# Characterization and exploration of secondary metabolites from two *Streptomyces* species for enhanced antibacterial activity against selected human pathogens

by

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Under the supervision of
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**Jesmina A S**

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# Abbreviations

| | |
|---|---|
| °C | Degree Celsius |
| % | Percent |
| µg | Micro gram |
| µl | Micro litre |
| µm | Micro metre |
| µM | Micro molar |
| ABC | ATP-binding cassette |
| ANOVA | Analysis of Variance |
| AGOS | Artificial Gene Operon System |
| AMR | Anti-Microbial Resistance |
| BBD | Box Benhken Design |
| BGC | Biosynthetic Gene Cluster |
| CCD | Central Composite Design |
| CFU | Colony Forming Units |
| CFR | chloramphenicol–florfenicol resistance |
| CSIR | Council for Scientific and Innovative Research |
| DNA | Deoxyribonucleic acid |
| FDA | Food and Drug Administration |
| FICI | Fractional Inhibitory Concentration Index |
| g | gram |
| h | Hour |
| IMTECH | CSIR-Institute of Microbial Technology |

| | |
|---|---|
| ISP | International Streptomyces Project |
| kcal | kilo calorie |
| $K_2HPO_4$ | Dipotassium phosphate |
| M | Molar |
| MDR | Multi-Drug Resistant |
| mg | Milli gram |
| MHA | Muller Hinton Agar |
| MIC | Minimum Inhibitory Concentration |
| min | Minute |
| 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-diphenyltetrazolium bromide |
| MFS | Major Facilitator Superfamily |
| MATE | Multidrug and toxic compound extrusion |
| NaCl | Sodium Chloride |
| NIIST | National Institute for Interdisciplinary Science and Technology |
| nm | Nano metre |
| NRPS | Non-ribosomal |
| OD | Optical Density |
| OFAT | One Factor At a Time |
| PCR | Polymerase chain reaction |
| PDA | Potato dextrose agar |
| PI | Propidium Iodide |

| | |
|---|---|
| PKS | Polyketide Synthase |
| RNA | Ribonucleic acid |
| ROS | Reactive Oxygen Species |
| rpm | Rotations per Minute |
| rRNA | Ribosomal RNA |
| RSM | Response Surface Methodology |
| RND | Resistance Nodulation Division |
| SCA | Starch Casein Agar |
| Sec | Seconds |
| SEM | Scanning Electron Microscopy |
| SMR | Small Multidrug Resistance |
| TBE | Tris Borate EDTA |
| v/v | Volume per volume |
| WHO | World Health Organization |
| YMB | Yeast Malt Broth |
| ZoI | Zone of Inhibition |

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# CHAPTER 1

# Introduction and review of literature

## 1.1. INTRODUCTION

The significant breakthrough of the 20th century was the introduction of antibiotics to clinical use. Before the 20th century, the average longevity of humans was below 50 because of proliferating pandemics like smallpox, typhoid fever, tuberculosis, syphilis, and plaque. The revolution against these prevalent infections began with Sir Alexander Fleming's discovery of penicillin in 1928. Two Oxford scientists, Howard Florey, and Ernst Chain, became interested in Fleming's penicillin. Later, with a group of experts, they purified the first penicillin, penicillin G in 1942. In 1945, the drug was released to the public, especially in the US World War II period, for soldiers. When it was introduced, penicillin was a miracle medication. It could treat many infections and diseases, including pneumonia, scarlet fever, meningitis, syphilis, and gonorrhea. Thus, the invention of new drugs continued, and most of the drugs now in clinics are isolated from the golden era of antibiotics (1950–1961). The multiple roles of antibiotics include treating infectious diseases, cancer treatments, organ transplants, and surgeries(Hutchings, Truman, and Wilkinson 2019). Misuse of antibiotics and widespread antibiotic abuse lead to antibiotic pollution and the emergence of antibiotic resistance (Walsh 2013). This AMR is emerging as a severe threat to human health.

Moreover, the World Health Organization placed it among the top ten urgent threats in 2019 (Yu et al. 2020). Many bacteria are becoming resistant to current antibiotics and becoming more threatening, like ESKAPEE pathogens, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli*. In this scenario, to overcome these problems, new antibiotics want to be isolated, so a better choice is natural resources. Streptomyces is a well-known source of secondary metabolites, and many approved drugs were isolated from the same group. It started with the discovery of actinomycin in 1940. Later, several antibiotics were isolated from an actinomycete, like streptothricin, streptomycin, chloramphenicol,

tetracyclines, erythromycins, neomycin, novobiocin, oleandomycin, nystatin, etc. and the event continues. Actinomycete includes several genus-like pathogens, including Corynebacterium, Mycobacterium, Nocardia, Propionibacterium, and Tropheryma. Some phyla include soil dwellers such as Micromonospora and Streptomyces species. Frankia species have an association with plants. Some are associated with the gastrointestinal tract, like Bifidobacterium species (Barka et al. 2016). Important genera of actinomycete include actinomadura, actinoplanes, actinosporangium, chromobacterium, micromonospora, nocardia, ptilocaulis, streptomyces, and streptoverticillum, which are rich sources of bioactive molecules. These actinomycetes are considered prolific producers of bioactive compounds. Actinomadura is known for producing actinotinocin and spirocardin A (Balachandran 2012). Research Laboratories of Gruppo Lepetit S.p.A., in Milan, Italy, isolate bioactive compounds from actinoplanes, including lipiarmycin, gardimycin, and teichomycin (Kurtböke 2017). A protonophore antibiotic, pyrrolomycin D, isolated from actinosporangium, well-known actinobacteria, shows a broad spectrum activity at the nanomolar level (Valderrama et al. 2019). Another important class of Actinobacteria is Micromonospora, and these are well known for their contribution to biometabolite production. *Megalomicin* antibiotics *isolated from M. megalomicea*, and rosamicin isolated from *M. rosaria* are important macrolide antibiotics isolated from Micromonospora. Likewise, several important compounds are isolated from different actinomycete genera, showing species' importance in bioactive compound production. Biosynthetic gene clusters (BGC) are responsible for the diversity of compounds produced by actinomycetes.

Non-ribosomal peptide synthetases, type 1 polyketide synthases, terpenes, and lantipeptides are common BGCs.(Belknap et al. 2020). Type-I PKSs and NRPSs are large multifunctional enzymes with various catalytic domains, and the metabolites are synthesized according to the co-linearity rule of assembly lines (Komaki et al. 2020). Recent advances in genome mining have revealed that Streptomyces genomes possess many new silent secondary metabolite

biosynthetic gene clusters (smBGCs). However, recent advances in genomics-based approaches revealed that most of the secondary metabolite biosynthetic gene clusters (smBGCs) of Streptomyces are inactive under laboratory conditions, suggesting that the ability of Streptomyces to produce secondary metabolites has been under-estimated (Lee et al. 2020). Identification of BGCs in isolates is a significant step. For this, various bioinformatic tools like "antibiotics and secondary metabolite analysis shell-antiSMASH," ClusterMine360, MIBiG, Orphan assembly line polyketide synthases, IMG-ABC/ Integrated Microbial Genomes: Atlas of Biosynthetic Gene Clusters are some examples of tools that have played a vital role in the characterization of the biosynthetic gene cluster (Salwan and Sharma 2020). The availability of big genome data helps mine new antibiotic gene clusters for identifying novel antibiotic compounds. The plug-and-play strategy is being explored for using an "artificial gene operon assembly system" (AGOS) to arrange genes in operons and express them in streptomyces strains with controlled regulation (Salwan and Sharma 2020). Besides these, antibiotic resistance to these existing antibiotics creates a challenging situation, so from the current natural source, it is mandatory to identify new compounds, Next-generation genome sequencing, metagenomics, and CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated) (Salwan and Sharma 2020), gene editing, and artificial operon based techniques are the most fundamental approaches in the new age of bioactive compound research and may benefit human welfare (Achparaki et al. 2012).

### 1.1.1. Evolution and Ecology

The phylum actinobacteria is one of the wide-ranging taxonomic units among the major lineages within the *Bacteria* domain (Barka et al. 2016). The phylum includes pathogenic genus like c*orynebacterium, mycobacterium, nocardia,propionibacterium,* and *tropheryma.* Some phylum includes soil dwellers such as *Micromonospora* and *Streptomyces* species. *Frankia species* have an association with plants. And some are associated with the gastrointestinal tract,

like *Bifidobacterium* species (Barka et al. 2016). These actinobacteria are gram-positive organisms with high guanine and cytosine content in their genomes (Mobolaji Felicia Adegboye 2012). They have a different lifestyle with complex differentiation, and most of these are saprophytes that spend their predominant lives in semi-dormant spores in extreme conditions (Barka et al. 2016). The reproduction of streptomyces is by mycelial fragmentation and is a type of asexual reproduction (Goodfellow, Ferguson, and Sanglier 1992).

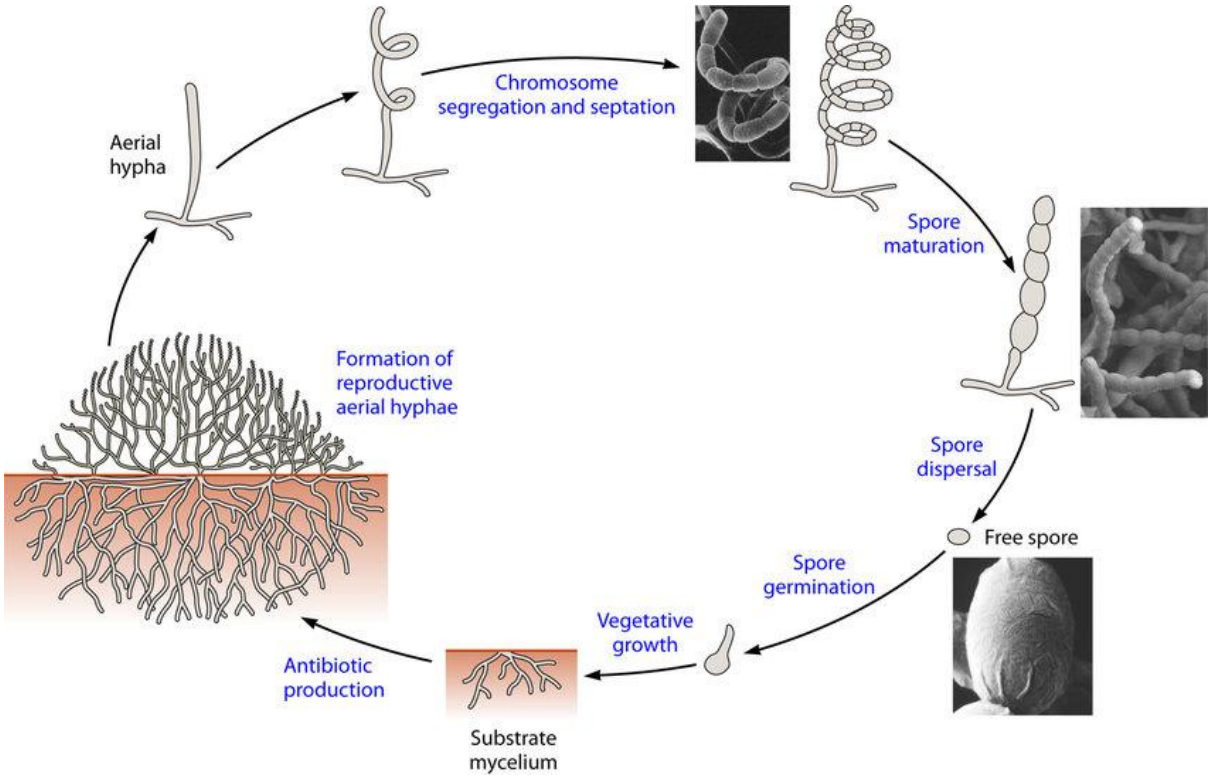


**Fig.1.1. Lifecycle of streptomyces (Barka et al. 2016).**

The group exhibits different morphological characteristics, such as other types of substrate mycelium, aerial mycelium, and colored mycelium, in producing melanoid pigments and types of spores. The spore chain morphology is quite an essential morphological character, and it occurs directly from the substrate mycelium. This spore chain structure can be used as an excellent morphological character to distinguish different types of *Streptomyces* like smooth, warty, hairy, spiny, and rugose (Barka et al. 2016). Physiologically and ecologically, most actinobacteria are aerobic, but there are exceptions. Further, they can be heterotrophic or

chemoautotrophic, but most are chemoheterotrophic and can use various nutritional sources, including different complex polysaccharides. The population density of actinobacteria depends on their habitat and the prevailing environmental conditions. They are typically present at densities on the order of $10^6$ to $10^9$ cells per gram of soil (Barka et al. 2016). Actinobacteria are mostly mesophilic, with optimal growth at temperatures between 25 and 30°C. Some thermophilic actinobacteria are there, which can grow from 50 to 60°C. Factors such as temperature, pH, and soil moisture also influence the growth of actinobacteria. The vegetative growth of actinobacteria in the soil is favoured by low humidity, especially when the spores are submerged in water. In the case of dry soils, the moisture tension is higher, and growth is minimal and may be halted. A humidity of aw 0.50 and above is necessary for good germination and elongation of germ pores (Doroshenko et al. 2005). Most aerotolerant acidophilic actinobacteria are found in habitats with pH 3.5, and most of the actinomycetes exhibit maximum growth around neutrality (Kim et al. 2004). The taxonomical details of the phylum actinobacteria increase with the accumulation of knowledge with the help of polyphasic taxonomic studies. As it is the largest taxonomical unit, it is challenging to discriminate closely related species or even genera. Genetic markers such as 16S rRNA, rpo B, ssgB, gyrB, and rec A are commonly used for molecular-level identification. Where rpo B, gyrB, and rec A are used explicitly for species-level identification. Where rpo B, gyrB, and rec A are specifically used for species-level identification. However, rRNA sequences do not discriminate well between closely related species or even genera, which can create ambiguity. Moreover, the massive recent increase in the availability of genome sequence information has provided detailed insights into genome evolution and made it possible to identify genes specific to organisms at the level of genera and family (Barka et al. 2016). Morphological characteristics are also considered a tool for identification. Microscopic morphology and chemotaxonomy are the primary tools used to depict the taxonomy of actinobacteria at the genus and species levels. Morphological

characteristics include aerial and substrate mycelium type, melanin or soluble pigment production in agar, and growth observation by changing different media compositions. The cell wall composition and cell sugar distribution play an essential role in the chemotaxonomical characterization (Barka et al. 2016). Actinomycetes can be classified into different groups, promicromonospora, micromonospora, and microbispora, which have similar cell wall compositions to those of actinomyces, actinoplanes, and streptosporangium (Yamaguchi 1965). Studies about the cell wall composition and classification of actinomycetes and their results concluded that actinomycetes are not included in fungi and can be classified under bacteria (Harris 1958). The whole-cell hydrolysate also has a vital role in the classification presence of mesodiaminopimelic acid identified as the main constituent in Nocardia and LL-diaminopimelic acid in Streptomyces sp.(Becker, B. et.al. 1964).

Streptomyces believed to have originated 4000 million years ago, are associated with plants and help compost dead plants, resulting in ancient soil formation (Chater 2016). The genus has so many essential features. Primarily, it is the most abundantly seen genus in soil and has a significant role in recycling organic debris. These are considered soil chemists, which can produce a range of bioactive molecules with various actions. The genome of streptomyces is composed of approximately 72%GC and has an average length of 8 megabases (Chevrette et al. 2019). Streptomyces has a complex lifecycle that starts with germinating spores that sprout to form vegetative hyphae and grow extensively into a branched mycelium. Following the compartmentalization of hyphae, each compartment possesses multiple copies of chromosomes; thus, these are called multicellular bacteria. The distribution of Streptomyces covers almost all habitats, including aquatic habitats, saline ecosystems, brackish and saline mangrove systems, limestones, associations with insects, and endophytic plant-associated Streptomyces. Hence, it proves the capability of Streptomyces to resist these adverse conditions. Investigating the different habitats needs to focus on exploring more antimicrobial agents beneficial for the current

scenario. Thus, it can produce novel drug producers and new antimicrobials functional against sprouting multidrug-resistant bacteria.

### 1.1.2. Contribution of Streptomyces to antibiotic world

Streptomyces has been proven to be an excellent source of bioactive compounds; these microbes produce over two third of clinically useful antibiotics. The discovery of antibiotics from Streptomyces began with isolating streptomycin from a culture of *Streptomyces griseus.* Streptomycin was the first aminoglycoside isolated in 1943 and the first antibiotic remedy for tuberculosis. This antibiotic is a protein inhibitor and a bactericidal antibiotic. Streptomycin is active against other pathogenic gram-negative bacteria, including *Brucella* spp, *Klebsiella granulomatis*, *Klebsiella pneumoniae*, *E. coli, Proteus spp., Aerobacter aerogenes, Francisella tularensis, Haemophilus ducreyi, Haemophilus influenzae,* and *Yersinia pestis,* as well as Gram-positive bacteria such as *Streptococcus viridians* and *Enterococcus faecalis* (Zaffiri, Gardner, and Toledo-pereyra 2012). Neomycin is another aminoglycoside antibiotic isolated from *Streptomyces radio*, and it was reported by Walksman in 1949 that it can be administered in different ways, like oral, ocular, and dermal (Balachandran 2012). Another aminoglycoside antibiotic synthesized by *Streptomyces hygroscopicus* is Hygromycin B consists of three rings, a hyosamine ring, a d-talose ring, and a destomic acid ring, and it is active against both prokaryotic and eukaryotic cells and is not currently used for clinical purposes, and used as a tool in the cloning of genes for both prokaryotes and eukaryotes in molecular-biology experiments (Takenoya et al. 2019). Waksman's work began the Golden Age of antibiotic discovery from the 1940s to the 1960s. In 1942, the broad-spectrum antibiotic Streptothricin F was isolated from *Streptomyces lavendulae* (Ji et al. 2009). Kanamycin A is an aminoglycoside that

interferes with protein biosynthesis in *Streptomyces kanamyceticus* (Hutchings, Truman, and Wilkinson 2019). A group headed by Benjamin Minge Duggar started digging for new isolates of Streptomyces. On their way, they got one active strain, and William Albrecht named it *Streptomyces aureofaciens*, the crude form of the same, showing good activity against rickettsias and typhus. They labeled it as a broad-spectrum antibiotic called aureomycin. Thus, after 1950, the enthusiasm of world researchers burst and sprouted for discovering new antibiotics from so-called ultra molds. Consecutively *Streptomyces rimosus* was identified, and a compound very similar to aureomycin was isolated, named terramycin.

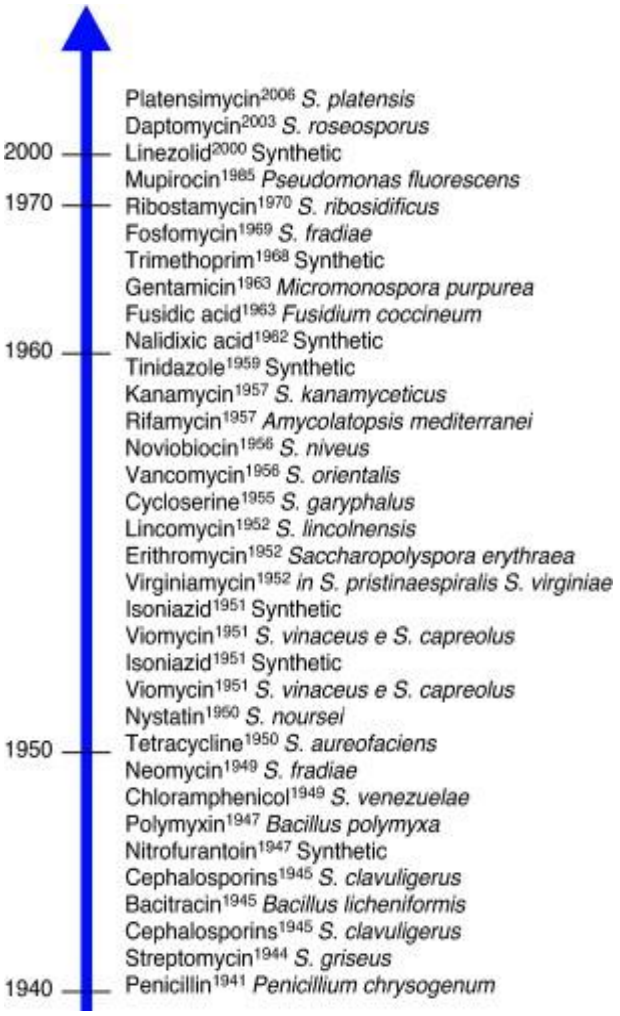


**Fig.1.2.Time line showing antibiotic discovery from 1940 to 2006 (Emerson et al. 2012)**

Succeeding scientists look forward to the chemical structure of both compounds. As we know, the limitation of the instruments for identifying the structure was their main problem. But fabled Haward University scientist Robert Woodward came forward and led a team for the discovery. They succeeded and discovered the first semisynthetic drug, tetracycline, which is a sub-class of polyketides having an octahydrotetracene-2-carboxamide skeleton, derivatives of polycyclic naphthacene carboxamide (Balachandran 2012) and approved by the FDA in 1954 (Nelson and Levy 2011). Many derivatives derived from tetracycline antibiotics were oxytetracycline, methacycline, doxycycline, and tigecycline (Balachandran 2012). Tigecycline is a glycylcycline antibiotic marketed by Wyeth under the brand name TYGACIL®. It was given a US Food and Drug Administration (FDA) fast-track approval on June 17, 2005 (Greer 2006).

This antibiotic is the first clinically available drug in a new class called glycylcycline (Greer 2006) It is structurally similar to the tetracyclines in that it contains a central four-ring carbocyclic skeleton (Balachandran 2012).

Chloramphenicol is a ribosome binding agent active against Gram-negative bacteria isolated from *Streptomyces venezuelae* in 1947 but is no longer used clinically due to rare side effects (Katz and Baltz 2016). Lincomycin, a lincosamide antibiotic isolated from *Streptomyces lincolnensis* and a semisynthetic derivative of Lincomycin, was made in 1966 called Clindamycin, a protein inhibitor active against several bacterial infections (Hutchings, Truman, and Wilkinson 2019). Vancomycin, a glycopeptide antibiotic discovered by Eli Lilly and Abbott laboratories in the mid-1950s, was produced by *Streptomyces Orientalis* and was first used clinically in 1959 (Gao 2002). Along with vancomycin, another antibiotic, ristocetin, was also identified, but it withdrew from use because it caused thrombocytopenia (Blaskovich et al. 2018). Vancomycin works by binding to the membrane lipids of the peptidoglycan layer and preventing cell wall formation. It is active against several gram-positive pathogens, including streptococci, enterococci, staphylococci, especially Methicillin-resistant *Staphylococcus aureus* infections (Gao 2002). Another cell wall inhibitor, seromycin, was isolated in 1955 from *Streptomyces orchidaceus* (Hutchings, Truman, and Wilkinson 2019). It belongs to the cycloserine antibiotic class of molecules and has been used to treat multidrug-resistant tuberculosis (MDR-TB) (Li et al. 2018). In the mid-1950s, a new Pristinamycin belonging to the streptogramin antibiotic class was isolated from *Streptomyces pristimespiralis* (Hutchings, Truman, and Wilkinson 2019). Pristinamycin is a protein synthesis inhibitor primarily used for treating staphylococcus infections. The gene cluster of Pristinamycin is considered the largest antibiotic supercluster (~210kb) (Mast et al. 2011). One of the major antibiotic class is macrolides these are polyketide class of antibiotics consist of 14 to 16 membered macrocyclic lactone rings to which sugar moieties will be attached especially deoxy sugars like erythreomycin

(Suzuki et al. 2001). Later, several semisynthetic macrolides are discovered using the structure of erythromycin, including azithromycin, clarithromycin, and dithromycin. Macrolides are bacteriostatic against *S. aureus. H. influenza* and streptococci, but may be bactericidal in high concentrations, recently azithromycin and clarithromycin were used to treat mycobacterium avium infection in children (Jaff and Bush 2001). The mechanism of action of macrolides is based on the inhibition of protein synthesis, and the target of macrolides is the peptidyl transferase center on the large 50S subunit of the bacterial ribosome (Olsufyeva and Yankovskaya 2020). Erythromycin and clarithromycin can prevent diffuse panbronchiolitis lung disease. Carbomycin is a macrolide antibiotic isolated from *Streptomyces halstedii* that inhibits gram-positive bacteria and some mycoplasma. Pikromycin-related macrolides have recently attracted significant research interest because they are structurally related to the semi-synthetic ketolide antibiotics that have demonstrated promising potential in combating multi-drug-resistant respiratory pathogens (Xue and Sherman 2001). Based on in-depth studies carried out by Xue et.al., using *Streptomyces venezuelae* proved to be a good tool for studying gene clusters and sequencing the responsible gene cluster for macrolide biosynthesis, including mitomycin, neomethymycin, pikromycin, and narbomycin. Thus, Xue and his coworkers opened a clear path toward the future for combinatorial biosynthesis of different antibiotics. *Mycoplasma genitalium* resistance is becoming prevalent due to high-dose usage of Fluoroquinolone and macrolide drugs (Xue and Sherman 2001). Pristinamycin at a daily dose of 2g or 4g combined with doxycycline shows effective treatment for macrolide-resistant *Mycoplasma gentitalium* infections (Read et al. 2018). Fosfomycin, belonging to the phosphonates class, isolated in 1969 from *Streptomyces fradiae*, is the only antibiotic in the clinic from the phosphonates group (Cao et al. 2020). It has been approved for treating urinary tract bacterial infections and was active against *E. coli*, Klebsiella, and Enterobacter (Hutchings, Truman, and Wilkinson 2019). Fosfomycin has a broad spectrum of bactericidal activity, and it inhibits cell wall formation by

inhibiting UDP-N-acetylglucosamine-3-enolpyruvyltransferase, also known as MurA. This enzyme catalyzes the first committed step in cell wall biosynthesis.

Carbapenems are β-lactam antibiotics possessing a β-lactam ring and a five-membered ring that differs from that of penicillin in being unsaturated and having a carbon atom rather than sulfur, thienamycin (1976), a naturally derived product of *Streptomyces cattleya*, was the first discovered carbapenem (Elshamy and Aboshanab 2020). An increasing trend is visible in the usage of penems in hospital settings. Their study concluded that meropenem is better because of its intrinsic activity against gram-negative bacilli and lower neurological toxicity in a higher dose. Rubimycinone, a new anthraquinone antibiotic isolated from streptomyces sp isolated from the root zone of *Yucca aloiafolia*. The compound has antibacterial activity against *Staphylococcus aureus* with a MIC of 5.2 µg/ml (Raju et al. 2013). Lipopeptides are a highly active antibiotic class against multi-resistant bacteria. Daptomycin, a lipopeptide antibiotic isolated from *Streptomyces roseus,* was approved in 2003 by the FDA to treat skin infections caused by Gram-positive pathogens (Baltz et al. 2006). Daptomycin, along with fosfomycin, provided a 12% higher success rate than daptomycin alone in the treatment of Methicillin-resistant *Staphylococcus aureus* (Pujol et al. 2021).

After the golden era of the discovery of antibiotics, a notable success was a Cubist pharma company's introduction of Cubicin (daptomycin) onto the market (Hutchings, Truman, and Wilkinson 2019). Several anti-MRSA antibiotics were isolated from Streptomyces species alone. According to the WHO, 95% of *Staphylococcus aureus* isolates worldwide are resistant to effective drugs, considered life-threatening. A series of novel glycopeptide antibiotics mannopeptimycins α-ε (**1**-**5**) separated from *Streptomyces hygroscopicus* exhibited moderate to good antibiotic activity against MRSA and VRSA (He et al. 2002). Nosiheptide, an anti-MRSA antibiotic isolated from marine sediment isolate Streptomyces sp. CNT 373 possesses Streptomyces species MC004 isolated from coral mines produced angumicynone B showed a better

MIC of 12.5 µg/ml. A potent anti-MRSA drug from the polyketomycin anthracycline group of antibiotics isolated from Streptomyces MK277-AF1 from the Kanagawa region in Japan with a high MIC of 0.025-0.2 µg/ml (Kemung et al. 2018). Novel compounds Munumbicin E4 and Munumbicin E5 produced from Streptomyces NRRL 30562 isolated from snake vine plant, both compounds are active against gram-positive and gram-negative pathogens.

Munumbicin E5 shows excellent activity against *Plasmodium falciparum,* and it also shows activity against MRSA (U. F. Castillo et al. 2006). Kakadumycin A, isolated from Streptomyces NRRL 30562, is a quinoxaline antibiotic that inhibits RNA synthesis. It shows broad-spectrum activity against gram-positive bacteria, significantly inhibiting *Bacillus anthracis* strains with MICs of 0.2–0.3 µg/ml (U. Castillo et al. 2003). Coronamycin, a novel peptide antibiotic isolated from an epiphytic vine associated with Streptomyces sp MSU-2110, shows a good MIC of 4µg/ml against the human fungal *pathogen Cryptococcus neoformans* (Ezra et al. 2004). Coronamycin also shows prominent anti-falciparum activity with an IC 50 value of 9 ng/ml. Similarly, chloroquine, the gold standard antimalarial compound, with an IC50 of 7 ng/ml. Chloroquine-resistant P. falciparum biotypes are sensitive to coronamycins, implying the role of coronamycin as a drug for malarial treatment. The hunt for antibiotic discovery continued El-Gendy et al. 2008 reported a triazolopyramidine antibiotic Essramycin was isolated from marine Streptomyces sp. Merv8102 with a MIC of 2 to 8 mg/ml against Gram-positive and Gram-negative bacteria showed no antifungal activity. It is the first report showing the presence of thiazolopyrimines in natural resources. Mainly thiazolopyramidines are used for the treatment of cardiovascular diseases, hypertension, and especially for atherosclerosis (El-Gendy et al. 2008). Warkmycine, isolated from Streptomyces sp. Acta 2930 is an angucycline antibiotic with antibacterial activity against Gram-positive bacteria *Staphylococcus lentus* and *Bacillus subtilis* (Helaly et al. 2013). A new benzoxazole antibiotic, carboxamycin, isolated from the deep-sea isolate Streptomyces sp. NTK 937, has antibacterial activity

against *Staphylococcus epidermidis* (DSM 20044), *Pseudomonas syringae* par. aptata (DSM 50252), and *Pseudomonas fluorescens* (NCIMB 10586) (Hohmann et al. 2009). Paromomycin is a broad-spectrum aminoglycoside antibiotic that is active against gram-positive, gram-negative, and especially against the treatment of certain life-threatening protozoal infections such as visceral leishmaniasis (Ibrahim et al. 2019). When isolating new antibiotics from natural resources, unexplored areas like mangrove habitats are the most dependent .Kock et al. 2005 explained that marine *Streptomyces* isolate B8005 produces a quinone-like antibiotic possessing antibacterial activity by inhibiting RNA polymerase activity. It mainly inhibits *E. coli, S. aureus*, and *C. albicans*. From the beginning, natural product discovery and novel antibiotic isolation from microbial sources were mainly focused on antitubercular antibiotics. Several antibiotics active against Mycobacterium tuberculosis were isolated from marine sponges, marine actinomycetes, endophytic fungi, and endophytic streptomyces. Heraclemycins A-D and Pluramycins were isolated from endophytic Streptomyces species showing very selective anti-tubercle activity and also showing activity against *S. aureus,* MRSA, and *B. subtilis* (Moloney 2016). Insects are the predominant class of the animal kingdom and have significant mutualism with microbes. Exploring more openup a wide range of microbial-associated natural product discoveries. Several antibiotics are showing activity against human bacterial pathogens. *Streptomyces sp. 1H-GS5* was isolated from the head of an ant (*Camponotus japonicas Mayr*), producing new cytotoxic spectabilin derivatives (Liu et al. 2016). Streptomyces associated with Solitary wasps provide antibacterial and antifungal chemical protection to their larvae through the production of streptochlorin, a variety of piericidin analogs, and other molecules (Chevrette et al. 2019). Poulsen et al. 2011 studied two species of solitary mud dauber wasps, *Sceliphron caementarium* and *Chalybion californicum*, for novel Streptomyces. They discovered several Streptomyces species associated with the wasp that produced bioactive compounds. Through chemical analysis of compounds of six different structural classes,

antimycins, bafilomycin A1 and B1, daunomycin, mycangimycin, streptazolin, and streptazon B, and the previously unknown macrocyclic lactam, sceliphrolactam, were noted. In the same period, unreported 26-membered polyene macrocyclic lactam, sceliphrolactam, was isolated from a Streptomyces sp., associated with the mud dauber, *Sceliphron caementarium* (Oh et al. 2011). Omics-based studies help to reveal new elaiophylin derivatives and rifamycins from termite-associated *Streptomyces sp. M56* (Klassen et al. 2019). Quercetin 3-O-glucoside, an antibiotic, was very recently isolated from the *Streptomyces antibiotics* strain essamA8 isolated from the insect *Tapinoma smoothie* (Nageh Sholkamy et al. 2020). Remali et al. 2017 reported a new Phenazine antibiotic 6-((2-hydroxy -4 methoxy phenoxy) carbonyl) Phenazine 1 carboxylic acid (HCPCA), and tubermycin B isolated from endophytic *Streptomyces kebasangaanensis* both compounds have antibacterial and antifungal activity.

