chen J, Chen J, Sun Y, Yan Y, Kong L, Li Y, et al. Cytotoxic Triterpenoids from *Azadirachta indica*. Planta Med. 2011;77:1844–7.
4. Chianese G, Yu H-B, Yang F, Sirignano C, Luciano P, Han B-N, et al. PPAR modulating polyketides from a Chinese *Plakortis simplex* and clues on the origin of their chemodiversity. J Org Chem. 2016;81:5135–43.
5. Díaz-Marrero AR, Cueto M, D'Croz L, Darias J. Validating an endoperoxide as a key intermediate in the biosynthesis of Elysiapyrones. Org Lett. 2008;10:3057–60.
6. Murray V, Moore AG, Matias C, Wickham G. The interaction of hedamycin and DC92-B in a sequence selective manner with DNA in intact human cells. Biochimica et Biophysica Acta (BBA) - Gene Struct Expr. 1995;1261:195–200.
7. Yasuzawa T, Saitoh Y, Sano H. Structures of the novel anthraquinone antitumor antibiotics, DC92-B and DC92-D. J Antibiotics. 1990;43:485–91.
8. Sato Y, Watabe H-O, Nakazawa T, Shomura T, Yamamoto H, Sezaki M, et al. Ankinomycin, a potent antitumor antibiotic. J Antibiotics. 1989;42:149–52.
9. Kawai Y, Furihata K, Seto H, Otake N. The structure of a new antibiotic, chromoxymycin. Tetrahedron Lett. 1985;26:3273–6.
10. Becke AD. Density-functional thermochemistry. III. The role of exact exchange. J Chem Phys. 1993;98:5648–52.
11. Runge E, Gross EKU. Density-functional theory for time-dependent systems. Phys Rev Lett. 1984;52:997–1000.
12. Miertuš S, Scrocco E, Tomasi J. Electrostatic interaction of a solute with a continuum. A direct utilizaion of AB initio molecular potentials for the prevision of solvent effects. Chem Phys. 1981; 55:117–29.
13. Kim H, Kim JH, Zuo G, Lim SS. Anti-obesity effect of *Melandrium firmum* Rohrbach extract in 3T3-L1 cells and high-fat diet-induced obese C57BL/6N mice. Food Sci Nutr. 2020;8:2251–61.
14. Poornima MS, Sindhu G, Billu A, Sruthi CR, Nisha P, Gogoi P, et al. Pretreatment of hydroethanolic extract of Dillenia indica L. attenuates oleic acid induced NAFLD in HepG2 cells via modulating SIRT-1/p-LKB-1/AMPK, HMGCR & PPAR-α signaling pathways. J Ethnopharmacol. 2022;292:115237.
15. Yang P-C, Mahmood T. Western blot: Technique, theory, and trouble shooting. North Am J Med Sci. 2012;4:429.
16. Ueda J, Hashimoto J, Nagai A, Nakashima T, Komaki H, Anzai K, et al. New aureothin derivative, alloaureothin, from streptomyces sp. MM23. J Antibiotics. 2007;60:321–4.
17. Padula D, Pescitelli G. How and how much molecular conformation affects electronic circular dichroism: the case of 1,1-Diarylcarbinols. Molecules. 2018;23:128.
18. Chen G, Gao H, Tang J, Huang Y, Chen Y, Wang Y, et al. Benzamides and quinazolines from a mangrove actinomycetes streptomyces sp. (No. 061316) and their inhibiting Caspase-3 catalytic activity in vitro. Chem Pharm Bull. 2011;59:447–51.

19. Yang X, Peng T, Yang Y, Li W, Xiong J, Zhao L, et al. Antimicrobial and antioxidant activities of a new benzamide from endophytic Streptomyces sp. YIM 67086. Nat Prod Res. 2015;29:331–5.
20. Zhang X, Shu C, Li Q, Lian XY, Zhang Z. Novel cyclohexene and benzamide derivatives from marine-associated Streptomyces sp. ZZ502. Nat Prod Res. 2019;33:2151–9.
21. Shaaban KA, Shepherd MD, Ahmed TA, Nybo SE, Leggas M, Rohr J. Pyramidamycins A-D and 3-hydroxyquinoline-2-carboxamide; Cytotoxic benzamides from Streptomyces sp. DGC1. J Antibiotics. 2012;65:615–22.
22. Chu MJ, Tang XL, Qin GF, Sun YT, Li L, de Voogd NJ, et al. Pyrrole derivatives and diterpene alkaloids from the South China Sea Sponge Agelas nakamurai. Chem Biodivers. 2017; 14. https://doi.org/10.1002/cbdv.201600446.
23. Shaaban KA, Saunders MA, Zhang Y, Tran T, Elshahawi SI, Ponomareva L. et al. Spoxazomicin D and Oxachelin C, potent neuroprotective carboxamides from the appalachian coal fire-associated isolate Streptomyces sp. RM-14-6 J Nat Prod. 2017;80:2–11.
24. König G, Wright A. Two new naturally occurring pyrrole derivatives from the tropical marine sponge Agelas Oroides. Nat Prod Lett. 1994;5:141–6.
25. Hamm JK, el Jack AK, Pilch PF, Farmer SR. Role of PPARgamma in regulating adipocyte differentiation and insulin-responsive glucose uptake. Ann N. Y Acad Sci. 1999;892:134–45.
26. Li KK, Liu CL, Shiu HT, Wong HL, Siu WS, Zhang C, et al. Cocoa tea (Camellia ptilophylla) water extract inhibits adipocyte differentiation in mouse 3T3-L1 preadipocytes. Sci Rep. 2016;6:20172.
27. Choi R-Y, Lee H-I, Ham JR, Yee S-T, Kang K-Y, Lee M-K. Heshouwu (Polygonum multiflorum Thunb.) ethanol extract suppresses pre-adipocytes differentiation in 3T3-L1 cells and adiposity in obese mice. Biomed Pharmacother. 2018;106:355–62.
28. Shyni GL, Sajin KF, Mangalam SN, Raghu KG. An in vitro study reveals the anti-obesity effects of 7- methoxy-3-methyl-5-((E)-prop-1-enyl)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran from Myristica fragrans. Eur J Pharmacol. 2021;891:173686.