ISOLATION AND CHARACTERIZATION OF ANTIBIOTICS FROM ACTINOMYCETES COLLECTED IN NEPAL

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF CHEMISTRY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN CHEMISTRY

BY

BIJAYA BAHADUR THAPA

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DECLARATION

Thesis entitled "**ISOLATION AND CHARACTERIZATION OF ANTIBIOTICS FROM ACTINOMYCETES COLLECTED IN NEPAL**" which is being submitted to the Central Department of Chemistry, Institute of Science and Technology (IoST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Niranjan Parajuli of Central Department of Chemistry, Tribhuvan University, and co-supervised by Associate Prof. Dr. Khaga Raj Sharma of Central Department of Chemistry.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

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शोधसार

माटो, पानी लगायत विभिन्न खाले प्राकृतिक स्रोतबाट निकालिने एक्टिनोमाइसेट्स नामक ब्याक्टेरिया ग्रामपोजिटिभ ब्याक्टेरियाको एउटा वर्ग हो जसमा ढुसीको जस्तै फिलामेन्टस माइसेलिया हुन्छ । धेरै जसो ती ब्याक्टेरियाहरूले जीवप्रतिरोधी यौगिकहरू उत्पादन गर्दछन् र तिनीहरू सङ्क्रामक रोगहरूसँग लड्न जैविक रूपमा सक्रिय यौगिकहरूका प्रमुख स्रोत मानिन्छन् । यसबाहेक बहुऔषधि प्रतिरोधी ब्याक्टेरियाले मानव र पशुचिकित्साका रोगहरूमा गम्भीर समस्याहरू निम्त्याइरहेका छन् । ती समस्याको समाधानका लागि रोग प्रतिरोधी एक्टिनोमाइसेट्स हानिकारक ब्याक्टेरियाको निर्मूलीकरण गर्न सक्ने नयाँ यौगिक पत्ता लगाउन आवश्यक देखिन्छ ।

नेपालका विभिन्न भूगोलबाट सङ्कलन गरिएका माटोबाट एक्टिनोमाइसेट्स पत्ता लगाउने कार्य यस शोधमा गरिएको छ । ब्याक्टेरियाको ग्रामपोजिटिभ वा नेगेटिभ भन्ने पहिचान ग्राम स्टेन (Gram Stain) विधिबाट पुष्टि गरिएको छ । विषम परिस्थितिमा उक्त एक्टिनोमाइसेट्स ब्याक्टेरियाहरूले द्वितीयक यौगिकहरू (Secondary metabolites) उत्पादन गर्दछन् र धेरै जसो ती यौगिकहरू एन्टिबायोटिक औषधिका रूपमा प्रयोग पनि हुन्छन् । ती यौगिकहरूलाई इथाइल एसिटेट नामक अर्ग्यानिक रसायनको प्रयोग गरी निकालिएको थियो । तिनीहरूका जीव प्रतिरोधी गतिविधिको वैज्ञानिक रूपमा परीक्षण गर्नका लागि तोकिएका विभिन्न ब्याक्टेरियाहरूको उपयोग गरिएको थियो । परिणामका रूपमा द्वितीयक यौगिकले *Staphylococcus aureus* ATCC ४३३०० भन्ने ब्याक्टेरियालाई बढी निषेध गरेको भेटियो ।

अन्य माटोबाट निकालिएका एक्टिनोमाइसेट्स तथा त्यही एक्टिनोमाइसेट्सबाट निकालिएका यौगिकहरूले माथि उल्लेख गरिएका ब्याक्टेरियाहरूलाई औसत निषेध गरेको भेटियो । सबै नमुनाहरूबाट प्राप्त Secondary metabolites को जीव प्रतिरोधी क्षमता पनि मापन गरियो जसलाई Minimum Inhibitory Concentration भनिन्छ । तिनीहरूले प्याथोजेनिक ब्याक्टेरियालाई औसत रूपमा निषेध गरेको भेटियो ।

प्रस्तुत शोधमा लिक्विड क्रोमाटोग्राफी - हाइ रिजुलेसन टेन्डम मास स्पेक्ट्रोमेट्री (LC-HRMS/MS) , MestreNova र SIRIUS सफ्टवेयर प्रयोग गरी जम्मा ५८ वटा यौगिकहरूको टिपोट गरिएको छ । ब्याक्टेरियाको वैज्ञानिक पहिचान गर्न तिनको डीएनए परीक्षण गरी सोको प्रजाति पहिचान गरिएको छ । सो विधिबाट एक्टिनोमाइसेट्सका पाँच वटा स्ट्रेप्टोमाइसेस प्रजातिहरू पहिचान गरियो । पत्ता लगाइएका ५८ वटा द्वितीयक यौगिकहरूमध्ये चार वटा नयाँ यौगिकहरू *Streptomyces* species प्रजातिहरूमा पहिलो पटक पत्ता लगाइयो ।

समग्रमा प्रस्तुत अनुसन्धानबाट नेपालका माटोमा धेरै प्रकारका स्ट्रेप्टोमाइसेस प्रजातिका ब्याक्टेरियाहरू पाइन्छन् र तिनीहरूबाट विशेष खालका एन्टिबायोटिकहरू पत्ता लगाउन सकिने देखिएको छ । प्रतिकूल वातावरणमा रहेका माटोमा पाइने ब्याक्टेरिया पहिचान गरी तिनीहरूबाट प्याथोजेनिक जीव प्रतिरोधी क्षमता भएका नयाँ एन्टिबायोटिकहरू पनि प्राप्त गर्न सकिने निष्कर्ष निकालिएको छ ।

ABSTRACT

Actinomycetes are a distinct class of Gram-positive bacteria with a filamentous mycelium-like that of fungi. They produce many potential antitumor, anticancer, and antimicrobials; a key source of biologically active compounds to combat infectious diseases and others. Moreover, multi-drug resistant (MDR) pathogens cause serious problems in both humans and animals. This issue demands a need for stronger antimicrobial agents. This research has examined the secondary metabolites produced by soil-based actinomycetes collected in various parts of Nepal.

Actinomycetes were isolated from soils by using the International *Streptomyces* Project 4 (ISP4) medium. Most of the aerial mycelia of bacterial isolates had a greyish-white color whereas some of them appeared bluish-white. The prominent colonies of isolated bacteria were cultured in a Tryptic Soy Broth (TSB) medium. The Gram-positive nature of the isolated bacteria was confirmed by Gram staining.

Ethyl acetate extracts of the isolates were prepared after shake flask fermentation in the TSB medium. The antimicrobial activity of the isolates was carried out against *Staphylococcus aureus* American Type Culture Collection (ATCC) 43300, *Shigella sonnei* ATCC 25931, *Salmonella typhi* ATCC 14028, *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 bacteria, and observed through primary and secondary microbiological screening. Primary screening of the isolates was performed by the perpendicular-streaking method. Most of the isolates showed strong antimicrobial activity against the tested pathogens. A total of 11 bacterial isolates exhibited significant zones of inhibition against both Gram-positive and Gram-negative pathogens. Isolates BT1, BT2, BT3, AB1, PT7, and PC1 showed greater antimicrobial efficacy against tested pathogens based on their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. Isolates BT5, BT13, BT33, BT36, and BT39 exhibited remarkable antimicrobial efficacy against tested pathogens. Among the isolates, isolate PC1 showed the highest zone of inhibition (32 mm. against *E. coli*, 30 mm. against *S. typhi*, 30 mm. against *S. sonnei*, and 30 mm. against *K. pneumoniae*) in all tested pathogens except in *S. aureus*.

The genomic deoxyribonucleic acid (DNA) of the isolated bacteria was extracted using the standard protocol of molecular biology. The amplified polymerase chain reaction

(PCR) product was then subjected to 16S Ribosomal ribonucleic acid (rRNA) sequencing. As such this study identified five *Streptomyces* species (*Streptomyces* sp.BT1, *Streptomyces* sp. BT2, *Streptomyces* sp. BT3, *Streptomyces* sp. BT5 and *Streptomyces* sp. PC1).

The metabolic profiles of the ethyl acetate extracts were investigated using liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS). Fifty-eight compounds were annotated from the raw data files of tandem mass using MestReNova (Mnova) software. Then, the annotated compounds were further validated through SIRIUS software. Out of the annotated compounds from LC-HRMS/MS, four of them were detected for the first time in *Streptomyces* species, namely Cyclo(Ile-Ser) from isolate BT2, 2-n-hexyl-5-n-propylresorcinol from isolate BT1, 3-((6-methylpyrazin-2-yl)methyl)-1H-indole from isolate PC1, and Cyclo(D-Leu-L-Trp) from isolate BT2. Total 7 antibiotics were annotated which were myxopyronin B, blasticidin H, flavofungin, xenocoumacin 2, okilactomycin A, butyrolactol A and SF2415-B2.

Accordingly, this research identified that Nepal's varied ecology offers ideal circumstances for the existence of several *Streptomyces* species from the soil habitat which can produce specialized secondary metabolites including antibiotics. This study set up a platform in Nepal, which inspired for isolation of rare actinomycetes, which could produce new therapeutics including antibiotics.

Keywords: *Actinomycetes-Multi-drug Resistant-Secondary Metabolites-Primary Screening-Mass Spectrometry-Streptomyces Species*

LIST OF ACRONYMS AND ABBREVIATIONS

AMR	: Antimicrobial Resistance
ATCC	: American Type Culture Collection
BGCs	: Biosynthetic Gene Clusters
BPC	: Base Peak Chromatograms
CLSI	: Clinical Laboratory Standard Institute
CRE	: Carbapenem-resistant Enterobacteriaceae
DKP	: Diketopiperazine
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic Acid
EA	: Ethyl Acetate
EDTA	: Ethylenediaminetetraacetic Acid
ESBL	: Extended Spectrum Beta-Lactamase
ESI	: Electrospray Ionization
EtBr	: Ethidium Dibromide
GC/MS	: Gas Chromatography Mass Spectrometry
HR	: High Resolution
HRMS	: High Resolution Mass spectrometry
ISP4	: International *Streptomyces* Project 4
LC-HRMS/MS	: Liquid Chromatography High-Resolution Tandem Mass Spectrometry
LC-MS	: Liquid Chromatography Mass Spectrometry
LC-MS/MS	: Liquid Chromatography Tandem Mass Spectrometry
LRMS	: Low-Resolution Mass spectrometry

MBC	: Minimum Bactericidal Concentration
MHA	: Mueller Hinton Agar
MHB	: Mueller Hinton Broth
MIC	: Minimum Inhibitory Concentration
MRSA	: Methicillin-resistant *Staphylococcus aureus*
MS	: Mass Spectrometry
MWAS	: Metabolome-wise Association Studies
NA	: Nutrient Agar
NRPs	: Non-ribosomal Peptide Synthetases
PA	: Potassium Acetate
PBPs	: Penicillin-Binding Proteins
PCI	: Phenol-Chloroform-Isoamyl Alcohol
PCR	: Polymerase Chain Reaction
PGPR	: Plant Growth-Promoting Rhizobacteria
PKS	: Polyketide Synthases
Q-TOF	: Quadrupole Time of Flight
RNA	: Ribonucleic Acid
RNase	: Ribonuclease
rRNA	: Ribosomal Ribonucleic Acid
Rt	: Retention Time
SAIF	: Sophisticated Analytical Instrument Facility
SDS	: Sodium Dodecyl Sulfate
TAE	: Tris Acetate EDTA
TB	: Tuberculosis

TE	: Tris-EDTA
TIC	: Total Ion Chromatogram
TMPD	: Tetramethyl Phenylene Diamine
TMP-SMX	: Trimethoprim-Sulfamethoxazole
TSB	: Tryptic Soy Broth
WGS	: Whole-Genome Sequencing
ZoI	: Zone of Inhibition

LIST OF SYMBOLS

%	: Percent
μ	: Micro
mL	: Millilitre
m/z	: Mass/charge
µL	: Microlitre
nm	: Nanometer
g	: Gram
^{o}C	: Degree Centigrade
hrs	: Hours
L	: Litre
m	: Meter
mg	: Milligram
mM	: Milli-molar
µM	: Micro-molar
rpm	: Revolutions Per Minute
sec	: Second
β	: Beta
∞	: Alpha

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CHAPTER 1

1. INTRODUCTION

1.1 History of Antibiotics

For millennia, individuals were rendered defenseless against diverse forms of illnesses, which frequently escalated to epidemic levels and claimed millions of lives. People then began to research the causes, consequences, and remedies of infectious diseases. However, for a long time, Mohr (2016) claimed that efforts to battle, treat, and prevent the spread of infectious diseases were fruitless due to ignorance. The first breakthrough in the history of antibiotic research began with debunking the abiogenesis theory. This theory triggered a debate over the nature of contagious diseases and the study of therapeutic qualities of (antibiotic-producing) molds contributing to further research. The first microscopic examination of microbes was undertaken in the 17th century. (Mohr, 2016). During research, the first antibiotic Salvarsan was introduced in 1910. Antibiotics have significantly contributed to modern medicine and increased human lifespan. The golden era of natural product antibiotic research started in 1928 with the discovery of penicillin concluded in the mid-1950s (Hutchings *et al.*, 2019).

The discovery of penicillin helped to understand the ability of natural substances to fight bacterial diseases and consequently saved countless lives (Wright, 2014). Penicillin previously had availability difficulties and was expensive. It became widely available during World War II due to Howard Florey and Ernst Boris Chain's efforts to purify it and initiate mass manufacturing methods in the 1940s (Ligon, 2004). Millions of Tuberculosis (TB) patients worldwide became hopeful after Selman Waksman and colleagues discovered streptomycin; another antibiotic in 1944. It marked a significant advancement in the treatment of this disease which did not have feasible medicine (Schatz *et al.*, 1944). Benjamin Duggar discovered tetracycline in 1948. Tetracycline is a highly functional antibiotic useful for the treatment of different types of illnesses. The number of antibiotics available for distribution increased significantly. However, the growth of drug resistance in bacteria due to the overuse and misuse of antibiotics has become a significant worldwide health issue. This demands a need for careful antibiotic usage and developing methods for fighting resistance (Laxminarayan *et al.*, 2013). The

isolation of Cephalosporin C was explained in 1956. This antibiotic had a diverse range of bacterial targets and offered options for patients who were allergic to penicillin (Abraham & Newton, 1961). This discovery opened a new horizon of antibiotic treatment. Novel antibiotics, including sulfonamides and chloramphenicol, were discovered in the 20th century. It altered the available treatments for bacterial infections and the source of producing antibiotics. Antibiotics no longer had to be originated only from natural sources (Emmerson & Jones, 2003). They have been chemically manufactured for multiple uses.

Antibiotics are widely used in agriculture to treat diseases and grow them well. This led to the new challenge of antibiotic-resistant bacteria in animal food. In the meantime, it helps to understand how animal and human health are related (Silbergeld *et al.*, 2008). Edmund Kornfeld isolated vancomycin in 1954 which is another milestone in the field of antibiotics for treating septicity due to bacteria that are resistant to traditional antibiotics, specifically Methicillin-resistant *Staphylococcus aureus* (MRSA) (Moellering, 2006). To address the issue of drug-resistant bacteria and ensure adequate treatment options for future generations, the current research on antibiotics is concerned with creating novel antibiotics and investigating alternative medicines (Ventola, 2015). The development of antibiotics in various timelines is presented in **Figure 1**.

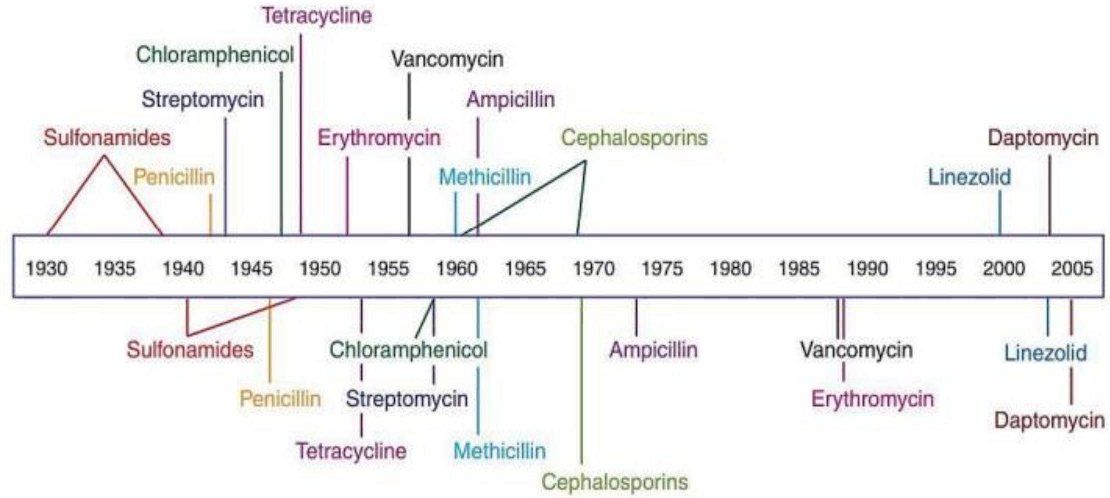


Figure 1: Development of antibiotics in various timelines (Clatworthy *et al.*, 2007).

1.1.1 Classification of Antibiotics

Classification of antibiotics is based on various factors and criteria such as their range of activities, the way they inhibit bacterial growth, and the makeup of their chemistry. Some of the common varieties of antibiotics are discussed here.

- **Penicillins:** Penicillins are the first antibiotic that humans discovered. They are usually used for combatting Gram-positive bacteria, penicillins work by restraining the penicillin-binding proteins (PBPs) of the bacterial cell wall thus completely blocking the formation of cell walls in those bacteria (Waxman & Strominger, 1983). The antibiotics with such working mechanisms are also called beta-lactam antibiotics. Penicillin G, ampicillin, and amoxicillin are common antibiotics that belong to the class penicillin.
- **Cephalosporins:** Cephalosporins work like β-lactam antibiotics which have three main working mechanisms to fight against bacteria: Penicillin-binding proteins (PBP) modification, decreased permeability and rise in efflux, and enzymatic inactivation (Prescott, 2013). Cephalosporins show strong activity against Gram-positive and Gram-negative bacteria. There are several antibiotics in the cephalosporin family, including cephalexin, ceftriaxone, and ceftazidime.
- **Macrolides:** By incorporating themselves into the 50S ribosomal subunit, macrolides prevent the production of proteins in bacteria (Nakajima, 1999). It's been known that they can effectively inhibit Gram-positive and Gram-negative bacteria. Drugs in this class include clarithromycin, azithromycin, and erythromycin.
- **Tetracyclines:** Tetracyclines stop the synthesis of proteins inside bacteria by binding themselves to their 30S ribosomal subunit (Roberts, 2005). Furthermore, they are efficient in suppressing certain protozoa and Gram-positive and Gram-negative bacteria. Tetracycline, doxycycline, and minocycline are some common examples.
- **Aminoglycosides:** Aminoglycosides control the protein synthesis of bacteria by specifically targeting the 30S component of the bacterial ribosome (Weisblum & Davies, 1968). They are primarily utilized against Gram-negative bacteria, although they are particularly effective against specific Gram-positive bacteria.

Streptomycin, amikacin, and gentamicin are some drugs that belong to this class.

- **Fluoroquinolones:** Fluoroquinolones block the action of the bacterial DNA gyrase and topoisomerase IV, this effect is very crucial for DNA replication and repair in bacteria (Walker, 1999). Gram-positive and Gram-negative bacteria are both subject to a variety of their effects. Drugs in this class include levofloxacine, moxifloxacine, and ciprofloxacine.

- **Sulfonamides:** Sulfonamides inhibit the enzyme dihydropteroate synthase which acts as antimetabolites in bacteria (Capasso & Supuran, 2019). They are also effective against both kinds of bacteria. Examples are trimethoprim-sulfamethoxazole (TMP-SMX), sulfadiazine, and sulfamethoxazole.

- **Glycopeptides:** Glycopeptides are big, stiff compounds that stop bacteria from synthesizing peptidoglycan at a later stage in their cell walls (Reynolds, 1989). Most often, they are enforced to treat diseases brought on by Gram-positive bacteria, particularly antibiotic-resistant *Staphylococcus aureus*. Vancomycin and teicoplanin are two examples of antibiotics in this class.

1.1.2 General Use of Antibiotics

Antibiotics are extensively employed in different fields and have a very flexible usage. The following are a few general uses of antibiotics:

- **Medicine:** Antibiotics are primarily used as medicine in humans and animals to treat bacterial infections. They are prescribed by physicians to eradicate or prevent the development of pathogenic bacteria that cause sore throat, pneumonia, urinary tract infections, and skin infections (Ventola, 2015). These antibiotics include Penicillin, Amoxicillin, Ciprofloxacin, and Azithromycin.

- **Surgery:** To lower the risk of problems following surgery and to prevent bacterial infections at the surgical site, antibiotics are frequently used in surgical procedures (Bratzler *et al.*, 2013). Cefazolin and Clindamycin are a few examples of such antibiotics.

- **Veterinary medicine:** Animals, including pets and cattle, who have bacterial illnesses are treated with antibiotics. Antibiotics help in the prevention of

outbreaks of infectious diseases among animals hence enhancing animal health. (Bengtsson & Greko, 2014). Tetracycline, Enrofloxacin, and Cephalexin are examples of such antibiotics.

- **Agriculture:** Antibiotics are used in agriculture to speed up animal growth and safeguard against bacterial illnesses in cattle. However, there is a great concern regarding antibiotic-resistant organisms due to their direct impact on human health (Ibrahim *et al.*, 2020). These antibiotics include Tylosin, Chlortetracycline, and Sulfamethazine.
- **Food Preservation:** Antibiotics are used in food preservation to stop bacteria from growing and extend the shelf life of degradable foods. However, their amounts should be properly regulated to maintain food safety (Grover *et al.*, 2016). Nisin and Natamycin are such antibiotics.
- **Biotechnology:** The selection and maintenance of bacterial cultures utilized in numerous scientific and industrial applications, including the synthesis of medications, enzymes, and biofuels, requires the usage of antibiotics (Demain & Sanchez, 2009). Ampicillin and Kanamycin are examples of such antibiotics.

The structures of some common antibiotics are shown in **Figure 2**.

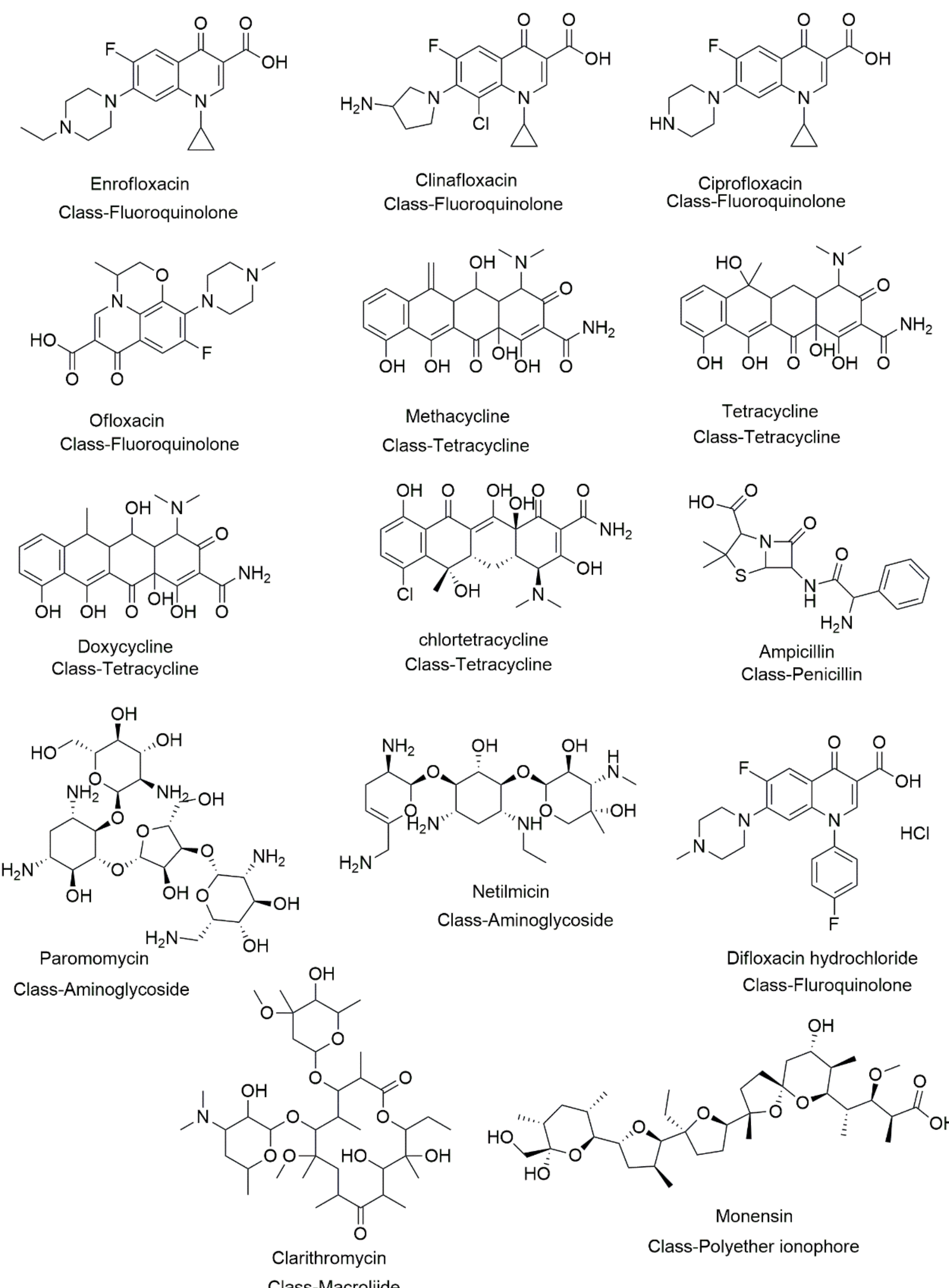


Figure 2: Some examples of antibiotics (Dawadi *et al.*, 2021).

Most of the antibiotics are isolated from actinomycetes. Soil, marine sediments, and dwelling water are rich sources of actinomycetes.

1.2 Actinomycetes

One of the classes of Gram-positive bacteria is the actinomycetes; with a filamentous shape that are renowned for their ability to produce various bioactive compounds. This is a diverse group of bacteria that are frequently encountered in nature, where they function as residents of soil ecosystems or contribute to nutrient cycling and processes across almost all natural environments such as freshwater, land, and marine systems. (Barka *et al.*, 2015). Their life cycle is complex and consists of branched filaments called hyphae that can form conidia or sporangia. Sporangia resembles spores (Bérdy, 2005). For such structures, they are categorized into families like Actinomycetaceae, Streptomycetaceae, and Micromonosporaceae (van Bergeijk *et al.*, 2020).

Actinomycetes have the potential to produce a large number of secondary metabolites, like immunosuppressants, antifungal agents, anticancer, and antibiotic drugs (Barka *et al.*, 2015). Most antibiotics come from the genus *Streptomyces* within the Actinomycetaceae family. It has been a significant antibiotic-producing potential (Bérdy, 2005). Actinomycetes have been widely used for biotechnological purposes in producing medicine, and industrial and agricultural purposes. Actinomycetes are a vital resource for producing drugs as they consist of bioactive molecules possessing a variety of pharmacological effects (Barka *et al.*, 2016).

Actinomycetes also accelerate environmental processes such as nitrogen fixation, and complex chemical molecules' decomposition and degradation (Bérdy, 2005). Recently, these microorganisms have been considered as essential components for bioremediation, and environmental management. Their metabolic plasticity and expansion of ecological niches contribute to pharmaceutical production and ecological management (van Bergeijk *et al.*, 2020). This study has focussed on the role of actinomycetes as major producers of bioactive compounds to combat contagious diseases. The discovery of such important bacteria has a long history.

1.2.1 Discovery of Actinomycetes

Dr. Selman Waksman, who is known as the "Father of Antibiotics," and his associates started a thorough investigation of soil microorganisms in the late 19th century, which marked the beginning of the study of actinomycetes (Kresge *et al.*, 2004). Their ground-breaking research provided the impetus for the identification of several bioactive substances made by actinomycetes, including antibiotics. Waksman had been

continuously screening fungi and bacteria in soil from 1914 to 1939 to discover an antibiotic for tuberculosis. He found out in 1939 how some fungi, particularly actinomycetes, affect the growth of bacteria. He succeeded in isolating actinomycin, a potent tuberculosis antibiotic, in 1940 and 1944 with the discovery of streptomycin. For his efforts, he was granted the 1952 Noble Prize in Physiology and Medicine (Sharma *et al.*, 2014).

The investigation into the diversity of actinomycetes has led to the discovery of a huge reservoir of secondary metabolites. Particularly *Streptomyces* species have become important sources of immunosuppressants, anticancer drugs, antibiotics, and enzyme inhibitors (Barka *et al.*, 2015). Numerous other genera, including Micromonospora, Nocardia, and Actinomyces, were also added to the collection of bioactive substances. Molecular biology and genomics developments have helped to understand the mechanisms underlying the production of the bioactive compounds in actinomycetes. The identification of new metabolites from actinomycetes has been made possible by the elucidation of biosynthetic gene clusters (BGCs) and focused genome mining techniques. Furthermore, metagenomic research has uncovered the unrealized potential of actinomycetes in a variety of settings, such as hot springs, marine sediments, and harsh ecosystems (Barka *et al.*, 2015). Genome sequencing, biotechnology, and bioinformatics have led to the study of actinomycetes in diverse ways. Actinomycetes are a source of novel bioactive substances, and their discovery could help tackle new problems in biotechnology, agriculture, and healthcare. *Streptomyces* is an important type of actinomycetes that belongs to the genus of phylum Actinobacteria.

1.3 *Streptomyces*

Gram-positive bacteria, *Streptomyces* are used in industry to synthesize secondary metabolites like antibiotics, which are important in many fields (Barka *et al.*, 2015). It belongs to the phylum actinobacteria and is a majorly studied genus in the actinomycetes order, with over 800 species currently recognized (Bérdy, 2012).

1.3.1 Taxonomy and Classification

Streptomyces is classified as a member of the Actinomycetales order and family Streptomycetaceae (Bérdy, 2012). They have a varied number of divisions separated based on their morphological, physiological, and metabolic traits.

On the morphology and growth characteristics basis, species of *Streptomyces* have filamentous structures, producing spore-bearing structures known as sporophores and branching aerial mycelia (Chater & Chandra, 2006). They are set apart by their slow growth on solid media. These bacteria also have an earthy odor caused by volatile metabolites. On a metabolic capabilities basis, *Streptomyces* species are recognized for their abundant secondary metabolism, which yields a large variety of bioactive materials including antifungals, antibiotics, and anticancer medicines (Barka *et al.*, 2015). Biosynthetic gene clusters (BGCs) encode complicated biosynthetic pathways that produce these secondary metabolites. Moreover, the biotechnological application and ecological significance of such bacteria and their secondary metabolites include the biocontrol agents in agriculture and environmental cleanup (Bérdy, 2012).

Streptomyces are crucial for soil ecology, nitrogen cycling, and plant health (Barka *et al.*, 2015). These bacteria engage in resource competition with other microorganisms in the soil and generate antimicrobial compounds that affect the interactions between microbes and plants as well as the dynamics of the soil (Chater & Chandra, 2006). The antimicrobial compounds consist of secondary metabolites.

1.4 Metabolites

The intermediates or final products of metabolic processes in living things are known as metabolites. These tiny molecules are essential to many biological functions, such as signaling pathways, growth, maintenance, and energy production. The function of a wide range of metabolites helps us understand the intricacy of cellular metabolism.

1.4.1 Classification of Metabolites

Based on their chemical makeup, roles in metabolic processes, and other characteristics, metabolites can be divided into several groups.

- **Primary Metabolites:** A crucial component of regular growth is the synthesis of primary metabolites, which are microbial products produced during the exponential growth phase. Cells utilize these molecules as the foundation for crucial macromolecules such as amino acids and nucleotides, or they transform them into coenzymes like vitamins. The intermediates and end-products of anabolic metabolism are also included in primary metabolites. Cellular components are not built using some primary metabolites, like ethanol, citric

acid, and acetic acid, despite their necessity for energy production and substrate utilization during development (Sanchez & Demain, 2008).

- **Secondary Metabolites:** Secondary metabolites are a broad class of naturally occurring metabolic products that are not imperative for vegetative growth. Instead, they perform adaptive roles, such as acting as signaling molecules or defense compounds in symbiotic relationships, competition, ecological interactions, and metal transport, among other contexts (Thirumurugan *et al.*, 2018).
- **Endogenous Metabolites:** Endogenous metabolism takes place inside a living cell when certain exogenous substrates are absent. The products of the process are called endogenous metabolites. The partial byproduct of this metabolism may be discharged into the surrounding medium. They are frequently taken up by the cells for their regeneration (Dawes & Ribbons, 1964).
- **Exogenous Metabolites:** Exogenous metabolites help metabolic modulation by increasing membrane potential. They stimulate proton motive force, engage in central carbon metabolism and cellular respiration, enhance host immune defense, and change the gut microbiome (Chung *et al.*, 2022).
- **Catabolites and Anabolites:** Anabolites are intermediate or final products created during the synthesis of complex molecules from simpler ones, whereas catabolites are metabolic byproducts generated after the breakdown of larger molecules. Anabolites are macromolecules like proteins, carbohydrates, and nucleic acids, whereas catabolites are molecules like carbon dioxide, ammonia, and urea (Kaur & Debnath, 2015).

The study has focused on secondary metabolites produced by actinomycetes.

1.5 Secondary Metabolites

Organic substances produced by organisms primarily for uses other than growth, development, or reproduction are known as secondary metabolites. In contrast to primary metabolites, which are necessary for fundamental biological functions like energy production, development, and maintenance, secondary metabolites are frequently generated in reaction to external stressors like resource rivalry or defense against infections, predators, or unfavorable environments. These substances have a

wide range of structures and functions and are essential to pharmacology, industry, agriculture, and ecological interactions.

1.5.1 Classification of Secondary Metabolites

One common method of classifying secondary metabolites is based on their chemical structure. This classification divides secondary metabolites into many key classes:

- **Alkaloids:** Alkaloids are frequently generated from amino acids. They are extensively found in plants, particularly in families like Solanaceae, Papaveraceae, and Apocynaceae, and are well-known for their wide range of pharmacological properties. Morphine, caffeine, and nicotine are a few examples (Dalton *et al.*, 2003).

- **Terpenoids:** Terpenoids are among the most numerous and varied classes of natural products, which are derived from the 5-carbon isoprene unit. They are present in many different plant species and possess a range of biological tasks. Based on several isoprene units, are further sorted into monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), and triterpenes (C30) (Tholl, 2015).

- **Phenolic Compounds:** These compounds are derivatives of aromatic compounds. The attachment of one or more hydroxyl groups with aromatic rings produces phenolic compounds. They are mostly dispensed throughout the plants and have a role in pollinator attraction, ultraviolet (UV) protection, and disease defense. Flavonoids, phenolic acids, lignans, and tannins are examples of phenolic chemicals (Crozier *et al.*, 2008).

- **Polyketides:** Acetyl, propionyl, and coenzyme molecules condense to form polyketides, a structurally varied class of chemicals. They are frequently produced by fungi, bacteria, and plants and have a variety of biological functions. Examples of polyketides are erythromycin, lovastatin, and doxorubicin (Fischbach & Walsh, 2006).

- **Glycosides:** Glycosides with their range of structural forms as a significant role in the pharmaceutical industry due to their bioactivities and widespread use. The structure of all glycosides is similar. A lipophilic aglycone (genin) unit and a hydrophilic glycone unit comprising one or more sugar components constitute this compound (Bartnik & Facey, 2024).

- **Quinones:** Quinones are a group of naturally occurring and synthetic compounds that have many peculiar characteristics. They are an essential component of photosynthesis as electron transporters. They belong to a class of chemicals known as vitamins that are focused on treating and eradicating disorders, including cardiovascular disease and osteoporosis. They have an antioxidant effect that helps with general health issues. Numerous cancer-fighting medications that have received clinical approval or are undergoing clinical studies are connected to quinones. Additionally, their existence as photoproducts of air pollution has toxicological effects. They are compounds that exhibit rapid redox cycling and can bind to hydroxyl, amine, and thiol groups (El-Najjar *et al.*, 2011).

The distinct chemical characteristics and biological activity exhibited by each class of chemicals are indicative of their varied evolutionary histories and ecological roles.

1.5.2 Significance of Secondary Metabolites

Secondary metabolites have numerous uses. The significance of the secondary metabolites is summarized below:

- **Ecological Function:** Secondary metabolites are essential to mediate interactions between organisms and their surroundings. Plants create secondary metabolites as a defense against infections, herbivores, and rival plants (Wink, 2018). These substances' toxicity, repulsiveness, or anti-feedant qualities may serve as a deterrent to predators. They can also prevent rival plants from growing by strengthening the producer's capabilities for the consumption of the resources.

- **Pharmacological Significance:** Many secondary metabolites have been used in both conventional medicine and contemporary drug development due to their medicinal qualities. Secondary metabolites alkaloids, including caffeine and morphine, have been utilized for stimulation and pain treatment due to their strong physiological effects on humans (Cordell & Colvard, 2012).

- **Industrial Applications:** Beyond medicine, secondary metabolites have a wide range of industrial uses. For instance, many antibiotics, antifungals, and anticancer medications are derived from bacteria and fungi's polyketides

(Fischbach & Walsh, 2009). Terpenoids enhance the flavor, aroma, and therapeutic qualities of essential oils that are used in aromatherapy, food additives, and perfumery (Bakkali *et al.*, 2008). In the food and beverage business, several secondary metabolites are also used as natural colors, pesticides, and flavor enhancers.

- **Regulation of BioSynthesis:** Complex genetic and environmental variables control the biosynthesis of secondary metabolites. The genes encoding secondary metabolic enzymes are modulated by signal transduction pathways, transcription factors, and epigenetic changes (Pauwels & Goossens, 2011). Changes in secondary metabolite production can be triggered by environmental factors such as light, temperature, nutrition availability, and biotic interactions. This allows organisms to respond to stressors and adapt to their surroundings.
- **Biosynthetic Pathways:** Complex biosynthesis pathways are used to create secondary metabolites by involving several enzyme processes. These pathways frequently diverge from primary metabolic pathways by using precursor molecules from primary metabolism (Weng & Noel, 2012). Variations in the enzymes, substrates, and regulatory processes involved in the production of secondary metabolites give rise to their diversity.
- **Evolutionary Significance:** Organisms that produce secondary metabolites have selection benefits that impact their evolutionary paths and ecological interactions (Ehrlich & Raven, 1964). Through coevolutionary processes, organisms can diversify their chemical defenses and counter-adaptations. For that, they resist or tolerate secondary metabolites produced by rivals or predators. The dynamic interplay between species in natural ecosystems is driven by ecological forces, genetic variety, and interspecific interactions, each of which impacts the secondary metabolism.

1.6 Secondary Metabolites Produced by Actinomycetes

The following bioactive compounds are among the many different secondary metabolites that actinomycetes produce.

- **Antibiotics:** The most well-known use of actinomycetes is probably in the manufacture of antibiotics. In 1943, Waksman and Schatz discovered

streptomycin—the first antibiotic that effectively treated tuberculosis—from *Streptomyces* griseus. Since then, actinomycetes have been the source of several antibiotics that have greatly improved human health and medicine, including tetracycline, erythromycin, vancomycin, and rifamycin (Bérdy, 2005).

- **Antifungals:** Numerous antifungal substances produced by actinomycetes have been used in both medicine and agriculture. For instance, humans and animals with fungal infections can be treated with nystatin, which was derived from *Streptomyces noursei* (Peláez, 2006).
- **Antitumor Agents:** Compounds with possible anticancer effects are abundant in actinomycetes. For instance, bleomycin, which was derived from *Streptomyces verticillus*, is used in cancer chemotherapy (Demain & Vaishnav, 2011).
- **Immunosuppressants:** Compounds produced by actinomycetes can alter the immune system. In organ transplantation, tacrolimus (FK506), an immunosuppressant derived from *Streptomyces tsukubaensis*, is used to stop organ rejection (Kino *et al.*, 1987).
- **Enzymes:** Proteases, lipases, amylases, and cellulases are among the industrially valuable enzymes derived from actinomycetes (Kumar & Takagi, 1999).

Complex regulatory networks and environmental factors control the production of these secondary metabolites. Actinomycetes primarily produce these materials to give themselves a competitive advantage in their natural habitats by impeding the growth of rival microbes or by providing extra ecological benefits (Bérdy, 2005).

1.7 Actinomycetes: The Antibiotic Producers

Actinomycetes create a large range of bioactive secondary metabolites, including antibiotics. Researchers and the pharmaceutical industries have taken a keen interest in actinomycetes due to their therapeutic uses. Within this class, the genus *Streptomyces* is an important source of antibiotics. It is the source of several antibiotics that are therapeutically significant such as vancomycin, erythromycin, and streptomycin (Genilloud, 2017).

Biosynthetic gene clusters (BGCs) are specialized genomic areas that encode metabolic pathways and networks that control the manufacture of antibiotics in actinomycetes. Genes involved in the synthesis, modification, and control of antibiotic biosynthesis are found in these clusters (van der Meij *et al.*, 2017). Actinomycetes' adaptive mode of producing antibiotics is reflected in the fact that BGC activation is frequently impacted by environmental conditions such as pH, temperature, availability of nutrients, and interactions with other microbes (Genilloud, 2017).

Actinomycetes go through several developmental phases during their life cycle, such as vegetative growth, aerial hyphae formation, sporulation, and secondary metabolite synthesis. When bacteria face more competition for resources in their surroundings during the shift from vegetative development to sporulation, antibiotic production usually takes place (Barka *et al.*, 2015). The development of aerial hyphae gives the generating strain in the surrounding environment by serving as a scaffold for the spatial organization of the antibiotic biosynthesis machinery (van der Meij *et al.*, 2017).

Though actinomycetes have historically been a useful source of antibiotics, the rediscovery of known compounds and challenges in obtaining unique chemical diversity has slowed the pace of antibiotic discovery in recent decades (Genilloud, 2017). However, the investigation of new genetic reservoirs, the finding of novel bioactive compounds, and the developments in genome sequencing, bioinformatics, and culturing techniques have rekindled efforts to discover antibiotics (van der Meij *et al.*, 2017). Actinomycetes are known for producing antibiotics, but they also play a vital role in the functioning of ecosystems by interacting with other microbes and participating in processes including nitrogen fixation, cellulose degradation, and promoting plant development (Barka *et al.*, 2015).

1.8 Antibiotic Resistance and Novel Antibiotics

Bacteria make antibiotics useless when they develop defense mechanisms (World Health Organisation, 2022). Antibiotic abuse and overuse in human medicine, animal husbandry, and agriculture naturally lead to the development of resistance over time. (Goldman, 2004).

1.8.1 History and Emergence of Antibiotic Resistance

Antibiotic resistance has roots in the early years of antibiotic discovery and application. Bacteria have demonstrated an amazing capacity for adaptation and the development of resistance mechanisms against these life-saving medications since the introduction of the first antibiotics.

1.8.1.1 Early Antibiotic Era

The antibiotic era began with Alexander Fleming's discovery of penicillin. The first antibiotic used extensively in medicine was penicillin, which had a significant influence on how bacterial infections were treated. Antibiotics such as erythromycin, tetracycline, and streptomycin were later discovered, which increased the number of antibiotics that might be used medicinally (Podolsky, 2018). These medications saved numerous lives and transformed the way bacterial infections were treated.

1.8.1.2 Emergence of Resistance

Reports of germs resistant to penicillin started to surface a few years after the drug's introduction. Bacterial strains like *Staphylococcus aureus* produce enzymes called beta-lactamases, which can render penicillin and other beta-lactam antibiotics inactive. The overuse and widespread application of such antibiotics in human, livestock medicine, and agriculture were responsible for accelerating the emergence of antibiotic resistance (Ventola, 2015). Antibiotics were frequently administered insufficiently or indiscriminately for viral infections, which put resistant microorganisms under selective pressure to survive.

1.8.1.3 Modern Era of Antibiotic Resistance

Multidrug-resistant bacteria have grown to be a serious public health hazard in recent decades (Podolsky, 2018). Treatment is greatly hampered by bacterial strains that are resistant to several antibiotic classes, such as carbapenem-resistant Enterobacteriaceae (CRE) and MRSA.

Antibiotic resistance is a worldwide problem that transcends national boundaries (Ventola, 2015). Travel, trade, healthcare, and environments are mainly responsible for the rapid global spread of resistant genes and bacteria.

The history of antibiotics and the development of antibiotic resistance are shown in **Figure 3**.

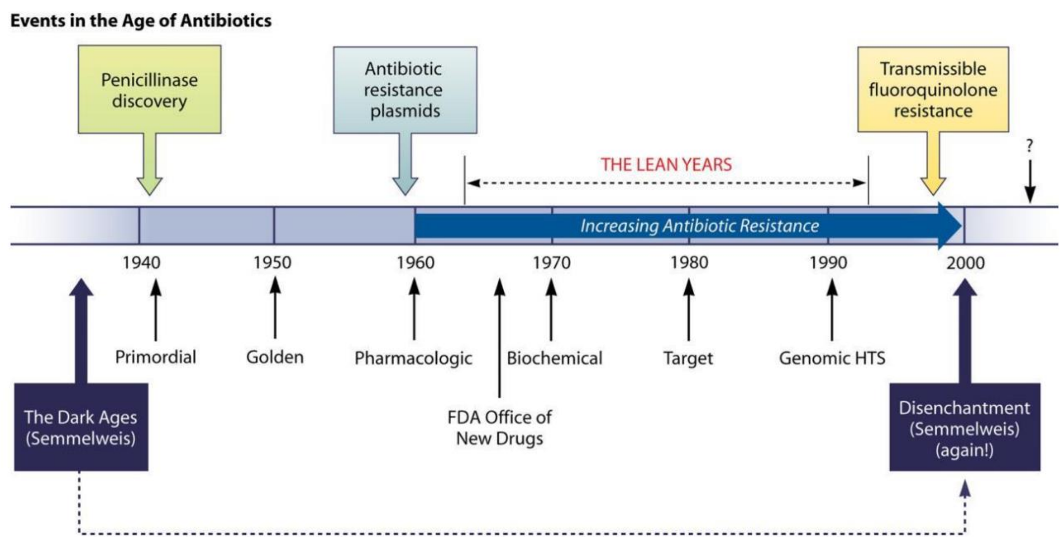


Figure 3: History of antibiotics and development of antibiotic resistance (Sharma *et al.,* 2014)

1.8.2 Mode of Resistance

There are several ways that antibiotic resistance can be developed that allow bacteria to withstand the effects of antibiotics. Some of them are described below.

- **Target Modification:** By altering the locations where medicines bind to their targets, bacteria can resist the medications. For instance, changes to the binding site of macrolide antibiotics like erythromycin can result from mutations in the bacterial ribosomal RNA (Poehlsgaard & Douthwaite, 2005).
- **Enzymatic Inactivation:** Antibiotics can be rendered inactive by certain bacteria's production of enzymes that alter or break them down. For example, many bacteria create beta-lactamase enzymes, which may hydrolyze the beta-lactam ring of cephalosporins and penicillins, resulting in the development of resistance to these antibiotics (Drawz & Bonomo, 2010).
- **Efflux Pumps:** Using efflux pump systems, bacteria can pump antibiotics out of their cells and build resistance. Antibiotics are actively transported across the bacterial membrane by these pumps, which lowers their intracellular concentration and effectiveness. Fluoroquinolones and tetracyclines are two

examples of the many antibiotics against which efflux pumps contribute significantly to resistance (Blair *et al.*, 2015).

- **Reduced Permeability:** Some bacteria become resistant to drugs by making their cell walls less permeable. Bacteria can lessen their vulnerability to antibiotics by controlling the amount of antibiotics that enter the cell. Gram-negative bacteria are known to demonstrate this process. They have an outer membrane that acts as a barrier against antibiotics (Nikaido, 2003).
- **Formation of Biofilms:** Cells arranged into ordered communities and covered in a barrier matrix are called biofilms and produced by bacteria. Treatment of infections is made more challenging by the greater resistance that biofilms give bacteria-to-host immune responses and medications (Hall-Stoodley *et al.*, 2004).

1.8.3 Tackling Antibiotic Resistance – Novel Antibiotics

Antibiotics have proven invaluable in the fight against infectious diseases. Many classes of potent antibiotics have been developed throughout the past century (Donadio *et al.*, 2010). However, pathogenic microbes are always changing and adapting, which causes antimicrobial resistance (AMR). The emergence of infections that are resistant to several drugs presents a noteworthy worldwide health concern, impeding the efficient application of antibiotics. The WHO has classified AMR as a genuine problem to global public health due to its severity. Thus, the main area of antibiotic research at the moment is the development of novel antibiotics that can combat pathogenic bacteria that have developed drug resistance.

Novel therapeutic medications are desperately needed to stop the spread of resistant pathogenic organisms. Even though current antibiotics can still be used, discovering new antibiotics is essential to treat bacterial infections more effectively (Bérdy, 2005). Metabolite production by different microorganisms was investigated and analyzed in the quest for new beneficial chemicals. Among them, soil microorganisms produce a wide range of bioactive chemicals. Since bacteria are potent in producing useful compounds, such as antibiotics, antivirals, herbicides, hydrolytic enzymes, anti-tumors, fungicides, and immune suppressants, people are interested in using them in medicines and agriculture (Khamna *et al.*, 2009).

1.9 Metabolomic Study

Metabolomic is the study of the metabolome in cells or organisms, which aims to identify and measure every molecule in a high-throughput manner. Metabolomics finds numerous uses in the study of health disorders, such as metabolome-wise association studies (MWAS), single-cell, epidemiologic population research, metabolic phenotyping, and precision/personalized medicine.

One analytical technique that can be used to analyze secondary metabolites is Mass Spectrometry (MS). To survive in harsh conditions, a tiny number of secondary metabolites that are produced by cells are important and beneficial to humans. In a similar vein, a great deal of these metabolites are produced by soil actinomycetes, which may be distinguished and identified in several ways. Using the mass-to-charge (*m/z*) ratio, different software in MS determines the compounds. The two main types of MS are Low-Resolution Mass Spectroscopy (LRMS) and High-Resolution Mass Spectroscopy (HRMS).

Mass accuracy: the precision with which the mass is determined distinguishes HRMS from LRMS. In contrast to LRMS systems, which are limited to a nominal mass of ± 1 Da, HRMS systems are capable of distinguishing substances based on their exact masses. Whereas LRMS cannot differentiate between separate isobaric compounds and/or share fragments and retention times (Rt), HRMS's high selectivity for the identification of precursors in MS1 and fragments in MS2. However, a significant disadvantage of HRMS is that its dynamic ranges and sensitivity are typically lower (Zarrouk *et al.*, 2023).

HRMS has developed into a potent analytical tool for identifying compounds. It is for achieving clear-cut, sufficiently sensitive qualitative and quantitative results. There is a wide range of high-resolution mass analyzers, varying in terms of instrument performance, cost, and complexity. These analyzers are commonly available in labs (Arrebola-Liébanas *et al.*, 2017).

Liquid Chromatography High-Resolution Tandem Mass spectrometry (LC-HRMS/MS) and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) are used to analyze ethyl acetate extracts from bacterial cultures. By comparing unknown substances to an online database using *m/z* value, this approach assists in determining their structure (Wang *et al.*, 2010a).

1.9.1 Metabolomic Study of Secondary Metabolites Produced by Actinomycetes

Streptomyces strains account for 90% of actinomycetes isolated from soil and rare actinomycetes obtained from plant roots. Actinomycetes, primarily *Streptomyces*, produce around two-thirds of all known antibiotics. Actinomycetes are the producers of around 61% of bioactive compounds out of all bioactive compounds produced by microorganisms. The "rare actinomycetes" account for 16% of this total, primarily from the *Micromonosporaceae*, with little additions also coming from the *Pseudonocardiaceae* and *Thermomonosporaceae* (Takahashi & Nakashima, 2018).

Metabolomics-guided examination of secondary metabolites isolated from soil actinomycetes is an emerging field of microbiology and drug discovery. Small molecules, or metabolites, inside biological systems, are the subject of in-depth analysis in the field of metabolomics (Fiehn, 2002). Here, metabolomics methods are used to investigate the metabolic profiles of actinomycetes, a common source of bioactive secondary metabolites (Bérdy, 2005).

Known for their capacity to generate a large number of secondary metabolites with different chemical compositions and biological activities, actinomycetes are a varied type of bacteria that are found in soil habitats (Bérdy, 2005). These secondary metabolites frequently have important medicinal qualities like immunosuppressive, antiviral, anticancer, and antibacterial effects. Many of these bioactive substances, meanwhile, are yet unidentified or poorly studied.

Actinomycetes cultures' metabolite profiles are thoroughly analyzed using cutting-edge analytical methods like mass spectrometry and nuclear magnetic resonance spectroscopy as part of metabolomics-guided research (Fiehn, 2002). Researchers can find new bioactive chemicals or maximize the production of recognized secondary metabolites by linking changes in metabolite profiles with certain environmental circumstances, genetic modifications, or culture conditions (Cheng *et al.*, 2005).

This method can identify silent or cryptic biosynthetic gene clusters, which code to produce secondary metabolites that are not expressed in typical laboratory settings (Katz & Baltz, 2016). Additionally, bioactive molecules with distinct chemical structures or modes of action can be identified by metabolomics-guided research. It may lead to the discovery of novel medication candidates with enhanced efficacy or decreased adverse effects (Netzker *et al.*, 2015). LC-HRMS/MS analytical technique is

used for the metabolomics study of secondary metabolites produced by soil actinomycetes.

1.9.1.1 LC-HRMS/MS Analytics

Since the mid-1990s, liquid chromatography-mass spectrometry (LC-MS) has grown in popularity as a tool for hyphenated analysis. Although it is a relatively new method, it has developed swiftly with applications ranging from inorganics to biological macromolecules. LC-MS simplifies sample preparation by allowing the study of non-volatile compounds of interest without derivatization procedures (Lu *et al.*, 2008).

The LC-HRMS/MS is an effective instrument for the simultaneous quantitative and qualitative study of organic contaminants. It allows for the quantification of these pollutants as well as the identification of unknown chemicals or the search for metabolites and transformation products. LC–HRMS/MS gives more information for each sample than low-resolution (LR) MS because it can precisely calculate the mass of the parent ions and daughter ions. Another advantage is its ability to process the data using target analysis, non-target screening, suspicious screening, or retrospective analysis (Aydoğan, 2020).

There are many benefits to using the LC-HRMS/MS technology for metabolomic research. It allows for the analysis of a broad range of metabolites with different chemical characteristics, such as polar and non-polar molecules, allowing for thorough metabolome coverage. Because of these benefits, LC-HRMS/MS is now a vital tool in metabolomics research. It facilitates an in-depth examination of the metabolome, enhancing our comprehension of biological systems and their reactions to diverse stimuli or disturbances (Wang *et al.*, 2010).

1.10 Unveiling the Bioactive Potential of Soil Actinomycetes in Nepalese Ecosystems

The study of biotic chemicals from soil actinomycetes in Nepal has great potential. Some of the reasons are discussed below.

- **Biodiversity Hotspot:** From the high Himalayas to the lowland Terai, Nepal is known for its abundant biodiversity, which spans a variety of ecosystems. A multitude of microorganisms, such as actinomycetes flourish in the soil of this diversified environment.

- **Untapped Microbial Resources:** Nepal's microbial biodiversity, especially its soil microbiome, is still largely unexplored despite its ecological significance. The microbiological diversity of many parts of the nation, particularly the isolated and mountainous regions, has not yet been adequately investigated. This offers a chance to find new strains of actinomycetes that have special metabolic properties and the capacity to produce bioactive substances.
- **Environmental Factors:** There are different types of environmental niches in Nepal due to its varied topography and climate with varied microbial communities and ecological dynamics. Microbial diversity and the metabolic capability of soil actinomycetes are influenced by various factors, including height, temperature, humidity, and soil composition. Researchers can access a wide range of microbial resources and improve the chance of finding physiologically active chemicals by sampling across different ecosystems and habitats.
- **Global Importance of Antibiotic Discovery:** The study of novel antimicrobial drugs is essential since antibiotic resistance poses a threat to world health. For a considerable amount of time, actinomycetes have been a major source of antibiotics, including well-known medications like streptomycin, erythromycin, and tetracycline.

1.11 Objectives

1.11.1 General Objective

- To isolate actinomycetes from the soils of Nepal to identify secondary metabolites including antibiotics.

1.11.2 Specific Objective

- To characterize bacterial strains based on partial sequencing of 16S rRNA gene.
- To screen bacterial strains based on antimicrobial and biochemical assays.
- To isolate secondary metabolites including antibiotics from shake flask fermentation.
- To annotate secondary metabolites computationally using tandem mass spectrometry.

1.12 Rationale of the Study

Human beings have been fighting many deadly diseases caused by microbes and even the rise of multidrug-resistant infections since the beginning of human civilization. Furthermore, bacterial infections are the cause of many premature mortality instances in third-world countries. The development of innovative broad-spectrum biomolecules offers a possible solution for this problem. The resources for such research are natural compounds, particularly those derived from microbial origins. Therefore, the thorough screening, isolation, and identification of novel actinomycetes strains and their bioactive compounds mark a significant advancement for industry and academia in the useful application of microorganisms.

1.13 Significance of the Study

The diverse topography of Nepal has rendered it an exceptional repository for a wide range of microorganisms. The unexplored habitats of actinomycetes from Nepal may contain new antibiotics. A novel bioactive substance with potential medical applications originates from actinomycetes found in Nepal's various habitats. Microbiological screening is a less explored area in Nepal. Still, not a single pharmaceutical company has installed a fermentation system to extract antibiotics from microbes. This work marks a significant advancement in Nepal's efforts to produce antibiotics from soil microbes. The numerous actinomycetes from diverse parts of Nepal have been examined in this study.

1.14 Hypothesis

Actinomycetes isolated from the soil of hilly regions of Nepal are very diverse, which aids in the production of emerging antibiotics that may contribute to the treatment of MDR infections.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Actinomycetes: Taxonomy, Phylogeny and Diversity

2.1.1 Introduction

Researchers have long been interested in actinomycetes: a diverse group of Gram-positive bacteria due to their vast ecological, industrial, and medical relevance (Bérdy, 2012). These filamentous bacteria are capable of producing a wide variety of antibacterial compounds (Goodfellow & Williams, 1983). Waksman and Henrici officially defined the biggest and most significant actinomycetes genus, Streptomyces, in 1923. This finding paved the way for the study of actinomycetes as a source of natural compounds, notably antibiotics (Waksman, 1953) .

Actinomycetes were initially identified in 1875 by Fording and Cohn. Actinomycetes were thought to be fungi until the 1950s. When technologies to examine morphology at the macroscopic and microscopic levels emerged, it was discovered that the microbes were filamentous prokaryotes. They exhibited close kinship to mycobacteria and coryneforms but they did not display any affinity to fungi (Stackebrandt *et al.*, 1997). Advances in microbe isolation and growing techniques accelerated actinomycete research. Modern molecular biology tools have improved the detection and characterisation of new species and strains with distinct metabolic profiles. In recent years, marine-derived actinomycetes have gained interest for their ability to create novel bioactive chemicals that are not often seen in terrestrial isolates (Subramani & Aalbersberg, 2012). These are unicellular, branching organisms that develop substrate and aerial mycelium; they are also acid-sensitive. Actinomycetes can be distinguished from fungi by their smaller filaments, measuring only 1µ smaller. They generate spores or conidia or multiply via binary fusion (Hesseltine, 2018).

Despite their importance, actinomycetes' systematics and classification have been continuously updated due to the discovery of new species and advancements in molecular biology.

2.1.2 Taxonomy and Classification

The inclusion of molecular data has resulted in considerable adjustments to the previous classification system, which separated actinomycetes into the groups namely Streptomycetaceae, Actinomycetaceae, and Micromonosporaceae (Stackebrandt *et al.*, 1997). Gene sequencing: 16S rRNA was used to determine the phylogenetic relationships of actinomycetes, leading to the identification of new families, genera, and species (Labeda *et al.*, 2012). For example, the variety of actinomycetes was extended beyond the classical taxonomy with the creation of the genera *Nocardiopsis* and *Saccharopolyspora*, as well as the family *Pseudonocardiaceae*.

Actinobacteria have been the subject of phylogenetic investigation primarily using 16S Ribosomal ribonucleic acid (rRNA) sequences. The phylum Actinobacteria is divided into 6 classes, 6 orders, and 14 suborders. In addition to the 16S rRNA gene, other standard markers such as RpoB, SecY, RecA, RpoB, GyrB, DnaK, and GrpE have also been used to build phylogenetic trees of certain Actinobacterium families, including *Streptomyces*, *Bifidobacterium*, and *Mycobacterium*. Furthermore, entire genome sequences have been used in Actinobacteria taxonomy research (Hazarika & Thakur, 2020).

The widely researched genus in this group is *Streptomyces*. They are distinguished by their filamentous development pattern, which results in branching hyphae that differentiate into aerial mycelia that carry spores (Barka *et al.*, 2015). The distinctive moldy look of *Streptomyces* colonies on agar plates is a result of the development of aerial hyphae and spores.

2.1.3 Phylogeny and Evolution

Actinomycetes' evolutionary relationships and patterns of diversification have been revealed by phylogenetic analyses based on molecular markers (van Bergeijk *et al.*, 2020). Using 16S rRNA gene sequences, phylogenetic trees were constructed, revealing that the phylum Actinobacteria is divided into several clades, with actinomycetes being a monophyletic group (Hazarika & Thakur, 2020). Moreover, research on comparative genomics has clarified the evolutionary processes and genomic characteristics of actinomycetes (Bentley *et al.*, 2002).

2.1.4 Ecological Significance

Actinomycetes are essential components of several biogeochemical cycles, supporting soil fertility, organic matter breakdown, and nutrient cycling (Fiedler *et al.*, 2005). Their significance in carbon recycling and soil organic matter turnover is highlighted by their capacity to break down complex organic molecules such as cellulose, chitin, and lignin (Goodfellow & Williams, 1983). Additionally, as plant growth-promoting rhizobacteria (PGPR), actinomycetes have symbiotic relationships with plants in which they aid in nutrient uptake, improve stress tolerance, and inhibit plant diseases (Bhattacharyya & Jha, 2012).

2.2 Actinomycetes Ecology

An isolated bioactive actinomycetes that generates a cytotoxic chemical was found in marine sediments. Based on the pure compound's spectrum data, resistoflavine: an antibiotic linked to quinone was found. It showed high cytotoxic action against the in vitro cultures of the gastric adenocarcinoma and hepatic carcinoma cell lines, HMO2 and HePG2 along with modest antibacterial activity hostile to both types of bacteria (Gorajana *et al.*, 2007).

A similar isolation of 140 actinomycetes was made from soil samples taken in several Iranian northwest regions. Through 16S rRNA sequencing, twelve of these isolates were recognized as *Streptomyces* spp. The isolates K36C5 and G614C1 exhibited similarities with *Streptomyces coelicolor* and *albogriseolus*, respectively (Maleki *et al.*, 2013). One hundred thirty-five unique *Streptomyces* sp. were isolated from symbionts, marine sediments, terrestrial soils, river sediments, severe habitats, and marine invertebrates. Out of them, 28 are found in marine habitats and 107 are found in terrestrial ones. 279 new secondary metabolites were extracted from these isolates (Donald *et al.*, 2022). Ten distinct strains of *Streptomyces* species were obtained from the feathers of domestic birds such as pigeons, *Larus michahellis*, sparrows, and seagulls (Sarmiento-Vizcaíno *et al.*, 2023).

Furthermore, Antido & Climacosa (2022) used an adapted integrated method to isolate many species of *Streptomyces* from various soil environments. Starch casein agar was used for the isolation process, and several biochemical assays and morphological characteristics were used for characterization. The soil sample taken from the dumpsite

has the highest proportion of the microbial population among the several soil habitats, whereas the forest has the lowest (Antido & Climacosa, 2022).