Even today, digging for novel compounds from Streptomyces continues one side, and on the other side, microbial-derived compound scaffolds are used for synthesizing new antibiotics. Recently many synthetic analogs of antibiotics isolated from Streptomyces have been approved by FDA for clinal fields like daptomycin, biapenem, etrapenem, etc. (Kemung et al. 2018). A reduction in the interest of pharma companies in microbial natural product isolation deserted the field. At last, in 2003, daptomycin was approved for use. Rejuvenation of the area is required by focused and sound research to awaken microbial natural product research and novel antibiotic inventions.

### 1.1.3. Antimicrobial Resistance

After Sir Alexander Fleming's epoch-making discovery, Ernst and Florey made a fruitful innovation by purifying Fleming's penicillin into penicillin G in 1942 and using it in the Second World War. Countless antibiotics were later identified, mainly from 1950 to 1970 and the golden era. It is to be noted that the usage of antibiotics was not started in this particular period; traces of tetracycline were found in human skeletal remains from ancient Sudanese Nubia in

the past 350–550 CE, and the presence of tetracycline was confirmed from the sample taken from the femoral midshafts of skeletons from Egypt (Aminov 2010). These antibiotics played an essential role at that time in healthcare systems. Unfortunately, the arrival of antimicrobial resistance is a significant threat to the field. The discovery of new chemicals helps to overcome some microbial infections, which seems to be the greatest accomplishment of the 20th century. As per the WHO, antimicrobial resistance is considered a global health challenge. By the 1940s, the first antimicrobial-resistant *Staphylococcus aureus* was isolated, and it had shown resistance to penicillin. At one time, *Staphylococcus aureus* was considered a significant nosocomial pathogen that acquired resistance to major antibiotics like tetracyclines, macrolides, methicillin, and vancomycin. The emergence and spreading of AMR are mainly caused by the groundless usage of antibiotics in human health, animal husbandry, poultry, and various other sectors (Yu et al. 2020).

### 1.1.4. Mechanisms of Antimicrobial resistance in pathogens

Bacterial pathogens adopt several mechanisms to nullify the effects of antibiotics. The resistance mechanisms adopted by bacteria reduce the concentration of the drug able to reach the target site by preventing or destroying it, and bacteria also inactivate the drug before reaching the target. Also, bacteria modify the target so that the drug can bind; thus, the bacteria prevent the drug from reaching the target site (Abdelrahman et al. 2021). Efflux pumps have a prominent role in the resistance mechanism. Mainly, five different classes of pumps are present in various gram-positive and gram-negative pathogenic bacteria, including efflux pumps of resistance nodulation division (RND) seen in gram-negative bacteria. An example is the AcrAB–TolC pump, initially discovered in *Escherichia coli. The* next class of pumps is found in gram-negative and gram-positive bacteria, the Major Facilitator Superfamily (MFS) pumps, and they are Qac A, Nor A. Lmr S are the three main pumps associated with Staphylococcus aureus and then comes Small multidrug resistance (SMR) transporters Examples include Qac C from *S.*

*aureus* and EmrA from *E. coli* (Baylay, Piddock, and Webber 2019). Multidrug and toxic compound extrusion (MATE) efflux pumps are commonly found in Gram-negative bacteria. Examples include VcrM from *Vibrio cholera*, MepA from *S. aureus,* and PmpM from *Pseudomonas aeruginosa*, which transport a variety of substrates, including fluoroquinolones and benzalkonium chloride. The last class is ATP-binding cassette (ABC) transporters such as PatAB from *Streptococcus pneumoniae*, which transports fluoroquinolones, and MacAB from *E. coli,* which exports macrolides (Baylay, Piddock, and Webber 2019). Another unique resistance mechanism prevents access to the target by reducing permeability. Outer membrane porins help the transport of hydrophilic antibiotics into the bacterial cell so bacteria down-regulate the expression of porins and thus create an intrinsic resistance in bacteria (Blair et al. 2015). Another known mechanism of resistance shown by bacteria is by enzymatic degradation of antibiotics Lactamases-mediated hydrolysis of β lactam antibiotic penicillin is one example of the enzymatic degradation of antibiotics (Abdelrahman et al. 2021). Carbapenemases, metallo-*β*-lactamase 1 (NDM-1), and macrolide esterase enzymes are other hydrolysis enzymes found in *K. pneumoniae, E. coli,* and *S. aureus* (Baylay, Piddock, and Webber 2019). Mutations in the target site and antibiotic binding prevention are other resistance mechanisms. For example, erythromycin ribosome methylase (*erm*) family of genes methylates 16S rRNA and alters the drug-binding site, and prevents binding of macrolides, lincosamines, and streptogramins, and, in the case of chloramphenicol–florfenicol resistance (*CFR*) methyltransferase, which methylates A2503 in the 23S rRNA explicitly and resist phenicols, pleuromutilins, streptogramins, lincosamides and oxazolidinones (including linezolid) (Blair et al. 2015). Andrade et al. 2020 in their review discussing the global health issue of antimicrobial-resistant bacteria in drinking water sources. Their detailed study investigated several microbes, especially *E. coli*, and pseudomonas, found to be abundant in groundwater and isolates resistant to different potent antimicrobial agents. This can be considered a severe problem of the coming era.

Recently, Browne et al. 2020 discussed the trend of antimicrobial resistance in fluoroquinolone drugs used for enteric fever. They thoroughly reviewed the AMR shown by *Salmonella enteric* serovar Typhi and Para typhi worldwide, especially in developing countries.

## 1.2. CONCLUSION

AMR is an increasingly severe threat to global public health that requires urgent attention. More antibiotics are needed to be isolated from natural resources. Here comes the importance of Streptomyces focusing on underexplored territories for more active metabolites. Since the isolation of antibiotics is not easy as it is so, we must focus on other strategies like modification of antibiotics, augmenting existing antibiotics by increasing their potency, and the usage of combination antibiotic therapies. Resistance mechanisms in pathogenic bacteria are growing, so properly elucidating the resistance mechanisms can help to design the appropriate solution. Strict measures must be taken to control and monitor the unwanted usage of antibiotics, like the usage and dispensing of antibiotics without prescription, etc.

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# CHAPTER 2

## Selection of *Streptomyces* species from the culture collection of our lab and screening through *in vitro* antagonism against human bacterial pathogens

## 2.1. INTRODUCTION

Streptomyces fall under actinobacteria, one of the largest bacterial phyla distributed ubiquitously (Barka et al. 2016). These microorganisms possess a mycelial lifestyle and produce bioactive secondary metabolites. Vegetative hyphae will form branched mycelium, differentiate, and mature to form spores on aerial hyphae. When the spores grow and disperse into the environment, they germinate, vegetative hyphae form, and the life cycle continues (Goodfellow, Ferguson, and Sanglier 1992). Morphological characteristics like spore structures are used for the taxonomical identification of actinobacteria. Spores are covered inside special vesicles called sporangia connected to aerial or substrate mycelium. Usually, these spores are single or seen as multiple chain-like structures, and in *Streptomyces* species, long spore chains with up to 100 spores are present. For determining the morphology, spore chain structures are visually observed through scanning electron microscopy, and these spore structures are classified into rectus-flexible, open loops or closed spirals, and verticillate (Barka et al. 2016). Streptomyces are well known for pigment production based on melanin pigment production and are classified into melanoid producers and non-melanoid producers. Streptomyces species produce many colored secondary metabolites, and these pigments are not directly involved in their growth and development but are bioactive compounds with significant properties.

One of the main aspects of exploring the Streptomyces species is the tremendous production of secondary metabolites capable of fighting against bacteria, fungi and with antioxidant activities. Exclusive studies are carried out on these microorganisms with the expectation that these isolated compounds will be used against bacterial infections mainly caused by multi-drug-resistant bacteria. *Streptomyces venezuelae* and *S. coelicolor* are the two species that provide new insights into the detailed study of antibiotic production, genes involved in the secondary metabolite production and point out the role of antibiotic production as regulatory ligands mediating autoregulation, cross-regulation, and interspecies interactions (Chater 2016).

Streptomyces possesses a complex life cycle with high G+C content; genomic approaches revealed that most secondary metabolite biosynthetic gene clusters are inactive in laboratory conditions. Each streptomyces can produce 30 different bioactive molecules, an invaluable source for novel drug discovery (Lee et al. 2020). Utilizing other genome mining technologies helps identify the genes responsible for secondary metabolite production. Recent developments in genome mining include offline and online possibilities like antiSMASH (antibiotics and secondary metabolite analysis shell), ClusterMine360, MIBiG, Orphan assembly line polyketide synthases, and IMG-ABC/Integrated Microbial Genome: Atlas of the Biosynthetic Gene Cluster (Salwan and Sharma 2020). Streptomycin from *S. griseus* isolated by Waksman et al. in 1943 paved a new path of discovery of natural products from streptomyces species. Later many antibacterial compounds were isolated, including cephalosporins from *S. clavuligerus*, neomycin and fosfomycin from *S. fradia*, tetracyclins from *S. aureofaciens*, vancomycin from *S. orientalis*, kanamycin from *S. kanamyceticus*, daptomycin from *S. roseosporus* and platensimycin from *S. platensis* (Emerson et al. 2012).

Over the years, antibiotics have saved many lives and decreased the suffering caused by many diseases and associated conditions. As a result, the demand for new antibacterial agents was expanding to keep up with the constant increase in antimicrobial resistance. Many kinds of research are going on, like isolating novel antibiotics from under-explored niches, augmentation of existing antibiotics, combinational therapies, and isolating novel antibacterial compounds from microbial resources. In the present study, an exploration of two streptomyces species was carried out to isolate antibacterial compounds. 16S rRNA sequencing and spore morphology were carried out to confirm the species, and a phylogenetic tree was constructed. The antibacterial activity of crude organic metabolites was tested to ensure the presence of antibacterial compounds.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Chemicals and reagents

Various culture media used for microbiological studies were procured from Hi-Media Laboratories Pvt. Limited, Mumbai, India. The solvents were purchased from Merck Life Sciences, Mumbai, India.

### 2.2.2. Test bacterial pathogens

Test bacterial pathogens used for *in vitro* antibiosis were *Bacillus cereus* MTCC 1305, *Mycobacterium smegmatis* MTCC 993, *Staphylococcus aureus* MTCC 902, (Gram-positive), *Escherichia coli* MTCC 2622, *Proteus mirabilis* MTCC 425, *Pseudomonas aeruginosa* MTCC 2642, and *Salmonella typhi* MTCC 3216, (Gram-negative). Bacterial strains were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India, and maintained on Luria-Bertani agar slants and kept at 4 °C.

### 2.2.3. Sample collection

Two Streptomyces species selected for the studies are collected from the culture collection of our lab. *Streptomyces* NIIST G132 was isolated from soil samples collected from Amalagiri, Kottayam district (9.4874° N, 76.548° E), and *Streptomyces* NIIST D75 was isolated from the soil collected from Malampuzha, Palakkad district (10.8281° N, 76.368° E).

### 2.2.4. Antibacterial activity

Antibacterial activity of the Streptomyces NIIST G132 and NIIST-D75 strain were determined by the agar overlay method (Jacob et al. 2017) and the crude organic extract by disc diffusion (Jacob et al. 2017) method against seven test bacterial pathogens. Streptomyces NIIST G132 and NIIST-D75 strains were spot inoculated in the centre of the ISP-2 agar Petri plate and

grown at 30 °C for 7 days in the Incubator (Rotek-RCI 12S, B & C Industries, India). The test pathogens in Luria-Bertani broth (LBB) were incubated at 37 °C for 18 h in the Incubator (Sanyo $CO_2$ Incubator MCO-20 A/C, Sanyo Electric Co Ltd, Japan), and adjusted to 1 x $10^6$ CFU/mL using the spectrophotometric method at 600 nm (Synergy4, BioTek Instruments, USA), which is equivalent to 0.5 McFarland standard. The ISP-2 Petri plates containing spotted Streptomyces NIIST G132 and NIIST-D75 strains were overlaid with the inoculum in 10 mL of Mueller Hinton agar (MHA) medium, and incubated the plates for 24 h at 37 °C and zone of inhibition (ZoI) were recorded (in mm). Petri plates without Streptomyces NIIST G132 and NIIST-D75 strains served as the control.

Antibacterial activity of the crude organic extract was carried out by inoculating test bacteria in LBB, which was incubated for 18 h at 37 °C and adjusted to 1 x $10^6$ CFU/mL, and swabbed to MHA agar plates. To the centre of Petri plates was introduced 6 mm disc impregnated with 30 µL of 10 mg/mL of crude organic extract (dissolved in 100% methanol) and incubated, and the ZoI was recorded (in mm) after 24 h. Disc with only methanol served as the control.

### 2.2.5. Molecular identification by 16S rRNA sequencing

#### 2.2.5.1 Genomic DNA Isolation from Bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A portion of streptomyces culture was taken from culture slant and placed in a microcentrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K were added and incubated at 56 ºC in a waterbath util thoroughly lysed. After complete lysis 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. After adding 200 µl of B3 buffer incubate at 70 ºC for 10 minutes. Later add 210 µl of 100% ethanol and mix by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a

new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. Following cleaning the NucleoSpin® Tissue column by put it in a clean 1.5 ml tube and eluted out DNA using 50 µl of BE buffer.

#### 2.2.5.2. Agarose Gel Electrophoresis

The purity of extracted DNA was checked using agarose gel electrophoresis. 5 µl of DNA was mixed with 6 X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0). The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were examined in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

#### 2.2.5.3. PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume that contained 1X PCR buffer (100 mM Tris HCl, pH-8.3; 500 mM KCl), 0.2 mM each dNTP (dATP, dGTP, dCTP, and dTTP), 2.5 mM MgCl2, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO. Forward primer (CAGGCCTAACACATGCAAGTC) and reverse primer (GGGCGGWGTGTACAAGGC) was used for the reaction. PCR reaction consisting of three steps including: a pre-denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 40 sec, extension at 72 °C for 60 sec and a repair extension at 72 °C for 7 min. PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

### 2.2.5.4. Agarose Gel electrophoresis of PCR product

1.2% agarose gel was prepared in 0.5X TBE buffer containing 0.5 g/ml ethidium bromide and used to test the PCR products. One microlitre of 6X loading dye was combined with 5 µl of PCR products and loaded, and electrophoresis was performed for around 1-2 hours at 75 V power supply with 0.5X TBE as electrophoresis buffer, until the bromophenol blue front had migrated to virtually the bottom of the gel. A 2-log DNA ladder (NEB) was utilized as the molecular standard. The gels were examined with a UV transilluminator (Genei), and the image was recorded under UV light with a Gel documentation system (Bio-Rad).

### 2.2.5.5. ExoSAP-IT Treatment

ExoSAP-IT (USB) is a specifically prepared buffer containing two hydrolytic enzymes, Exo-nuclease I and Shrimp Alkaline Phosphatase (SAP), for the elimination of undesired primers and dNTPs from a PCR product. 5 µl of PCR product and 0.5 µl of ExoSAP-IT mixture was incubated at 37 $^{o}$C for 15 minutes followed by enzyme inactivation at 85 $^{o}$C for 5 minutes.

### 2.2.5.6. Sequencing using BigDye Terminator v3.1

The sequencing reaction was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA). The PCR mixture consist of 10-20 ng of ExoSAP treated PCR product, 3.2 pM primer (either Forward or Reverse), 0.28 µl Sequencing Mix, and 1.86 µl Reaction buffer. Sterile distilled water was used to make up to 10 µl of the mixture. The sequencing PCR tem-perature profile included a first cycle at 96 °C for 2 minutes, followed by 30 cycles at 96 °C for 30 seconds, 50 °C for 40 seconds, and 60 °C for 4 minutes.

### 2.2.5.7. Post sequencing PCR clean up

Master mix I (10 µl milli Q and 2 µl 125 mM EDTA) per reaction and master mix II (2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol) were prepared. Followed that, 12 µl of master

mix I was added to each reaction comprising of 10 µl of reaction contents and mixed thoroughly and 52 µl of master mix II was added to each reaction. The contents were mixed properly and incubated at room temperature for 30 minutes and spun at 14,000 rpm for 30 minutes. The supernatant was decanted and added 100 µl of 70 % ethanol and spun at 14,000 rpm for 20 minutes and again supernatant was decanted and repeated ethanol wash. After spun for one more time the supernatant was decanted and air dried the pellet. The dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

#### 2.2.5.8. Sequence Analysis

The Quality of sequence was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 (Drummond et al. 2012)

### 2.2.6. Phylogenetic tree

The 16S rRNA sequence was deposited in GenBank data library with assigned Accession numbers. The sequences were compared with other similar sequence retrieved from the GenBank data library was used to construct the phylogenetic tree using MEGA version 11 (Tamura, Stecher, and Kumar 2021). Sequences were aligned using the multiple sequence alignment tool ClustalW and were analysed to determine the relationships between isolates by the neighbour-joining method Bootstrap values were generated using 100 replicates.

### 2.2.7. Microscopic characterization of spore structures

Sterilized circular aluminium stubs were inserted in an angle of about 45 °C into the PDA plate. The Petri plate with stub was incubated for 24 h at 37 °C to check any contamination. After 24 h, the Streptomyces were spread using a inoculation loop along the line were stub met the medium and incubate 28 °C for 5 days. After incubation, carefully remove the stub and

dehumidified for 20 min under vacuum and sputter-coated with gold for SEM analysis (Carl Zeiss EVO 18 Research, Germany) (Jacob et al. 2017).

## 2.3. RESULTS

### 2.3.1. Sample collection

Two streptomyces species, viz. *Streptomyces* NIIST G132 and *Streptomyces* NIIST D75, isolated from two different places, were selected from the culture collection of our lab for further studies. *Streptomyces* NIIST G132 possess a white aerial mycelium with brown substrate mycelium producing diffusible pigment towards the agar, and *Streptomyces* NIIST D75 possess a pale-yellow aerial mycelium and olive brown pigmented substrate mycelium producing diffusible yellow pigment in medium.

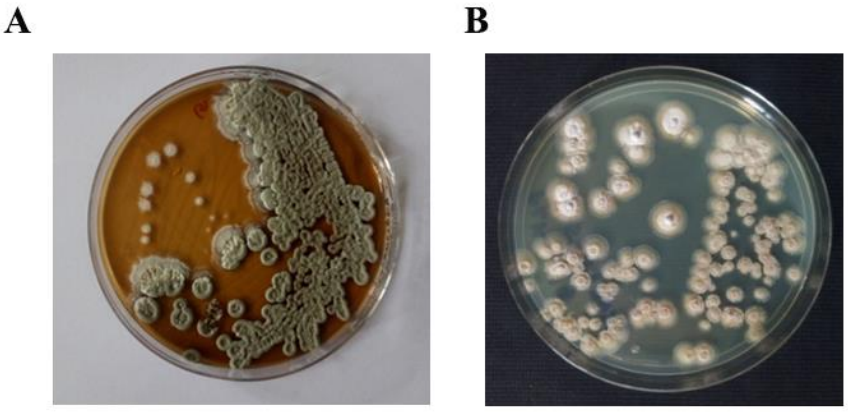


**Fig 2.1: Petri plate showing morphology of (A) *Streptomyces* NIIST G132 and (B) *Streptomyces* NIIST D75**

### 2.3.2. Antibacterial activity

The antibacterial activity of the crude organic metabolite of NIIST G132 exhibited a broad spectrum of activity against all bacterial pathogens. Maximum antibacterial activity was displayed against *P. mirabilis* (35.66 ± 0.57 mm), followed by *B. cereus* (26.66 ± 0.57 mm).

The crude organic extract of NIIST D75 exhibited broad-spectrum antibacterial efficacy against all the test pathogens. Among them, the crude organic extract showed a maximum ZoI of 27 mm against *B. cereus*, followed by ZoI of 25 mm against *S. aureus*.

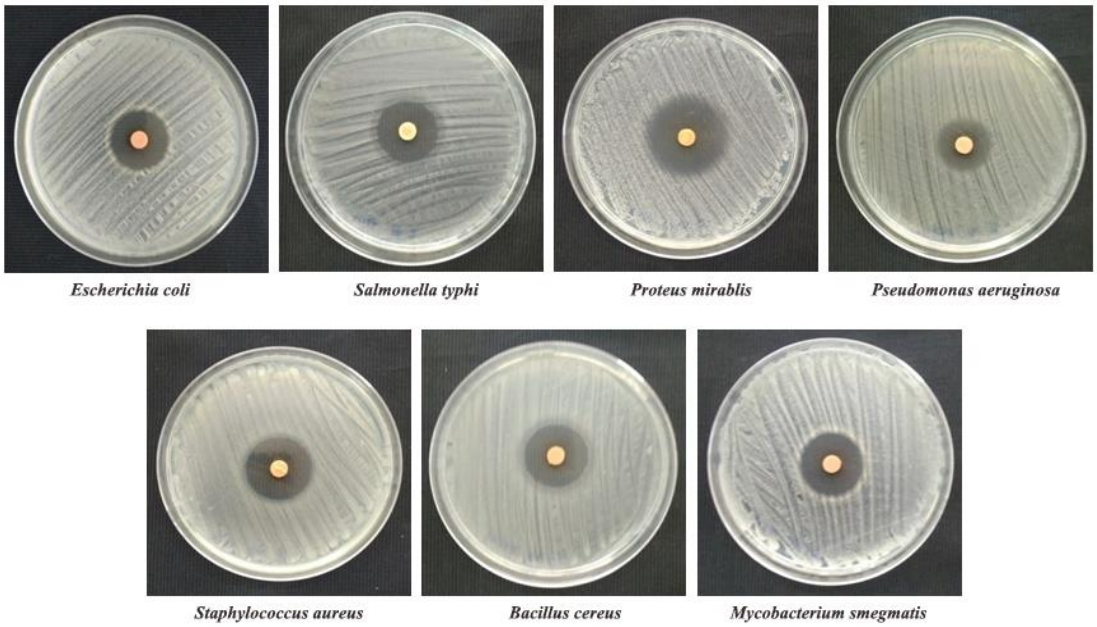


**Fig 2.2: Antibacterial activity of crude organic metabolite of *Streptomyces* NIIST G132**

**Table 2.1: Antibacterial activity of crude organic extract of Streptomyces NIIST G132**

| Test bacteria | Zone of Inhibition (mm) |
|---|---|
| *Escherichia coli* MTCC 2622 | 23.33 ± 0.30 |
| *Salmonella typhi* MTCC 3216 | 25.00 ± 0.00 |
| *Pseudomonas aeruginosa* MTCC 2642 | 20.66 ± 0.57 |
| *Proteus mirablis* MTCC 425 | 35.66 ± 0.57 |
| *Bacillus cereus* MTCC 1305 | 26.66 ± 0.57 |
| *Staphylococcus aureus* MTCC 902 | 25.00 ± 0.00 |
| *Mycobacterium smegmatis* MTCC 993 | 26.00 ± 0.00 |

Values are the average of triplicate and represented in mean ± SD

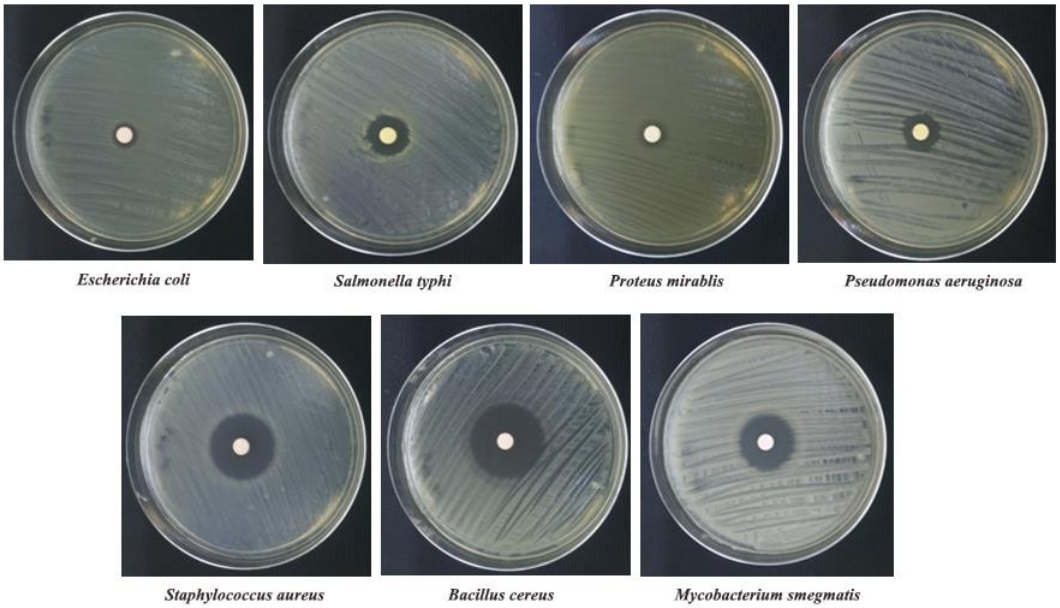


**Fig 2.3: Antibacterial activity of crude organic metabolite of *Streptomyces* NIIST D75**

**Table 2.2: Antibacterial activity of crude organic extract of *Streptomyces* NIIST D75**

| Test bacteria | Zone of Inhibition (mm) |
|---|---|
| *Escherichia coli* MTCC 2622 | 16.00 |
| *Salmonella typhi* MTCC 3216 | 21.00 |
| *Pseudomonas aeruginosa* MTCC 2642 | 20.00 |
| *Proteus mirablis* MTCC 425 | 16.00 |
| *Bacillus cereus* MTCC 1305 | 27.00 |
| *Staphylococcus aureus* MTCC 902 | 25.00 |
| *Mycobacterium smegmatis* MTCC 993 | 24.00 |

### 2.3.3. Molecular identification by 16S rRNA sequencing

The 16S rRNA sequence of our isolate and similar sequences retrieved from GeneBank were aligned using ClustalW software, and the phylogenetic tree was constructed with MEGA 11 software using the neighbour joining method.

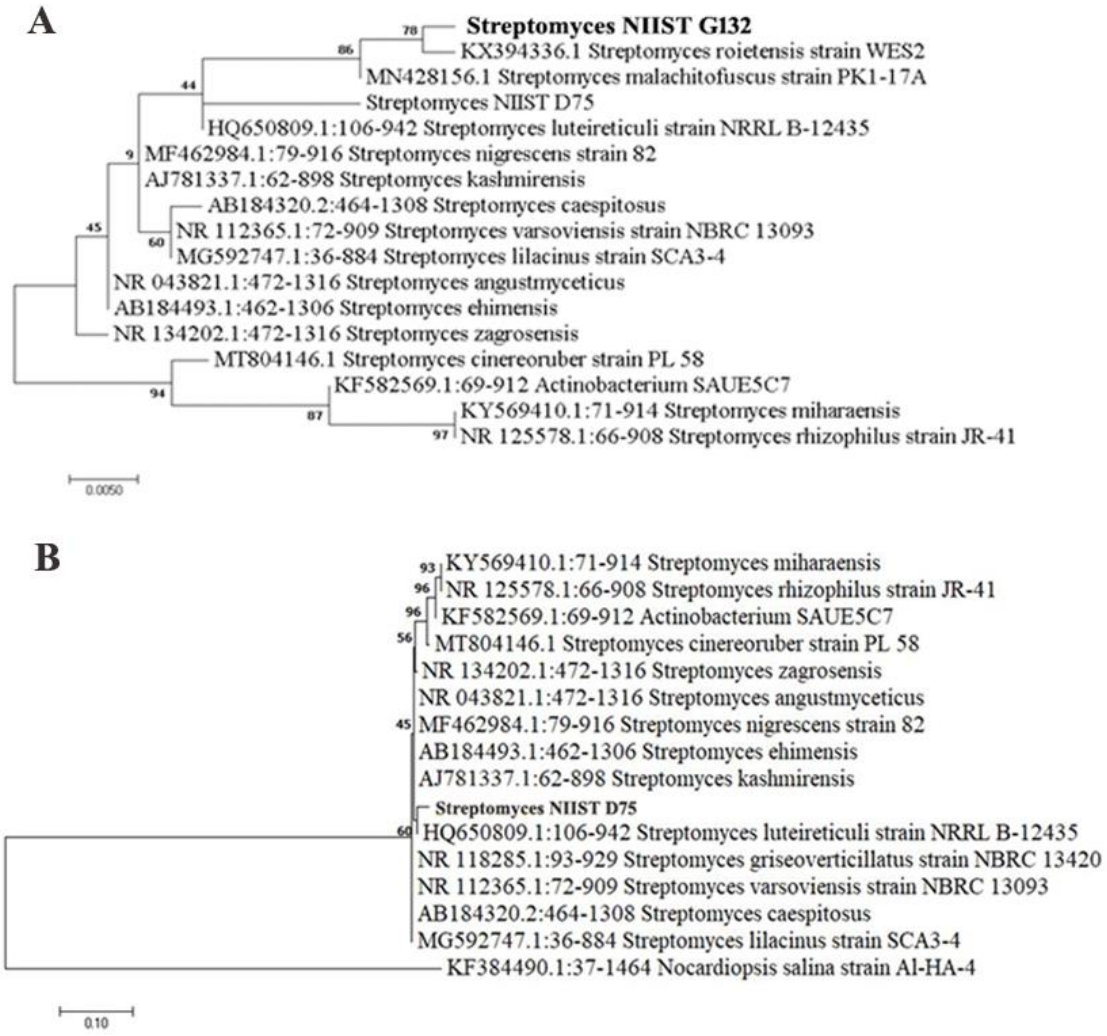


**Fig 2.4: Phylogenetic tree of (A) *Streptomyces* NIIST G132 (B) *Streptomyces* NIIST D75**

### 2.3.4. 16srRNA sequence of Streptomyces NIIST G132 and Streptomyces NIIST D75 submitted in GeneBank

>ON046673.1:1-942 Streptomyces sp. strain NIIST G132 16S ribosomal RNA gene, partial sequence

AGGGGGGAGGGGGGGGGAGGAGAGGAGGGAGTTTTTTTTGGTTTCTTTTTCAAAATTAAGAC-
CTCGGAGGGATATGGCGACGGGTGAGTACACGTGGGCAATCTGCCCTGCACTCTGGGACAAG
CCCTGGAAACGGGGTCTAATACCGGATACGTTCACCGGCATCGGTGATTCTGGAAA-
GCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAA
GGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACAC-
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAA-
GCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTT-
GTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTA

GAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG-
GAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGG
GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGGTGGGCACTAGGTGTGGGG-
CAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCCGCCCTGGGGGAGTACG
GCCGCAAGGCTAAAAACTCAAAAGGAATTGAACGGGGGCCCGCACAAAGCGGCGGAG-
CATGTTGGGCTTTAAATTTCCGAAACCGGCCCAA

>ON054043.1:1-888 Streptomyces sp. strain NIIST D75 16S ribosomal RNA gene, partial sequence
GAAGATACGCAGTTCAGGTGCTATTACATGCGAGTCGGGTGACTAACACGTGGG-
CAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACCGCAG
ACCGCATGGTTGGTGGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTT-
GTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA-
TATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTT
GTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAA-
GCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATT
ATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGATGTGAAA-
GCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATC
GGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCG-
GATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGTTGGGCACTAGGTGTGGGCGACATTCCAC-
GTCTTCCGTGCCGCAGCCTACGCATTTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTTA
AACCTCAAATGATATTGACTGGAGGCCCGCACCAGCCAAGCGAGAGCTATGTT-
GGCGTTACTTCCACCGC

### 2.3.5. Microscopic analysis of spore structure

Microscopic observations showed that NIIST G132 formed extensively branched, non-fragmented substrate and aerial mycelia. The spore chain morphology of the strain was straight and recti-flexible with a smooth surface and less than 50 spores in a chain. Microscopic observation of NIIST D75 also formed branched, non-fragmented mycelium and smooth round spore at the tip of the aerial mycelia were observed.

