Identification of natural antimicrobial agent against *Staphylococcus aureus* from the Himalayan forest soil

SUMMARY OF THESIS

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Ladakh, Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, West Bengal, Manipur, Meghalaya, Mizoram, Nagaland, Tripura, Assam, and Arunachal Pradesh are the thirteen Indian states and union territories that make up the Indian Himalayan region. Himalayas have a pristine ecological environment that is a treasure trove for microbial diversity**.** The Himalayan region including the highlands and foothills, are a tremendous source of natural products. However, less anthropogenic activity and unreachable terrain make it unique. The Forest is a major part of any country that plays a vital role in its economic, historical, cultural, and social development. The total forest of Himalayas is 3,466 thousand hectares and Uttarakhand covers 62.27%. The natural resources of Uttarakhand forests are important environmentally and economically. An abundance of vegetation and microbial diversity in Himalayan soil is a significant source of natural antimicrobials due to intense cold and climatic stress conditions. Its habitat is fostered by mild to low temperature, that trigger to stimulate microbial systems to produce a variety of secondary metabolites in similar species**.** Genus *Streptomyces* were investigated for the first time nearly eight decades ago by Waksman for the isolation of the first antibiotic named actinomycin. Most of the antibiotic class currently belongs to this genus such as ß-lactams, aminoglycosides, tetracyclines, and macrolides. Although more than 625 genomes of the genus *Streptomyces* are known only less than 10% of the genetic potential has been investigated for antibiotic production. That suggests a huge reservoir of antimicrobials remains unidentified and seeks to be investigated for better antibiotics in the future. Bacterial metabolites exhibit a variety of physiologically active chemicals, including those with antibiotic qualities. It is commonly known that *Bacillus* can be a useful source of bioactive secondary metabolites. Additionally, *Bacillus* species are the most common and plentiful bacteria that may be found in every type of environment in which soil serves as a major source. *Bacillus* spp. are also ubiquitous and the most abundant bacterium found in every environmental condition while soil acts as a prominent habitat. *Bacillus* spp. are rod-shaped, Gram positive, and endospore-producing bacteria. These spp. can produce complex antibiotics including lipopeptide bio-surfactants and lipopeptides. It has been observed that *Bacillus* strains produce a wide range of lipopeptide antibiotics that can

suppress the growth of Gram positive and Gram negative bacteria with other phytopathogens. *Bacillus* species are diverse that produce different varieties of broad-spectrum antibiotics. The naturally occurring bacterium *Brevibacillus laterosporus* is widely recognized as *Bacillus laterosporus*. A recent whole genome sequencing study revealed that this species is capable of producing toxins, polyketides, and non-ribosomal peptides. Investigation of microbial sources for secondary metabolites as potent antimicrobial agents for application in agriculture, industries, pharmaceuticals, and research has grown rapidly due to the emergence of antibiotic-resistant pathogens.

Staphylococcus aureus is a common inhabitant of the human body and an opportunistic pathogen, which became resistant against penicillin in 1940. *S. aureus* remains a worldwide concern, Gram positive, catalase-positive, cocci-shaped, and facultative anaerobic bacteria found commonly in human and animal bodies. Manifestation of *S. aureus* can cause serious fatal infections that can lead to death, *S. aureus* spreads and colonizes superficial to internal tissue and can infect the organs. This organism mainly causes skin-to-soft tissue infections (SSTIs) like endocarditis, device-related infections, bacteremia, osteoarticular infections, and pneumonia. The most prevalent type of *S. aureus* infection is skin infections like impetigo, folliculitis, cellulitis, and more serious, invasive soft-tissue infections are just a few examples of how this may present. Major bone and joint infections, such as osteomyelitis, septic arthritis, and infections following replacement joint procedures, are frequently caused by *S. aureus.* The invasion of an infectious agent (*S. aureus*) that causes joint inflammation is known as acute septic arthritis, infectious arthritis, suppurative arthritis, and pyogenic arthritis.

S. aureus is frequently discovered in biofilms that have developed on human tissue or implanted medical equipment. *S. aureus* biofilm is the main culprit behind orthopedic implant-related infections, although it can also be discovered on different catheters, cardiac implants, vascular grafts, and implants used in aesthetic surgery. However, *S. aureus* biofilms play a significant role in the development of illness because they can result in antibiotic resistance and immune system evasion. So, natural antimicrobial agents could be the best option for infection management, they can't interfere with human and animal systems.