Several actinomycetes, primarily from the *Streptomyces* and *Microbispora* genera, have been suggested to have symbiotic relationships with numerous plants. By generating a variety of bioactive compounds, including antibiotics, plant growth regulators, and advantageous enzymes, these symbionts appeared to aid in the host plant's development (Hasegawa *et al.*, 2006).

By using phylogenetic analysis, Djebbah and colleagues isolated a new strain of *Streptomyces*, known as GLD22, from the limestone of Gueldaman Cave in Algeria. Analyzing isolates, Gas chromatography-mass spectrometry (GC-MS) disclosed the presence of several compounds, including cyclo(leucyloprolyl), 2-tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl)phenol, and dibutyl phthalate. These compounds displayed strong antibacterial action against both types of bacteria (Djebbah *et al.*, 2021).

2.3 Historical Background of Streptomyces

Streptomyces was first discovered in the early twentieth century. Russian scientist Olga Krassilnikov first observed the genus in 1916, but it was not until 1943 that Selman Waksman and his colleagues at Rutgers University identified the antibiotic streptomycin from Streptomyces griseus (Waksman, 1953). This was a watershed moment in the history of microbiology and medicine, when streptomycin became the first successful therapy for tubercuiosis, which had previously been believed incurable. Waksman's discovery of streptomycin not only transformed tuberculosis therapy, but also established Streptomyces as a major role in antibiotic manufacture. In 1952, Waksman was awarded the Nobel Prize in Physiology or Medicine in recognition of his remarkable discovery.

Hopwood found several antibiotics from diverse *Streptomyces* species, including erythromycin, tetracycline, and neomycin. These antibiotics were critical in fighting bacterial illnesses in the mid-twentieth century and are being used today (Hopwood, 2007).

Berdy studied the genus *Streptomyces* in 2005, and it is now recognised to be one of the biggest bacterial genera, with over 600 species reported to date. These species are mostly soil-dwelling microorganisms with a complicated life cycle that involves the

production of aerial mycelium and spore chains. *Streptomyces* species have an extraordinary capacity to create secondary metabolites, accounting for more than two-thirds of therapeutically effective antibiotics (Bérdy, 2005).

In 2006, Chater explored the importance of *Streptomyces* beyond medicines. *Streptomyces* species also generate enzymes, anticancer agents, antifungal chemicals, and immunosuppressive medicines. The identification of novel molecules from *Streptomyces* remains an important area of study, especially in light of developing antibiotic resistance and the need for new therapeutic medicines (Chater & Chandra, 2006).

In 2017, Baltz researched the application of genetic engineering and genome mining, which has opened new pathways for finding previously inaccessible chemicals from *Streptomyces* species (Baltz, 2017).

2.4 Bioactive Compounds from Actinomycetes and Their Applications

Actinomycetes, a genus of bacteria noted for its prolific synthesis of bioactive compounds, produce two-thirds of the antibiotics used in medicine. (Bérdy, 2012). Actinomycetes produce these tiny compounds, known as secondary metabolites, which are not directly related to their main metabolic processes. Rather, they frequently carry out ecological tasks such as defense against other microbes, communication, and competition (Barka *et al.*, 2015).

Bioactive compounds isolated from actinomycetes belong to several chemical groups, such as alkaloids, terpenoids, polyketides, nonribosomal peptides, and others. Nonribosomal peptide synthetases (NRPS) synthesize nonribosomal peptides. Polyketide synthases (PKS) produce polyketides. Alkaloids are produced from amino acids, whereas terpenoids come from the mevalonate or methylerythritol phosphate pathways (Bérdy, 2012).

Many biological effects, including antibacterial, antifungal, antiviral, antiparasitic, anticancer, immunosuppressive, and enzyme-inhibitory actions are exhibited by actinomycete's secondary metabolites (Genilloud, 2017). For instance, actinomycetes generate the well-known antibiotics streptomycin and tetracycline, which are very effective against a wide range of bacteria. Similarly, vancomycin is a strong antibiotic used to cure illnesses brought on by Gram-positive bacteria. It is processed by *Amycolatopsis orientalis* (Ling *et al.*, 2015). Actinomycete's secondary metabolites are

widely used in many different fields because of their broad biological activity. They are used in various fields which are discussed below.

- **Pharmaceutical Industry:** Secondary metabolites produced by actinomycete's fermentation have been used in the production of several antibiotics and other medicinal products. For example, respiratory tract infections are frequently treated with erythromycin: a macrolide antibiotic made by *Saccharopolyspora erythraea* (Donadio *et al.*, 2010). In a similar vein, *Streptomyces antibioticus* produces actinomycin D, which is utilized in cancer chemotherapy as an antineoplastic drug (Piel *et al.*, 2005).

- **Agriculture:** As biocontrol agents and plant growth boosters, actinomycete's secondary metabolites have been used in agriculture. For instance, bacterial infections in crops are managed using tetracycline and streptomycin (Palaniyandi *et al.*, 2013). Actinomycetes also generate substances like siderophores and indole acetic acid that help plants develop and absorb nutrients better (IAA) (Barka *et al.*, 2015).

- **Bioremediation:** Actinomycetes produce secondary metabolites with biodegradative qualities that aid in the breakdown of numerous environmental contaminants. For instance, actinomycetes assist environmental cleanup efforts by breaking down pesticides, hydrocarbons, and other xenobiotic chemicals (Pham & Kim, 2012). Antibiotics producing actinomycetes and their application are shown in **Table 1**.

Table 1: Antibiotics producing actinomycetes and their application.

Actinobacteria	Antibiotic compound	Application
Streptomyces sp.	1,8-Dihydroxy-2-ethyl-3-methyl anthraquinone	Antitumor
Schisandra chinensis	1-Hydroxy-1-norresistomycin	Antimicrobial; anticancer
Streptomyces sp.	2-Allyloxyphenol	Antibacterial; food preservative; oral disinfectant
S. galileus	Anthracyclines	Antitumor
Salinispora arenicola	Arenicolides A–C	Mild cytotoxicity

S. arenicola	Arenimycin	Antimicrobial; anticancer
Streptomyces avermitilis	Avermectin	Antiparasitic
S. griseus, *Streptomyces halstedii*	Bafilomycin	ATPase determent of microorganisms, living cells
Streptomyces sp.	Bisanthraquinone	Antibacterial
Streptoverticillium	Butenolides	Antitumor
Streptomyces sp.	Carboxamycin	Antibacterial; anticancer
Streptomyces sp.	Chinikomycins	Anticancer
Streptomyces venezuelae	Chloramphenicol	Antibacterial, an inhibitor of protein Biosynthesis
Solieria pacifica	Cyanospraside A	Unknown
Streptomyces sp.	Daryamides	Antifungal; anticancer
S. griseus	Frigocyclinone	Antibacterial
Streptomyces sp.	Glaciapyrroles	Antibacterial
Streptomyces hygroscopicus	Hygromycin	Antimicrobial, immunosuppressive
Streptomyces Nodosus	Lajollamycin	Antibacterial
Streptomyces lincolnensis	Lincomycin	Antibacterial, an inhibitor of protein Biosynthesis
Thermoactinomyces	Mechercharmycins	Anticancer
Streptomyces lavendulae	Mitomycin C	Antitumor, binds to double-stranded DNA
S. pacifica	Pacificanones A & B	Antibacterial
Streptomyces sp.	Piericidins	Antitumor
Verrucosispora sp.	Proximicins	Antibacterial; anticancer
S. hygroscopicus	Rapamycin	Immunosuppressive, antifungal
Streptomyces sp.	Resistoflavin methyl ether	Antibacterial; antioxidative
S. arenicola	Saliniketal	Cancer chemoprevention
S. pacifica	Salinispyrone	Unknown

S. pacifica	Salinispyrone A & B	Mild cytotoxicity
Salinispora tropica	Salinosporamide A	Anticancer; antimalarial
Streptomyces sp.	Salinosporamide B & C	Cytotoxicity
Streptomyces sp.	Sesquiterpene	Unknown
S. tropica	Staurosporinone	Antitumor; phycotoxicity
Streptomyces sp.	Streptokordin	Antitumor
S. griseus	Streptomycin	Antimicrobial
S. achromogenes	Streptozotocin	Diabetogenic
Streptomyces achromogenes, Streptomyces rimosus	Tetracyclines	Antimicrobial
Streptomyces sp.	Tirandamycins	Antibacterial
S. griseus	Valinomycin	Ionophor, toxic for prokaryotes and eukaryotes
Actinomadura sp.	ZHD-0501	Anticancer
Streptomyces sp. BK 190	Elaiomycins B and C	Antitumor
Nocardia dassonvillei	N-[2-hydroxyphenyl)-2-phenazinamine (NHP)	Anticancer; antifungus
Streptomyces coelicolor	Chromomycin B, A2, A3	Antitumor
Streptomyces sp. RAUACT-1	1,4-dihydroxy-2-(3-hydroxybutyl)-9, 10-anthraquinone 9, 10-anthrac	Antibacterial

(Anandan *et al.*, 2016)

2.5 Tandem Mass Analysis for Secondary Metabolites

Several analyzers including time of flight mass spectrometers, magnetic sector spectrometers, quadrupole ion trap mass spectrometers, electrostatic sector spectrometers, and ion cyclotron resonance were used to analyze the secondary metabolites that were extracted from the actinomycetes (Eliuk & Makarov, 2015). LC-HRMS/MS has been utilized in a non-targeted manner to analyze metabolomics. A hybrid quadrupole time-of-flight mass analyzer and an Ultra-Performance LC system were employed in the investigation of metabolomics (Hemmer *et al.*, 2020).

Streptomyces sp.VN1 was used in another study to identify several metabolites, including cinnamamide, Cyclo-L-proline-L-tyrosine, and the spirotetronate antibiotic lophophorin A. The synthesis of lobophorin analogs was confirmed by High Resolution Quadrupole Time of Flight (HR-Q-TOF) ESI/MS/MS analysis for the crude extract in conjunction with genome mining. This study suggests that the *Streptomyces* sp. VN1 strain holds a promise to produce novel natural chemicals. Additionally, Zhao (2019) recovered two new glutarimide antibiotics namely hydroxyliso-9-methylstreptimidone and 9-methylstreptimidone 2-D-glucopyranoside from *Streptomyces* sp. HS-NF-780 and structures were identified by spectroscopic analysis, which included ESI/MS and 1D and 2D NMR methods (Zhao *et al.*, 2019)

The three tumor cell lines were subjected to moderate cytotoxic activity from both drugs hydroxyliso-9-methylstreptimidone and 9-methylstreptimidone 2-D-glucopyranoside. By this, five known quinomycins and mono-sulfoxide quinomycin were recovered from the culture broth of *Streptomyces* sp. HCCB11876, after two novel quinomycins were found by UPLC-MS. All seven substances exhibited notable cytotoxic and antibacterial properties (Yang *et al.*, 2013).

Peng and colleagues isolated four antifungal chemicals from *Streptomyces* sp. of rhizospheric soils like fungichromin, antifungalmycin, actinomycin, and thailandin B (Peng *et al.*, 2020). Elshafie (2023) used LC-MS analysis to identify 20 secondary metabolites that were separated from actinomycetes of the rhizosphere. In the investigation, molecules were ionized in mass spectrometry using the ESI method, and secondary metabolites were separated using liquid chromatography (Elshafie et al., 2023).

2.6 Study Conducted in Nepal

Actinomycetes that produce antibiotics have been less explored in Nepal. It is not extensively documented in scholarly publications. Seventy-nine actinomycetes were identified from the soil of Kalapatthar, which is close to Mount Everest. In the primary screening process, out of 27 isolates, two Gram-positive and nine Gram-negative bacteria indicated antimicrobial characteristics hostile to at least one of the test bacteria. Secondary screening revealed antibacterial activity in 48.15% of cases. Three isolates showed broad-spectrum efficacy, which included inhibiting *Staphylococcus aureus* that was resistant to methicillin (Gurung *et al.*, 2009).

Forty *Streptomyces* that produced antibiotics were separated and examined from soil gathered near the *Alnus nepalensis* root. The cross-streak method was used for primary screening to identify methicillin-resistant *Staphylococcus aureus*, while antimicrobial activity was performed through the agar well diffusion method. Using rota vapor on a solid-state fermentation substrate, antibiotics were recovered using solvent extraction. The results of the experiment demonstrated the isolates' efficacy against MDR pathogens (Baniya *et al.*, 2018).

From a soil sample taken from Everest Base Camp, Yadav (2008) isolated *Streptomyces* sp. (Lob18.2b) that inhibited *Salmonella paratyphi*, *Salmonella typhi*, *Proteus mirabilis*, *Proteus vulgaris*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*, demonstrating antibacterial agent highly effective against all susceptible Gram-negative bacteria except *Proteus* spp. Thin-layer chromatography demonstrated that the antibacterial agent's chemical properties were similar to those of streptomycin (Yadav *et al.*, 2008).

Similarly, Khadayat (2020) used bioinformatics methods to extract *Streptomyces* species and characterize them based on similarity in morphology and 16s rRNA sequence. The potential activity against *E. coli* that yields Extended Spectrum Beta-Lactamase (ESBL) was demonstrated by the *Streptomyces* species (Khadayat *et al.*, 2020).

According to Sapkota (2020), out of 41 actinomycete isolates, 43.34% of them were shown to be highly effective antimicrobial producers against test organisms that were Gram-positive and 12.19% against those that were Gram-negative. Secondary analysis was done by agar well diffusion method after the perpendicular streaking method for the first screening was performed. Morphological analysis, various protein and sugar utilization, hydrolysis tests, temperature, and pH tolerance were used to identify the isolates (Sapkota *et al.*, 2020).

Shrestha et al. (2021) examined eight actinomycetes for efficiency against bacteria in water and soil sediments from Nepal, using both primary and secondary screening. Actinomycetes isolated from a water source had more antibacterial activity than soil samples. The most prevalent isolates were *Micromonospora*, followed by *Nocardia* and *Streptomyces,* which exhibited better antibacterial properties (Shrestha et al., 2021).

Rai et al. (2018) did research on soil in Chitwan, Nepal, and found twenty five distinct actinomycetes from two genera, *Streptomyces* (n=15) and *Micromonospora* (n=10), from specified soil locations. Primary and secondary screening of isolates revealed good antibacterial activity (Rai et al., 2018).

Bhandari et al. (2022) studied soil to characterise *Streptomyces* species and validate the antimicrobial activity of their metabolites using Molecular Docking.The most powerful *Streptomyces* species had higher MIC and MBC efficacy against both *Staphylococcus aureus* and *Shigella sonnei*. The extract of S. species SB10 contained significant metabolites by LC-HRMS. Studies on binding energy, docking viability, and protein-ligand molecular interactions revealed that these compounds are responsible for antibacterial activities (Bhandari et al., 2022).

Bhattarai et al. (2022) investigated the untargeted metabolomics of *Streptomyces* species isolated from Nepalese soils. To anticipate secondary metabolites, molecular annotation was conducted using the GNPS server, the SIRIUS platform, and accessible databases. The sequence of the 16S rRNA gene indicated that the isolates BN6 and BN14 are closely related to Streptomyces species (Bhattarai et al., 2022).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Research Design

The research strategy for the isolation of soil actinomycetes and examining the secondary metabolites produced by them was illustrated in the flowchart in **Figure 4**.

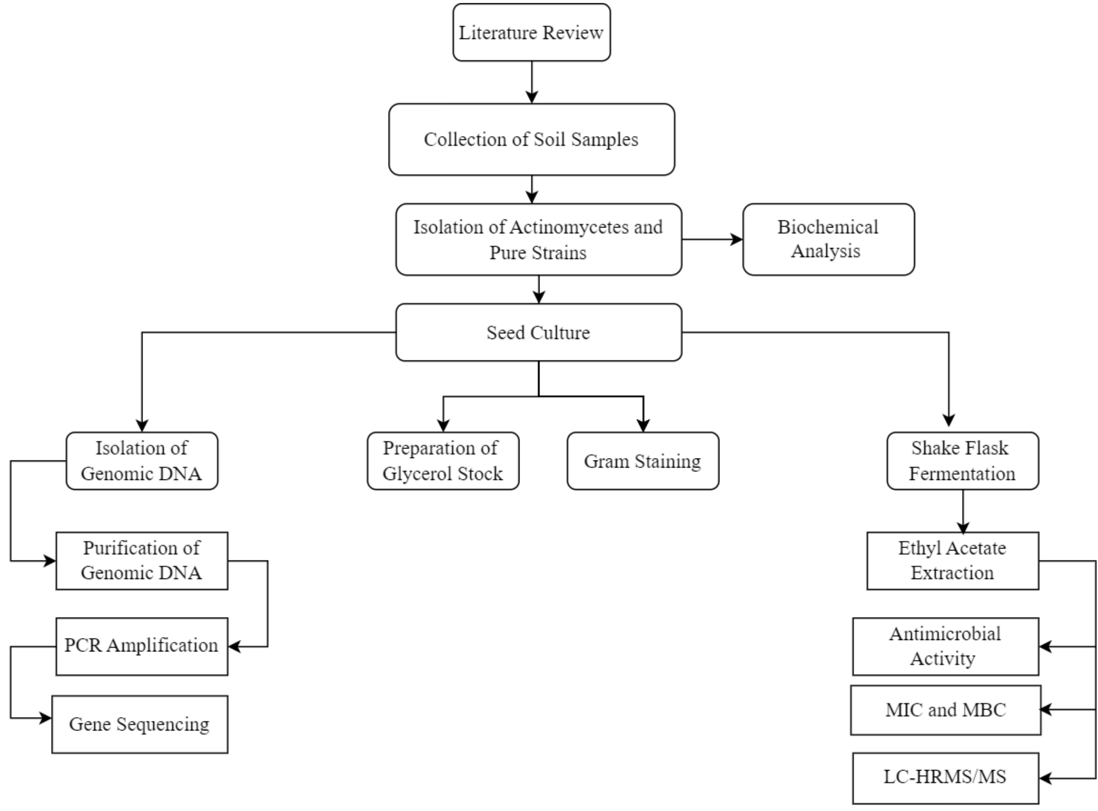


Figure 4: Workflow of research

3.2 Collection of Soil Samples

Different habitats of actinomycetes from Nepal were selected for the soil sample. A depth of three to five inches below the topsoil layer was chosen for this investigation due to the area's abundant microbial life. Using a dry, clean zip bag and a sterilized spatula, about 300 g of each soil sample was collected and the collected samples were air-dried. After that, the soil samples were transported to the Central Department of Chemistry laboratory, Tribhuvan University, Nepal. The samples were kept in storage at 4 ^{o}C.

There were 11 soil samples collected from different parts of Nepal. Sampling sites are selected based on the altitude from 1010 m to 4919 m which almost belongs to the hilly region of Nepal. The sample names, their locations, and altitudes are given in **Table 2**:

Table 2: Locations of soil sample collection

Location	Sample Name	Altitude (meter)
Low Camp (Kaski)	BT1	2680
Jomsom (Mustang)	BT2	2743
Galkot (Baglung)	BT3	1010
Pritam Deurali (Kaski)	BT5	2370
Badal Dada (Kaski)	BT13	3420
Tame (Ramechhap)	BT33	2173
Tilicho (Manang)	BT36	4919
Jaljala (Rolpa)	BT39	3234
Khaptad National Park	PC1	2500
Barpak (Gorkha)	AB1	1900
Upper Tamghas (Gulmi)	PT7	2000

3.3 Preparation of Culture Media

Various media were used for the isolation and culture of the bacteria from the collected sample in the laboratory under direct observation.

3.3.1 Preparation of ISP4 Media

International Streptomyces Project 4 (ISP4) medium is particularly designed for the culture of Streptomyces and other actinomycetes. For 1-liter ISP4 medium in distilled water, it contained 10g of soluble starch, which is the principal carbon source, 1g dipotassium phosphate, 1g magnesium sulphate, 1g sodium chloride, and 1g calcium carbonate all contain essential elements. 2g of ammonium sulphate is also supplied as a nitrogen source. Ferrous sulphate, manganese chloride, and zinc sulphate, each containing 1 mg, provide trace elements necessary for microbial development and metabolism. To solidify the medium, 18 g of bacto agar is supplemented. This balanced

composition provides an excellent environment for actinomycetes' development and secondary metabolite synthesis. The broth was then autoclaved at 121 ºC for 15 minutes. A 20 mg/L solution of nalidixic acid and 50 mg/L solution of cycloheximide were supplemented to the ISP4 broth when it reached a temperature of about 40 ºC to avoid the development of Gram-negative bacteria and fungal species respectively. After that, 25-30 mL of ISP4 broth was poured into a petri dish. Then, it was covered and kept at 0 ºC for preservation (Kharel *et al.*, 2010).

3.3.2 Preparation of TSB Media

Seed culture and fermentation of isolated actinomycetes strains were accomplished using Tryptic Soy Broth (TSB) medium (tryptone 17.0 g, soytone 3.0 g, glucose 2.5 g, sodium chloride 5.0 g, dipotassium phosphate 2.5 g, pH 7.3 ± 0.2 at 28 ◦C; volume 1 L water). 30 g of TSB powder was weighed, dissolved in 1000 mL of distilled water, and boiled for two to three minutes at 50 ºC. For seed cultivation, 20 mL of TSB medium was added to a dry, clean 100 mL capacity conical flask. Eight or ten sterile glass beads were placed inside the flask, which was then lightly packed with sterile cotton and covered with aluminum foil. The flask fermentation was carried out using a dry, sterile baffle flask that held 100 mL of the TSB medium. Finally, for 15 minutes the flask was autoclaved at 121 ºC.

3.3.3 Preparation of MHA Media

The primary and secondary screening of the ethyl acetate extract was done by using Mueller Hinton Agar (MHA) media. In 1000 mL of distilled water, 38 g of MHA powder was dissolved, and the broth was heated to make the mixture homogeneous. The media was then sterilized at 121 ºC for 15 minutes in an autoclave. The sterilized media was cooled, and 25-30 mL of the media was poured into the sterile plate. The media plates were refrigerated at 0 ºC.

3.3.4 Preparation of MHB Media

It was necessary to create a bacterial suspension of the tested bacteria to undertake antimicrobial screening. For 1L of Mueller Hinton Broth (MHB) media in distilled water, 21g MHB media was dissolved and autoclaved at 121 ºC for 15 minutes. Then, 5 mL of MHB medium was added to test tubes, each of which was then inoculated with

the tested bacterium. The test tubes were then loosely sealed with aluminum foil and cotton. Lastly, it was incubated in an incubator for 24 hours at 37 ^{o}C.

3.3.5 Preparation of NA Media

Nutrient Agar (NA) media was used for the determination of Minimum Bactericidal Concentration (MBC). 28 g of NA powder was dissolved in 1000 mL of distilled water and autoclaved for 15 minutes at 121 ^{o}C. Then, 25-30 mL of NA media was poured into the sterile Petri dishes and refrigerated at 0 ^{o}C.

3.4 Isolation of Actinomycetes

Each soil sample weighing 1 g was serially diluted with 10 mL of sterile water. The liquid was vortexed to create a uniform mixture. Three-fold serial dilutions were performed to reduce bacterial concentration. The concentration of the suspension prepared from three-fold dilution is 10^{-3} g/mL. 0.1 mL of the suspension resulting from the dilution was then uniformly distributed onto ISP4 plates. Afterward, these plates were kept in an incubator at 28 ^{o}C for 7-10 days (Kharel *et al.*, 2010).

3.5 Morphological Characterization and Subculture

A distinct colony was subcultured in the ISP4 medium when the bacteria had grown sufficiently. A colony was chosen based on morphological characteristics, including color, roughness, and the mycelium of the substrate and aerial parts. After choosing an appropriate colony, it was streaked over the ISP4 medium and incubated at 28 ^{o}C for 7 days (Takahashi & Omura, 2003).

3.6 Biochemical Analysis

Biochemical tests were used to identify isolated microorganisms. Different biochemical studies were conducted using standard procedures for biochemical characterization (Singh *et al.*, 2019). The following biochemical tests were administered in this research.

3.6.1 Catalase Test

This test was performed to ascertain the presence of the catalase enzyme in actinomycetes. Initially, the culture of the target actinomycete species was cultivated on an agar medium and incubated under optimal conditions. When the culture attained the desired growth stage, the aseptic technique was employed to transfer a small aliquot of the culture into a sterile microscope slide. Subsequently, a precise volume of

hydrogen peroxide solution (3 %) was introduced into the slide containing the actinomycete culture at a 1:1 ratio. Following the addition of hydrogen peroxide, the test tube was promptly observed for the appearance of bubbles: indicative of oxygen gas production within the designated time frame of 15 to 30 seconds. Positive catalase activity was denoted by the rapid formation of bubbles within the culture. It signals the breakdown of hydrogen peroxide into water and oxygen by the catalase enzyme (Anjem & Imlay, 2012).

Conversely, the absence of bubbles within the stipulated timeframe led to the conclusion that the tested organism lacked catalase activity, resulting in a negative test outcome (Quinones *et al.*, 2006). The catalase test served as a pivotal tool in elucidating the biochemical attributes of the examined actinomycete species, particularly in discerning their capacity to produce the catalase enzyme (Baltch *et al.*, 1999).

3.6.2 Oxidase Test

The oxidase test was performed to detect the presence of the cytochrome c oxidase enzyme in actinomycetes; a vital component of the electron transport chain involved in aerobic respiration (Gray & Doolittle, 1982). Initially, the test reagent Tetramethyl Phenylene Diamine (TMPD) was prepared and a small amount of the actinomycete culture was transferred onto a sterile filter paper or directly onto the culture. Subsequently, a drop of the oxidase reagent was applied directly to the culture or filter paper containing the actinomycetes culture. The test was promptly observed for any color changes, particularly a transition to purple-blue, within 10 to 30 seconds (Clark & Lubs, 1947). A change into purple-blue indicated the presence of the oxidase enzyme and thus a positive oxidase response. Actinomycetes that exhibited a positive oxidase response included various species of *Streptomyces* and *Nocardia* (Gray & Doolittle, 1982). Conversely, if there was no discernible change in color within the specified time frame, the test result was negative, indicating the absence of the oxidase enzyme in the tested organism.

3.6.3 Nitrate Reduction Test

This is a crucial test to find out if an organism can convert from nitrate to nitrites. The ability of the organism to convert from nitrate to nitrite is indicated by the production of red colour in the presence of nitrite. The presence of red colour implies that the test organism has not reduced the nitrate. The organism has converted the nitrate to nitrogen

gas if the solution does not change colour. The necessary amount of nitrate broth was made and sterilised. The suspected bacterial culture was appended to the nitrate broth, which was then incubated for five days at 37 °C. One millilitre each of sulphanilic acid and α-Naphthylamine was added to the nitrate broth tubes after incubation. When nitrite was present, a red colour was produced, suggesting that the organism could convert from nitrate to nitrite. A small amount of zinc was added to broths which showed a detrimental effect. The red coloration suggested that the test organism had not reduced the nitrate (Chauhan & Jindal, 2020).

3.6.4 Starch Hydrolysis Test

Starch hydrolysis test is performed to identify the amylase enzyme which breaks down starch. Gram's iodine is poured into the plate after the incubation of the isolates. A reddish-colored clear zone appeared around the bacterial growth indicating hydrolyzed starch. Conversely, a blue or black patch appears if the starch has not been completely hydrolyzed. After being streaked on an NA slant, the bacteria were cultured for 24 hours at 37 °C. The test organism was cultured on the Starch Agar plate that had already solidified. For 48 hours, the plates were incubated aerobically at 37 °C. After taking the plate out of an incubator, the growth's location and appearance were documented before iodine was added. Gram’s iodine was present throughout the growth and its surroundings. Then, the remaining iodine from the plate was removed, and the effect was analyzed (Chauhan & Jindal, 2020).

3.6.5 Indole Test

The motive of the indole test is to ascertain whether bacteria possess the ability to convert tryptophan; an amino acid into indole using tryptophanase. A dark pink colour ring appears on the surface that contains indole when Kovac's reagent (hydrochloric acid, p-dimethylaminobenzaldehyde, and amyl alcohol) is introduced. The indole test needs to be read within 48 hours of incubation because prolonged incubation can induce more indole degradation. Test tubes were filled with 5 mL of the medium, which was then autoclaved. A suspected colony extracted from the nutrient agar plates was introduced into a tube. Incubate the tube at 37 °C for 24 hours, then add a few drops of Kovac's reagent (Chauhan & Jindal, 2020).

3.6.6 Mobility Test

Examining the tubes after incubation allows us to immediately witness bacterial mobility. If the organism is mobile, growth extends beyond the line of inoculation. Growth is provided throughout the tube by highly mobile organisms. Non-motile creatures grow only along the stab line. After streaking a loopful of culture on an NA slant in serialized test tubes, it was incubated at 37 °C for 24 hours. Cultured bacteria were introduced into these tubes by piercing the medium column's center down to more than half the depth of the tubes. The tubes were kept at 37 °C for 48 hours. Following incubation, the tubes' development concerning the stab line was noted. Motile organisms expanded out from the line of inoculation and may have grown throughout the medium, whereas non-motile organisms only developed along the line of inoculation (Chauhan & Jindal, 2020).

3.7 Seed Culture

Seed culture was performed in Tryptic Soy Broth (TSB) media to acquire the pure culture of isolated colonies. Using a sterile inoculating loop, a suitable colony was chosen from the Petri plate based on its morphological characteristics. Then, 20 mL of autoclaved TSB media was added to a 100 mL conical flask containing 10-12 glass beads offering a broad surface area for bacterial adhesion and growth. The mouth of the flask was closed with cotton and wrapped with aluminum foil. The injected medium was then shaken at 180 revolutions per minute for 5-7 days at 28 ˚C (Shirling & Gottlieb, 1966).

3.8 Bacterial Growth Curve

After fermentation, the ideal period to collect secondary metabolites is usually determined by examining a growth curve, which is often accomplished by using a method called the dry mass method. With this technique, the evolution of the bacterial population in culture over time was monitored. 1 mL of bacterial culture was centrifuged in an Eppendorf tube for 10 minutes, at a specific speed of 4000 rpm. After centrifugation, the liquid culture medium-containing supernatant was carefully removed, leaving the bacterial precipitate. After that, the bacterial precipitate was completely dried to remove any remaining moisture. When the bacterial precipitate had completely dried, its dry mass was measured and recorded. Over several days, measurements were taken at precise 24-hour intervals. This methodological technique

provides valuable insights into the growth dynamics of the bacterial culture and helps researchers identify the optimal time point for harvesting secondary metabolites, based on growth curve analysis (McGinnis, 2012).

3.9 Microscopic Characterization

Gram staining was used to perform microscopic characterization of the isolated strains. After the TSB media had been allowed for adequate growth, a loop full of colonies was extracted and a bacterial smear was made on a dry and clean glass slide. Subsequently, a minute was spent leaving the smear submerged in crystal violet. After that, the slide was cleaned with distilled water and the leftover water was removed by shaking the slide gently. Gram's iodine was applied to the smear and allowed to sit for 30 seconds. After giving it a thorough cleaning with distilled water, 95% ethanol was applied for 30 seconds. Then, ethanol was cleaned with distilled water and safranin was added as a counterstain and allowed to soak into the smear for 30 seconds. Finally, the glass slide was cleaned with distilled water, and the remaining water was shaken off. After drying, the slide was examined under a microscope at 10 X and subsequently at 100 X using immersion oil. The slide was observed whether it was contaminated for mycelia or not (Bartholomew & Mittwer, 1952). The isolates were taken for further processing if it was not contaminated.

3.10 Preparation of Glycerol Stock

To ensure their long-term preservation, the isolated actinomycetes were stored in stock solutions containing 50% glycerol. The glycerol stock was prepared by dissolving 500 µL of glycerol in an equivalent volume of autoclaved distilled water. Then, the mixture was vortexed. Next, 1 mL of the bacterial suspension from the seed culture was added to the 50% glycerol solution and vortexed to ensure equal dispersion. The resulting suspension was tagged, packaged with parafilm, and stored at -20 °C to avoid contamination for future use (Shepherd *et al.*, 2010).

3.11 Shake Flask Fermentation

The dry mass method was used to analyze the bacterial growth curve. This gives information regarding the best time for harvesting secondary metabolites after fermentation. In this method, 1 mL of bacterial culture was taken in an Eppendorf tube and centrifuged at 4000 rpm for 10 minutes. Then, the supernatant liquid layer was

removed, and the precipitate was dried. After complete drying, the dry mass was measured and noted. The same procedure was repeated for several days and measured exactly at intervals of 24 hours, till constant mass was observed (Li & Mira de Orduña, 2010).

Before fermentation, the seed culture of individual isolated colonies was carried out in TSB media at 28 °C for 5-7 days at 180 rpm in a shaking incubator. After sufficient growth of the isolates, 1 mL of bacterial suspension was taken and put into 100 mL of autoclaved TSB media for fermentation. The fermentation was carried out at 28 °C for 7 days at 180 rpm in a shaking incubator.

3.12 Ethyl Acetate Extraction

The ethyl acetate extraction method was used to produce the crude extracts. To do this, the bacterial suspension and an equal volume (100 mL) of ethyl acetate were completely mixed in the separating funnel. To get the crude bacterial extract, the clear supernatant was moved to a dry and clean beaker and let to evaporate in a water bath at 37 °C for 2-3 days. The extract was then dissolved in 2 mL of ethyl acetate and put into an Eppendorf tube which allowed it to evaporate for 24 hours at 37 °C in a water bath. Following that, the dried extract was kept at -4 ˚C until it was needed. (Bhattarai *et al.*, 2022).

3.13 Antimicrobial Activity

The extract was tested against a variety of Gram-positive pathogens, including *Staphylococcus aureus* ATCC 25293, and Gram-negative pathogens, including *Salmonella typhi* ATCC 14028, *Shigella sonnei* ATCC 25931, *Klebsiella pneumoniae* ATCC 700603, and *Escherichia coli* ATCC 3292, to determine its potency. Primary and secondary screening were used to assess the bacterial extracts' capacity to eradicate or stop the growth of harmful pathogens.

3.13.1 Primary Screening

The perpendicular streaking method was used for the primary screening of isolate on Mueller Hinton Agar (MHA) medium. After being separated, the bacteria were streaked linearly across the diameter of the plate and cultivated for five to seven days at 28 °C. The pathogens under investigation were grown in MHB medium for 24 hours at 37 °C. Their turbidity was then adjusted to standard 0.5 McFarland (1.5×10^8 CFU/mL) for

future use. The pure suspension was streaked perpendicular to the isolates when they had reached complete growth. Then, at 37 °C for 24 hours, the plates were incubated and the antibacterial activity of the isolates was observed by the suppression of test organisms (Bizuye *et al.*, 2013).

3.13.2 Secondary Screening

Agar-well diffusion technique was employed for the secondary screening of the crude extracts. Using sterile cotton buds, the test organisms' standard culture was swabbed over the MHA media. Tests were conducted on the Gram-positive pathogen *Staphylococcus aureus* ATCC 43300 as well as the Gram-negative pathogens *Salmonella typhi* ATCC 14028, *Shigella sonnei* ATCC 25931, *Klebsiella pneumoniae* ATCC 700603, and *Escherichia coli* ATCC 25922. The pathogens that were being studied were cultured for 24 hours at 37 °C in MHB medium. Their turbidity was subsequently matched to standard 0.5 McFarland (1.5×10^8 CFU/mL). Next, a sterilized cork borer of 6 mm diameter was taken to make the wells. Then, the wells were filled with the extract working solution diluted in 50 % Dimethyl Sulfoxide (DMSO), the positive control (1 mg/mL Neomycin), and the negative control (50% DMSO). Eventually, the clear zone of inhibition was seen after the plates were incubated for 24 hours at 37 °C (Sapkota *et al.*, 2020).

3.13.3 MIC and MBC

The resazurin-based broth microdilution method was used to assess the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the potent extracts, in compliance with the Clinical Laboratory Standard Institute (CLSI) (Andrews, 2001).

Two types of bacteria, namely *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300 were cultured on MHB medium for 24 hours at 37 °C in an incubator. 100 µL MHB was added to all the wells of 96 micro-dilution plates except the twelfth column. The extract with the same concentration of 100 µL was taken twice and loaded to the adjacent wells of the plate for the first row. The same process was repeated for four different samples on each plate. The ninth well of the first row was filled with 100 µL Neomycin (1 mg/mL) which was used as a positive control. After matching the turbidity of bacterial suspension with 0.5 McFarland, it was further diluted 100 times to reach a final concentration of 1.5×10^8 CFU/mL. 100 µL bacterial inoculum

was added to the tenth well of the first row of 96 well plates. The eleventh column of the micro-dilution plate which already had the MHB media was used as a negative control. 30 µL of bacteria was added to all the wells except the negative control. The plate was covered with a sterile lid and incubated for 24 hours at 37 °C. After the microtiter plate was incubated for 24 hours, 5 µL of Resazurin (0.015%) was added to each well, and the plate was incubated for 3–4 hours at 37 °C. The color of the wells with bacterial growth was pink, whereas the wells without bacterial growth were blue. The MIC is the lowest concentration of the extract that controls the visible growth of a bacteria whereas the MBC is the the minimal amount of a bacterial agent required to eliminate the test bacterium. The MBC was measured by streaking the contents of the wells just above the pink color onto NA plates, which were then incubated at 37 ºC for more than 18 hours (Elshikh *et al.*, 2016).

3.14 Molecular Identification by LC-HRMS/MS

Ethyl acetate (EA) extracts of four samples (BT1, BT2, BT3, and PC1) were then subjected to liquid chromatography-high resolution tandem mass spectrometric analysis (LC–HRMS/MS) using an Agilent G6545B Q-TOF mass spectrometer (Agitech, Santa Clara, CA, USA) at Sunkyunkwan University, Suwon, Republic of Korea. The LC–HRMS/MS analysis of the extracts PT7 and AB1 was conducted at the Central Drug Research Institute (CSIR) in Lucknow, India.

After dissolving EA extracts at a concentration of 1 mg/mL in HPLC-grade solvent, four samples were ready for MS/MS analysis. An HPLC autosampler vial was filled with 150 µL of each sample. A 150 mm × 2.1 mm, 1.7 µM Acquity® UPLC BEH reverse-phase C18 column was used to achieve chromatographic separation. H_2O (A) and acetonitrile (B) were the mobile phases that had been acidified using 0.1% formic acid. The organic solvent's composition was as follows: 5% from 0.00 to 2.00 minutes, 20% at 5.00 minutes, 100% at 20.00 minutes, and 5% from 23.00 to 25.00 minutes. A consistent flow rate of 0.5 mL/min was maintained, and each sample's injection volume was kept at 3 µL. The electrospray ionization (ESI) technique in positive ion mode was used to acquire the MS/MS data, with a full width at half maximum (FWHM) of 3000 and the *m/z* range of 50–1200 Da. The collision energies were set at 15V and 40V.

The .d format of the raw data was changed to the .mzXML format and then further annotated using the CSI: FingerID graphical interface included in the SIRIUS software.

The MestReNova software (version 12.0) was used to calculate the mass, absolute error, RDBE, and molecular formulas. These results were compared to the formula produced by SIRIUS. Additionally, the annotated compounds were verified through the SciFinder literature survey, natural products-based databases like ChemSpider (https://www.chemspider.com/), Natural Products Atlas (https://www.npatlas.org/), LOTUS (https://lotus.naturalproducts.net/), PubChem (https://pubchem.ncbi.nlm.nih.gov/), and library searches using SIRIUS software. A higher SIRIUS score denotes greater confidence in the molecular annotation. The SIRIUS score is a metric that is generated by software to measure molecular annotation confidence.

3.15 Identification of *Streptomyces* Species

3.15.1 Isolation of Genomic DNA

Strains with uncontaminated, hair-like thin mycelia under the microscope were chosen for the isolation of genomic DNA. 15 mL of bacterial suspension in a falcon tube cultivated in TSB media was centrifuged at 4000 rpm for 10 minutes which produced a cell pellet from a pure seed culture. The pellets were washed again by centrifugation using a 10 % sucrose solution. The clear supernatant layer was discarded. The resultant cell pellet was preserved at -20 °C after being dried for 24 hours at 37 °C in an incubator and was used later.

Using the Phenol-Chloroform-Isoamyl alcohol (PCI) technique, genomic DNA was extracted from the collected cell pellet. In a falcon tube, the dry cell pellet was mixed with an equal volume of lysis buffer (10 % sucrose solution) and vigorously vortexed. After adding a small amount of lysozyme (about 1 mg), the tube was gently vortexed to mix completely. After that, the tube was kept for 45 minutes at 37 °C in a water bath, with the lysis process being checked in every 15 minutes. 20 µL proteinase K and 250 µL 0.5 M Ethylenediaminetetraacetic acid (EDTA) were added. Then, the tube was incubated for 5 minutes at 37 °C. Next, 200 µL of 10 % Sodium Dodecyl Sulfate (SDS) was added, mixed thoroughly, and allowed to incubate for 10 minutes at 70 °C. After that, the tube was quickly placed in a water bath for 15 minutes. 500 µL of 5M potassium acetate was added. The tube was then placed in an ice bath for 15 minutes.

After that, the same amount of PCI (25:24:1) was added and properly mixed. The tube was then centrifuged for 10 minutes at 4000 rpm. A sterilized falcon tube was used to properly receive the clear supernatant. After adding double-volume chloroform to the

supernatant, the tube was centrifuged once again for 10 minutes at 4000 rpm. After transferring the upper clear supernatant to a dry and clean Eppendorf tube, 40 μL of 1 mg/mL of RNase was added. The tube was incubated at 37 °C in the water bath for 1 hour.

Following the RNase treatment, the Eppendorf tube was filled with two volumes of 100% ethanol and one volume of isopropanol and shaken slowly. The mixture was then kept at -20 °C for 30 minutes to precipitate the DNA. The supernatant was disposed of after the tube was centrifuged for 5 minutes at 10,000 rpm. After washing and air-drying the DNA pellet with 70% ethanol, it was dissolved in 20 μL of Tris-EDTA (TE) buffer. It was preserved at -20 °C to use the DNA sample for later (Saunders, 1993).

3.15.2 Purification of Genomic DNA

The ethanol precipitation technique is used in genomic DNA purification after the DNA is extracted in a TE solution. To evaluate purity, 10 μL of genomic DNA was combined with roughly 2.5 volumes of absolute ethanol (25 μL). The mixture was mixed with 0.1 volume of 3 M sodium acetate (pH 5.2) to aid in precipitation. After that, the mixture was kept at 20 °C overnight to allow the DNA to precipitate. DNA pellets were cleaned twice with 70% ethanol and allowed to air dry before being used for further procedures such as agarose gel electrophoresis.

3.15.3 Gel Electrophoresis

Agarose gel electrophoresis was used to observe and quantify the genomic DNA that was separated from cell pellets. 0.08 g of agarose was dissolved in 20 mL of TE buffer (1 X) to prepare a 0.4% agarose gel. After the solution was heated to dissolve the agarose gel, 2 μL of ethidium-bromide (EtBr) was added and thoroughly mixed. A comb was placed over the gel cassette and agarose gel solution was poured into it. The gel was stored in an electrophoresis container filled with 1X Tris Acetate EDTA (TAE) buffer after the agarose solution was allowed to set. Subsequently, a clean aluminum foil was used to mix 1 μL of 6X loading dye (bromophenol blue and Glycerol) with 3 μL of DNA solution. The combined solution was then pipetted and added to the well. After that, 1.5 μL of loading dye and 3 μL of a 1kb DNA (Sigma, D3937) ladder were combined to make a DNA ladder, which was then loaded into another well. Electrophoresis was then run for an hour at 75 V (P. Y. Lee *et al.*, 2012; Friedman &

Brewer, 1995). Following electrophoresis, Gel-Doc (UVITEC, Cambridge) was used to observe genomic DNA.

3.15.4 PCR Amplification

Using these set of universal primers, 27F: AGAGTTTGATCMTGGCTCAG and 1492R: ACGGYTACCTTGTTACGACTT (Merck), the 16S rRNA gene was amplified by PCR (Applied Biosystems, Thermo Fisher Scientific) (Heuer *et al.*, 1997). Primers were diluted with nuclease-free water to a working concentration of 10 μM. Then, amplification was performed by using 5X premix (Solis Biodyne) in a 50 μL reaction mixture including Taq-polymerase in 29 cycles at an annealing temperature of 51.4 °C. After electrolysis, the amplification products were examined and purified. And a band of 1.5kb size was produced.

The PCR product was sent to Macrogen in Seoul, South Korea for sequencing after it was purified (using the Monarch® PCR & DNA clean-up kit, Biolabs T1030S) and validated by electrophoresis.

The PCR reaction mixture of 50 μL was prepared by mixing 10 μL PCR premix (5X), 2 μL Forward primer (10 pM), 2 μL Reverse primer (10 pM), 2 μL DNA template (125 ng) and 34 μL Nuclease free water. The final concentration of the constituents was 1X, 0.4 pM, 0.4 pM, and 5 ng respectively.

The PCR reaction mixture was then loaded into the PCR machine using a PCR tube. Inside the PCR machine, the mixture is initially denaturated at 95 ^{o}C for 3 minutes. It is then denaturated at 95 ^{o}C for 30 seconds, annealed at 51.4 ^{o}C for 30 seconds, and extended at 72 ^{o}C for 50 seconds. The process of denaturation, annealing, and extension is carried out for 29 cycles in series. The final extension of the mixture takes place at 72 ^{o}C for 10 minutes. The mixture is then held for a certain amount of time at 4 ^{o}C which is indicated in the PCR machine.

3.15.5 Gene Sequencing

3.15.5.1 Purification of Amplified DNA and 16S rRNA Sequencing

The PCR product was filtered to get rid of contaminants such as leftover primers, dNTPs, magnesium ions, DNA polymerase, and unamplified products. This was accomplished using a New England Biolab purification kit (Monarch® PCR & DNA clean-up kit, Biolabs T1030S). With a few minor adjustments, the manufacturer's

instructions were followed. The PCR product and DNA binding buffer were added to a 1.5 mL microcentrifuge tube in a 5:1 ratio, and the mixture was vortexed. After that, it was moved to a column within a collection tube, and it was centrifuged at 13,000 rpm for two minutes. The column was filled with 200 µL of DNA wash buffer, centrifuged for two minutes, and then cleaned. At last, the column matrix was directly filled with 12 µL of DNA elution buffer, and it was allowed to sit at room temperature for 5 minutes. Then, the column was moved to a 1.5 mL microcentrifuge tube, and the DNA was eluted by centrifuging for two minutes. By using electrophoresis, the PCR product purifications were confirmed.

Thus, purified products were sent for sequencing to GenoTech Inc., Daejeon, South Korea. All the laboratory experiments were performed in the Biological Chemistry Laboratory, Central Department of Chemistry, Tribhuvan University, Kritipur, Nepal.

3.15.5.2 Basic Local Alignment Search Tool (BLAST) Analysis

Sequences were read in FASTA format and subjected to analysis using the BioEdit Sequence Alignment Editor (Foley *et al.*, 2019). The BLAST search engine was utilized to compare DNA sequences with sequences that were obtained from the NCBI database management system (Johnson *et al.*, 2008). Sequencing was done for this dissertation using only the forward primer. The consensus sequences were deposited to NCBI and were incorporated during publication.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Isolation of Actinomycetes and Pure Strains of Isolates

Eleven soil samples were collected from various ecosystems of Nepal across different regions and altitudes. After 7 days of incubation, a significant number of colonies were seen in the isolation plates. A total of 110 actinomycetes colonies, 10 from each sample were cultured. Based on the color, roughness, and characteristics, a colony of each sample was selected and subcultured on ISP4 media. Pure strains were obtained from the subculture which were incubated for 7 days (Kharel *et al.*, 2010). Substrate and aerial mycelia of isolated actinomycetes are shown in **Appendix B**.

Soil is a diverse and nourishing habitat that supports a vast range of microorganisms. Molecular phylogenetic analysis has shown bacterial species per gram of soil (Uroz *et al.*, 2010). The soil ecosystem is a valuable resource for isolating *Streptomyces*, which produce a wide variety of metabolites and produce novel molecules in response to varying environmental or nutritional conditions (Abdella *et al.*, 2023). Accurate species-level identification of bacterial isolates is crucial because it offers important information about the microbe, its potential bioactive compounds, and its uniqueness (Olubukola, 2014). Actinomycetes' growth and maintenance in agar media, such as ISP4, and broth medium, such as TSB, are important for secondary metabolite synthesis. Most actinomycetes sporulate effectively in the ISP4 medium. ISP4 medium contains nalidixic acid and cyclohexamide, which prohibit the development of Gram-negative bacteria and fungi, respectively (Kharel *et al.*, 2010).

4.2 Gram Staining

From the Gram staining, it was observed that the mycelium of the selected isolates had hair-like structures and flagellated bacteria. The mycelium retained the color of the primary strain; crystal violet after gram staining which indicated that the isolates were Gram-positive (Schleifer & Kilpper-Bälz, 1987).

Additionally, morphology is crucial for identifying various *Streptomyces* species within the genus and for setting *Streptomyces* apart from other spore-forming actinomycetes.

Eleven bacterial strains (BT1, BT2, BT3, BT5, BT13, BT33, BT36, BT39, AB1, PT7, and PC1) were isolated from soils of different environments for this study. The results showed that the mycelia had a smooth, filamentous structure and bore spores on the aerial mycelia, indicating the presence of *Streptomyces* species (Kumar & Takagi, 1999).

Under microscopic examination using Gram staining, all of the isolates showed a variety of morphological characteristics and were identified to be Gram-positive. Mycelia of the selected isolates are shown in **Appendix C**.

4.3 Shake Flask Fermentation

After fermentation of actinomycetes for 7 days in a shaking incubator, an equal volume of ethyl acetate was added. About 20-40 mg of crude extract of each isolate was obtained after the whole process. Several parameters must be optimized during shake flask fermentation in order to maximize bioactive metabolite yields. These include the growth medium's composition, temperature, pH, and agitation rate. The medium and its components (such as carbon and nitrogen sources) have a substantial influence on the bacteria's metabolic pathways, influencing the quantity and quality of metabolites generated. The most efficient antibacterial crude extract was determined to be ethyl acetate crude extract among various solvents (Murtaza *et al.*, 2024).

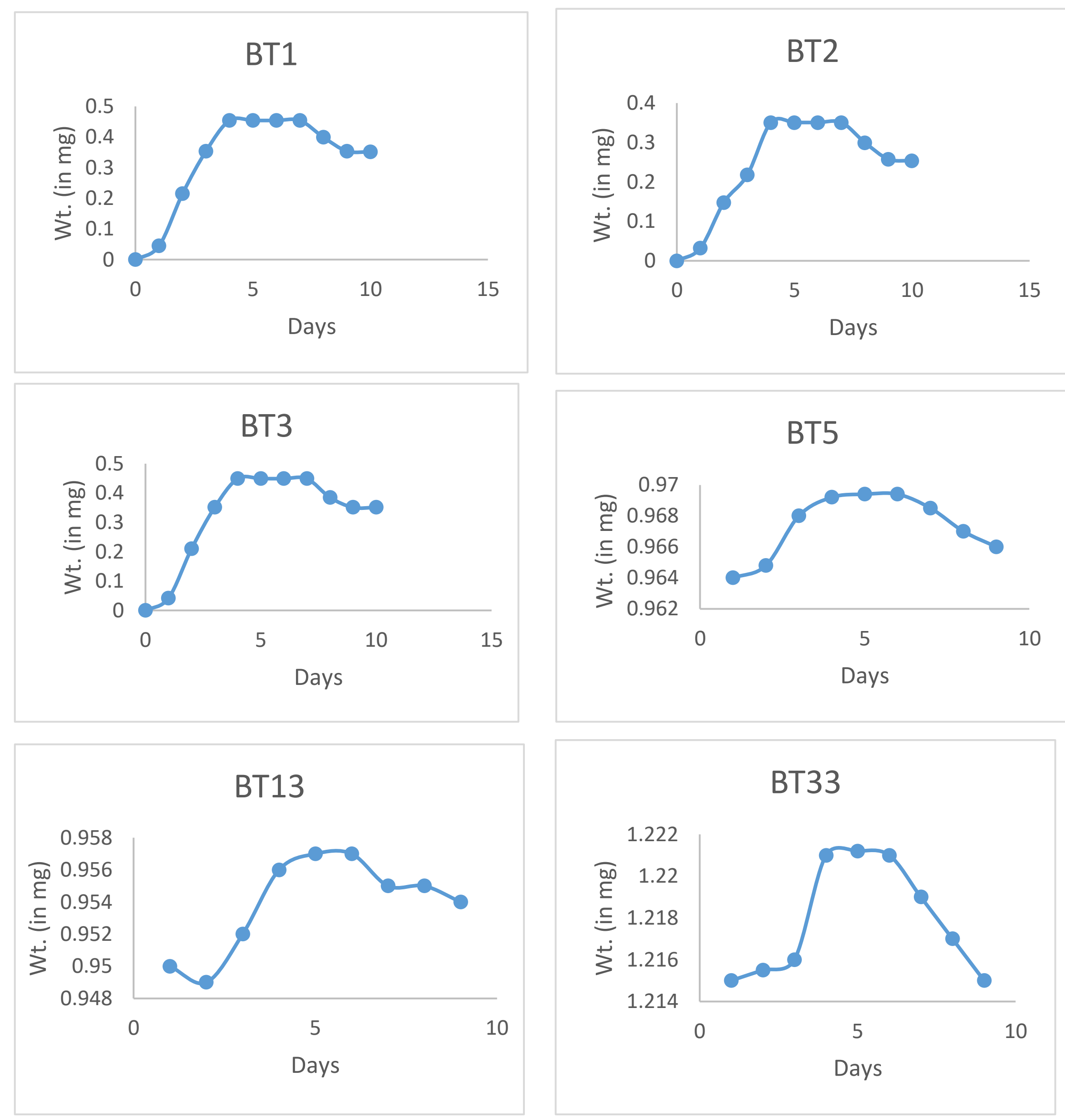
4.4 Growth Curve

The population's development was characterized in four ways. They are i. Cell concentration (cell count/mL), ii. Dry weight (mg/mL), iii. Colony-forming unit (CFU) (colonies/mL), and iv. Optical density measures (Jaishankar & Srivastava, 2017). Using the dry weight method, the growth kinetics of bacterial strains were examined to measure biomass production over time. After being injected into a medium rich in nutrients, bacterial colonies were continuously stirred and incubated. Samples were taken and analyzed to find the dry weight of the bacterial biomass at predefined intervals (e.g., every 24 hours).

The generated growth curve showed the typical stages of bacterial development: lag phase, exponential phase, stationary phase, and decline phase. During the lag phase, there was little increase in dry weight while the bacterial cells acclimated to the growth medium.

Fast biomass expansion during the exponential phase was a sign of aggressive cell division and metabolic activity. This phase showed a sharp rise in dry weight, which was consistent with the bacterial population's exponential growth rate.

The culture entered the stationary phase after the exponential phase. The stationary phase was characterized by a dry weight plateau as cell growth balanced with food depletion and cell death (Ughy *et al.*, 2023). In the bacterial population, the stationary phase denoted an equilibrium state when the rates of cell division and cell death were similar. At last, the decline phase was noted, during which a progressive drop in dry weight signified the death of cells and a reduction in viability because of nutrient exhaustion or the buildup of hazardous byproducts. Growth curves of isolated actinomycetes are shown in **Figure 5**.



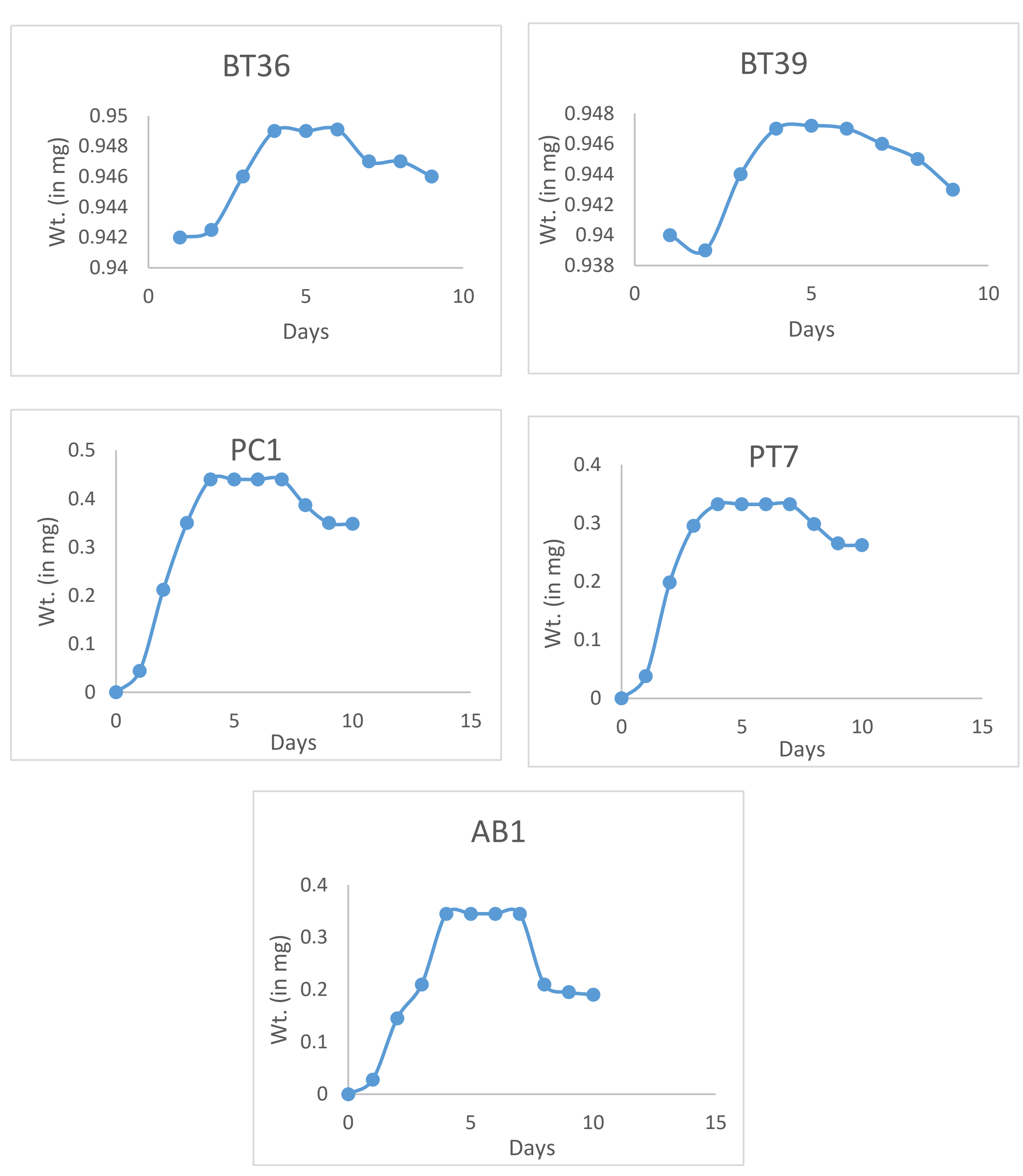


Figure 5: Growth curve study of various actinomycetes at TSB media.

4.5 Antimicrobial Activity

4.5.1 Primary Screening

Eleven actinomycetes were isolated from different ecosystems in Nepal. For the initial screening, microbial colonies exhibiting unique morphological traits were chosen. Those colonies demonstrated remarkable antimicrobial activity against Gram-positive bacteria *S. aureus* ATCC 25293 and Gram-negative bacteria *S. typhi* ATCC 14028, *K. pneumonia* ATCC 700603, *S. sonnei* ATCC 25931, *A. baumanii* ATCC 19606 and *E. coli* ATCC 3292 on the first screening of each strain using the perpendicular streaking method. Isolate PC1 showed strong antimicrobial efficacy against *S.sonnei* and *S.*

aureus. Isolate BT36 exhibited remarkable antimicrobial efficacy against all tested pathogens which are shown in **Appendix D**. A secondary screening was additionally performed on the active strains.

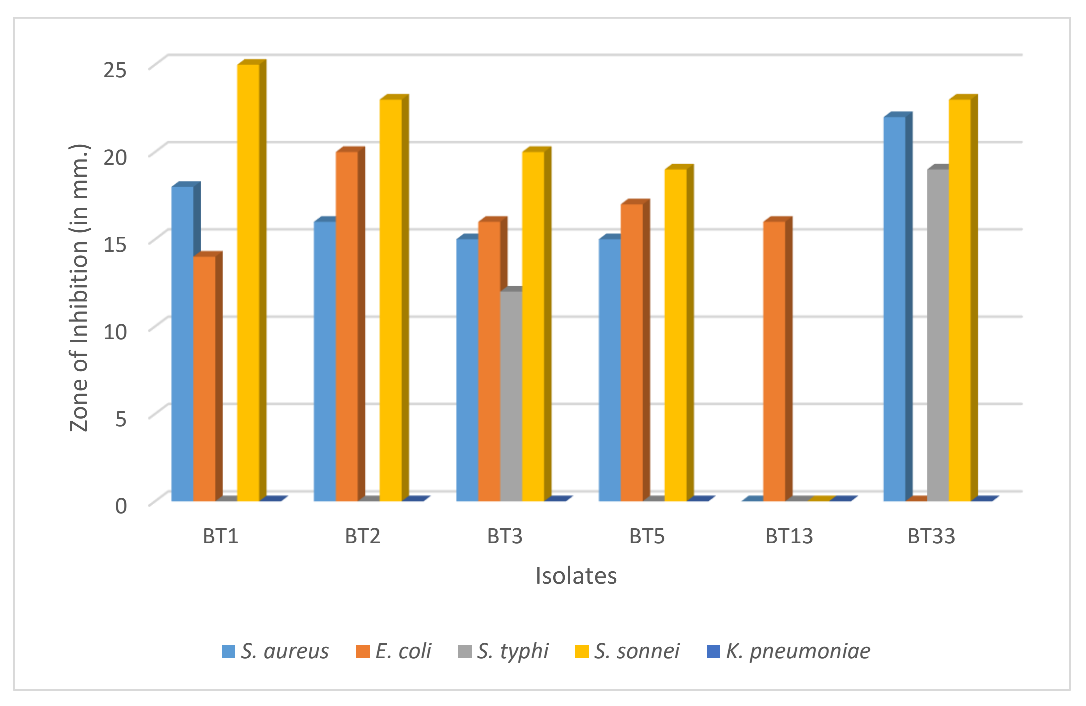
The survival of microbes even in very hostile and demanding habitats might be owing to their adaptability to the environment and capacity to develop resistant structures. Temperature is one of the environmental constraints and growth variables that impact actinomycete development and variety. It has a significant impact on the physiology, morphology, sporulation, biochemistry, and antimicrobial metabolite synthesis of organisms (Sapkota *et al.*, 2020).

4.5.2 Secondary Screening

A total of 11 bacterial isolates exhibited a significant zone of inhibition against both the Gram-positive pathogen *Staphylococcus aureus* ATCC 43300 as well as the Gram-negative pathogens *Salmonella typhi* ATCC 14028, *Shigella sonnei* ATCC 25931, *Klebsiella pneumoniae* ATCC 700603, and *Escherichia coli* ATCC 25922. The bacterial isolates were tested against different known strains of bacteria. The highest zone of inhibition was seen in PC1 against almost all tested pathogens except in *Staphylococcus aureus* ATCC 43300. The respective zones of inhibition for those isolates are listed below in **Table 3** and in **Figure 6.** Neomycin at a concentration of 1 mg/mL served as the positive control, while a 50% DMSO solution served as the negative control. For our extracts, the same concentration of 20 mg/mL was employed. When tested against the Gram-positive bacteria *Staphylococcus aureus* ATCC 43300, the majority of the extracts showed an average zone of inhibition; however, these extracts appeared to be less efficient against *Escherichia coli* ATCC 25922. Actinomycetes' genetic composition and growing circumstances impact their ability to produce bioactive metabolites. Variations in food availability, pH, temperature, and incubation time can all influence the types and quantities of antimicrobial compounds generated, resulting in varying degrees of bacterial inhibition (Berdy, 2005).