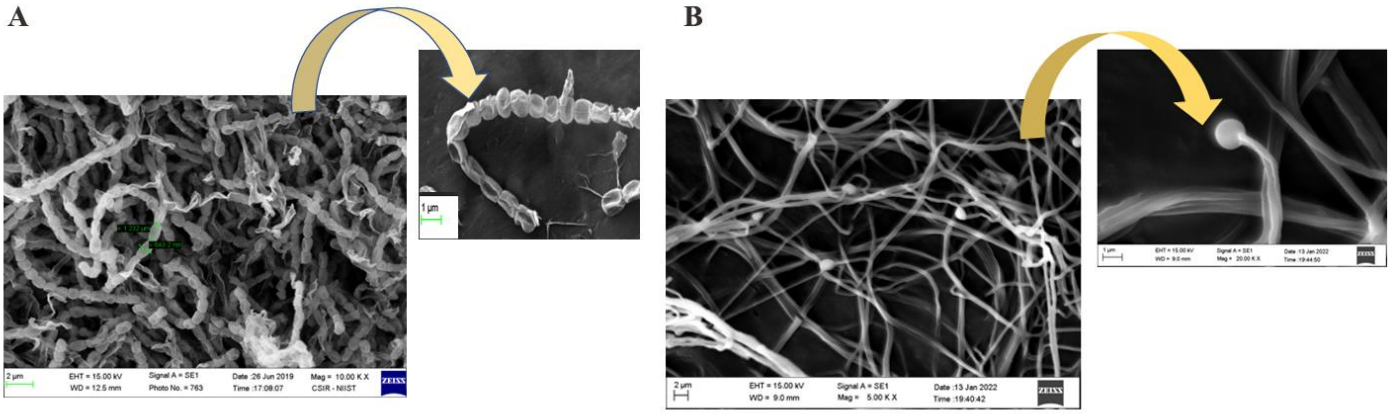


**Fig 2.5: SEM analysis of mycelia and spore structure of (A) *Streptomyces* NIIST G132 (B) *Streptomyces* NIIST D75**

## 2.4. DISCUSSION

Antibiotics underpin modern medicine through their multidisciplinary action. As the number of antimicrobial-resistant bacteria increases, new antibiotics are needed. In previous World Economic Forum Global Risks reports, antibiotic resistance has been identified as one of the most severe dangers to human health (Blair et al. 2015). Streptomyces are the active producers of biologically active compounds, including antibacterial compounds. In the present study, we selected two Streptomyces species from the culture collection in our lab. Morphological analysis of the spore was carried out by observing the streptomyces under a scanning electron microscope. Two Streptomyces are morphologically distinct; Streptomyces NIIST G132 has beads like aerial mycelium with recti flexible spores attached as a chain structure. Streptomyces NIIST D75 was observed as non-fragmented aerial mycelium with a smooth, round spore at the tip of the mycelium. Molecular identification was also carried out, and these 16S rRNA sequences were deposited in GenBank. The phylogenetic tree constructed with the 16S rRNA sequence and similar sequences retrieved from GeneBank shows similarity towards the most identical sequence. Streptomyces NIIST G132 showed 99.36% similarity

towards *Streptomyces roietensis* strain WES2, and Streptomyces NIIST D75 showed 95.64% similarity towards *Streptomyces luteireticuli* strain NRRL B-12435. The presence of antibacterial compounds in the crude organic extract of two selected Streptomyces was confirmed by disc diffusion assay, resulting in a broad - spectrum activity against seven selected bacterial pathogens. Thus, both Streptomyces ensued for further studies.

Streptomyces are well known for bioactive compound production; a diverse range of compounds spanning many different chemical classes have been identified, including cyclic and linear peptides, linear polyketides, terpenoids, polyaromatics, macrocycles, and furans were isolated from streptomyces (Lacey and Rutledge 2022). For the last few decades, the number of compounds isolated from streptomyces was minimal, possibly due to the repeated isolation of compounds from similar niches and the difficulty in isolating compounds. The rising public health burden associated with AMR in clinics and the public emphasizes the need to isolate novel active agents. The genes encoding secondary metabolites are clustered into biosynthetic gene clusters. Genome mining of these genes helped identify potentially active compound codes in silent genes. Most BGCs are non-ribosomal peptide synthetases, type 1 polyketide synthases, terpenes, and lantipeptides (Belknap et al. 2020). Thus, the discovery of numerous and diverse compounds demonstrates streptomyces continued potential as a source of new and exciting antibacterial compounds

## 2.5. CONCLUSION

In this chapter, two morphologically distinct streptomyces were selected from the culture collection of our lab. 16S rRNA sequencing of two streptomyces was carried out, confirming that the chosen streptomyces belonged to two species. *Streptomyces* NIIST G132 showed a similarity to *Streptomyces roietensis* WES2, and *Streptomyces* NIIST D75 was similarity to *Streptomyces luteireticuli* strain NRRL B-12435. The 16S rRNA sequences of individual species were deposited in GeneBank (NIIST G132 with accession number ON046673 and NIIST D75

with accession number ON054043). The antibacterial activity of the crude organic extract was tested to see the extent of antibiosis against seven bacterial pathogens. Both streptomyces showed a broad-spectrum activity and confirmed the presence of antibacterial compounds in the crude organic extract.

## 2.6. REFERENCE


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# CHAPTER 3

# Enhanced production of the metabolites from *Streptomyces* NIIST G132 through response surface methodology (RSM) employing Box-Behnken design

## 3.1. INTRODUCTION

A well-optimized culture medium is a preliminary requirement for a microbe to grow and produce secondary metabolites. Media components and cultural conditions play an important role in secondary metabolite production. Minor changes in these significantly affect the production and quality of metabolites (Yan et al. 2018). Hence, optimizing the culture conditions and media components to enhance metabolite production and improve antibacterial activity is essential. Therefore, properly designing media optimization is needed, which is the most challenging phase of fermentation studies. Before the 1970s, media optimization was carried out using classical methods, which were more time-consuming, expensive, and required many experiments that produced less accuracy. The advent of modern statistical tools and their application in the optimization of media components and standardization became easier with high precision and efficiency (Singh et al. 2017). The selection of variables from a vast number is a challenging process. The choice of critical factors from many process variables is difficult. These modern statistical experimental designs are thus quite useful in preliminary studies where the main goal is to select variables that can be fixed or eliminated in subsequent optimization processes (Patel et al. 2020).

Apart from culture media, other factors like pH, temperature, and incubation period must also be optimized. These factors were optimized through the OFAT (One Factor At a Time) method. The technique can be helpful where the process is influenced by a smaller number of variables (Rajeswari et al. 2014). However, it never depicts the net effect of interactions between the different variables in the process (Ju et al. 2018). For better results, experimental design should be done to precisely evaluate variables, their interactions, and their effects, which will satisfy the objectives. In this regard, media optimization can be carried out using response surface methodology (RSM), which allows quick screening of an extensive experimental domain (Ju et al. 2018), demonstrates the role of each component, give insight solely into the interactions

between the process variables and confer multiple responses at the same time with a significant reduction in a total number of experiments (Latha, Sivaranjani, and Dhanasekaran 2017).

Response surface methodology was developed by Box and collaborators in 1951, and the term was derived after the introduction of the fitness of the mathematical model (Bezerra et al. 2008). RSM is a group of techniques that are used to study the relations between one or more measured dependent factors (responses) and several input (independent) factors (Patel et al. 2020). For RSM, linear or square polynomial functions are employed to describe the system studied and, consequently, to explore (modelling and displacing) experimental conditions until its optimization (Bezerra et al. 2008). RSM can be used to assess the relative significance of numerous independent variables and their interactions with the parameters. It can be done mainly based on using the Plackett-Burman design to identify factors that significantly impact the fermentation process, determining the path of steepest ascent to ascertain the approximate range of the best fermentation conditions using the key elements, and also performing the Box-Behnken test design to establish the fermentation model and determine the optimal fermentation conditions (Yan et al. 2018). The application of RSM has successfully worked in various aspects of increased metabolite production, increased antibacterial activity, and novel metabolite isolation. Maximum metabolite production with increased antifungal activity was optimized in *Streptomyces sparsus* VSM-30 employing the Central Composite Design (CCD) of RSM (Managamuri et al. 2017). Optimization of media for increased production of tacrolimus, a 23 - membered polyketide secondary metabolite produced by various species of *Streptomyces* used widely as medicine in graft rejection, was carried out by employing CCD of RSM using *Streptomyces tsukubaen*sis NBRC 108819 (Patel et al. 2020). Optimized culture media for enhanced antibacterial compound production by *Streptomyces sp*. JAJ13 is carried out with statistical methodology based on the Box-Behnken design (BBD) of RSM (Rajeswari et al. 2014). A 12.33% increase in antibacterial activity was observed for the metabolites

from *Streptomyces* sp. 1–14 optimized through RSM employing Plackett-Burman and Box-Behnken designs (Yan et al. 2018). Standardization of fermentation conditions and media components was carried out using the Plackett-Burman design and Box-Behnken design of RSM in *Streptomyces* A30 and A32, resulting in increased metabolite production with increased antibacterial activity (Jacob et al. 2017).

In the present study, the optimum condition and media components of *Streptomyces* NIIST G132 were optimized by selecting five different media and physiological conditions, such as pH, incubation period, and temperature. RSM was employed to maximize the media components in the media chosen for enhanced metabolite production in Streptomyces NIIST G132. The interactive effect of different media components, such as yeast extract, malt extract, peptone, and dextrose, was investigated using the Box-Behnken design.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Test bacterial pathogens

Gram-positive bacterial strain *Staphylococcus aureus* MTCC 902 and Gram-negative *Escherichia coli* MTCC 2622 were used here. These two test pathogens were procured from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India.

### 3.2.2. Standardization of fermentation medium

NIIST G132 was grown in five different mediums, including Tryptone Yeast Extract Broth and Yeast Malt Broth. Potato Dextrose Broth, Starch Caesin Broth, and Sabouraud Dextrose Broth. To this fermentation medium, 3-day old homogenized culture of NIIST G132 was inoculated and incubated for 7 days at 28 ± 2 ºC. After the incubation period, the culture was centrifuged, and the crude extract was collected by solvent extraction, and antibacterial activity of the individual fermentation medium was screened against seven bacterial pathogens, and results were

recorded as ZOI in mm, and the best fermentation medium was then selected for further optimization studies.

### 3.2.3. Optimization of pH, temperature, and incubation time

ISP2 media was selected for enhanced metabolite production, and optimum temperature, pH, and incubation time were determined. Different temperatures (25, 30, 35, 40, and 45 ºC) at pH 7.0 were selected to optimize temperature for maximum metabolite production. After 7 days of incubation, the antibacterial activity was checked by disc diffusion assay. Optimization of pH was determined by selecting a range of pH (5, 5.5, 6, 6.5, 7, and 7.5) was established and studied for 7 days at 30 ºC. Optimization of the incubation period was also carried out for 3 to 10 days at 30 ºC for 7 days.

### 3.2.4. Optimization of medium components and maximum antibacterial compound production in ISP2 medium by response surface methodology using Box-Behnken design (BBD)

The optimum level of four media components was determined by BBD of response surface methodology using the statistical software MINITAB. The variables were studied at three different levels: low (-1), medium (0), and high (+1), respectively, in a total of 27 runs. Multiple regression analysis was used to analyze the data using a second-degree polynomial equation. $Yi = \beta o + \Sigma \beta iXi + \Sigma \beta iiXi\ 2 + \Sigma \beta ij XiXj$ where *Yi* is the predicted response, *xixj* are input variables that influence the response variable *Y*; βo is the offset term; βi is the *i*th linear coefficient, β*ii* the *i*th quadratic coefficient, and β*ij* is the *ij*th interaction coefficient. A statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the Fisher's *F*-test (overall model significance), its associated probability *p(F), the* correlation coefficient *R,* and the determination coefficient *R*2, which measures the goodness of fit of the regression model. The quadratic models were represented as contour plots (3D) for each variable, and the software generated response surface curves.

### 3.2.5. Validation of the model

The correlation between the experimental and predicted values was determined by doing validation experiments for these four experiments and comparing the predicted and observed values. After incubation time, the crude metabolite from four experiments was assayed for antibacterial activity in the zone of inhibition was recorded.

## 3.3. RESULTS

### 3.3.1. Standardization of fermentation medium

Among the five-fermentation media selected for better metabolite production with increased antibacterial activity, *Streptomyces* NIIST G132 exhibited maximum metabolite production (120 mg/l) in Yeast Malt Broth. Crude organic extract was isolated using ethyl acetate: methanol solvent system in a ratio of 95:5 and the antibacterial activity was tested against *E. coli* and *S. aureus.* Here *E. coli* (12.3 ± 0.57 mm) was inhibited more than *S. aureus* (10.3 ± 0.57 mm) (Table 3.1.).

**Table 3.1. Antibacterial activity of crude organic extract isolated from *Streptomyces* NIIST G132 grown in different fermentation media**

| Test Bacteria | Zone of Inhibition in mm | | | | |
|---|---|---|---|---|---|
| | TYB | YMB | PDB | SCB | SDB |
| *E. coli* | 11.3 ± 0.28 | 12.3 ± 0.57 | 9.3 ± 0.57 | 6.8 ± 0.28 | 6.8 ± 0.57 |
| *S. aureus* | 8.0 ± 0.00 | 10.3 ± 0.57 | 10.0 ± 1.00 | 9.0 ± 0.00 | 9.3 ± 0.57 |

Values are average of triplicate and represented in mean ± SD

### 3.3.2. Optimization of pH, temperature and incubation time

Optimization of parameters was determined in YMB medium selected as the better fermentation medium for further studies by observing the response, antibacterial activity through disc diffusion assay against *S. aureus* (Fig.1.1). Maximum antibacterial activity observed against *S. aureus* in pH 6 at 30 ºC for 7 days and thus parameters are optimized.

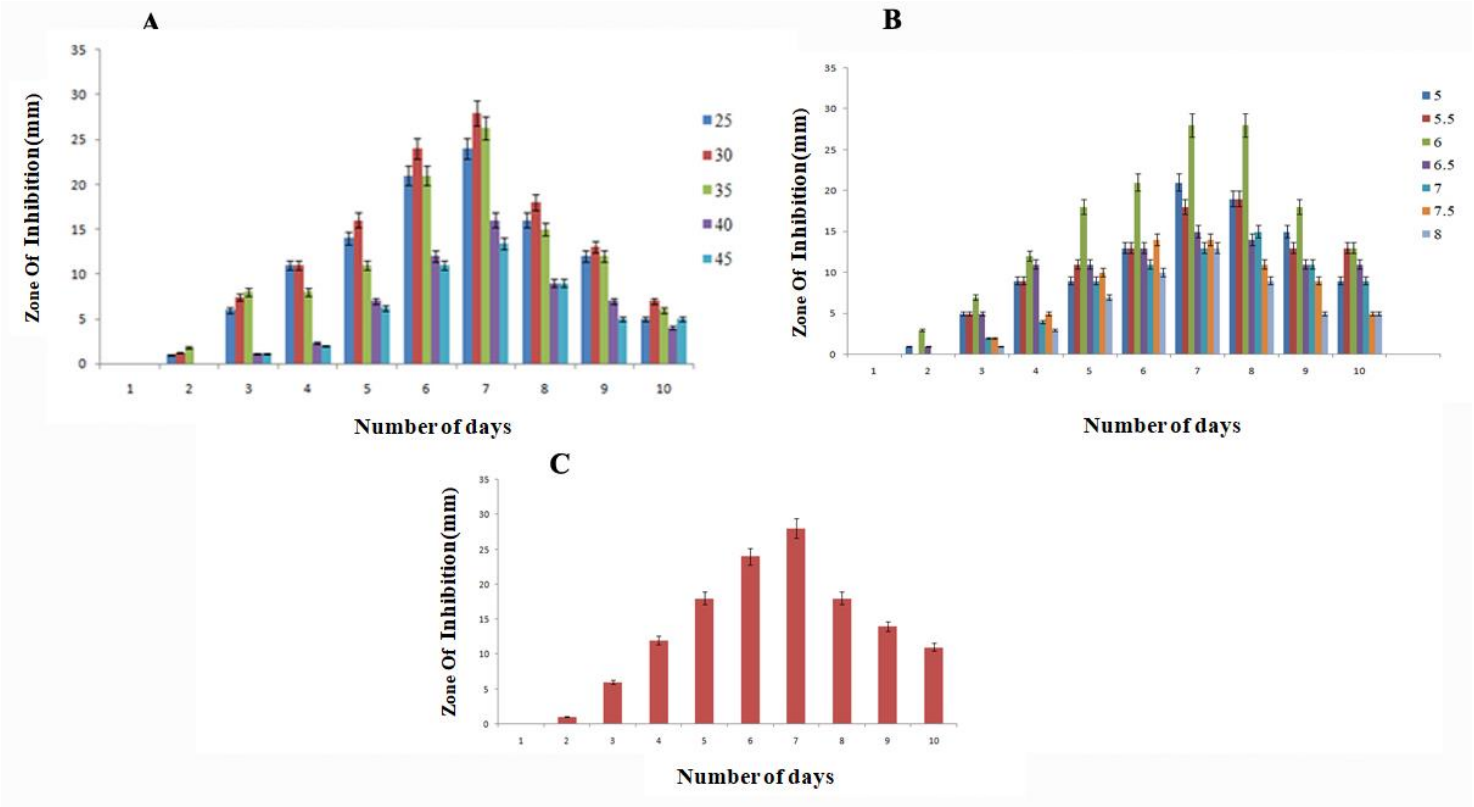



**Fig. 3.1. Optimization of parameters for RSM (A) Temperature, (B) pH and (C) Incubation time**

### 3.3.3. Optimization of media components using Box-Behnken Design

The model F value of 60.15 implies the model is significant and shows there is negligible or no chance that the model F value could occur due to noise. The R2 value (multiple correlation coefficient) closer to 1 denotes a better correlation between the observed and predicted values. The P values indicate the significance of the coefficients and are also crucial in understanding the pattern of the mutual interactions between the variables. Multiple regression analysis was

used to analyze the antibacterial activity, and thus, a polynomial equation was derived from regression analysis as follows.

Y = -4.58 + 0.931 X1 + 0.473 X2 + 0.535 X3 + 0.308 X4 - 0.06250 X12 - 0.02188 X22 - 0.03750 X32 - 0.00937 X42 - 0.0250 X1X2 - 0.0063 X1X3+ 0.0125 X1X4 - 0.0250 X2X3- 0.0125 X2X4 - 0.0125 X3X4

Here Y denotes antibacterial activity in the zone of inhibition in mm against *S. aureus*, and the values suggest that among the three variables studied, X1 (Peptone), X3 (malt extract), and X4 (dextrose) showed maximum interaction. The predicted R2 of 98.6% is in reasonable agreement with the adjusted R2 value of 96.96%.

**Table.3.2. Range of variables used for Box-Behnken Design**

| Media components | -1 (g/l) | 0 (g/l) | +1 (g/l) |
|---|---|---|---|
| PEPTONE | 3 | 5 | 7 |
| YEAST EXTRACT | 1 | 3 | 5 |
| MALT EXTRACT | 1 | 3 | 5 |
| DEXTROSE | 8 | 10 | 12 |

**Table.3.3. Results of Box-Behnken Design**

| Runs | Peptone (g/l) | Yeast extract (g/l) | Malt extract (g/l) | Dextrose (g/l) | Antibacterial activity (mm) |
|---|---|---|---|---|---|
| 1 | 5 | 3 | 5 | 8 | 22.33 ± 0.57 |
| 2 | 5 | 1 | 3 | 12 | 25.00 ± 1.00 |
| 3 | 5 | 3 | 3 | 10 | 23.66 ± 0.57 |
| 4 | 5 | 1 | 5 | 10 | 24.00 ± 1.00 |
| 5 | 5 | 3 | 1 | 12 | 21.56 ± 0.51 |
| 6 | 3 | 3 | 5 | 10 | 13.00 ± 0.20 |
| 7 | 7 | 1 | 3 | 10 | 26.93 ± 0.30 |
| 8 | 3 | 3 | 1 | 10 | 10.66 ± 0.57 |
| 9 | 3 | 3 | 1 | 10 | 10.33 ± 0.57 |
| 10 | 7 | 3 | 5 | 10 | 11.66 ± 0.57 |
| 11 | 3 | 3 | 3 | 8 | 12.26 ± 0.30 |
| 12 | 5 | 5 | 3 | 8 | 21.13 ± 0.81 |

| | | | | | |
|---|---|---|---|---|---|
| 13 | 7 | 3 | 3 | 8 | 25.56 ± 0.56 |
| 14 | 5 | 1 | 3 | 8 | 18.63 ± 0.60 |
| 15 | 5 | 3 | 5 | 12 | 24.26 ± 0.30 |
| 16 | 5 | 1 | 1 | 10 | 18.43 ± 0.25 |
| 17 | 3 | 1 | 3 | 10 | 12.33 ± 0.20 |
| 18 | 5 | 3 | 1 | 8 | 18.10 ± 0.45 |
| 19 | 7 | 3 | 1 | 10 | 25.70 ± 0.30 |
| 20 | 7 | 3 | 3 | 12 | 31.00 ± 0.91 |
| 21 | 7 | 5 | 3 | 10 | 25.33 ± 0.41 |
| 22 | 5 | 5 | 5 | 10 | 21.63 ± 0.51 |
| 23 | 3 | 3 | 3 | 12 | 15.40 ± 0.52 |
| 24 | 5 | 3 | 3 | 10 | 22.00 ± 1.00 |
| 25 | 5 | 5 | 1 | 10 | 20.50 ± 0.50 |
| 26 | 5 | 3 | 3 | 10 | 23.83 ± 0.28 |
| 27 | 5 | 5 | 3 | 12 | 25.10 ± 0.10 |

### 3.3.4. Response surface and contour plots

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The response surface curves are represented in (Fig.3.2); the interaction between yeast extract and malt extract shows that low levels of both these media components did not result in a higher antibacterial effect. The shape of the response surface curves showed an interaction between these tested variables, and a maximum antibacterial effect was found between yeast extract concentrations of 2–4 g/l and malt extract concentrations of 4-5 g/l. The yeast extract and dextrose interaction indicate a moderate interaction between the factors. The maximum antibacterial effect was recorded at the middle levels of yeast extract and higher levels of dextrose. Between maltose and dextrose, the best antibacterial effect was recorded at malt extract concentrations of 3-5 g/l and higher levels of dextrose concentration.

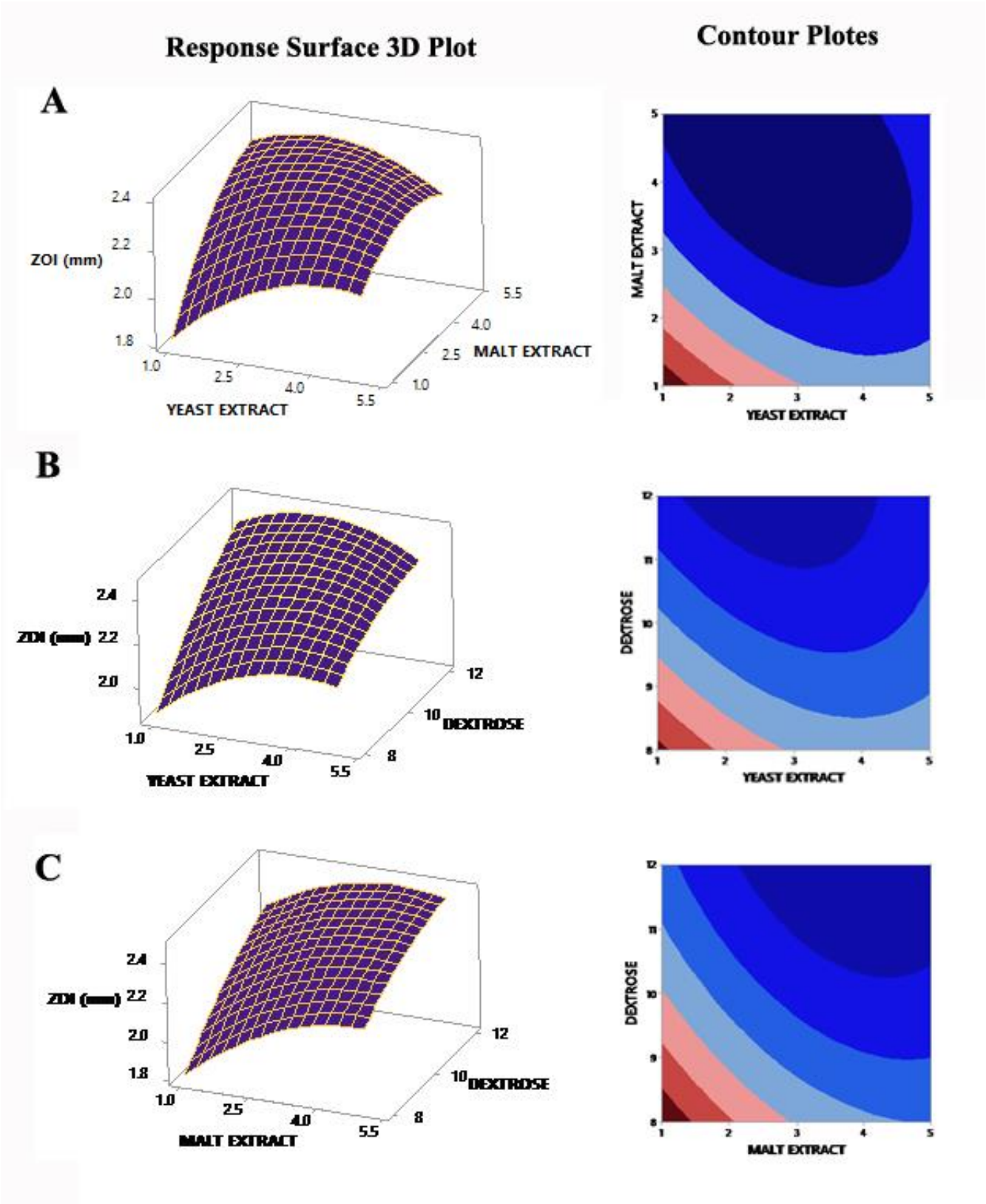


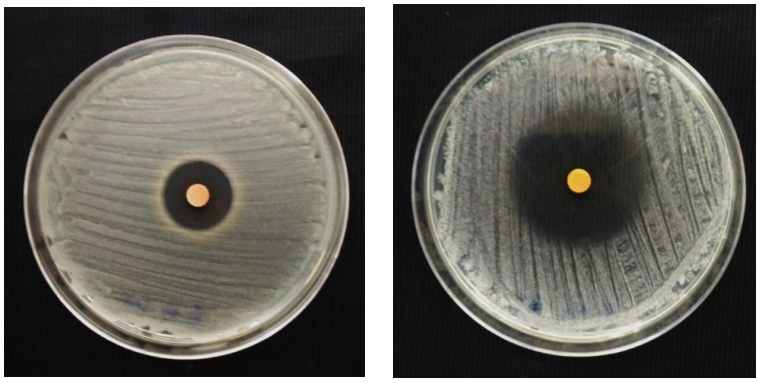

**Fig.3.2. Response surface 3D plot and contour plots showing independent and interactive effect of variables on antibacterial activity of NIIST G132 (A) showing interaction between yeast extract and malt extract (B) Effect of yeast extract and dextrose (C) Interaction between malt extract and dextrose**

### 3.3.5. Validation of model

Validation was carried out under conditions predicted by the model. The conditions are represented in (Table 3.4.) along with the responses obtained. A close correlation of 0.92 was observed between the experimental and predicted values which indicate significance on the model. The study revealed that the optimal levels of the significant process variables for maximum antibacterial effect were (i) peptone (7 g/l), (ii) yeast extract (1 g/l), (iii) malt extract (5 g/l) and (iv) dextrose (12 g/l).

**Table 3.4. Antibacterial activity obtained from validation experiment**

| RUNS | PEPTONE(g/l) | YEAST EXTRACT (g/l) | MALT EXTRACT (g/l) | DEXTROSE (g/l) | PREDICTED VALUE (mm) | OBSERVED VALUE (mm) |
|---|---|---|---|---|---|---|
| 1 | 7 | 1 | 5 | 9.5 | 27 | 25 |
| 2 | 4.7 | 3 | 3 | 12 | 24 | 25 |
| 3 | 7 | 4 | 3 | 12 | 28 | 28 |
| 4 | 5 | 3 | 5 | 8 | 21 | 20 |



Unoptimized media Optimized media

**Fig. 3.3. Antibacterial activity of unoptimized and optimized media**

### 3.4. DISCUSSION

Statistical optimization of media components for enhanced production of metabolites with increased antibacterial activity was carried out through RSM employing BBD. Fermentation parameters are the other essential factors that need to be optimized. In our study, optimum pH, temperature, and incubation days were optimized for the proper production of active antibacterial metabolites in NIIST G132. Statistical optimization through RSM is a strenuous process; many trials and errors are needed, and we often need help optimizing the fermentation conditions. The optimization of antibacterial production was done according to RSM; the significant variables were optimized by employing Box Behnken Design; four selected variables, peptone, yeast extract, malt extract, and dextrose, were analyzed at three levels low, medium, and high as -1, 0, +1 in a total of 27 runs. The maximum antibacterial production was achieved in run number 20, and the minimum output was observed in runs 8 and 9, respectively. The statistical significance of the model was determined by the coefficient of determination, R2. In this study, R2 was found to be 0.986 and close to 1.0, showing a solid response. Optimization validation was done, the correlation coefficient was found to be 0.92, and the model is considered valid. The study revealed that the optimal levels of the significant process variables for maximum antibacterial effect were (i) peptone (7 g/l), (ii) yeast extract (1 g/l), (iii) malt extract (5 g/l), and (iv) dextrose (12 g/l).

After optimization through RSM, the antibacterial activity has notably increased compared to the unoptimized medium, and antibacterial activity against *S. aureus* in terms of zone of inhibition increased from 20.33 ± 0.05 to 33.66 ± 0.05 mm. Hence, the current statistical *e*xperimental design accurately optimizes the significant media components. From the previous studies carried out by (jubi et al. 2017) it is evident that modification of the existing fermentation medium can change the production of metabolites. The nutritional requirement for better antibacterial activity was optimized in *Streptomyces rimosus* AG-P1441 using RSM (Ju et al.

2018). Optimization of actinomycin D present in a novel strain of *Streptomyces* GSBNT10 by RSM employing Plackett-Burman design (PBD). Compared to unoptimized media, a 58.56 % increase in metabolite production was observed in optimized media (Djinni et al. 2019). Similarly, a 1.23-fold increased production of actinomycin was achieved by optimizing the media composition of *Streptomyces parvulus* K-R1 through RSM using central composite design (CCD) (Chandrakar and Gupta 2019). A 25 % increased production of Kalafungin, a β-lactamase inhibitor isolated from marine streptomyces species, was optimized by RSM utilizing PBD and BBD (Mary et al. 2021).

### 3.5. CONCLUSION

In this chapter, the design and standardization of fermentation media for Streptomyces NIIST G132 were carried out. Standardization of fermentation conditions like temperature, incubation time, and pH was carried out, and optimum conditions were systematized. Here, optimum conditions for proper growth and secondary metabolite production with more antibacterial activity were a temperature of 30 ºC and a pH of 6 for an incubation period of 7 days. Five different media were selected, and growth and secondary metabolite production was estimated by observing the antibacterial activity against one Gram-positive (*S. aureus*) and one Gram-negative (*E. coli*) pathogen. Better antibiosis was shown against bacterial pathogens with the crude organic metabolite collected from yeast malt broth; thus, the media was selected as the proper media with optimum physiological conditions for the better growth of Streptomyces NIIST G132. The media component standardization was carried out by RSM employing a Box Behnken design of 27 runs. The media optimization for NIIST G132 by statistical approaches increased antibacterial activity from $20.33 \pm 0.57$ to $33.66 \pm 0.57$ mm compared to that obtained in the unoptimized media. Thus, the statistical trial using RSM for optimizing medium compounds by Streptomyces NIIST G132 was validated and considered a potent and valuable tool.