Hence, in this study, we have explored the potential of Himalayan soil samples for the production of natural antimicrobial agents against several Gram positive and Gram

negative pathogens. We have collected the soil samples from the forest range of the Himalayan region in Uttarakhand, India, and subjected them to exhaustive primary, secondary, and tertiary screening for isolation of potential isolates that showed antimicrobial activity against *Escherichia coli* MTCC 1304, *Bacillus subtilis* ATCC 6633, *Salmonella typhi* MTCC 581, *Shigella flexneri* MTCC 9543, *Pseudomonas aeruginosa* ATCC 2785 and *Staphylococcus aureus* ATCC 12598 and *Staphylococcus aureus* ATCC 23925.

This thesis is compiled into NINE CHAPTERS. CHAPTER FIRST is a general introduction related to the research topic. This chapter has explained the detailed background information, the Himalayan region is the natural hub of diverse flora and fauna, due to its unique climate conditions, forests found in the Himalayan region such as alpine, sub-tropical, temperate and tropical regions are ~~a~~ tremendous source of natural products. *S. aureus* serves as a commensal bacterium, colonizing roughly 30% of the human population asymptomatically, it can occasionally lead to illness. One of the most frequent causes of bacteremia and infective endocarditis, in particular, is *S. aureus.* NAMPs (natural antimicrobial peptides) are generally small family polypeptides synthesized by microorganisms and exhibit broad-spectrum antimicrobial activity against viruses, fungi, bacteria, and parasites, have a low toxic and cause low mutation rate in pathogenic microbes, so best known as next-generation antibiotics. In CHAPTER SECOND, information related to the entire Himalayan region, natural resources and biodiversity of Uttarakhand forest is important as well as environmentally and economically.

The general information of *S. aureus* regarding infections, *S. aureus* mainly causes skin-to-soft tissue infections (SSTIs) like endocarditis, device-related infections, bacteremia, osteoarticular infections, and pneumonia. The development of extra-chromosomal genetic components containing genes that impart resistance to certain antibiotics is the mechanism through which antimicrobial resistance is genetically based. However, investigations of microbial sources for secondary metabolites as potent antimicrobial agents for application in pharmaceuticals and research.

However, microorganisms have enormous potential to produce a variety of antimicrobial agents such as bacteriocins (nisin), bacterial metabolism-derived compounds (organic acid, reuterin, hydrogen peroxide, carbon dioxide), and antifungal

compounds. A potent antibacterial agent producing actinomycetes including *Streptomyces* spp. is a rich source of antibiotics and about 80% of antibiotics are obtained from it. While *Bacillus* is a Gram positive, spore-forming, aerobic bacteria, can produce ribosomal and non-ribosomal natural antimicrobial peptides also reviewed in this chapter.

Moreover, CHAPTER THREE has written all four objectives related to my thesis topic. CHAPTER FOUR mainly focused on materials and methods used in sample collection, isolation, and screening of antimicrobial agent-producing bacteria by well diffusion method. Characterization of most potential isolates RM-1(13), RM-1(5), KD-4(7) and RM-1(12) through 16S rRNA method. Growth kinetics was done in 10 to 14 days and active compounds were extracted by an n-butanol solvent system. Active compounds have been separated by TLC and HPLC, and identified by FTIR and ESI-MS technique. Separated compounds MIC was calculated by 96 well plate method and all compound's antibiofilm efficacy was tested against *S. aureus*. The bacterial diversity of the RM1 sample was also done by metagenomics approach in which DNA of the soil sample was extracted by using Xploregen DNA extraction Kit (Xploregen Discoveries, India) according to the manufacturer's instructions and the functional metabolic pathway identified by Microbiomeanalyst.