Table 3: Zone of inhibition of the isolates

Isolates	Bacterial Strains				
	S. aureus	***E. coli***	***S. typhi***	***S. sonnei***	***K. pneumoniae***
	Zone of Inhibition (in mm.)				
BT1	18	14	-	25	-
BT2	16	20	-	23	-
BT3	15	16	12	20	-
BT5	15	17	-	19	-
BT13	-	16	-	-	-
BT33	22	-	19	23	-
BT36	34	27	19	20	-
BT39	18	20	9	20	-
PT7	25	15	-	19	-
AB1	22	23	10	12	-
PC1	31	32	30	30	30
Neomycin	28	25	25	25	23



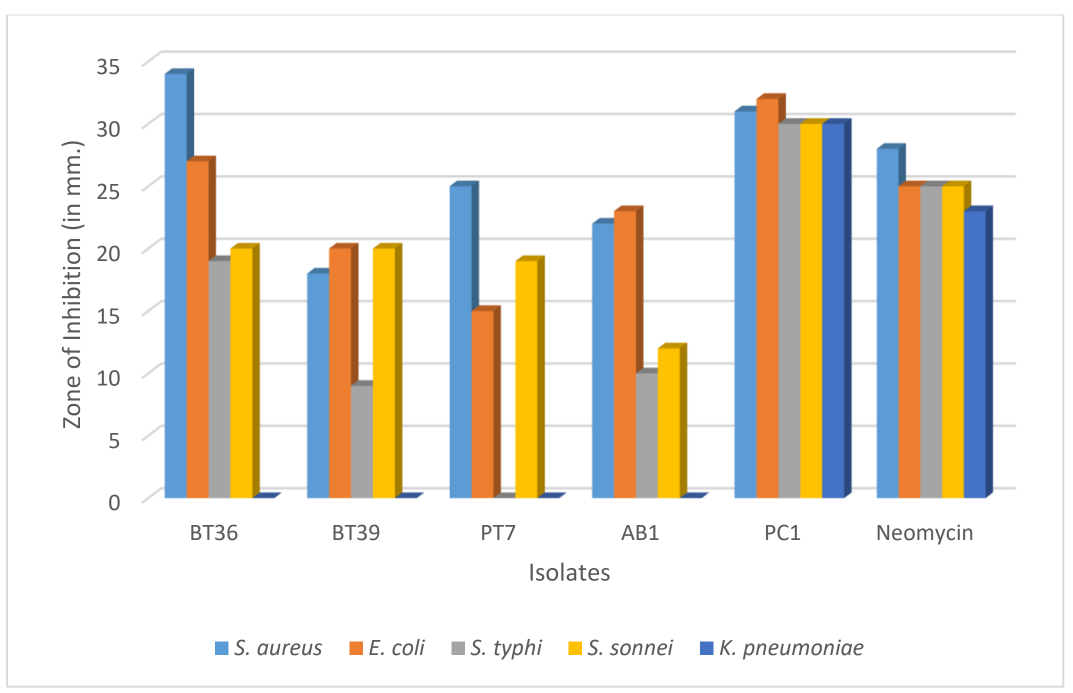


Figure 6: Bar diagram of zone of inhibition

The agar well diffusion assay's standardized technique offers reasonably consistent findings, allowing for easy comparison between research and facilities. Furthermore, this approach is particularly adaptable since it may be used to evaluate a wide range of antimicrobial agents such as antibiotics, plant extracts, and synthetic compounds against various microbes (Hossain, 2024).

4.6 Determination of MIC and MBC

The MIC and MBC of all 11 isolates were assessed against ATCC bacterial strains for both Gram-positive (*Staphylococcus aureus* ATCC 43300) and Gram-negative (*Escherichia coli* ATCC 25922) bacteria. MIC and MBC values of all of those isolates are shown in **Table 4** below. Neomycin and MHB broth were used as a positive and negative control respectively. The resazurin assay is a flexible and dependable technique for determining antibacterial characteristics. It has various benefits, including simplicity, sensitivity, speed, and robustness (Chakansin *et al.*, 2022). When tested against *Escherichia coli* and *Staphylococcus aureus*, the MIC and MBC of all extracts showed greater efficacy. PC1 showed a lower MIC value (0.658 mm) against *S. aureus* than all the isolates. It indicated that PC1 has a higher potency than other isolates. PT7 showed a lower MIC value (0.012 mm.) against *E. coli* but its zone of inhibition against *E. coli* found lesser value (15 mm.) than isolate PC1. The lower the MIC and MBC value of isolates against the tested pathogens, the higher will be the potency.

Table 4: MIC and MBC values of isolates

Isolates	*E. coli*		*S. aureus*	
	MIC value(mg/mL)	**MBC value(mg/mL)**	**MIC value(mg/mL)**	**MBC value(mg/mL)**
BT1	1.090	2.188	2.295	4.590
BT2	1.738	3.477	1.960	3.920
BT3	0.937	1.875	1.875	3.750
BT5	0.638	1.276	2.032	4.064
BT13	2.472	4.945	1.573	3.144
BT33	3.141	6.281	1.958	3.917
BT36	1.952	3.905	1.202	2.405
BT39	1.653	3.307	1.115	2.231
PT7	0.012	1.213	1.017	1.240
AB1	0.029	1.312	1.213	1.452
PC1	1.500	3.000	0.658	2.635
Neomycin	0.625	1.253	0.783	1.562

Consequently, some were conducted for antibacterial activity and others for various additional screening procedures. Some of them were thrown out since they showed no signs of growth during the culturing process. Numerous clinical conditions are caused by the Gram-positive *Staphylococcus aureus* bacterium. Because MRSA and other MDR strains are becoming more common, treating the infection is still difficult (Boucher & Corey, 2008).

4.7 Biochemical Analysis

To determine whether the isolated bacteria were Gram-positive or Gram-negative, several biochemical tests were performed. Oxidase, starch hydrolysis, nitrate reduction, mobility, indole, and catalase tests were used to identify Gram-positive bacteria. The biochemical assay results are shown in **Table 5**.

Table 5: Biochemical test

Isolates	Catalase	Oxidase	Starch Hydrolysis	Nitrate reduction	Mobility	Indole test
BT1	+	-	-	-	+	-
BT2	+	+	+	-	-	-
BT3	+	+	-	-	-	-
BT5	+	+	-	-	-	-
BT13	+	+	+	-	-	-
BT33	-	-	+	+	-	-
BT36	-	+	+	+	-	-
BT39	+	+	-	-	-	-
PT7	-	+	-	-	+	-
AB1	+	+	-	-	-	-
PC1	+	+	+	-	+	-

Physiological and biochemical parameters indicate important features for biotechnological applications in addition to aiding in the identification of microorganisms for phenotypic characterization (Chater & Chandra, 2006). By the catalase test, most bacterial extracts had positive results. In the indole test, all of them had negative results. Similar biochemical analysis results were published in a study by Sadiqi, Muhammad, and Farooq (Sadiqi *et al.*, 2022).

4.8 LC-HRMS/MS Analysis

4.8.1 Metabolic Comparison of *Streptomyces* Species

MestReNova software was used to acquire and analyze raw data. Every peak was identified, examined, positioned, and labeled. The total ion chromatograms (TIC) of BT1, BT2, BT3, and PC1 were stacked together as indicated in **Figure 7** to observe the differences in metabolites while the TIC of AB1 and PT7 were stacked in **appendix H**. Lastly, a comparison between the outcomes and literature including the database libraries was made. A total of 58 metabolites, including diketopiperzine and alkyl resorcinol, were found in the samples. **Table 6** and **Figure 38** display the secondary metabolites annotated from LC-HRMS/MS analysis.

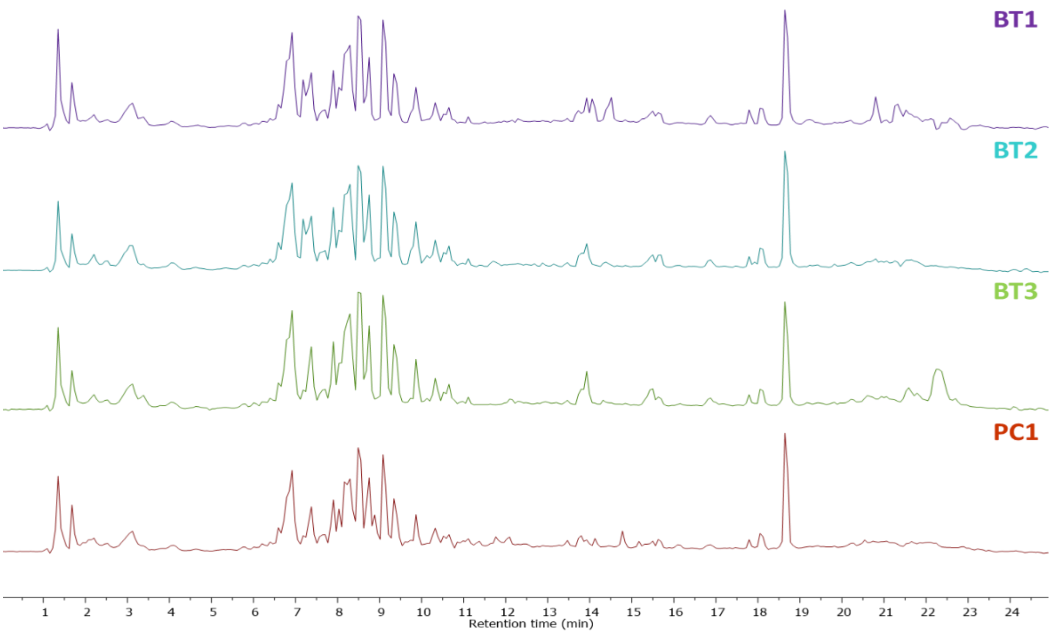


Figure 7: Total Ion chromatograms of BT1, BT2, BT3, and PC1.

The BPC and MS profiles of the annotated compounds detected for the first time in *Streptomyces* species are shown in the **Figures 8-11**.

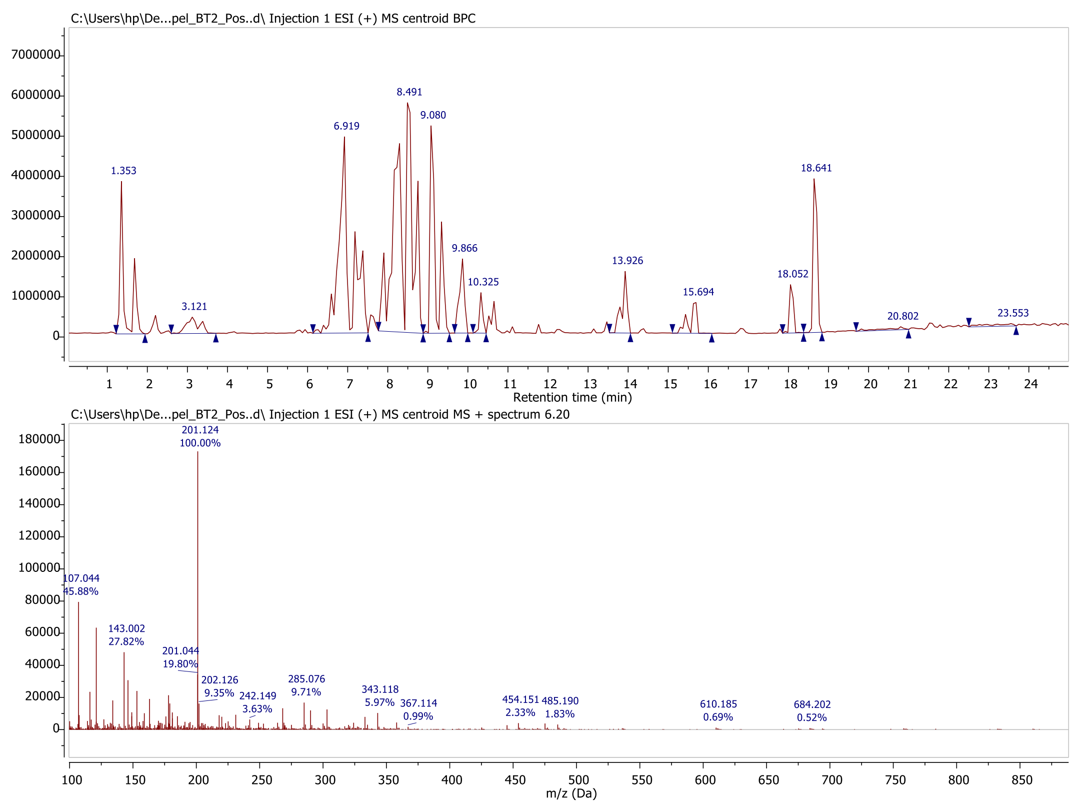


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Figure 8: BPC and MS profile of Cyclo(Ile-Ser)

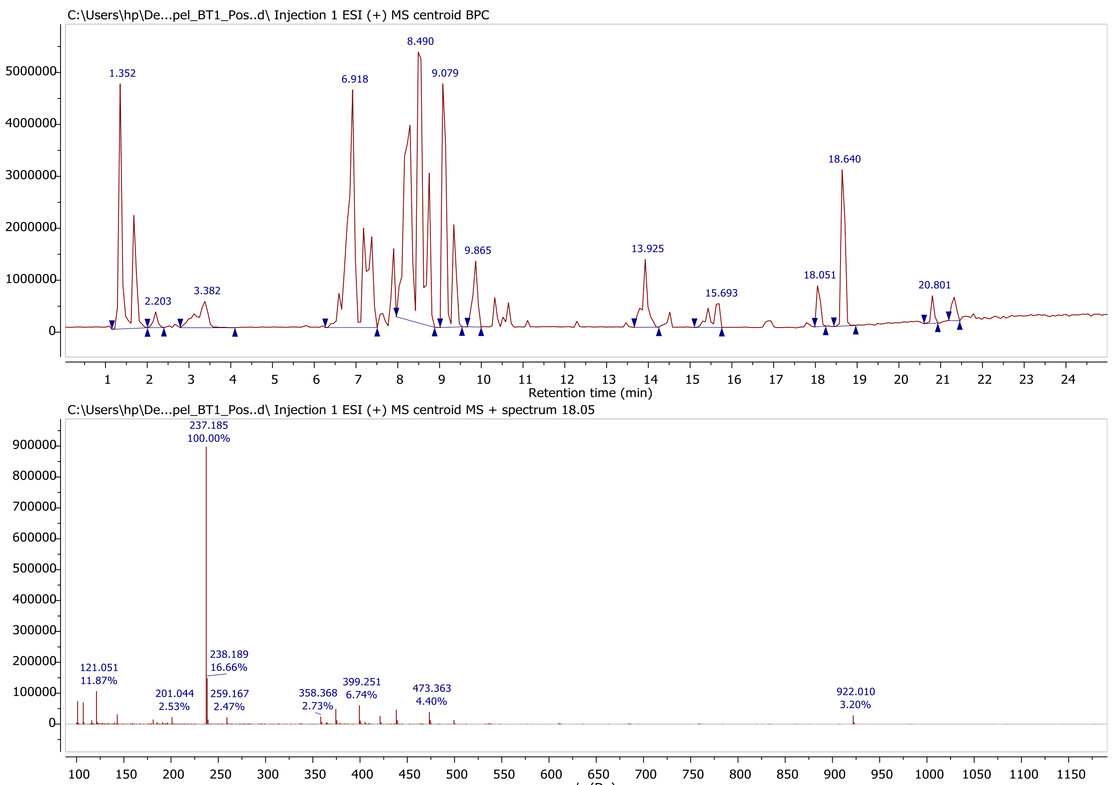


Figure 9: BPC and MS profile of 2-n-hexyl-5-n-propylresorcinol

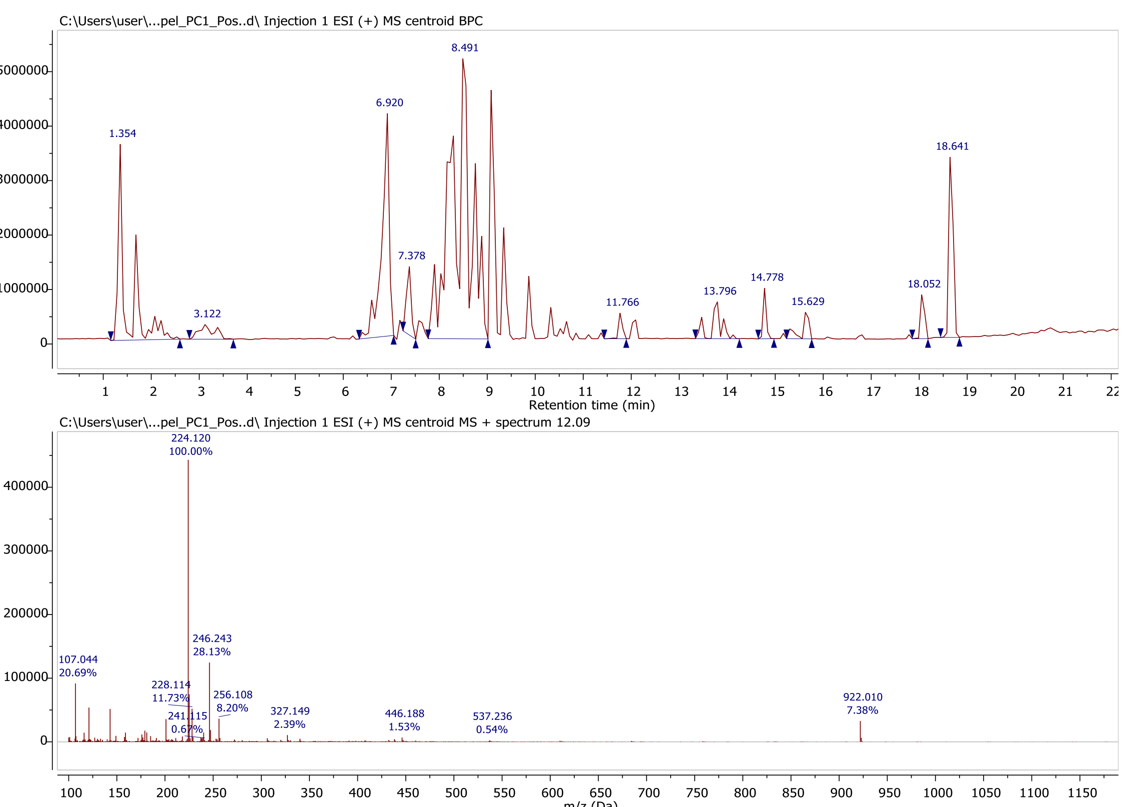


Figure 10: BPC and MS profile of 3-[(6-methylpyrazin-2-yl) methyl]-1H-indole.

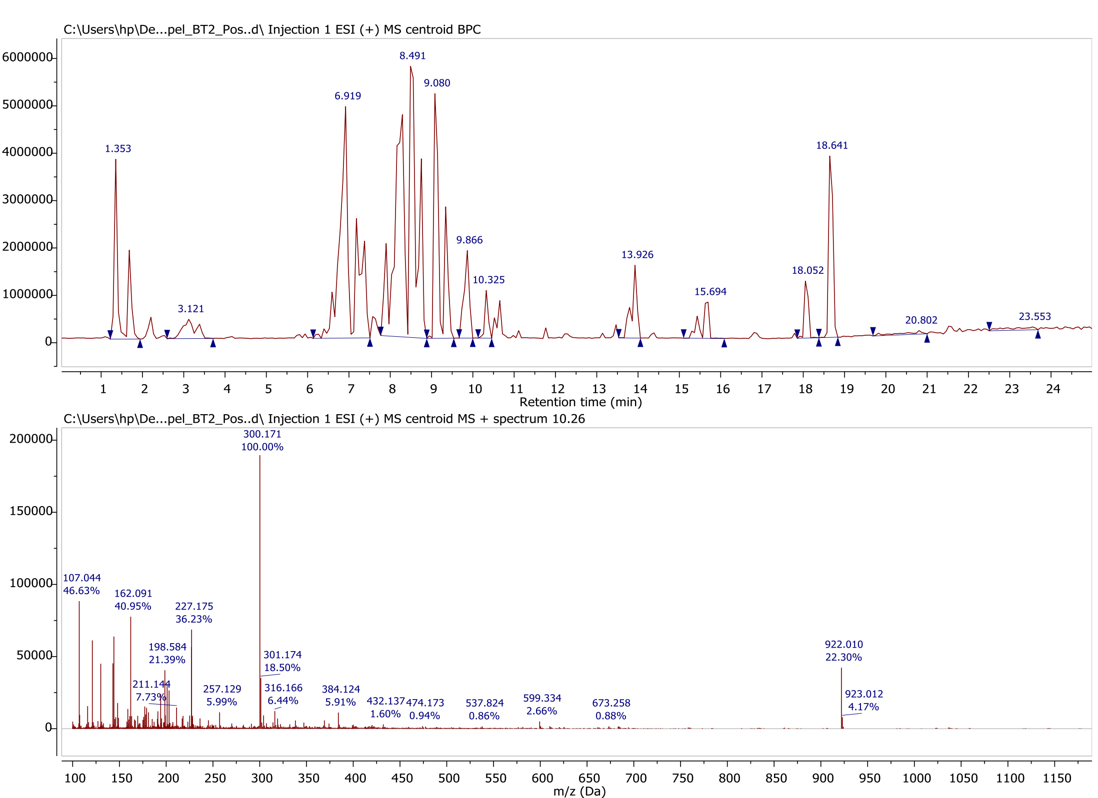


Figure 11: BPC and MS profile of Cyclo(D-Leu-L-Trp)

Table 6: Annotated compounds from LC-HRMS/MS

C. N.	**Annotated compound**	**Exact Mass *m/z***	**Observed mass *m/z***	**Detected ion**	**Molecular formula**	**RDBE**	**Absolute Error (ppm)**	**Retention time(min)**	**Sources**	**CSI: Finger ID Score (%)**	**References**
1	Cyclo(D-Pro-D-Phe)	244.120	245.129	$[M+H]^+$	$C_{14}H_{16}N_2O_2$	8.0	3.20	8.76	BT1	99.24	(Singh *et al.*, 2019)
2	Cyclo(L-Val-L-Leu)	212.159	213.160	$[M+H]^+$	$C_{11}H_{20}N_2O_2$	3.0	1.84	9.34 (BT1)	BT1, BT2	92.71	(X. Li *et al.*, 2006)
3	Maculosin	260.115	261.123	$[M+H]^+$	$C_{14}H_{16}N_2O_3$	8.0	0.57	6.59 (BT1)	PC1, BT1, BT3	98.00	(Paudel *et al.*, 2021)
4	Neomarinone	424.225	425.233	$[M+H]^+$	$C_{26}H_{32}O_5$	11.0	4.70	14.02	BT1,PT7,AB1	48.47	(Hardt *et al.*, 2000)
5	Dibutyl phthalate	278.152	279.160	$[M+H]^+$	$C_{16}H_{22}O_4$	6.0	2.18	18.64 (BT1)	BT1, BT2	95.91	(Roy *et al.*, 2006)
6	Pyridoxine	169.073	170.081	$[M+H]^+$	$C_8H_{11}NO_3$	4.0	1.34	1.35 (BT1)	BT1, BT2	99.45	(Dempsey, 1966)
7	Cyclo(L-Pro-L-OMet)	244.087	245.10	$[M+H]^+$	$C_{10}H_{16}N_2O_3S$	4.0	0.70	2.53	BT2	76.71	(Yang *et al.*, 2013a)

8	Surfactin C13	1007.651	1008.660	[M+H]$^+$	$C_{51}H_{89}N_7O_{13}$	11.0	3.394	21.29	BT1	99.72	(Hoefler *et al.*, 2012)
9	Cyclo(L-Valyl-L-Phenyl alanyl)	246.136	247.144	[M+H]$^+$	$C_{14}H_{18}N_2O_2$	7.0	0.74	9.87	BT2	93.04	(Dashti *et al.*, 2014)
10	Di-*n*-butyl terephthalate	278.151	279.159	[M+H]$^+$	$C_{16}H_{22}O_4$	6.0	1.76	18.58	BT3	54.11	(Zhou *et al.*, 2014)
11	Phthalic anhydride	148.015	149.023	[M+H]$^+$	$C_8H_4O_3$	7.0	0.63	18.64	BT3	99.24	(Sriragavi *et al.*, 2023)
12	Phytoceramide	555.522	556.531	[M+H]$^+$	$C_{34}H_{69}NO_4$	11.0	1.22	20.68	BT3	71.70	(Stankeviciute *et al.*, 2022)
13	Cyclo(Pro-Gly)	154.073	155.081	[M+H]$^+$	$C_7H_{10}N_2O_2$	4.0	3.27	2.20 (BT3)	PC1, BT1, BT2, BT3	97.50	(Ser *et al.*, 2015)
14	Cyclo(L-Leu-L -Pro) or Gancidin W	210.136	211.144	[M+H]$^+$	$C_{11}H_{18}N_2O_2$	4.0	1.41	8.03 (BT2)	PC1, BT1, BT2, BT3	98.69	(Zin *et al.*, 2017)
15	Cyclo(L-Phenylalanyl-*trans*-4-hydroxy-L-Proline)	260.112	261.119	[M+H]$^+$	$C_{14}H_{16}N_2O_3$	8.0	1.32	6.72 (PC1)	PC1, BT1, BT2, BT3	62.83	(Wattana-Amorn *et al.*, 2016)
16	Coronafacoyl-L-isoleucine	321.194	344.183	[M+Na]$^+$	$C_{18}H_{27}NO_4$	6.0	0.02	10.85 (PC1)	PC1, BT3	36.16	(Fyans *et al.*, 2015)

17	Cyclo(D-Pro-L -Tyr)	260.112	261.119	$[M+H]^+$	$C_{14}H_{16}N_2O_3$	4.0	1.85	6.79 (BT2)	PC1, BT1, BT2, BT3	87.08	(Wattana-Amorn *et al.*, 2016)
18	Cyclo(Pro-Val)	196.120	197.129	$[M+H]^+$	$C_{10}H_{16}N_2O_2$	4.0	2.10	6.59 (BT2)	PC1, BT1, BT2, BT3	95.58	(Pettit *et al.*, 2006)
19	N-Acetyltyramine	179.094	180.102	$[M+H]^+$	$C_{10}H_{13}NO_2$	5.0	2.48	7.18 (BT2)	PC1, BT1, BT2, BT3	73.66	(Driche *et al.*, 2022)
20	Cyclo(L-Ala-L -Leu)	184.120	185.129	$[M+H]^+$	$C_9H_{16}N_2O_2$	3.0	1.25	7.57 (BT2)	BT1, BT2, BT3	97.87	(CHO *et al.*, 2012)
21	Cyclo(Tyr-Leu)	276.147	277.155	$[M+H]^+$	$C_{15}H_{20}N_2O_3$	7.0	1.07	7.84 (BT2)	PC1, BT1, BT2, BT3	94.12	(Wei *et al.*, 2017)
22	Cyclo(L-Phe-L -Ala)	218.104	219.114	$[M+H]^+$	$C_{12}H_{14}N_2O_2$	7.0	4.91	7.97 (BT2)	PC1, BT1, BT2, BT3	95.63	(Bhandari *et al.*, 2022)
23	Cyclo(Phenylalanyl-Prolyl)	244.121	245.129	$[M+H]^+$	$C_{14}H_{16}N_2O_2$	8.0	1.00	8.69 (BT2)	PC1, BT1, BT2, BT3	99.62	(Macherla *et al.*, 2005)
24	Brevianamide F or Cyclo-L -Trp-L -Pro	283.131	284.139	$[M+H]^+$	$C_{16}H_{17}N_3O_2$	10.0	0.47	9.41 (BT2)	PC1, BT1, BT2, BT3	99.40	(Elleuch *et al.*, 2010)

25	N-Phenethylacetamide	163.099	164.107	$[M+H]^+$	$C_{10}H_{13}NO$	5.0	1.63	9.74 (BT2)	PC1, BT1, BT2, BT3	99.24	(Ding *et al.*, 2013a)
26	Cyclo(L-Leucyl-L -Leucyl)	226.168	227.176	$[M+H]^+$	$C_{12}H_{22}N_2O_2$	3.0	0.83	10.32 (BT2)	PC1, BT1, BT2, BT3	94.97	(Al-Dhabi *et al.*, 2019)
27	Cyclo(D-Leu-L -Trp)	299.163	300.171	$[M+H]^+$	$C_{17}H_{21}N_3O_2$	9.0	1.61	10.26 (BT2)	PC1, BT1, BT2, BT3	85.35	(Kimura *et al.*, 2005)
28	Cyclo(Phenylalanyl-Phenylalanyl)	294.139	295.147	$[M+H]^+$	$C_{18}H_{18}N_2O_2$	11.0	1.55	11.11 (BT2)	PC1, BT1, BT2, BT3	69.32	(Srivastava *et al.*, 2018)
29	Cyclo(Tyr-Val)	262.133	263.141	$[M+H]^+$	$C_{14}H_{18}N_2O_3$	7.0	0.27	7.71 (BT2)	BT1, BT2, BT3	76.92	(Zhou *et al.*, 2014)
30	1-Acetyl-3-methoxycarbonyl-β-carboline	268.086	269.093	$[M+H]^+$	$C_{15}H_{12}N_2O_3$	11.0	1.95	14.38 (BT2)	BT1, BT2	81.21	(Kornsakulkarn *et al.*, 2018)
31	Cyclo(D-Ala-L -Pro)	168.089	169.097	$[M+H]^+$	$C_8H_{12}N_2O_2$	4.0	0.56	2.99 (BT2)	PC1, BT1, BT2, BT3	98.56	(Tan *et al.*, 2019)
32	Cyclo(Ile-Ser)	200.125	201.124	$[M+H]^+$	$C_9H_{16}N_2O_3$	3.0	1.02	6.20 (BT2)	PC1, BT1, BT2, BT3	83.49	(Feng *et al.*, 2019)

33	(S)-3-Isobutylpiperazine-2,5-dione or Cyclo(Gly-Leu)	170.105	171.113	$[M+H]^+$	$C_8H_{14}N_2O_2$	3.0	1.11	6.46 (BT2)	PC1, BT1, BT2, BT3	76.44	(Wei *et al.*, 2017)
34	3-((6-methylpyrazin-2-yl)methyl)-1H-indole	223.110	224.118	$[M+H]^+$	$C_{14}H_{13}N_3$	10.0	0.84	12.09	PC1	84.57	(Yang *et al.*, 2013a)
35	N-Lauryldiethanolamine	273.266	274.275	$[M+H]^+$	$C_{16}H_{35}NO_2$	0.0	2.34	13.80 (BT2)	BT1, BT2, BT3	99.17	(Chai *et al.*, 2019)
36	2-n-Hexyl-5-n-propylresorcinol	236.177	237.185	$[M+H]^+$	$C_{15}H_{24}O_2$	4.0	1.02	18.05 (BT1)	BT1, BT2, BT3	76.54	(Calderón *et al.*, 2019)
37	2-Hexyl-5-methylresorcinol	208.146	209.154	$[M+H]^+$	$C_{13}H_{20}O_2$	4.0	2.33	15.63	BT1	71.60	(Álvarez-Álvarez *et al.*, 2017)
38	Epopromycin A	412.256	413.266	$[M+H]^+$	$C_{21}H_{36}N_2O_6$	5	4.29	15.17	PT7 and AB1		(Tsuchiya *et al.*, 1997)
39	SF2415B2 antibiotic	424.225	425.233	$[M+H]^+$	$C_{26}H_{32}O_5$	11.0	4.70	0.189	PT7 and AB1		(Gomi *et al.*, 1987)
40	Gilvocarcin HE	511.163	512.171	$[M+H]^+$	$C_{27}H_{28}O_{10}$	14.0	3.72	0.108	PT7 and AB1		(Hou *et al.*, 2012)
41	Butyrolactol A	526.314	527.324	$[M+H]^+$	$C_{28}H_{46}O_9$	6.0	4.89	0.64	PT7		(Harunari *et al.*, 2017)
42	Epoxomicin	554.368	555.376	$[M+H]^+$	$C_{28}H_{50}N_4O_7$	7.0	2.96	9.87	PT7 and AB1		(Nihei *et al.*, 1993)

43	Aldgamycin L	700.403	701.411	$[M+H]^+$	$C_{36}H_{60}O_{13}$	7.0	2.96	2.158	PT7 and AB1	(Wang *et al.*, 2010b)
44	Blasticidin H	440.213	441.221	$[M+H]^+$	$C_{17}H_{28}N_8O_6$	8.0	4.13	0.63	PT7 and AB1	(Svidritskiy *et al.*, 2013)
45	Phoxalone	424.209	425.217	$[M+H]^+$	$C_{22}H_{32}O_8$	7	0.72	0.286	AB1 and PT7	(Guo & Tao, 2008)
46	Okilactomycin A	434.229	435.239	$[M+H]^+$	$C_{24}H_{34}O_7$	8	1.89	0.288	AB1	(Imai *et al.*, 1987)
47	SF2809-IV antibiotic	440.173	441.179	$[M+H]^+$	$C_{27}H_{24}N_2O_4$	17	4.48	0.292	AB1 and PT7	(Tani *et al.*, 2004)
48	(4R)-4,5-dihydro-4-hydroxygeldanamycin	578.283	579.293	$[M+H]^+$	$C_{29}H_{42}N_2O_{10}$	10	2.37	18.68	PT7 and AB1	(E. Li & Mira de Orduña, 2010b)
49	Janthinopolyenemycin B	412.260	413.267	$[M+H]^+$	$C_{26}H_{36}O_4$	9	2.98	15.17	AB1 and PT7	(Rani *et al.*, 2021)
50	8-deoxyheronamide C	433.297	434.500	$[M+H]^+$	$C_{29}H_{39}NO_2$	11	3.69	19.16	AB1 and PT7	(Sugiyama *et al.*, 2014)
51	Actinoallolide C	546.356	547.364	$[M+H]^+$	$C_{32}H_{50}O_7$	12.5	1.33	18.64	AB1 and PT7	(Xu *et al.*, 2022)
52	Glucopiericidin A	577.325	578.330	$[M+H]^+$	$C_{31}H_{47}NO_9$	9	3.80	9.12	AB1	(Shaaban *et al.*, 2012)
53	Gombapyrone A	406.214	407.206	$[M+H]^+$	$C_{26}H_{30}O_4$	8	1.80	15.17	AB1 and PT7	(Helaly *et al.*, 2009)
54	Xenocoumacin 2	406.479	407.206	$[M+H]^+$	$C_{21}H_{30}N_2O_6$	8	1.80	15.17	AB1	(Park *et al.*, 2009)
55	7,3-di-(γ,γ-dimethylallyloxy)-5-	436.188	437.199	$[M+H]^+$	$C_{26}H_{28}O_6$	13	2.96	0.158	AB1	(Ding *et al.*, 2013b)

	hydroxy-4-methoxyflavone									
56	Flavofungin	664.419	665.427	$[M+H]^+$	$C_{37}H_{60}O_{10}$	8	0.11	0.116	AB1	(Uri & Bekesi, 1958)
57	Myxopyronin B	431.230	432.953	$[M+H]^+$	$C_{24}H_{33}NO_6$	11.5	3.47	0.75	AB1 and PT7	(Yakushiji *et al.*, 2013)
58	5,7-Trihydroxy-3,4-dimethoxy isoflavone	314.079	353.042	$[M+K]^+$	$C_{17}H_{14}O_6$	5.0	2.3	8.49	AB1 and PT7	(Hosny & Rosazza, 1999)

(Thapa et al., 2024)

Streptomyces has already produced about 7600 bioactive compounds, with the potential to uncover more than 100,000 additional ones. It has been proposed to examine and broaden the scope of microbial natural product research (Newman & Cragg, 2016).

In this work, we investigated the antibiotics produced by Streptomyces from Nepalese biodiversity. Brevianamide F, which was previously isolated from *Streptomyces sp.* TN262, was recognized as the molecular ion at *m/z* 284.139 $[M+H]^+$ (Elleuch *et al.*, 2010). It was found at a retention time of 9.41 min in our MS/MS analysis which is shown in **Figure 12**.

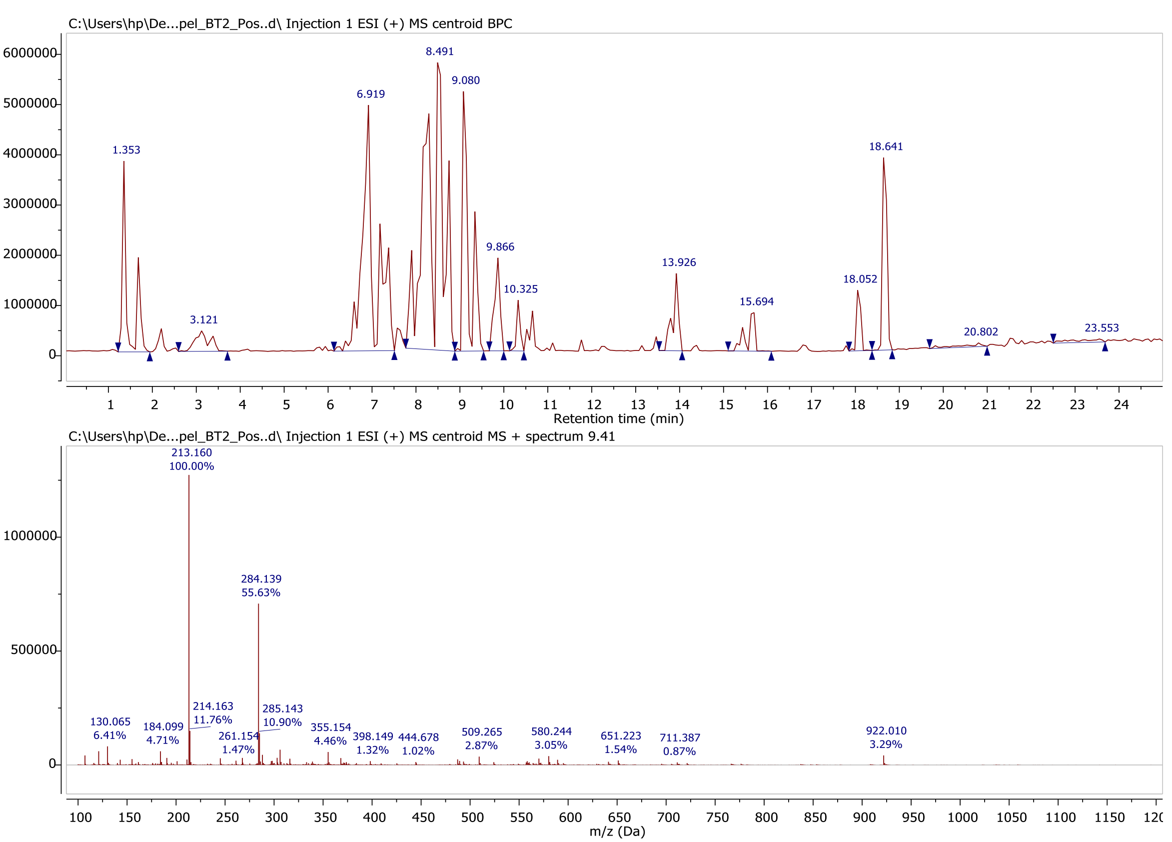


Figure 12: BPC and MS profile of Brevianamide F

This investigation found Cyclo(Phenylalanyl-Prolyl), which was previously isolated and characterized in *Streptomyces* sp., as a protonated ion at *m/z* 245.129 $[M+H]^+$ (Macherla *et al.*, 2005) which is shown in **Figure 13**.

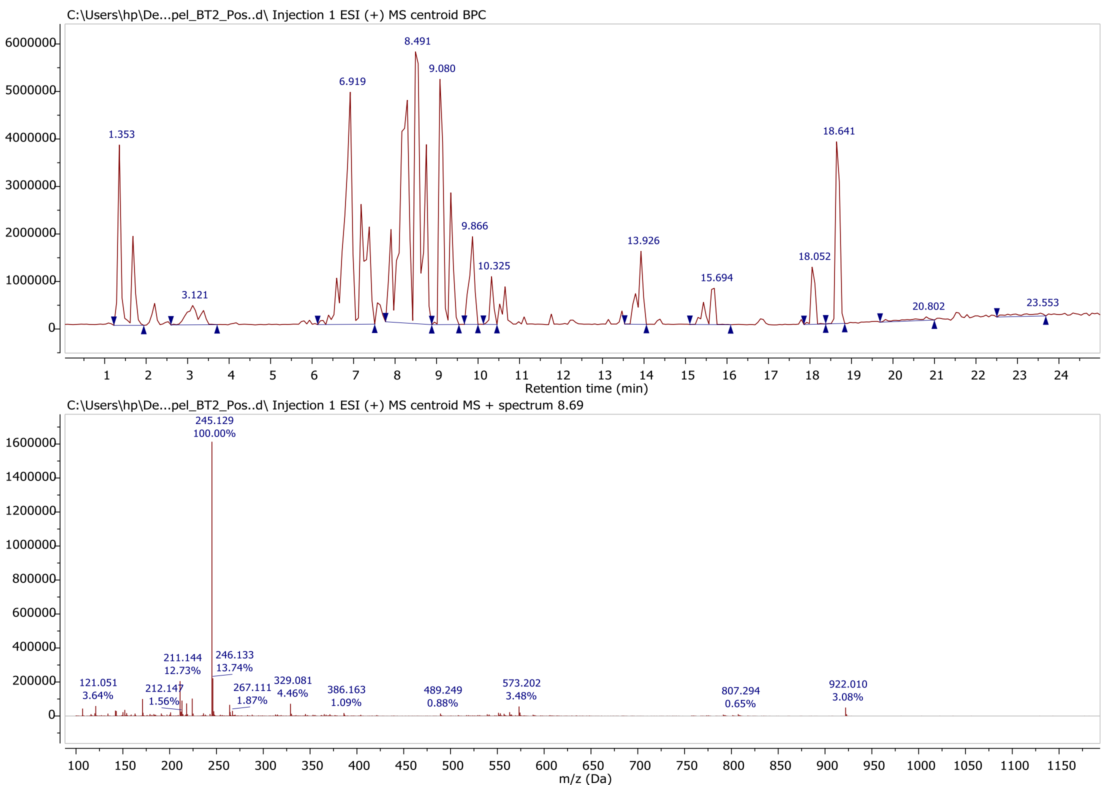


Figure 13: BPC and MS profile of Cyclo(phenylalanyl-prolyl)

Similarly, maculosin, which was previously found in *Streptomyces* sp. KTM18 was recognized as a molecular ion at *m/z* 261.123 $[M+H]^+$ observed at a retention time of 6.59 min. which is shown in **Figure 14**.

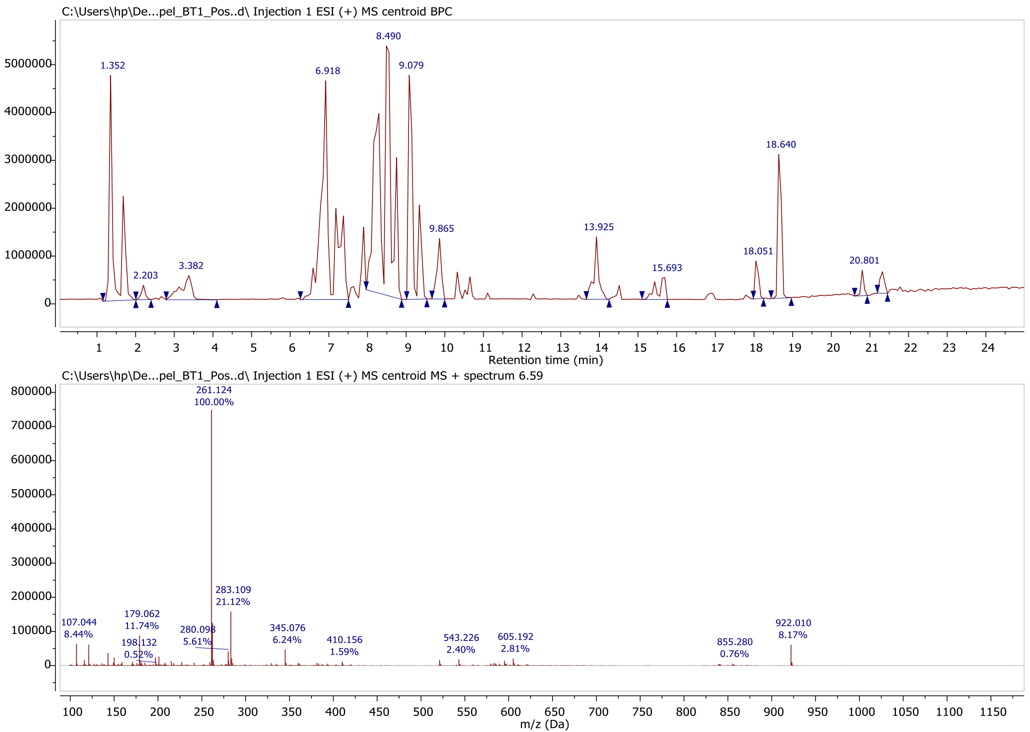


Figure 14: BPC and MS profile of maculosin

After 6.46 minutes of retention, a molecular ion at *m/z* 171.113 [M+H]$^+$ was found. It was recognised as Cyclo(Gly-Leu), which has previously been identified in *Streptomyces xanthophaeus* (Wei *et al.*, 2017).

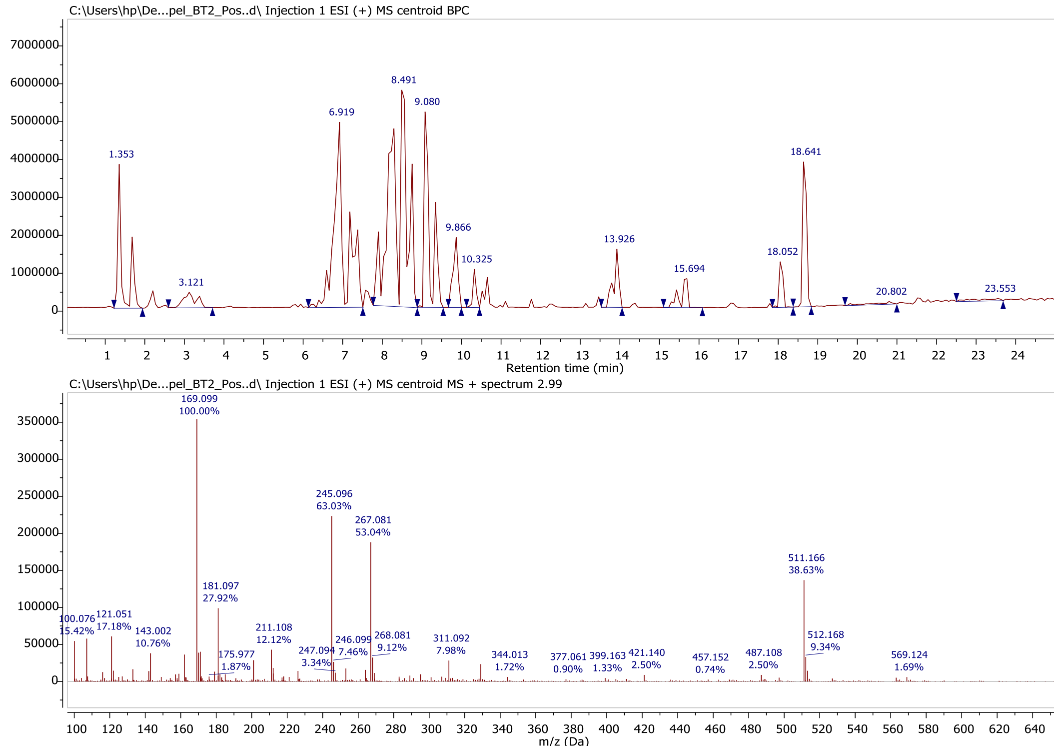


Figure 15: BPC and MS profile of Cyclo(D-Ala-L-Pro).

At a retention duration of 2.99 minutes, Cyclo(D-Ala-L-Pro) was found with *m/z* 169.097 [M+H]$^+$. Tan *et al.* (2019) which is shown in **Figure15** have previously identified this species in *Streptomyces* sp. isolated from mangroves.

At *m/z* 263.141 [M+H]$^+$, Cyclo(Tyr-Val), another cyclic dipeptide molecule, was found at a retention time of 7.71 min which is shown in **Figure 16**. It has been previously reported in *Streptomyces* sp. (Zhou *et al.*, 2014).

Additionally, the precursor ion identified as Cyclo(Phenylalanyl-Phenylalanyl), previously reported in *Streptomyces chrestomyceticus*, was found at *m/z* 295.147 [M+H]$^+$ (Srivastava *et al.*, 2018) which is shown in **Figure 17**.

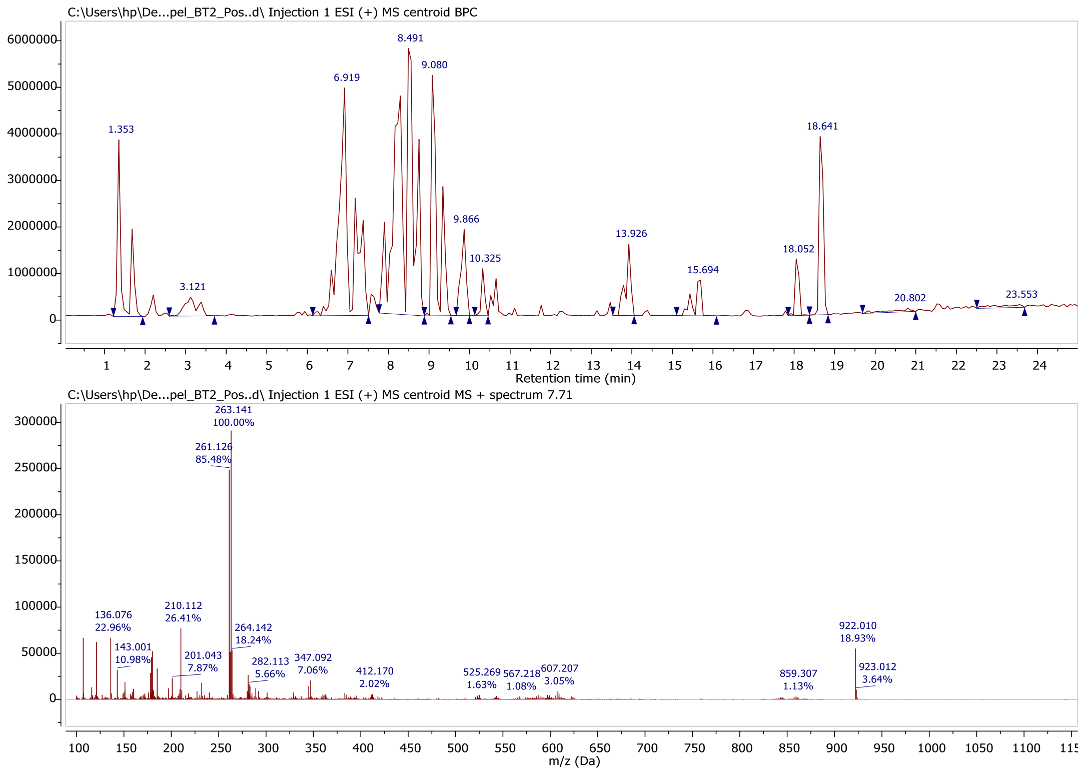


Figure 16: BPC and MS profile of Cyclo(Tyr-Val)

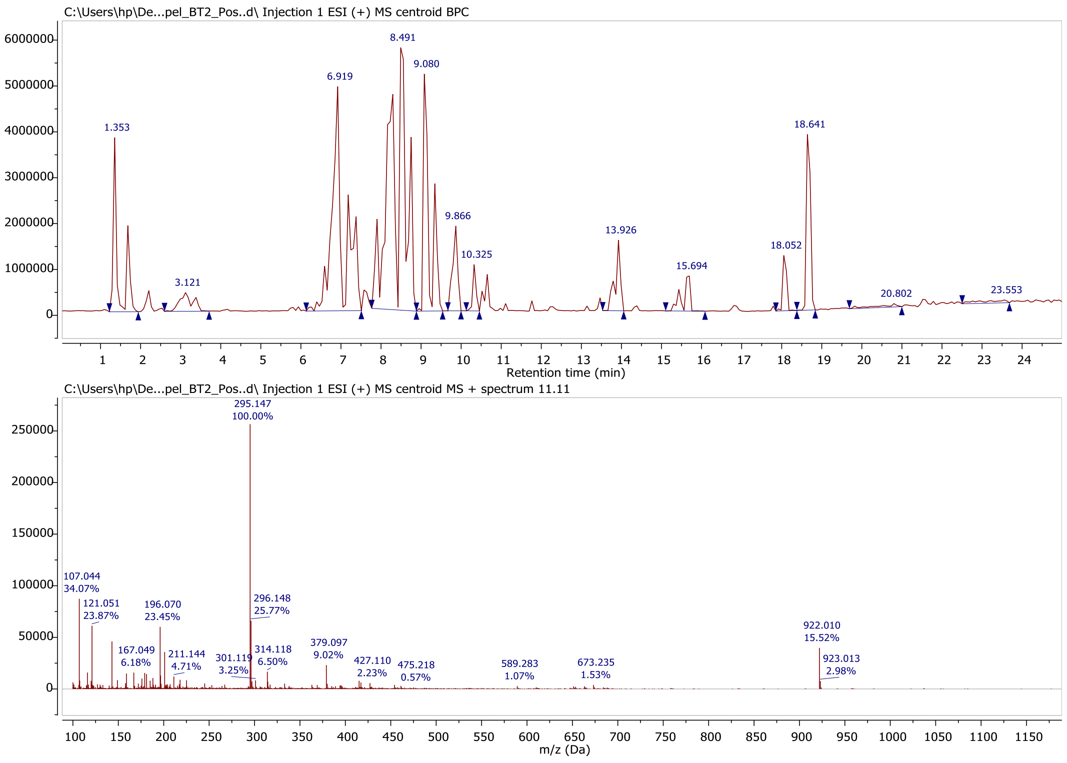


Figure 17: BPC and MS profile of Cyclo(phenylalanyl-phenylalanyl)

Cyclo(L-Leucyl-L-Leucyl), which was previously found in *Streptomyces* sp., as a precursor ion at *m/z* 227.176 $[M+H]^+$ (Al-Dhabi *et al.*, 2019) was detected.

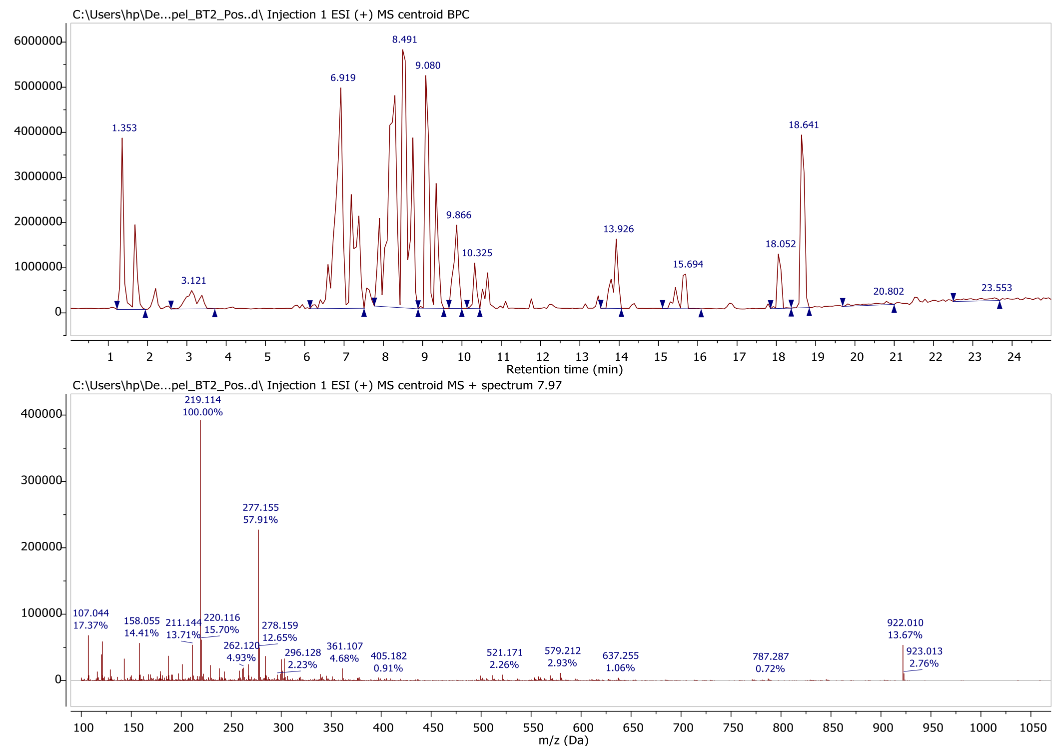


Figure 18: BPC and MS profile of Cyclo(L-Phe-L -Ala)

After a retention period of 7.97 minutes, a molecular ion at *m/z* 219.114 $[M+H]^+$ was recognized as Cyclo(L-Phe-L-Ala), which had previously been found in *Streptomyces sp.* (Bhandari *et al.*, 2022) which is shown in **Figure 18**.

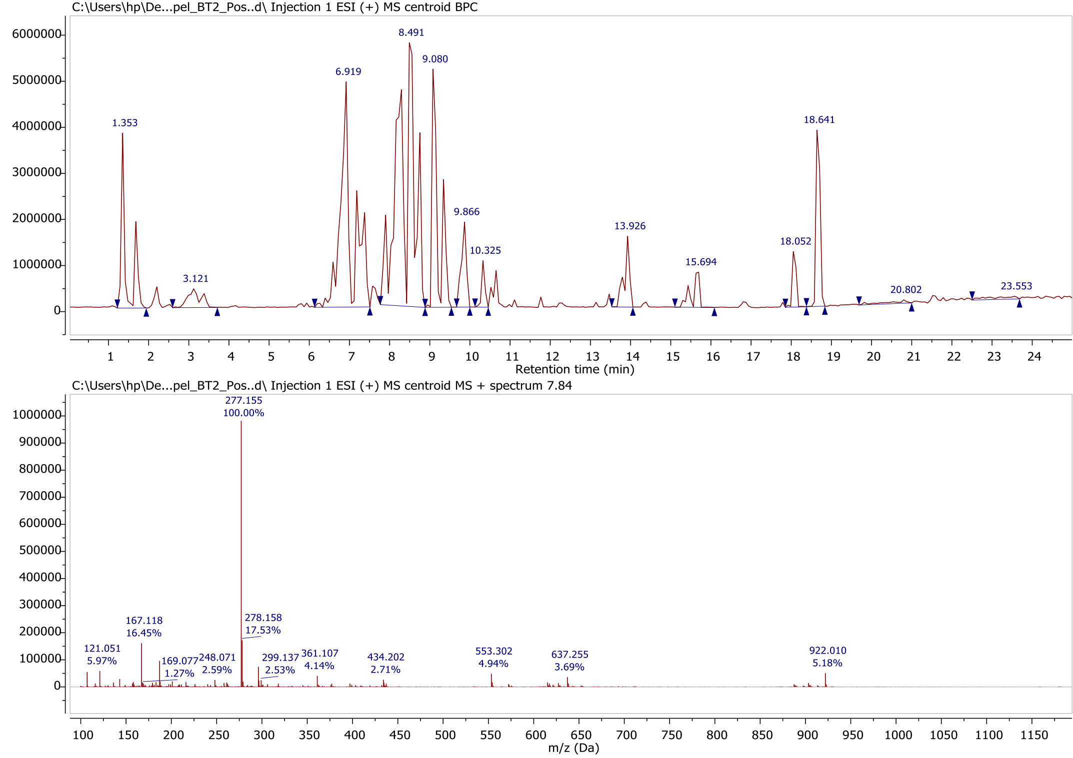


Figure 19: BPC and MS profile of Cyclo(Tyr-Leu)

A protonated ion found at *m/z* 277.155 $[M+H]^+$ was also identified as Cyclo(Tyr-Leu), which was previously found in *Streptomyces kunmingensis*, a soil-derived organism (Wei *et al.*, 2017) whose BPC and MS profile is shown in **Figure 19**.

In addition, a molecular ion at *m/z* 164.107 $[M+H]^+$ was annotated for N-phenethylacetamide. Neomarinone was discovered as the precursor ion at *m/z* 425.233 $[M+H]^+$ that was found at a retention time of 14.06 minutes which is given in **Figure 20**.

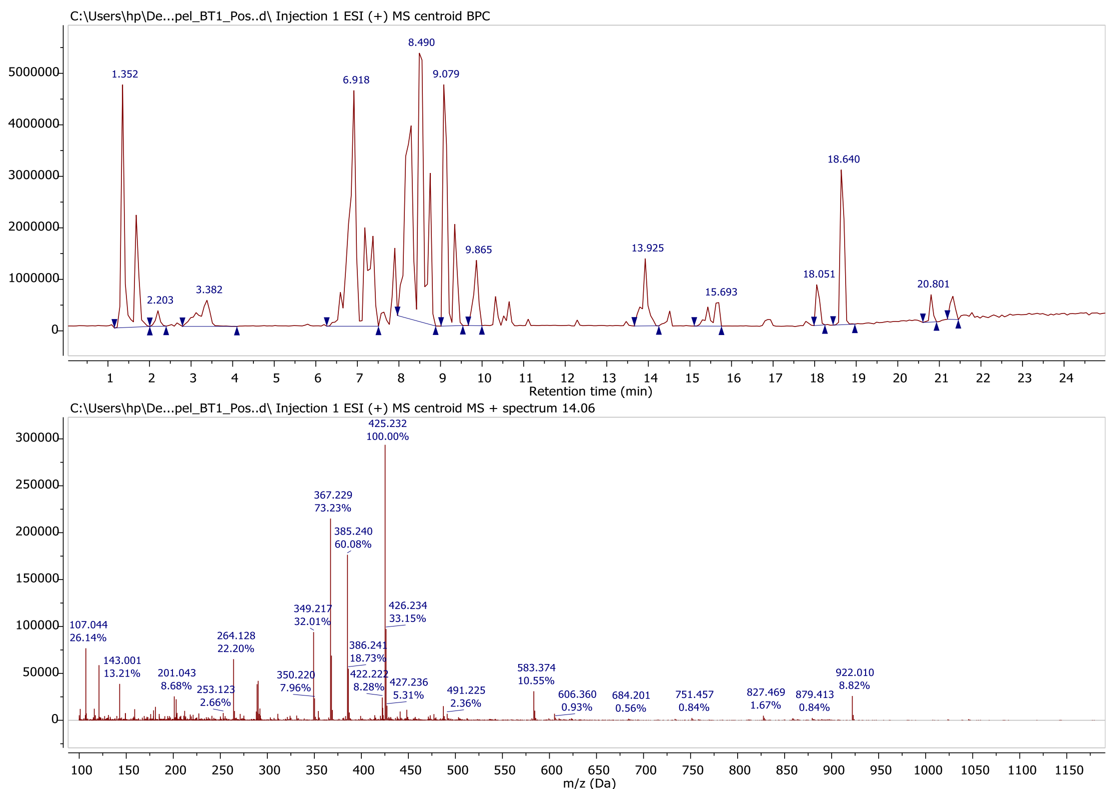


Figure 20: BPC and MS profile of neomarinone

Similar to this, Cyclo(D-Pro-D-Phe), which was previously described by Alshaibani *et al.* in *Streptomyces* sp. SUK 25, was found as a molecular ion at *m/z* 245.129 $[M+H]^+$ (Alshaibani *et al.*, 2017). Cyclo (L-Val-L-Leu) was identified at *m/z* 213.160 $[M+H]^+$ as a protonated ion in *Streptomyces xiamenensis* MCC A01570 (X. Li *et al.*, 2006).