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# CHAPTER 4

## Production, extraction, and characterization of the metabolites produced by the two *Streptomyces* species through various analytical methods

## 4.1. INTRODUCTION

Microbial natural products, incredibly isolated from streptomyces, play an essential role in developing new therapeutic strategies in the medicinal field. Streptomyces can produce many active compounds, including antibiotics, antifungal, anticancer, antiviral, antimalarial, anti-termite, various enzymes, immunosuppressants, and other industrially essential compounds (Mobolaji Felicia Adegboye 2012). Streptomyces is the major contributor of secondary metabolites; nearly two-thirds of the antibiotics are discovered from the source (Salwan and Sharma 2020). The discovery of antibiotics from streptomyces began with isolating streptomycin from a culture of *Streptomyces griseus*. Streptomycin was the first aminoglycoside isolated in 1943 and the first antibiotic remedy for tuberculosis. This antibiotic is a protein inhibitor and a bactericidal antibiotic. It is active against other pathogenic gram-negative bacteria, including *Brucella* spp, *Klebsiella granulomatis*, *Klebsiella pneumonia*, *E. coli*, *Proteus* spp., *Aerobacter aerogenes*, *Francisella tularensis*, *Haemophilus ducreyi*, *Haemophilus influenza*, and *Yersinia pestis*, as well as Gram-positive bacteria such as *Streptococcus viridian's* and *Enterococcus faecalis* (Zaffiri, Gardner, and Toledo-pereyra 2012). Aminoglycoside antibiotics like kanamycin from *Streptomyces kanamyceticus*, neomycin from *Streptomyces fradia*, and Hygromycin B from *Streptomyces hygroscopicus* were also isolated (Emerson et al. 2012). A group headed by Benjamin Minge Duggar started digging for new isolates of streptomyces; on their way, they got one active strain, and William Albrecht named it *Streptomyces aureofaciens*, the crude from the same, which showed good activity against rickettsia and typhus. They labelled it a broad-spectrum antibiotic called aureomycin (Rusu and Buta 2021). Thus, after 1950, the enthusiasm of world researchers burst and sprouted for the discovery of new antibiotics from so-called ultra-molds.

Consecutively, *Streptomyces rimosus* was identified, and a compound very similar to aureomycin was isolated, named terramycin. Succeeding scientists look forward to the chemical

structure of both compounds. As we know, the limitation of the instruments for identifying the structure was their main problem. However, the fabled Haward University scientist Robert Woodward came forward. He led a team for the discovery. They succeeded and discovered the first semisynthetic drug tetracycline, a subclass of polyketides with an octa hydro tetracene-2-carboxamide skeleton, derivatives of polycyclic naphthacene carboxamide (Rusu and Buta 2021). Tigecycline is a glycylcycline antibiotic marketed by Wyeth under the brand name TYGACIL®. It was given a U.S. Food and Drug Administration (FDA) fast-track approval on June 17, 2005. It is the first clinically available drug in a new class of antibiotics called glycylcycline (Greer 2006). Chloramphenicol is a ribosome binding agent active against Gram-negative bacteria isolated from *Streptomyces venezuelae* in 1947 but is no longer used clinically due to rare side effects (Katz and Baltz 2016). Carbomycin is a macrolide antibiotic isolated from *Streptomyces halstedii*, which inhibits gram-positive bacteria and some mycoplasma (Zhong et al. 2017). Daptomycin, a lipopeptide antibiotic isolated from *Streptomyces roseosporus*, was approved in 2003 by the FDA to treat skin infections caused by Gram-positive pathogens (Baltz et al. 2006). After the golden era of the discovery of antibiotics, a notable success was the introduction of Cubicin (daptomycin) to the market by Cuba's pharma company (Hutchings, Truman, and Wilkinson 2019). Even today, digging for novel compounds from streptomyces continues one side, and on the other side, microbial-derived compound scaffolds are used for synthesizing new antibiotics. Recently, many synthetic analogs of antibiotics isolated from streptomyces have been approved by the FDA in the clinical field, like daptomycin, biapenem, and ertapenem (Kemung et al. 2018). A reduction in the interest of pharma companies in microbial natural product isolation deserted the field. So, rejuvenation of the field is required by focused and sound research to awaken microbial natural product research and novel antibiotic inventions.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Culture conditions and isolation of crude organic extract of Streptomyces NIIST G132 and NIIST D75

Streptomyces NIIST G132 (10 ml) was inoculated in 100 ml of YMB media and incubated for 7 days at 160 rpm and 30 °C in an Incubator Shaker (Lab Companion IS97IR, Jeio Tech, Korea). After fermentation, the cell-free extract was collected after centrifugation at 10,000 g for 10 min in a centrifuge (Kubota 7780, Kubota Corporation, Tokyo) and extracted thrice with ethyl acetate: methanol (95:5, 100 ml). The organic layer was concentrated in a Büchi Rotavapor®, and the crude organic extract was obtained. Fermentation was carried out repeatedly for 3 different batches 8 l of the first batch afforded 2.2 g, 8 l of the second batch afforded 2.3 g and 15 l of the third batch afforded 5.2 g of crude organic extract.

Homogenized inoculum of NIIST-D75 (5 ml), grown in Yeast Malt Broth (YMB) medium for 6 days, was inoculated into a 500-ml flask containing 150 ml of YMB medium and incubated for 12 days at 160 rpm and 30 °C in an incubator shaker. Cell-free culture filtrate was collected after centrifugation at 10,000 g for 10 min in a centrifuge and extracted three times with ethyl acetate: methanol (95:5, 150 ml). The organic layer was concentrated, and repetition of culture conditions up to 7 l of fermentation broth afforded 1.39 g of crude organic extract as a first batch; similarly, a second batch yielded 2.2 g.

### 4.2.2. Purification and structural characterization of crude organic extracts from Streptomyces NIIST G132 and NIIST D75

The crude organic extract from the first batch was subjected to silica column chromatography (100–200 mesh) using chloroform: methanol acetate (v/v) gradient solutions of 100:0, 95:5, 90:10, 85:15, 80:20, and 70:30. Later, C18 column purification, preparative HPLC and preparative TLC were performed for isolating pure compounds.

The crude organic extract from the first batch was subjected to preparative TLC (TLC Silica gel 60 F254), developed using ethyl acetate: hexane (7:3), to obtain a major band visualized by short wavelength UV. Repetition of preparative TLC afforded a total of 500 mg of this major band, which was subjected to purification by silica gel column chromatography (100–200 mesh) using hexane: ethyl acetate (v/v) gradient elution of 100:0, 95:5, 90:10, 85:15 and 80:20, which afforded four major compounds designated as compound 1, compound 2, compound 3, and compound 4. However, for the second batch, silica gel column chromatography was adopted for obtaining the compounds. The isolated compounds were dissolved in CDCl3, and recorded 1 H and 13C NMR 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, dd for double doublet, etc. The HR-ESI-MS data were obtained from a Thermo Scientific Exactive mass spectrometer with Orbitrap analyzer, and the ions are given in m/z.

### 4.2.3. GC-MS analysis of crude organic extract of Streptomyces NIIST G132

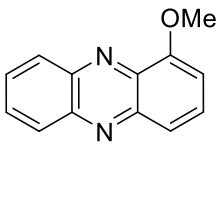
Gas Chromatograph- spectrometry analyzed the crude extract dissolved in methanol to identify the possible compounds in the crude organic extract. The crude organic extract was analyzed by Gas chromatography-mass spectrometry (SHIMADZU QP2020). The Rxi-5Sil MS fused silica column (Restek, USA) with 60 m length, 0.25 mm internal diameter, and 0.25 µm thickness was used for GC-MS analysis. The analysis started at 70 °C and was increased to 140 °C at a rate of 10 °C per minute and to 240 °C at a rate of 3 °C/min. After reaching 240 °C the temperature was held stable for 15 min before the analysis was terminated. The analysis was performed using the NIST 17 mass spectral library, and the individual fatty acid was identified based on mass spectrum and retention time.

## 4.3. RESULTS

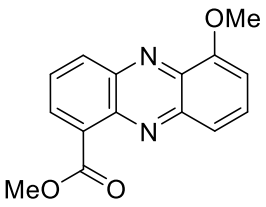
### 4.3.1. Purification and characterization of compounds from Streptomyces NIIST G132 and NIIST D75

Various purification methods were tried to isolate pure compounds from Streptomyces NIIST G132, including silica gel column chromatography, C18 column purification, preparative HPLC, and TLC. No pure compounds were isolated after all these purification methods. So, we tried the purification and characterization of the second organism selected.

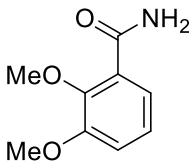
Purification of the major band (500 mg), from the crude organic extract of the first batch, by silica gel column chromatography afforded 2.0 mg of compound 1, 1.3 mg of compound 2, 6.0 mg of compound 3, and 3.0 mg of compound 4 (Fig.4.1.). From the second batch, 8.0 mg of compound **1** (Fig.4.2.), 3.2 mg of compound **2** (Fig.4.3.), 5.0 mg of compound **3** (Fig.4.4.) and compound **4** (Fig.4.5) were obtained. 1 H and 13C NMR analysis suggested compound 1 as 1-methoxy phenazine, compound 2 as methyl 6-methoxyphenazine-1-carboxylate, compound 3 as 2,3-dimethoxy benzamide, and compound 4 as 1,6- dimethoxy phenazine, which was further confirmed by HR-ESI-MS analysis.



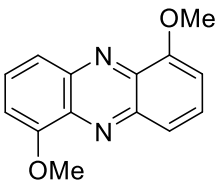

**1-Methoxyphenazine (1)**




**Methyl 6-methoxy-phenazine-1-carboxylate (2)**




**2,3-Dimethoxy benzamide (3)**




**1,6-Dimethoxyphenazine (4)**

**Fig.4.1. Chemical structures of the four compounds isolated from NIIST-D75**

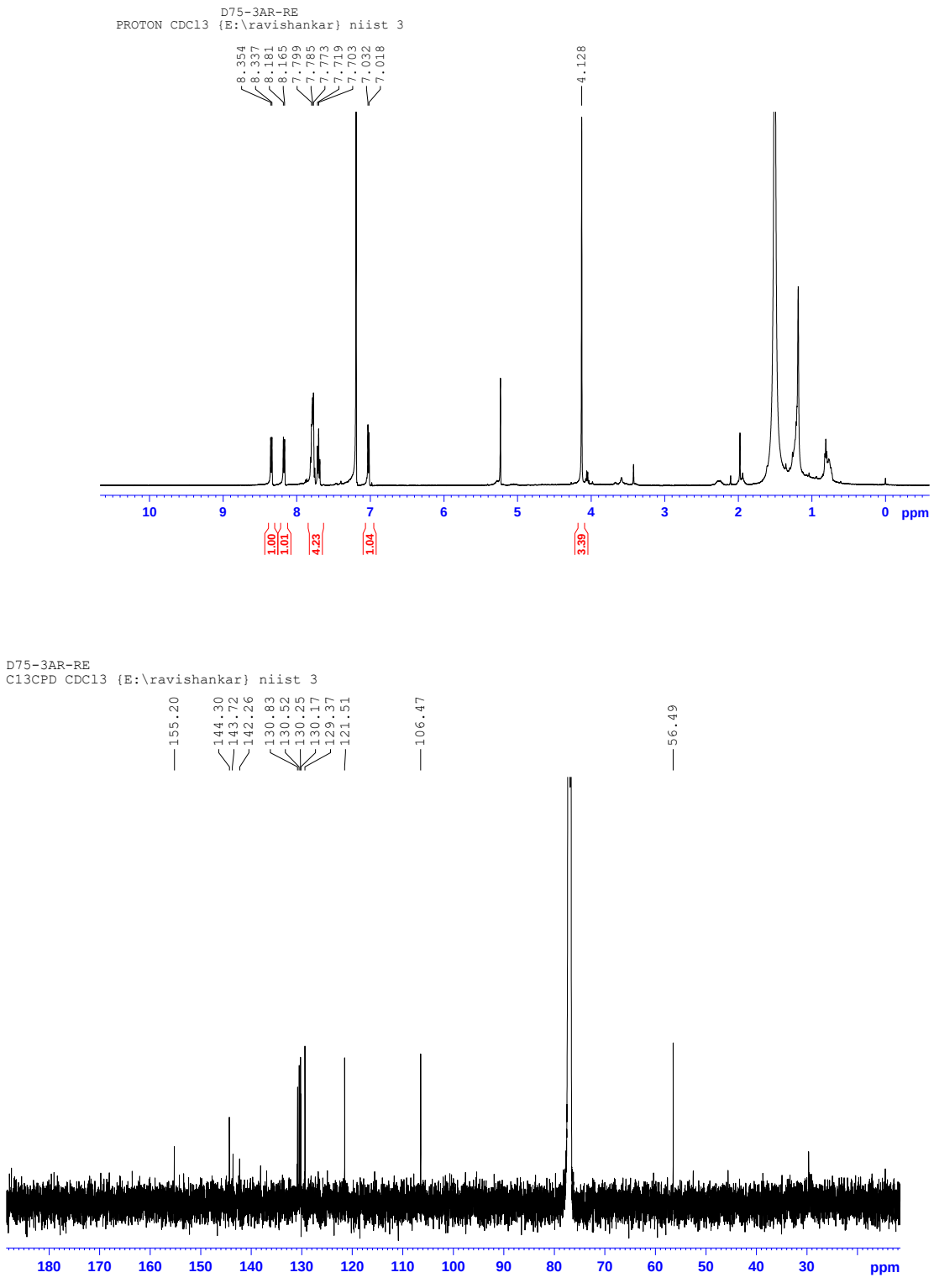



**Fig.4.2. $^{1}H$ and $^{13}C$ NMR of Compound 1**

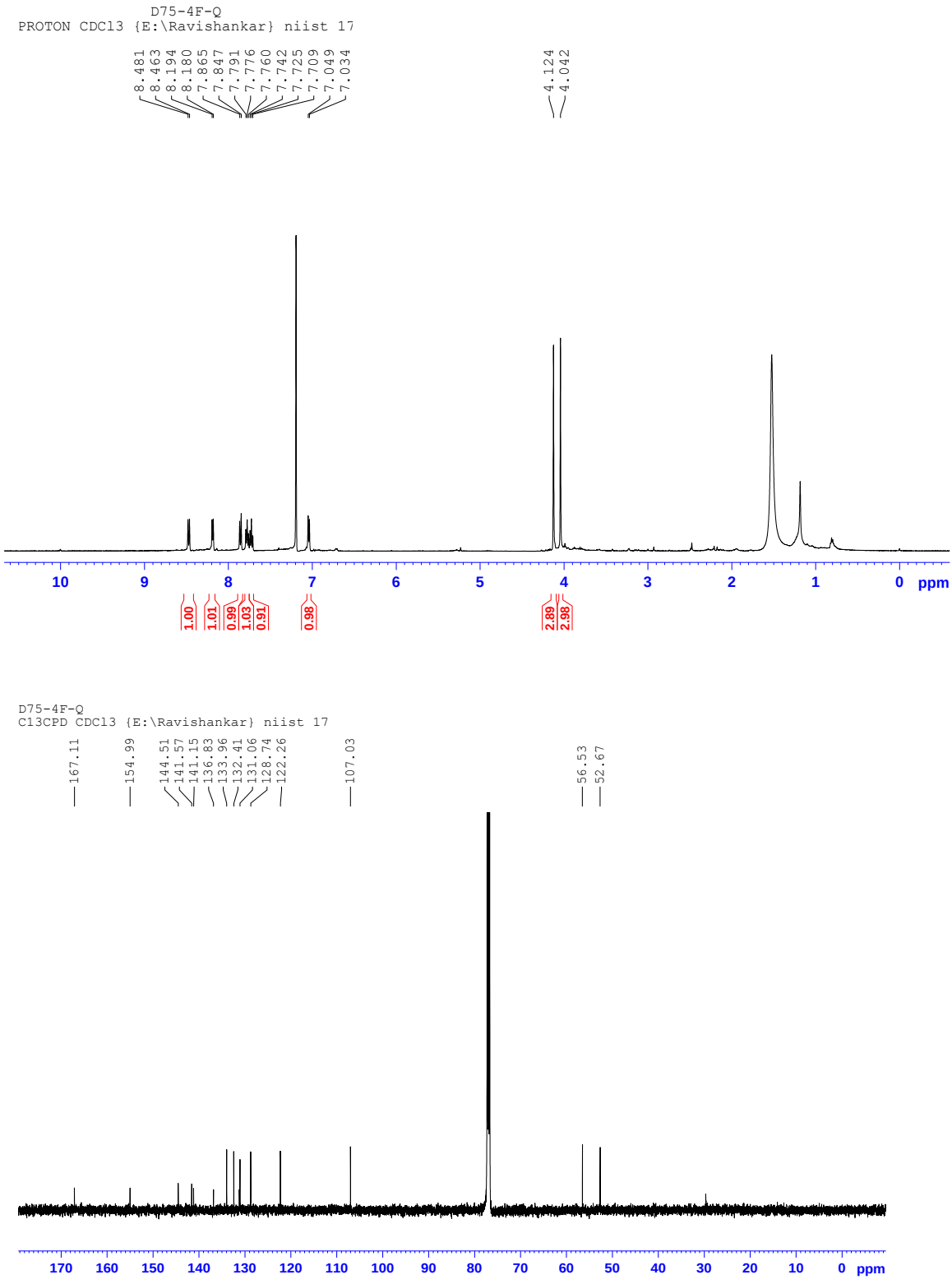



**Fig.4.3. $^{1}H$ and $^{13}C$ NMR of Compound 2**

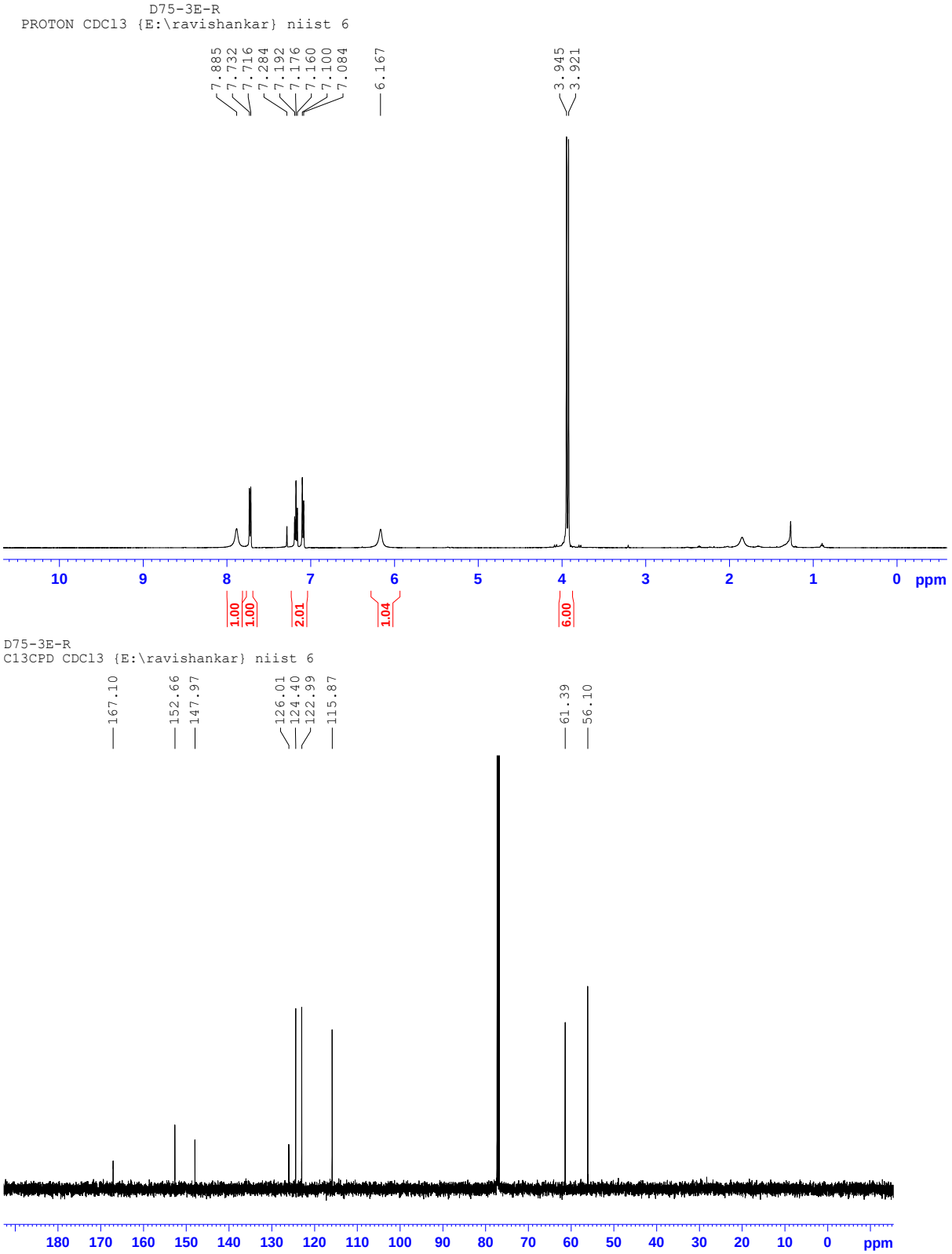



**Fig.4.4. $^{1}H$ and $^{13}C$ NMR of Compound 3**

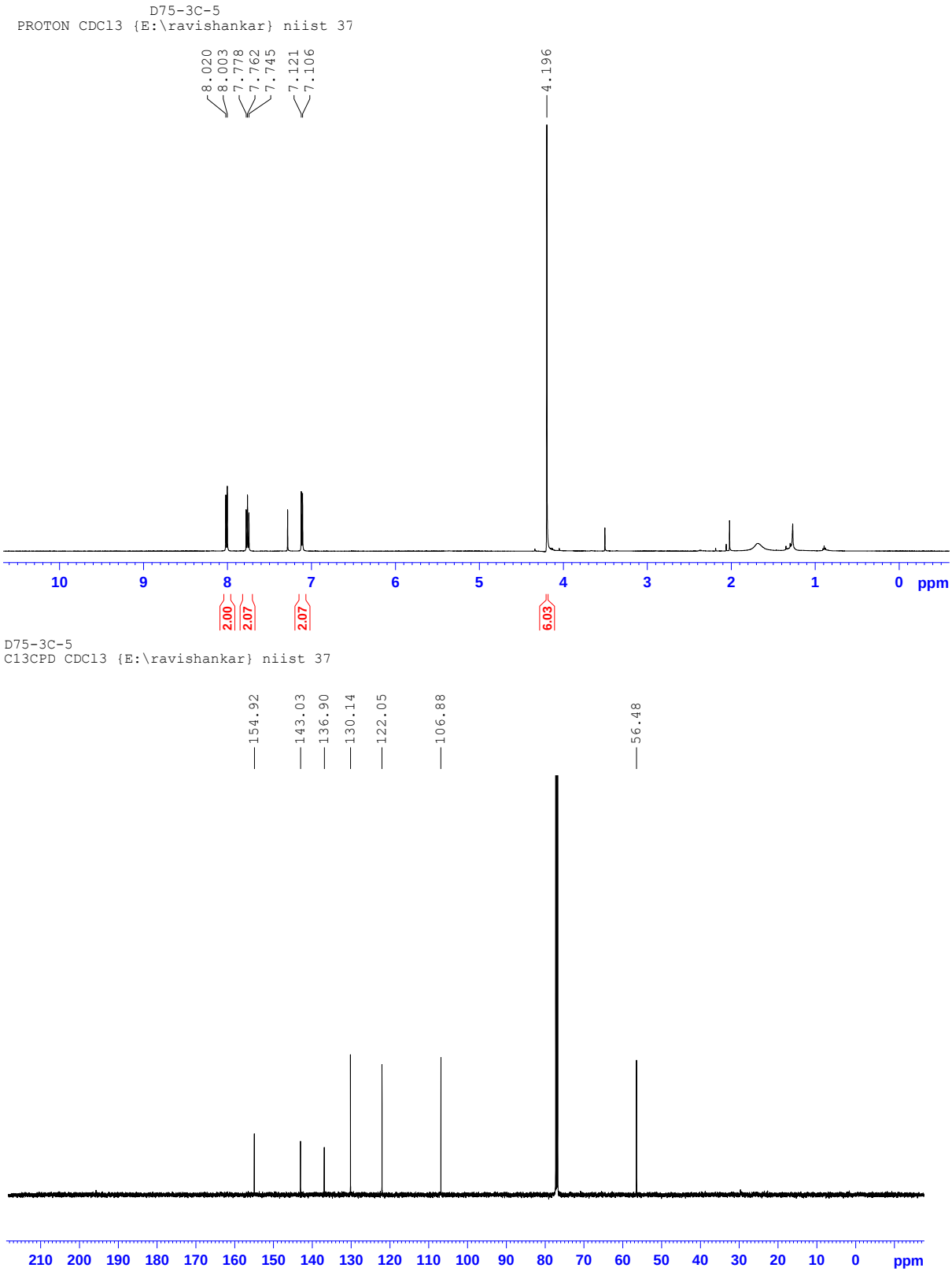



**Fig.4.5. $^{1}H$ and $^{13}C$ NMR of Compound 4**

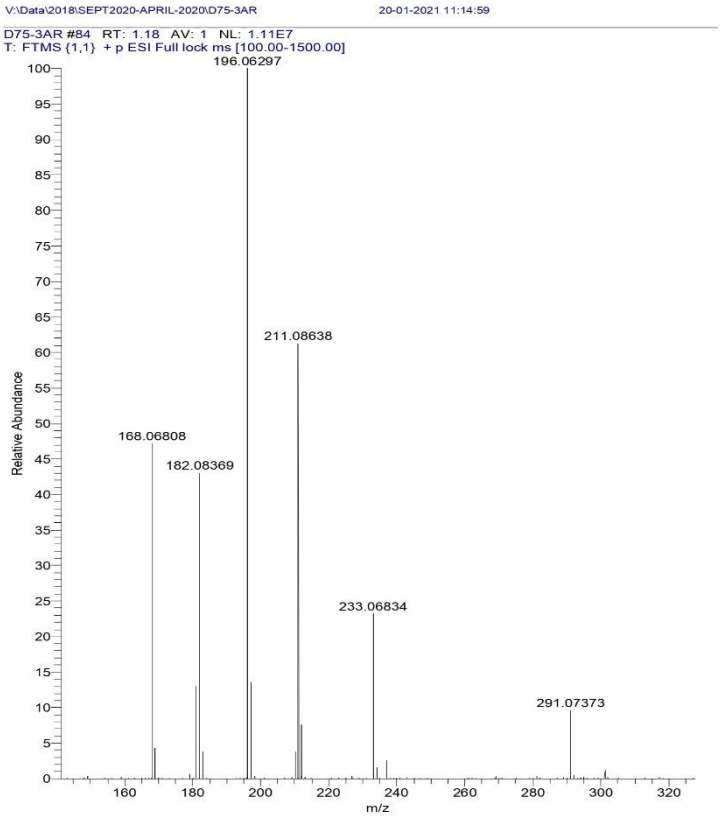



**Fig.4.6. HR-MS data of Compound 1**

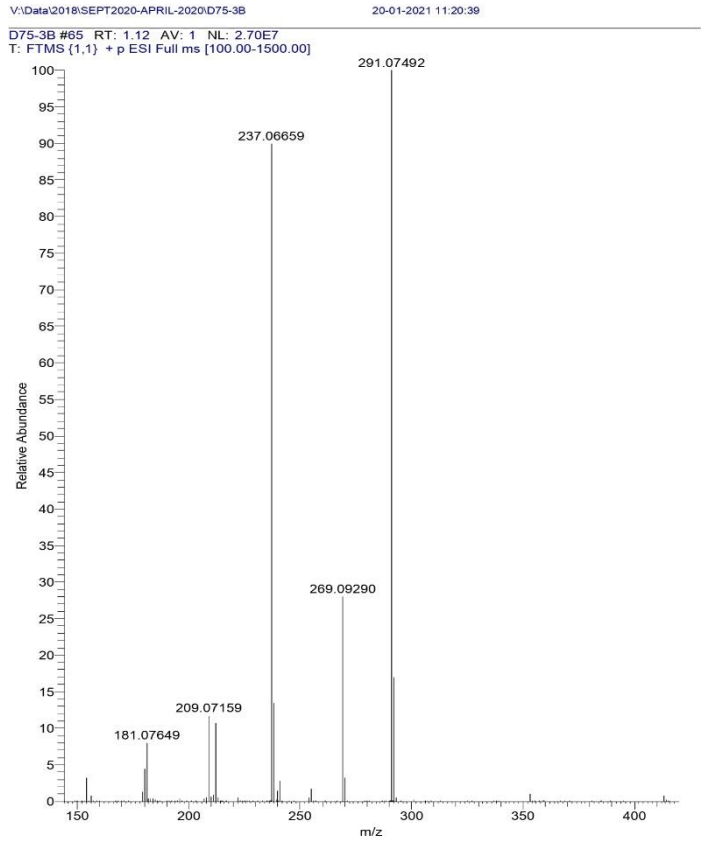



**Fig.4.7. HR-MS data of Compound 2**

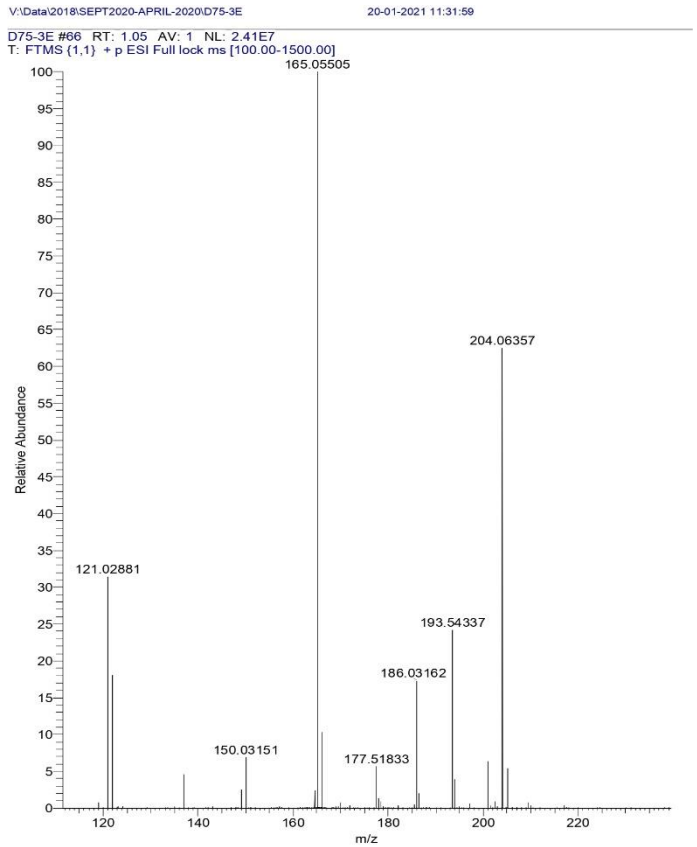



**Fig.4.8. HR-MS data of Compound 3**

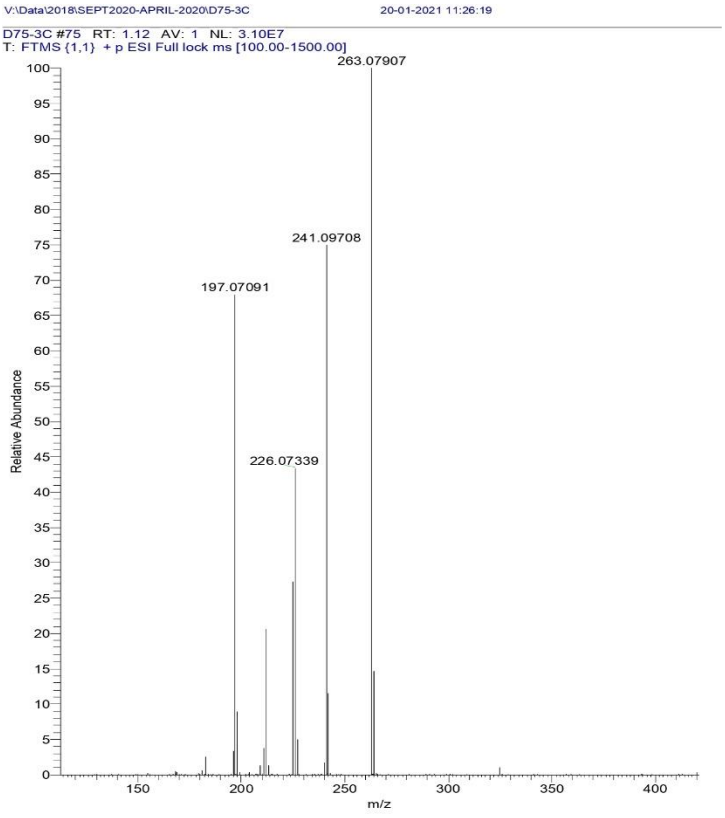



**Fig.4.9. HR-MS data of Compound 4**

### 4.3.2. GC-MS analysis of crude organic extract of Streptomyces NIIST G132

The GC-MS analysis revealed 15 major compounds present in the crude organic extract of streptomyces NIIST G132 (Table 4.1.). The chromatogram is illustrated in the (Fig.4.10.).