The CHAPTER FIVE has described the results of all methods. The findings of the study revealed that screened most potential isolates RM-1(13), RM-1(5), KD-4(7) and RM-1(12) by molecular methods identified as *Streptomyces albus* strain RG1012 (0M780276), KD-4(7) as *Brevibacillus laterosporus* strain RG1007 (OP653881) and RM-1(12) as *Bacillus tequilensis* strain RG1009 (OP672437). FTIR and ESI-MS analysis suggested the bioactive compound belongs to Emycin-E, Pamamycin, Brevibacillin, and Monamycin with molecular weight 310.1, 578.1, 1583.1, and 678.1. We further calculated the MIC of Emycin-E, (0.31 µg/ml) Pamamycin (0.31 µg/ml), Brevibacillin (0.62 µg/ml), and Monamycin (1.25 µg/ml) against *S. aureus*. Further, the metagenomics of the RM1 soil sample revealed that Himalayan forest soil showed a higher abundance of phylum Proteobacteria (28.86%) and Actinobacteria (26.70%). The dominancy of amino acid biosynthesis (14.22%) followed by carbon metabolism (11.53%) and methane metabolism (9.40%) present in RM1.

The SIXTH CHAPTER has discussion and conclusion of all my research. In this

chapter, we have discussed: four potent isolates RM-1(13), RM-1(5), KD-4(7), and RM-1(12) in which all isolates were Gram positive. Isolate RM-1(13) identified as *Streptomyces griseus* strain RG1011 which produced black color melanin-like pigment and RM-1(5) as *Streptomyces albus* strain RG1012 producing orange-brown pigment. Other hand KD-4(7) identified as *Brevibacillus laterosporus* strain RG1007 and RM-1(12) as *Bacillus tequilensis* strain RG1009 showed colorless growth in nutrient broth media. *Streptomyces* generally do not propagate in simple media, so, we have optimized the production of pigment by using modified media with 2 gm/L of tyrosine in a nutrient broth medium, since most of the microbial melanin is formed by the conversion of tyrosine to dopaquinone by tyrosinase enzyme present in bacteria. The results of HPLC suggest major peaks of *S. griseus* at 7.747 and *S. albus* at 9.68 in the UV range which suggests the cyclic unsaturated aromatic compound. *S. griseus's* HPLC fraction was further confirmed by the UV-visible spectroscopy where two peaks at 226 nm and 275 nm were found that suggest the presence of two pairs of cyclic unsaturated aromatic rings. While UV-vis absorbance of *S. albus* HPLC fraction at 224 and 277 nm gave the information that the compound was highly saturated and alicyclic. The unknown compounds of KD-4(7) and RM-1(12) were separated at retention time (RT) at 15.147 and 10.045 respectively indicating the peptidic nature of the compounds**.**

The molecular weight of the pure compound was determined by ESI-MS analysis. The mass/charge ratio of RM-1(13) was calculated as 310.1 which is similar to the previously reported antimicrobial compound Emycin-E, belonging to class polyketides. RM-1(5) was confirmed by the ESI-MS as $(M-H)^-$, m/z was determined 578.1 which is exactly similar to previously found pamamycin that is an antibacterial agent, which is macrodiolides of polyketide in origin. KD-4(7) was calculated as 1583.1 was similar to the previously reported Brevibacillin compound. Whereas the purified compound of RM-1(12) was found as 678.1 indicating a highly abundant peak, similar to monamycin oligopeptide. The quantitative analysis of biofilm inhibition in the presence of Emycin-E and pamamycin at MIC 0.15 µg/mL brevibacillin at 0.62 µg/mL and monamycin at 1.25 µg/mL suggests that *S. aureus* was not able to form biofilm as compared to positive control.

So all extracted bioactive compounds had good antibiofilm and bactericidal activity that might be used to prevent bacterial biofilm and bacterial infections in hospitals. These

compounds would be useful for industries and research as future antibiotics that would replace synthetic antibiotics. This assumes that it follows a special mode of action against pathogenic bacteria where its target will not be interfering with the eukaryote system. Bacterial diversity analysis also uncovers a new perspective on land use management for sustainable development.

CHAPTER SEVEN has summarized the entire thesis work, results, and findings with scientific output systematically. CHAPTER EIGHT contains all the references cited in the thesis chapter related to the topic. I have cited recent references in each chapter. The cited references are related to natural antimicrobial agents against *Staphylococcus aureus* from the Himalayan forest soil, *Streptomyces,* and *Bacillus.* There are 307 references cited throughout the entire thesis.

The last NINTH CHAPTER has compiled and listed all the scientific output. I have published seven research papers, three review papers, and one chapter in reputed Journals. At international conferences, I have received certificates of participation and awards for oral presentations.