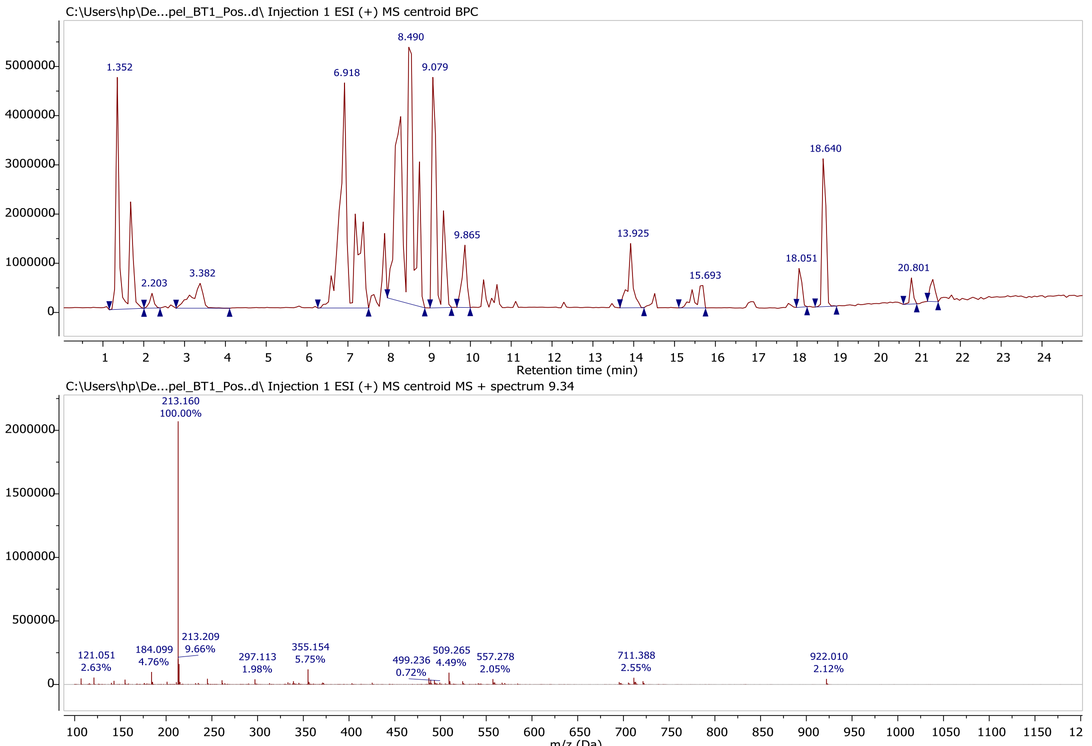


Figure 21: BPC and MS profile of Cyclo(L-val-L-Leu)

Furthermore, a molecular ion at *m/z* 249.024 $[M+H]^+$ was annotated for dibutyl phthalate which is given in **Figure 22**.

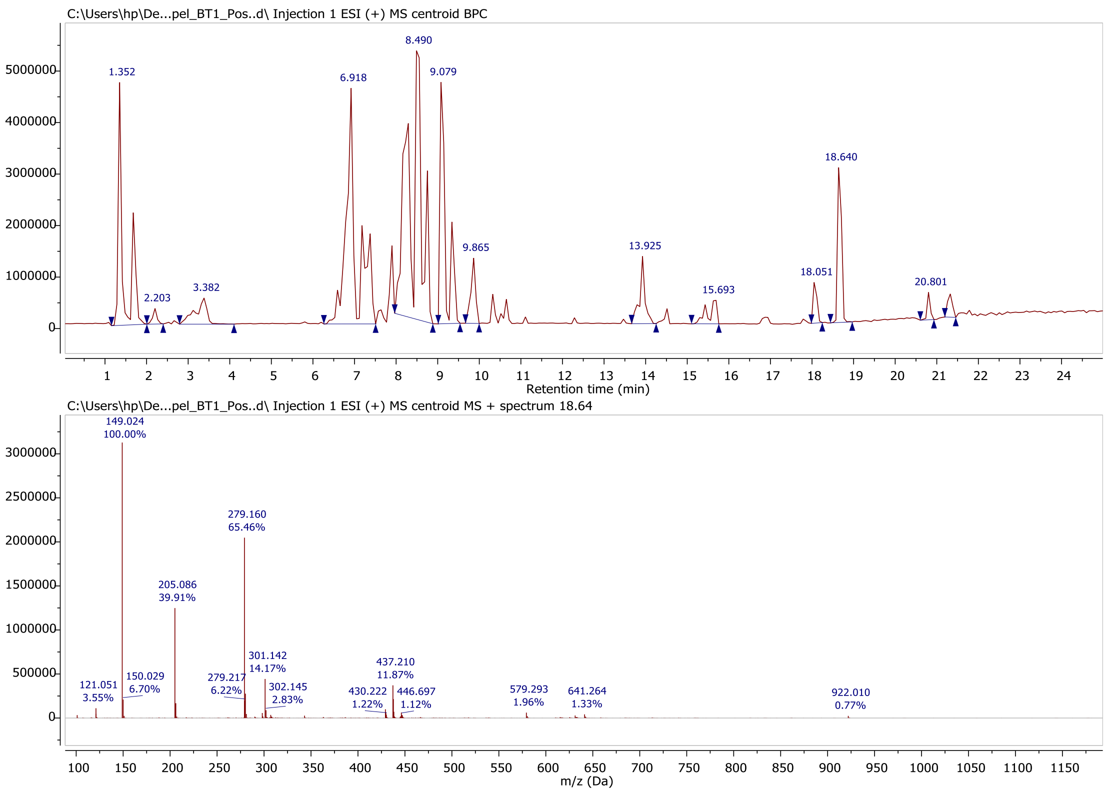


Figure 22: BPC and MS profile of dibutyl phthalate

At a retention time of 14.38 minutes, a precursor ion $[M+H]^+$ at *m/z* 269.093 was discovered; it was identified as 1-acetyl-3-methoxycarbonyl-β-carboline. *Ophiocordyceps sphecocephala* BCC 2661 was the fungus from which this chemical was originally isolated (Kornsakulkarn *et al.*, 2018) which is shown in **Figure 23.**

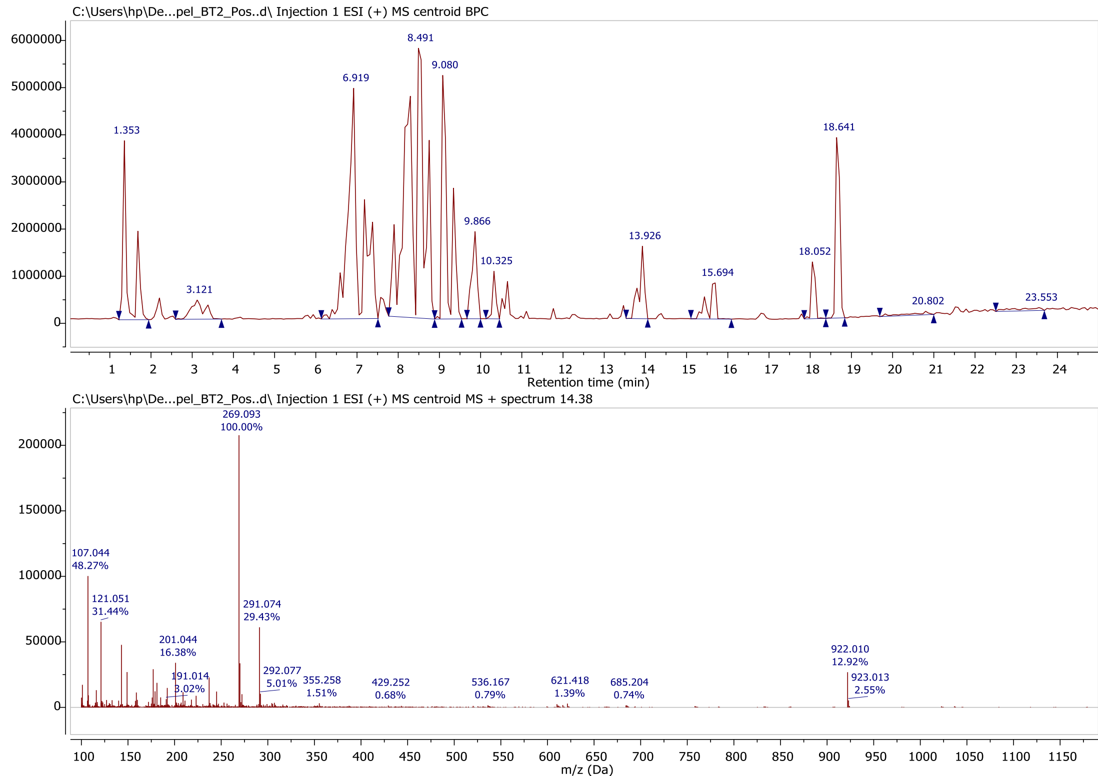


Figure 23: BPC and MS profile of 1-acetyl-3-methoxycarbonyl-β-carboline

Pyridoxine was identified as the molecule displaying a molecular ion peak at *m/z* 170.081 $[M+H]^+$ which is given in **Figure 24**.

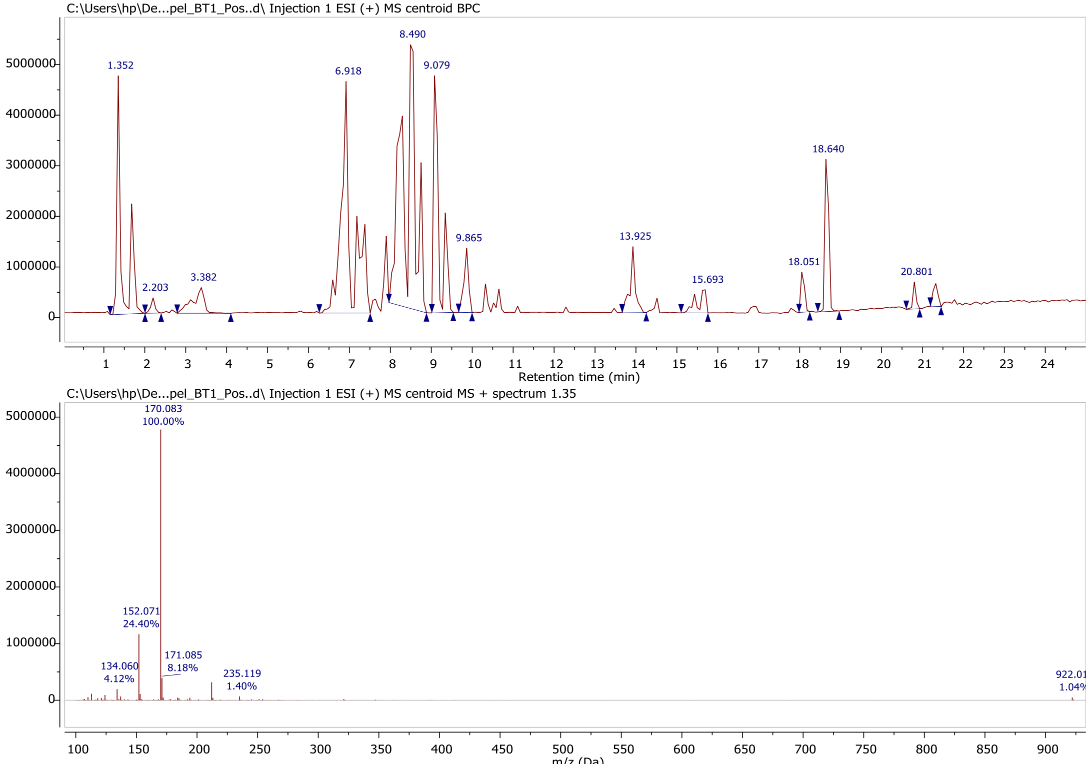


Figure 24: BPC and MS profile of pyridoxine.

Moreover, spectral data analysis by Yang *et al.* (2013) revealed that the precursor ion at *m/z* 245.096 $[M+H]^+$ was identified as Cyclo(L-Pro-L -OMet), found with a retention duration of 2.53 min which is shown in **Figure 25**.

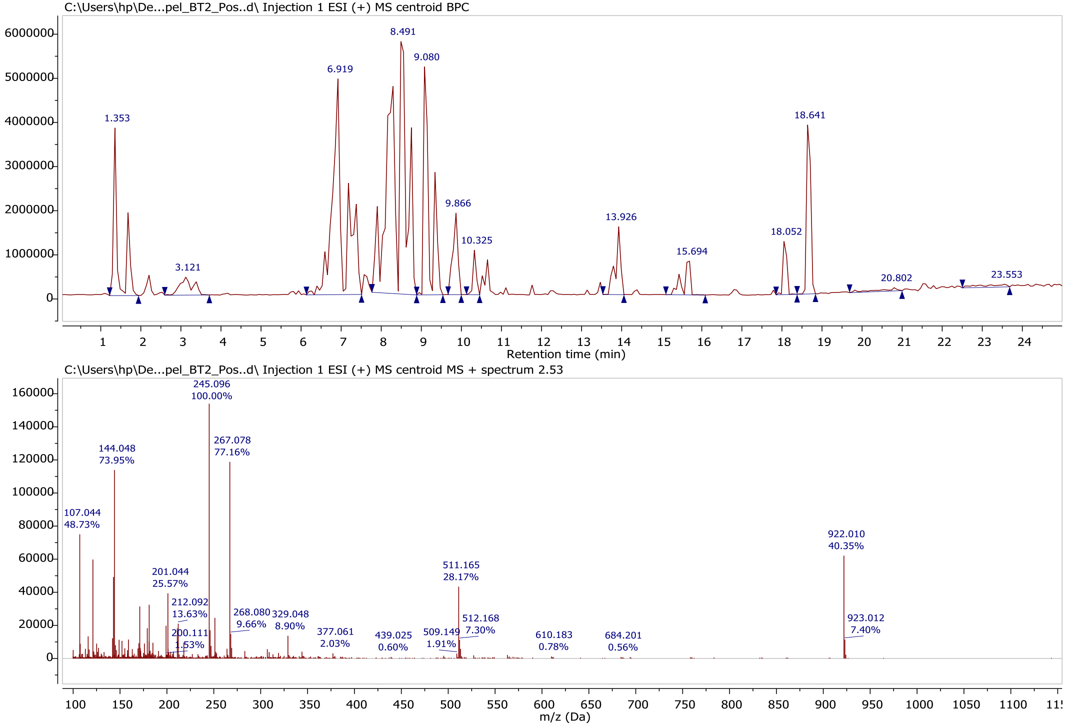


Figure 25: BPC and MS profile of Cyclo(L-Pro-L-OMet)

Surfactin C13 was identified as the molecular ion at *m/z* 1008.660 $[M+H]^+$ that was found at a retention time of 2.53 min (Hoefler *et al.*, 2012), but it was detected at a retention time of 21.32 in our chromatogram which is given in **Figure 26**.

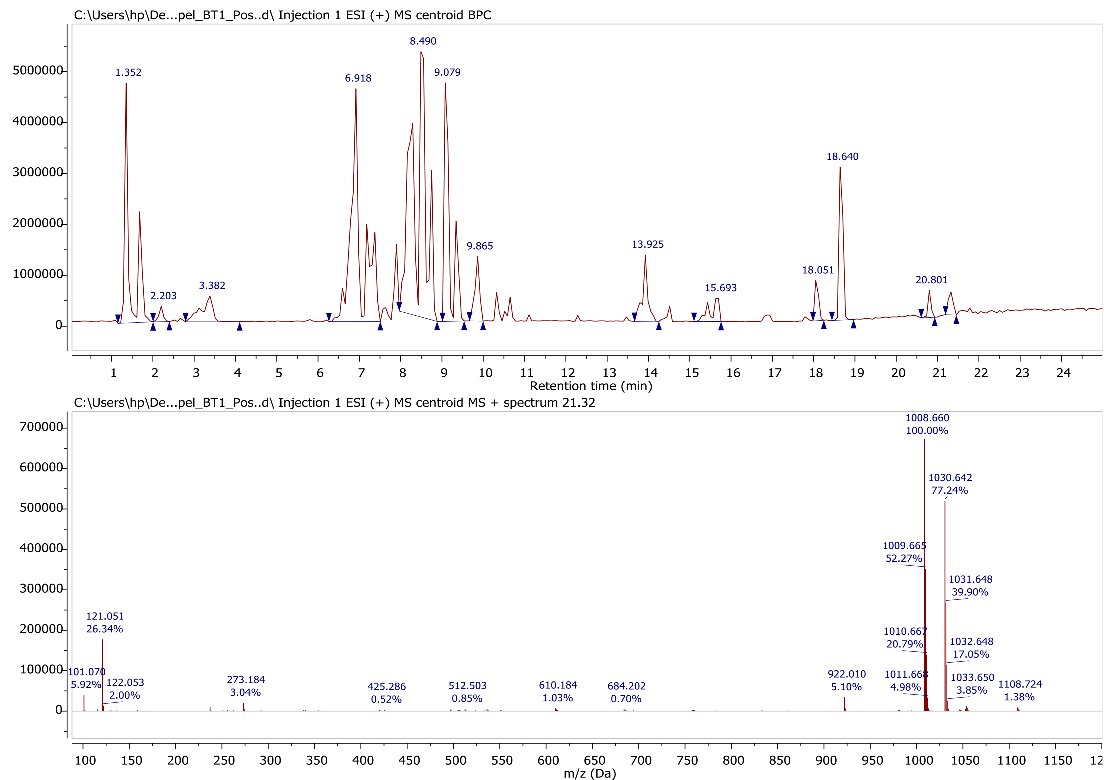


Figure 26: BPC and MS profile of Surfactin C13

Similarly, phthalic anhydride was identified as a molecular ion at *m/z* 149.023 $[M+H]^+$ that was found with a retention duration of 18.64 minutes which is given in **Figure 27**.

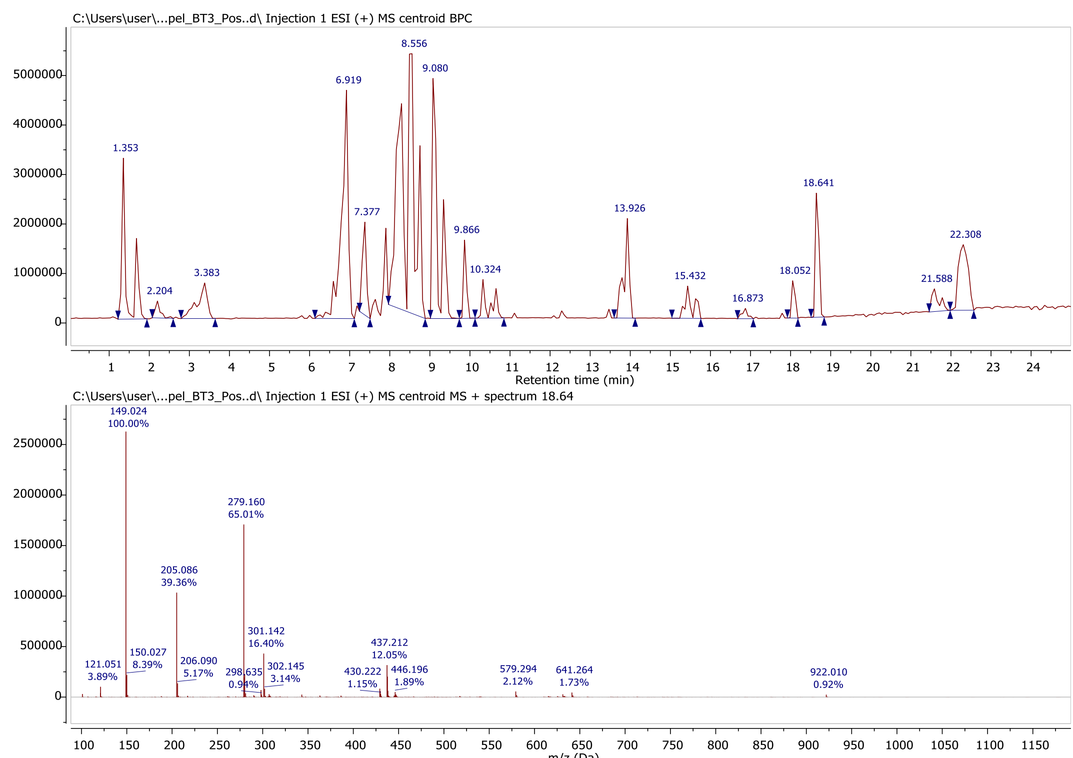


Figure 27: BPC and MS profile of phthalic anhydride

Phytoceramide was found as another molecular ion at *m/z* 556.531 $[M+H]^+$ that was detected at a retention time of 20.67 minutes which is shown in **Figure 28**.

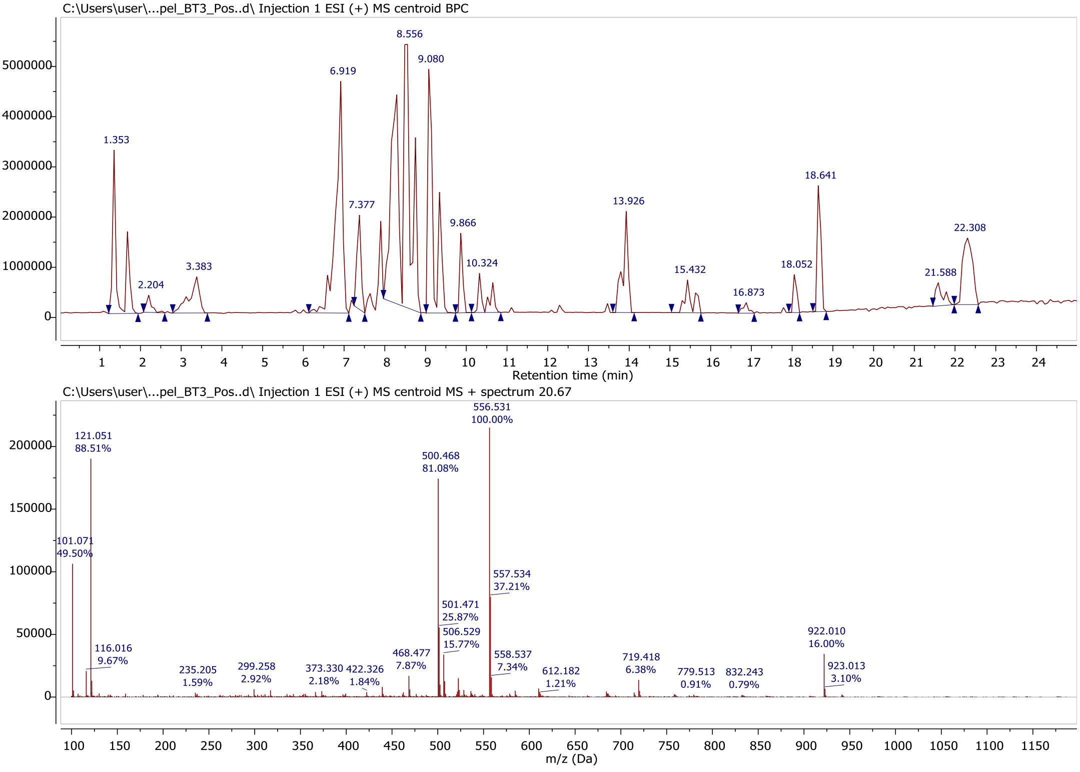


Figure 28: BPC and MS profile of phytoceramide

Likewise, using the body of existing research, the $[M+H]^+$ at *m/z* 155.081 $[M+H]^+$ observed at a retention time of 2.20 min was determined to be Cyclo(Pro-Gly) which is given in **Figure 29**.

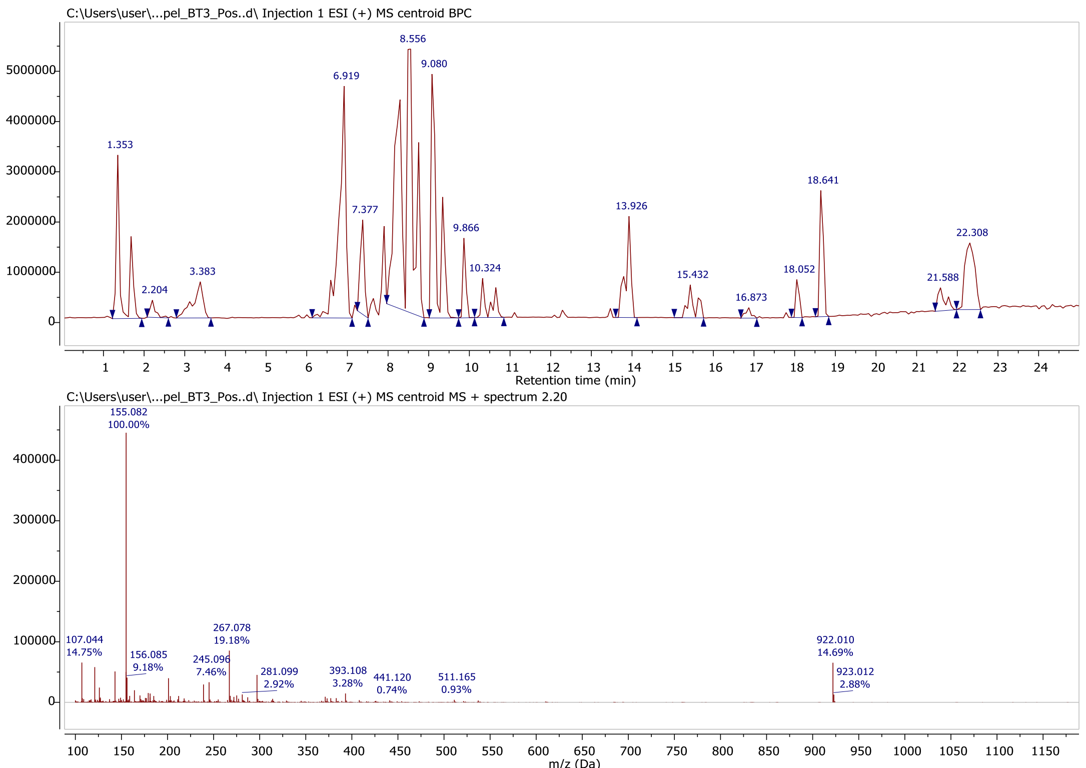


Figure 29: BPC and MS profile of Cyclo(Pro-Gly)

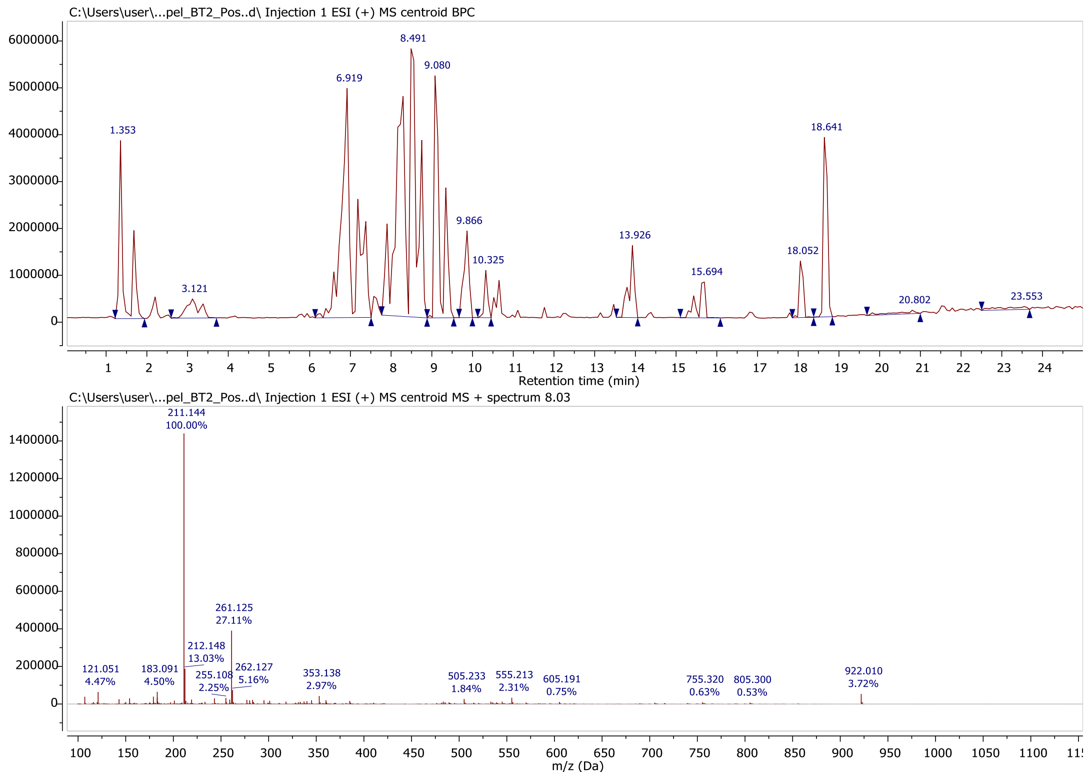


Figure 30: BPC and MS profile of Cyclo(L-leu-L -pro)

After being identified at a retention time of 8.03 minutes, a chemical ion at *m/z* 211.144 $[M+H]^+$ was recognized as Cyclo(L-Leu-L-Pro), which Zin *et al.* had previously described (Zin *et al.*, 2017) which is shown in **Figure 30.**

Additionally, Abdelkader *et al.* have previously isolated the molecular ion at *m/z* 261.124 $[M+H]^+$ from *Streptomyces asenjonii*, and it was identified as Cyclo(L-Phenylalanyl-trans-4-hydroxy-L-Proline). This molecular ion was found as a protonated ion at a retention time of 6.72 min (Abdelkader *et al.*, 2018) which is given in **Figure 31**.

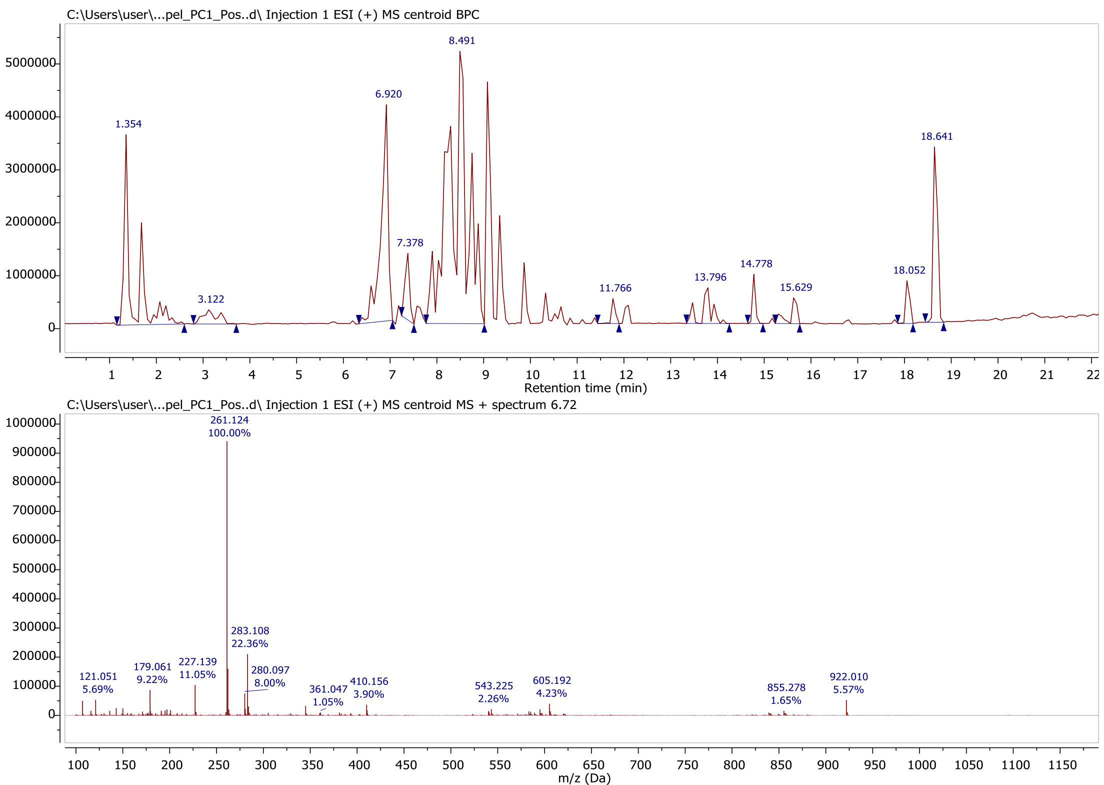


Figure 31: BPC and MS profile of Cyclo(L-phenylalanyl-trans-4-hydroxy-L -proline)

Coronafacoyl-L-isoleucine was recognized as the sodium adduct produced at *m/z* 344.176 $[M+Na]^+$, as reported in the literature, and discovered at a retention time of 10.85 min. This metabolite was previously identified in *Pseudomonas syringae* and *Streptomyces scabies* (Bown *et al.*, 2017) which is shown in **Figure 32**.

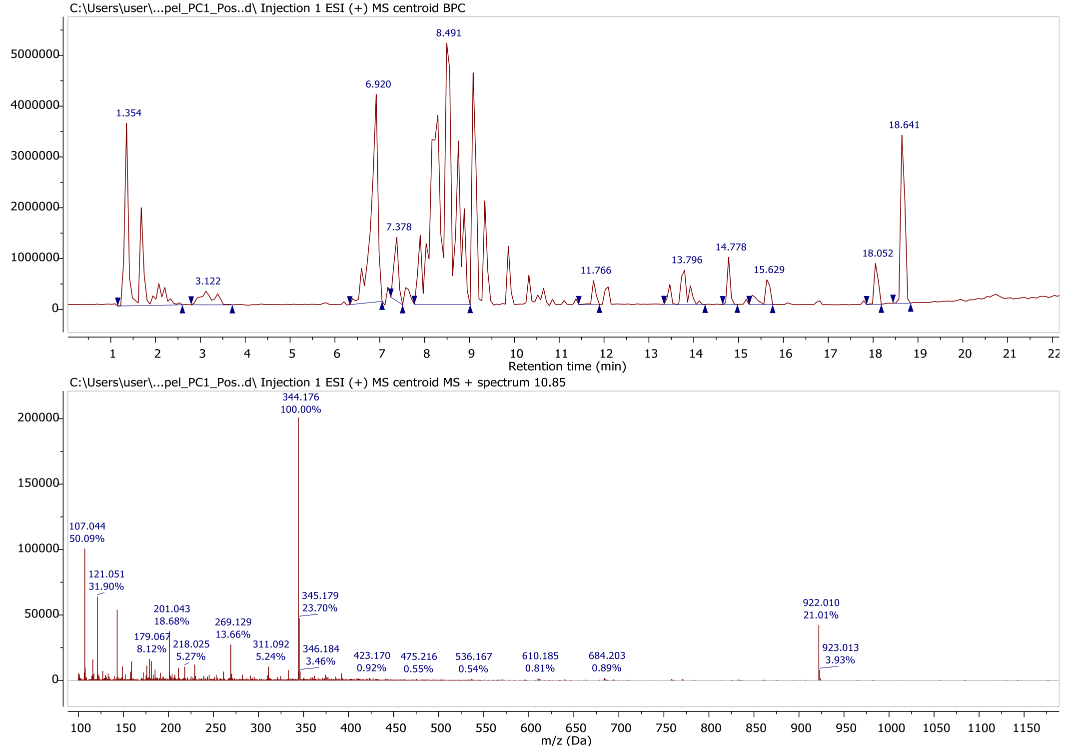


Figure 32: BPC and MS profile of coronafacoyl-L-isoleucine

Another molecular ion peak, identified as Cyclo(D-Pro-L-Tyr), was found at a retention time of 6.79 min with *m/z* 261.123 $[M+H]^+$. This peak was previously identified at *Streptomyces sp.* strain 22-4 and its BPC and MS profile is given in **Figure 33**.

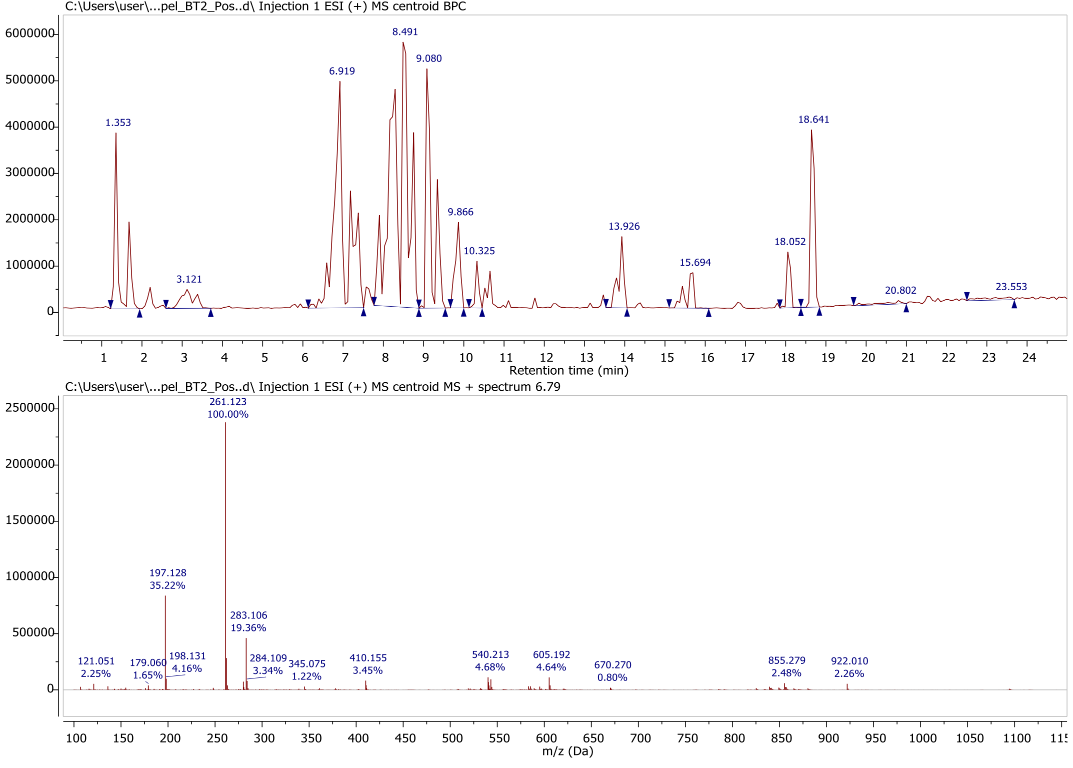


Figure 33: BPC and MS profile of Cyclo(D-Pro-L -Tyr)

Furthermore, a metabolite was identified as Cyclo(Pro-Val) in accordance with the extant literature after being discovered at *m/z* 197.12 $[M+H]^+$ as a protonated ion in the retention duration of 6.85 min (Pettit *et al.*, 2006). It was found in isolate BT2 at the same retention time and its BPC and MS profile is given in **Figure 34**.

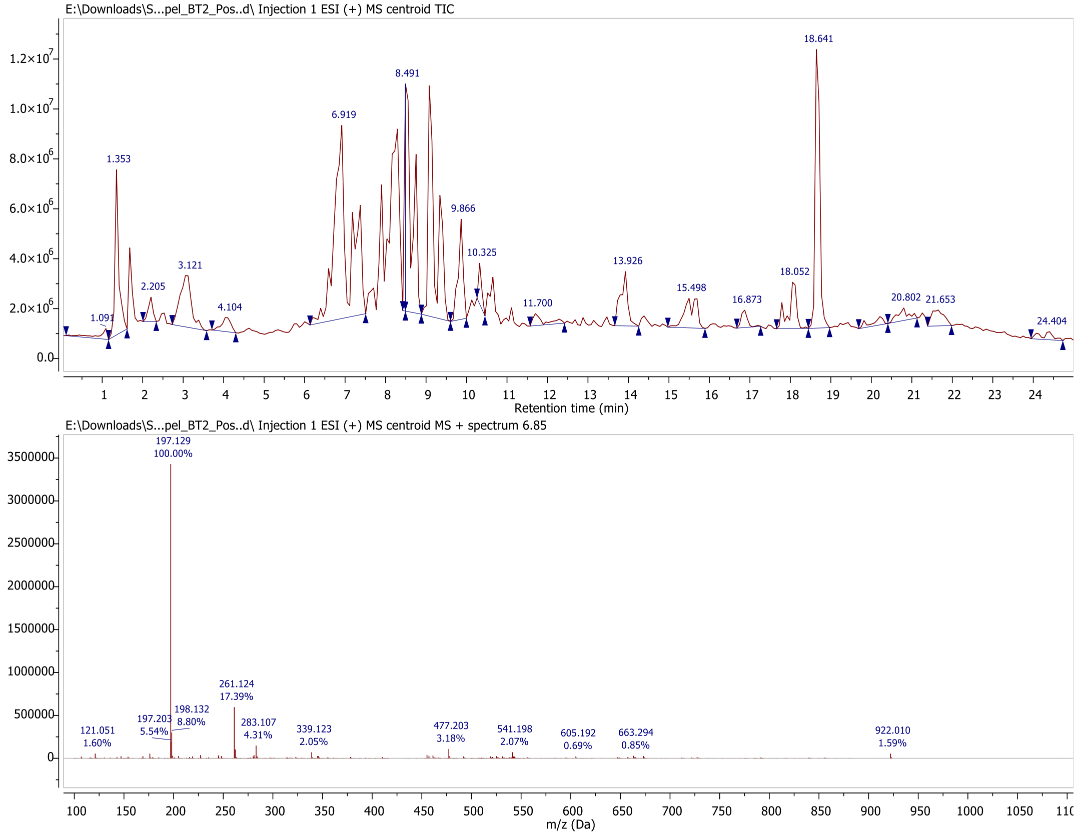


Figure 34: BPC and MS profile of Cyclo(Pro-Val)

Likewise, the chemical ion identified as N-acetyltyramine from the spectrum analysis performed by Driche *et al.* (2022) was found at a retention time of 7.18 min with *m/z* 180.102 $[M+H]^+$. It was found in isolate BT2 at the same retention time which is given in **Figure 35**.

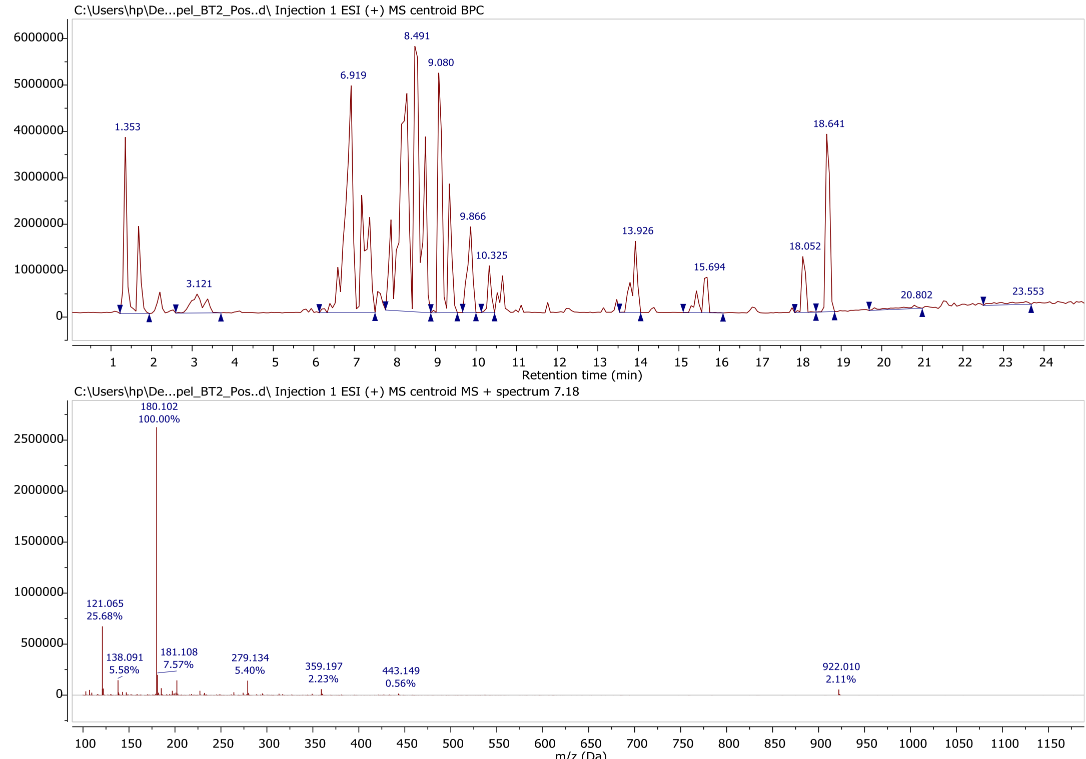


Figure 35: BPC and MS profile of N-acetyltyramine

After being previously identified from *Streptomyces* species in the soil, the metabolite Cyclo (L-Ala-L-Leu) was found at *m/z* 185.129 $[M+H]^+$ as a protonated ion during the retention duration of 7.57 min (Wang *et al.*, 2010) which is given in **Figure 36**.

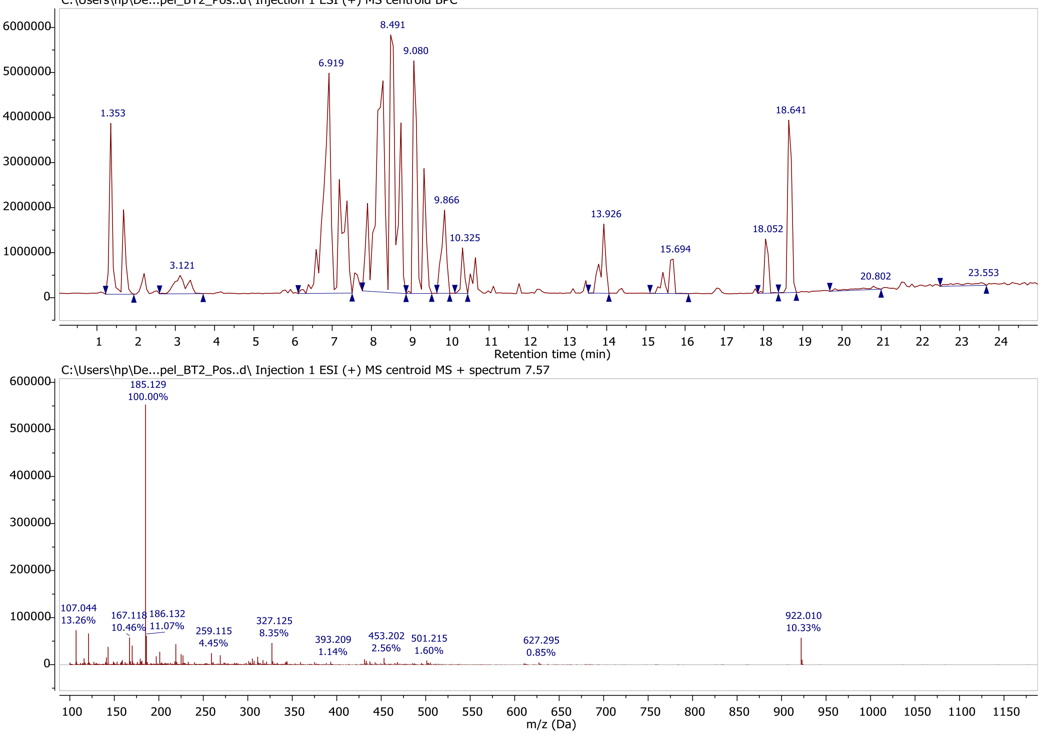


Figure 36: BPC and MS profile of Cyclo(L-Ala-L-Leu)

N-lauryldiethanolamine was the molecule that was identified as the other one found at *m/z* 274.275 $[M+H]^+$ which is shown in **Figure 37**.

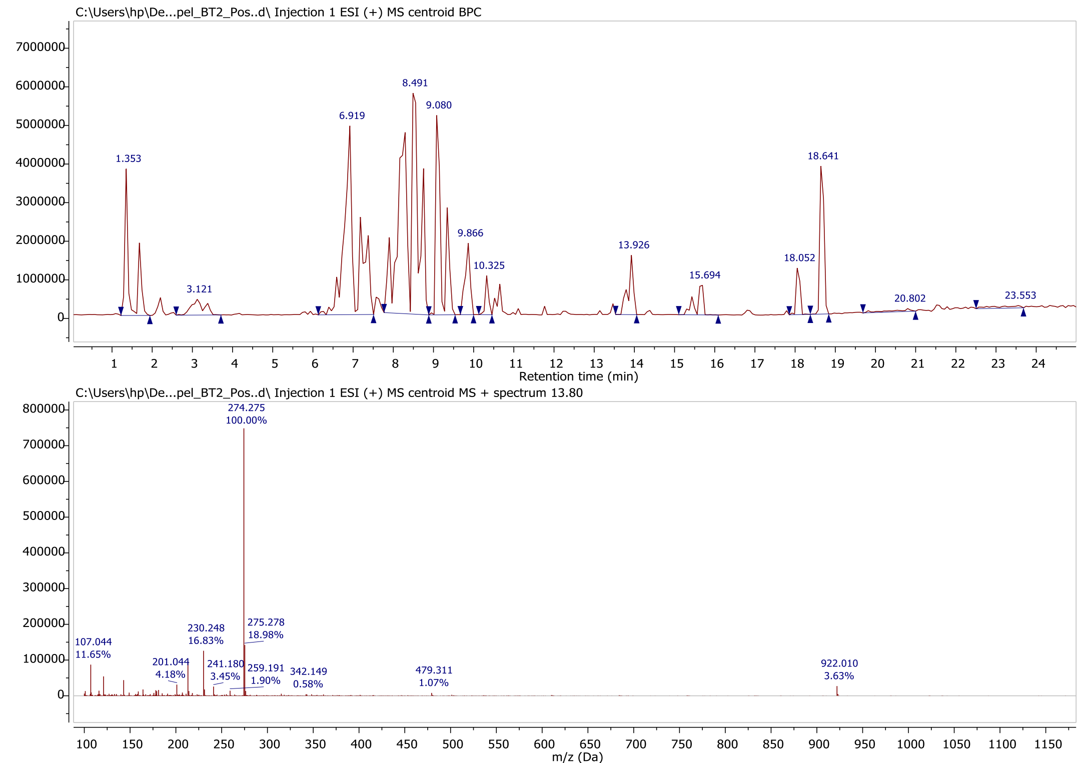
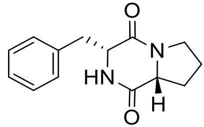
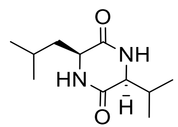


Figure 37: BPC and MS profile of N-lauryl diethanolamine

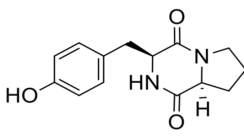
Similarly, 2-hexyl-5-methylresorcinol, which has previously been described in *S. clavuligerus*, was suspected to be the protonated ion observed at *m/z* 237.185 $[M+H]^+$ at a retention time of 18.05 min (Álvarez-Álvarez *et al.*, 2017).



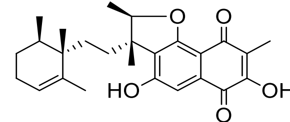
Cyclo(D-pro-D-phe)
Exact Mass: 244.1212
$C_{14}H_{16}N_2O_2$



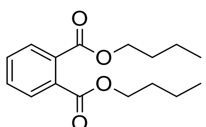
Cyclo(L-val-L-leu)
Exact Mass: 212.1525
$C_{11}H_{20}N_2O_2$



Maculosin
Exact Mass: 260.1161
$C_{14}H_{16}N_2O_3$



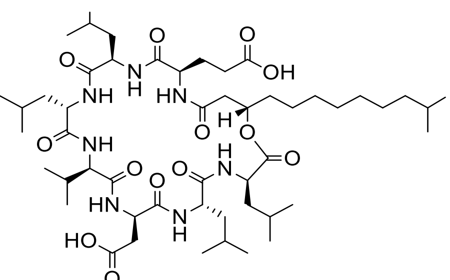
Neomarinone
Exact Mass: 424.2250
$C_{26}H_{32}O_5$



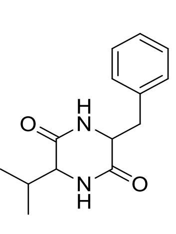
Dibutyl phthalate
Exact Mass: 278.1518
$C_{16}H_{22}O_4$

Pyridoxine
Exact Mass: 169.0739
$C_8H_{11}NO_3$

Cyclo(L-Pro-L-OMet)
Exact Mass: 244.0882
$C_{10}H_{16}N_2O_3S$

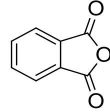


Surfactin C13
Exact Mass: 1007.6518
$C_{51}H_{89}N_7O_{13}$

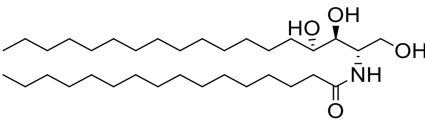


Cyclo(L-valyl-L-phenylalanyl)
Exact Mass: 246.136
$C_{14}H_{18}N_2O_2$

Di-*n*-butyl terephthalate
Exact Mass: 278.1518
$C_{16}H_{22}O_4$



Phthalic anhydride
Exact Mass: 148.0160
$C_8H_4O_3$

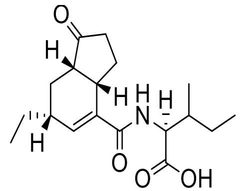


Phytoceramide
Exact Mass: 555.5227
$C_{34}H_{69}NO_4$

Cyclo (Pro-Gly)
Exact Mass: 154.0742
$C_7H_{10}N_2O_2$

Cyclo(L-leu-L-pro)
Exact Mass: 210.1368
$C_{11}H_{18}N_2O_2$

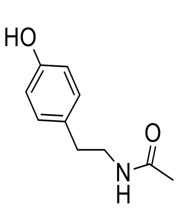
Cyclo(L-phenylalanyl-*trans*-4-hydroxy-L-proline)
Exact Mass: 260.1161
$C_{14}H_{16}N_2O_3$



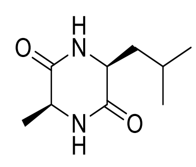
Coronafacoyl-L-isoleucine
Exact Mass: 321.1940
$C_{18}H_{27}NO_4$

Cyclo(D-Pro-L-Tyr)
Exact Mass: 260.1161
$C_{14}H_{16}N_2O_3$

Cyclo(Pro-Val)
Exact Mass: 196.121
$C_{10}H_{16}N_2O_2$

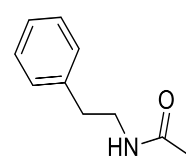


N-acetyltyramine
Exact Mass: 179.095
$C_{10}H_{13}NO_2$

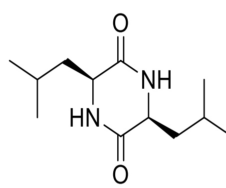


Cyclo(L-Ala-L-Leu)
Exact Mass: 184.121
$C_9H_{16}N_2O_2$

Cyclo(Tyr-Leu)
Exact Mass: 276.147
$C_{15}H_{20}N_2O_3$

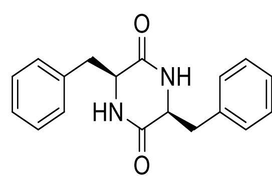


N-Phenethylacetamide
Exact Mass: 163.10
$C_{10}H_{13}NO$

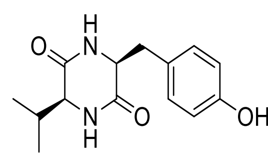


Cyclo(L-leucyl-L-leucyl)
Exact Mass: 226.17
$C_{12}H_{22}N_2O_2$

Cyclo-(D-Leu-L-Trp)
Exact Mass: 299.16
$C_{17}H_{21}N_3O_2$



Cyclo(phenylalanyl-phenylalanyl)
Exact Mass: 294.14
$C_{18}H_{18}N_2O_2$



Cyclo(Tyr-Val)
Exact Mass: 262.13
$C_{14}H_{18}N_2O_3$

1-Acetyl-3-methoxy-carbonyl-beta-carboline
Exact Mass: 268.08
$C_{15}H_{12}N_2O_3$

(4R)-4,5-dihydro-4-hydroxygeldanamycin
Exact Mass: 578.283
$C_{29}H_{42}N_2O_{10}$

Xenocoumacin 2
Exact Mass: 406.479
$C_{26}H_{30}O_4$

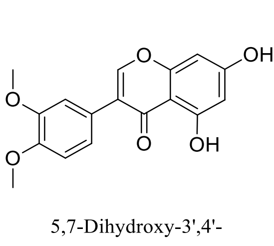
SF2809-IV
Exact Mass: 440.173
$C_{27}H_{24}N_2O_4$

Blasticidin H
Exact Mass: 440.213
$C_{17}H_{28}N_8O_6$

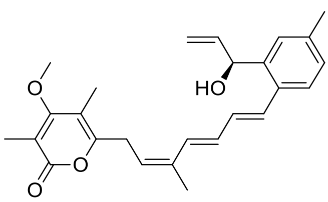
Janthinopolyenemycin B
Exact Mass: 412.260
$C_{26}H_{36}O_4$

Butyrolactol A
Exact Mass: 526.314
$C_{28}H_{46}O_9$

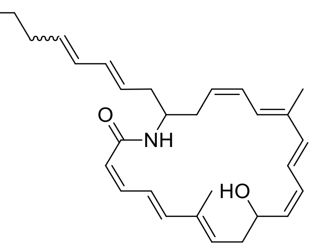
SF2415B2
Exact Mass: 424.225
$C_{26}H_{32}O_5$



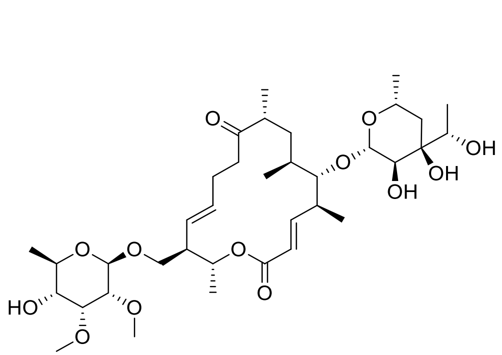
5,7-Dihydroxy-3',4'-dimethoxyisoflavone
Exact Mass: 314.079
$C_{17}H_{14}O_6$



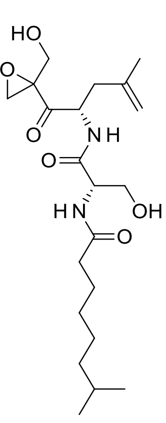
Gombapyrone A
Exact Mass: 406.214
$C_{26}H_{30}O_4$



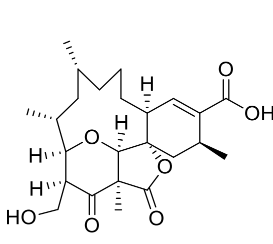
8-deoxyheronamide C
Exact Mass: 433.297
$C_{29}H_{39}NO_2$



Aldgamycin L
Exact Mass: 700.403
$C_{36}H_{60}O_{13}$



Epopromycin A
Exact Mass: 412.256
$C_{21}H_{36}N_2O_6$



Okilactomycin A
Exact Mass: 434.229
$C_{24}H_{34}O_7$

Phoxalone
Exact Mass: 424.209
$C_{22}H_{32}O_8$

Epoxomicin
Exact Mass: 554.368
$C_{28}H_{50}N_4O_7$

Gilvocarcin HE
Exact Mass: 512.168
$C_{27}H_{28}O_{10}$

Myxopyronin B
Exact Mass: 431.230
$C_{24}H_{33}NO_6$

Glucopiericidin A
Exact Mass: 577.325
$C_{31}H_{47}NO_9$

Actinoallooide C
Exact Mass: 546.356
$C_{32}H_{50}O_7$

Flavofungin
Exact Mass: 664.419
$C_{37}H_{60}O_{10}$

Figure 38: Structural formula of annotated compounds

Using LC-HRMS/MS analysis, a total of 58 metabolites were annotated from isolates BT1, BT2, BT3, AB1, PT7, and PC1. A total of 7 antibiotics were identified which were myxopyronin B, blasticidin H, flavofungin, xenocoumacin 2, okilactomycin A, butyrolactol A, and SF2415-B2. Most of the compounds fell into the diketopiperazine class. Microorganisms involved in fermentation processes produce a unique family of organic chemicals called diketopiperazine (DKP), which is particularly important in the food and beverage industry. DKP resembles piperazine and has two amide linkages. Due to its wide range of biological functions, it has recently attracted a great deal of attention. Most indole DKPs exhibited noteworthy bioactivities, such as antiviral,

antibacterial, anti-inflammatory, and cytotoxic properties. Hence, these compounds could potentially be used as building blocks for the development of new drugs (Kingston, 2000). Most of the metabolites identified in this study had a strong antimicrobial effect, which helped the bacterial strain exhibit good antimicrobial activity. Cyclo(D-Pro-D-Phe) showed antibacterial action against, a phytopathogenic organism (Cimmino *et al.*, 2021). Likewise, Cyclo(L-Pro-L-OMet) showed antifungal activity with a 50 µg/mL MIC value and antimicrobial activity against *E. Coli, Pseudomonas aeruginosa, and Staphylococcus aureus* with a 100 µg/mL MIC value (Abdelhameed *et al.*, 2019). Cyclo(L-Leu-L-Pro) has broad therapeutic applicability because of its strong antibacterial action and synergistic effect in the development of enterococci, which are resistant to vancomycin and have a MIC of 0.25 to 0.5 µg/mL (Rhee, 2004). Cyclo(L-Phenylalanyl-trans-4-hydroxy-L-Proline) is a promising candidate with potential therapeutic applications because it can exhibit antibacterial activity against *Vibrio anguillarum* with a MIC value of 2.68×10^{-7} µg/mL (Fdhila *et al.*, 2003). PrAMPs, also known as proline-rich antimicrobial peptides, exhibit strong antibacterial activity and low cytotoxicity, rendering them promising therapeutic agents for infections resistant to many medications (Mardirossian *et al.*, 2019). Similarly, Cyclo(D-Pro-L-Tyr) exhibits potential antibacterial activity against a range of plant pathogenic bacteria (Wattana-Amorn *et al.*, 2016). Cyclo(Pro-Val) demonstrated antibacterial activity against *Vibrio anguillarum*, with a MIC of 7.14×10^{-7} µg/mL (Fdhila *et al.*, 2003). However, the proliferation of cancer cells was not inhibited by this metabolite. With a MIC value of 30 mg/mL, another metabolite found in this work, N-acetyltyramine has tremendous potential for further study against drug-resistant bacteria (Driche *et al.*, 2022).

The antibacterial activities of maculosin, a cyclic dipeptide derived from *Pseudomonas rhizosphaerae*, have been demonstrated against many marine bacteria, such as *Bacillus cereus*, *Ruegeria* sp., and *Pseudoalteromonas piscida*. A glycoside of maculosin that was discovered from marine *Streptomyces* sp. ZZ446 showed strong antibacterial action (26–37 µg/mL) against *Candida albicans, Escherichia coli*, and methicillin-resistant *Staphylococcus aureus* (Paudel *et al.*, 2021). Therefore, the majority of the diketopiperzines that were found in this study consist of antibacterial potential. Previous studies have suggested that Brevianamide F may be used to treat bacterial infections and cardiovascular dysfunction (Tangerina *et al.*, 2020; T. Mehetre *et al.*,

2019). In addition to its action as a glycopeptide-like antibiotic, Cyclo(Phenylalanyl-Prolyl) can be utilized for preventing membrane permeability and slowing down the production of DNA (Husain & Wardhani, 2021). Maculosin exhibits strong antioxidant properties and is non-toxic, making it a viable option for a variety of medicinal and cosmetic uses (Paudel *et al.*, 2021). Previous studies have demonstrated the interaction between Cyclo(Gly-Leu) and dopamine receptors, indicating a possible role for central dopamine receptors in the pathophysiology of hypertension (Bhargava, 1984). Nevertheless, it was noted that Cyclo(D-Ala-L-Pro) isolated from the fungus *Colpoma* sp. has weak antibacterial action (S. Lee *et al.*, 2016). Cyclo(Tyr-Leu) could promote H. marmoreus mycelial growth at an optimal dose of 10 µg/mL (J. Cao *et al.*, 2023). N-phenethylacetamide was found to inhibit the TGF-β/Smad pathway, which in turn limited the metastasis of A549 cells by affecting the epithelial-mesenchymal transition (EMT) generated by TGF-β (M. J. Lee *et al.*, 2021). Furthermore, in response to an attack by *Pseudomonas syringae,* Cyclo(L-Ala-L-Leu) develops disease resistance; nevertheless it does not directly block the growth of fungi (Noh *et al.*, 2017). Surfactin C13 has been shown in the literature to have cytotoxic capability against a range of cancer cell lines (Liu *et al.*, 2010). The creation of phthalate esters, which can be used as plasticizers, is one of the most popular applications for phthalic anhydride (Lorz *et al.*, 2007). Phytoceramide can be utilised in the cosmetic industry to make skin barrier moisturisers because it increases moisture and speed up the repair of damaged human stratum corneum (Oh *et al.*, 2017). Furthermore, in a recent study of Monanchora clathrata, it was shown that phytoceramides were cytotoxic to the MES-SA, MCF-7, and HK-2 cell lines. These findings have further demonstrated the utility of phytoceramides in the prevention of neurodegeneration in both in vivo and in vitro settings (Santalova *et al.*, 2023). Cyclo(Pro-Gly) exhibits both anxiolytic and neuroprotective properties; its capacity to reduce motor neuronal death following brain injury has been demonstrated (Bellezza *et al.*, 2019; Guan, 2008). Similarly, a metabolic intermediary of coronatine called coronafacoyl-L-isoleucine appears to increase the severity of disease symptoms caused by pathogenic bacteria during host infection (Mitchell & Young, 1985; Bignell *et al.*, 2018). Due to its strong proton affinity, N-lauryldiethanolamine is a plastic antistatic agent that is detected in positive ionisation mode and is reported to leach from plastic micro-tubes during sample pretreatment (Chai *et al.*, 2019). According to reports, neomarinone exhibited

cytotoxicity in vitro against HCT-116 colon cancer cells, with an IC_{50} value of 8 µg/mL (Hardt *et al.*, 2000).