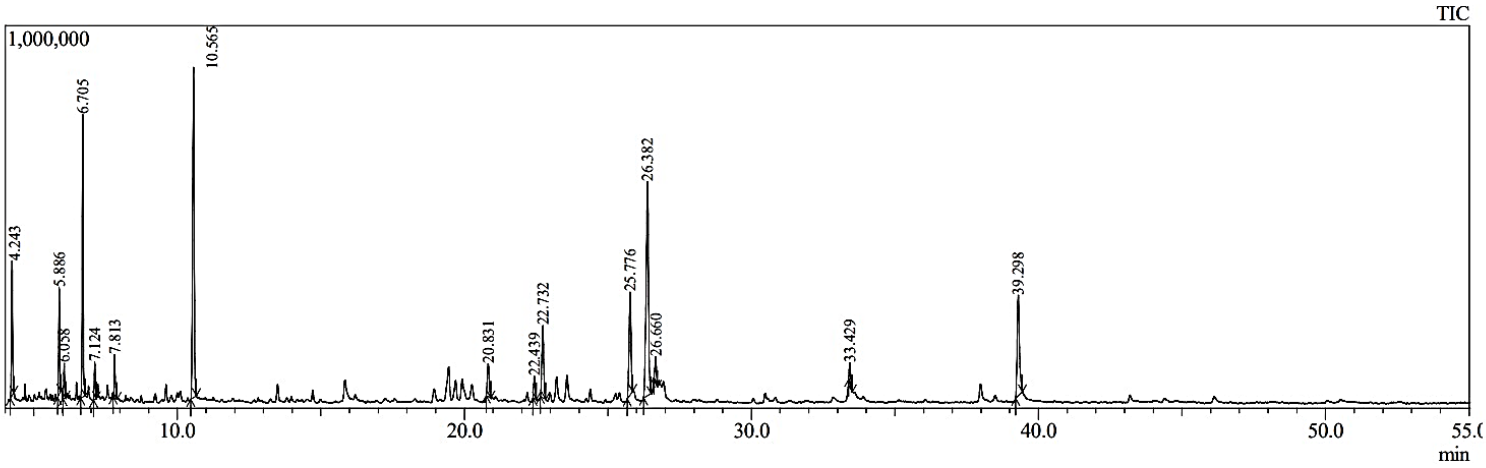



**Fig.4.10. Chromatogram of GC-MS analysis of crude organics extract from *Streptomyces* NIIST G132**

**Table 4.1. Details of compounds identified by GC-MS analysis**

| Retention Time | compound | Area % | Activity Reported | Reference |
|---|---|---|---|---|
| 4.243 | Butanal, 3-methyl-, oxime | 5.96 | | (Schettino et al. 2017) |
| 5.886 | Phenylethyl Alcohol | 4.21 | Antimicrobial and antioxidant | (Cheng et al. 2020) |
| 6.705 | Benzeneacetic acid, methyl ester | 11.82 | Antimicrobial | (Abdullah et al. 2015) |
| 10.565 | Benzeneacetamide | 21.90 | Antimicrobial and antitumor | (Balachandran 2012) |
| 20.831 | Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- | 2.51 | Antifungal | (Manimaran and Kannabiran 2017) |
| 22.732 | Cyclo(L-prolyl-L-valine) | 5.54 | Antimicrobial | (Khedr et al. 2015) |
| 25.776 | Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- | 8.40 | Antifungal | (Manimaran and Kannabiran 2017) |

| | | | | |
|---|---|---|---|---|
| 26.382 | Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- | 21.00 | Antifungal | (Manimaran and Kannabiran 2017) |
| 39.298 | Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- | 9.48 | Antifungal | (Manimaran and Kannabiran 2017) |

## 4.4. DISCUSSION

For decades, efforts to isolate natural products from microbial resources have continued. Several active microbial natural products were isolated and are currently being used. But the search for isolating natural microbial products is still an enthusiastic field where novel active compounds can be encountered. In search for isolating compounds, many researchers may need help in isolating them in pure form or lack of technique to isolate compounds quickly. This chapter isolates compounds from two streptomyces species, Streptomyces NIIST G132 and Streptomyces NIIST D75. Different methods were adopted to isolate compounds from the selected microorganism.

The streptomyces was cultured in a fermentation medium for secondary metabolite production for a particular period depending on the microorganism type. Here streptomyces NIIST G132 was cultured in YMB media for 7 days, and after incubation days, the culture-free extract was collected, and the crude organic extract was extracted. For streptomyces, NIIST D75 YMB medium was used as fermentation media, and incubation was for 12 days. After the incubation period, the cell-free extract was collected, and the crude organic extract was collected. The crude organic extract was extracted in ethyl acetate: methanol (95:5). Streptomyces NIIST D75 was purified using silica gel column chromatography and the preparative TLC method. From these methods, four pure compounds were isolated. The structural characterization of compounds was carried out through NMR and HR-MS data. But compound isolation was difficult in the case of Streptomyces NIIST G132. Various methods were tried to isolate pure

compounds from the crude organic extract, including silica gel column chromatography, preparative TLC, C18 column purification, and preparative HPLC. At least for the identification of compounds present in the crude organic extract, GC-MS analysis was carried out, and 15 significant compounds were identified. The literature data on compounds identified suggests these compounds possesses antimicrobial activity.

## 4.5. CONCLUSION

In this chapter, compound isolation from two selected streptomyces was carried out. From streptomyces NIIST D75, four compounds were isolated, including three phenazines and one benzamide. The compounds were 1-methoxy phenazine, methyl 6-methoxyphenazine-1-carboxylate, 2,3-dimethoxy benzamide, and 1,6-dimethoxyphenazine. These compounds were selected further to explore their biological activity studies. From streptomyces, NIIST G132 compounds were identified through GC-MS analysis. Major 15 compounds were identified and possess antimicrobial activity. Some compounds include Butanal, 3-methyl-, oxime, phenylethyl alcohol, benzene acetic acid, methyl ester, benzene acetamide, diketopiperazines, etc. The presence of antibacterial active compounds gave some hope for selecting the two streptomyces for further studies.

## 4.6. REFERENCE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# CHAPTER 5

## MIC, Synergistic activity studies, SEM analysis, Time-kill assay, Cytotoxicity assay, and docking analysis of isolated pure compounds from *Streptomyces* NIIST-D75 with selected antibiotics

## 5.1. INTRODUCTION

A decline in the approval of new antibiotics has resulted in a possibility for combination therapy, using existing drugs, as a potential strategy. Studies of synergism with existing antibiotics and approved drugs or novel antimicrobials can efficiently combat invasive antimicrobial drug resistance. Reports of combinations of antibiotics with phages (Li et al. 2021) polymers, (Lin et al. 2020) quorum sensing inhibitors, (Shaw and Wuest 2020) non-antibiotic drugs (Cheng, Williamson, and Zheng 2019), silver, (Möhler et al. 2018) polymyxins, (Ni et al. 2014) essential oils, (Soo et al. 2014) aminoglycosides, (Jackson, Chen, and Buising 2013) displayed potent antimicrobial synergism and facilitated a reduced dose of antibiotics. Combination of ciprofloxacin antibiotic, induced synergy, with other antibiotics viz. fosfomycin against ciprofloxacin-resistant *Shigella flexneri* isolates, tigecycline against *Vibrio vulnificus* sepsis (kim et.al. 2019) clarithromycin against *P.aeruginosa*, (Elkhatib and Noreddin 2014) meropenem against multidrug-resistant (MDR) *P. aeruginosa*, (Cardoso et al. 2009) cefotaxime against *V. vulnificus* sepsis and *Salmonella bacteremia*,(Jang et al. 2014) (Chang et al. 2006) rifampicin and azithromycin against *S. aureus*, (Beach and Champney 2014) amikacin against extended-spectrum β-lactamase-producing *Escherichia coli*, (Drago et al. 2013) and ceftazidime in the treatment of *P. aeruginosa*-infected orthopedic implants (Brouqui et al. 1995). Among the non-antibiotic drugs explored in combination with ciprofloxacin are celecoxib against *S. aureus*, (Dey et al. 2012) ambroxol, protamine sulfate, ivacaftor against *P. aeruginosa* (Cho et al. 2018), (Soboh et al. 1995) and (Lu et. al. 2013).

On the other hand, a synergistic combination of existing antibiotics with natural products is a promising route to obtain leads in antibacterials. The synergism of curcumin, baicalein, esculetin, and cinnamaldehyde with ciprofloxacin was demonstrated at sub-inhibitory concentrations against *P. aeruginosa* biofilms (Kart et.al. 2021). Synergistic activity of an inhalable powder by co-spray drying *Pseudomonas* phage PEV20 with ciprofloxacin was shown *in vivo* in

acute lung infection by *P. aeruginosa* (Lin et al. 2020). Thyme and peppermint essential oils exhibited promising biofilm inhibitory effects in *Klebsiella pneumoniae* in combination with ciprofloxacin (Mohamed et al. 2018) Synergistic antibacterial effect was observed for the combination of ciprofloxacin with berberine against MDR *K. pneumoniae* with activities of 18% synergy and 77% additivity(Zhou et al. 2016). Synergistic effects of baicalein with ciprofloxacin were reported against 12 out of 20 clinical ciprofloxacin resistant strains (Chan et al. 2011). Synergy of methanolic extract of *Punica granatum* fruit pericarp combination with ciprofloxacin was demonstrated against Gram-negative bacilli (Dey et al. 2012). A combination effect of maggot metabolites of *Luciliacuprina blowfly* and ciprofloxacin was demonstrated at sub-MIC levels on *S. aureus* (Arora, Baptista, and Lim 2011). 2-(2-Aminophenyl) indole, a microbe-derived natural product, in combination with ciprofloxacin exhibited promising activity as bacterial efflux pump inhibitor for *S. aureus* infections (Tambat et al. 2019). Surprisingly, synergistic antibacterial effects of microbe-derived natural products in combination with ciprofloxacin is less explored.

Actinomycetes are considered prolific antimicrobials producers, but their vast untapped diversity awaits exploitation (Genilloud 2018). In this regard, a promising area for discovering new antibiotics is the isolation of potent microbes from ecologically important niches. The 180,000 $km^2$ stretch of Western Ghats region of India is renowned for its rich flora and fauna (Nampoothiri, Ramkumar, and Pandey 2013); however, the microbial wealth from this area for novel antimicrobials remain underexploited (Vasava 2021) In our quest for isolation of novel antibiotics from the Malampuzha forest area of Western Ghats region of Kerala, (Drissya 2022) the present study deals with the isolation of secondary metabolites from a *Streptomyces luteireticuli* NIIST-D75 and exploration of their antibacterial effects. Together with the NIIST-D75 strain, 300 more *Streptomyces* strains were isolated from the same area, of which 25 recorded potential *in vitro* antagonism (unpublished data), which beckon the Malampuzha forest area of

Western Ghats region of Kerala as the 'promised land' for potent antimicrobial strains. Recently, we published the isolation of a novel aureothin derivative from *Streptomyces sp.* NIIST-D31(Drissya 2022). Three phenazines, 1-methoxyphenazine, methyl-6-methoxyphenazine-1-carboxylate, 1,6-dimethoxyphenazine, and a 2,3-dimethoxy benzamide were isolated from *S. luteireticuli* NIIST-D75, and the antibacterial effects of the isolated compounds in combination with ciprofloxacin were explored against selected human pathogenic bacteria.

## 5.2. MATERIALS AND METHODS

### 5.2.1. Test bacterial pathogens

Gram-positive bacterial strain *S. aureus* MTCC 902, and three Gram-negative bacteria viz. *E. coli* MTCC 2622, *P. aeruginosa* MTCC 2642, *Salmonella typhi* MTCC 3216 were used in the present study. All the test bacterial pathogens were procured from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India.

### 5.2.2. Minimum Inhibitory Concentration (MIC) Assay

The antibacterial activity of compounds and their combination were carried out by microdilution method against *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi*,(Jacob 2017) with an indicator dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to determine the MIC in 96-well plates. A serial three-fold dilution using Milli-Q® water resulted in test concentrations of 333.33 to 0.017 µg/mL in 10 wells from a stock solution of 1000 µg/mL in methanol for compounds **2** and **3**. Similarly, test concentrations were prepared from a 1000 µg/mL stock solution for ampicillin in distilled water, ciprofloxacin in 0.1N HCl, and nalidixic acid in ethanol. A serial three-fold dilution using Milli-Q® water resulted in 1000.00 to 0.05 µg/mL test concentrations in 10 wells from a stock solution of 3000 µg/mL in methanol for compound **1**. Methanol with three-fold dilution in 10 wells horizontally was used as a control. The test pathogens in LBB (Luria-Bertani broth) were incubated at 37 °C for 18 h in the

Incubator (Sanyo $CO_2$ Incubator MCO-20 A/C, Sanyo Electric Co Ltd, Japan), and adjusted to 1 x $10^6$ CFU/mL using the spectrophotometer (Synergy4, BioTek Instruments, USA) at 600 nm, which is equivalent to 0.5 McFarland standard. Bacterial inoculum (150 µL) was then added to the wells containing 150 µL of the test concentration and incubated at 37 °C. After 24 h of incubation, 20 µL of MTT dye (5 mg/mL in distilled water) was added, and the color change is visually noted, wherein yellow indicates inhibition and purple indicates bacterial viability. MIC is the lowest concentration at which there was no visible bacterial growth. The assay was performed in duplicate along with standard antibiotics. At the working test concentrations, we found that compound **4** is partially soluble; hence, this compound was not considered for antibacterial studies.

**5.2.3. Checkerboard assay**

The interaction between three compounds individually and their combination with ciprofloxacin was evaluated through checkerboard assay against *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi* with a modified method (Fadwa et al. 2021). The stock solution of 333.33 µg/mL for three compounds and their combination in methanol, and 2 µg/mL for ciprofloxacin in 0.1N HCl was prepared. The compounds and ciprofloxacin were subjected to three-fold serial dilution using Milli-Q® water separately in 2 mL tubes and 100 µL from each tube were added to 96 well plate horizontally with ciprofloxacin (0.666 to 0.0009 µg/mL) and vertically with individual compounds and their combination (111.11 to 0.050 µg/mL), and then 50 µL of 1 x $10^6$ CFU/mL bacterial inoculum was added. Column eleven was kept as a growth control where 50 µL of 1 x $10^6$ CFU/mL bacterial inoculum was added to 200 µL of Milli-Q® water, and column twelve contain 200 µL Milli-Q® water as a sterility control. The plates were incubated at 37 °C after 24 h of incubation, 20 µL of MTT dye (5 mg/mL in distilled water) was added, and MIC was determined as above. The interaction between three individual compounds and their

combination with ciprofloxacin was interpreted by Fractional Inhibitory Concentration Index (FICI), calculated by the equation:

FICI = FIC of compound/s + FIC of ciprofloxacin; FIC of compound/s = (MIC of combination of compound/s with ciprofloxacin ÷ MIC of compound/s alone) and FIC of ciprofloxacin= (MIC of combination of ciprofloxacin with compound/s ÷ MIC of ciprofloxacin alone).

FICI interpretation: FICI ≤ 0.5: Synergy; FICI > 0.5 and < 4.0: Indifference; FICI ≥ 4.0: Antagonism.

**5.2.4. Time-kill assay**

The rate of killing of four test bacterial pathogens viz. *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* by ciprofloxacin and three compounds at concentrations of MIC, ½MIC, and MIC of individual combination of compounds with ciprofloxacin were carried out by time-kill assay (Kumar and Lankalapalli 2014). The respective MICs of compounds, ciprofloxacin, and combinations were prepared by dilution with LBB from a stock solution of 3 mg/mL of compound **1** in methanol, 1 mg/mL of compound **2** and **3** in methanol, 10 µg/mL of ciprofloxacin in 0.1N HCl. 100 µL of bacterial inoculum in LBB, adjusted to $1 \times 10^6$ CFU/mL, as above, was added to 1 mL of LBB containing the respective concentration of compounds, ciprofloxacin, and combinations in 1.5 mL *Eppendorf® microcentrifuge tubes*. The control contains 1 mL of LBB with 100 µL of bacterial inoculum in LBB. The resulting tubes were then incubated at 37 °C for 48 h, and 100 µL was aliquoted at different time intervals (0, 2, 4, 8, 10, 12, 24, and 48 h). Each aliquot was tenfold serially diluted seven times with Milli-Q® water. A volume of 100 µL from each dilution was plated on LBA and incubated for 24 h at 37 °C, and colonies were counted manually. The colony counts represented as $\log_{10}$CFU/mL were plotted on Y-axis on a logarithmic scale and the time interval on X-axis. CFU/mL was calculated as follows:

CFU/mL = (No. of colonies x dilution factor)/(volume of sample plated in mL)

A ≥ 3 $\log_{10}$CFU/mL reduction in colony counts from the original inoculum (control) is considered bactericidal, and a ≤ 3 $\log_{10}$CFU/mL reduction is bacteriostatic.

### 5.2.5. Scanning Electron Microscopy (SEM) analysis

The changes in the morphology of *S. aureus* and *E. coli* by NIIST-D75, crude organic extract, MICs of compounds, their combination, ciprofloxacin, and synergistic combinations were analyzed by SEM (Carl Zeiss EVO 18 Research, Germany) (Mal et al. 2020). The SEM analysis results were compared against the morphology of *S. aureus* and *E. coli*. The MICs were prepared by dilution with LBB from a stock solution of 333.33 µg/mL for three compounds and their combination in methanol and 2 µg/mL for ciprofloxacin in 0.1N HCl. 100 µL of bacterial inoculum in LBB, adjusted to $1 \times 10^6$ CFU/mL, as above, was added to 1 mL of LBB containing the respective MICs of compounds (**1** to **3**), their combination, ciprofloxacin, and synergistic combinations in 1.5 mL *Eppendorf® microcentrifuge tubes.* The control contains 1 mL of LBB with 100 µL of bacterial inoculum in LBB. The resulting tubes were then incubated at 37 °C for 24 h.

Agar overlay method (Jacob 2017) was carried out by spot inoculation of NIIST-D75 in the center of the ISP-2 (International Streptomyces Project-2) agar Petri plate and grown at 30 °C for 7 days in the Incubator (Rotek-RCI 12S, B & C Industries, India). The respective ISP-2 agar Petri plates containing spotted NIIST-D75 were overlaid with the bacterial inoculum in LBB, adjusted to $1 \times 10^6$ CFU/mL, as above, in 10 mL of MHA (Mueller Hinton agar) medium, and incubated. For the disc diffusion method,(Bauer 1966) bacterial inoculum in LBB, adjusted to $1 \times 10^6$ CFU/mL, as above, was swabbed to MHA agar plates, and to the centre of Petri plates was introduced 6 mm disc impregnated with 30 µL of 10 mg/mL of crude organic extract (dissolved in 100% methanol), and incubated. After incubation for 24 h at 37 °C, the bacterial cells were collected using a sterile inoculation loop from the edge of the ZoI and added to 1 mL of LBB in 1.5 mL *Eppendorf® microcentrifuge tubes.*

After incubation, the respective tubes with bacteria were resuspended in 500 µL of 0.1 M phosphate-buffered saline (PBS) at pH 7.4 and centrifuged at 3000 g for 5 min. After decanting the supernatant, the cells were then fixed with 50 µL of 2% glutaraldehyde and incubated for 12 h at 4 °C, followed by resuspending cells in PBS and centrifugation as above, and post-fixation, a 50 µL of 1% osmium tetroxide was added and incubated for 1 h at 4 °C. Subsequently, the cells were treated with 500 µL of a series of ethanol concentrations of 30%, 50%, 75%, 95%, and 100% for removal of water by centrifuging at 3000 g for 10 minutes. The resulting cells were stored in 100% ethanol, and 20 µL from each tube were drop-casted over the stub, dehumidified for 20 min under vacuum, and sputter-coated with gold for SEM analysis. The change in morphology of *S. aureus* and *E. coli* upon treatment with individual combinations of compounds (**1** to **3**) with ciprofloxacin at their synergistic concentrations were quantified using respective SEM images by ImageJ microscopic image analyzing tool. The SEM image was first calibrated using the scale bar, and a selected area was analyzed by setting a threshold. Then the surface area was found by running the analyze particle command, which was used to construct the histogram.

**5.2.6. Cytotoxicity assay**

Cytotoxicity of three compounds (**1-3**), their individual combination with ciprofloxacin at synergistic concentrations observed against *S. aureus* and *E. coli*, and ciprofloxacin alone were evaluated with Hep G2 cell line, procured from National Centre for Cell Sciences (NCCS), Pune, India. The cell line was cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), and antibiotics (100 µg/mL of penicillin and 100 µg/mL of streptomycin) in a humidified incubator with 95% air and 5% $CO_2$ at 37 °C.(Anupama et al. 2018). Cell viability was evaluated through MTT assay by seeding in a 96-well plate at a density of 5 x $10^3$ cells per well. Cells after 60-70% confluence were exposed to different concentrations of compounds ranging from 1 – 500 µM and incubated in a humidified

incubator (New Brunswick Galaxy 170S, Eppendorf, Germany) for 24 h with 95% air and 5% $CO_2$ at 37 °C. A 10 mM stock solution of compounds was diluted with Milli-Q® water and further diluted with EMEM. The final working concentration contains 0.1% methanol, and the stock solution for ciprofloxacin was 10 mM in 0.1N HCl. A 10 µg/mL stock solution of compounds, ciprofloxacin, was diluted with EMEM to arrive at MIC concentrations of the synergistic combination. After incubation, the medium was removed, cells were washed with Hank's balanced salt solution (HBSS), incubated for 4 h with 5 mg/mL of MTT, and dissolved in serum free EMEM. Then cells were again washed with 100 µL HBSS, and 100 µL DMSO was added, gently shaken for 20 minutes for complete dissolution, and absorbance was recorded at 570 nm using a microplate spectrophotometer (Synergy 4, Biotek, USA).

**5.2.7. Docking analysis**

The structure of DNA gyrase was taken from RCSB PDB (PDB ID: 2XCT), and docking analysis was done in Autodock vina. The protein was prepared by deleting the water and other unwanted molecules using the Autodock program. Polar hydrogens were added and gave gasteiger charges to the protein. The size of the grid box for docking was kept as 126x126x126. After preparing three ligand molecules downloaded from the Pubchem database, Autodock vina was used for the docking through shell script. The pose with a best binding affinity of each ligand was taken further from the nine poses of ligand generated by Vina. The energy minimization of each docked complex was done separately by YASARA energy minimization server. The docking of ciprofloxacin to each docked complex was done with Autodock vina. The binding affinity of the ciprofloxacin with DNA Gyrase and its complexes are calculated using PRODIGY webserver, and the ligand interactions diagrams of ciprofloxacin with protein and complexes were plotted using Ligplot +.

## 5.3. RESULTS

### 5.3.1. MIC Assay

MIC values recorded for compounds **1**, **2** and **3**, individually, against *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi* were in the range of 111.11 to 1000.00 µg/mL, whereas, the combination of all three compounds exhibited an MIC of 37.03 µg/mL (Table 5.1.). In comparison, the commercial antibiotic ampicillin exhibited MIC of 1.37 µg/mL against *S. aureus*, *P. aeruginosa* and *S. typhi*, and 37.03 µg/mL against *E. coli*. Ciprofloxacin exhibited MIC of 0.46 µg/mL against *P. aeruginosa* and *S. typhi*, 0.05 µg/mL against *E. coli*, and 1.37 µg/mL against *S. aureus*. Nalidixic acid exhibited MIC of 12.35 µg/mL against *S. aureus* and *P. aeruginosa*, 1.37 µg/mL against *E. coli*, and 333.33 µg/mL against *S. typhi*.

**Table 5.1. MIC of compounds 1, 2, 3, and commercial antibiotics.**

| **Test bacteria** | **MIC in µg/mL** | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ampicillin | Ciprofloxacin | Nalidixic acid | Compound **1** | Compound **2** | Compound **3** | Compound **1+2+3** |
| *S. aureus* | 1.37 | 1.37 | 12.35 | 1000.00 | 111.11 | 333.33 | 37.03 |
| *E. coli* | 37.03 | 0.05 | 1.37 | 1000.00 | 333.33 | 333.33 | 37.03 |
| *P. aeruginosa* | 1.37 | 0.46 | 12.35 | 1000.00 | 111.11 | 333.33 | 37.03 |
| *S. typhi* | 1.37 | 0.46 | 333.33 | 1000.00 | 111.11 | 333.33 | 37.03 |

### 5.3.2. Checkerboard assay

MIC and calculated FICI values against *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi* obtained through checkerboard assay for combination of compounds **1**, **2**, **3** with ciprofloxacin are shown (Table 5.2.). The respective values of MIC in combination of compounds with ciprofloxacin are as follows: MIC of compound **1** was 0.05 µg/mL against all four test bacterial pathogens. MIC of compound **2** was 0.15 µg/mL against *S. aureus*, *E. coli* and *S. typhi*, and 1.37 µg/mL against *P. aeruginosa*. MIC of Compound **3** was 0.45 µg/mL against *S. aureus*, *E. coli* and *S. typhi*, and 1.37 µg/mL against *P. aeruginosa*. MIC of sum of three compounds was 0.15 µg/mL against all four test bacterial pathogens; and ciprofloxacin exhibited MIC values

of 0.02, 0.07 and 0.22 µg/mL. The FICI values in these combination studies, calculated from MIC, were as follows: compound **1** exhibited 0.16, 1.40, 0.48, and 0.48 for *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi*, respectively. Compound **2** and **3** each exhibited 0.16, 0.40, 0.16, and 0.48 for *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi*, respectively. The sum of all three compounds exhibited 0.01, 0.40, 0.04, and 0.04 for *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi*, respectively.

**Table.5.2.FICI of combination of compounds 1, 2, and 3 with ciprofloxacin**

| **Test bacteria** | **Combination** | **MIC** | | **Fold reduction in MIC** | **FICI** | **Interpretation** |
|---|---|---|---|---|---|---|
| | | **Alone** | **Combination** | | | |
| | | **µg/mL** | **µg/mL** | | | |
| *S. aureus* | Compound **1** | 1000 | 0.05 | 20000 | 0.16 | Synergism |
| | Ciprofloxacin | 1.37 | 0.22 | 6.2 | | |
| | Compound **2** | 111.11 | 0.15 | 740.7 | 0.16 | Synergism |
| | Ciprofloxacin | 1.37 | 0.22 | 6.2 | | |
| | Compound **3** | 333.33 | 0.45 | 740.7 | 0.16 | Synergism |
| | Ciprofloxacin | 1.37 | 0.22 | 6.2 | | |
| | Compound **1+2+3** | 37.03 | 0.15 | 246.9 | 0.01 | Synergism |
| | Ciprofloxacin | 1.37 | 0.02 | 68.5 | | |
| *E. coli* | Compound **1** | 1000 | 0.05 | 20000 | 1.40 | Indifference |
| | Ciprofloxacin | 0.05 | 0.07 | * | | |
| | Compound **2** | 333.33 | 0.15 | 2222.2 | 0.40 | Synergism |
| | Ciprofloxacin | 0.05 | 0.02 | 2.5 | | |
| | Compound **3** | 333.33 | 0.45 | 740.7 | 0.40 | Synergism |
| | Ciprofloxacin | 0.05 | 0.02 | 2.5 | | |
| | Compound **1+2+3** | 37.03 | 0.15 | 246.9 | 0.40 | Synergism |
| | Ciprofloxacin | 0.05 | 0.02 | 2.5 | | |
| *P. aeruginosa* | Compound **1** | 1000 | 0.05 | 20000 | 0.48 | Synergism |
| | Ciprofloxacin | 0.46 | 0.22 | 2.1 | | |
| | Compound **2** | 111.11 | 1.37 | 81.10 | 0.16 | Synergism |
| | Ciprofloxacin | 0.46 | 0.07 | 6.6 | | |
| | Compound **3** | 333.33 | 1.37 | 243.3 | 0.16 | Synergism |
| | Ciprofloxacin | 0.46 | 0.07 | 6.6 | | |
| | Compound **1+2+3** | 37.03 | 0.15 | 246.9 | 0.04 | Synergism |
| | Ciprofloxacin | 0.46 | 0.02 | 23 | | |
| *S. typhi* | Compound **1** | 1000 | 0.05 | 20000 | 0.48 | Synergism |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Ciprofloxacin | 0.46 | 0.22 | 2.1 | | |
| | Compound **2** | 111.11 | 0.15 | 740.73 | 0.48 | Synergism |
| | Ciprofloxacin | 0.46 | 0.22 | 2.1 | | |
| | Compound **3** | 333.33 | 0.45 | 740.73 | 0.48 | Synergism |
| | Ciprofloxacin | 0.46 | 0.22 | 2.1 | | |
| | Compound **1+2+3** | 37.03 | 0.15 | 246.9 | 0.04 | Synergism |
| | Ciprofloxacin | 0.46 | 0.02 | 23 | | |

Values are average of two readings. *Increase in MIC by 1.4-fold.

### 5.3.3. Time kill analysis

The time-kill assay colony counts for the test bacterial pathogens *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* are shown in (Fig. 5.1.). The $\log_{10}$CFU/mL value of the original inoculum (control) displayed a steady increase in growth from 6.04 (0 h) to 7.80 (48 h), 6.52 (0 h) to 6.96 (48 h), 6.34 (0 h) to 6.96 (48 h), and 6.27 (0 h) to 6.96 (48 h) for *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi*, respectively. In general, the individual compounds **1**, **2** and **3** exhibited bacteriostatic activity against all the four test bacterial pathogens for the entire time period up to 48 h at MIC and ½MIC, compound **2** exhibited bactericidal activity against *S. aureus* between 24 to 48 h at MIC and at 48 h at ½MIC. Ciprofloxacin showed bactericidal activity at MIC between 8 to 48 h, 10 to 48 h, 6 to 10 h, and 10 to 48 h against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi*, respectively. Additionally, at ½MIC, ciprofloxacin showed bactericidal activity between 8 to 48 h, 8 to 10 h, and 10 to 48 h against *S. aureus*, *P. aeruginosa*, and *S. typhi*, respectively, however, bacteriostatic activity was observed up to 48 h against *E. coli*. In general, individual combination of compounds with ciprofloxacin at synergistic concentrations (Table 5.2.) showed bactericidal action against all the four test bacterial pathogens. The time period where the bactericidal action was observed in combinations were 8 to 48 h with compound **1**, and 6 to 48 h with compound **2** or **3** against *S. aureus*; 8 to 48 h with compound **2**, and 4 to 48 h with compound **3** against *E. coli*; 2 to 48 h, 6 to 48 h, and 4 to 48 h with compounds **1**, **2**, and **3**, respectively, against *P. aeruginosa*; 8 to 48 h with compound **1** or **2**, 2

to 48 h with compound **3** against *S. typhi*. As an exception in combinations, a bacteriostatic activity was observed for compound **1** up to 48 h against *E. coli*.