Cyclo(D-Pro-D-Phe) has antifungal, quorum sensing, and anti-microbial properties. It does not have adverse effects on human cell lines up to 200 µg/mL (Nishanth Kumar *et al.*, 2014). Hela S3 and PANC-1 cancer cells are 50% inhibited by the metabolite Cyclo(L-Val-L-Leu) (Lin *et al.*, 2020). Dibutyl phthalate chemical showed harmful effects in humans, including headaches and dizziness, and it may have serious effects on the nose, throat, and eye. Additionally, this substance harms the developing foetus and testicles over the long run (Yuan *et al.*, 2022; Wu *et al.*, 2024). However, it exhibits antibacterial and antifungal properties in spite of all the detrimental effects (Khatiwora *et al.*, 2012). Strong antibacterial action was shown by dibutyl phthalate, which was extracted from *Streptomyces albidoflavus* (Roy *et al.*, 2006). Pyridoxine prevents homocysteine formation and builds immunity against cancer by helping the body maintain the right ratio of potassium to sodium and by increasing the development of red blood cells (T3DB online).

Class β-carboline compounds possess biological actions such as antiviral, antibiotic, anticancer, and antimalarial characteristics (Patel *et al.*, 2012; R. Cao *et al.*, 2007). Alkyl resorcinol, a significant structural component of amphiphilic phenolic lipids, has been shown to exhibit a range of biological activities, including genotoxic, antioxidative, cytotoxic, and signalling properties (Stasiuk & Kozubek, 2010). The 2-n-hexyl-5-n-propylresorcinol (HPR) has mild antibacterial and fungal effects (Kanda *et al.*, 1975). A number of bacteria in the cell create HPR, a small molecule that has some antibacterial properties in the environment (Calderón *et al.*, 2019). This study has discovered Cyclo(D-Leu-L-Trp) in *Streptomyces* sp. and studies have suggested that it may promote seedling root growth (Kimura *et al.*, 2005). Nevertheless, it was noted that 3-((6-methylpyrazin-2-yl)methyl)-1H-indole had weak antibacterial activity and had little cytotoxic effect on human liver cell lines (Yang *et al.*, 2013). Consequently, previously documented compounds' activities have identified the component causing such antimicrobial activities, and additional research on these annotated compounds may result in the identification of new antibiotics.

Several literature sources state that erythromycin A is a necessary antibiotic that is frequently used in clinical practice to treat infections caused by Gram-positive bacteria.

Additionally, many lung infections, including Legionnaire's disease, are treated primarily with it (Kingston, 2000).

4.8.2 Identification of New metabolites in *Streptomyces* Species

Four of the metabolites found in this study were discovered in *Streptomyces* species for the first time. At a retention time of 6.20 min, a molecular ion $[M+H]^+$ was found at *m/z* 201.124. Its MS^2 peaks (**Figure 41a**) revealed fragments ions at *m/z* 173 $[M+H-28]^+$ owing to $[C_8H_{17}N_2O_2]^+$ ion formed by loss of a CO molecule from precursor ion, *m/z* 114 $[M+H-59-28]^+$ due to $[C_6H_{12}NO]^+$ ion formed by departure of a neutral C_2H_5NO unit and a CO molecule at the same time, and $[C_6H_{12}NO]^+$ ion further lost a CO molecule to give a clear peak at *m/z* 86 attributed to $C_5H_{12}N^+$ ion. Moreover, as **Figure 39** illustrates, the loss of two CO molecules and a neutral $C_5H_{11}N$ unit resulted in the observation of an ion at *m/z* 60 $[M+H-85-28-28]^+$. As a result, it was concluded that this substance is Cyclo(Ile-Ser), which was previously isolated from *Ophiocordyceps sobolifera* (Feng *et al.*, 2019).

Exact Mass: 201.123

-CO

Chemical Formula: $C_8H_{17}N_2O_2^+$
Exact Mass: 173.128

Chemical Formula: C_2H_5NO
Exact Mass: 59.037

Exact Mass: 27.995

Chemical Formula: $C_6H_{12}NO^+$
Exact Mass: 114.091

-CO

Chemical Formula: $C_5H_{12}N^+$
Exact Mass: 86.096

Chemical Formula: $C_5H_{11}N$
Exact Mass: 85.089

Exact Mass: 88.039

-CO

Chemical Formula: $C_2H_6NO^+$
Exact Mass: 60.044

Figure 39: Observed fragmentation pattern of Cyclo(Ile-Ser) in (+)-ESI mode.

At a retention period of 12.09 min, a second precursor ion with *m/z* 224.118 $[M+H]^+$ was found. As seen in **Figure 40**, its MS^2 profile (**Figure 41b**) revealed a clear peak at *m/z* 130.065 $[M+H-94]^+$ caused by the $C_9H_8N^+$ ion, which is linked to the loss of a neutral $C_5H_6N_2$ unit. Thus, 3-((6-methylpyrazin-2-yl)methyl)-1H-indole was the proposed name for this chemical, which Yang *et al.* (2013) had previously isolated and characterised as a novel alkaloid from marine *Serinicoccus profundi* sp. The first evidence of this chemical comes from *Streptomyces* sp (Yang *et al.*, 2013).

Exact Mass: 224.118

Chemical Formula: $C_9H_8N^+$
Exact Mass: 130.065

Chemical Formula: $C_5H_6N_2$
Exact Mass: 94.053

Figure 40: Observed fragmentation pattern of 3-((6-methylpyrazin-2-yl)methyl)-1H-indole in (+)-ESI mode.

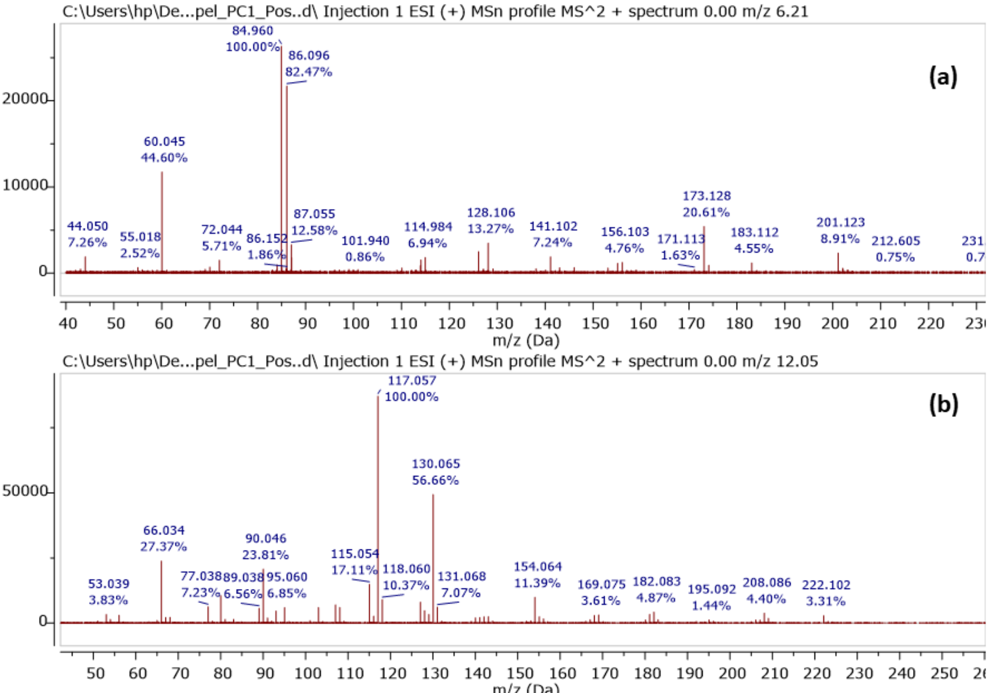


Figure 41: Observed MS/MS profiles of the precursor protonated molecules at m/z 201.124 (a), and m/z 224.118 (b)

In addition, a molecular ion was detected at *m/z* 237.185 $[M+H]^+$ after 10.27 minutes of retention time. The molecular ion peak $[C_{15}H_{25}O_2]^+$, which appeared as a base peak in its MS^2 spectrum (**Figure 43d**), might have resulted from incomplete fragmentation. Additionally, as **Figure 42** illustrates, fragment ions were found at *m/z* 124 $[C_7H_8O_2]^{\bullet+}$ due to $[M+H{-}C_5H_{11}{-}C_3H_6]^{\bullet+}$ and *m/z* 137 due to $[M+H{-}C_5H_{11}{-}C_2H_5]^{2\bullet+}$. As a result, 2-n-hexyl-5-n-propylresorcinol was the provisional identification given to this chemical. This alkyl resorcinol was initially discovered in *Streptomyces* and was previously described in *Pseudomonas chlororaphis* PCL1606 (Calderón *et al.*, 2019).

$\overset{+}{O}H_2$, OH

$-C_5H_{11}^{\cdot}$

Chemical Formula: $C_{15}H_{25}O_2^+$
Exact Mass: 237.18

Chemical Formula: $C_{10}H_{15}O_2^+$
Exact Mass: 167.107

Chemical Formula: C_5H_{12}
Exact Mass: 72.094

$-C_2H_5^{\cdot}$

$-C_3H_6$

Chemical Formula: $C_8H_9O_2^{2\cdot+}$
Exact Mass: 137.06

Chemical Formula: $C_7H_8O_2^{\cdot+}$
Exact Mass: 124.05

Chemical Formula: C_3H_6
Exact Mass: 42.047

Figure 42: Observed fragmentation pattern of 2-n-hexyl-5-n-propylresorcinol in (+)-ESI mode.

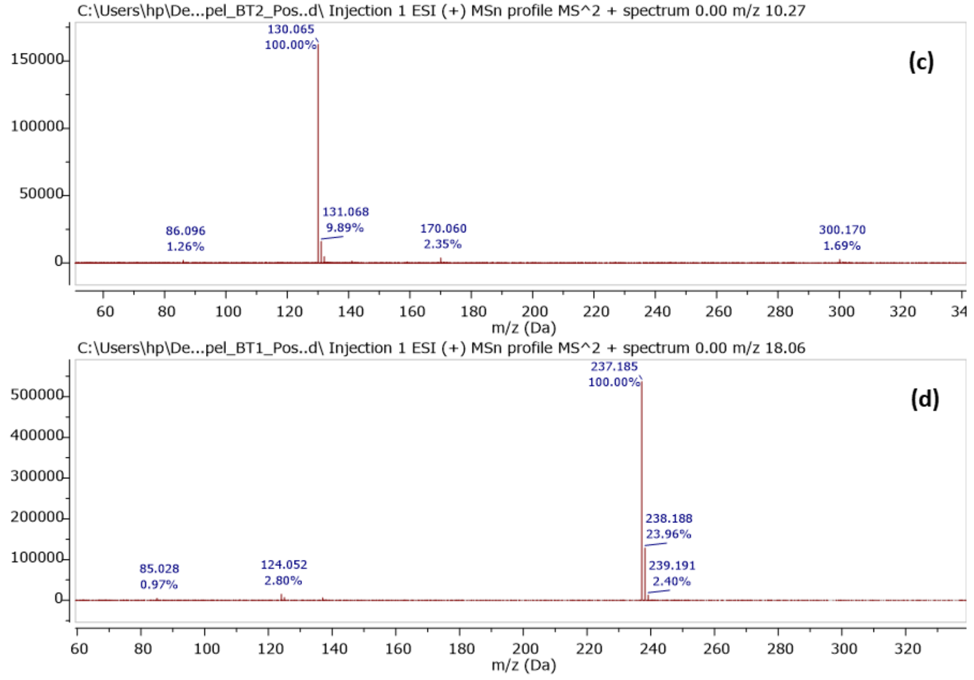


Figure 43: Observed MS/MS profiles of the precursor protonated molecules at m/z 300.171 (c), and m/z 237.185 (d)

At *m/z* 300.171 $[M+H]^+$, a protonated ion was also found after a 10.26 minute retention period. The base peak with *m/z* 130[M+H−170] on its MS^2 profile (**Figure 43c**) was caused by the $C_9H_9N^+$ ion, which was generated when the $C_8H_{14}N_2O_2$ unit was lost, as seen in **Figure 44**. As a result, cyclo(D-Leu-L-Trp) was the tentative identification of

this molecule. This chemical has been described before in *Penicillium brevicompactum*, but *Streptomyces* sp. is the first organism to have it (Kimura *et al.*, 2005).

Exact Mass: 300.171

Chemical Formula: $C_9H_8N^+$
Exact Mass: 130.065

Chemical Formula: $C_8H_{14}N_2O_2$
Exact Mass: 170.106

Figure 44: Observed fragmentation pattern of Cyclo(D-Leu-L-Trp) in (+)-ESI mode

The misuse and overprescription of medications have resulted in drug resistance, a severe worldwide problem (Zhai *et al.*, 2023). One bacterial adaptation mechanism for drug resistance is the structural change of the drug molecule. Finding a secondary metabolite that offers the same therapeutic advantages helps in the drug discovery process and is an alternate approach to address the issue of drug resistance (Zhang & Cheng, 2022).

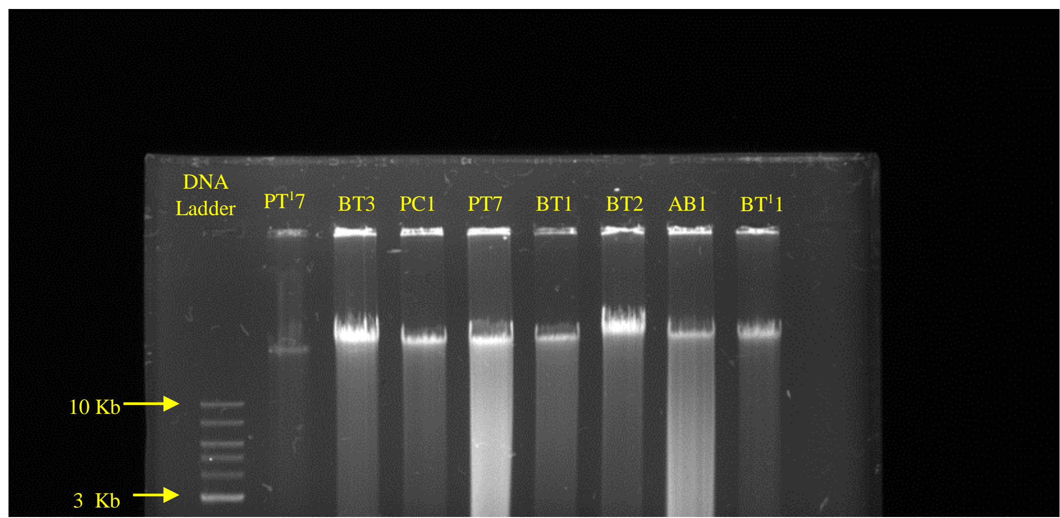
Out of 58 annotated compounds from LC-HRMS/MS analysis, four of them were identified for the first time in *Streptomyces* species which were shown in **Figure 8-11**.

4.9 Identification of *Streptomyces* Species

4.9.1 Isolation of Genomic DNA

To treat the RNA and protein contamination, respectively, proteinase K and RNase were employed. Using a much of Proteinase K could cause the DNA to degrade. The concentration of the band is dependent on a number of factors, such as the amount of ethanol present, the concentration of gel, voltage, and buffer conditions. Over the course of the genomic DNA separation procedure, the bacterial cell membrane was gradually destroyed using lysis buffer and lysozyme, frequently in conjunction with EDTA and a detergent such as SDS. EDTA causes the outer membrane of bacteria to become unstable by eliminating divalent cations from the cell. Additionally, SDS solubilizes the membrane lipids although EDTA and magnesium ions bind to stop DNase from damaging DNA. The normally circular bacterial chromosome will split into linear parts. After the cell wall has been destroyed, the DNA is extracted from the combination of proteins, lipids, RNA, and DNA using enzymes and the phenol-

chloroform technique. The proteinase K with phenol chloroform extraction technique yields more diluted DNA. DNA is precipitated with ethanol and then dissolved in the appropriate proportion of TE to concentrate it. Electrophoresis of genomic DNA of actinomycetes is given in **Figure 45**.



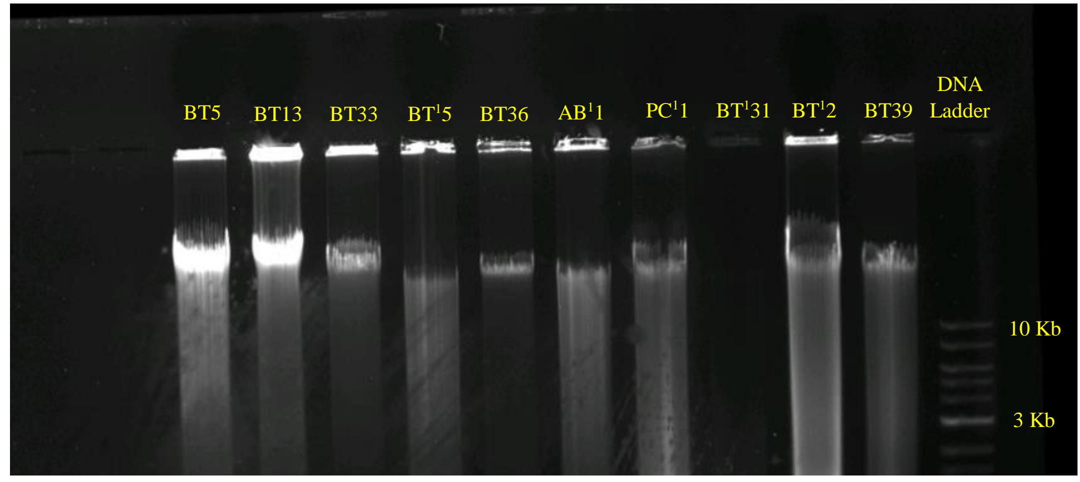


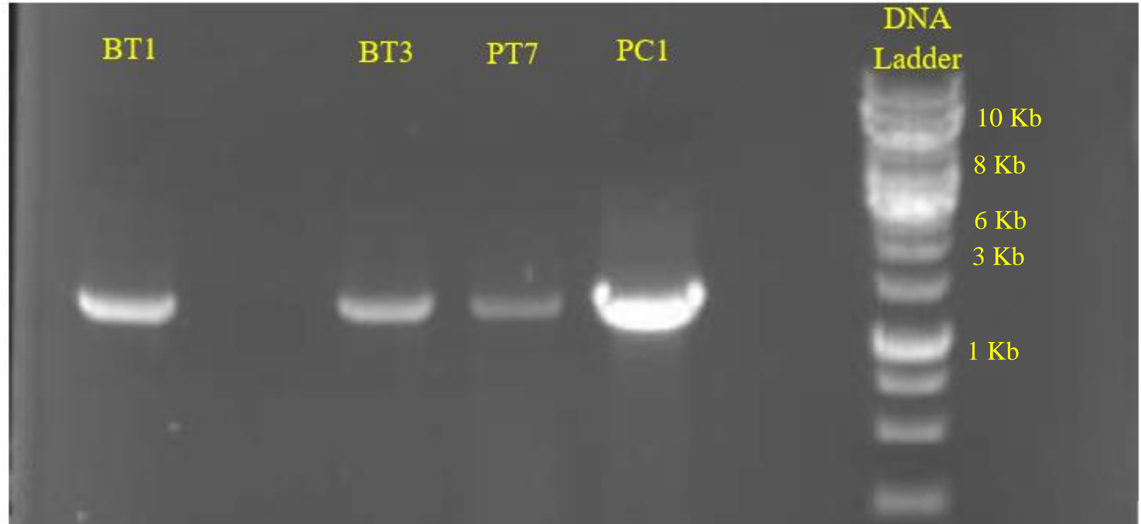
Figure 45: Electrophoresis of genomic DNA of actinomycetes

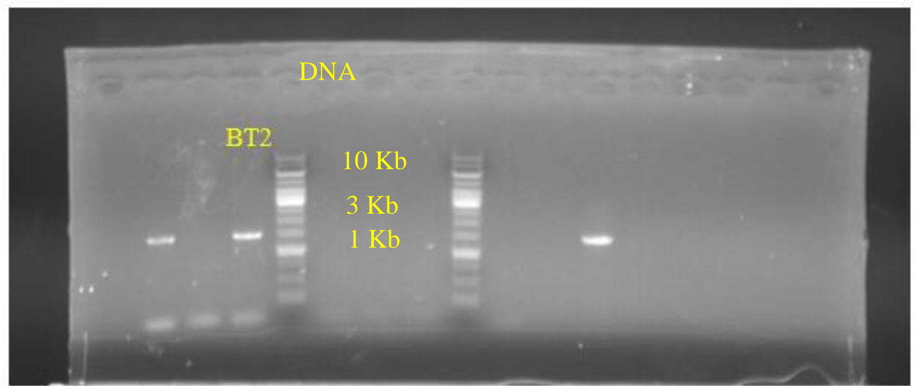
4.9.2 PCR Amplification of 16S rRNA

Using universal primers 27F and 1492R along with Taq DNA Polymerase, the 16S ribosomal DNA was amplified by the PCR technique. After 45 minutes of running the amplified products on 0.8% agarose gel at 100 volts with a 1 kb ladder, it was found that amplified product's size was around 1.5 kb smaller than the 1 kb ladder which is shown in the **Figure 46**.

A novel environment was devised for *Streptomyces* 16s rRNA, which included denaturation, annealing, and extension at 95 °C, 51.4 °C, and 72 °C respectively. The

PCR product was purified to remove primers, dNTPs, unamplified products, polymerase, and DNA fragments. PCR Amplification of 16S rRNA is given in **Figure 46.** Hence, these compounds could be building blocks for developing new drugs.





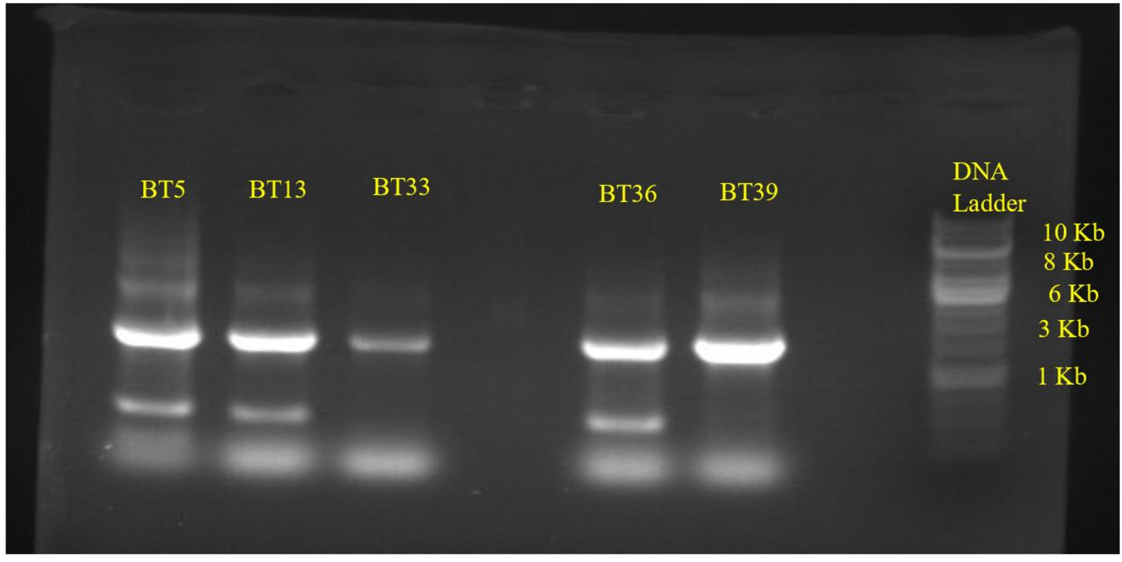


Figure 46: PCR Amplification of 16S rRNA

4.9.3 Gene Sequencing

After the 27F primer sequencing, BLAST analysis was performed. The result of the analysis is displayed in **Table 7.** 16S rRNA sequence of identified *Streptomyces* sp. was shown in **Appendix** I

Table 7: Basic Local Alignment Search Tool (BLAST) analysis

Bacterial Isolates	GenBank Accession Number	Blast Search	% Similarity	New name given
BT1	OR578351	*Streptomyces purpureus* strain LMG 19368.	96%	*Streptomyces* sp.BT1
BT2	PP106255	*Streptomyces* sp. strain MHUB12027	99%	*Streptomyces* sp.BT2
BT3	OR905603	*Streptomyces silvae* strain For3	99%	*Streptomyces* sp.BT3
PC1	OR577614	*Streptomyces cyaneofuscatus* strain CSSP436	99%	*Streptomyces* sp.PC1
BT5	PP379909	Streptomyces rhizosphaerihabitans strain JR-35	98%	*Streptomyces* sp.BT5

The 16S rRNA gene sequences of the five bioactive actinomycetes were homologously searched for using the NCBI's BLAST tool. All of the sequences showed strong similarity (more than 99%) with numerous *Streptomyces* species sequences in the GenBank. Our sequences underwent multiple sequence alignments with multiple NCBI 16S rRNA gene sequences, and the ClustalX 2.1 program was used to create a phylogenetic tree. Because of their similarity to several sequences of distinct Streptomyces, all five were identified as *Streptomyces* sp. The phylogenetic tree of 16S rRNA of identified *Streptomyces* sp. is shown in **Figure 47**.

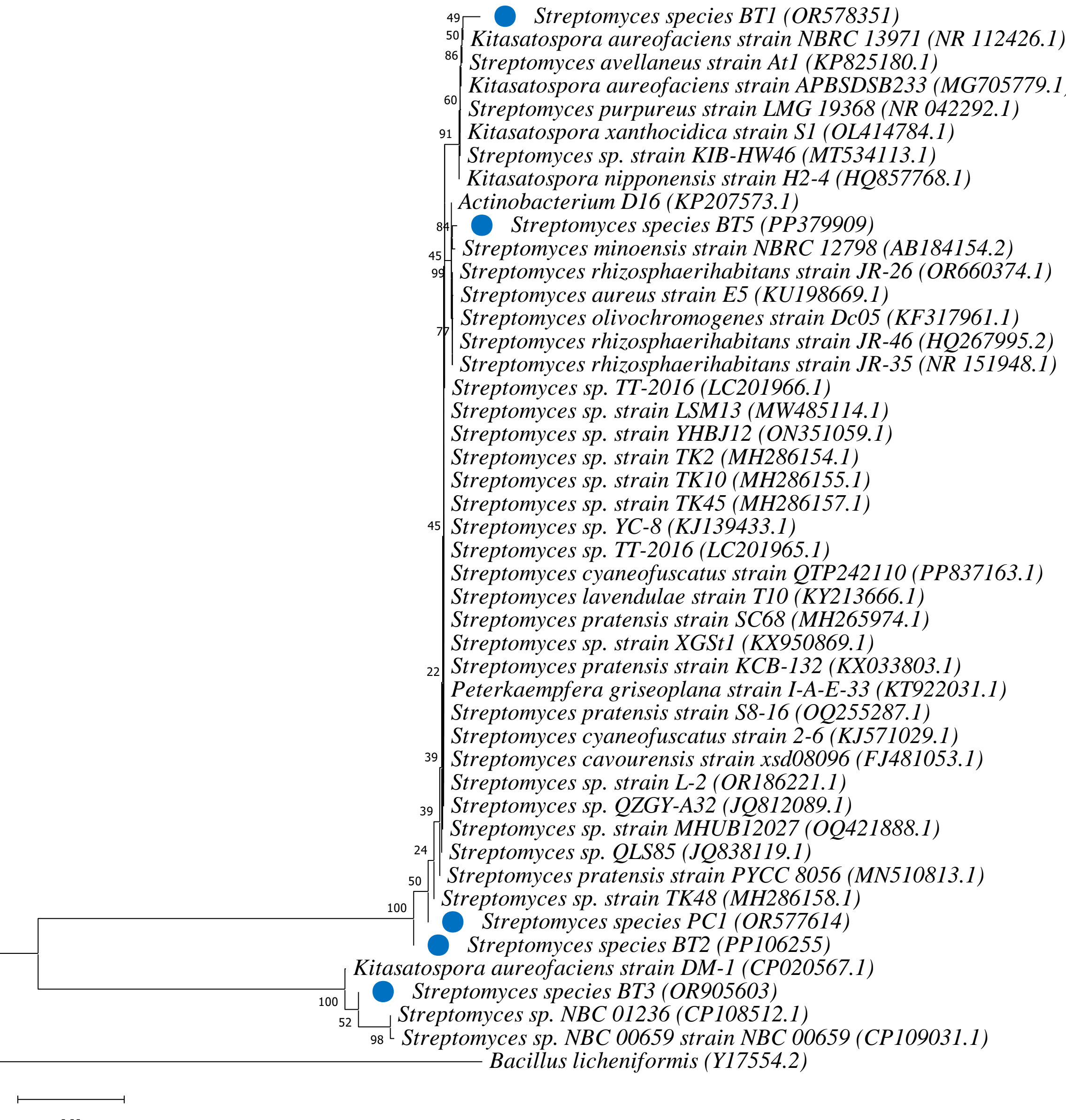


Figure 47: Phylogenetic tree of 16S rRNA of isolates (Streptomyces sp. BT1, BT2, BT3, BT5 and PC1)

Above and alongside the internal branches, horizontal branch lengths correspond to the estimated number of nucleotide changes, as well as bootstrap probabilities (as percentages) calculated for 1000 resamplings. The bar in the lower-left corner shows 0.20 substitutions per nucleotide location. *Bacillus licheniformis* was used as an outgroup to root the tree.

16S rRNA sequencing was done to accurately identify these strains, and the results indicated that the isolates were *Streptomyces* species, including sp. PC1, sp. BT1, sp. BT2, sp. BT3, and sp BT5. PC1 had the strongest antibacterial activity against

antibiotic-resistant bacteria as mentioned by WHO, and its measured MIC and MBC suggest that it can be effective against multidrug-resistant pathogens.

In this research, partial 16S rRNA gene sequencing was performed. Hence, bacterial species were identified at the genus level but not at the species level. To confirm whether the *Streptomyces* species is novel or not, complete tests 1.6 Kb 16S rRNA gene sequencing in both directions, amplification of other housekeeping genes, FAME (Fatty Acid Methyl Ester) analysis, and DNA-DNA hybridization have to be performed.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Actinomycetes can produce a variety of bioactive secondary metabolites that can effectively combat several MDR infections. *Streptomyces* is a treasure trove of bioactive compounds with enormous potential for therapeutic development and discovery. The bacterial genus deserves more investigation and utilization because of its high degree of adaptability, ecological significance, potential for biosynthesis, and potential applications in medical research.

In this research, 11 soil samples were collected from different environments in Nepal. They were found to be effective against MDR pathogens after various analysis steps. The LC-HRMS/MS analysis was performed on the extract of samples BT1, BT2, BT3, AB1, PT7, and PC1, demonstrating a notable zone of inhibition against MDR pathogens.

Among the examined pathogens, *Streptomyces* sp. PC1 exhibited the highest level of inhibition (32 mm. against *E. coli*, 30 mm. against *S. typhi*, 30 mm. against *S. sonnei*, and 30 mm. against *K. pneumoniae*). A total of fifty-eight secondary metabolites were annotated using LC-HRMS/MS analysis. Out of fifty-eight secondary metabolites, four secondary metabolites were found to be reported for the first time in *Streptomyces* species: Cyclo(Ile-Ser) from isolate BT2, 2-n-hexyl-5-n-propylresorcinol from isolate BT1, 3-((6-methylpyrazin-2-yl) methyl)-1H-indole from isolate PC1, and Cyclo(D-Leu-L-Trp) from isolate BT2. A total of 7 antibiotics were identified from the bacterial extracts which were Myxopyronin B, Blasticidin H, Flavofungin, Xenocoumacin 2, Okilactomycin A, Butyrolactol A, and SF2415-B2. Five *Streptomyces* species were identified and characterized as *Streptomyces* sp. BT1, *Streptomyces* sp. BT2, *Streptomyces* sp. BT3, *Streptomyces* sp. BT5 and *Streptomyces* sp. PC1.

Accordingly, this research identified that Nepal's varied ecology offers ideal circumstances for the existence of several *Streptomyces* species from the soil habitat which can produce specialized antibiotics. The isolation of new biologically diverse

organisms indicates the chances of getting new valuable compounds including antibiotics.

5.2 Recommendations

This research work offers a way to identify antibacterial substances from soil actinomycetes. Antibiotics are among the many bioactive secondary metabolites that actinomycetes are known to produce. These compounds have proven to be extremely useful in combating infectious diseases. This work has highlighted the unexplored microbial diversity in the area and made a significant contribution to the worldwide effort in antibiotic discovery by isolating and characterizing drugs from actinomycetes strains indigenous to Nepal.

In this research, a significant number of isolated actinomycetes were obtained from several soil samples collected from the different ecological habitats of Nepal. Four new secondary metabolites were identified using the LC-HRMS/MS analysis and five strains of actinobacteria, *Streptomyces* sp. were identified through gene sequencing. This study further supports the point that the diverse habitats of Nepal are an ideal place for those species from which antibiotics could be extracted. Therefore, it is strongly recommended to continue the research on actinobacteria in different parts of Nepal and harvest the secondary metabolites produced by them. New antibiotics could be discovered and brought to clinical trials. The efficacy of the antibiotics against different diseases can be tested through trials and the potential antibiotics can be sent for mass manufacturing. It will help to meet the demand for antibiotics through national production.

Another recommendation arising from this study is the investigation of the identified antibiotics' effectiveness against multidrug-resistant infections. Finding new antimicrobial drugs is essential since the emergence of antibiotic resistance poses a serious global issue to public health. The antibiotics that were isolated from actinomycetes in Nepal may be able to help fight drug-resistant bacteria.

Additionally, the study creates opportunities for bioprospecting in Nepal and partnerships related to it. There is significant potential for further research into the nation's microbial resources for biotechnological applications due to its tremendous biodiversity. Partnerships between research institutions, pharmaceutical firms, and community-based organisations can enable the long-term use of Nepal's microbiological diversity for novel drug discovery and biotechnology advancements.

Regarding prospects, the results of this study may facilitate the establishment of an actinomycetes-focused bioresource centre in Nepal. A facility of this kind would be instrumental for microbial research, offering the tools, knowledge, and resources needed to isolate, identify, and characterize bioactive substances from strains of actinomycetes. Furthermore, it might encourage programs aimed at developing local scientists' skills in cutting-edge biotechnological methods, enhancing Nepal's capacity for natural product discovery research.

Overall, there is great potential for scientific progress and societal influence in studying the isolation and characterization of antibiotics from actinomycetes collected in Nepal. This work adds to the global pipeline of antimicrobial drugs by utilizing the vast microbial variety of the area. It also offers prospects for biotechnological innovation, capacity building, and sustainable development in Nepal.

CHAPTER 6

6. SUMMARY

This research work has been completed in four sections namely isolation, screening, metabolic profiling, and identification of *Streptomyces* species.

The project started with the collection of soil samples from different ecosystems in Nepal. Different colonies of actinomycetes from these samples were isolated. Ten actinomycetes colonies were isolated from each soil sample, but most of them were discarded based on their morphological characteristics. Only one peculiar bacterial colony was selected from each sample. The isolates were cultured in different media and preserved for further use. Aerial mycelia of most of the bacteria were greyish-white in color while some also appeared to be bluish-white and other colors. The purity of Gram-positive bacteria isolated from the samples was verified by Gram staining. Gram staining revealed that the isolates were composed of hair-like mycelium and flagellated Gram-positive bacteria.

The screening process involves a series of primary and secondary screenings. The antimicrobial activity of the pure isolates has been determined. Primary screening was performed using perpendicular streaking and secondary screening was conducted using Agar well diffusion method. Primary screening of the isolated actinomycetes strains showed remarkable efficacy against tested pathogens. *Streptomyces* species PC1 showed the highest zone of inhibition (32 mm. against *E. coli*, 30 mm. against *S. typhi*, 30 mm. against *S. sonnei*, and 30 mm. against *K. pneumoniae*) against the tested pathogens. The isolates that showed a significant zone of inhibition were subjected to further study. Resazurin-based broth microdilution method was used to determine the MIC and MBC values of the isolates. When tested against *Escherichia coli* and *Staphylococcus aureus*, the MIC and MBC of all extracts showed greater efficacy.

Metabolic profiling of the secondary metabolites produced by the isolates was performed through the mass spectrometry technique LC-HRMS/MS. The spectra were examined using MestreNova software to determine the chemical masses and *m/z* values by comparing them to the literature collection. SIRIUS software was used to analyse mass spectrometry data at high resolution for the identification of annotated

compounds. Total fifty eight compounds were annotated from the isolates, including, valinomycin, epopromycin A, myxopyronin B, gilvocarcin HE, and okilactomycin A, in which four of them were detected for the first time in *Streptomyces* species, namely Cyclo(Ile-Ser) from isolate BT2, 2-n-hexyl-5-n-propylresorcinol from isolate BT1, 3-((6-methylpyrazin-2-yl)methyl)-1H-indole from isolate PC1, and Cyclo(D-Leu-L-Trp) from isolate BT2.

PCR amplification was performed on the genomic DNA extracted from the isolated bacteria. The pure amplified product obtained from the amplification was subjected to partial 16S rRNA gene sequencing for the identification of the *Streptomyces* species. Five *Streptomyces* species namely *Streptomyces* sp.BT1, *Streptomyces* sp.BT2, *Streptomyces* sp.BT3, *Streptomyces* sp.PC1 and *Streptomyces* sp.BT5 was identified at the genus level through morphology and DNA sequencing.

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APPENDIX

Appendix A: List of chemicals and instruments

Table A1. List of chemicals.

S.N.	Chemicals	Company/Brands	Country Name	Lot No.
1	Mueller Hinton Agar (MHA)	HiMedia	India	0000543399
2	Mueller Hinton Broth (MHB)	HiMedia	India	0000543388
3	Tryptic Soya Broth (TSB)	HiMedia	India	0000580396
4	Nutrient Agar (NA)	HiMedia	India	0000600819
5	Agar-Agar	HiMedia	India	3643490819
6	Bacto Agar	Qualigens	India	3643490819
7	Ammonium Sulphate	Qualigens Fine Chemicals	India	21495
8	Zinc Sulphate	Fischer Scientific	USA	28985
9	Ferrous Sulphate	Fischer Scientific	USA	1199850816
10	Sodium Chloride	Marck Life Science	Germany	DK8D682766
11	Manganese Chloride	Fischer Scientific	USA	-
12	Magnesium Sulphate	Marck Life Science	Germany	-
13	Dipotassium Phosphate	Fischer Scientific	USA	0000215661
14	Calcium Carbonate	Fischer Scientific	USA	1678040117
15	Soluble Starch	Marck Life Science	Germany	
16	Neomycin	HiMedia	India	000021167
17	Resazurin	Loba Chemie	India	0555200001
18	Dimethyl sulfoxide (DMSO)	Loba Chemie	India	6954391221
19	Ethyl acetate	Marck Life Science	Germany	RM/035/19
20	Cycloheximide	Loba Chemie	India	0063000001
21	Nalidixic acid	Sigma-Aldrich	USA	#BCCB7376
22	Gram's Iodine	Thermo Fisher Scientific	USA	7467511122

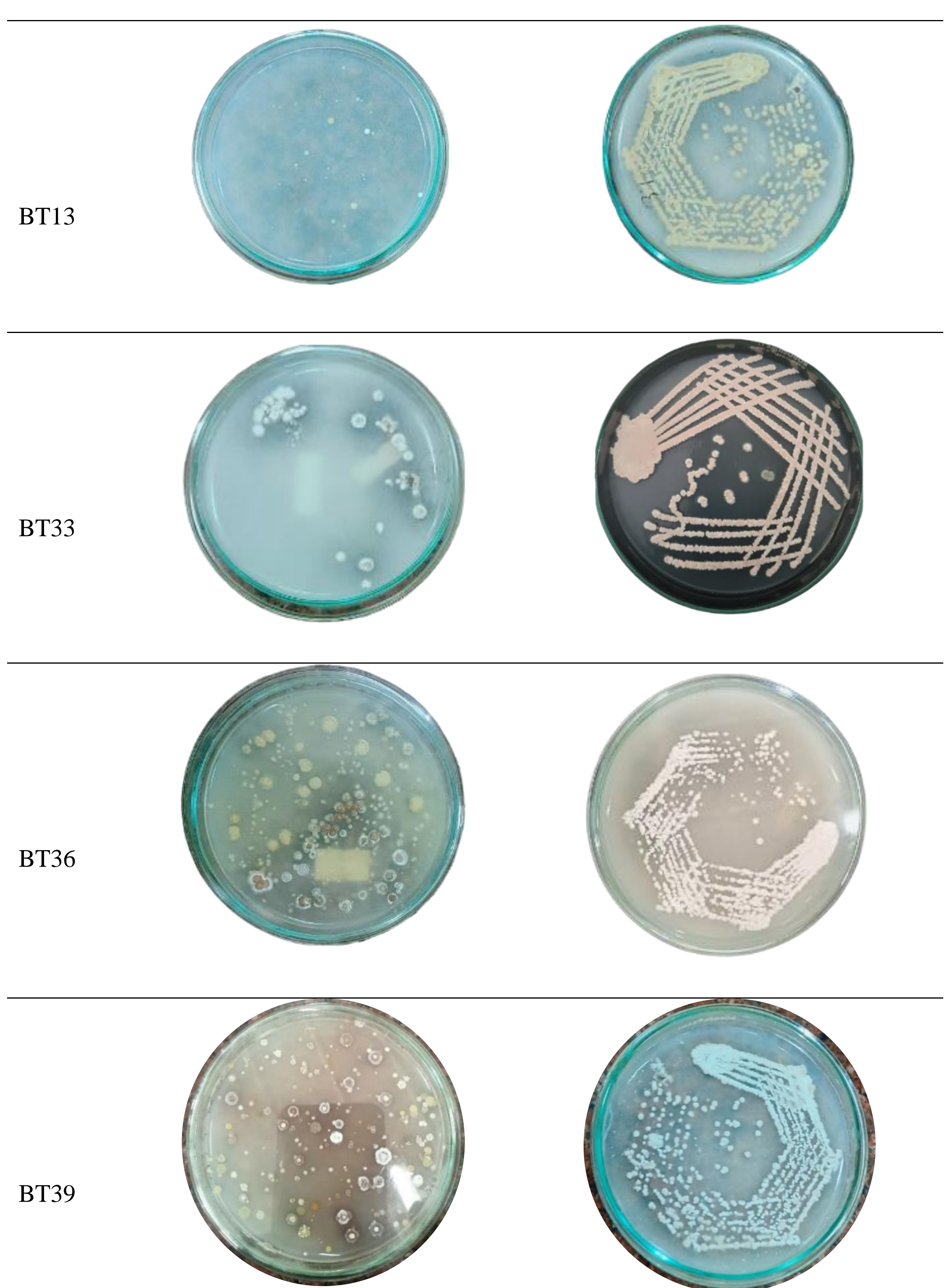
23	Universal primers: 27F and 1492R	GenoTech	Korea	
24	PCR premix	Solis Biodyne	Korea	
25	Loading dye	New England Bio Lab	USA	10055713
26	1kb DNA ladder	New England Bio Lab	USA	10055731

Table A2. List of instruments.

S.N.	Instruments	Companies/Brands	Country Name
1	Biosafety Cabinet	Sanjeev Scientific Udyog	India
2	Incubator	JEIO Tech Scientific	Korea
3	Refrigerator	Whirlpool and Samsung	India
4	Shaking Incubator	Shivaki	-
5	Vortex	Swirlex	-
6	Centrifuging Machine	Thermo Scientific	Germany
7	Water Bath	Medico	-
8	Autoclave	Thermo Scientific	USA
9	PCR	Thermo Scientific	USA
10	PCR & DNA clean-up kit	Biolabs T1030S	USA
11	G6545B Q-TOF mass spectrometer	Agilent Technologies	Korea
12	Gel-Doc	UVITEC, Cambridge	UK

Appendix B: Substrate and aerial mycelia of bacteria

Samples	Isolation	Pure Strain of Isolates
BT1		
BT2		
BT3		
BT5		


BT13
BT33
BT36
BT39

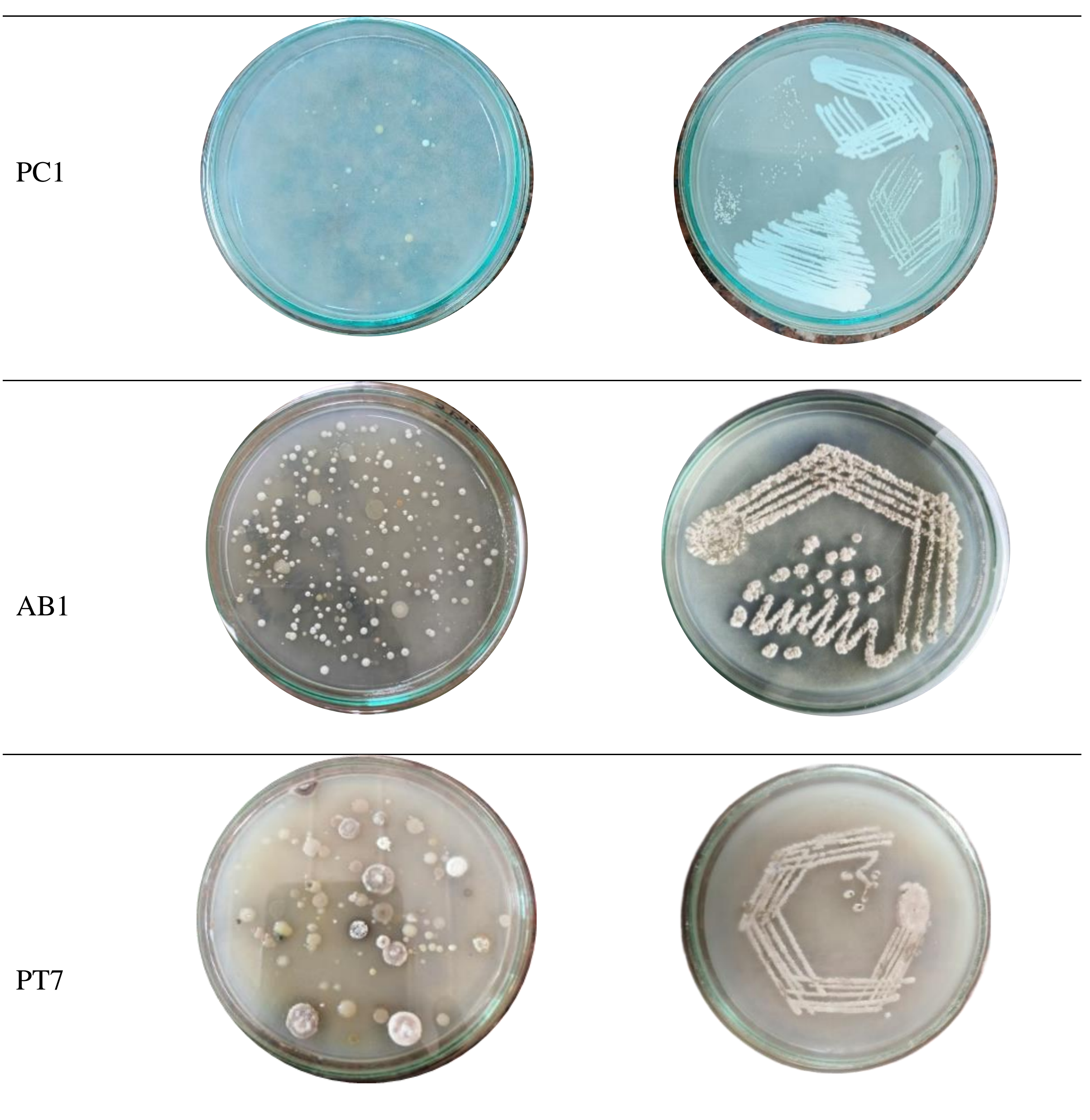


Figure B: Isolation plates and pure strain of actinomycetes

Appendix C: Hair-like mycelia of actinomycetes in Gram staining

Sample	Gram Staining	Sample	Gram Staining
BT1		BT2	
BT3		BT5	
BT13		BT33	

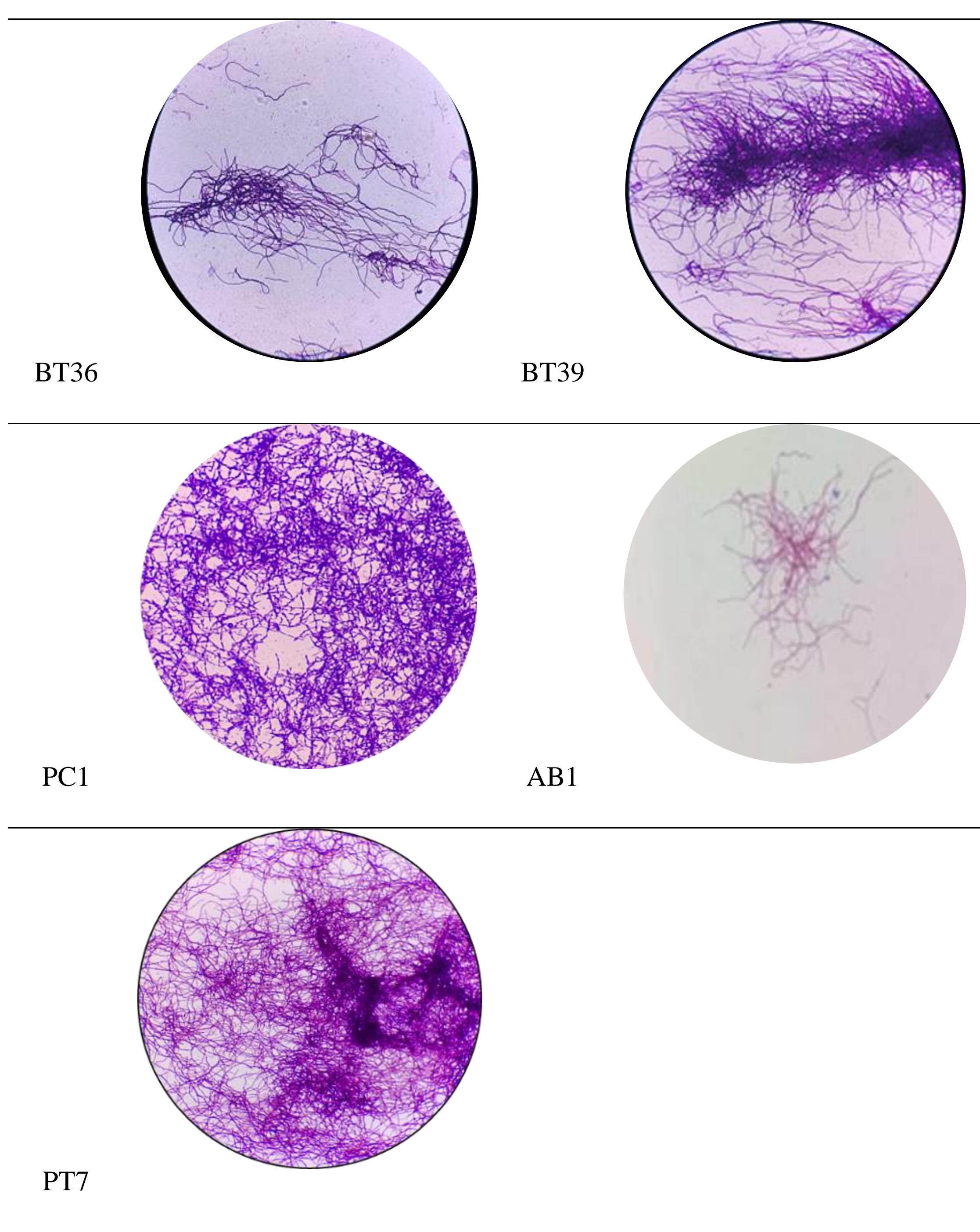
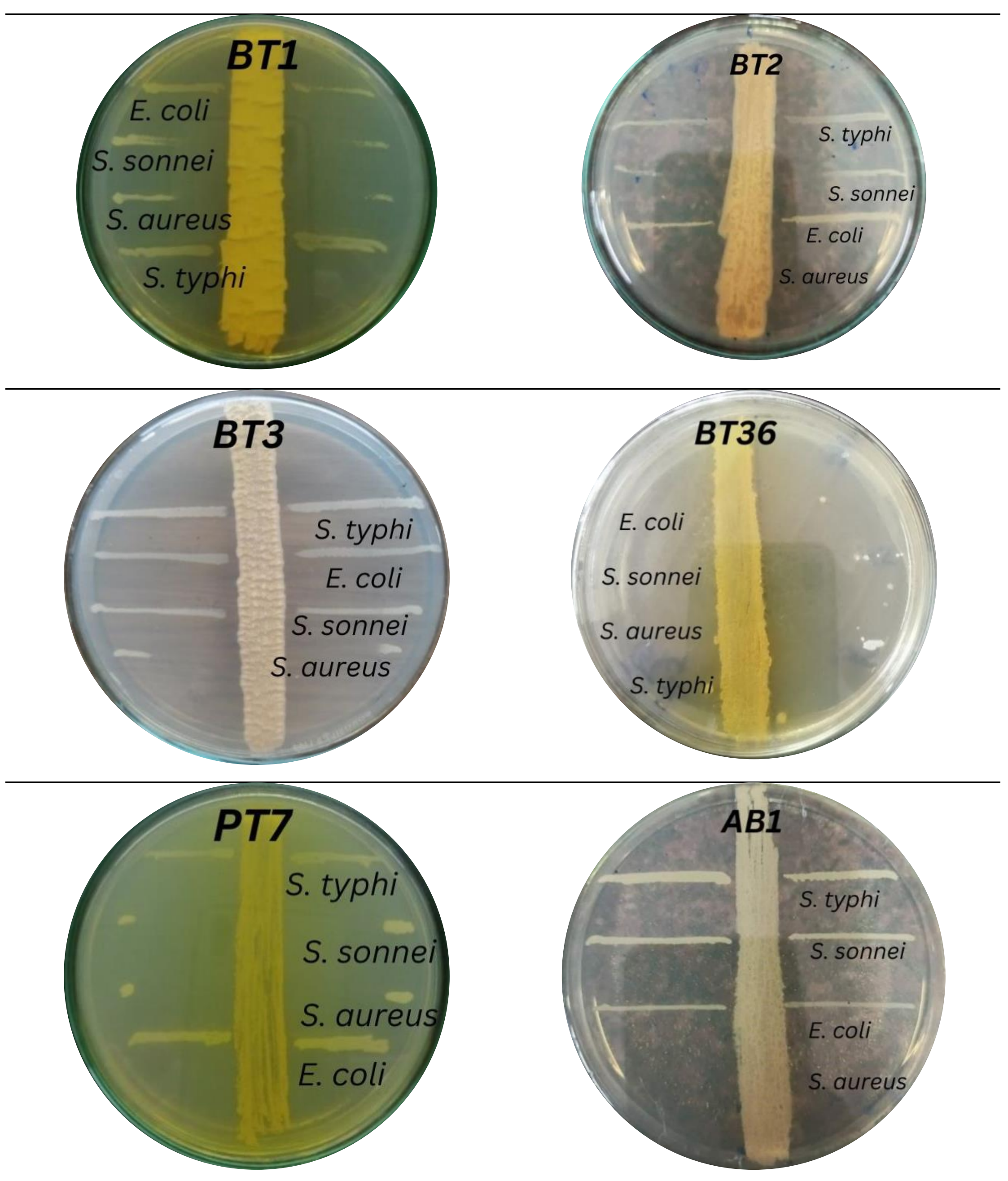


Figure C: Mycelium of the isolates seen via gram staining

Appendix D: Primary screening of potent isolates



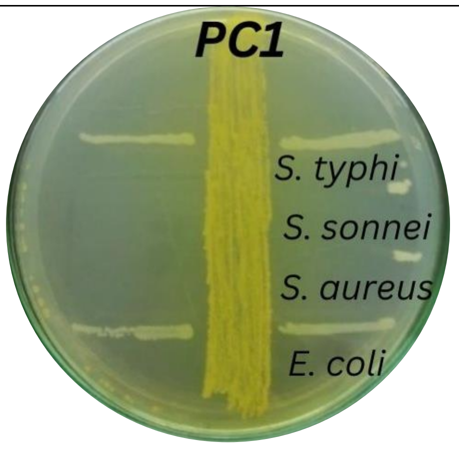
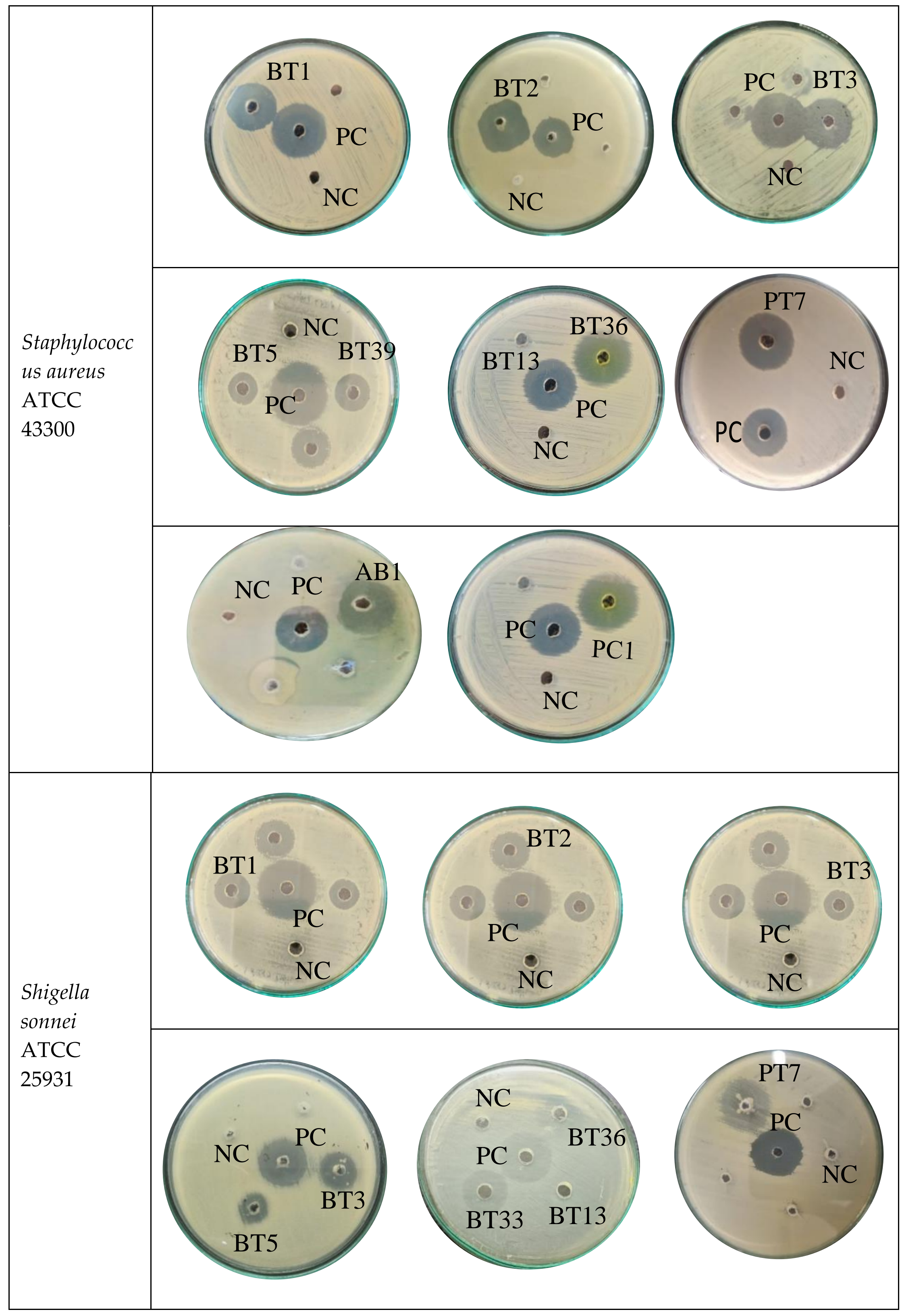
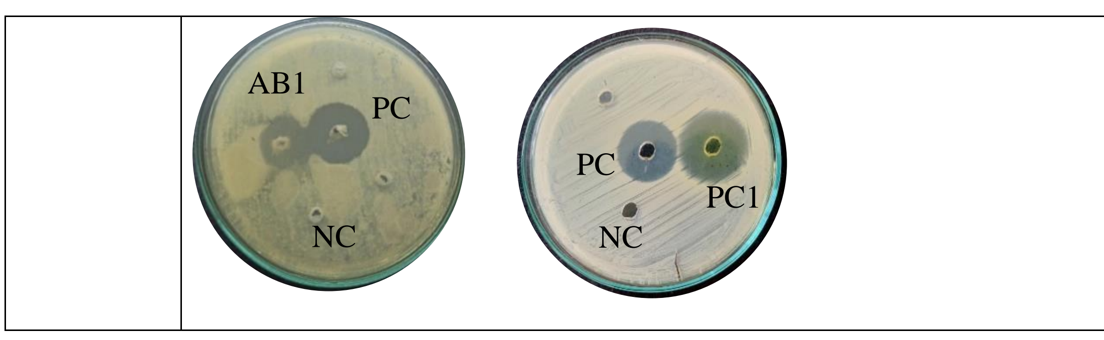


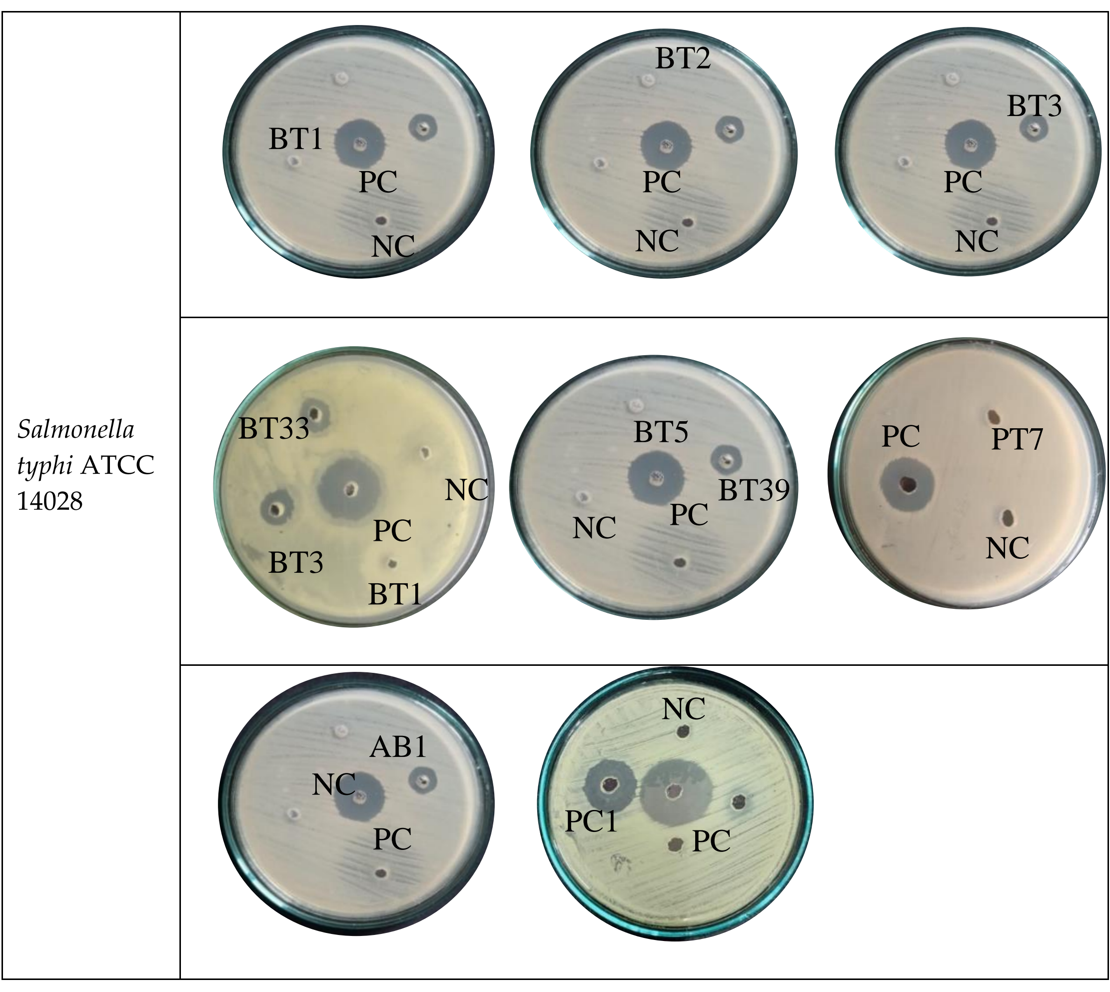
Figure D: Primary screening of some potent isolates

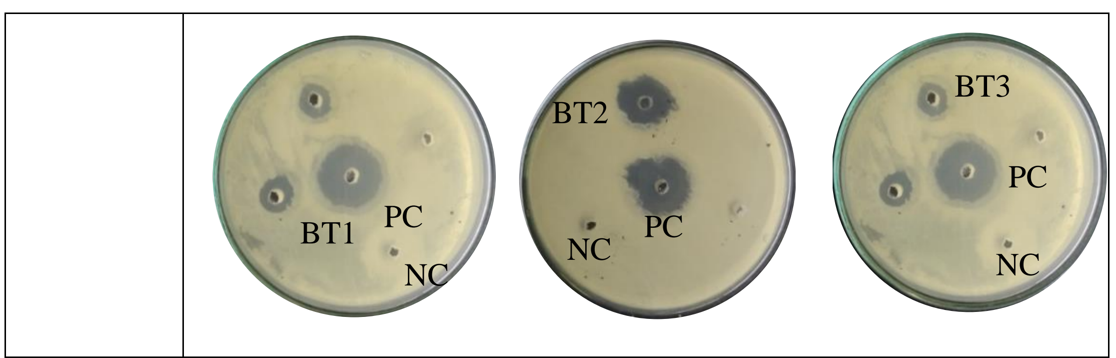
Appendix E: Zone of innibition in MHA plate





Salmonella typhi ATCC 14028





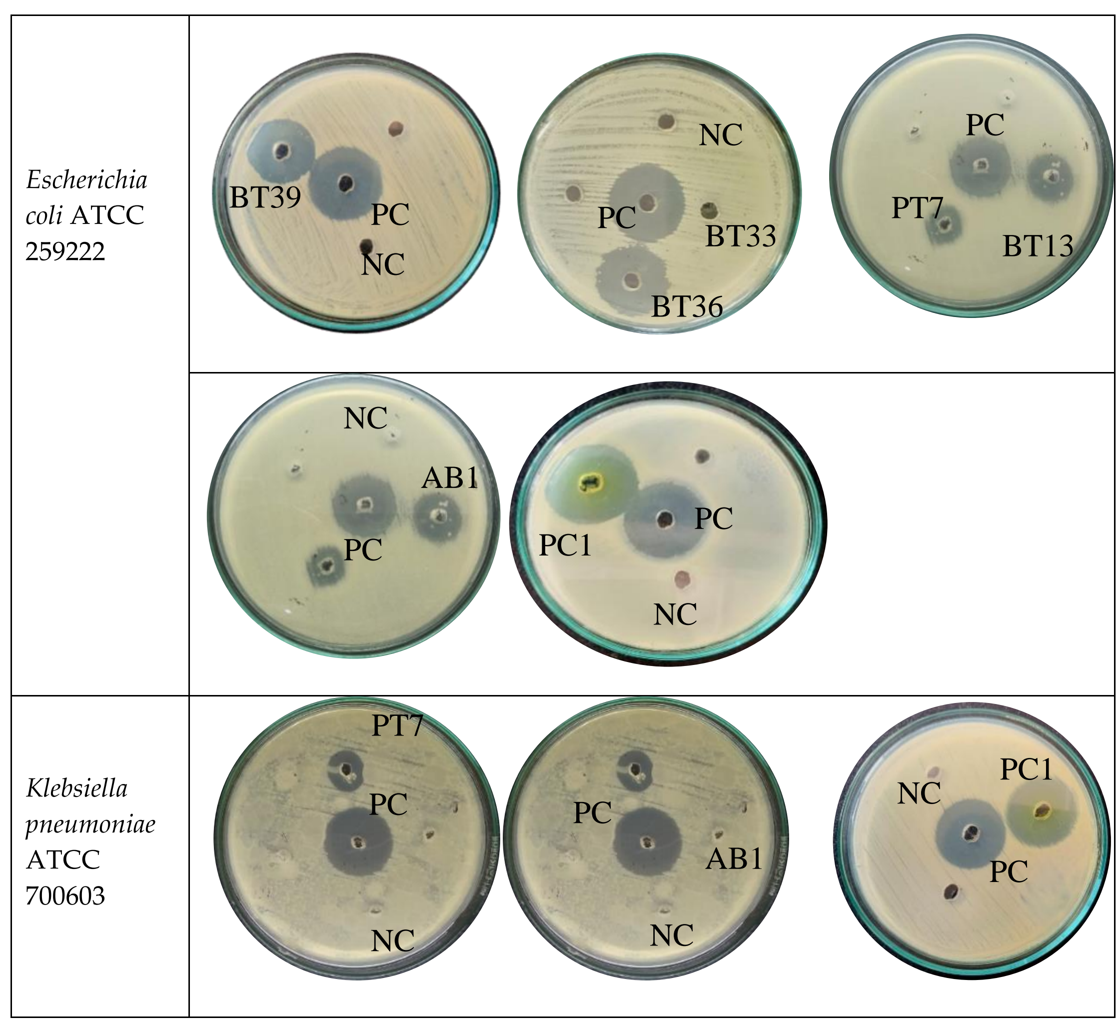
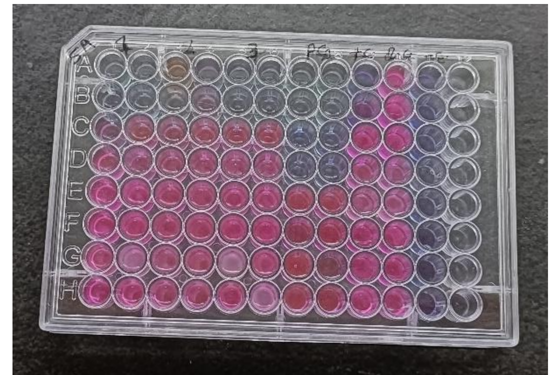
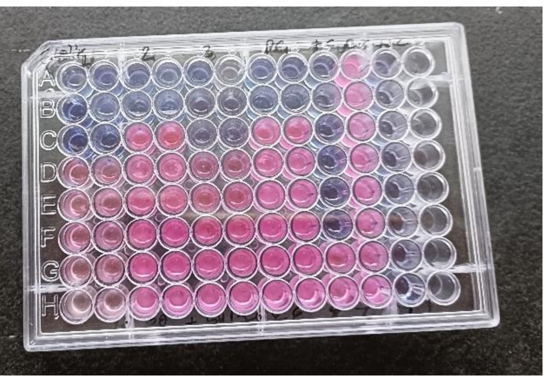


Figure E: MHA plates showing zone of inhibition

Appendix F: MIC of potent isolates



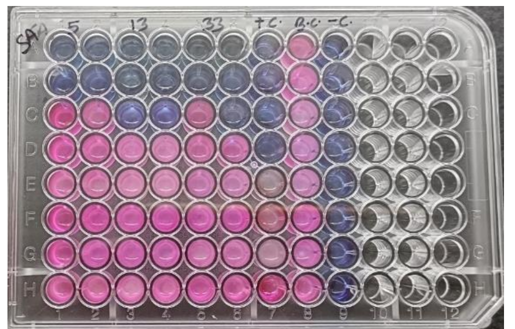
1st and 2nd column: BT1
3rd and 4th column: BT2
5th and 6th column: BT3
7th and 8th column: PC1
9th column: Positive control (Neomycin)
10th column: Bacterial control (*S.aureus*)
11th column: Negative control (MHB)



1st and 2nd column: BT1
3rd and 4th column: BT2
5th and 6th column: BT3
7th and 8th column: PC1
9th column: Positive control (Neomycin)
10th column: Bacterial control *(E. coli)*
11th column: Negative control (MHB)

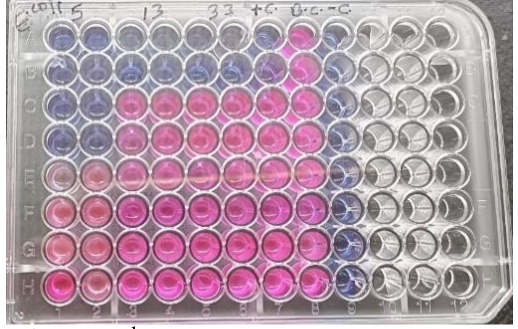
1st, 2nd, and 3rd column: PT7
4th, 5th, and 6th column: AB1
7th, 8th, and 9th column: other sample
10th column: Positive control (Neomycin)
11th column: Bacterial control (*S.aureus*)
12th column: Negative control (MHB)

1st, 2nd, and 3rd column: PT7
4th, 5th, and 6th column: AB1
7th, 8th, and 9th column: other sample
10th column: Positive control (Neomycin)
11th column: Bacterial control (*E. coli*)
12th column: Negative control (MHB)

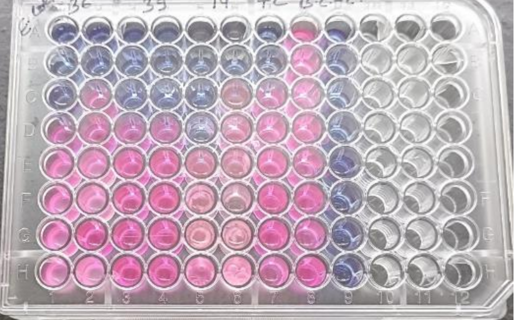


1st and 2nd column: BT5
3rd and 4th column: BT13
5 th and 6 th column: BT33
7th column: Positive control (Neomycin)
8th column: Bacterial control (*S. aureus*)
9th column: Negative control *(MHB)*

1st and 2nd column: BT36
3rd and 4th column: BT39
5 th and 6 th column: other sample
7th column: Positive control (Neomycin)
8th column: Bacterial control (*S. aureus*)
9th column: Negative control *(MHB)*



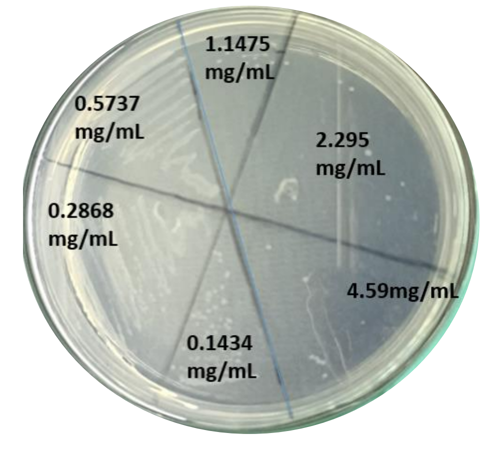
1st and 2nd column: BT5
3rd and 4th column: BT13
5 th and 6 th column: BT33
7th column: Positive control (Neomycin)
8th column: Bacterial control (*E. coli*)
9th column: Negative control *(MHB)*



1st and 2nd column: BT36
3rd and 4th column: BT39
5 th and 6 th column: other sample
7th column: Positive control (Neomycin)
8th column: Bacterial control (*E. coli*)
9th column: Negative control *(MHB)*

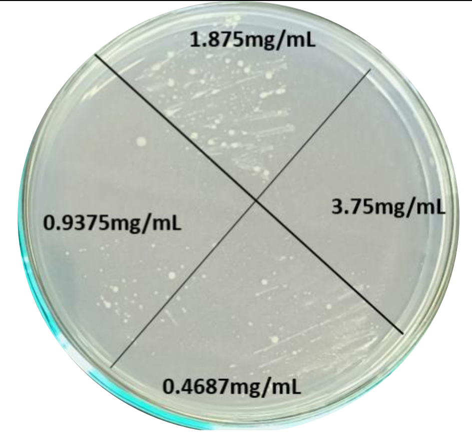
Figure F: MIC in 96 well plate

Appendix G: MBC of potent isolates

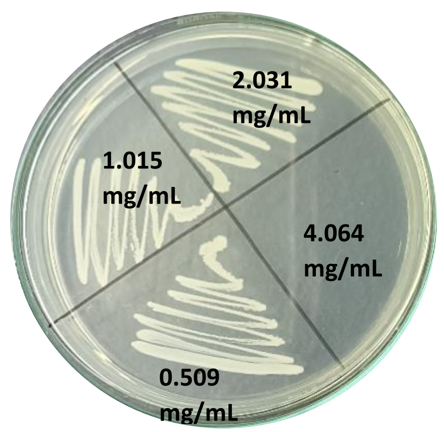


MBC of BT1 (4.59 mg/mL) in *S. aureus*

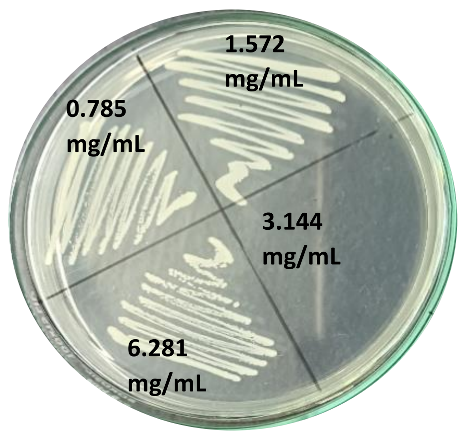
MBC of BT2 (3.92 mg/mL) in *S.aureus*



MBC of BT3 (3.75 mg/mL) in *S. aureus*

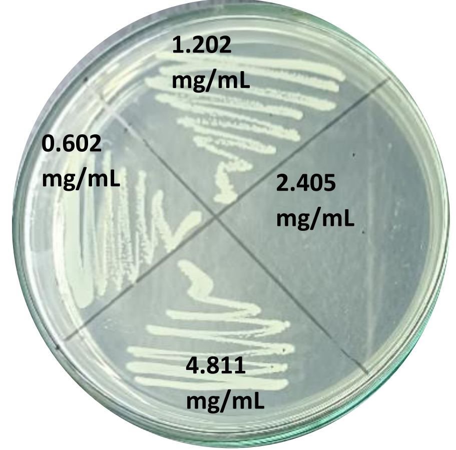


MBC of BT5 (4.064 mg/mL) in *S. aureus*

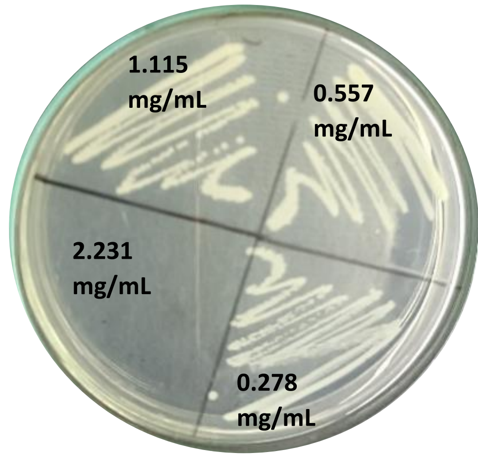


MBC of BT13 (3.144 mg/mL) in *S. aureus*

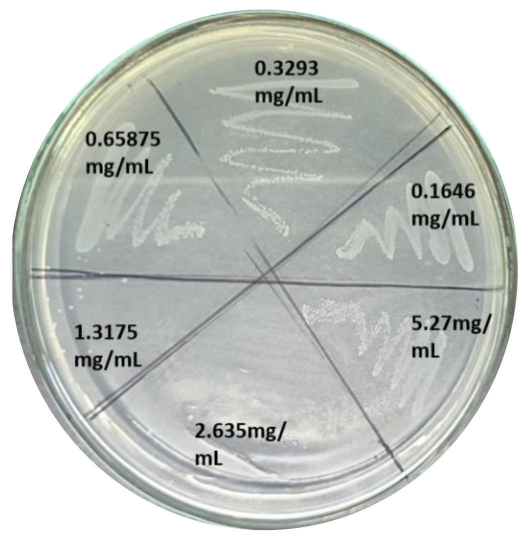
MBC of BT33 (3.917 mg/mL) in *S. aureus*



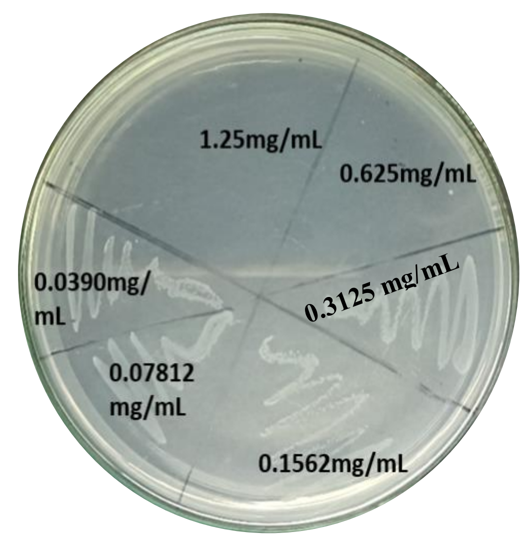
MBC of BT36 (2.405 mg/mL) in *S. aureus*



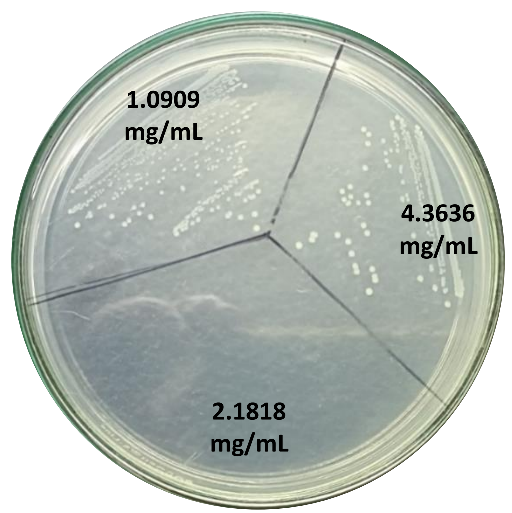
MBC of BT39 (2.231 mg/mL) is *S. aureus*



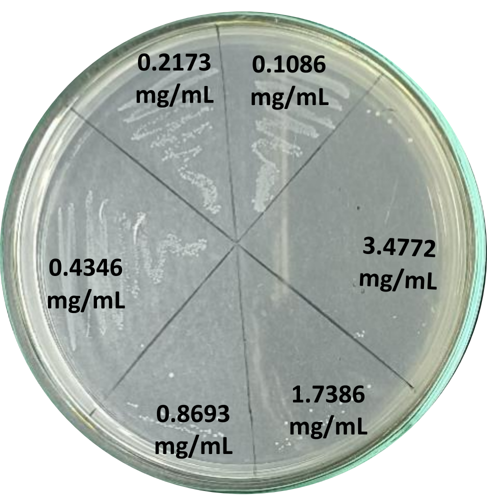
MBC of PC1 (2.635 mg/mL) in *S. aureus*



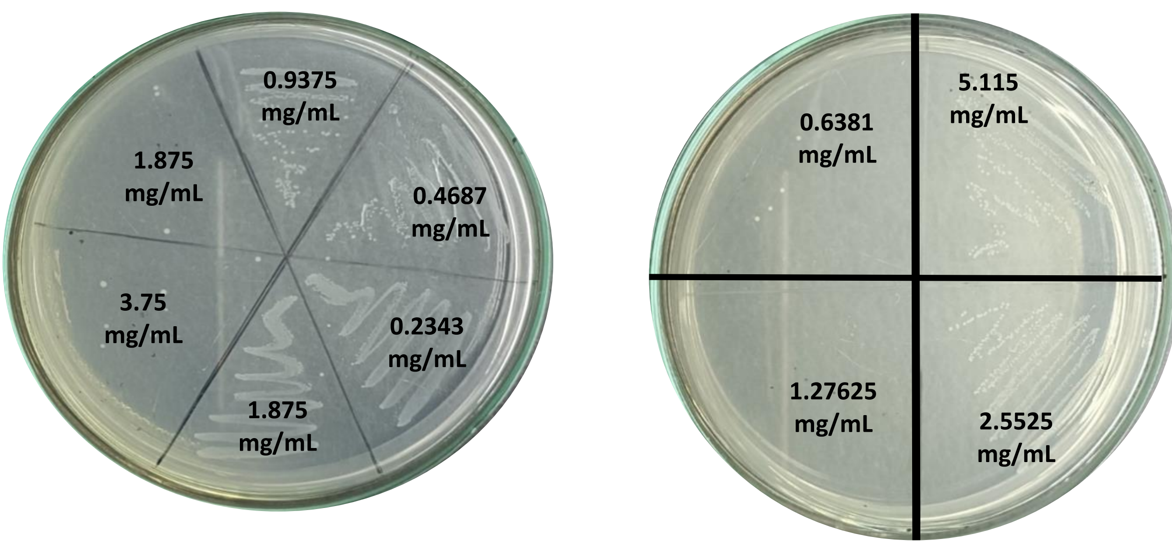
MBC of Neomycin (1.25 mg/mL) in *S. aureus*



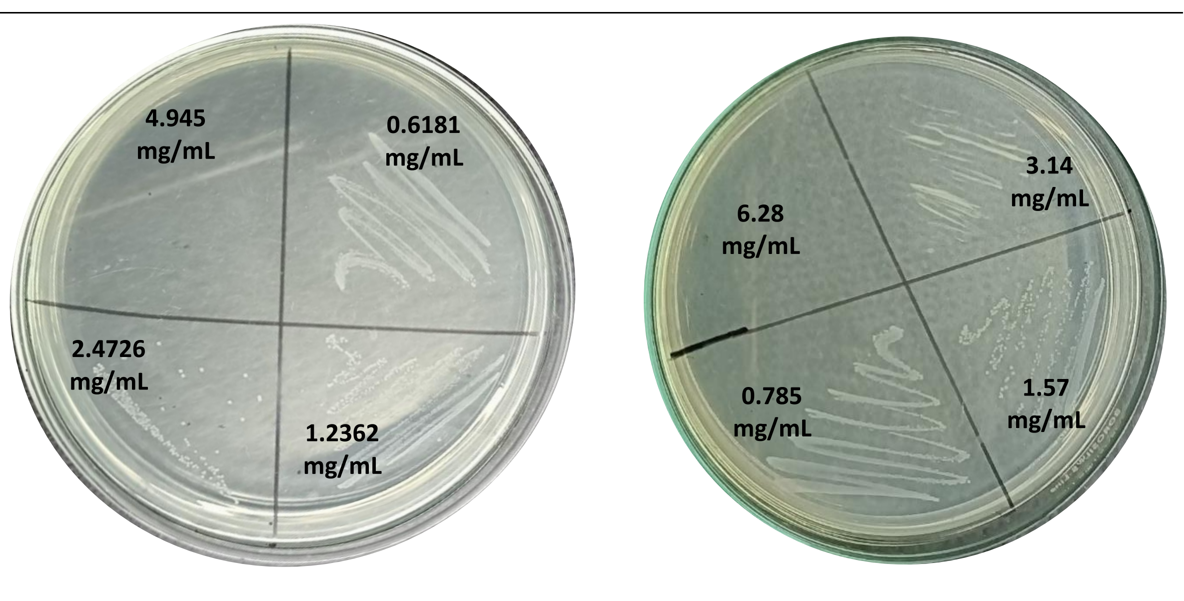
MBC of BT1 (2.1818 mg/mL) in *E. coli*



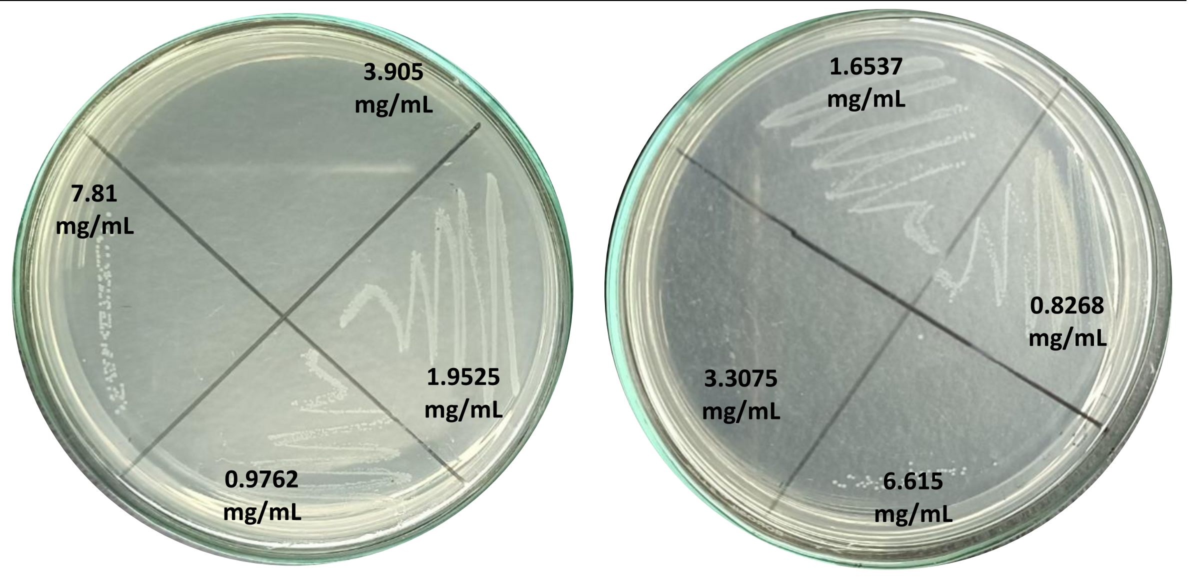
MBC of BT2 (3.4772 mg/mL) in *E. coli*



MBC of BT3 (1.875 mg/mL) in *E. coli* MBC of BT5 (1.27625 mg/mL in *E. coli*

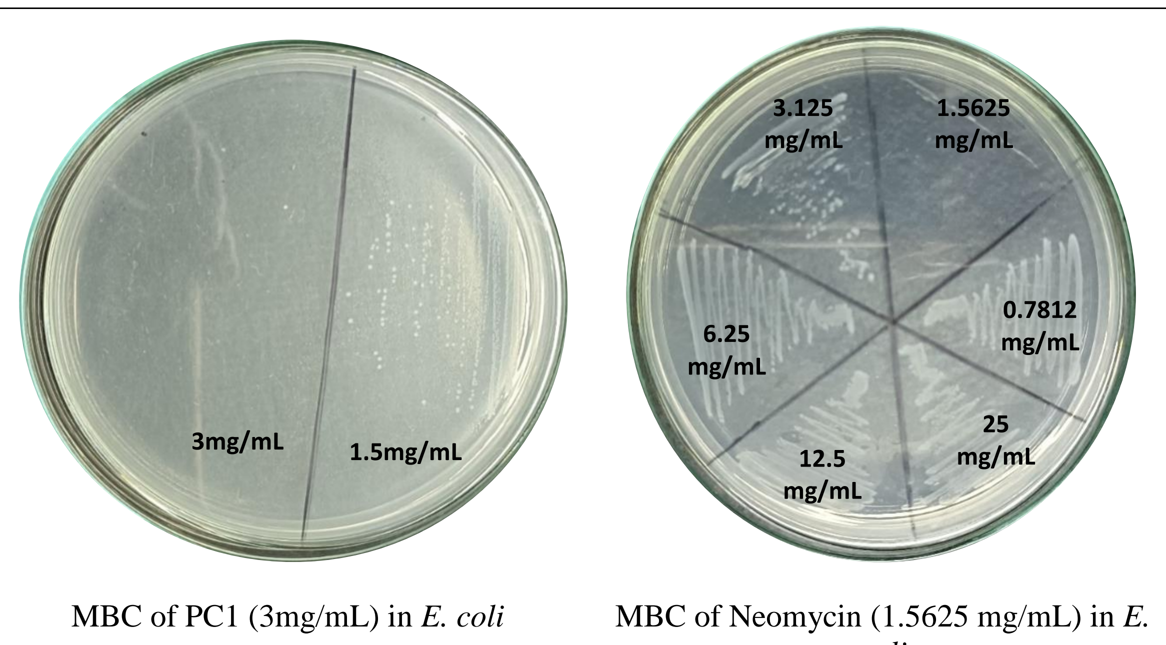


MBC of BT13 (4.945 mg/mL) in *E. coli* MBC of BT33 (6.28 mg/mL) in *E. coli*



MBC of BT36 (3.905 mg/mL) in *E. coli*

MBC of BT39 (3.3075 mg/mL) in *E. coli*



MBC of PC1 (3mg/mL) in *E. coli*

MBC of Neomycin (1.5625 mg/mL) in *E. coli*

Figure G: MBC in NA plate

Appendix H: Base peak chromatogram (BPC) and mass spectra (MS) of compounds obtained from LC-HRMS/MS

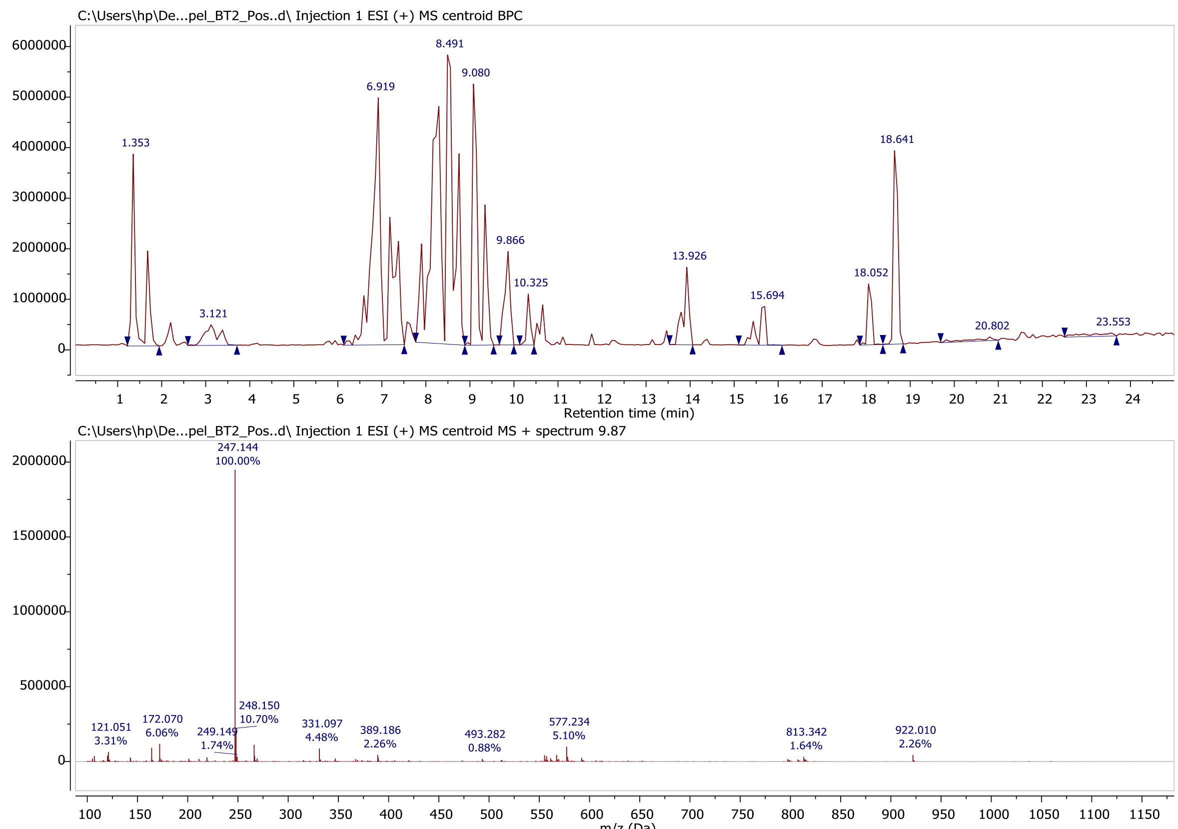


Figure H1. BPC and MS profile of Cyclo(L-valyl-L-phenyl alanyl).

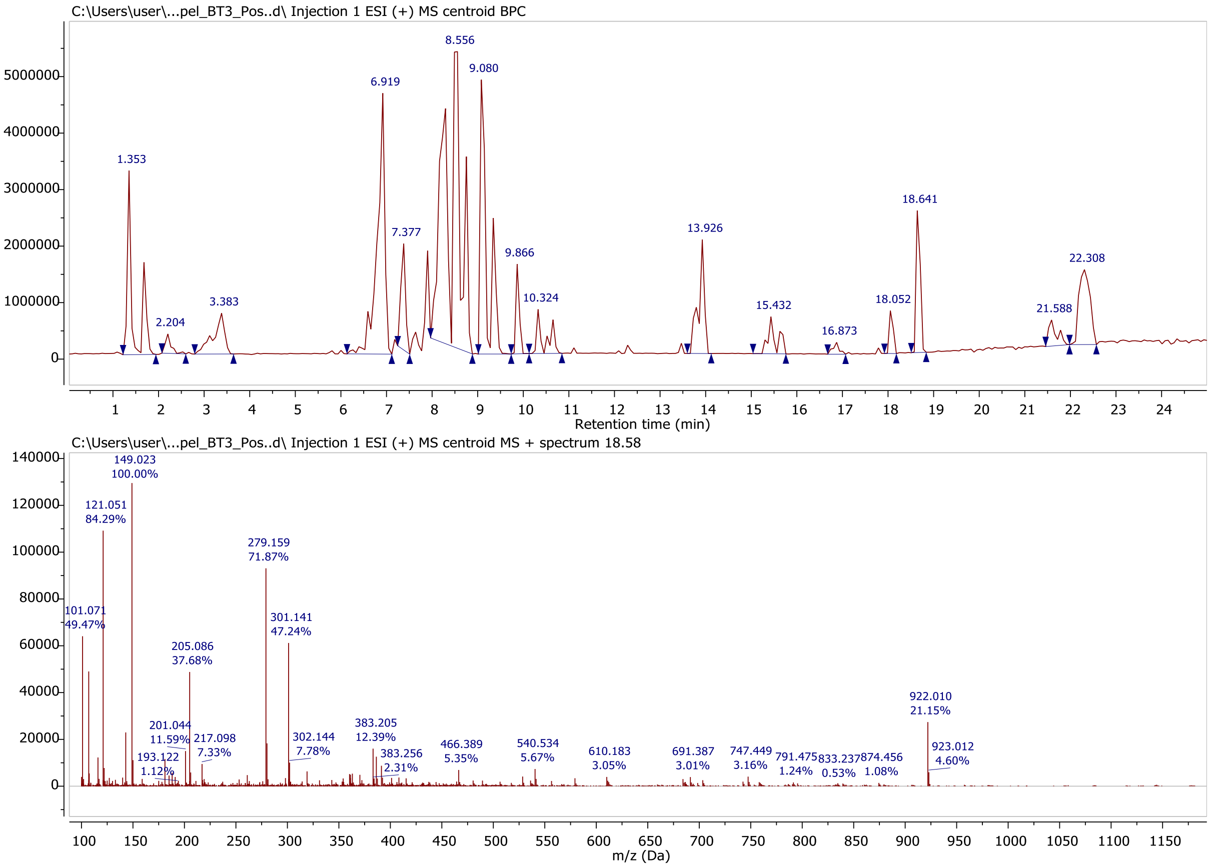


Figure H2. BPC and MS profile of di-n-butyl terephthalate.

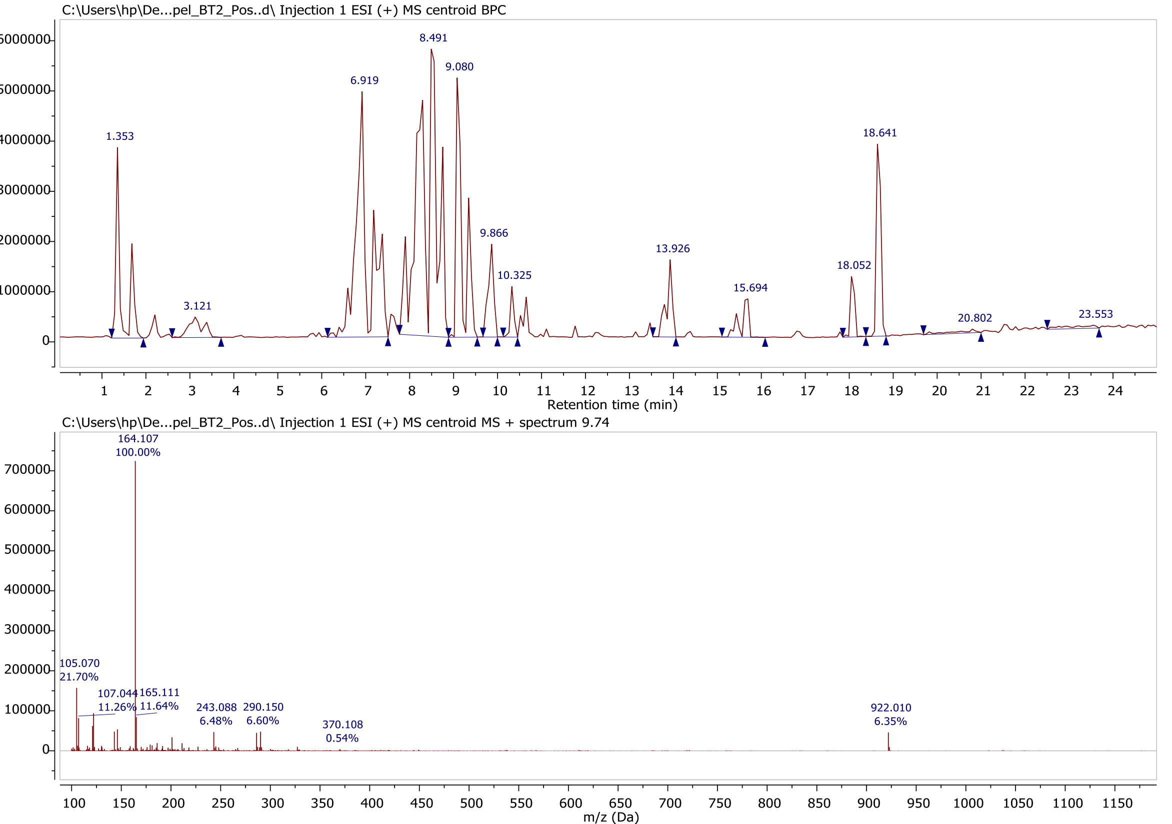


Figure H3. BPC and MS profile of N-phenethylacetamide

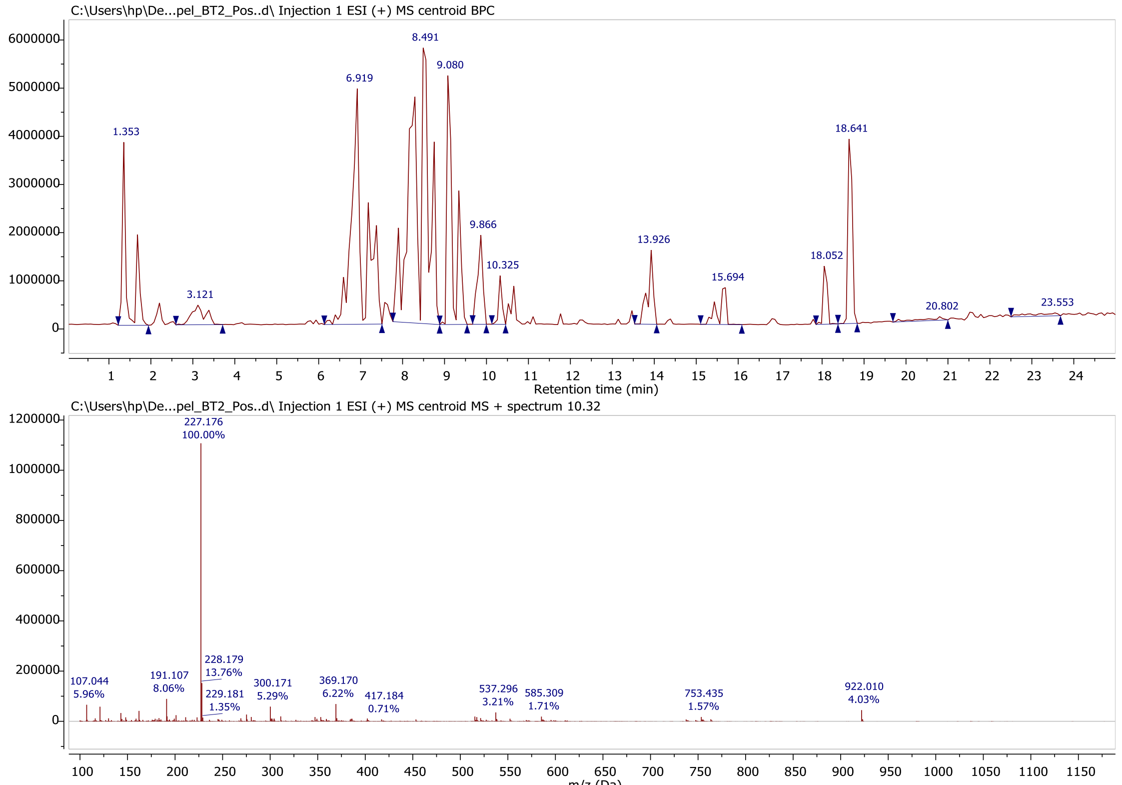


Figure H4. BPC and MS profile of Cyclo(L-leucyl-L-leucyl).

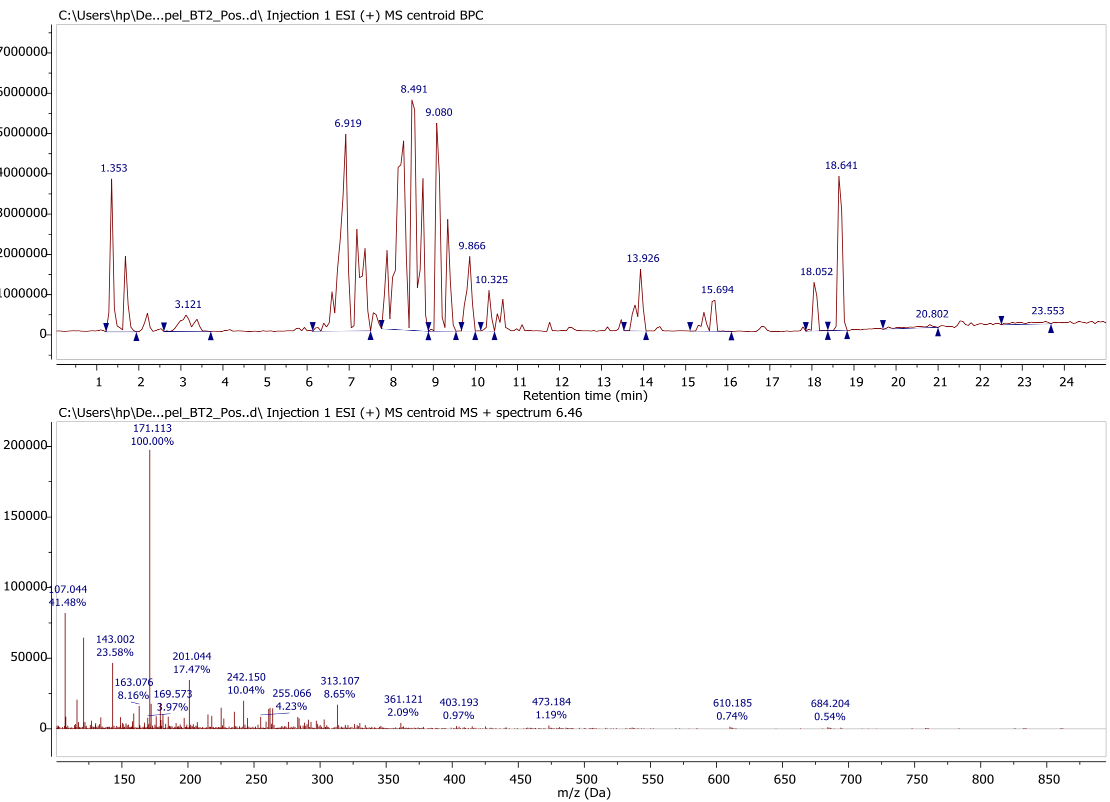


Figure H5. BPC and MS profile of (S)-3-isobutylpiperazine-2,5-dione.

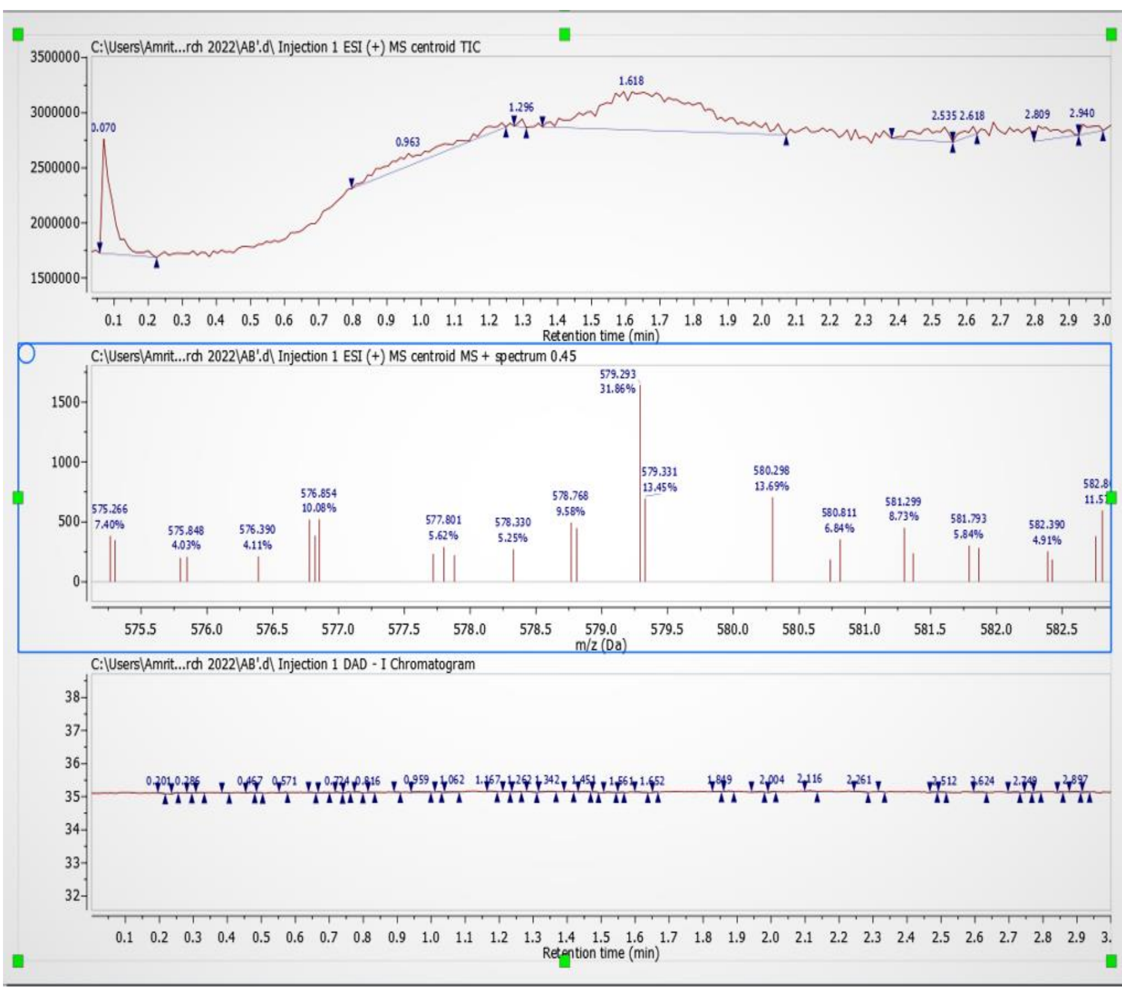


Figure H6. BPC and MS profile of (4R)-4,5-dihydro-4-hydroxygeldanamycin.

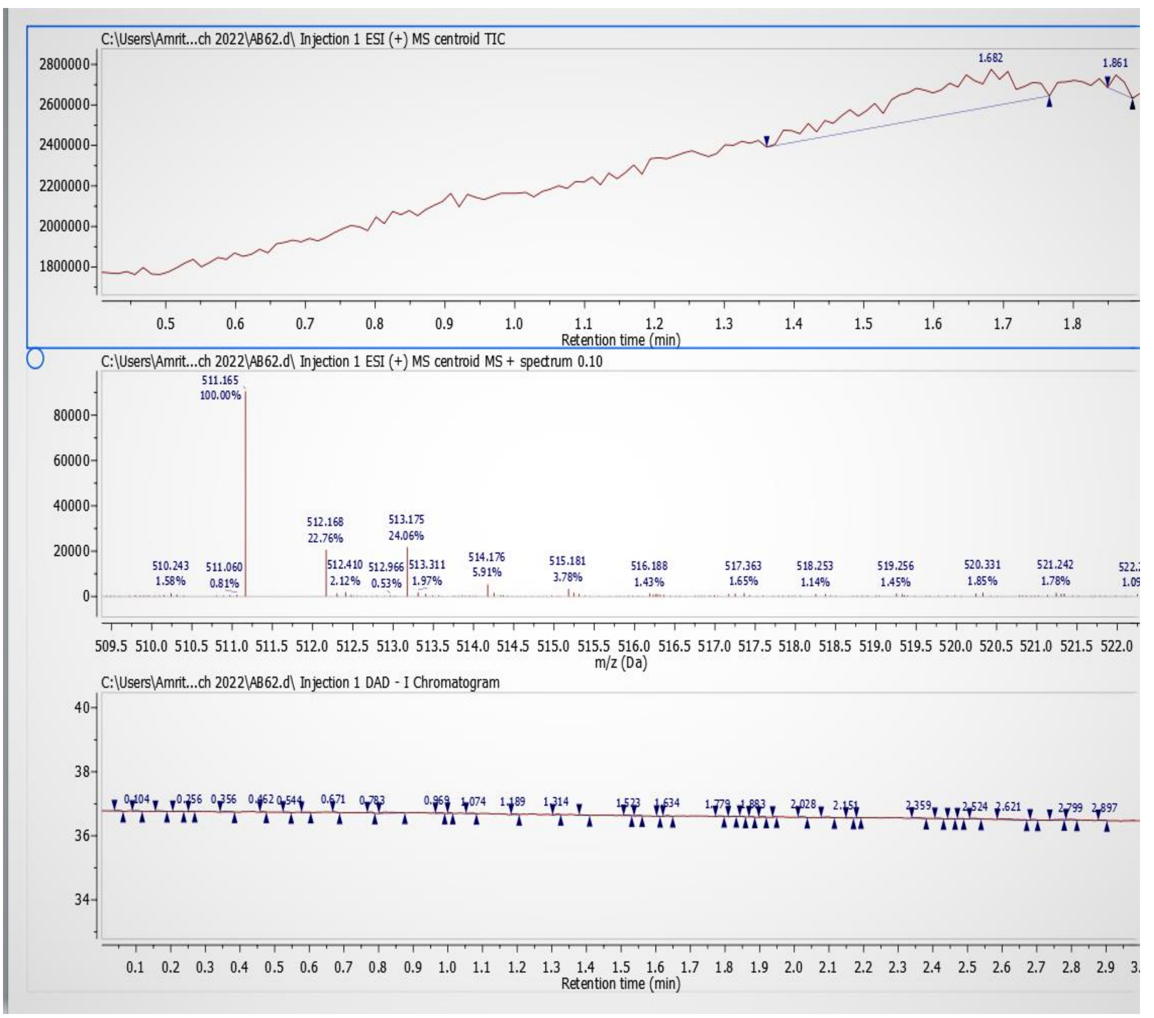
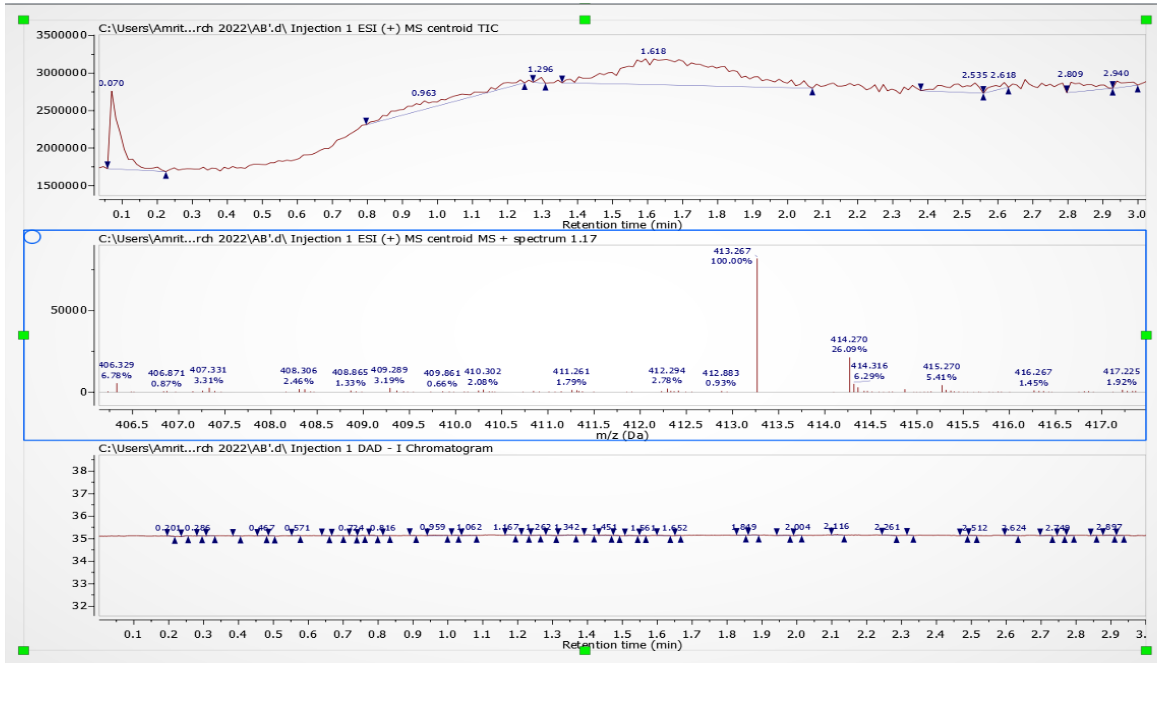


Figure H7. BPC and MS profile of Gilvocarcin A.



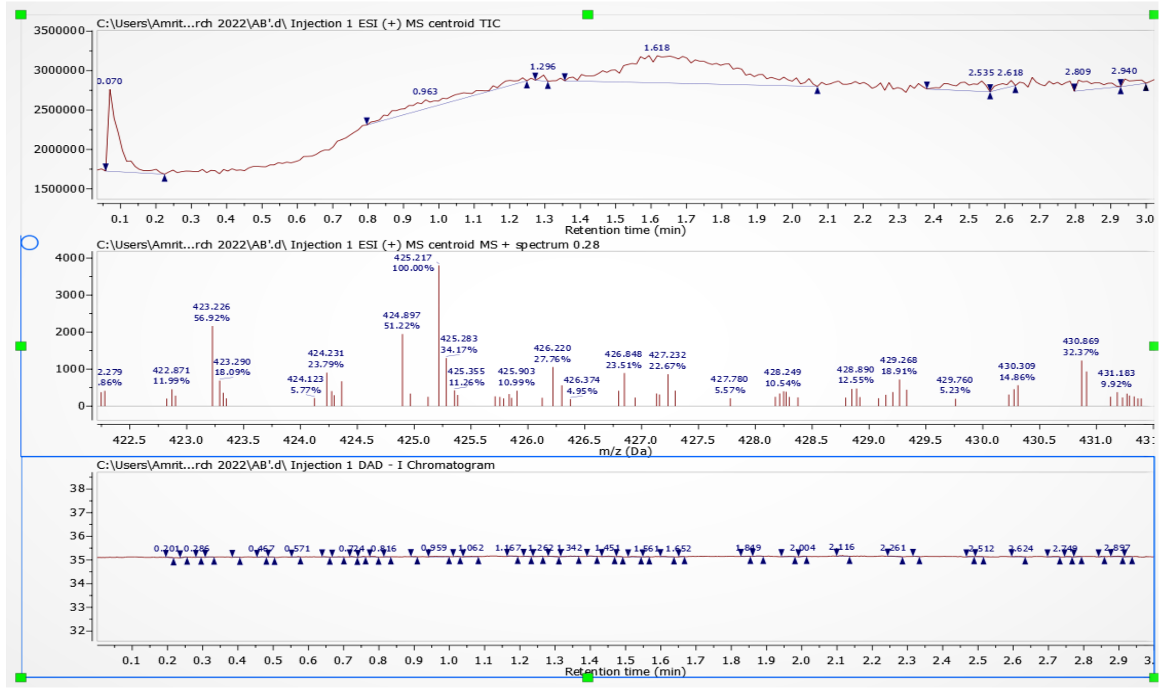


Figure H8. BPC and MS profile of Phoxalone and Janthinopolyenemycin B respectively.

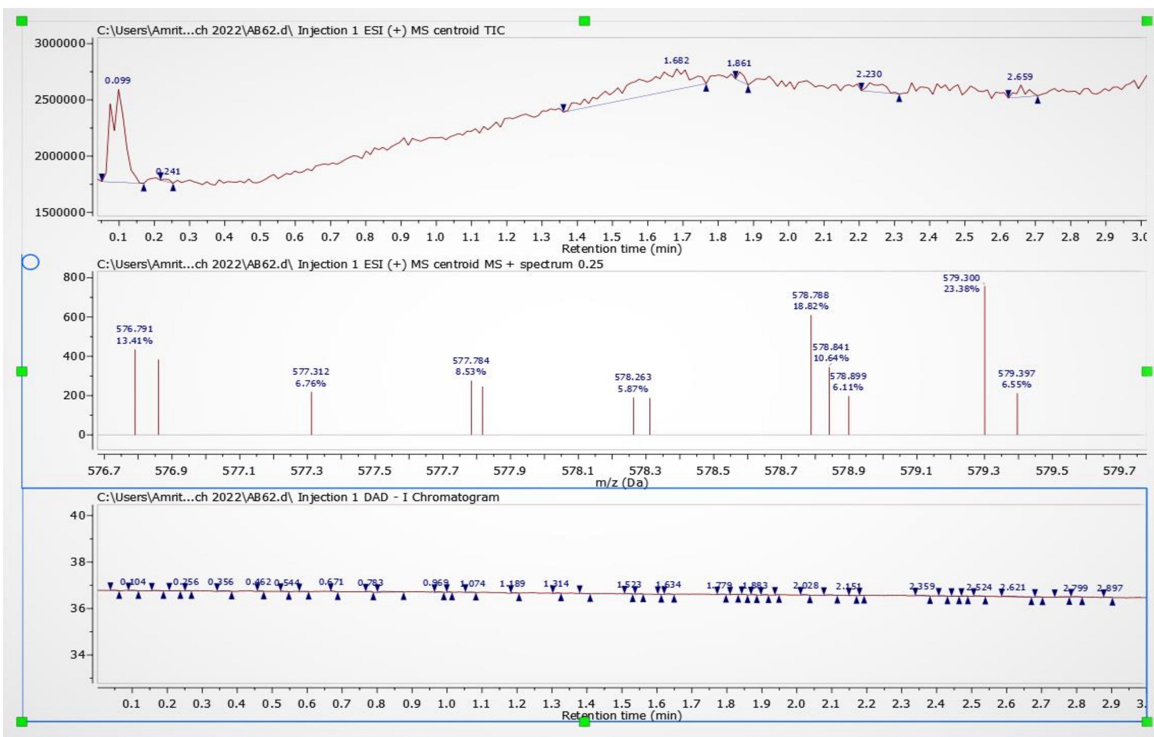


Figure H9. BPC and MS profile of Glucopiericidin A.

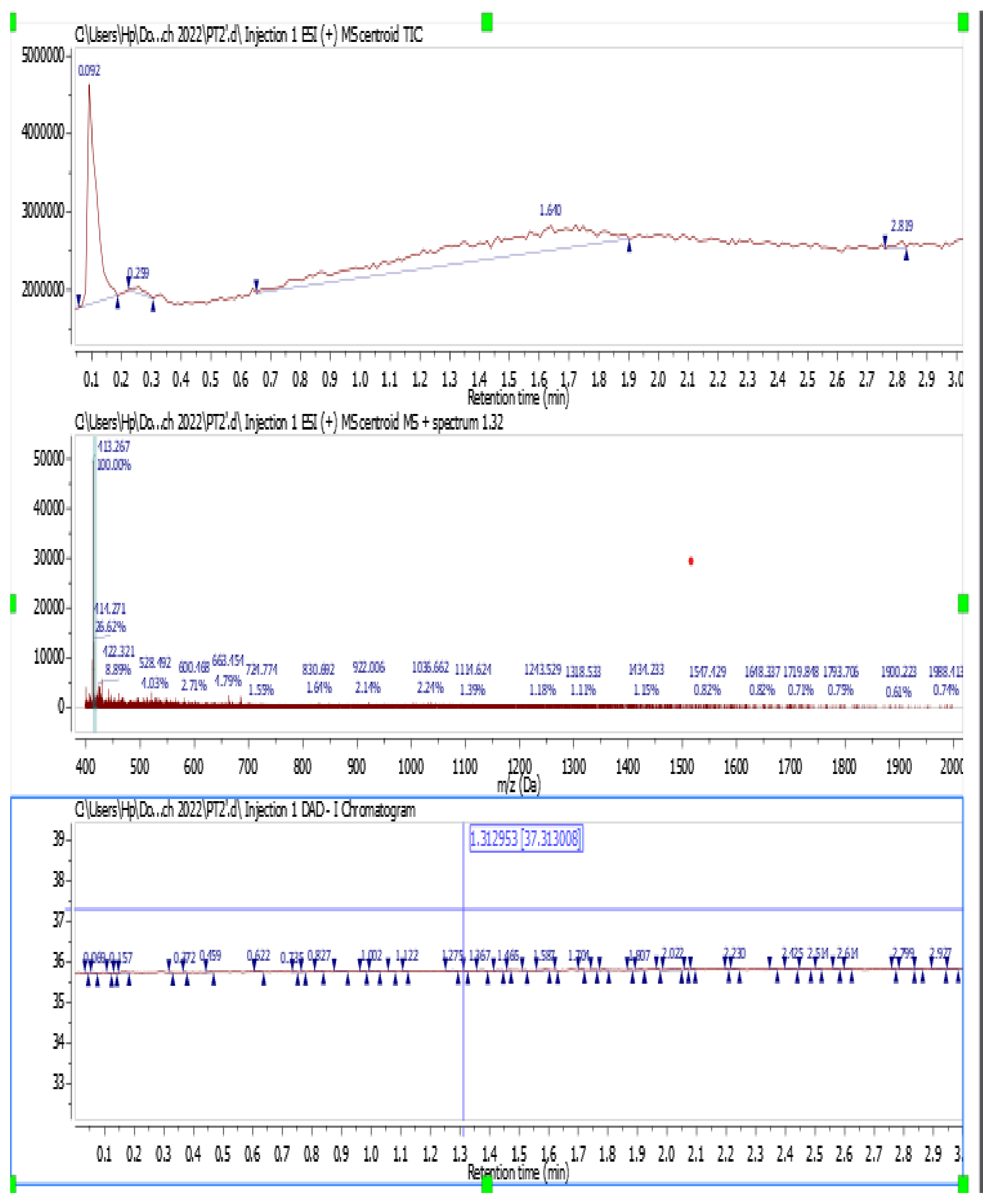


Figure H10. BPC and MS profile of Epopromycin A.