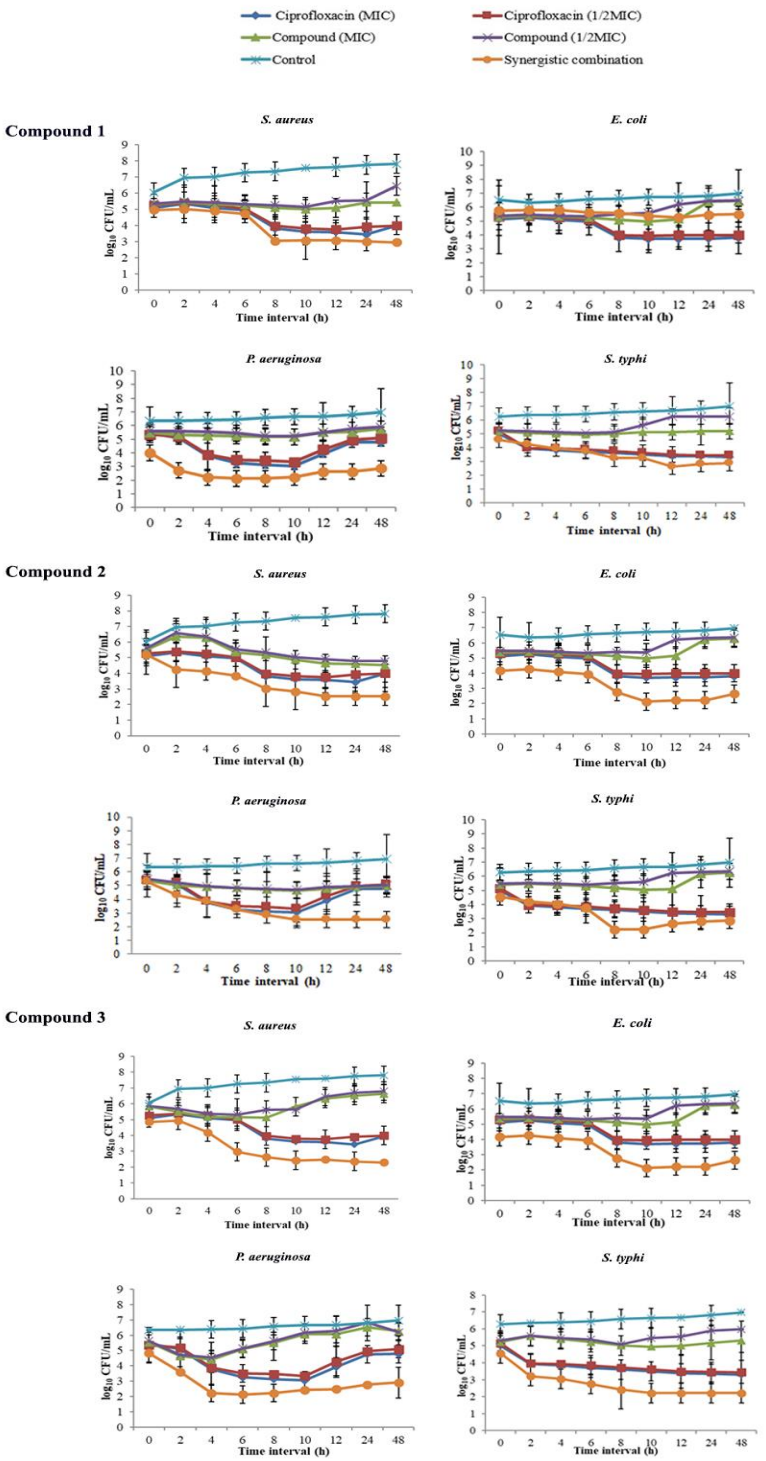


**Fig. 5.1. Time-kill kinetics of compounds 1, 2, and 3 in combination with ciprofloxacin against *S. aureus, E. coli, P. aeruginosa* and *S. typhi.* Each data points at a different hour represents the mean number of viable bacterial counts for triplicate experiments, and the error bar represents the standard deviations.**

### 5.3.4. SEM analysis

Treatment of *S. aureus* and *E. coli* with NIIST-D75 resulted in dent formation and surface depression, respectively (Fig.5.2.), whereas, the crude organic extract resulted in a change of surface morphology of the organisms (Fig.5.2.). SEM image of *S. aureus* displayed a round, smooth surface cells with normal structural integrity and cell size (593.2 nm) and *E. coli* displayed a rod shape with a continuous smooth outer membrane (1.46 µm length), and both possessing a normal surface morphology serves as the control (Fig. 5.2.).

Mild changes in morphology in *S. aureus* were observed with compounds such as dent formation with compound **1**, doughnut like formation with compound **2**, and change of surface morphology with compound **3** as well as in the combination of three compounds (Fig. 5.2.). Similarly, treatment of *S. aureus* with ciprofloxacin resulted in a mild dent formation (Fig. 5.2.). In synergistic combination of compound **1** with ciprofloxacin, a mild dent formation was observed (Fig.5.2.), whereas drastic changes in morphologies were observed with synergistic combinations pertaining to compound **2**, compound **3** and in the combination of three compounds such as biofilm disruption, cell bursting and doughnut like formation. The change in surface morphology in *S. aureus* displayed a decrease in surface area drastically from compound **1** to **3** with synergistic combinations compared to ciprofloxacin alone (Fig.5.3.).

Mild changes in morphology in *E. coli* were observed with compounds such as change in surface integrity with compound **1**, cell surface depression with compound **2**, compound **3** as well as in the combination of three compounds (Fig.5.2.). Treatment of *E. coli* with ciprofloxacin resulted in cell elongation of 4 µm (Fig.5.2.). However, drastic changes in morphologies with synergistic combination of compounds with ciprofloxacin were observed (Fig.5.2.). The synergistic combination of compound **1** exhibited cell elongation (9.07 µm) with cell burst, compound **2** exhibited cell elongation (10 µm), compound **3** exhibited cell elongation (20.46 µm) with ruptured cell, and in the combination of three compounds cell elongation with change in

surface morphology was observed. The change in surface morphology in *E. coli* with synergistic combinations of **1** or **2** were in par with ciprofloxacin surface area, however, the combination with **3** displayed a drastic increase in surface area (Fig. 5.3.).

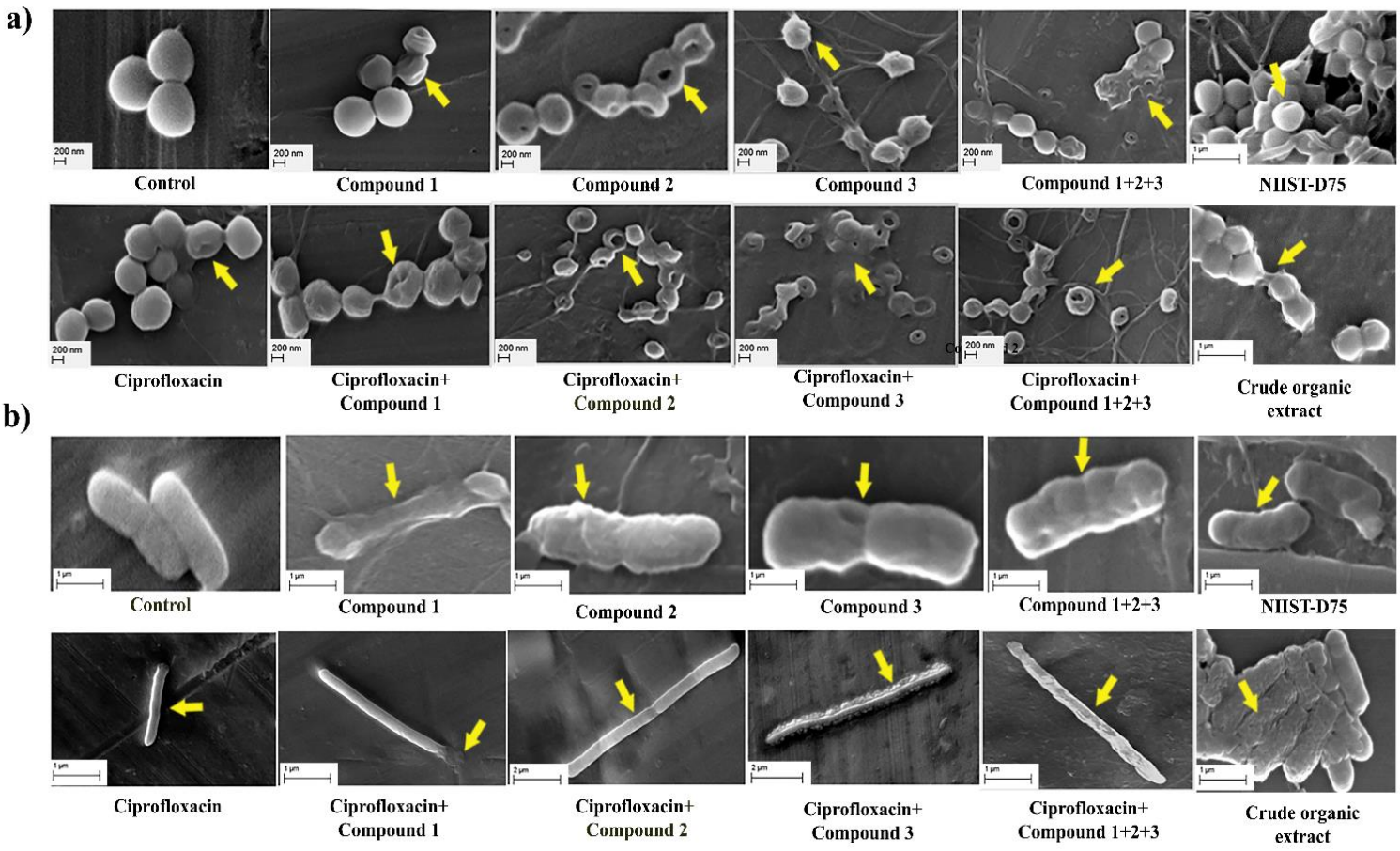


**Fig.5.2. Changes in morphology of a) *S. aureus* and b) *E. coli* upon treatment with MIC concentrations of compounds 1, 2, 3, their combination, ciprofloxacin, synergistic combinations, NIIST-D75, and crude organic extract.**

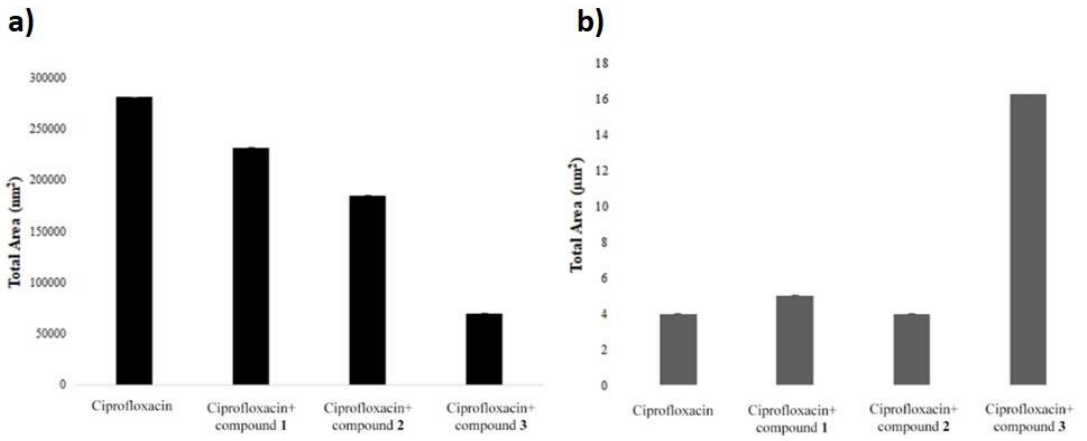


**Fig. 5.3. Changes in cell surface area (measured from the corresponding SEM images) upon treatment with ciprofloxacin and its combination with individual compounds 1 to 3 for a) *S. aureus*, and b) *E. coli*.**

### 5.3.5. Cytotoxicity assay

Cytotoxicity studies with Hep G2 cell line confirmed that all the compounds displayed ~90% cell viability up to 250 µM but ciprofloxacin displayed ~70%. However, at 500 µM, ciprofloxacin recorded 55.59% cell viability, whereas compound **1**, **2**, and **3** exhibited 75%, 94%, and 85%, respectively (Fig.5.4.). The synergistic combination of individual compounds with ciprofloxacin at their respective concentrations (MIC, Table 5.2) revealed 88 to 100% cell viability (Fig.5.5.).

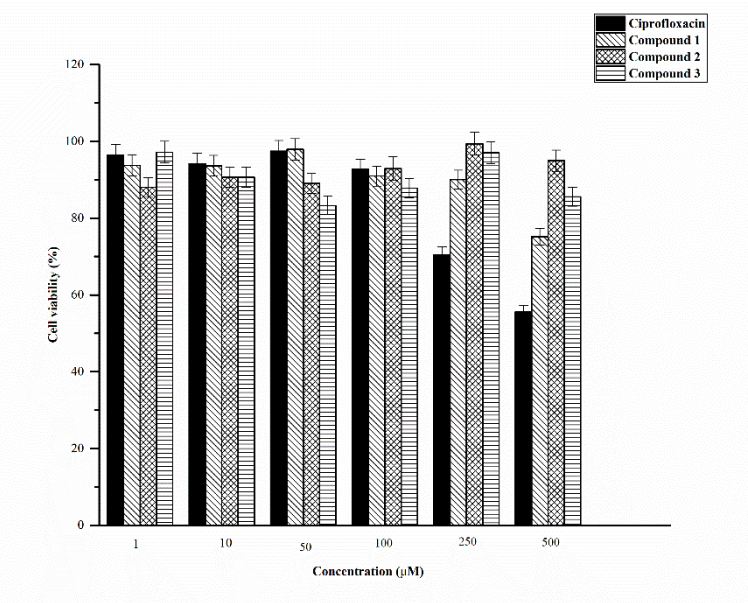


**Fig.5.4. Cytotoxicity assay of the compounds with Hep G2 cell line.**

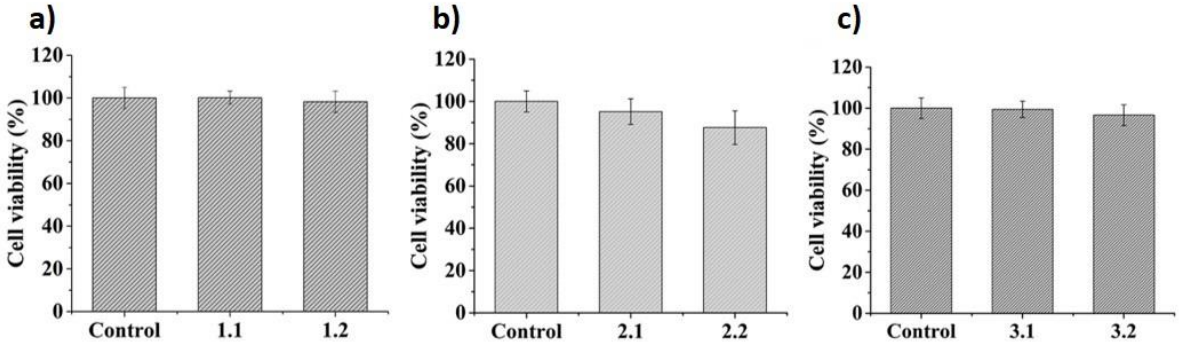


**Fig. 5.5. Cell viability assay on Hep G2 cells treated with synergistic concentrations (MIC, Table 1) of individual combination of compounds 1, 2, and 3 with ciprofloxacin. a) 1.1 (MICs of compound 1, ciprofloxacin against *E. coli*), 1.2 (MICs of compound 1, ciprofloxacin against *S. aureus*); b) 2.1 (MICs of compound 2, ciprofloxacin against *E. coli*), 2.2 (MICs of compound 2, ciprofloxacin against *S. aureus*); and c) 3.1 (MICs of compound 3, ciprofloxacin against *E. coli*), 3.2 (MICs of compound 3, ciprofloxacin against *S. aureus*). All data are represented as mean ± SD (n = 6).**

### 5.3.6. Docking Analysis

It is evident from the data obtained from docking studies that the binding affinity of ciprofloxacin is more in the DNA Gyrase complexed with all three compounds than DNA Gyrase alone. Ciprofloxacin is shown a higher binding affinity (-8.0 Kcal/mol)when bound with DNA Gyrase -compound **3** complex (Table 5.3., Fig.5.6.).

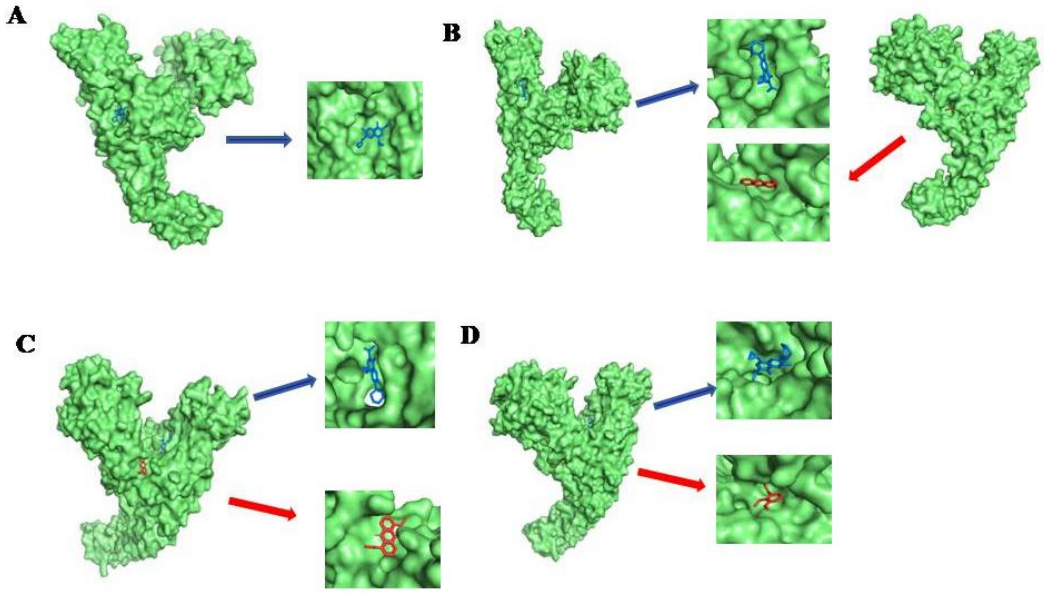


**Fig.5.6.Docking of compounds with DNA gyrase (A) docking of ciprofoxacin alone with DNA gyrase,(B) docking of ciprofloxacin and D75-3B,(C) docking of ciprofloxacin and D75-3E with DNA gyrase and (D ) shows ciprofloxacin and D75-AR with DNA gyrase**

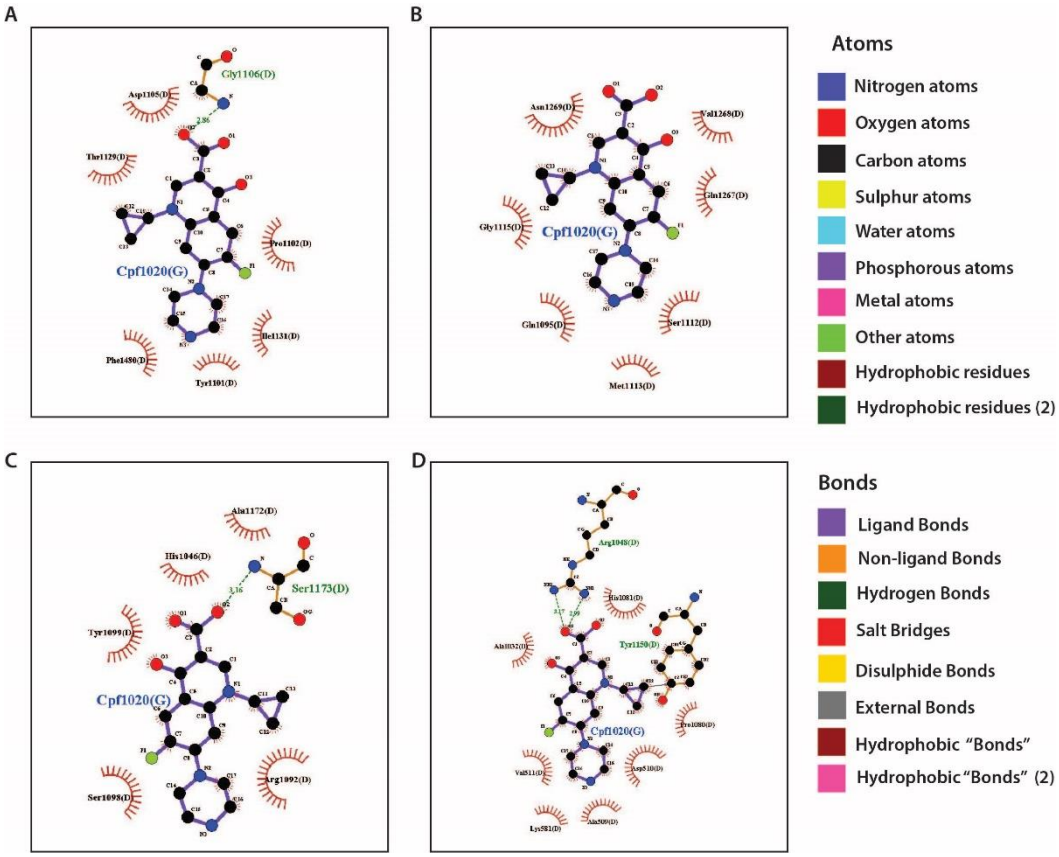


**Fig.5.7. The ligand interaction diagrams of Ciprofloxacin with DNA Gyrase and its complexesA. Interactions between Ciprofloxacin and DNA Gyrase alone. B. Interactions between Ciprofloxacin and DNA Gyrase complexed with Compound 2 C. Interactions between Ciprofloxacin and DNA Gyrase complexed with Compound 3 D. Interactions between Ciprofloxacin and DNA Gyrase complexed with Compound 1**

**Table.5.3. Binding Affinity of Ciprofloxacin with DNA Gyrase and its complexes**

| Structure | Docking Score | Binding Affinity (Kcal/mol) |
|---|---|---|
| DNA Gyrase + Ciprofloxacin | -6.0 | -7.3 |
| (DNA Gyrase + D75_3B)+ Ciprofloxacin | -6.8 | -7.4 |
| (DNA Gyrase + D75_3E)+ Ciprofloxacin | -6.9 | -8.0 |
| (DNA Gyrase + D75_AR)+ Ciprofloxacin | -6.6 | -7.7 |

## 5.4. DISCUSSION

Four compounds were isolated from the crude organic extract of the *S. luteireticuli*, three phenazines (compound **1**, **2** and **4**) and one benzamide (**3**). Compound **1**, **2** (a.k.a. mycomethoxin B) and **4** were reported from *S. luteoreticuli* (Yamanaka 1972) (Yamagishi S, 1971) We recently reported compound **3** from another *Streptomyces* sp. NIIST-D31,(Drissya 2022) from the same soil location where NIIST-D75 was collected. Interestingly, related benzamides were reported from *Streptomyces* species with antibacterial activity (Shaaban et al. 2012) (Yang et al. 2014). We have demonstrated earlier that phenazines in combination with azoles exhibit synergistic activity against *Candida* species (Nishanth Kumar et al. 2014). Accordingly, motivated by sheer curiosity, phenazines were tested for synergistic activity in combination with ciprofloxacin in the present study. Antibacterial activity of isolated compounds (**1-3**) and their individual combination with ciprofloxacin was carried out against four bacterial pathogens viz. *S. aureus, E. coli, P. aeruginosa,* and *S. typhi*, which are known to cause nosocomial infections. A significant reduction in MIC values were noted (Table 5.2.) in combination studies for both ciprofloxacin and compounds, checkerboard assay, compared to individual treatment to test bacterial pathogens (Table 5.1.). FICI of 0.16 for the individual combination of compounds with ciprofloxacin and FICI of 0.01 for the mixture of compounds **1**+**2**+**3** with ciprofloxacin indicate synergism against *S. aureus* (Table 5.2.). Combination of natural product baicalein with ciprofloxacin that exhibited a 2- to 4-fold reduction in MIC of ciprofloxacin against community-associated, ciprofloxacin-susceptible (CA)-MRSA strains was reported,[46] in the present study, a 6.2-fold reduction in MIC of ciprofloxacin in combination with compound **1** to **3** against ciprofloxacin-susceptible *S. aureus* was observed. In combination studies against *E. coli*, FICI of 1.40 for combination of compound **1** with ciprofloxacin indicate indifference and FICI of 0.40 for the rest of the combinations indicate synergism against *E. coli* (Table 5.2.). Synergistic action with amikacin or cefepime antibiotics in combination with ciprofloxacin

against extended-spectrum β-lactamase-producing *E. coli* strains with a 2-fold reduction in MIC of ciprofloxacin was reported,(Drago et al. 2013) herein, a 2.5-fold reduction in MIC of ciprofloxacin was observed in combination with compound **2** or **3** against ciprofloxacin-susceptible *E. coli*. In combination studies against *P. aeruginosa*, FICI of 0.48 and 0.16 for combination of ciprofloxacin with individual compounds **1** and **2** or **3**, respectively, and FICI of 0.04 for mixture of compounds with ciprofloxacin indicate synergism against *P. aeruginosa* (Table 5.2.). A 37-fold reduction in MIC of ciprofloxacin was reported in its combination with ivacaftor against a PAO-1 strain of *P. aeruginosa*,(Cho et al. 2018) however, ivacaftor is an expensive drug that is used for the treatment of cystic fibrosis. In combination studies against *S. typhi*, FICI of 0.48 for combination of all individual compounds with ciprofloxacin and FICI of 0.04 for the combined compounds **1**+**2**+**3** with ciprofloxacin indicate synergism against *S. typhi* (Table 5.2.). A 1.3-fold reduction in MIC of ciprofloxacin was reported in its combination with cefotaxime antibiotic against nalidixic acid-susceptible as well as nalidixic acid-resistant *S. typhi*(Kim et al. 2010) .We have obtained a MIC of 333.33 µg/mL for nalidixic acid and 0.46 µg/mL for ciprofloxacin against *S. typhi* (Table 5.1.), which suggest that the *S. typhi* MTCC 3216 strain used in the present study is a nalidixic acid-resistant *S. typhi*. However, in the present study, a 2.1-fold reduction in MIC of ciprofloxacin was observed in combination with either of the compounds **1** to **3** against *S. typhi*. Thus, based on the checkerboard assay results, a drastic reduction in MIC of ciprofloxacin was observed in individual combinations with compounds and a several fold reductions in MIC was observed by combination with all the three compounds, indicating the potentiating effect of compounds **1** to **3**. Though the synergistic studies of the present nature with phenazines and antibiotics is not reported for antibacterial studies, a combination study with redox active phenazine, pyocyanin, and polymyxin drug was shown to be detrimentally cytotoxic at clinically relevant concentrations (100 to 150 µM) (Mossine 2018). However, cytotoxicity evaluation using Hep G2 cell line confirmed that

phenazines (**1**, **2**) and benzamide (**3**) did not record any significant detrimental effects up to 250 µM on cell viability but at the same concentration ciprofloxacin exhibited 70% cell viability (Fig. 5.4.). Cell viability of 88 to 100% in Hep G2 cell lines was observed at the synergistic concentrations (MIC, Table 5.2.), observed against *S. aureus* and *E. coli*, for the individual combination of compounds (**1-3**) with ciprofloxacin (Fig. 5.3.).

The bactericidal and bacteriostatic activities, based on time-kill curves, were assessed by the reduction in $\log_{10}$CFU/mL, which represents the colony count of the test bacterial pathogens. An early bactericidal response in the combination studies of ciprofloxacin at synergistic concentrations (Table 5.2.) with individual compounds in comparison with ciprofloxacin alone was considered as the basis for efficacy of the combination results. Ciprofloxacin showed bactericidal activity at MIC between 8 to 48 h against *S. aureus*, however, in combination with compounds **2** or **3**, an early bactericidal response between 6 to 48 h was observed. A time-kill bactericidal effect was reported from 4 to 24 h for a combination of baicalein with ciprofloxacin against *S. aureus* (Chan et al. 2011), however, the concentration of ciprofloxacin in their combination was 4 µg/mL compared to 0.22 µg/mL of ciprofloxacin in combination with compounds **2** or **3**. Ciprofloxacin showed bactericidal activity at MIC between 10 to 48 h against *E. coli*, however, an early bactericidal response was observed in combination with compound **2** between 8 to 48 h, and in combination with compound **3** between 4 to 48 h. A synergistic bactericidal action was reported for combination of ciprofloxacin with amikacin or cefepime between 6 to 24 h against extended-spectrum β-lactamase-producing *E. coli* strains,(Drago et al. 2013) however, the studies were conducted with a final inoculum of 0.5 x $10^6$ CFU/mL compared to our present study with 1 x $10^6$ CFU/mL. Ciprofloxacin showed bactericidal activity at MIC between 6 to 10 h against *P. aeruginosa*, but remained bacteriostatic between 10 to 48 h. However, the bactericidal response lasted up to 48 h in combination with compounds, an early bactericidal response was observed in combination with compound **1** from 2 h and

compound **3** from 4 h, however, for compound **2**, the bactericidal activity was observed from 6 h. A synergistic bactericidal action was reported for combination of ciprofloxacin with meropenem against MDR *P. aeruginosa* strains between 6 to 24 h with MIC of ciprofloxacin being 32 µg/mL and with a final inoculum of 1 x $10^5$ CFU/mL(Cardoso et al. 2009),while ciprofloxacin with MIC between 0.07-0.22 µg/mL was used in combination studies with **1** to **3** with a final inoculum of 1 x $10^6$ CFU/mL of ciprofloxacin-susceptible *P. aeruginosa*. Ciprofloxacin showed bactericidal activity at MIC between 10 to 48 h against *S. typhi*, however, in combination with compounds **1** or **2**, an early bactericidal response between 8 to 48 h, and with compound **3** between 2 to 48 h was observed. Combination of ciprofloxacin (0.012–0.375 µg/mL) and cefotaxime was reported with bactericidal activity at 24 h against nalidixic acid-susceptible as well as nalidixic acid-resistant *S. typhi* (0.5 x $10^6$ CFU/mL)(Kim et al. 2010). Based on the time-kill assay results, the early bactericidal response in the combination studies of ciprofloxacin at synergistic concentrations (Table 5.2.) with individual compounds **1** to **3** were superior to the published reports *vide supra*.

In SEM analysis, mild changes in morphology such as dent formation, doughnut like formation, and change of surface morphology in *S. aureus* were observed in the case of treatment with NIIST-D75, crude organic extract, and isolated compounds, whereas in the case of *E. coli*., surface depression, change of surface morphology was observed (Fig. 5.2.). Similarly, mild dent formation was observed for the treatment of *S. aureus* with ciprofloxacin and cell elongation in *E. coli*. Next, we explored the effect of individual combination of ciprofloxacin at synergistic concentrations (MIC, Table 5.2.) with compounds **1** to **3** against *S. aureus* and *E. coli* with respect to changes in morphology by SEM analysis.

The combination of compound **1** and ciprofloxacin against *S. aureus* exhibited dent formation in some cells due to mechanical rupture of the membrane and cell wall (Qian et al. 2020),(Hartmann et al. 2010). The combination of ciprofloxacin and compound **2** or **3** or the

combination of three compounds exhibited severe changes in morphology like biofilm disruption, cell burst, and doughnut-like cell formation (Zhang et al. 2020),(Akbari et al. 2018),(Sarkar et al. 2016). Biofilms are clusters of bacterial cells adhering to a surface composed of exopolysaccharides, which give stability to biofilm. Detached cells observed in synergistic combinations of the present study indicate a disruption in biofilms. Another morphological change observed in this study was a doughnut-shaped ghost cell-bearing hole (Akbari et al. 2018). Doughnut-shaped cells with holes in *S. aureus* by the action of ciprofloxacin is attributed to production of reactive oxygen species, which first causes lipid membrane damage and eventually leads to DNA damage (Becerra, P, and Lar 2006).

Filamentation, a continuous elongation of *E. coli* cells was observed with ciprofloxacin (4 µm) in the present study and an enhanced elongation was detected with combinations. Elongation of *E. coli* cells is due to halting in DNA replication by inhibiting DNA gyrase, which induces SOS response and leads to inhibition of cell division (Diver 1986). Combinations of ciprofloxacin with compound **1** resulted in more elongation (9.07 µm) compared to ciprofloxacin, along with a ruptured cell, and compound **2** caused an elongation of 10 µm. Interestingly, the combination of compound **3** with ciprofloxacin exhibited a maximum elongation of 20.46 µm, causing complete distortion of cells with release of intracellular contents. The combination of all three compounds with ciprofloxacin resulted in elongation of 10.98 µm and change in membrane integrity. Protein leakage assay and cell membrane permeability evaluation suggests the cell rupturing, cell burst and leakage of intracellular contents are due to irreversible damages in the interior of cells that affects both DNA and cell membranes (Sun 2018).Thus, the combination of the compounds in the present study with ciprofloxacin and the drastic changes in morphology observed in SEM analysis (Fig. 5.2.), and the change in total surface area of the selected cells (Fig.5.3. ) against *S. aureus* and *E. coli* corroborate synergism.

## 5.5. CONCLUSION

In the present study, we described individual combinations of ciprofloxacin with phenazines (**1** and **2**) and benzamide (**3**) exerted significant inhibitory effect against *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi* with bactericidal activity. Further, by SEM analysis, we showed that the combinations displayed severe changes in morphology in *S. aureus* and *E. coli*. Combinational therapy employs an effective treatment strategy for many nosocomial bacteria, including multi-drug resistant microorganisms. Most importantly, if indiscriminate antibiotic usage is the main cause of antimicrobial drug resistance, a lower dose of antibiotics combined with the potentiators, revealed in the current study, by synergistic action harbinger disrupting the status quo in antibiotic therapeutic modalities. In conclusion, compounds **1**, **2** and **3** were shown explicitly with elaborate *in vitro* experiments as potentiators of ciprofloxacin for antibacterial activity with highly reduced dosage, which warrants validation of their combination in the *in vivo* studies.

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# CHAPTER 6

# Summary and conclusion

## 6.1. SUMMARY AND CONCLUSION

Antimicrobial resistance is currently a severe concern for public health, exacerbated by the Covid-19 outbreak, and requires an immediate response. Unquestionably, antibiotics are the best remedy to cope with the situation, but an increase in resistance and decreased number of new active antibiotic discoveries are the main problems. There are several different methods to increase the potential of existing antibiotics. This thesis mainly describes the utilization of naturally isolated compounds from Streptomyces crude organic metabolites and their enhanced antibacterial activity in combination with ciprofloxacin. The importance of streptomyces in natural compound isolation was proved many years ago, and here we utilized the same. The key findings of the current study are summarized as follows.