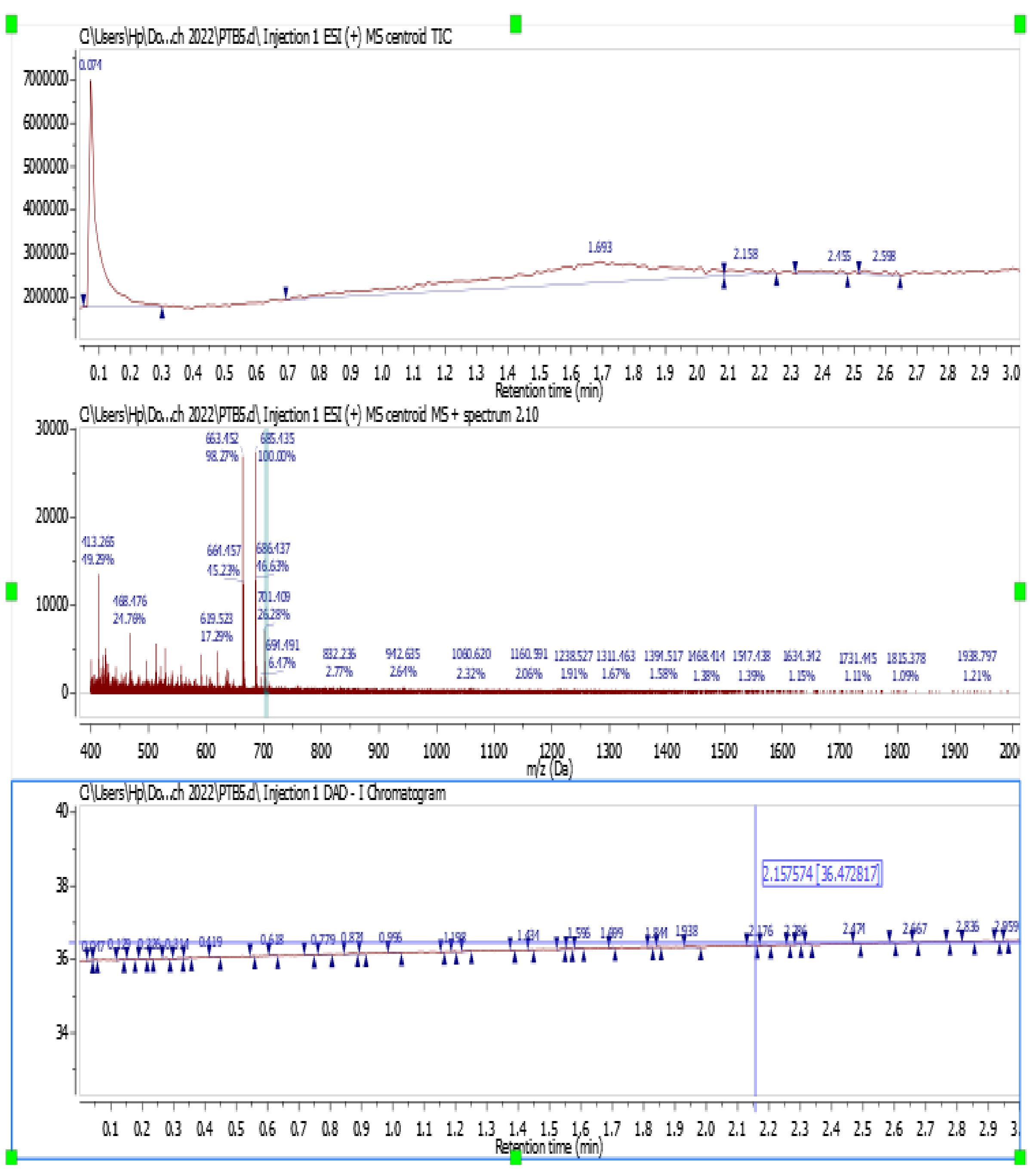


Figure H11. BPC and MS profile of Aldgamycin L.

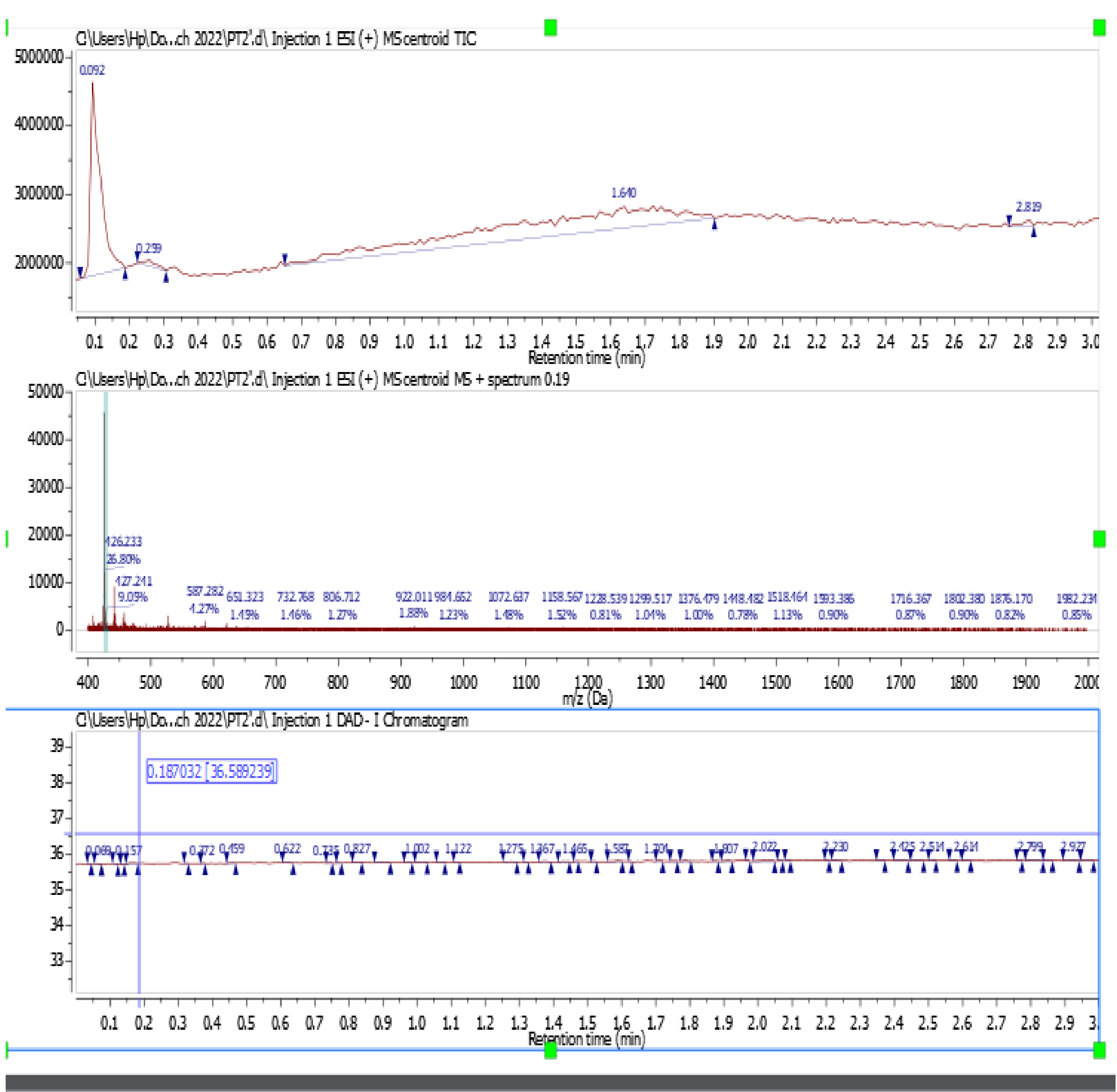


Figure H12. BPC and MS profile of SF2415B2.

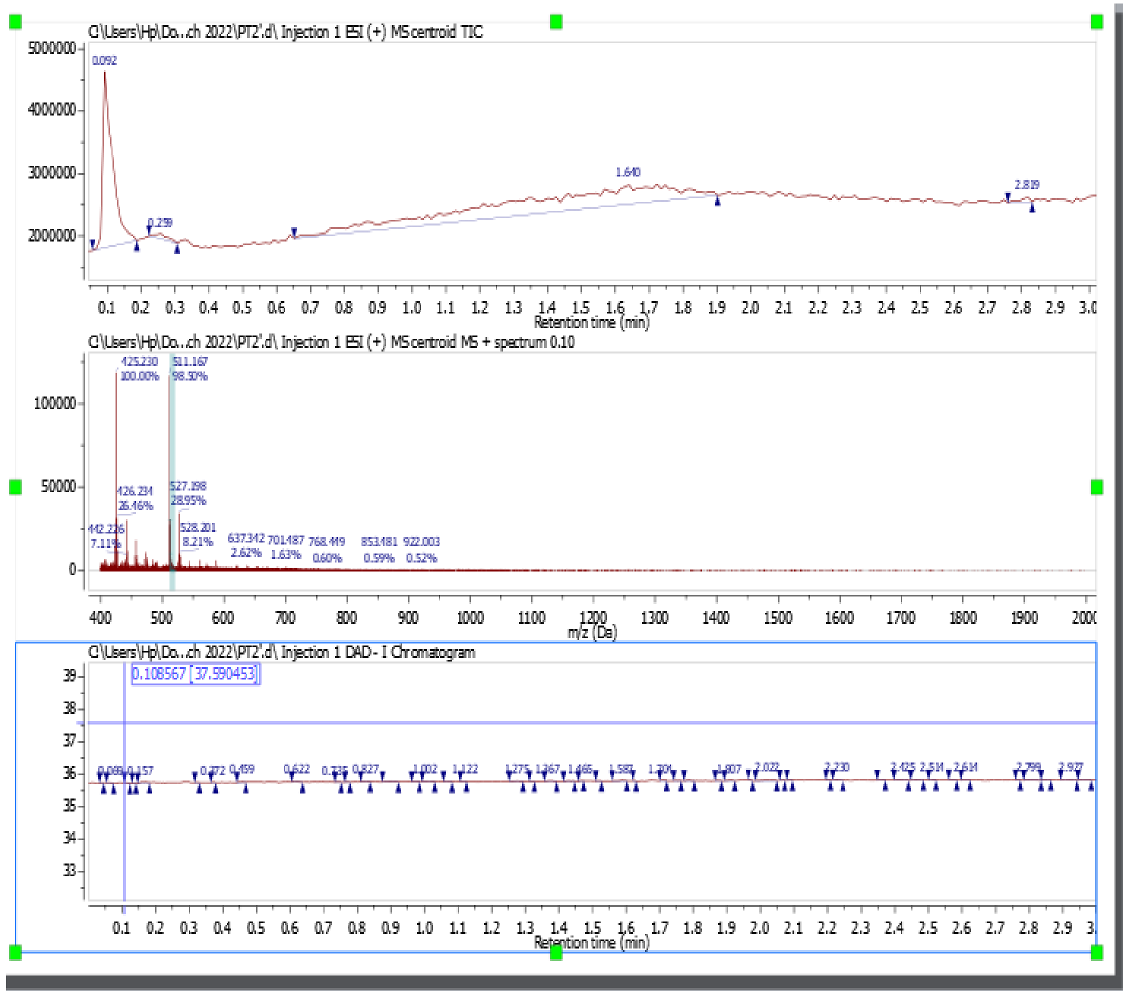


Figure H13. BPC and MS profile of Gilvocarcin HE.

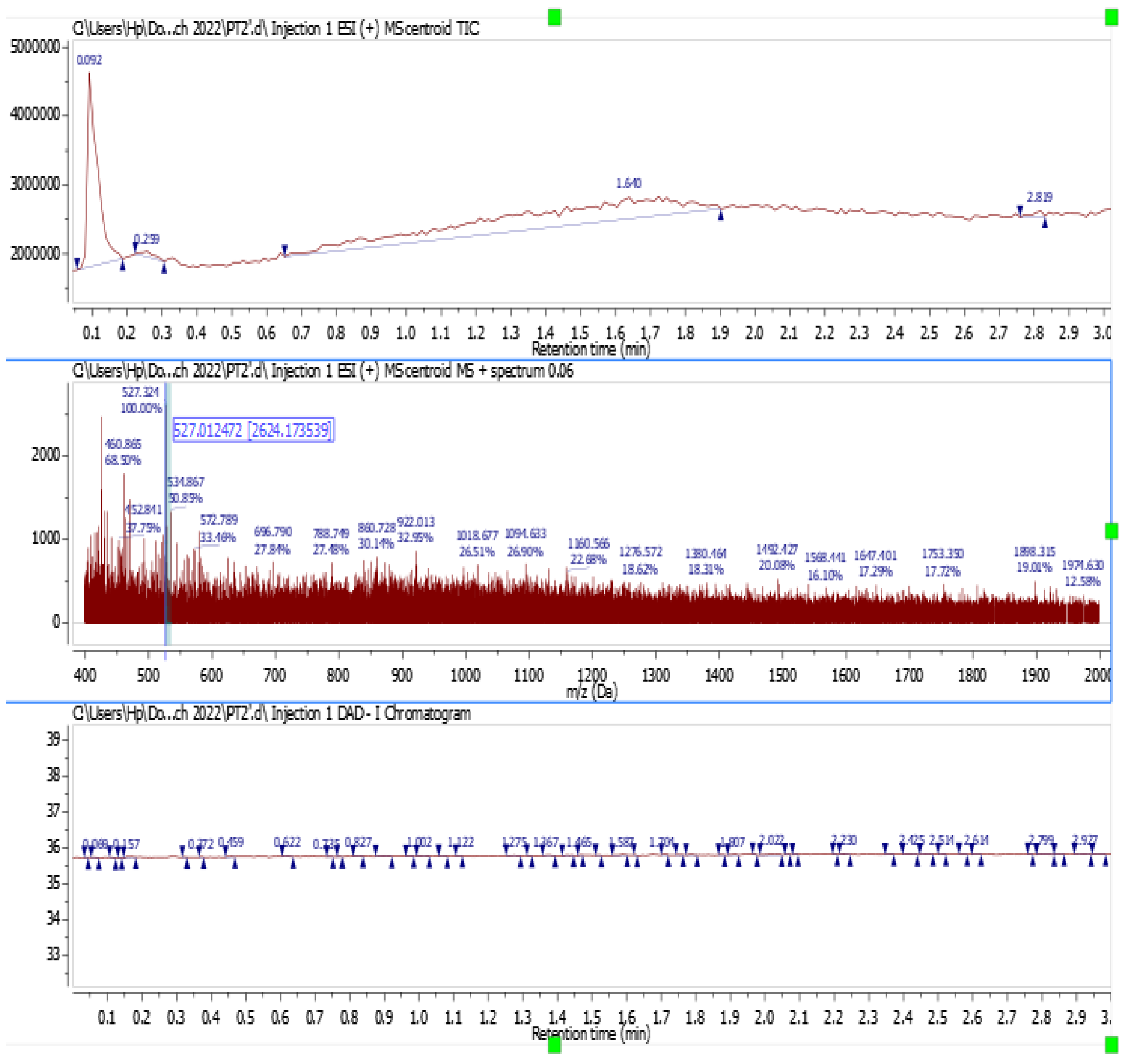


Figure H14. BPC and MS profile of compound Butyrolactol A.

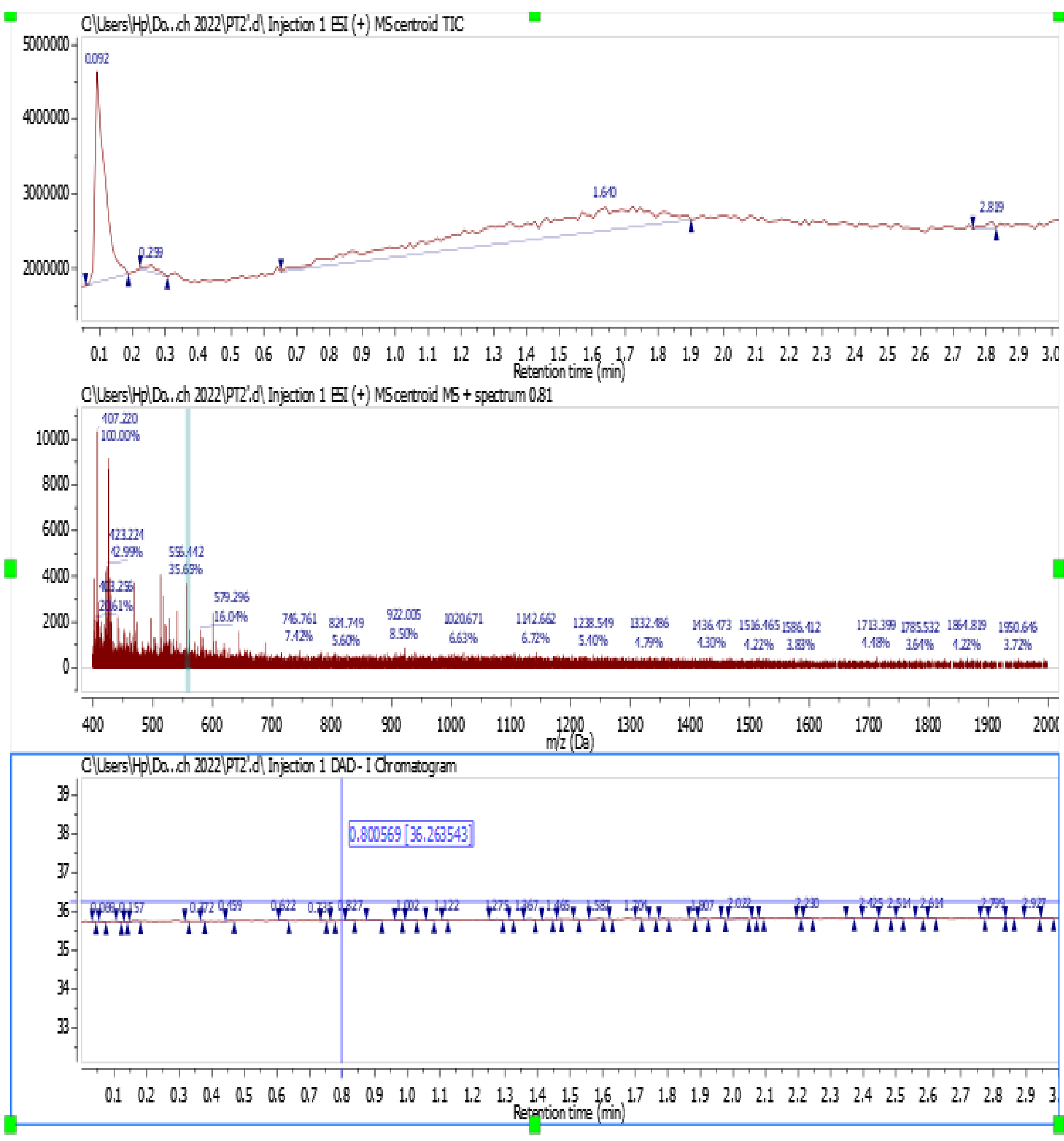


Figure H15. BPC and MS profile of Epoxomicin.

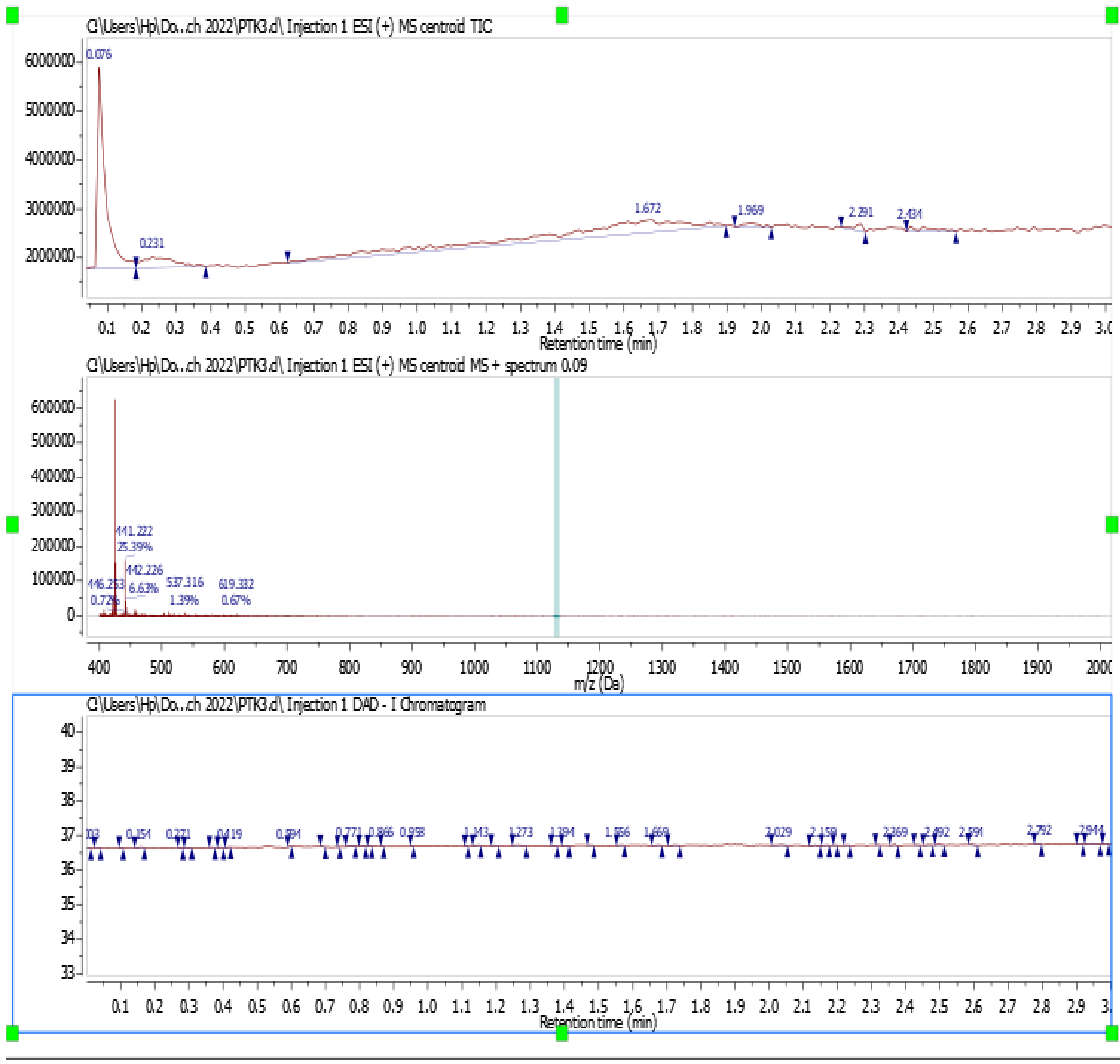


Figure H16. BPC and MS profile of Blasticidin H.

Appendix I: 16S rRNA sequence of identified *Streptomyces* sp.

>BT1 (Accession number: OR578351**)**

CTGCATGCGCTGCTACACATGCAAGTCGTACCAGTGAAGACCCGTTACAGGGGTGGATCAGTGGCGAAC
GGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGG
ATATGACCTTCCTCTGCATGTTGTCGGGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCA
GCTTGTTGGTGGGGTAACGGCCCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAG
CCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAG
CGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
TGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCCTGTCGCGTCGGATGTGAAAGC
CCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCC
TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTA
CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
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GGGAGTACCGCCGCAAGGCTAAAACTCACAGGAATTGACGGGGGCCCGCACAAACAGCGGAGCATGTGG
CTTAATTCGACGCAACGCGCAGAACCTTACCAAGGCTTGACATATGCCGGAGACACACAGACATGAGTG
CGCCCTTGTGGTCGGTGTACAGATGATGCATGGTTGTCGTCTGCTCGTGTCGTGAGATGTTGGGTTTAG
TCCTTCAGCGAAGACAACACTTCATCTGTGGTGGCGGCGTGCCCTTCCGGGCGATGGGGAGTGATGGGG
AACTAC

>BT2 (Accession number: PP106255**)**

CTACCTGCAGTCGACGATGAAGCCTTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAA
TCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACTCTGTCCCGCATGG
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GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT
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CATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTA
AAACTCAAAGGAATTGACAGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGA
AGAACCTTACCAAGGCTTGACATATACCAGGAAAGCATCACACCC

>BT3 (Accession number: OR905603**)**

GCTCCTCCACAGGGGTTGGGCCACCGGCTTCGGGTGTTACCGACTTTCGTGACGTGACGGGCGGTGTGT
ACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACGAGCAACTCCGACTTCATGGG
GTCGAGTTGCAGACCCCAATCCGAACTGAGACCGGCTTTTTGAGATTCGCTCCGCCTCGCGGCATCGCA

GCTCATTGTACCGGCCATTGTAGCACGTGTGCAGCCCAAGACATAAGGGGCATGATGACTTGACGTCGT
CCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCTGTGAGTCCCCATCACCCCGAAGGGCATGCTGGC
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CATGCACCACCTGTATACCGACCACAAGGGGGGCACCATCTCTGATGCTTTCCGGTATATGTCAAGCCT
TGGTAAGGTTCTTCGCGTTGCGTCGAATTAAGCCACATGCTCCGCTGCTTGTGCGGGCCCCCGTCAATT
CCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGAACTTAATGCGTTAGCTGCGGCACCGAC
GACGTGGAATGTCGCCAACACCTAGTTCCCAACGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT
TCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAATGGCCCAGAGATCCGCCTTCGCCACCGGTGTTCC
TCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCGATCTCCCCTACCACACTCTAGCTAGCCC
GTATCGAATGCAGACCCGGGGTTAAGCCCCGGGGCTTTCACATCCGACGTGACAAGCCGCCTACGAGCT
CTTTACGCCCNAATAATTCCGGACAACGCTTGGCGCCCTACGTATTACCGCGGCTGC

>PC1 (Accession number: OR577614**)**

TGCGCGACTACCATGCAGTCGAACGATGAAGCCTTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACA
CGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACTCTGT
CCCGCAAGGGACGGGGTTAAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGG
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TGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCC
GCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACG
CAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCCATTGTGG
TCGGTATACAGGTGGTGCATGGCTGTCGTCAGCCGTGTCGTGAGATGTTGGGTTTAAT

>**BT5 (Accession number:** PP379909**)**

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TGAATACGTTCCCCGGGCCTTGTATCATCACCCGCCCGTCAGCGTCACGAAAGGTCGGTAAGCACCCGA
AGACACGGTGGCCCAATCCCCTTGTGGGAGGCACGCTTCAACGTGAT

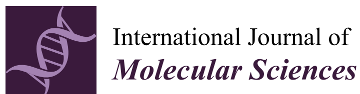
Appendix J: Scientific publications and Conferences/ Seminar participation.

Appendix J1: List of published articles

- **Thapa, B.B**., Huo, C., Budhathoki, R., Chaudhary, P., Joshi, S., Poudel, P.B., Magar, R.T., Parajuli, N., Kim, K.H., & Sohng, J.K. (2024). Metabolic comparison and molecular networking of antimicrobials in streptomyces species. *Int. J. Mol. Sci.*, **25**: 4193. https://doi.org/10.3390/ijms25084193
- **Thapa, B. B.** , Shakya, S., Shrestha, N., Yadav, R. P. ., Sharma, K. R. ., Mishra, A. D. ., Basnyat, R. C. ., & Parajuli, N. . (2024). Identification of secondary metabolites from actinomycetes isolated from the hilly region of Nepal. *Prithvi Academic Journal*, **7**: 20–40. https://doi.org/10.3126/paj.v7i1.65745
- Dawadi, S., Thapa, R., Modi, B., Bhandari, S., Timilsina, A.P., Yadav, R.P., Aryal, B., Gautam, S., Sharma, P., **Thapa, B.B.**, *et al.* (2021). Technological advancements for the detection of antibiotics in food products. *Processes*, **9**: 1500. https://doi.org/10.3390/pr9091500.
- Bhandari, S., Bhattarai, B.R., Adhikari, A., Aryal, B., Shrestha, A., Aryal, N., Lamichhane, U., Thapa, R., **Thapa, B.B.,** Yadav, R.P., *et al.* (2022). Characterization of streptomyces species and validation of antimicrobial activity of their metabolites through molecular docking. *Processes*, **10**: 2149. https://doi.org/10.3390/pr10102149.

Appendix J2: Participation in Conferences/ Seminar

- **International Conference on Applied Science and Engineering in a Changing World (ICASECW-24)** held on June 11-13, 2024 at Hotel Grande, Pokhara, Nepal (As an oral presenter).
- **Ph.D. Festival 2023** held on 9-10 October, 2023 (22-23 Ashoj 2080) in University Campus, Kritipur, Nepal (As a poster presenter).
- **First National Biotechnology Conference 2023** held on May 11-12, 2023 at Kathmandu University (As a poster presenter).
- **Second Research Project Dissemination Seminar** held at the Central Department of Chemistry, Tribhuvan University on December 27, 2022 (As an oral presenter).
- **Biological Chemistry Research Seminar** organized by UGC Supported Research Project, Central Department of Chemistry, 2022, Tribhuvan University (As an oral presenter).
- **Eighth Graduate Conference 2022** held in Mid-West University, Birendranagar, Surkhet, Nepal on April 4-5, 2022, organized by Himalayan Knowledge Conclave (As an oral presenter).



Article

Metabolic Comparison and Molecular Networking of Antimicrobials in *Streptomyces* Species

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Abstract: *Streptomyces* are well-known for producing bioactive secondary metabolites, with numerous antimicrobials essential to fight against infectious diseases. Globally, multidrug-resistant (MDR) microorganisms significantly challenge human and veterinary diseases. To tackle this issue, there is an urgent need for alternative antimicrobials. In the search for potent agents, we have isolated four *Streptomyces* species PC1, BT1, BT2, and BT3 from soils collected from various geographical regions of the Himalayan country Nepal, which were then identified based on morphology and 16S rRNA gene sequencing. The relationship of soil microbes with different *Streptomyces* species has been shown in phylogenetic trees. Antimicrobial potency of isolates was carried out against *Staphylococcus aureus* American Type Culture Collection (ATCC) 43300, *Shigella sonnei* ATCC 25931, *Salmonella typhi* ATCC 14028, *Klebsiella pneumoniae* ATCC 700603, and *Escherichia coli* ATCC 25922. Among them, *Streptomyces* species PC1 showed the highest zone of inhibition against tested pathogens. Furthermore, ethyl acetate extracts of shake flask fermentation of these *Streptomyces* strains were subjected to liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis for their metabolic comparison and Global Natural Products Social Molecular Networking (GNPS) web-based molecular networking. We found very similar metabolite composition in four strains, despite their geographical variation. In addition, we have identified thirty-seven metabolites using LC-MS/MS analysis, with the majority belonging to the diketopiperazine class. Among these, to the best of our knowledge, four metabolites, namely cyclo-(Ile-Ser), 2-n-hexyl-5-n-propylresorcinol, 3-[(6-methylpyrazin-2-yl) methyl]-1H-indole, and cyclo-(D-Leu-L-Trp), were detected for the first time in *Streptomyces* species. Besides these, other 23 metabolites including surfactin B, surfactin C, surfactin D, and valinomycin were identified with the help of GNPS-based molecular networking.

Keywords: antibiotics; microbial natural products; metabolomics; mass spectrometry

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1. Introduction

Pathogens, which are causes of infectious diseases, acquire resistance to commonly used antibiotics due to excessive and improper application, leading to a growing necessity to combat the escalating issue of drug resistance. In 2015, the World Health Organization (WHO) officially recognized a worldwide health crisis, stating that 700,000 deaths occur annually due to infections caused by MDR. The WHO also projected a staggering 10 million deaths by the year 2050 if the issue is not effectively addressed [1].

For that reason, researchers around the world are currently focusing on the search for potent antimicrobials from natural sources with the help of different tools and techniques. Currently, metabolomics is emerging as a promising avenue for the analysis of the metabolites present in extracts under definite conditions and can facilitate and speed up the search for alternative bioactive molecules [2]. Metabolomics based on liquid chromatography-high resolution mass spectrometry (LC-HRMS) is an effective analytical approach for evaluating mixtures of metabolites in a biological system to aid in the dereplication and discovery of novel natural products [3]. In addition, a more recent approach in drug discovery programs is molecular networking, a tandem mass spectrometry (MS/MS) data organizational approach. It has been demonstrated that molecular networking can be used to analyze complex MS/MS data utilizing GNPS web-based platform, which can aid in the identification of various compounds because of the network formed due to similarity in collision-induced dissociation-tandem mass spectrometry (CID-MS/MS) fragmentation pattern [4]. This, in turn, contributes to the identification of precursor ions or molecules towards the discovery of lead compounds.

Streptomyces, the largest genus within the phylum *Actinomycetota*, is renowned for being a major source of a broad spectrum of bioactive secondary metabolites including antibiotics, anticancer and antiviral agents, and various enzymes [5]. These are usually isolated from the soil and marine environment using a variety of growth media, such as a series of International *Streptomyces* Project (ISP) media, casein starch agar (CSA) media, chitin agar, and starch nitrate agar. Earlier studies have reported that the genus *Streptomyces* has contributed to the production of more than 74% of the antibiotics currently available such as streptomycin, tetracycline, chloramphenicol, erythromycin, neomycin, nystatin, etc. [6,7].

The secondary metabolites produced from the *Streptomyces* species were mostly well known for their structural diversity and novelty. The search for novel lead compounds from conventional soil-isolated *Streptomyces* has experienced a downturn due to the rediscovery of the same or known compounds [8]. Despite this fact, some researchers have been reporting novel lead compounds from soil-derived *Streptomyces* species. Yang et al. isolated and identified eight new fasamycin-type antibiotics and streptovertimycins A–H from *Streptomyces morookaense* strain derived from soil [9]. Two new cyclic thiopeptides, geninthiocins E and F with antiviral activities were isolated from soil-derived *Streptomyces* sp. CPCC 200267 by Fang et al. [10]. Similarly, dipimprinine E and dipimprinine F two alkaloids showing significant anticancer activity were isolated from soil-derived *Streptomyces* sp. 44414B [11]. Picolinamycin, a novel antibiotic showing antibacterial activity against multidrug resistant bacterial strains was isolated from soil-derived *Streptomyces* sp. SM01 [12]. Moreover, Hu et al. isolated and identified two new phenazines with antimicrobial activity; 6-hydroxyphenazine-1-carboxamide and methyl 6-carbamoylphenazine-1-carboxylate from soil-derived *Streptomyces* species [13]. In addition, a novel pranonaphthoquinone antibiotic, xiakemycin A, was isolated from *Streptomyces* sp. CC8-201 [14]. Thus, the above evidence indicates that there are still chances of finding new bioactive molecules from soil microbes.

Nepal's varied ecological landscape spans a spectrum of climatic zones, stretching from tropical conditions in the lowlands to alpine environments in the lofty Himalayan peaks. This broad diversity of habitats provides a range of niches for microbial communities, including the genus *Streptomyces*. The capacity of *Streptomyces* species to generate a variety of physiologically active secondary metabolites, which are very powerful against microbial pathogens, makes them one of the most influential groups of bacteria [15]. In this study, we have collected four soil samples from different niches of Nepal with varying altitudes to isolate *Streptomyces* species. The primary goal of this study is to explore and analyze the potent antimicrobial metabolites present in *Streptomyces* isolated from soil by tandem mass spectrometry and GNPS-based molecular networking.

2. Results

2.1. Isolation of Streptomyces Species

Four soil samples PC1, BT1, BT2, and BT3 were collected from different ecosystems in Nepal, ranging from 1010 m to 2743 m above sea level (Table S1). As suggested by Bergey's Manual of Systematic Bacteriology, bacterial strains PC1, BT1, BT2, and BT3 were initially identified based on biochemical assays, morphology, growth pattern, and Gram staining [16]. They were grown on ISP4 media, and their secondary metabolites harvesting was determined by the growth curve, which varied in individual bacterial strains. The color of aerial mycelia of the isolates BT1, BT2, and BT3 were greyish-white in appearance, while isolate PC1 was found to be whitish, which is presented in Figure S1. The Gram-staining revealed that the isolates were composed of hair-like mycelium and flagellated Gram-positive bacteria. We isolated more than twenty actinomycetes strains from each soil sample PC1, BT1, BT2, and BT3, respectively, but most of them were discarded based on their similar morphological characters, antimicrobial properties, and molecular sequencing. Finally, only one peculiar actinomycetes strain was selected from each soil sample. Further research on those soils may lead to the isolation of additional actinomycetes in the future.

2.2. PCR of 16S rRNA and Molecular Sequencing

About 1.5 kb 16S rRNA gene was amplified using universal oligonucleotides (27F and 1492R) from the genomic DNA of soil microbes (PC1, BT1, BT2, and BT3). Then, it was subjected to molecular sequencing as methods described earlier. The homology search using the BLAST tool in NCBI for the 16S rRNA gene sequences of these actinomycetes revealed that all their sequences exhibited high similarity (greater than 99.0%) with several sequences of *Streptomyces* species in the GenBank. Their 16S rRNA gene sequences were deposited in the GenBank (PC1, accession number OR577614; BT1, accession number OR578351; BT2, accession number PP106255; BT3, accession number OR905603). Multiple sequence alignment was performed for our sequences with twelve 16S rRNA genes showing the highest sequence similarity from NCBI, and a phylogenetic tree was generated using the MEGA software (version 11.0.13) (https://www.megasoftware.net/) [17]. All four were assigned as *Streptomyces* species considering their closeness to many sequences of different *Streptomyces.* Figure S3 displays the link between the isolates and the nearest phylogenetic neighbors as well as a comparison of the sequencing findings of *Streptomyces* isolates.

2.3. Antimicrobial Assays

The antimicrobial activity of *Streptomyces* species was tested against various Gram-positive and Gram-negative bacteria. The zone of inhibition of respective bacterial fermented extracts against tested pathogenic bacteria is listed below in Table S2. Among four isolates, *Streptomyces* sp. PC1 showed the highest zone of inhibition against all tested pathogenic bacteria. Thus, the MIC and MBC values (Table 1) of the extract of *Streptomyces* sp. PC1 was determined against *S. aureus* and *E. coli* along with *Streptomyces* species BT1, BT2, and BT3. The MICs of *Streptomyces* sp. PC1 against *S. aureus* and *E. coli* were 0.65 mg/mL and 1.5 mg/mL, respectively, while positive control neomycin exhibited MICs of 0.62 µg/mL and 0.78 µg/mL against *S. aureus* and *E. coli*, respectively. The MBCs of *Streptomyces* sp. PC1 against *S. aureus* and *E. coli* were 2.63 mg/mL and 3.0 mg/mL, respectively, compared to positive control (neomycin), which had MBCs of 1.25 µg/mL and 1.56 µg/mL against *S. aureus* and *E. coli*, respectively (Figure S2).

Table 1. MIC and MBC of various extracts against *Staphylococcus aureus* and *Escherichia coli*.

Streptomyces Species Extracts	BT1 (mg/mL)	BT2 (mg/mL)	BT3 (mg/mL)	PC1 (mg/mL)	Neomycin (µg/mL)
MIC (*Staphylococcus aureus*)	2.295	1.960	1.875	0.658	0.625
MIC (*Escherichia coli*)	1.090	1.738	0.937	1.500	0.781
MBC (*Staphylococcus aureus*)	4.590	3.920	3.750	2.635	1.250
MBC (*Escherichia coli*)	2.188	3.477	1.875	3.000	1.562

2.4. Metabolic Comparision in Streptomyces Species

Mass spectrometry generated raw data were analyzed through MestReNova software 12.0.0, Spain (https://mestrelab.com/, accessed on 10–30 December 2023). Each peak was analyzed, detected, aligned, and annotated. To visualize any difference in metabolites, the total ion chromatograms (TIC) of ethyl acetate (EA) extracts of *Streptomyces* species BT1, BT2, BT3, and PC1 were stacked together as shown in Figure 1. Finally, the results were compared with literature and database libraries. Thirty-seven metabolites were detected in all samples of classes such as diketopiperazine and alkyl resorcinol. The secondary metabolites annotated through LC-HRMS/MS analysis are shown in Table 2 and Figure S4. The base peak chromatograms (BPC) and MS profiles of identified metabolites are displayed in Supplementary Figures S5–S41.

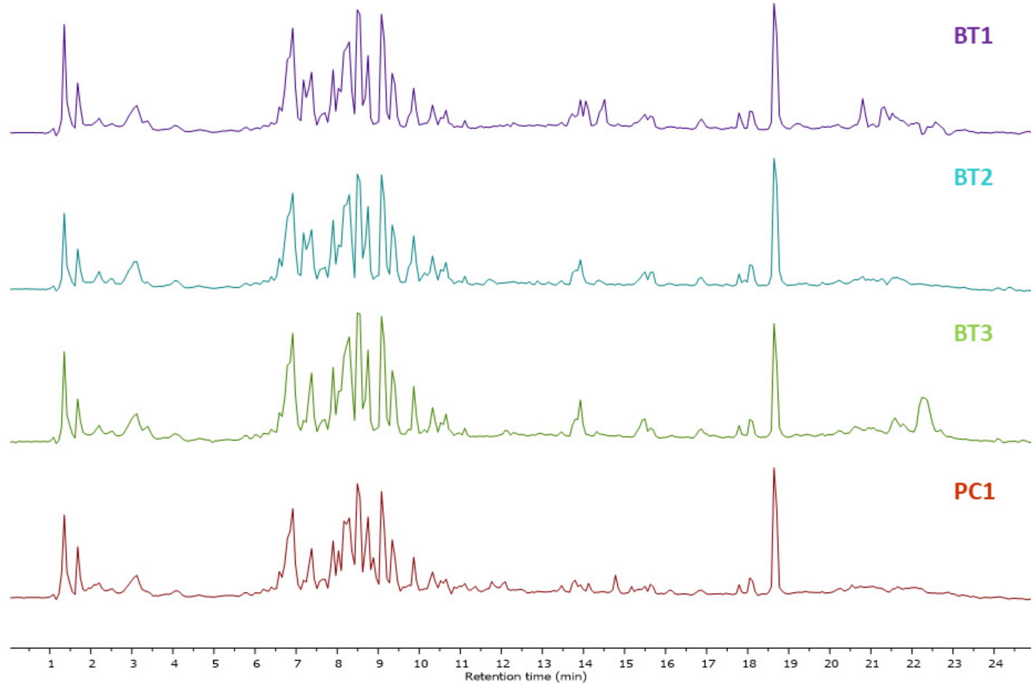

Figure 1. Total ion chromatograms obtained in EA extracts of *Streptomyces* species BT1, BT2, BT3, and PC1 in a stacked format.

Table 2. The list of annotated compounds in ethyl acetate fermentation extracts of *Streptomyces* species BT1, BT2, BT3, and PC1.

C.N	Annotated Compound	Exact Mass *m/z*	Observed Mass *m/z*	Detected Ion	Molecular Formula	RDBE	Absolute Error (ppm)	RetentIon Time (min)	Bacterial Source	CSI:FingerID Score (%)	References
1	Cyclo-(D-Pro-D-Phe)	244.120	245.129	$[M+H]^+$	$C_{14}H_{16}N_2O_2$	8.0	3.20	8.76	BT1	99.24	[18]
2	Cyclo-(L-Val-L-Leu)	212.159	213.160	$[M+H]^+$	$C_{11}H_{20}N_2O_2$	3.0	1.84	9.34 (BT1)	BT1, BT2	92.71	[19]
3	Maculosin	260.115	261.123	$[M+H]^+$	$C_{14}H_{16}N_2O_3$	8.0	0.57	6.59 (BT1)	PC1, BT1, BT3	98.00	[20]
4	Neomarinone	424.225	425.233	$[M+H]^+$	$C_{26}H_{32}O_5$	11.0	4.70	14.02	BT1	48.47	[21]
5	Dibutyl phthalate	278.152	279.160	$[M+H]^+$	$C_{16}H_{22}O_4$	6.0	2.18	18.64 (BT1)	BT1, BT2	95.91	[22]
6	Pyridoxine	169.073	170.081	$[M+H]^+$	$C_8H_{11}NO_3$	4.0	1.34	1.35 (BT1)	BT1, BT2	99.45	[23]
7	Cyclo-(L-Pro-L-OMet)	244.087	245.10	$[M+H]^+$	$C_{10}H_{16}N_2O_3S$	4.0	0.70	2.53	BT2	76.71	[24]
8	Surfactin C13	1007.651	1008.660	$[M+H]^+$	$C_{51}H_{89}N_7O_{13}$	11.0	3.394	21.29	BT1	99.72	[25]
9	Cyclo-(L-Valyl-L-Phenyl alanyl)	246.136	247.144	$[M+H]^+$	$C_{14}H_{18}N_2O_2$	7.0	0.74	9.87	BT2	93.04	[26]
10	Di-*n*-butyl terephthalate	278.151	279.159	$[M+H]^+$	$C_{16}H_{22}O_4$	6.0	1.76	18.58	BT3	54.11	[27]
11	Phthalic anhydride	148.015	149.023	$[M+H]^+$	$C_8H_4O_3$	7.0	0.63	18.64	BT3	99.24	[28]
12	Phytoceramide	555.522	556.531	$[M+H]^+$	$C_{34}H_{69}NO_4$	11.0	1.22	20.68	BT3	71.70	[29]
13	Cyclo-(Pro-Gly)	154.073	155.081	$[M+H]^+$	$C_7H_{10}N_2O_2$	4.0	3.27	2.20 (BT3)	PC1, BT1, BT2, BT3	97.50	[30]
14	Cyclo-(L-Leu-L-Pro) or Gancidin W	210.136	211.144	$[M+H]^+$	$C_{11}H_{18}N_2O_2$	4.0	1.41	8.03 (BT2)	PC1, BT1, BT2, BT3	98.69	[31]
15	Cyclo-(L-Phenylalanyl-*trans*-4-hydroxy-L-Proline)	260.112	261.119	$[M+H]^+$	$C_{14}H_{16}N_2O_3$	8.0	1.32	6.72 (PC1)	PC1, BT1, BT2, BT3	62.83	[32]
16	Coronafacoyl-L-isoleucine	321.194	344.183	$[M+Na]^+$	$C_{18}H_{27}NO_4$	6.0	0.02	10.85 (PC1)	PC1, BT3	36.16	[33]
17	Cyclo-(D-Pro-L-Tyr)	260.112	261.119	$[M+H]^+$	$C_{14}H_{16}N_2O_3$	4.0	1.85	6.79 (BT2)	PC1, BT1, BT2, BT3	87.08	[32]
18	Cyclo-(Pro-Val)	196.120	197.129	$[M+H]^+$	$C_{10}H_{16}N_2O_2$	4.0	2.10	6.59 (BT2)	PC1, BT1, BT2, BT3	95.58	[34]
19	*N*-Acetyltyramine	179.094	180.102	$[M+H]^+$	$C_{10}H_{13}NO_2$	5.0	2.48	7.18 (BT2)	PC1, BT1, BT2, BT3	73.66	[35]
20	Cyclo-(L-Ala-L-Leu)	184.120	185.129	$[M+H]^+$	$C_9H_{16}N_2O_2$	3.0	1.25	7.57 (BT2)	BT1, BT2, BT3	97.87	[36]
21	Cyclo-(Tyr-Leu)	276.147	277.155	$[M+H]^+$	$C_{15}H_{20}N_2O_3$	7.0	1.07	7.84 (BT2)	PC1, BT1, BT2, BT3	94.12	[37]
22	Cyclo-(L-Phe-L-Ala)	218.104	219.114	$[M+H]^+$	$C_{12}H_{14}N_2O_2$	7.0	4.91	7.97 (BT2)	PC1, BT1, BT2, BT3	95.63	[38]
23	Cyclo-(Phenylalanyl-Prolyl)	244.121	245.129	$[M+H]^+$	$C_{14}H_{16}N_2O_2$	8.0	1.00	8.69 (BT2)	PC1, BT1, BT2, BT3	99.62	[39]
24	Brevianamide F or Cyclo-L-Trp-L-Pro	283.131	284.139	$[M+H]^+$	$C_{16}H_{17}N_3O_2$	10.0	0.47	9.41 (BT2)	PC1, BT1, BT2, BT3	99.40	[40]
25	*N*-Phenethylacetamide	163.099	164.107	$[M+H]^+$	$C_{10}H_{13}NO$	5.0	1.63	9.74 (BT2)	PC1, BT1, BT2, BT3	99.24	[41]
26	Cyclo-(L-Leucyl-L-Leucyl)	226.168	227.176	$[M+H]^+$	$C_{12}H_{22}N_2O_2$	3.0	0.83	10.32 (BT2)	PC1, BT1, BT2, BT3	94.97	[42]
27	Cyclo-(D-Leu-L-Trp)	299.163	300.171	$[M+H]^+$	$C_{17}H_{21}N_3O_2$	9.0	1.61	10.26 (BT2)	PC1, BT1, BT2, BT3	85.35	[43]
28	Cyclo-(Phenylalanyl-Phenylalanyl)	294.139	295.147	$[M+H]^+$	$C_{18}H_{18}N_2O_2$	11.0	1.55	11.11 (BT2)	PC1, BT1, BT2, BT3	69.32	[44]
29	Cyclo-(Tyr-Val)	262.133	263.141	$[M+H]^+$	$C_{14}H_{18}N_2O_3$	7.0	0.27	7.71 (BT2)	BT1, BT2, BT3	76.92	[45]
30	1-Acetyl-3-methoxycarbonyl-β-carboline	268.086	269.093	$[M+H]^+$	$C_{15}H_{12}N_2O_3$	11.0	1.95	14.38 (BT2)	BT1, BT2	81.21	[46]
31	Cyclo-(D-Ala-L-Pro)	168.089	169.097	$[M+H]^+$	$C_8H_{12}N_2O_2$	4.0	0.56	2.99 (BT2)	PC1, BT1, BT2, BT3	98.56	[47]

Table 2. *Cont.*

C.N	Annotated Compound	Exact Mass m/z	Observed Mass m/z	Detected Ion	Molecular Formula	RDBE	Absolute Error (ppm)	RetentIon Time (min)	Bacterial Source	CSI:FingerID Score (%)	References
32	Cyclo-(Ile-Ser)	200.125	201.124	$[M+H]^+$	$C_9H_{16}N_2O_3$	3.0	1.02	6.20 (PC1)	PC1, BT1, BT2, BT3	83.49	[48]
33	(*S*)-3-Isobutylpiperazine-2,5-dione or Cyclo(Gly-Leu)	170.105	171.113	$[M+H]^+$	$C_8H_{14}N_2O_2$	3.0	1.11	6.46 (BT2)	PC1, BT1, BT2, BT3	76.44	[49]
34	3-((6-methylpyrazin-2-yl)methyl)-1H-indole	223.110	224.118	$[M+H]^+$	$C_{14}H_{13}N_3$	10.0	0.84	12.09	PC1	84.57	[50]
35	*N*-Lauryldiethanolamine	273.266	274.275	$[M+H]^+$	$C_{16}H_{35}NO_2$	0.0	2.34	13.80 (BT2)	BT1, BT2, BT3	99.17	[51]
36	2-*n*-Hexyl-5-*n*-propyl resorcinol	236.177	237.185	$[M+H]^+$	$C_{15}H_{24}O_2$	4.0	1.02	18.05 (BT1)	BT1, BT2, BT3	76.54	[52]
37	2-Hexyl-5-methyl resorcinol	208.146	209.154	$[M+H]^+$	$C_{13}H_{20}O_2$	4.0	2.33	15.63	BT1	71.60	[53]

Note: C.N (Compound Number), RDBE (Ring Double Bond Equivalents). The isolate name has been given with Retention time for better tracking.

A molecular ion at m/z 284.139 $[M+H]^+$ detected at retention time 9.41 min was identified as brevianamide F, isolated previously from *Streptomyces* sp.TN262 [40]. Cyclo-(Phenylalanyl-Prolyl), previously isolated and identified in *Streptomyces* sp. [39], was also identified in this study at m/z 245.129 $[M+H]^+$ as a protonated ion. Likewise, a molecular ion at m/z 261.123 $[M+H]^+$ detected at retention time 6.59 min was annotated as maculosin, which was identified previously in *Streptomyces* sp. KTM18 [20]. We detected a molecular ion at m/z 171.113 $[M+H]^+$ in retention time 6.46 min and identified it as cyclo-(Gly-Leu), already reported in *Streptomyces xanthophaeus* [49]. Cyclo-(D-Ala-L-Pro) was detected at a retention time of 2.94 min with m/z 169.097 $[M+H]^+$, already identified in mangrove-derived *Streptomyces* sp. by Tan et al. [47]. Another cyclic dipeptide compound, cyclo-(Tyr-Val), was detected at m/z 263.141 $[M+H]^+$ and reported previously in *Streptomyces* sp. [45]. Moreover, a precursor ion detected at m/z 295.147 $[M+H]^+$ was identified as cyclo-(Phenylalanyl-Phenylalanyl), reported previously in *Streptomyces chrestomyceticus* [44]. Cyclo-(L-Leucyl-L-Leucyl), previously identified in *Streptomyces* sp. [42], was also identified in our study at m/z 227.176 $[M+H]^+$ as a precursor ion. A molecular ion detected at m/z 219.114 $[M+H]^+$ at retention time 7.97 min was identified as cyclo-(L-Phe-L-Ala), previously detected in *Streptomyces* sp. [38]. Moreover, a protonated ion detected at m/z 277.155 $[M+H]^+$ was identified as cyclo(Tyr-Leu), previously identified from soil-derived *Streptomyces kunmingensis* [37]. In addition, *N*-phenethylacetamide was annotated for a molecular ion at m/z 164.107 $[M+H]^+$. A precursor ion at m/z 425.233 $[M+H]^+$ detected at a retention time of 14.02 min was identified as neomarinone [21]. Similarly, a molecular ion at m/z 245.129 $[M+H]^+$ was identified as cyclo-(D-Pro-D-Phe), previously reported by Alshaibani et al. in *Streptomyces* sp. SUK 25 [54]. Based on the literature survey, cyclo-(L-Val-L-Leu) was identified at m/z 213.160 $[M+H]^+$ as a protonated ion, already reported in *Streptomyces xiamenensis* MCC A01570 [19]. In addition, dibutyl phthalate was annotated for a molecular ion at m/z 279.160 $[M+H]^+$. A precursor ion $[M+H]^+$ at m/z 269.093 detected at retention time 14.38 min was putatively identified as 1-acetyl-3-methoxycarbonyl-β-carboline. This compound was previously isolated from the fungus *Ophiocordyceps sphecocephala* BCC 2661 [46].

A compound exhibiting a molecular ion peak at m/z 170.081 $[M+H]^+$ was annotated as pyridoxine [23]. Furthermore, the precursor ion at m/z 245.10 $[M+H]^+$ was annotated as cyclo-(L-Pro-L-OMet), detected at a retention time of 2.53 min, as per the analysis of spectral data of Yang et al. [24]. A molecular ion at m/z 1008.660 $[M+H]^+$ detected at retention time 2.53 min was identified as surfactin C13 [25]. Likewise, a molecular ion at m/z 149.023 $[M+H]^+$ detected at a retention time of 18.64 min was annotated as phthalic anhydride. Another molecular ion at m/z 556.531 $[M+H]^+$ detected at a retention time of 20.68 min was identified as phytoceramide. Similarly, the $[M+H]^+$ at m/z 155.081 $[M+H]^+$ detected at retention time 2.20 min was identified as cyclo-(Pro-Gly), based on the existing

literature [30]. A molecular ion at *m*/*z* 211.144 [M+H]$^+$ detected at retention time 8.03 min was identified as cyclo-(L-Leu-L-Pro), previously reported by Zin et al. [31]. Furthermore, the molecular ion at *m*/*z* 261.119 [M+H]$^+$ detected as a protonated ion at retention time 6.79 min was previously isolated from *Streptomyces asenjonii* by Abdelkader et al. and was annotated as cyclo-(L-Phenylalanyl-trans-4-hydroxy-L-Proline) [55]. As per the literature, the sodium adduct formed at *m*/*z* 344.183 [M+Na]$^+$ detected at retention time 10.85 min was identified as coronafacoyl-L-isoleucine. This metabolite was previously isolated from *Streptomyces scabies* [56]. Another molecular ion peak detected at retention time 6.79 min having *m*/*z* 261.119 [M+H]$^+$ was annotated as cyclo-(D-Pro-L-Tyr), previously detected at *Streptomyces* sp. strain 22-4 [32]. In addition, a metabolite was detected at *m*/*z* 197.12 [M+H]$^+$ as a protonated ion in the retention time of 6.85 min, and identified as cyclo-(Pro-Val) as per the existing literature [34]. Similarly, the molecular ion detected at a retention time of 7.18 min with *m*/*z* 180.102 [M+H]$^+$ was annotated as N-acetyl tyramine, from the spectral analysis carried out by Driche et al. [35]. The metabolite cyclo-(L-Ala-L-Leu), previously isolated from *Streptomyces* species from the soil environment, was detected at *m*/*z* 185.129 [M+H]$^+$ as a protonated ion at the retention time of 7.57 min [57]. We identified another compound detected at *m*/*z* 274.275 [M+H]$^+$ as *N*-lauryl diethanolamine. Likewise, a protonated ion detected at *m*/*z* 237.185 [M+H]$^+$ at retention time 18.05 min was tentatively identified as 2-hexyl-5-methylresorcinol which was reported already in *S. clavuligerus* [53].

Identification of Metabolites New to *Streptomyces* Species

Among 37 metabolites, we identified four metabolites for the first time in *Streptomyces* species. A molecular ion [M+H]$^+$ was detected at *m*/*z* 201.124 at a retention time of 6.20 min, with its MS^2 peaks shown in Figure 2a.

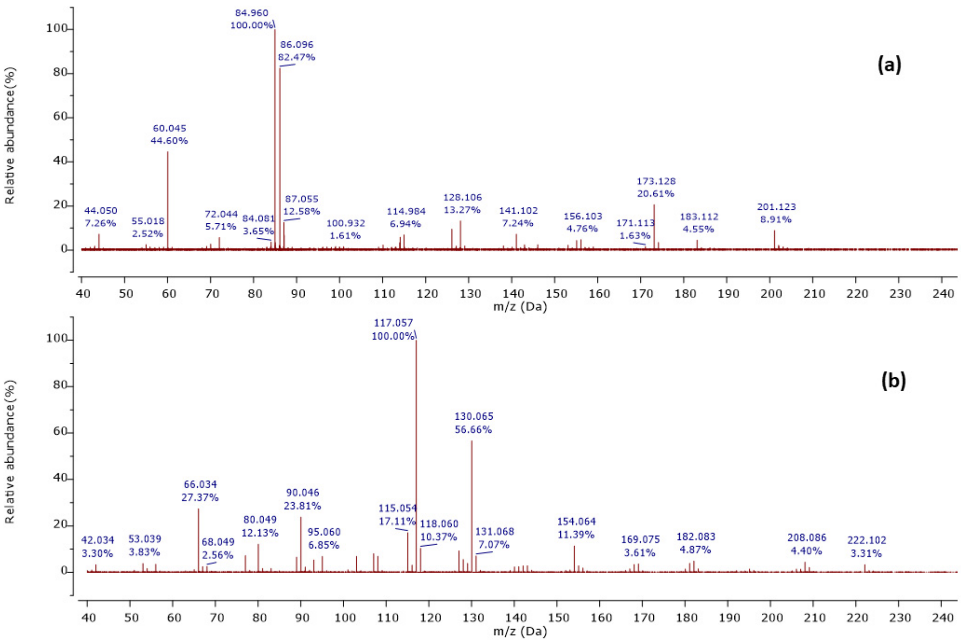

Figure 2. Observed MS/MS profiles of the precursor protonated molecules at *m*/*z* 201.124 (**a**) and *m*/*z* 224.118 (**b**).

The MS^2 spectrum showed the fragment ions at *m*/*z* 173 [M+H-28]$^+$ due to $[C_8H_{17}N_2O_2]^+$ ion formed by loss of a CO molecule from precursor ion, *m*/*z* 114 [M+H-59-28]$^+$ owing to $[C_6H_{12}NO]^+$ ion formed by departure of a neutral C_2H_5NO unit and a CO molecule simultaneously, and $[C_6H_{12}NO]^+$ ion further lose a CO molecule to give a distinct peak at *m*/*z* 86 attributed to $C_5H_{12}N^+$ ion. Furthermore, an ion at *m*/*z* 60 [M+H-85-28-28]$^+$ was observed due to the loss of a neutral $C_5H_{11}N$ unit and two CO molecules as shown in Figure 3. Thus, we identified this compound as cyclo-(Ile-Ser), isolated previously from *Ophiocordyceps sobolifera* [48].

Figure 3. Observed fragmentation pattern of cyclo-(Ile-Ser) in (+)-ESI mode.

Another precursor ion was detected at a retention time of 12.09 min with m/z 224.118 $[M+H]^+$. Its MS^2 profile (Figure 2b) displayed a distinct peak at m/z 130.065 $[M+H-94]^+$ due to the $C_9H_8N^+$ ion attributed to the loss of a neutral $C_5H_6N_2$ unit, as shown in Figure 4. Thus, this compound was putatively identified as 3-[(6-methylpyrazin-2-yl) methyl]-1H-indole, isolated and identified previously as a new alkaloid from marine *Serinicoccus profundi* sp. by Yang et al. [50]. To the best of our knowledge, this compound was observed for the first time in *Streptomyces* sp.

Exact Mass: 224.118

Chemical Formula: $C_9H_8N^+$
Exact Mass: 130.065

Chemical Formula: $C_5H_6N_2$
Exact Mass: 94.053

Figure 4. Observed fragmentation pattern of 3-[(6-methylpyrazin-2-yl) methyl]-1H-indole in (+)-ESI mode.

Furthermore, a molecular ion was observed at m/z 237.185 $[M+H]^+$ in a retention time of 10.27 min, with its MS^2 spectrum shown in Figure 5a.

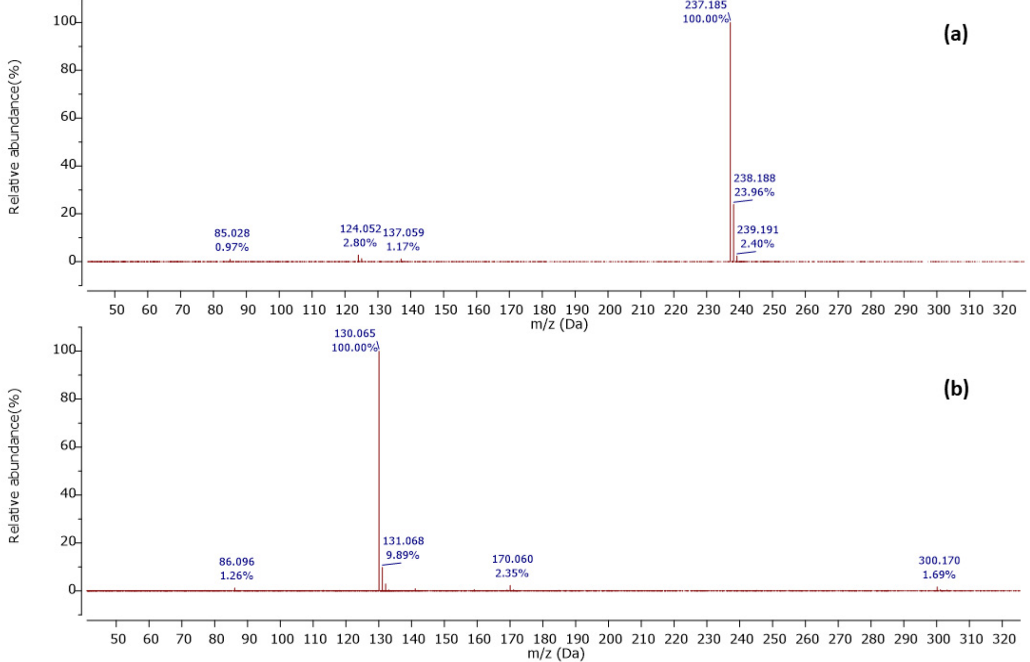

Figure 5. Observed MS/MS profiles of the precursor protonated molecules at m/z 237.185 (**a**) and m/z 300.170 (**b**).