For the study, streptomyces NIIST G132 and streptomyces NIIST D75 were selected. The two selected bacteria were characterized; spore chain morphology was visualized by scanning electron microscopy. Streptomyces NIIST G132 possess nearly 50 spores in a chain with recti flexible and smooth surfaces. While streptomyces NIIST D75 possess non-filamentous aerial hyphae with a round, smooth-surfaced spore at the tip of each hypha. The colony morphology of two selected species was also observed; streptomyces NIIST G132 has white aerial mycelia with brown pigmented substrate mycelia producing diffusible pigment to agar. Streptomyces NIIST D75 possesses a yellow aerial mycelium with olive-brown pigmented substrate mycelium and diffusible yellow pigment in the medium. The study aimed to isolate antibacterial compounds from the selected organism to confirm the antibacterial activity; the crude organic extract was separated and tested against seven human pathogens. As expected, the crude organic extracts showed a broad spectrum of activity isolated from the two different organisms. Thus, the selected organisms were pursued further studies.

The next step was to investigate a better fermentation for streptomyces NIIST G132 for enhanced metabolite production with antibacterial activity. Five different fermentation media were selected, and fermentation was carried out. Yeast malt broth was shown to have better antibacterial activity compared to other media. Next, we standardized optimum physiological conditions like pH, incubation period, and temperature. Here, an optimum temperature of 30 ºC, pH of 6, and an incubation period of 7 days was observed for better antibacterial production. The significant media components of yeast malt broth were peptone, yeast extract, malt extract, and dextrose. So to statistically standardize the media components, we utilized the statistical software Minitab by using Response Surface Methodology employing Box Behnken Design. The media optimization of streptomyces NIIST G132 enhanced antibacterial activity of 33.66 ± 0.57 mm compared to unoptimized media with 20.33 ± 0.57 mm against *S. aureus*. Enhance metabolite production was also observed from 120 mg/l to 350 mg/l.

Further, GC-MS analysis was carried out to confirm and identify the active antibacterial compounds in the crude organic metabolite of streptomyces NIIST G132 characterization of methanolic extract. The presence of 15 major peaks was observed. Most of the compounds were antimicrobial 21.90% was covered by benzene acetamide which has already been reported for antimicrobial activity. 21% of area is shown by Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- which is a diketopiperazine known for antifungal activity. Then comes benzene acetic acid, with 11.82% also possessing antimicrobial activity. Thus, the identification of compounds shows the presence of antimicrobially active compounds. Crude organic extract of streptomyces NIIST D75 was purified through preparative TLC and silica gel column chromatography and afforded four compounds. The structural confirmation of isolated compounds was analyzed by NMR and confirmed by HRMS analysis. The isolated compounds include three phenazines and one benzamide. The compounds include compound **1** - 1 methoxy phenazine, compound **2**- methyl 6-methoxyphenazine-1-carboxylate, compound **3-** 2,3-

dimethoxy benzamide, and compound **4**-1,6-dimethoxyphenazine from these four compounds; compound **4** was not selected for further studies due to some solubility issues.

The minimum inhibitory concentration assay was carried out to analyze the antibacterial activity of compounds. Compared with some commercial antibiotics, the isolated compounds showed less antibacterial activity with higher MIC values in the 1000-111 µg/ml range. So, we decide to go for a combinational treatment by enhancing the antibacterial activity of compounds in combination with some known antibiotics. Here for the study, we selected ciprofloxacin because it is well-known and widely used, and WHO categorized the drug under the group of essential drugs. Combinational studies were carried out using a checkerboard assay against four selected human bacterial pathogens, including *S. aureus*, *E. coli*, *P. aeruginosa,* and *S. typhi*. Except for compound **1** in combination with ciprofloxacin against *E. coli,* all other combinations against selected bacterial species showed synergism. A significant reduction in the fold of ciprofloxacin was also observed. These results indicated that combining the compound with ciprofloxacin can be a choice against treating selected bacterial pathogens. The time-dependent activity study of the combinations with ciprofloxacin compared to ciprofloxacin was carried out to confirm the bactericidal or bacteriostatic activity. Except for compound **1** with ciprofloxacin, all combinations showed bactericidal activity. Here early/ extended bactericidal activity was observed for the synergistic combination compared to ciprofloxacin alone.

The SEM analysis was carried out to observe the extent of damage caused by the combination of compounds with ciprofloxacin against *S. aureus* and *E. coli*. Severe damages were observed for both cells in combinations compared to ciprofloxacin alone. Compared to ciprofloxacin-treated *S. aureus* cells, the damage was much more in cells treated with combinations. In ciprofloxacin-treated cells, dent formations were only observed, but the cells treated with combinations showed considerable damages like dent formations, deep depressions, hole formations, and biofilm disruptions. In the case of *E. coli,* the damages were more on the combinations

than on ciprofloxacin alone. Cells treated with ciprofloxacin showed an elongation of cells of 4 μm that increased to 20 μm in combinations. Thus, the enhanced activity of synergistic combinations was confirmed. Later cytotoxic studies of compounds alone and in combinations with ciprofloxacin against Hep G2 cell lines were carried out. Compounds alone showed 90% cell viability up to 250 μM, and 88–100% cell viability was observed for compounds in combination with ciprofloxacin, which confirmed the drugs are safe for further studies. A docking experiment was carried out to observe the interaction of these small drug molecules in combination with ciprofloxacin with the target DNA gyrase. Here, more docking scores and binding affinity were observed for compounds in combination with ciprofloxacin than ciprofloxacin alone. Thus, from the whole study, we observed the importance of natural compounds from streptomyces and their applications as antibacterial agents against human bacterial pathogens.

However, additional characterization of these combinations of compounds against multidrug-resistant bacteria is a future possibility. In the future, this may lead the way for new combination therapy for many human bacterial pathogens. Animal studies also need to be conducted to see the activity of the combination of drugs *in vivo* conditions. An increased spectrum of activity, less chance of resistance, a lower dose of antibiotics, and decreased side effects are essential features of combinational therapy. Thus, it is considered one of the therapy choices for treating increased antibiotic resistance.

## ANNEXURE I- Media composition

### Yeast Malt Broth (YMB) $gL^{-1}$

| | |
|---|---|
| Dextrose | 10 |
| Peptone | 5 |
| Malt extract | 3 |
| Yeast extract | 3 |
| Final pH 6.2 ± 0.2 at 25ºC | |

### Luria Bertani Broth (LBB) $gL^{-1}$

| | |
|---|---|
| Tryptone | 10 |
| Yeast Extract | 5 |
| Sodium chloride | 10 |
| Final pH 7.5 ± 0.2 at 25ºC | |

### Muller Hinton Agar (MHA) $gL^{-1}$

| | |
|---|---|
| Beef infusion | 300 |
| Casein acid hydrolysate | 17.5 |
| Starch | 1.5 |
| Agar | 17 |
| Final pH 7.3 ± 0.1 at 25ºC | |

### Tryptone Yeast Extract Broth (ISP 1) $gL^{-1}$

| | |
|---|---|
| Casein enzyme hydrolysate | 6 |
| Yeast Extract | 3 |
| Final pH 7.2 ± 0.2 at 25ºC | |

### Potato Dextrose Broth (PDB) $gL^{-1}$

| | |
|---|---|
| Potato infusion | 200 |
| Dextrose | 20 |
| Final pH 5.1 ± 0.2 at 25ºC | |

**Starch Casein Broth (SCB) $gL^{-1}$**

| | |
|---|---|
| Starch | 10 |
| $K_2HPO_4$ | 2.0 |
| $KNO_3$ | 2.0 |
| Casein | 0.3 |
| $MgSO_4.7H_2O$ | 0.05 |
| $CaCO_3$ | 0.02 |
| $FeSO_4.7H_2O$ | 0.01 |
| Agar | 17 |
| Final pH 7.2 ± 0.1 at 25ºC | |

**Sabouraud's Dextrose Broth (SDB) $gL^{-1}$**

| | |
|---|---|
| Dextrose | 20 |
| Meat Peptone | 10 |
| Final pH 5.6 ± 0.2 at 25ºC | |

## 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

| | |
|---|---|
| Name of the student : **Jesmina A S**<br>Faculty of study : **Science** | Registration No : **10BB17A39029**<br>Year of Submission : **2023** |
| AcSIR Academic Centre/CSIR Lab<br>Name of the Supervisor (s) | : CSIR NIIST, Thiruvananthapuram<br>: Dr. Ravi Shankar L, Dr. Dileep Kumar B S |
| Title of the thesis: Characterization and exploration of secondary metabolites from two *Streptomyces* species for enhanced antibacterial activity against selected human pathogens | |

Streptomyces NIIST G132 and streptomyces NIIST D75 were explored for the isolation of active metabolites. Statistical optimization of fermentation medium and media compositions of streptomyces NIIST G132 was carried out and increased metabolite production with increase in antibacterial activity was achieved through the statistical optimized medium compare to un-optimized media. GC-MS analysis of crude organic metabolite of streptomyces NIIST G132 was analysed and 15 antimicrobial active compounds were identified. Four compounds were isolated from the crude organic extract of streptomyces NIIST D75. Antibacterial activity of the three isolated compounds with ciprofloxacin was carried out to enhance the antibacterial activity of less active isolated compounds. Combinations showed low MIC with increased antibacterial activity than compounds alone. Influence of compounds in combinations was observed through SEM analysis against *S. aureus* and *E. coli*. Drastic morphological changes were observed compared to ciprofloxacin alone. 88-100% viable cells were observed while treatment with Hep G2 cell lines in combinations. For observing the interaction of ciprofloxacin with the compounds towards the target DNA gyrase was analyzed using docking analysis. The combination of compound **3** with ciprofloxacin shows maximum binding affinity and docking score compare to ciprofloxacin alone. Thus, the combination of compounds with ciprofloxacin shows increased antibacterial activity with enhanced or prolonged bactericidal

activity. Thus, the combination therapy designed through the work shows best activity compared to the known antibiotic alone.

## List of publications

### List of Publications from the thesis work

**A. 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.

### List of Publications not directly linked to the thesis

Dehannath Induja., **Jesmina A.R.S**.,Manu Joseph.,Shanmugan Shamjith.,Nagaraja Ingaladal.,Kaustabh Maithi., Kumar,B.D. and Ravi S. Lankalapalli. Isolation of two new stereochemical variants of streptophenazine by cocultivation of Streptomyces NIIST-D31, Streptomyces NIIST-D47and Streptomyces NIIST-D63 strains in 3C2 combinations. *The Journal of Antibiotics* 2023. DOI: 10.1038/s41429- 023-00638-7.

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. DOI: 10.1038/s41429-022-00547-1

Mohan, B., Salfeena, C.T.F., Ashitha, K.T., Krishnan, G.V., **Jesmina, A.R.S**., Varghese, A.M., Patil, S.A., Kumar, B.N.S.D. and Sasidhar, B.S. 2018. Functionalized pyrimidines from alkynes and nitriles: application towards the synthesis of marine natural product meridianin analogs. *ChemistrySelect*, *3*(23), pp.6394-6398.DOI: 10.1002/slct.201801126.

**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.

**Seminar Attended**

- International Seminar on "Life Sciences for Sustainable Development- Issues and Challenges" held at University College, Thiruvananthapuram, on October 3-5,2019 (Best poster award)

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Japan Antibiotics Research Association
The Society for Actinomycetes Japan

ARTICLE

# In vitro antibacterial effects of combination of ciprofloxacin with compounds isolated from *Streptomyces luteireticuli* NIIST-D75

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## Abstract

Three phenazines, 1-methoxyphenazine (**1**), methyl-6-methoxyphenazine-1-carboxylate (**2**), 1,6-dimethoxyphenazine (**4**), and a 2,3-dimethoxy benzamide (**3**) were isolated from the *Streptomyces luteireticuli* NIIST-D75, and the antibacterial effects of compounds **1-3**, each in combination with ciprofloxacin, were investigated. The in vitro antibacterial activity was assessed by microdilution, checkerboard, and time-kill assay against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*. According to the checkerboard assay results, each combination of compounds **1**, **2** and **3** with ciprofloxacin resulted in a significantly lower minimum inhibitory concentrations (MICs) of 0.02–1.37 μg $ml^{-1}$, suggesting synergistic combinations by fractional inhibitory concentration index, and displayed bactericidal activity in time-kill kinetics within 48 h. SEM analysis was carried out to determine the changes in morphology in *S. aureus* and *E. coli* during treatment with individual combination of ciprofloxacin and compounds (**1-3**), which revealed drastic changes in the cells such as dent formation, biofilm disruption, cell bursting, and doughnut-like formation, change in surface morphology in *S. aureus*, and cell elongation, cell burst with ruptured cell, and change in surface morphology in *E. coli*. Hep G2 cell viability was not affected by the compounds (**1-3**) that were tested for cytotoxicity up to 250 μM.

## Introduction

A decline in the approval of new antibiotics has resulted in a possibility for combination therapy, using existing drugs, as a potential strategy. Studies of synergism with existing antibiotics and approved drugs or novel antimicrobials can be an efficient route to combat invasive antimicrobial drug resistance. Reports of combinations of antibiotics with phages [1], polymers [2], quorum sensing inhibitors [3], non-antibiotic drugs [4], silver [5], polymyxins [6], essential oils [7], aminoglycosides [8], displayed potent antimicrobial synergism and facilitated a reduced dose of antibiotics. Combination of ciprofloxacin antibiotic, induced synergy, with other antibiotics viz. fosfomycin against ciprofloxacin-resistant *Shigella flexneri* isolates [9], tigecycline against *Vibrio vulnificus* sepsis [10], clarithromycin against *P.aeruginosa* [11], meropenem against multidrug-resistant (MDR) *P. aeruginosa* [12], cefotaxime against *V. vulnificus* sepsis and *Salmonella bacteremia* [13, 14], rifampicin and azithromycin against *S. aureus* [15], amikacin against extended-spectrum β-lactamase-producing *Escherichia coli* [16], and ceftazidime in the treatment of *P. aeruginosa*-infected orthopedic implants

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[17]. Among the non-antibiotic drugs, which were explored in combination with ciprofloxacin include celecoxib against *S. aureus* [18], ambroxol, protamine sulfate, ivacaftor against *P. aeruginosa* [19–21].

On the other hand, synergistic combination of existing antibiotics with natural products is a promising route to obtain leads in antibacterials. Synergism of curcumin, baicalein, esculetin, and cinnamaldehyde with ciprofloxacin was demonstrated at sub-inhibitory concentrations against *P. aeruginosa* biofilms [22]. Synergistic activity of an inhalable powder by co-spray drying *Pseudomonas* phage PEV20 with ciprofloxacin was shown in vivo in acute lung infection by *P. aeruginosa* [23]. Thyme and peppermint essential oils exhibited promising biofilm inhibitory effect in *Klebsiella pneumoniae*, in combination with ciprofloxacin [24]. Synergistic antibacterial effect was observed for the combination of ciprofloxacin with berberine against MDR *K. pneumoniae* with activities of 18% synergy and 77% additivity [25]. Synergistic effects of baicalein with ciprofloxacin was reported against 12 out of 20 clinical ciprofloxacin resistant strains [26]. Synergy of methanolic extract of *Punica granatum* fruit pericarp combination with ciprofloxacin was demonstrated against Gram-negative bacilli [27]. A combination effect of maggot metabolites of *Luciliacuprina blowfly* and ciprofloxacin was demonstrated at sub-MIC levels on *S. aureus* [28]. 2-(2-Aminophenyl)indole, a microbe-derived natural product, combination with ciprofloxacin exhibited promising activity as bacterial efflux pump inhibitor for *S. aureus* infections [29]. Surprisingly, synergistic antibacterial effects of microbe-derived natural products in combination with ciprofloxacin is less explored.

Actinomycetes are regarded as prolific producers of antimicrobials, but their vast untapped diversity awaits exploitation [30]. In this regard, a promising area for the discovery of new antibiotics is the isolation of potent microbes from ecologically important niches. The 180,000 $km^2$ stretch of Western Ghats region of India is renowned for its rich flora and fauna [31], however, the microbial wealth from this area for novel antimicrobials remain underexploited [32]. In our quest for isolation of novel antibiotics from the Malampuzha forest area of Western Ghats region of Kerala [33], the present study deals with the isolation of secondary metabolites from a *Streptomyces luteireticuli* NIIST-D75 and exploration of their antibacterial effects. Together with the NIIST-D75 strain, 300 more *Streptomyces* strains were isolated from the same area, of which 25 recorded potential in vitro antagonism (unpublished data), which beckon Malampuzha forest area of Western Ghats region of Kerala as the 'promised land' for potent antimicrobial strains. Recently, we published the isolation of a novel aureothin derivative from *Streptomyces sp*. NIIST-D31 [33]. Three phenazines, 1-methoxyphenazine, methyl-6-methoxyphenazine-1-carboxylate, 1,6-dimethoxyphenazine, and a 2,3-dimethoxy benzamide were isolated from *S. luteireticuli* NIIST-D75, and the antibacterial effects of the isolated compounds in combination with ciprofloxacin was explored against selected human pathogenic bacteria.

## Materials and methods

### Microorganism

The strain, designated as NIIST-D75 was isolated from the topsoil sample collected from the Malampuzha forest area (10°83′78.19″N, 76°66′19.96″E) of Palakkad district in Kerala of the Western Ghats region of India. Observation of morphology of the strain was carried out by Compound Microscope (Olympus CX41RF, Olympus Corporation, Japan) and by Scanning Electron Microscope (SEM, Carl Zeiss EVO 18 Research, Germany). Identification of NIIST-D75 strain was carried out by 16 S rRNA sequencing and phylogenetic analysis. The strain was maintained in potato dextrose agar (PDA) slants at 4 °C for further studies.

### Culture conditions and isolation of crude organic extract

Homogenized inoculum of NIIST-D75 (5 ml), grown in Yeast Malt Broth (YMB) medium for 6 days, was inoculated to 500-ml flask containing 150 ml of YMB medium and incubated for 12 days at 160 rpm and 30 °C in an Incubator Shaker (Lab Companion IS97IR, Jeio Tech, Korea). Cell free culture filtrate was collected after centrifugation at 10,000 *g* for 10 min in a Centrifuge (Kubota 7780, Kubota Corporation, Tokyo) and extracted thrice with ethyl acetate:methanol (95:5, 150 ml). The organic layer was concentrated in a Büchi Rotavapor® and the crude organic extract was obtained. Repetition of culture conditions up to 7 l of fermentation broth afforded 1.39 g of crude organic extract as a first batch, similarly, a second batch yielded 2.2 g.

### Purification and structural characterization

The crude organic extract from the first batch was subjected to preparative TLC (TLC Silica gel 60 $F_{254}$), developed using ethyl acetate:hexane (7:3), to obtain a major band that was visualized by short wavelength UV. Repetition of preparative TLC afforded a total of 500 mg of this major band, which was subjected to purification by silica gel column chromatography (100–200 mesh) using hexane:ethyl acetate (v/v) gradient elution of 100:0, 95:5, 90:10, 85:15 and 80:20, which afforded four major compounds designated as compound **1**, compound **2**, compound **3**, and compound **4**. However, for the second batch, silica

gel column chromatography was adopted for obtaining the compounds. The isolated compounds were dissolved in $CDCl_3$, 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 ($\delta$) 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. 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.

## Test bacterial pathogens

Gram-positive bacterial strain *S. aureus* MTCC 902, and three Gram-negative bacteria viz. *E. coli* MTCC 2622, *P. aeruginosa* MTCC 2642, *Salmonella typhi* MTCC 3216 were used in the present study. All the test bacterial pathogens were procured from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India.

## Minimum inhibitory concentration (MIC) assay

The antibacterial activity of compounds and their combination were carried out by microdilution method against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* [34], with an indicator dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to determine the MIC in 96-well plates. A serial three-fold dilution using Milli-Q® water resulted in test concentrations of 333.33 to 0.017 μg ml$^{-1}$ in 10 wells from stock solution of 1000 μg ml$^{-1}$ in methanol for compound **2** and **3**. Similarly, test concentrations were prepared from stock solution of 1000 μg ml$^{-1}$ for ampicillin in distilled water, ciprofloxacin in 0.1 N HCl, and nalidixic acid in ethanol. A serial three-fold dilution using Milli-Q® water resulted in test concentrations of 1000.00 to 0.05 μg ml$^{-1}$ in 10 wells from stock solution of 3000 μg ml$^{-1}$ in methanol for compound **1**. Methanol with three-fold dilution in 10 wells horizontally was used as a control. The test pathogens in LBB (Luria-Bertani broth) were incubated at 37 °C for 18 h in the Incubator (Sanyo $CO_2$ Incubator MCO-20 A/C, Sanyo Electric Co Ltd, Japan), and adjusted to $1 \times 10^6$ CFU ml$^{-1}$ using the spectrophotometer (Synergy4, BioTek Instruments, USA) at 600 nm, which is equivalent to 0.5 McFarland standard. Bacterial inoculum (150 μl) was then added to the wells containing 150 μl of the test concentration and incubated at 37 °C. After 24 h of incubation, 20 μl of MTT dye (5 mg ml$^{-1}$ in distilled water) was added and the colour change is visually noted, wherein, yellow colour indicates inhibition and purple colour indicates bacterial viability. MIC is the lowest concentration at which there was no visible bacterial growth. The assay was performed in duplicate along with standard antibiotics. At the working test concentrations, we found that compound **4** is partially soluble, hence, this compound was not considered for antibacterial studies.

## Checkerboard assay

The interaction between three compounds individually and their combination with ciprofloxacin was evaluated through checkerboard assay against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* with a modified method [35]. The stock solution of 333.33 μg ml$^{-1}$ for three compounds and their combination in methanol, and 2 μg ml$^{-1}$ for ciprofloxacin in 0.1 N HCl was prepared. The compounds and ciprofloxacin were subjected to three-fold serial dilution using Milli-Q® water separately in 2 ml tubes and 100 μl from each tube were added to 96 well plate horizontally with ciprofloxacin (0.666 to 0.0009 μg ml$^{-1}$) and vertically with individual compounds and their combination (111.11 to 0.050 μg ml$^{-1}$), and then 50 μl of $1 \times 10^6$ CFU ml$^{-1}$ bacterial inoculum was added. Column eleven was kept as a growth control where 50 μl of $1 \times 10^6$ CFU ml$^{-1}$ bacterial inoculum was added to 200 μl of Milli-Q® water and column twelve contain 200 μl Milli-Q® water as a sterility control. The plates were incubated at 37 °C, after 24 h of incubation, 20 μl of MTT dye (5 mg ml$^{-1}$ in distilled water) was added and MIC was determined as above. The interaction between three individual compounds and their combination with ciprofloxacin was interpreted by Fractional Inhibitory Concentration Index (FICI), calculated by the equation:

FICI = FIC of compound/s + FIC of ciprofloxacin; FIC of compound/s = (MIC of combination of compound/s with ciprofloxacin ÷ MIC of compound/s alone) and FIC of ciprofloxacin = (MIC of combination of ciprofloxacin with compound/s ÷ MIC of ciprofloxacin alone).

FICI interpretation: FICI ≤0.5: Synergy; FICI > 0.5 and <4.0: Indifference; FICI ≥4.0: Antagonism.

## Time-kill assay

The rate of killing of four test bacterial pathogens viz. *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* by ciprofloxacin and three compounds at concentrations of MIC, ½MIC, and MIC of individual combination of compounds with ciprofloxacin were carried out by time-kill assay [36]. The respective MICs of compounds, ciprofloxacin and combinations were prepared by dilution with LBB from stock solution of 3 mg ml$^{-1}$ of compound **1** in methanol, 1 mg ml$^{-1}$ of compound **2** and **3** in methanol, 10 μg ml$^{-1}$ of ciprofloxacin in 0.1 N HCl. 100 μl of bacterial inoculum in LBB, adjusted to $1 \times 10^6$ CFU ml$^{-1}$, as above, was added to 1 ml of LBB containing the respective concentration of compounds, ciprofloxacin, and combinations in 1.5 ml Eppendorf® microcentrifuge tubes. The control contains 1 ml of LBB with 100 μl of bacterial inoculum in LBB. The resulting tubes

were then incubated at 37 °C for 48 h and 100 µl was aliquoted at different time intervals (0, 2, 4, 8, 10, 12, 24, and 48 h) and each aliquot was tenfold serially diluted for seven times with Milli-Q® water and a volume of 100 µl from each dilution was plated on LBA and incubated for 24 h at 37 °C and colonies were counted manually. The colony counts represented as $\log_{10}$CFU $ml^{-1}$ were plotted on *Y*-axis on a logarithmic scale and the time interval on X-axis. CFU $ml^{-1}$ was calculated as follows:

CFU $ml^{-1}$ = (No. of colonies x dilution factor)/(volume of sample plated in ml)

A ≥3 $\log_{10}$CFU $ml^{-1}$ reduction in colony counts from the original inoculum (control) is considered bactericidal and a ≤3 $\log_{10}$CFU $ml^{-1}$ reduction is bacteriostatic.

## Scanning electron microscopy (SEM) analysis

The changes in the morphology of *S. aureus* and *E. coli* by NIIST-D75, crude organic extract, MICs of compounds, their combination, ciprofloxacin, and synergistic combinations were analyzed by SEM (Carl Zeiss EVO 18 Research, Germany) [37]. The SEM analysis results were compared against the morphology of *S. aureus* and *E. coli*. The MICs were prepared by dilution with LBB from a stock solution of 333.33 µg $ml^{-1}$ for three compounds and their combination in methanol, and 2 µg $ml^{-1}$ for ciprofloxacin in 0.1 N HCl. 100 µl of bacterial inoculum in LBB, adjusted to $1 \times 10^6$ CFU $ml^{-1}$, as above, was added to 1 ml of LBB containing the respective MICs of compounds (**1** to **3**), their combination, ciprofloxacin and synergistic combinations in 1.5 ml Eppendorf® microcentrifuge tubes. The control contains 1 ml of LBB with 100 µl of bacterial inoculum in LBB. The resulting tubes were then incubated at 37 °C for 24 h.

Agar overlay method [38] was carried out by spot inoculation of NIIST-D75 in the center of the ISP-2 (International Streptomyces Project-2) agar Petri plate and grown at 30 °C for 7 days in the Incubator (Rotek-RCI 12 S, B & C Industries, India). The respective ISP-2 agar Petri plates containing spotted NIIST-D75 was overlaid with the bacterial inoculum in LBB, adjusted to $1 \times 10^6$ CFU $ml^{-1}$, as above, in 10 ml of MHA (Mueller Hinton agar) medium, and incubated. For disc diffusion method [39], bacterial inoculum in LBB, adjusted to $1 \times 10^6$ CFU $ml^{-1}$, as above, was swabbed to MHA agar plates and to the centre of Petri plates was introduced 6 mm disc impregnated with 30 µl of 10 mg $ml^{-1}$ of crude organic extract (dissolved in 100% methanol), and incubated. After incubation for 24 h at 37 °C, the bacterial cells were collected using a sterile inoculation loop from the edge of the ZoI and added to 1 ml of LBB in 1.5 ml Eppendorf® microcentrifuge tubes.

The respective tubes with bacteria after incubation were resuspended in 500 µl of 0.1 M phosphate-buffered saline (PBS) at pH 7.4, and centrifuged at 3000 *g* for 5 min. After decanting the supernatant, the cells were then fixed with 50 µl of 2% glutaraldehyde and incubated for 12 h at 4 °C, followed by resuspending cells in PBS and centrifugation as above, and post-fixation, a 50 µl of 1% osmium tetroxide was added and incubated for 1 h at 4 °C. Subsequently, the cells were treated with 500 µl of a series of ethanol concentrations of 30%, 50%, 75%, 95%, and 100% for removal of water by centrifuging at 3000 *g* for 10 min. The resulting cells were stored in 100% ethanol, and 20 µl from each tube were drop-casted over the stub and dehumidified for 20 min under vacuum and sputter-coated with gold for SEM analysis. The change in morphology of *S. aureus* and *E. coli* upon treatment with individual combination of compounds (**1** to **3**) with ciprofloxacin at their synergistic concentrations were quantified using respective SEM images by ImageJ microscopic image analyzing tool. The SEM image was first calibrated using the scale bar, a selected area was analyzed by setting a threshold, and then by running the analyze particle command the surface area was found, which was used to construct the histogram.

## Cytotoxicity assay

Cytotoxicity of three compounds (**1–3**), their individual combination with ciprofloxacin at synergistic concentrations observed against *S. aureus* and *E. coli*, and ciprofloxacin alone were evaluated with Hep G2 cell line, procured from National Centre for Cell Sciences (NCCS), Pune, India. The cell line was cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), and antibiotics (100 µg $ml^{-1}$ of penicillin and 100 µg $ml^{-1}$ of streptomycin) in a humidified incubator with 95% air and 5% $CO_2$ at 37 °C [40]. Cell viability was evaluated through MTT assay by seeding in 96 well plate at a density of $5 \times 10^3$ cells per well. Cells after 60–70% confluence were exposed to different concentrations of compounds ranging from 1–500 µM and incubated in a humidified incubator (New Brunswick Galaxy 170 S, Eppendorf, Germany) for 24 h with 95% air and 5% $CO_2$ at 37 °C. A 10 mM stock solution of compounds were diluted with Milli-Q® water and further diluted with EMEM such that the final working concentration contains 0.1% methanol, and the stock solution for ciprofloxacin was 10 mM in 0.1 N HCl. A 10 µg $ml^{-1}$ stock solution of compounds, ciprofloxacin was diluted with EMEM to arrive at MIC concentrations of the synergistic combination. After incubation time, medium was removed and cells were washed with Hank's balanced salt solution (HBSS) and incubated for 4 h with 5 mg/mL of MTT, dissolved in serum free EMEM. Then cells were again washed with 100 µl HBSS and 100 µL DMSO was added, gently shaken for 20 min for complete dissolution and absorbance

was recorded at 570 nm using microplate spectrophotometer (Synergy 4, Biotek, USA).

## Results

### Microorganism

Based on the morphology, NIIST-D75 strain was a Gram-positive, spore forming with pale yellow aerial mycelium, and olive brown pigmented substrate mycelium producing diffusible yellow pigment in the medium. SEM analysis confirmed smooth round spore at the tip of the aerial mycelia (Fig. S1, Supplementary Material). The 16 S rRNA sequencing (NCBI Accession number: ON054043) and phylogenetic tree construction identified the NIIST-D75 strain as *Streptomyces luteireticuli* (Fig. S2, Supplementary Material).

### Purification and characterization of compounds

Purification of the major band (500 mg), from the crude organic extract of the first batch, by silica gel column chromatography afforded 2.0 mg of compound **1**, 1.3 mg of compound **2**, 6.0 mg of compound **3**, and 3.0 mg compound **4**. From the second batch 8.0 mg of compound **1**, 3.2 mg of compound **2**, and 5.0 mg of compound **3** were obtained. $^{1}H$ and $^{13}C$ NMR analysis (Fig. S3–6, Supplementary Material) suggested compound **1** as 1-methoxyphenazine, compound **2** as methyl 6-methoxyphenazine-1-carboxylate, compound **3** as 2,3-dimethoxy benzamide, and compound **4** as 1,6-dimethoxyphenazine (Fig. 1), which were further confirmed by HR-ESI-MS analysis.

### MIC assay

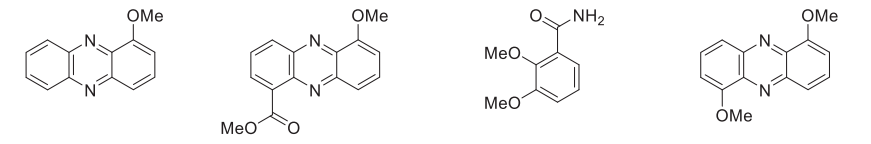
MIC values recorded for compounds **1**, **2** and **3**, individually, against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* were in the range of 111.11 to 1000.00 μg ml$^{-1}$, whereas, the combination of all three compounds exhibited an MIC of 37.03 μg ml$^{-1}$ (Table S1, Supplementary Material). In comparison, the commercial antibiotic ampicillin exhibited MIC of 1.37 μg ml$^{-1}$ against *S. aureus*, *P. aeruginosa* and *S. typhi*, and 37.03 μg ml$^{-1}$ against *E. coli*. Ciprofloxacin exhibited MIC of 0.46 μg ml$^{-1}$ against *P. aeruginosa* and *S. typhi*, 0.05 μg ml$^{-1}$ against *E. coli*, and 1.37 μg ml$^{-1}$ against *S. aureus*. Nalidixic acid exhibited MIC of 12.35 μg ml$^{-1}$ against *S. aureus* and *P. aeruginosa*, 1.37 μg ml$^{-1}$ against *E. coli*, and 333.33 μg ml$^{-1}$ against *S. typhi*.

### Checkerboard assay

MIC and calculated FICI values against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* obtained through checkerboard assay for combination of compounds **1**, **2**, **3** with ciprofloxacin are shown in Table 1. The respective values of MIC in combination of compounds with ciprofloxacin are as follows: MIC of compound **1** was 0.05 μg ml$^{-1}$ against all four test bacterial pathogens. MIC of compound **2** was 0.15 μg ml$^{-1}$ against *S. aureus*, *E. coli* and *S. typhi*, and 1.37 μg ml$^{-1}$ against *P. aeruginosa*. MIC of Compound **3** was 0.45 μg ml$^{-1}$ against *S. aureus*, *E. coli* and *S. typhi*, and 1.37 μg ml$^{-1}$ against *P. aeruginosa*. MIC of sum of three compounds was 0.15 μg ml$^{-1}$ against all four test bacterial pathogens; and ciprofloxacin exhibited MIC values of 0.02, 0.07 and 0.22 μg ml$^{-1}$. The FICI values in these combination studies, calculated from MIC, were as follows: compound **1** exhibited 0.16, 1.40, 0.48, and 0.48 for *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi*, respectively. Compound **2** and **3** each exhibited 0.16, 0.40, 0.16, and 0.48 for *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi*, respectively. The sum of all three compounds exhibited 0.01, 0.40, 0.04, and 0.04 for *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi*, respectively.

### Time-kill assay

The time-kill assay colony counts for the test bacterial pathogens *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* are shown in Fig. 2, and details of the reduction in $\log_{10}$CFU ml$^{-1}$ are provided in Table S3 (Supplementary Material). The $\log_{10}$CFU ml$^{-1}$ value of the original inoculum (control) displayed a steady increase in growth from 6.04 (0 h) to 7.80 (48 h), 6.52 (0 h) to 6.96 (48 h), 6.34 (0 h) to 6.96 (48 h), and 6.27 (0 h) to 6.96 (48 h) for *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi*, respectively (Table S2, Supplementary Material). In general, the individual compounds **1**, **2** and **3** exhibited bacteriostatic activity against all the four test bacterial pathogens for the entire time period up to 48 h at MIC and ½MIC, compound **2** exhibited bactericidal activity against *S. aureus* between 24 to 48 h at MIC and at 48 h at ½MIC.




1-Methoxyphenazine (1) | Methyl 6-methoxy-phenazine-1-carboxylate (2) | 2,3-Dimethoxy benzamide (3) | 1,6-Dimethoxyphenazine (4)

**Fig. 1** Chemical structures of the four compounds isolated from NIIST-D75

**Table 1** FICI of combination of compounds **1**, **2**, and **3** with ciprofloxacin

| Test bacteria | Combination | MIC | | Fold reduction in MIC | FICI | Interpretation |
|---|---|---|---|---|---|---|
| | | Alone μg $ml^{-1}$ | Combination μg $ml^{-1}$ | | | |
| *S. aureus* | Compound **1** | 1000 | 0.05 | 20,000 | 0.16 | Synergism |
| | Ciprofloxacin | 1.37 | 0.22 | 6.2 | | |
| | Compound **2** | 111.11 | 0.15 | 740.7 | 0.16 | Synergism |
| | Ciprofloxacin | 1.37 | 0.22 | 6.2 | | |
| | Compound **3** | 333.33 | 0.45 | 740.7 | 0.16 | Synergism |
| | Ciprofloxacin | 1.37 | 0.22 | 6.2 | | |
| | Compound **1** + **2** + **3** | 37.03 | 0.15 | 246.9 | 0.01 | Synergism |
| | Ciprofloxacin | 1.37 | 0.02 | 68.5 | | |
| *E. coli* | Compound **1** | 1000 | 0.05 | 20,000 | 1.40 | Indifference |
| | Ciprofloxacin | 0.05 | 0.07 | * | | |
| | Compound **2** | 333.33 | 0.15 | 2222.2 | 0.40 | Synergism |
| | Ciprofloxacin | 0.05 | 0.02 | 2.5 | | |
| | Compound **3** | 333.33 | 0.45 | 740.7 | 0.40 | Synergism |
| | Ciprofloxacin | 0.05 | 0.02 | 2.5 | | |
| | Compound **1** + **2** + **3** | 37.03 | 0.15 | 246.9 | 0.40 | Synergism |
| | Ciprofloxacin | 0.05 | 0.02 | 2.5 | | |
| *P. aeruginosa* | Compound **1** | 1000 | 0.05 | 20000 | 0.48 | Synergism |
| | Ciprofloxacin | 0.46 | 0.22 | 2.1 | | |
| | Compound **2** | 111.11 | 1.37 | 81.10 | 0.16 | Synergism |
| | Ciprofloxacin | 0.46 | 0.07 | 6.6 | | |
| | Compound **3** | 333.33 | 1.37 | 243.3 | 0.16 | Synergism |
| | Ciprofloxacin | 0.46 | 0.07 | 6.6 | | |
| | Compound **1** + **2** + **3** | 37.03 | 0.15 | 246.9 | 0.04 | Synergism |
| | Ciprofloxacin | 0.46 | 0.02 | 23 | | |
| *S. typhi* | Compound **1** | 1000 | 0.05 | 20,000 | 0.48 | Synergism |
| | Ciprofloxacin | 0.46 | 0.22 | 2.1 | | |
| | Compound **2** | 111.11 | 0.15 | 740.73 | 0.48 | Synergism |
| | Ciprofloxacin | 0.46 | 0.22 | 2.1 | | |
| | Compound **3** | 333.33 | 0.45 | 740.73 | 0.48 | Synergism |
| | Ciprofloxacin | 0.46 | 0.22 | 2.1 | | |
| | Compound **1** + **2** + **3** | 37.03 | 0.15 | 246.9 | 0.04 | Synergism |
| | Ciprofloxacin | 0.46 | 0.02 | 23 | | |

Values are average of two readings

*Increase in MIC by 1.4-fold

Ciprofloxacin showed bactericidal activity at MIC between 8 to 48 h, 10 to 48 h, 6 to 10 h, and 10 to 48 h against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi*, respectively. Additionally, at ½MIC, ciprofloxacin showed bactericidal activity between 8 to 48 h, 8 to 10 h, and 10 to 48 h against *S. aureus*, *P. aeruginosa*, and *S. typhi*, respectively, however, bacteriostatic activity was observed up to 48 h against *E. coli*. In general, individual combination of compounds with ciprofloxacin at synergistic concentrations (Table 1) showed bactericidal action against all the four test bacterial pathogens. The time period where the bactericidal action was observed in combinations were 8 to 48 h with compound **1**, and 6 to 48 h with compound **2** or **3** against *S. aureus*; 8 to 48 h with compound **2**, and 4 to 48 h with compound **3** against *E. coli*; 2 to 48 h, 6 to 48 h, and 4 to 48 h with compounds **1**, **2**, and **3**, respectively, against *P. aeruginosa*; 8 to 48 h with compound **1** or **2**, 2 to

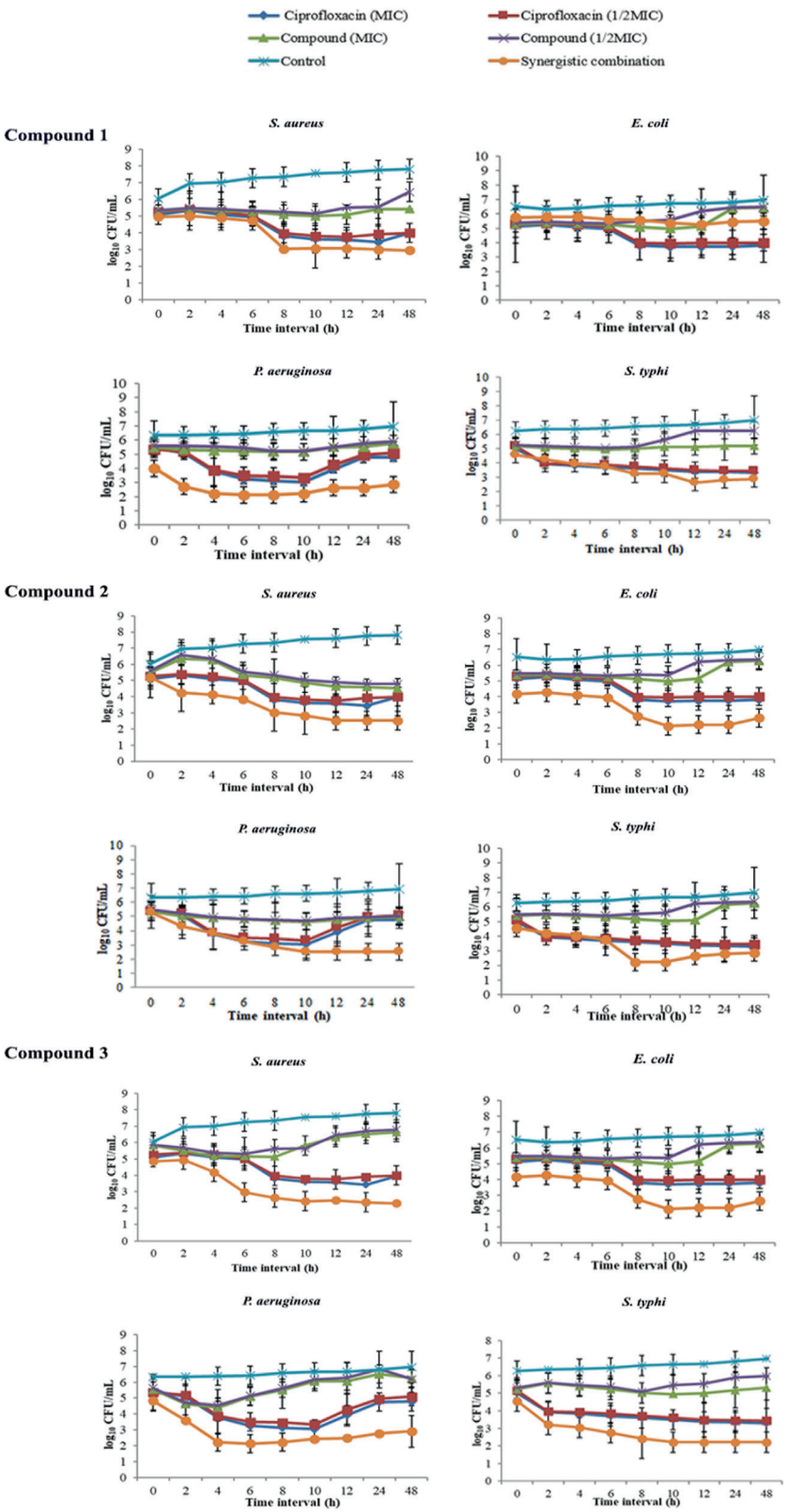



**Fig. 2** Time-kill kinetics of compounds **1**, **2**, and **3** in combination with ciprofloxacin against *S. aureus, E. coli, P. aeruginosa* and *S. typhi*. Each data points at a different hour represents the mean number of viable bacterial counts for triplicate experiments, and the error bar represents the standard deviations

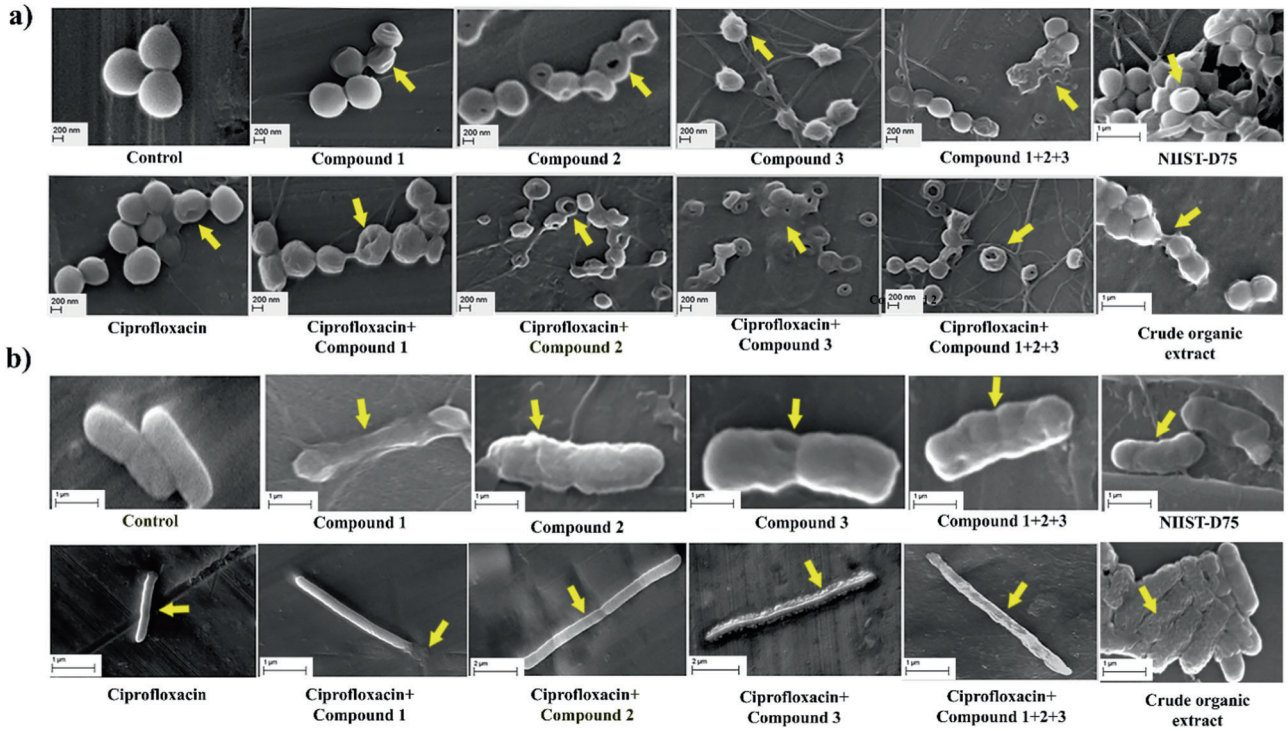



**Fig. 3** Changes in morphology of (**a**) *S. aureus* and (**b**) *E. coli* upon treatment with MIC concentrations of compounds **1**, **2**, **3**, their combination, ciprofloxacin, synergistic combinations, NIIST-D75, and crude organic extract

48 h with compound **3** against *S. typhi*. As an exception in combinations, a bacteriostatic activity was observed for compound **1** up to 48 h against *E. coli*.

## SEM analysis

Treatment of *S. aureus* and *E. coli* with NIIST-D75 resulted in dent formation and surface depression, respectively (Fig. 3a, b), whereas, the crude organic extract resulted in a change of surface morphology of the organisms (Fig. 3a, b). SEM image of *S. aureus* displayed a round, smooth surface cells with normal structural integrity and cell size (593.2 nm) and *E. coli* displayed a rod shape with a continuous smooth outer membrane (1.46 µm length), and both possessing a normal surface morphology serves as the control (Fig. 3a, b).

Mild changes in morphology in *S. aureus* were observed with compounds such as dent formation with compound **1**, doughnut like formation with compound **2**, and change of surface morphology with compound **3** as well as in the combination of three compounds (Fig. 3a). Similarly, treatment of *S. aureus* with ciprofloxacin resulted in a mild dent formation (Fig. 3a). In synergistic combination of compound **1** with ciprofloxacin, a mild dent formation was observed (Fig. 3a), whereas drastic changes in morphologies were observed with synergistic combinations pertaining to compound **2**, compound **3** and in the combination of three compounds such as biofilm disruption, cell bursting and doughnut like formation. The change in surface morphology in *S. aureus* displayed a decrease in surface area drastically from compound **1** to **3** with synergistic combinations compared to ciprofloxacin alone (Fig. 4a).

Mild changes in morphology in *E. coli* were observed with compounds such as change in surface integrity with compound **1**, cell surface depression with compound **2**, compound **3** as well as in the combination of three compounds (Fig. 3b). Treatment of *E. coli* with ciprofloxacin resulted in cell elongation of 4 µm (Fig. 3b). However, drastic changes in morphologies with synergistic combination of compounds with ciprofloxacin were observed (Fig. 3b). The synergistic combination of compound **1** exhibited cell elongation (9.07 µm) with cell burst, compound **2** exhibited cell elongation (10 µm), compound **3** exhibited cell elongation (20.46 µm) with ruptured cell, and in the combination of three compounds cell elongation with change in surface morphology was observed. The change in surface morphology in *E. coli* with synergistic combinations of **1** or **2** were in par with ciprofloxacin surface area, however, the combination with **3** displayed a drastic increase in surface area (Fig. 4b).

## Cytotoxicity assay

Cytotoxicity studies with Hep G2 cell line confirmed that all the compounds displayed ~90% cell viability up to 250 µM

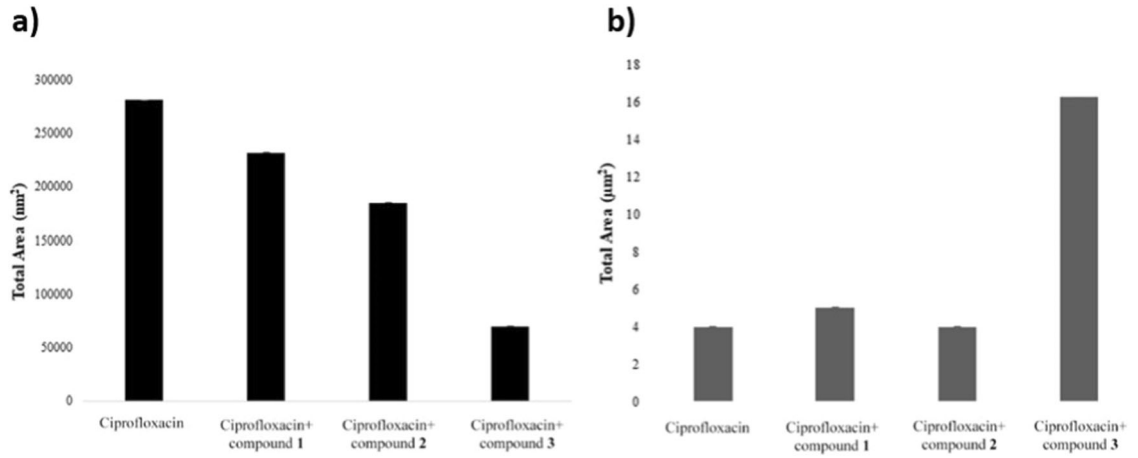



**Fig. 4** Changes in cell surface area (measured from the corresponding SEM images) upon treatment with ciprofloxacin and its combination with individual compounds **1** to **3** for (**a**) *S. aureus*, and (**b**) *E. coli*

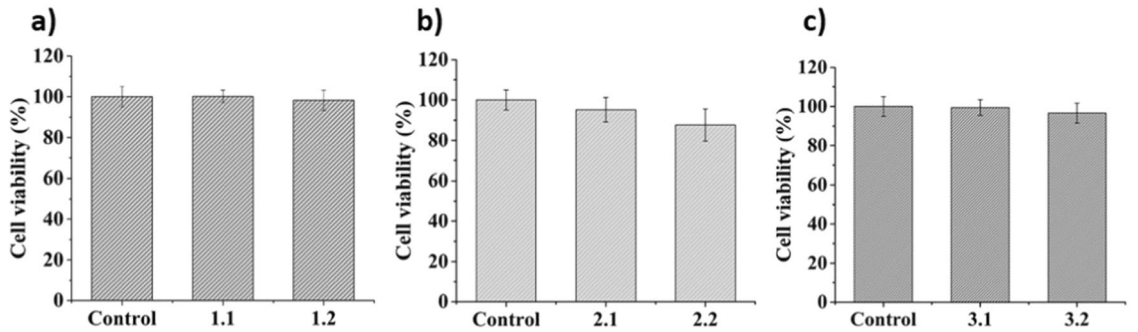



**Fig. 5** Cell viability assay on Hep G2 cells treated with synergistic concentrations (MIC, Table 1) of individual combination of compounds **1**, **2**, and **3** with ciprofloxacin. **a**) **1.1** (MICs of compound **1**, ciprofloxacin against *E. coli*), **1.2** (MICs of compound **1**, ciprofloxacin against *S. aureus*); **b**) **2.1** (MICs of compound **2**, ciprofloxacin against *E. coli*), **2.2** (MICs of compound **2**, ciprofloxacin against *S. aureus*); and **c**) **3.1** (MICs of compound **3**, ciprofloxacin against *E. coli*), **3.2** (MICs of compound **3**, ciprofloxacin against *S. aureus*). All data are represented as mean ± SD ($n = 6$)

but ciprofloxacin displayed ~70%. However, at 500 µM, ciprofloxacin recorded 55.59% cell viability, whereas compound **1**, **2**, and **3** exhibited 75%, 94%, and 85%, respectively (Fig. S7, Supplementary Material). The synergistic combination of individual compounds with ciprofloxacin at their respective concentrations (MIC, Table 1) revealed 88 to 100% cell viability (Fig. 5a–c).

## Discussion

Four compounds were isolated from the crude organic extract of the *S. luteireticuli*, three phenazines (compound **1**, **2** and **4**) and one benzamide (**3**). Compound **1**, **2** (a.k.a. mycomethoxin B) and **4** were reported from *S. luteoreticuli* [41, 42]. We recently reported compound **3** from another *Streptomyces* sp. NIIST-D31 [33], from the same soil location where NIIST-D75 was collected. Interestingly, related benzamides were reported from *Streptomyces* species with antibacterial activity [43, 44]. We have demonstrated earlier that phenazines in combination with azoles exhibit synergistic activity against *Candida* species [45]. Accordingly, motivated by sheer curiosity, phenazines were tested for synergistic activity in combination with ciprofloxacin in the present study. Antibacterial activity of isolated compounds (**1–3**) and their individual combination with ciprofloxacin was carried out against four bacterial pathogens viz. *S. aureus, E. coli, P. aeruginosa*, and *S. typhi*, which are known to cause nosocomial infections.

A significant reduction in MIC values were noted (Table 1) in combination studies for both ciprofloxacin and compounds, checkerboard assay, compared to individual treatment to test bacterial pathogens (Table S1, Supplementary Material). FICI of 0.16 for the individual combination of compounds with ciprofloxacin and FICI of 0.01 for the mixture of compounds **1** + **2** + **3** with ciprofloxacin indicate synergism against *S. aureus* (Table 1). Combination of natural product baicalein with ciprofloxacin that

exhibited a 2- to 4-fold reduction in MIC of ciprofloxacin against community-associated, ciprofloxacin-susceptible (CA)-MRSA strains was reported [46], in the present study, a 6.2-fold reduction in MIC of ciprofloxacin in combination with compound **1** to **3** against ciprofloxacin-susceptible *S. aureus* was observed. In combination studies against *E. coli*, FICI of 1.40 for combination of compound **1** with ciprofloxacin indicate indifference and FICI of 0.40 for the rest of the combinations indicate synergism against *E. coli* (Table 1). Synergistic action with amikacin or cefepime antibiotics in combination with ciprofloxacin against extended-spectrum β-lactamase-producing *E. coli* strains with a 2-fold reduction in MIC of ciprofloxacin was reported [16], herein, a 2.5-fold reduction in MIC of ciprofloxacin was observed in combination with compound **2** or **3** against ciprofloxacin-susceptible *E. coli*. In combination studies against *P. aeruginosa*, FICI of 0.48 and 0.16 for combination of ciprofloxacin with individual compounds **1** and **2** or **3**, respectively, and FICI of 0.04 for mixture of compounds with ciprofloxacin indicate synergism against *P. aeruginosa* (Table 1). A 37-fold reduction in MIC of ciprofloxacin was reported in its combination with ivacaftor against a PAO-1 strain of *P. aeruginosa* [19], however, ivacaftor is an expensive drug that is used for the treatment of cystic fibrosis. In combination studies against *S. typhi*, FICI of 0.48 for combination of all individual compounds with ciprofloxacin and FICI of 0.04 for the combined compounds **1** + **2** + **3** with ciprofloxacin indicate synergism against *S. typhi* (Table 1). A 1.3-fold reduction in MIC of ciprofloxacin was reported in its combination with cefotaxime antibiotic against nalidixic acid-susceptible as well as nalidixic acid-resistant *S. typhi* [47]. We have obtained a MIC of 333.33 μg/ml$^{-1}$ for nalidixic acid and 0.46 μg ml$^{-1}$ for ciprofloxacin against *S. typhi* (Table S1, Supplementary Material), which suggest that the *S. typhi* MTCC 3216 strain used in the present study is a nalidixic acid-resistant *S. typhi*. However, in the present study, a 2.1-fold reduction in MIC of ciprofloxacin was observed in combination with either of the compounds **1** to **3** against *S. typhi*. Thus, based on the checkerboard assay results, a drastic reduction in MIC of ciprofloxacin was observed in individual combinations with compounds and a several fold reductions in MIC was observed by combination with all the three compounds, indicating the potentiating effect of compounds **1** to **3**. Though the synergistic studies of the present nature with phenazines and antibiotics is not reported for antibacterial studies, a combination study with redox active phenazine, pyocyanin, and polymyxin drug was shown to be detrimentally cytotoxic at clinically relevant concentrations (100 to 150 μM) [48]. However, cytotoxicity evaluation using Hep G2 cell line confirmed that phenazines (**1**, **2**) and benzamide (**3**) did not record any significant detrimental effects up to 250 μM on cell viability but at the same concentration ciprofloxacin exhibited 70% cell viability (Fig. S7). Cell viability of 88 to 100% in Hep G2 cell lines was observed at the synergistic concentrations (MIC, Table 1), observed against *S. aureus* and *E. coli*, for the individual combination of compounds (**1-3**) with ciprofloxacin (Fig. 5a–c).

The bactericidal and bacteriostatic activities, based on time-kill curves, were assessed by the reduction in $\log_{10}$CFU ml$^{-1}$ (Table S3, Supplementary Material), which represents the colony count of the test bacterial pathogens. An early bactericidal response in the combination studies of ciprofloxacin at synergistic concentrations (Table 1) with individual compounds in comparison with ciprofloxacin alone was considered as the basis for efficacy of the combination results. Ciprofloxacin showed bactericidal activity at MIC between 8 to 48 h against *S. aureus*, however, in combination with compounds **2** or **3**, an early bactericidal response between 6 to 48 h was observed. A time-kill bactericidal effect was reported from 4 to 24 h for a combination of baicalein with ciprofloxacin against *S. aureus* [46], however, the concentration of ciprofloxacin in their combination was 4 μg/ml$^{-1}$ compared to 0.22 μg ml$^{-1}$ of ciprofloxacin in combination with compounds **2** or **3**. Ciprofloxacin showed bactericidal activity at MIC between 10 to 48 h against *E. coli*, however, an early bactericidal response was observed in combination with compound **2** between 8 to 48 h, and in combination with compound **3** between 4 to 48 h. A synergistic bactericidal action was reported for combination of ciprofloxacin with amikacin or cefepime between 6 to 24 h against extended-spectrum β-lactamase-producing *E. coli* strains [16], however, the studies were conducted with a final inoculum of $0.5 \times 10^6$ CFU ml$^{-1}$ compared to our present study with $1 \times 10^6$ CFU ml$^{-1}$. Ciprofloxacin showed bactericidal activity at MIC between 6 to 10 h against *P. aeruginosa*, but remained bacteriostatic between 10 to 48 h. However, the bactericidal response lasted up to 48 h in combination with compounds, an early bactericidal response was observed in combination with compound **1** from 2 h and compound **3** from 4 h, however, for compound **2**, the bactericidal activity was observed from 6 h. A synergistic bactericidal action was reported for combination of ciprofloxacin with meropenem against MDR *P. aeruginosa* strains between 6 to 24 h with MIC of ciprofloxacin being 32 μg ml$^{-1}$ and with a final inoculum of $1 \times 10^5$ CFU ml$^{-1}$, [12] while ciprofloxacin with MIC between 0.07–0.22 μg ml$^{-1}$ was used in combination studies with **1** to **3** with a final inoculum of $1 \times 10^6$ CFU ml$^{-1}$ of ciprofloxacin-susceptible *P. aeruginosa*. Ciprofloxacin showed bactericidal activity at MIC between 10 to 48 h against *S. typhi*, however, in combination with compounds **1** or **2**, an early bactericidal response between 8 to 48 h, and with compound **3** between 2 to 48 h was observed. Combination of ciprofloxacin (0.012–0.375 μg ml$^{-1}$) and cefotaxime was reported with bactericidal activity at 24 h against nalidixic

acid-susceptible as well as nalidixic acid-resistant *S. typhi* ($0.5 \times 10^6$ CFU $ml^{-1}$) [47]. Based on the time-kill assay results, the early bactericidal response in the combination studies of ciprofloxacin at synergistic concentrations (Table 1) with individual compounds **1** to **3** were superior to the published reports *vide supra*.

In SEM analysis, mild changes in morphology such as dent formation, doughnut like formation, and change of surface morphology in *S. aureus* were observed in the case of treatment with NIIST-D75, crude organic extract, and isolated compounds, whereas in the case of *E. coli*., surface depression, change of surface morphology was observed (Fig. 3). Similarly, mild dent formation was observed for the treatment of *S. aureus* with ciprofloxacin and cell elongation in *E. coli*. Next, we explored the effect of individual combination of ciprofloxacin at synergistic concentrations (MIC, Table 1) with compounds **1** to **3** against *S. aureus* and *E. coli* with respect to changes in morphology by SEM analysis.

The combination of compound **1** and ciprofloxacin against *S. aureus* exhibited dent formation in some cells due to mechanical rupture of the membrane and cell wall [49, 50]. The combination of ciprofloxacin and compound **2** or **3** or the combination of three compounds exhibited severe changes in morphology like biofilm disruption, cell burst, and doughnut-like cell formation [51–53]. Biofilms are clusters of bacterial cells adhering to a surface composed of exopolysaccharides, which give stability to biofilm. Detached cells observed in synergistic combinations of the present study indicate a disruption in biofilms. Another morphological change observed in this study was a doughnut-shaped ghost cell-bearing hole [52]. Doughnut-shaped cells with holes in *S. aureus* by the action of ciprofloxacin is attributed to production of reactive oxygen species, which first causes lipid membrane damage and eventually leads to DNA damage [54].

Filamentation, a continuous elongation of *E. coli* cells was observed with ciprofloxacin (4 μm) in the present study and an enhanced elongation was detected with combinations. Elongation of *E. coli* cells is due to halting in DNA replication by inhibiting DNA gyrase, which induces SOS response and leads to inhibition of cell division [55]. Combinations of ciprofloxacin with compound **1** resulted in more elongation (9.07 μm) compared to ciprofloxacin, along with a ruptured cell, and compound **2** caused an elongation of 10 μm. Interestingly, the combination of compound **3** with ciprofloxacin exhibited a maximum elongation of 20.46 μm, causing complete distortion of cells with release of intracellular contents. The combination of all three compounds with ciprofloxacin resulted in elongation of 10.98 μm and change in membrane integrity. Protein leakage assay and cell membrane permeability evaluation suggests the cell rupturing, cell burst and leakage of intracellular contents are due to irreversible damages in the interior of cells that affects both DNA and cell membranes [56]. Thus, the combination of the compounds in the present study with ciprofloxacin and the drastic changes in morphology observed in SEM analysis (Fig. 3), and the change in total surface area of the selected cells (Fig. 4) against *S. aureus* and *E. coli* corroborate synergism.

## Conclusion

In the present study, we described individual combinations of ciprofloxacin with phenazines (**1** and **2**) and benzamide (**3**) exerted significant inhibitory effect against *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* with bactericidal activity. Further, by SEM analysis, we showed that the combinations displayed severe changes in morphology in *S. aureus* and *E. coli*. The combinational therapy employs an effective treatment strategy for many nosocomial bacteria including multi-drug resistant microorganisms. Most importantly, if indiscriminate antibiotic usage is the main cause of antimicrobial drug resistance, a lower dose of antibiotics combined with the potentiators, revealed in the current study, by synergistic action harbinger disrupting the status quo in antibiotic therapeutic modalities. In conclusion, compounds **1**, **2** and **3** were shown explicitly with elaborate in vitro experiments as potentiators of ciprofloxacin for antibacterial activity with highly reduced dosage, which warrants validation of their combination in the in vivo studies.

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### Compliance with ethical standards

**Conflict of interest** The authors declare no competing interests.

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