The MS^2 spectrum displayed a molecular ion peak $[C_{15}H_{25}O_2]^+$ as a base peak which may be due to incomplete fragmentation. Further, fragment ions were detected at m/z 124 $[C_7H_{10}O_2]^{\bullet+}$ because of $[M+H-C_5H_{11}-C_3H_6]^{\bullet+}$ and m/z 137 owing to $[M+H-C_5H_{11}-C_2H_5]^{2\bullet+}$, as shown in Figure 6. Thus, this compound was tentatively identified as 2-n-hexyl-5-n-propylresorcinol. This alkyl resorcinol, previously reported in *Pseudomonas chlororaphis* PCL1606, has now been identified in *Streptomyces* sp. for the first time [52].

In addition, a protonated ion was detected at m/z 300.171 $[M+H]^+$ in a retention time of 10.26 min. Its MS^2 profile (Figure 5b) revealed a base peak with m/z 130 $[M+H-170]^+$ due to the $C_9H_9N^+$ ion formed by the loss of the $C_8H_{14}N_2O_2$ unit as shown in Figure 7. Thus, this compound was tentatively identified as cyclo-(D-Leu-L-Trp). Although this compound was previously reported in *Penicillium brevicompactum* [43], this is the first time it was observed in *Streptomyces* sp.

Figure 6. Observed fragmentation pattern of 2-n-hexyl-5-n-propylresorcinol in (+)-ESI mode.

Figure 7. Observed fragmentation pattern of cyclo-(D-Leu-L-Trp) in (+)-ESI mode.

2.5. GNPS-Based Molecular Networking

To comprehensively investigate the detailed metabolite profile of four isolates, namely *Streptomyces* sp. BT1, BT2, BT3, and PC1, we conducted MS^2 and GNPS metabolic profiling as illustrated in Figure 8. A total of 378 molecular ions were observed to have MS^2 spectra of four samples represented by nodes that were connected by 788 edges in the molecular network. Out of these 378 ions, 62 ion pairs formed two-node clusters, while the remaining 335 molecular ions were self-looped. We successfully identified and dereplicated 25 known compounds through the GNPS library (Table 3), and you can find more detailed information on the GNPS website [58]. The GNPS approach revealed that the extracts from the four isolates consist of various compounds, including diketopiperazines, lipopeptides, dodecylamines, and tyramine alkaloids.

The extracts of the four samples (*Streptomyces* sp. BT1, BT2, BT3, and PC1) underwent metabolic profiling through MS^2 data with positive ion mode and built a network using GNPS (Figure 9A). In Figure 9B, the ions with m/z 1022.680, 1036.690, and 1050.710 corresponded to surfactins B, C, and D, respectively. Intriguingly, the presence of an ion with m/z 1008.660, 16 Da lower than m/z 1022.680, suggests the removal of a methylene group from surfactin B, with a similar pattern observed for the ion at m/z 994.645 relative to m/z 1008.660, indicating structural similarities and related metabolic pathways. Notably, it was revealed that the BT1 isolate extract contained lipopeptides, as indicated by the base peak ion MS chromatograms. Additionally, Figure 9C revealed that the BT2

extract contained a significant amount of tyramine alkaloid, as suggested by the presence of annotated compounds isorugulosuvine and brevianamide F. Furthermore, the cluster associated with BT3 displayed an ammonium-adduct ion peak $[M+NH_4]^+$ at m/z 1128.670, corresponding to valinomycin (Figure 9D). In contrast to BT1, BT2, and BT3, PC1 was found to lack large molecules of lipopeptides. Instead, it contained smaller nitrogen-containing compounds, including pyridoxine, penciclovir, and β-indoleacetic acid (Figure 9E). These findings collectively highlight the diverse and distinct chemical profiles of the four isolates, shedding light on their unique metabolic pathways and chemical compositions.

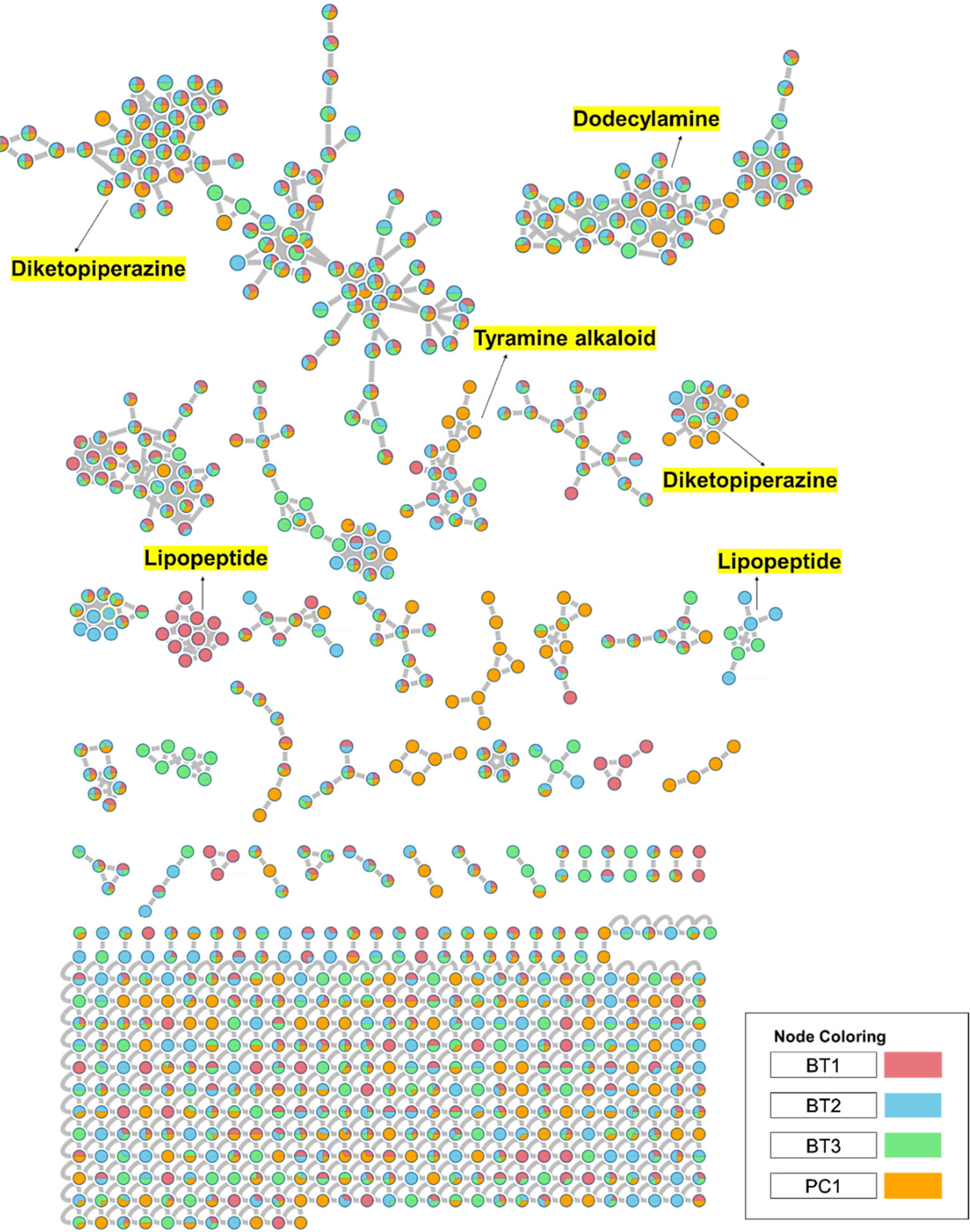

Figure 8. Full molecular networking was created using MS/MS data in positive mode from extracts of *Streptomyces* species BT1, BT2, BT3, and PC1. Nodes are labeled with parent mass. The networking is displayed as a pie chart with pink, blue, green, and orange indicating the chemical composition distribution of *Streptomyces* species BT1, BT2, BT3, and *Streptomyces* sp.PC1 extracts, respectively.

Table 3. The list of annotated compounds using GNPS in *Streptomyces* species BT1, BT2, BT3, and PC1.

S.N.	Comp. Name	Accurate Mass (Da)	Precursor Ion	Adduct Type	MS^2 Fragmentation Pattern	Molecular Formula	Retention Time (min)	Bacterial Source	Error (ppm)	Reference
1	3-*epi*-Xestoaminol C	229.241	230.247	$[M+H]^+$	212.237, 66.070, 55.054, 44.050	$C_{14}H_{31}NO$	13.85	BT1, BT2, BT3, PC1	4.4	[59]
2	4-Aminobenzoic acid	137.048	138.055	$[M+H]^+$	138.049, 120.040, 77.034, 65.035	$C_7H_7NO_2$	4.85	PC1	0.0	[60]
3	5-Aminovaleric acid	117.079	100.076	$[M+H-H_2O]^+$	100.076, 72.081, 56.050	$C_5H_{11}NO_2$	3.14	BT1, BT2, PC1	0.0	[61]
4	(−)-α-Bisabolol	222.372	205.195	$[M+H-H_2O]^+$	205.195, 121.100, 93.069, 81.069	$C_{15}H_{26}O$	18.84	BT2	0.1	[62]
5	Anthranilic acid	137.048	138.055	$[M+H]^+$	120.044, 92.049, 965.038	$C_7H_7NO_2$	8.43	BT1, BT2, PC1	7.2	[63]
6	Cyclo-[L-(4-hydroxy-pro)-L-leu]	226.132	227.138	$[M+H]^+$	199.153, 181.142, 86.078	$C_{11}H_{18}N_2O_3$	7.37	BT1, BT2, BT3, PC1	8.8	[64]
7	Cyclo(L-leu-trans-4-hydroxy-L-pro)	226.132	227.139	$[M+H]^+$	199.153, 181.142, 86.078	$C_{11}H_{18}N_2O_3$	6.92	BT1, BT2, BT3, PC1	4.3	[65]
8	Cyclo(D-6-Hyp-L-Phe)	260.116	261.124	$[M+H]^+$	120.080, 103.054, 86.060	$C_{14}H_{16}N_2O_3$	8.92	BT1, BT2, BT3,	3.8	[66]
9	Cyclo(L-Phe-D-Pro)	244.121	245.128	$[M+H]^+$	154.072, 120.080, 70.065	$C_{14}H_{16}N_2O_2$	9.40	BT1, BT2, BT3, PC1	4.0	[67]
10	Cyclo(L-Val-L-Pro)	196.121	197.129	$[M+H]^+$	124.112, 98.060, 70.066	$C_{10}H_{16}N_2O_2$	7.03	BT1, BT2, BT3, PC1	0.0	[68]
11	Tryptophol	161.084	162.091	$[M+H]^+$	143.072, 130.064, 115.053, 91.054	$C_{10}H_{11}NO$	10.29	BT1, BT3, PC1	6.1	[40]
12	Surfactin C1	1035.683	1036.690	$[M+H]^+$	1036.687, 699.466, 582.410, 455.286, 356.245	$C_{53}H_{93}N_7O_{13}$	22.59	BT1	2.94	[25]
13	Lipopeptide NO	993.636	994.644	$[M+H]^+$	994.647, 881.554, 554.371, 441.284	$C_{50}H_{87}N_7O_{13}$	20.80	BT1	1.04	[69]
14	Surfactin B	1021.667	1044.660	$[M+Na]^+$	931.574, 818.491, 728.496, 657.458, 594.348	$C_{52}H_{91}N_7O_{13}$	22.23	BT1	0.9	[70]
15	Lauryldiethanolamine	273.267	274.274	$[M+H]^+$	274.274, 256.263, 106.086, 88.075	$C_{16}H_{35}NO_2$	13.78	BT1, BT2, BT3, PC1	7.0	[71]
16	Tetradecyldiethanolamine	301.298	302.305	$[M+H]^+$	302.305, 284.294, 106.086, 88.075	$C_{18}H_{39}NO_2$	15.30	BT1, BT2, BT3, PC1	6.6	[72]
17	Indole-3-carbinol	147.068	130.066	$[M+H-H_2O]^+$	103.056, 95.050, 77.038	C_9H_9NO	9.43	BT1, BT2, BT3, PC1	7.6	[73]
18	Cyclo(leucylprolyl)	210.137	211.144	$[M+H]^+$	211.145	$C_{11}H_{18}N_2O_2$	9.48	BT1, BT2, BT3, PC1	4.7	[26]
19	*N*-acetyl-2-phenylethylamine	163.100	164.107	$[M+H]^+$	105.070	$C_{10}H_{13}NO$	9.78	BT1, BT2, BT3, PC1	6.1	[74]
20	*N*-acetyltyramine	179.095	180.102	$[M+H]^+$	121.065, 103.054, 93.070	$C_{10}H_{13}NO_2$	7.19	BT1, BT2, BT3, PC1	5.5	[35]
21	N6-(delta2-isopentenyl)adenine	203.117	204.124	$[M+H]^+$	148.063, 136.063, 69.071	$C_{10}H_{13}N_5$	8.10	BT1, BT3, PC1	9.86	[75]
22	*N*-[2-(1*H*-indol-3-yl)ethyl]acetamide	202.111	203.118	$[M+H]^+$	144.081	$C_{12}H_{14}N_2O$	10.21	BT1, BT2	0.0	[76]
23	Brevianamide F	283.132	284.139	$[M+H]^+$	130.064	$C_{16}H_{17}N_3O_2$	9.19	BT1, BT2, BT3, PC1	6.9	[73]
24	Normetanephrine	183.090	184.097	$[M+H]^+$	134.059, 106.065, 91.054, 77.038	$C_9H_{13}NO_3$	2.41	BT1, BT2, BT3, PC1	0.0	[77]
25	13-Docosenamide	337.334	338.342	$[M+H]^+$	149.132, 121.101, 97.101	$C_{22}H_{43}NO$	22.36	BT1, BT2, BT3, PC1	0.0	[78]

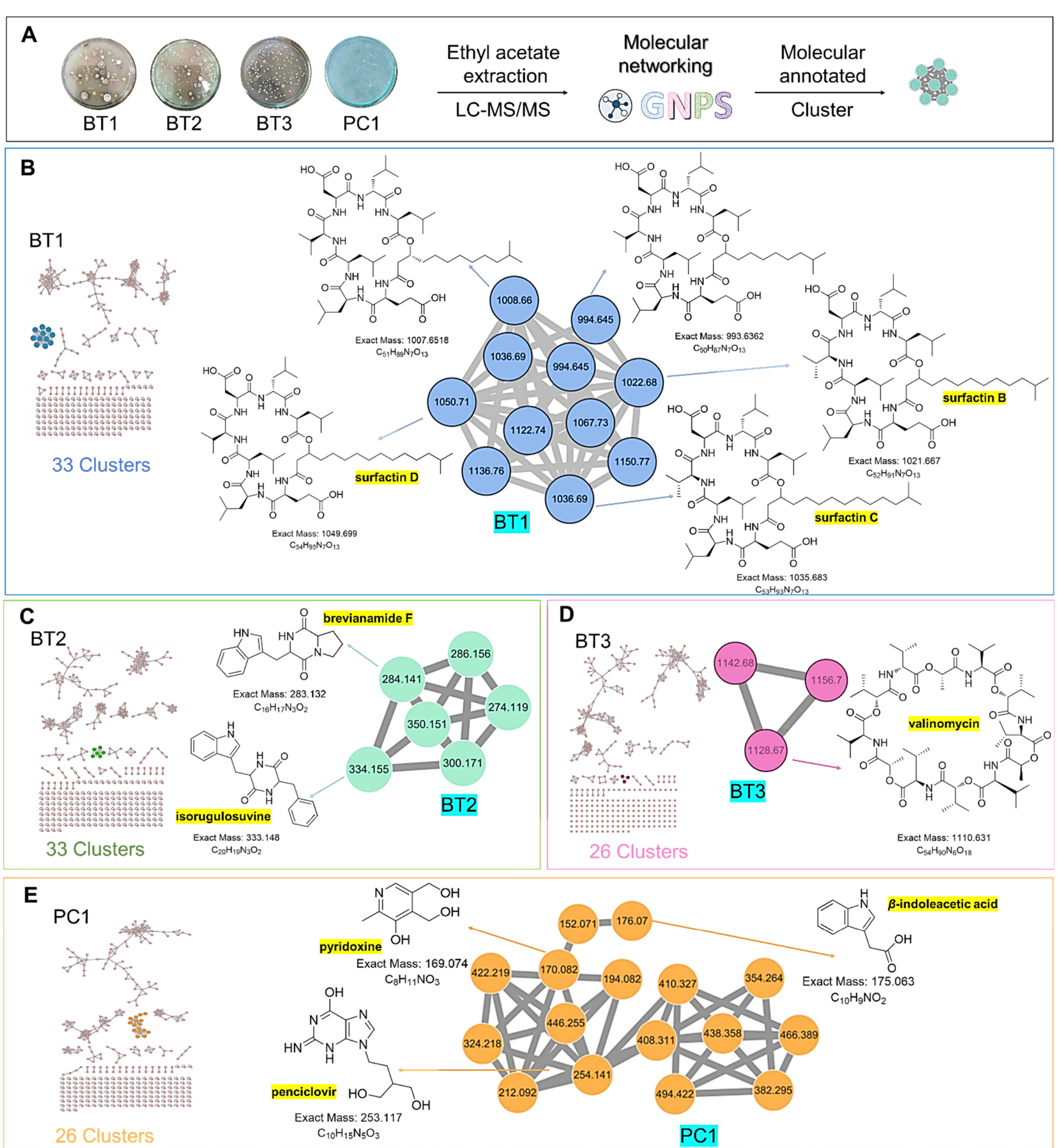

Figure 9. (**A**) Molecular networking analysis and identification of compounds in four samples. (**B**) Zoomed-in molecular networking of annotated compounds from BT1. (**C**) Zoomed-in molecular networking of annotated compounds from BT2. (**D**) Zoomed-in molecular networking of annotated compounds from BT3. (**E**) Zoomed-in molecular networking of annotated compounds from PC1.

3. Discussion

The improper usage and overprescription of pharmaceuticals have led to MDR pathogens, which have become a serious global issue [79]. Structural modification of a drug molecule is one adaptive strategy employed by bacteria to develop resistance to drugs. Thus, an alternative strategy to solve the problem of drug resistance could be the

discovery of secondary metabolites that have the same therapeutic benefits and therefore can aid in the drug discovery program.

Soil ecosystem serves as key sources for *Streptomyces* isolation, exhibiting a broad range of metabolites production, with novel compounds emerging in response to varying nutritional or environmental factors [80]. Precise identification of bacterial isolates at the species level is essential, providing valuable insights into the microorganism, potential bioactive chemicals, and its distinctiveness [81]. Morphology also proves to be an essential factor in differentiating *Streptomyces* from other spore-forming actinomycetes, and in characterizing distinct species within the *Streptomyces* genus. Further, sequencing of 16S rRNA was performed for accurate identification of these strains suggesting that isolates are *Streptomyces* species including *Streptomyces* sp. BT1, *Streptomyces* sp. BT2, *Streptomyces* sp. BT3, and *Streptomyces* sp. PC1.

The total ion chromatogram of ethyl acetate extracts of *Streptomyces* sp. BT1, BT2, BT3, and PC1, shown in Figure 1 illustrated an identical chromatogram, signifying the elution of the same type of metabolites in the particular retention time. Despite originating from distinct ecological niches, we observed quite similar metabolic profiles across the species studied. This intriguing finding may be attributed to the uniformity in cultivation conditions experienced by all species. Specifically, each species was grown under an identical culture media, maintaining consistent temperature, and environmental conditions throughout the cultivation process. This standardized approach ensured that external factors did not introduce variability, allowing us to confidently attribute the observed similarities in metabolomic profiles to intrinsic biological factors shared among the studied species. Changes in the metabolites have been reported in *Streptomyces* species when altering the growth media. For instance, the use of four different media in the culture resulted in the generation of many bioactive compounds, specifically, three new macrolides were discovered when using a YMG agar medium, five additional polyketides from sterilized Waksman synthetic medium, and three newly discovered naphthomycins from oatmeal medium [82–84].

In our study, 37 metabolites were identified in *Streptomyces* sp. BT1, BT2, BT3, and PC1 using LC-HRMS/MS analysis in which many compounds belong to the diketopiperazine (DKP) class. DKP is a unique class of organic compounds resembling piperazine with two amide linkages, produced by microorganisms involved in fermentation processes, particularly crucial in the food and beverage industry. It has gained a massive interest nowadays due to its variety of biological activities. The majority of indole DKPs showed notable bioactivities, including cytotoxic, antibacterial, anti-inflammatory, and antiviral activities; as a result, these substances may serve as the basis for novel drug development [85]. Almost all the metabolites identified in this study demonstrate a potent antimicrobial effect as per the existing literature, leading the bacterial strain towards a good antimicrobial activity. Cyclo-(D-Pro-D-Phe) showed antimicrobial activity against phytopathogenic *Rhodococcus fascians* [86]. Similarly, cyclo-(L-Pro-L-OMet) demonstrated antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* showing a 100 μg/mL MIC value along with antifungal activity with a 50 μg/mL MIC value [87]. Due to the potent antimicrobial activity and synergistic impact in the development of *enterococci*, which are resistant to vancomycin, with MIC between 0.25 and 0.5 μg/mL, cyclo-(L-Leu-L-Pro) has a wide therapeutic application [88]. The ability to demonstrate antimicrobial activity against *Vibrio anguillarum* with a MIC value of 2.68×10^{-7} μg/mL makes cyclo-(L-Phenylalanyl-trans-4-hydroxy-L-Proline) a successful candidate that might show therapeutic applications [89]. Proline-rich antimicrobial peptides, or PrAMPs, have a significant amount of antibacterial activity and minimal cytotoxicity, making them prospective agents against infections that are resistant to several drugs [90]. Likewise, cyclo-(D-Pro-L-Tyr) is reported to demonstrate a potential antimicrobial effect against various plant pathogenic bacteria [32]. Cyclo-(Pro-Val) exhibited an antimicrobial effect against *Vibrio anguillarum* with a MIC value of 7.14×10^{-7} μg/mL [89], but this metabolite did not stop the proliferation of cancer cells. Another metabolite *N*-

acetyltyramine detected in this work highlights a great potential to be used as a successful candidate for further study against drug-resistant bacteria as it shows a MIC value of 30 mg/mL [35]. A cyclic dipeptide called maculosin, which was isolated from *Pseudomonas rhizosphaerae*, has been shown to have antibacterial properties against a range of marine bacteria, including *Bacillus cereus*, *Ruegeria* sp., and *Pseudoalteromonas piscida* [91]. Strong antibacterial action (26–37 μg/mL) was demonstrated by a glycoside of maculosin that was isolated from marine *Streptomyces* sp. ZZ446, against methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* [20]. Hence, most of the diketopiperazines identified in our study might be responsible for the observed antimicrobial potential.

Earlier research indicates that brevianamide F could potentially be employed in the treatment of cardiovascular dysfunction, and bacterial infection [92,93]. Apart from functioning as a glycopeptide-like antibiotic, cyclo-(Phenylalanyl-Prolyl) has demonstrated various significant roles, including inhibiting membrane permeability and decelerating DNA synthesis [94]. Due to its potent antioxidant capabilities and non-toxic nature, maculosin could be a promising candidate for diverse applications in cosmetics and therapeutics [20]. Prior research revealed that cyclo-(Gly-Leu) interacts with dopamine receptors, suggesting a potential involvement of central dopamine receptors in the pathophysiology of hypertension [95]. However, cyclo-(D-Ala-L-Pro) isolated from the fungus *Colpoma* sp. was reported to show poor antimicrobial activity [96]. It was reported that at an optimal concentration of 10 μg/mL, cyclo-(Tyr-Leu) was able to enhance the mycelial growth of *H. marmoreus* [97]. It was reported that *N*-phenethylacetamide hindered the TGF-β/Smad pathway, restraining the metastasis of A549 cells by impacting TGF-β-induced epithelial-mesenchymal transition (EMT) [98]. Further, cyclo-(L-Ala-L-Leu) develops disease resistance contrary to *Pseudomonas syringae* attack, but it does not directly stop the growth of fungi [99].

As per the existing literature, surfactin C13 demonstrated cytotoxic potential against various cancer cell lines [100]. One of the most common uses of phthalic anhydride is the production of phthalate esters, which can be used as plasticizers [101]. Phytoceramide is reported to promote hydration and enhance the healing process of damaged human stratum corneum in human skin and can be used in the cosmetic industry for creating skin barrier moisturizers [102]. Moreover, phytoceramides were found to be cytotoxic to the MES-SA, MCF-7, and HK-2 cell lines in the previous study of *Monanchora clathrata*, and have further proven to be used in the prevention of neurodegeneration in both in vivo and in vitro [103]. The ability of cyclo-(Pro-Gly) to decrease motor neuronal death demonstrates the neuroprotective function after brain injury and this compound also shows anxiolytic activity [104,105]. Similarly, coronafacoyl-L-isoleucine is a biosynthetic intermediate of coronatine, which seems to amplify the degree of illness symptoms brought on by pathogenic microorganisms during host infection [106,107]. *N*-lauryl diethanolamine is a plastic antistatic agent with high proton affinity, thus detected in positive ionization mode and reported as an interference substance that leaches from plastic microtubes during sample pretreatment [51]. Neomarinone was reported to show in vitro cytotoxicity, with an IC_{50} value of 8 μg/mL against HCT-116 colon cancer cells [21]. Cyclo-(D-Pro-D-Phe) has been reported to show antifungal, quorum sensing, and antimicrobial activities, and it does not show any toxicity effect up to 200 μg/mL to human cell lines [108]. Cyclo-(L-Val-L-Leu) metabolite shows a 50% inhibition rate against PANC-1 and Hela S3 cancer cells [109]. Dibutyl phthalate demonstrated a toxic effect in humans causing headaches and vertigo and could affect the throat and nose severely. Furthermore, this compound has long-term negative effects on the developing fetus and testicles [110,111]. However, despite all the negative effects, it does show antimicrobial and antifungal activities [112]. Dibutyl phthalate isolated from *Streptomyces albidoflavus* exhibited potent antimicrobial activity against both Gram-positive and Gram-negative bacteria [22]. The ability of pyridoxine to maintain the proper ratio of potassium and sodium in the body followed by enhancement in the production of red blood cells aids in the prevention of homocysteine synthesis and develops immunity against cancer [113].

In general, compounds of class β-carboline are known to have biological activities including antiviral, antibiotic, anticancer, and antimalarial properties [114,115]. Similarly, alkyl resorcinol, an important structural group of amphiphilic phenolic lipids, is reported to show a variety of biological activities, such as cytotoxic, genotoxic, antioxidant, and signaling capabilities [116]. 2-n-hexyl-5-n-propylresorcinol (HPR) identified in our study is reported to demonstrate antimicrobial properties against both fungi and bacteria [117]. HPR is a tiny chemical produced by several bacteria from the cell that exhibits some antibacterial action in the surroundings [52]. In our research, we identified cyclo-(D-Leu-L-Trp) in *Streptomyces* sp. for the first time, and researchers have reported that it could enhance the root growth of seedlings [43]. However, 3-((6-methylpyrazin-2-yl)methyl)-1H-indole was reported to show a low cytotoxicity effect against human liver cell lines and demonstrated poor antibacterial activity [50]. Hence, previously reported activities of compounds have shown the factor responsible for those antimicrobial activities, and further studies on these annotated compounds can lead to the discovery of novel antimicrobials.

Furthermore, GNPS-based molecular networking was employed for the molecular annotation of metabolites in addition to manual interpretation of LC-MS/MS data. GNPS is a web-based mass spectrometry platform accessible to the public and offers several tools for analyzing MS/MS data. This platform enables the annotation of compounds either by using spectral library search or molecular networking-based grouping of compounds into families or clusters of molecules [92]. Different compounds were annotated from GNPS including different classes' majority of diketopiperazines, lipopeptides, dodecyl amine, and tyramine alkaloids (Figure 8). In the manual interpretation of MS/MS data, we used several natural product-based databases. Whereas, GNPS uses its library and some other reference libraries for spectral hitting, and approximately 1.8% to 2% of metabolites are annotated in untargeted metabolomics experiments due to a lack of sufficient chemical space coverage [118,119]. Moreover, ions with very low intensity lying near noise level are generally neglected in manual interpretation but GNPS workflow utilizes all ions including ions with low intensity for spectral hitting. Thus, these factors may be responsible for the variation in the number and metabolites detected in manual interpretation and GNPS spectral library hitting. 3-epi-xestoaminol C, a stereoisomer of xestoaminol C, was identified via molecular networking. This compound was reported to demonstrate IC_{50} values of 19.4 μM against *M. tuberculosis* H37Ra, 8.8 μM against HL-60 cells, and 18.0 μM against HEK cells, as reported in the literature [59]. These results emphasize the varied and distinct metabolite profiles of four isolates, providing insights into their metabolic pathways, chemical compositions, and bioactivities.

4. Materials and Methods

4.1. Isolation and Characterization of Streptomyces Species

Soil samples were collected from various ecosystems ranging up to 2743 m, altitudes in Nepal. The soils were taken from a depth of 10–15 cm beneath the earth's surface representing diverse sampling habitats including agriculture fields, forests, and hilly regions (Table S1). The collected soil samples were kept in a sterilized zip bag and stored at 4 °C in the laboratory.

Isolation of *Streptomyces* species was carried out using the spread plate technique developed by William and Davies, amended with antibiotics supplements [120]. One gram of each soil sample was dissolved in 9 mL of distilled and autoclaved water and thoroughly mixed by using a vertex shaker. A three-fold serial dilution was carried out to lower the bacterial population. Then, 100 μL of each serially diluted soil suspension was spread over the ISP4 medium with the help of a sterile glass spreader. 20 mg/L nalidixic acid and 50 mg/L cycloheximide were also added to the ISP4 medium to inhibit Gram-negative bacteria and fungus species, respectively. Finally, the plates were incubated at 28 °C for 7 days [121].

4.2. Genomic DNA Extraction and 16S rRNA Gene Sequencing

The genomic DNA of *Streptomyces* species was isolated using the phenol-chloroform method as described in the standard protocol of molecular biology [122]. For taxonomy identification, 16S rRNA gene was amplified using oligonucleotides 27F: 5′-AGAGTTTGAT CCTGGCTCAG-3′, and 1492R: 5′-GGTTACCTTGTTACGACTT-3′. The Polymerase Chain Reaction (PCR) was carried out in a 50 µL reaction mixture using 5x*Taq*-PCR Premix (GenoTech Corporation, Daejeon, Republic of Korea) containing *Taq*-polymerase within 30 cycles. A cycle was programmed with denaturation at 98 °C (10 s), an annealing at 54 °C (10 s), and an extension at 72 °C (2 min). The PCR products were isolated from low-melting agarose gel and were purified by using the QIAquick Gel Extraction Kit (Qiagen, Germantown, MD, USA). The purified PCR products were sequenced using the same primers (27F and 1492F) through the Sangar dideoxy method by GenoTech Co., Daejeon, Republic of Korea. Then, the BLAST tool of the National Center for Biotechnology Information (NCBI) was used to compare the 16S rRNA gene sequences of our isolates with those in the GenBank database [123]. Then, 12 sequences that were highly similar to our amplified 16S rRNA genes were subjected to multiple sequence alignment with our sequences, followed by the generation of a phylogenetic tree using the neighbor-joining method with the MEGA Software (version 11.0.13) (https://www.megasoftware.net/) [17].

4.3. Fermentation and Extraction of Metabolites

The seed culture of *Streptomyces* species was carried out in Tryptic Soy Broth (TSB) medium (Tryptone 17.0 g, Soytone 3.0 g, Glucose 2.5 g, Sodium Chloride 5.0 g, Dipotassium Phosphate 2.5 g, pH 7.3 ± 0.2 at 28 °C; volume 1 L water). After sufficient growth of *Streptomyces* species, 1 mL (1%) of bacterial suspension was transferred into 100 mL of freshly prepared TSB medium for fermentation (for production of secondary metabolites). The incubation was conducted at 28 °C for 5–7 days at 180 rpm in a shaking incubator until bacterial growth reached the stationary phase. Secondary metabolites were harvested by mixing an equal volume of ethyl acetate with culture broth. The clear supernatant was transferred into a clean and dry beaker and evaporated in a water bath at 37 °C for 2–3 days to obtain the crude bacterial extracts. Then, the dried extract was placed at 4 °C until use [124].

4.4. Antimicrobial Assays

The primary screening of the isolates was carried out in Mueller Hinton Agar (MHA) medium by a perpendicular streaking method. The agar-well diffusion method was used for the secondary screening of crude extracts [125]. In this method, the standard culture of test organisms was swabbed over the MHA medium with the help of sterile cotton buds. For this, Gram-positive pathogen *Staphylococcus aureus* ATCC 43300, and various Gram-negative pathogens, *Shigella sonnei* ATCC 25931, *Salmonella typhi* ATCC 14028, *Klebsiella pneumoniae* ATCC 700603, and *Escherichia coli* ATCC 25922 were tested. These tested pathogens were incubated in Mueller Hinton Broth (MHB) medium at 37 °C for 24 h. Then, their turbidity was adjusted to that of standard 0.5 McFarland (1.5×10^8 CFU/mL) for further use. Then, the wells were made with the help of sterile cork borers of 6 mm in diameter. Finally, the wells were filled with the positive control (1 mg/mL neomycin), negative control (50% DMSO), and a working solution of extract dissolved in 50% DMSO. At last, the plates were incubated at 37 °C for 24 h and observed for the clear zone of inhibition.

The MIC and MBC of the crude extract were determined by the broth microdilution method according to the Clinical Laboratory Standard Institute (CLSI) [126]. A series of two-fold dilutions of extract were prepared directly in sterile 96-well microdilution plates containing MHB to obtain a range of concentrations. The bacterial inoculum was added at a final concentration of 1.5×10^8 CFU/mL by diluting 1:100 after matching the turbidity of the 0.5 McFarland turbidity culture in MHB. Finally, 30 µL of bacteria were added to each well except for the negative control. The MBC was determined by streaking the good contents onto nutrient agar plates, followed by incubation for over 18 h at 37 °C [127].

4.5. Metabolic Comparison

Ethyl acetate (EA) extracts of *Streptomyces* species were then subjected to liquid chromatography-high resolution tandem mass spectrometric analysis (LC–HRMS/MS) by employing an Agilent G6545B quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a heated electrospray ion source at Sungkyunkwan University, Suwon, Republic of Korea. For MS/MS analysis, four samples (BT1, BT2, BT3, and PC1) were prepared by dissolving EA extracts in HPLC-grade solvent (methanol) at a concentration of 1 mg/mL. A volume of 150 µL from each sample was transferred to HPLC autosampler vials. Chromatographic separation was achieved using an Acquity® (Indio, CA, USA) UPLC BEH reverse-phase C18 column (150 mm × 2.1 mm, 1.7 µM). The mobile phases, acidified with 0.1% formic acid, consisted of H_2O (A) and acetonitrile (B). The composition of the organic solvent was used as follows: 5% from 0.00 to 2.00 min, 20% at 5.00 min, 100% at 20.00 min, and then returning to 5% from 23.00 to 25.00 min. The injection volume for each sample was maintained at 3 µL, and a constant flow rate of 0.5 mL/min was maintained. The MS/MS data acquisition was performed using the electrospray ionization (ESI) technique in positive ion mode with a m/z range of 50–1200 Da, collision energies set at 15 V and 40 V, and a full width at half maximum (FWHM) of 3000.

The raw data (.d format) were converted into .mzXML format [128] and further annotated using CSI: FingerID, which is a graphical interface incorporated in SIRIUS software (version 5.8.0) [129]. The calculated mass, absolute error, RDBE, and molecular formulae were generated by MestReNova software (version 12.0.0) (accessed on 10–30 December 2023) and were compared with the formula generated by SIRIUS. Furthermore, the annotated compounds were validated via the literature survey using SciFinder (https://scifinder-n.cas.org/, accessed on 12–14 January 2024), and natural products-based databases such as PubChem (https://pubchem.ncbi.nlm.nih.gov/, accessed on 25–30 December 2023), ChemSpider (https://www.chemspider.com/, accessed on 25–30 December 2023), Natural Products Atlas (https://www.npatlas.org/, accessed on 25–30 December 2023), LOTUS (https://lotus.naturalproducts.net/, accessed on 25–30 December 2023), and libraries search using SIRIUS software (version 5.8.0). SIRIUS score, generated by software, serves as a parameter for gauging the confidence of molecular annotation, with a higher score indicating greater confidence in the annotation.

4.6. GNPS-Based Molecular Networking Analysis

EA extracts of four *Streptomyces* species were prepared for MS/MS analysis by dissolving them in HPLC-grade MeOH (1 mg/mL), with 150 µL of each sample transferred to an HPLC autosampler vial. Metabolomic profiling was conducted using an Agilent G6545B quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a heated electrospray ion source (HESI). Chromatographic separation was achieved using an Acquity® UPLC BEH reverse-phase column C18 (150 mm × 2.1 mm, 1.7 µM). The mobile phase consisted of 0.1% formic acid in H_2O (A), and acetonitrile (B) in varying proportions: 5% (B) from 0 to 2 min, 5–20% (B) from 2 to 5 min, 20–100% (B) from 5 to 20 min, 100% (B) from 20 to 23 min, and 100–5% (B) from 23 to 25 min. Each sample was injected at a volume of 3 µL, with a flow rate of 0.3 mL/min maintained. MS/MS analysis was conducted using electrospray ionization (ESI) in positive ion mode. Spectral hits were performed using a modified version with an m/z range of 50–1700, with collision energies set at 15 V and 40 V, capillary voltage (2.5 kV), and a full width at half maximum (FWHM) of 3000 [129]. The raw data '.d format' files were first converted to '.mzXML' format using open-source MSConvert software (https://proteowizard.sourceforge.io/download.html) (Version: 3.0). To upload the files, the recommended FTP client WinSCP was utilized, and they were transferred to the GNPS platform. Visualizing the MS/MS data followed established GNPS-based procedures (accessed on 17 January 2023). Molecular networks generated in GNPS were further exported

to Cytoscape (version 3.9.1.) in '.graphml' format to enable customized visualization and additional analysis.

5. Conclusions

Streptomyces genus can produce a wide range of bioactive secondary metabolites that have good efficacy against several MDR pathogens. In this study, *Streptomyces* species were isolated from the soils collected from various habitats in Nepal and characterized by using 16S rRNA gene sequencing. Further, the ethyl acetate extracts of *Streptomyces* sp. were subjected to LC-HRMS/MS analysis and GNPS-based molecular networking. We found a similar metabolite profile in the mentioned species. Thirty-seven different secondary metabolites encompassing a range of compounds, including polypeptides, bacterial alkaloids, amino compounds, and diketopiperazines were annotated in our study. These metabolites, specifically diketopiperazines are reported to demonstrate an effective antimicrobial activity based on existing literature. In addition, to the best of our knowledge, four metabolites, namely cyclo-(Ile-Ser), 2-n-hexyl-5-n-propylresorcinol, 3-(6-methylpyrazin-2-yl) methyl)-1H-indole and cyclo-(D-Leu-L-Trp) were reported for the first time in *Streptomyces* species. In addition, these findings contribute new additional value by revealing new sources for the isolation of these metabolites, which can aid in the drug discovery process. Furthermore, despite being isolated from different ecological niches, we observed similar metabolome profiles in the studied species. These results could be supported by the fact that all the species were cultivated under identical culture media, temperature, and conditions. Therefore, further study can be conducted on the *Streptomyces* species by varying fermentation media and culture conditions to unveil new metabolites. Moreover, the newly annotated metabolites in this study can further be validated through NMR, followed by their biological evaluation, which could facilitate the drug discovery process.

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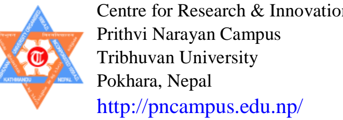

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RESEARCH ARTICLE

Identification of Secondary Metabolites from Actinomycetes Isolated from the Hilly Region of Nepal

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ABSTRACT

Actinomycetes are a unique class of Gram-positive bacteria with a fungus-like filamentous mycelium. They are often called 'antibiotics factories' because they can produce many potent secondary metabolites. At present, multidrug-resistant (MDR) pathogens are the main problem for humans and animals and to overcome this problem, more potent antibiotics are urgently required. Fortunately, Nepal has great geographical and climatic diversity serving as a home for various actinomycetes. This study identified several actinomycetes using morphological and biochemical assays from seven soil samples collected from different elevations in Nepal. The primary objective of this study was to screen the potent metabolites-producing actinomycetes. For this purpose, antimicrobial assays of seven isolates were carried out against *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 14028, *Shigella sonnei* ATCC 25931, and *Staphylococcus aureus* ATCC 43300. The sample BT36 demonstrated the highest zone of inhibition against the tested microorganisms among others. Similarly, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of all isolates were carried out. Among them, the MIC of samples BT36, PT7, and AB1 were found to be 1.952 mg/mL, 0.012 mg/mL, and 0.029 mg/mL, respectively. Ethyl acetate extracts of resulting potent isolates PT7 and AB1 were subjected to mass spectrometric analysis. A total of 22 bioactive compounds were annotated from liquid chromatography-high resolution mass spectroscopy (LC-HRMS), including epopromycin A, myxopyronin B, gilvocarcin HE, and okilactomycin A. It is concluded that this study will be useful in setting up a strategy to isolate useful secondary metabolites from soil microbes from the hilly region of Nepal.

KEYWORDS: Soil microbes, secondary metabolites, antimicrobial assays, mass spectrometry

INTRODUCTION

Soil microbes are an excellent source of therapeutically relevant compounds for isolation and screening. Various natural products have been important sources for creating and discovering new drugs from the early days (Newman & Cragg, 2020). Actinomycetales are a significant category of soil microbes. One of the biggest taxonomic groups in the bacterium domain is the phylum Actinobacteria, which includes the order Actinomycetales, containing the actinomycetes. Gram-positive bacteria in this phylum have high DNA Guanine-Cytosine (G-C) contents; these contents range from 51% in some *Corynebacterium* to over 70% in *Streptomyces* and *Frankia* (De Simeis & Serra, 2021). Even though actinomycetes are widely distributed, very few of them have been investigated and tested in the past.

A study recently carried out has demonstrated that interactions between different species of bacteria, facilitated by small-molecule natural products, produce a surprising range of makeup in bacteria that live in soil, especially in species of *Streptomyces* and *Bacillus* (Traxler & Kolter, 2015). Actinomycetes can reproduce binary fission and generate vegetative or aerial mycelia. Due to the formation of the volatile organic chemicals geosmin and 2-methylisoborneol, both *in vitro*, in cultivation, and natural environment, they have the familiar scent of moist soil (Cheng et al., 2020). *Streptomyces* species synthesize a large variety of naturally occurring chemicals with a broad range of structural variants, including glycopeptides, aminoglycosides, terpenes, tetracyclines, and macrolides. For example, *Streptomyces hygroscopicus* produces around 180 metabolites with different bioactivities (Salwan & Sharma, 2020). The *Streptomyces* genome has more than twenty gene clusters that code for specific secondary metabolites of more clinical significance, such as drugs that might slow the spread of antimicrobial resistance (Alam et al., 2022). *Streptomyces* is a well-known prolific generator of important bioactive compounds and typically makes up a significant fraction of the overall actinomycetes population in natural soil habitats. One of the major risks to modern development, food security, and global health is antibiotic-resistant bacteria. A global report on the surveillance of antimicrobial resistance of the World Health Organization (WHO) states that bacterial resistance to commonly prescribed drugs in the treatment of diseases has drastically developed in many regions of the world. Similarly, many studies have showed that actinomycetes provide 80% of the antibiotics used in medicine globally (Elbendary et al., 2018).

Most of the antimicrobials are found in actinomycetes, specifically in the *Streptomyces* genus. The produced chemicals encompass all major drug classes that are now used in clinics, such as glycopeptides, tetracyclines, macrolides, aminoglycosides, and beta-lactams (Mast & Stegmann, 2019). Only about 100 of over 5000 antibiotics that have been found in filamentous fungi, Gram-positive bacteria, and Gram-negative bacteria have been utilized in commercial medicine to treat illnesses in people, animals, and plants. However, because of their importance, natural compounds with intriguing bioactivities have recently piqued the curiosity of scientists (Aksenov et al., 2021). More than 95% of all actinomycetes found in soil are *Streptomyces*, which are often present at densities of 10^6 to 10^9 cells per gram of soil. While certain species pose serious health risks to higher plants and animals, many others do not (Ait Barka et al., 2015).

A major worldwide health problem is microorganisms that are resistant to antibiotics, known as anti-microbial resistance (AMR). Clinically relevant microorganisms with MDR strains and an alarmingly rapid pace of worldwide spread have been described in recent years (Andersson et al., 2014). The fast introduction of novel diseases and the development of MDR have increased the demand for new antimicrobial medicines more than ever (Clardy & Walsh, 2004). Actinomycetes are one

of the most significant bacterial groups due to their capacity to generate a large number of physiologically active secondary metabolites that are quite successful in preventing microbial infections (Ganesan et al., 2017).

In Nepal, very little research has been done on soil actinomycetes, and no new compounds have been isolated from soil actinomycetes. Nepal is rich in biological diversity; therefore, exploring those exploited habitats and unexplored soil can be an excellent source to produce antibiotics that can combat MDR. The study of the microbial class known as endophytic actinobacteria is still in its infancy. Endophytic actinobacteria are special in that their relationship with plants confers certain biological and chemical characteristics (Bernardi et al., 2019). Nepal is a diversely physically split nation with three distinct regions: the terai, the hills, and the mountains. Similar microflora may be observed in those soil types and altitudes, which raises the prospect of varying the distribution of actinomycetes that produce antimicrobials (Gurung et al., 2009).

Overall, to address the issues raised above, the primary objective of this study was to use LC-HRMS techniques to profile secondary metabolites obtained from actinomycetes isolated from the soil of the hilly region of Nepal, along with their antimicrobial activities. In this study, various secondary metabolites were listed, which can be further analysed and extracted for further study.

MATERIALS AND METHODS

Collection of Soil Samples

The soil samples were collected from various regions of Nepal, including drainage areas, riverbanks, woodlands, compost, and rhizospheres' soils. Different ecological places were selected for the isolation of potential actinomycetes. To isolate actinomycetes, the soil samples were taken at a depth of 10 to 15 cm where the microbial population is high. They were kept in a clear sterile zip bag and brought to the Central Department of Chemistry, Tribhuvan University, and were stored at 4°C till further use (Saadoun et al., 1999).

Isolation of Soil Actinomycetes

One gram of each soil sample was taken and serially diluted with 10 mL of sterile water. This activity was followed by a homogenous mixture, which was prepared by vortexing for 1-2 minutes. As the homogenous mixture consists of many bacteria, it must be diluted. To lower the bacterial concentration, three-fold serial dilutions were carried out. Afterward, a volume of 100 µL of the bacterial suspension was evenly spread on ISP4 (International Streptomyces Project medium 4) plates (ISP4: 10 g starch, 1 g calcium carbonate, 1 g dipotassium phosphate (K_2HPO_4), 1 g magnesium sulfate, 1 mg of ferrous sulfate, 1 mg zinc sulfate, 1 mg manganese chloride, 18 g Bacto agar, 2 g ammonium sulfate, 1 g sodium chloride, and 1000 mL autoclaved distilled water at pH 7.0 ± 0.1). Likewise, nalidixic acid (20 mg/L) and cycloheximide (50 mg/L) were added to the ISP4 medium to prevent the development of gram-negative bacteria and fungi, respectively. These plates were incubated at 28 °C for seven days (Kharel et al., 2010). Finally, after the growth of actinomycetes, they were preserved at 4°C till further use.

Biochemical Analysis of Isolates

The isolated actinomycetes colonies were examined under a microscope for pigmentation, color, substrate, and aerial mycelium. Similarly, the microscopic identification was carried out by the Gram-staining method as suggested by Bergey's Manual of Systematic Bacteriology, Second Edition, Vol, 5, The Actinobacteria, Part A. In addition, the isolated bacteria were identified using biochemical assays. A standard

protocol for biochemical characterization was followed when performing a variety of biochemical experiments (Singh et al., 2013). The oxidation of tetramethyl phenylenediamine (TMPD), catalase, and oxidase activities were examined, along with the generation of oxygen bubbles in a 3% hydrogen peroxide. On a plate with isolates that had been cultured for 6-7 days at 28 °C, Gram's iodine was flooded to check for starch hydrolysis (Bérdy, 2005). This activity was followed by the use of 1 % peptone water and phenol red, which was an indication for a test on the fermentation of carbohydrates. The physiological attribute was assessed thereafter.

Shake Flask Fermentation and Extraction of Secondary Metabolites

The dry mass method was used to analyze the bacterial growth curve. This gives information regarding the best time for harvesting secondary metabolites after fermentation. Using this technique, 1 mL of bacterial culture was taken in a 2 mL Eppendorf tube and centrifuged for 10 minutes at 4000 rotations per minute (rpm). The residue was dried and the supernatant was discarded. After it was completely dried, the dry mass was measured and noted. The identical process was carried out repeatedly over several days, with measurements made precisely every 24 hours until a steady mass was noticed (Li & Mira de Orduña, 2010). Before fermentation, the seed culture of individual isolated colonies was carried out in a Tryptic Soy Broth (TSB) medium at 28 °C for 5-7 days at 180 rpm in a shaking incubator. When there was sufficient growth of the isolates, 1 mL of bacterial suspension was taken and put into 100 mL of TSB medium for fermentation. In a shaking incubator, the fermentation was conducted at 28 °C for 5-7 days at 180 rpm.

Using the ethyl acetate extraction procedure, crude extracts were obtained. This was accomplished by adding an equivalent volume (100 mL) of ethyl acetate to the bacterial suspension and thoroughly mixing it in the separating funnel. The crude bacterial extract was obtained by transferring the clear supernatant into a dry and clean beaker and allowing it to evaporate in a hot water bath at 37 °C for 2-3 days. To get the desired extract, it was dissolved in 2 mL of ethyl acetate, moved to an Eppendorf tube, and then evaporated for 24 hours at 37 °C in a water bath. In the final step of this process, the dried extract was kept at 4 ˚C until further use.

Antimicrobial Activity of Extracts

Primary Screening

The primary screening of the isolates was performed by using the perpendicular streaking method in the Mueller Hinton Agar (MHA). The isolated bacteria were cultured at 28˚C after being streaked linearly across the plate's breadth for 5-7 days. After the growth of the isolates, the pure suspension of tested pathogens, compared with 0.5 McFarland, were streaked perpendicularly to the isolates. Then, these plates were incubated at 37 ˚C for 24 hours, and the antimicrobial assays of the isolates were carried out against test organisms (Bizuye et al., 2013).

Secondary Screening

The agar-well diffusion method was utilized for secondary screening of crude extracts. In this method, the standard culture of test organisms was swabbed over the MHA medium with the help of sterile cotton buds. To perform this test, Gram-positive pathogen *Staphylococcus aureus* ATCC 43300, and various Gram-negative pathogens, *Shigella sonnei* ATCC 25931, *Salmonella typhi* ATCC 14028, and *Escherichia coli* ATCC 25922 were tested. These tested pathogens were incubated in Mueller Hinton Broth (MHB) medium at 37 °C for 24 hours. Then, their turbidity was adjusted to that of

standard 0.5 McFarland ($1.5x10^8$ CFU/mL) for further use. After that, a sterilized cork borer was used to make the wells. Finally, the wells were filled with the positive control (1 mg/mL neomycin), negative control (50 % DMSO), and a working solution of extract dissolved in 50% DMSO. Finally, the distinct zone of inhibition was observed during the 24-hour incubation at 37 °C (Sapkota et al., 2020).

Determination of MIC and MBC

The MIC and MBC of the potent extract were ascertained by the Resazurin-based broth microdilution method according to the Clinical Laboratory Standard Institute (CLSI) (Andrews, 2001). To perform this activity, tested bacteria, both Gram-negative bacteria *(E. coli* ATCC 25922) and Gram-positive bacteria (*S. aureus* ATCC 43300) were grown in MHB media for 24 hours in an incubator at 37 °C. After that, a series of 100 µL extract in duplicate (two times, the first and second wells of the first column contained the same extract) were loaded by 100 µL MHB solution in sterile 96-well microdilution plates and, then, 8-fold serial dilution was performed to get various concentrations. After matching the turbidity of MHB with 0.5 McFarland, the bacterial inoculum concentration was made of $1.5x10^8$ CFU/mL by diluting 1:100. Finally, all the wells were filled with 30 µL bacteria, except for the negative control. In this process, the common medication that was employed as a positive control was neomycin. After placing a sterile lid on the plate, it was incubated at 37 °C for 24 hours. Following a 24-hour incubation, 5 µL of resazurin (0.015%) was introduced into each well of the 96-well plate, and it was then incubated for 3–4 hours at 37 °C. The wells that had bacterial growth were pink, while the wells that did not have bacterial growth were blue. MIC was found to be the one that prevents bacterial growth. The content of the wells was streaked onto nutrient agar (NA) plates, which were then incubated for more than 18 hours at 37 ºC, to measure the MBC, necessary to kill bacteria at a specified duration (Sambrook et al., 1989).

Analysis of Metabolites by LC-HRMS

The extracts PT7 and AB1 were subjected to LC–HRMS, which was performed at the CSIR-Central Drug Research Institute, Lucknow, India, at the Sophisticated Analytical Instrument Facility (SAIF). The samples were analyzed using the Agilent 6520 Accurate-Mass Q-TOF Mass Spectrometer, which was equipped with a G1311A quaternary pump, G1329A autosampler, and G1315D diode array detector (DAD). A 4 µL sample was employed for analysis. Acetonitrile (ACN), 5 mM acetate buffer, and water were added to a gradient system over 25 minutes at a flow rate of 0.5 mL/min in LC-MS separation. The starting condition was 5% ACN for 0.1 minutes, which proceeded by 30% ACN for 10 minutes, 80% ACN for 32 minutes, and then returning to the starting circumstances. The temperature of the column was maintained at 30 °C for the entire analysis. After passing through the DAD flow cell, the column elute was routed to the Q-TOF HRMS fitted through an electrospray interface (ESI). For the MS analysis, an ESI-positive ionization mode was employed, with mass ranges of 100–2000 Da.

RESULTS

Sample Collection and Isolation

A total of 7 soil samples were taken from 1900 m to 4919 m altitudes in various parts of Nepal. The list of soil samples and respective locations were presented in the supplementary data in Table S1. From soil samples taken from various locations, yet-to-be-explored, and unspoiled parts of Nepal, seven distinct actinobacteria were identified

after several rounds of routine screening among hundreds of bacterial colonies. The morphological traits resembled those of actinomycetes; they were rough, tough, and elevated; they were also slightly deep-grown. The bacterial isolates exhibited different colors: gray, and white, and are shown in Figure S1 (A and B). The presence of hair threads-like structures of mycelia observed in the Gram stain and similar colony characteristics to actinobacteria indicated that all strains were Gram-positive filamentous actinobacteria as shown in Figure S2. Based on the series of biochemical tests, morphological features, and macroscopic examination, the isolated colonies were found to be Gram-positive actinomycetes.

Biochemical Characterization

Several biochemical assays were performed to ascertain if the isolated bacteria were Gram-positive or Gram-negative. To identify Gram-positive bacteria, tests for oxidases, starch hydrolysis, nitrate reduction, mobility, and catalase were performed. Some of the representative colonies were selected for the tests, and the results are listed in Table 1 below.

Table 1

Showing Various Biochemical Tests for Selected Colonies (only representative results are shown)

Samples	Catalase	Oxidase	Starch hydrolysis	Nitrate reduction	Mobility
BT5	+	+	-	-	-
BT13	+	+	+	-	-
BT33	-	-	+	+	-
BT36	-	+	+	+	-
BT39	+	+	-	-	-
PT7	-	+	-	-	+
AB1	+	+	-	-	-

Note: Sign (+) for positive test results and sign (-) for negative test results

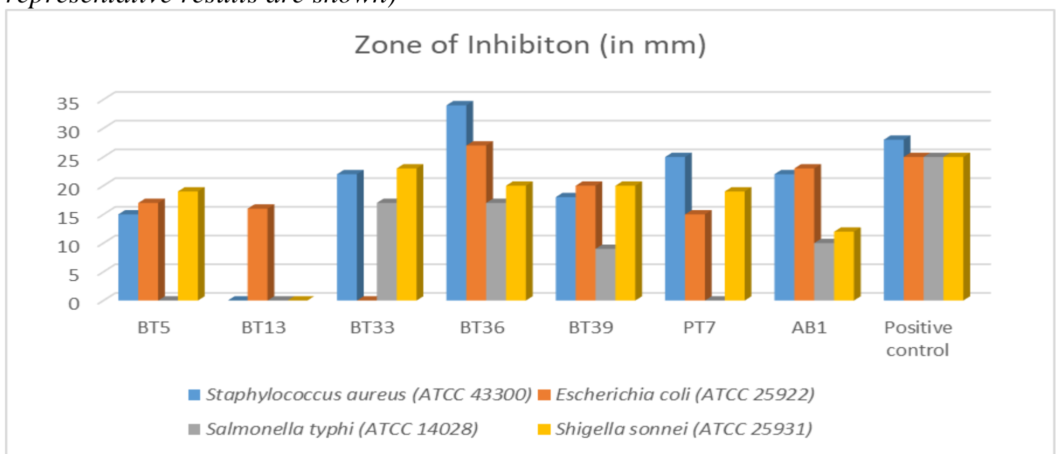
Antimicrobial Activity

All bacterial extracts showed an effective zone of inhibition against both Gram-negative and Gram-positive bacteria. The zones of inhibition of respective bacterial extracts against tested pathogenic bacteria are exhibited in Figure 1. For positive control, 1 mg/mL neomycin was used, and 50 % DMSO solution was used as a negative control. Similarly, a concentration of 20 mg/mL was used for our extracts. Supplementary data in Figure S3 demonstrated the antibacterial efficacy of potent extracts.

As shown in Figure 1 below, the bacterial extracts with the highest zone of inhibition against the ATCC strain of bacteria were BT36, PT7, AB1, and BT33. However, BT36 exhibited the maximum zone of inhibition against *S. aureus* among these extracts, while BT36, AB1, and BT39 showed the acceptable zone of inhibition against *S. typhi, E. coli*, and *S. sonnei*, respectively.

Figure 1

Zone of Inhibition of Bacterial Extracts against the Tested Strain Bacteria (only representative results are shown)



MIC and MBC

MIC and MBC of BT5, BT13, BT33, BT36, and BT39 were determined against the ATCC bacterial strain, Gram-positive bacteria (*S. aureus* ATCC 43300) and Gram-negative bacteria (*E. coli* ATCC 25922). MIC values of BT5, BT13, BT33, BT 36, BT39, PT7, and AB1 were exhibited 0.638, 2.472, 3.141, 1.952, 1.653, 0.012, and 0.029 mg/mL respectively and positive control neomycin showed MIC 0.625 µg/mL against *E. coli.* Similarly, the MIC values of BT5, BT13, BT33, BT36, BT39, PT7, and AB1 were found at 2.032, 1.573, 1.958, 1.202, 1.115, 1.017, and 1.213 mg/mL respectively and neomycin had MIC of 0.783 µg/mL against *S. aureus.* MBC of BT5, BT13, BT33, BT36, BT39, PT7, and AB1 was obtained 1.276, 4.945, 6.281, 3.905, 3.307, 1.213, and 1.312 mg/mL respectively and neomycin 1.253 µg/mL against *E. coli.* MBC of BT5, BT13, BT33, BT36, BT39, PT7, and AB1 were obtained at 4.064, 3.144, 3.917, 2.405, 2.231, 1.240, and 1.452 mg/mL respectively, and neomycin had MBC of 1.562 µg/mL against *S. aureus.* The results of MBC and MIC are shown in Table S4, Figure S4, and Figure S5.

LC-HRMS Annotation

The raw mass data were analyzed through MestreNova 12.0 software. Each peak was analyzed, detected, aligned, and annotated. Finally, the results were compared with literature and various online database libraries, such as PubMed, ChemSpider, Dictionary of Natural Products, etc. The secondary metabolites annotated through LC-HRMS are shown in Table 2 and Figure 2.

Table 2

Showing the List of Annotated Compounds in PT7 and AB1 Extracts

SN	Annotated Compound	Exact Weight	Observed Mass	Adduct Type	Molecular Formula	DBE	Absolute Error	Sources	References
1	Epopromycin A	412.257	413.265	$[M+H]^+$	$C_{21}H_{36}N_2O_6$	5	4.29	PT7 and AB1	(Tsuchiya et al., 1997)
2	SF2415B2 antibiotic	424.225	425.233	$[M+H]^+$	$C_{26}H_{32}O_5$	11.0	4.70	PT7 and AB1	(Gomi et al., 1987)
3	Gilvocarcin	511.168	512.176	$[M+H]^+$	$C_{27}H_{28}$	14.0	3.72	PT7 and	(Hou et

	HE				O_{10}			AB1	al., 2012)
4	Butyrolactol A	526.314	527.324	$[M+H]^+$	$C_{28}H_{46}O_9$	6.0	4.89	PT7	(Harunari et al., 2017)
5	Epoxomicin	554.368	555.376	$[M+H]^+$	$C_{28}H_{50}N_4O_7$	7.0	2.96	PT7 and AB1	(Nihei et al., 1993)
6	Aldgamycin L	700.403	701.411	$[M+H]^+$	$C_{36}H_{60}O_{13}$	7.0	2.96	PT7 and AB1	(Wang et al., 2010)
7	Neomarinone	424.225	425.233	$[M+H]^+$	$C_{26}H_{32}O_5$	11.0	4.70	PT7 and AB1	(Dickschat et al., 2005)
8	Blasticidin H	440.213	441.221	$[M+H]^+$	$C_{17}H_{28}N_8O_6$	8.0	4.13	PT7 and AB1	(Svidritskiy et al., 2013)
9	Phoxalone	424.209	425.217	$[M+H]^+$	$C_{22}H_{32}O_8$	7	0.72	AB1 and PT7	(Guo & Tao, 2008)
10	Okilactomycin A	434.229	435.239	$[M+H]^+$	$C_{24}H_{34}O_7$	8	1.89	AB1	(Imai et al., 1987)
11	SF2809-IV antibiotic	440.173	441.179	$[M+H]^+$	$C_{27}H_{24}N_2O_4$	17	4.48	AB1 and PT7	(Tani et al., 2004)
12	(4R)-4,5-dihydro-4-hydroxy geldanamycin	578.283	579.293	$[M+H]^+$	$C_{29}H_{42}N_2O_{10}$	10	2.37	PT7 and AB1	(Li & Mira de Orduña, 2010)
13	Janthinopolyenemycin B	412.261	413.269	$[M+H]^+$	$C_{26}H_{36}O_4$	9	2.98	AB1 and PT7	(Rani et al., 2021)
14	8-deoxyheronamide C	433.298	434.306	$[M+H]^+$	$C_{29}H_{39}NO_2$	11	3.69	AB1 and PT7	(Sugiyama et al., 2014)
15	Actinoallolide C	546.356	547.364	$[M+H]^+$	$C_{32}H_{50}O_7$	12.5	1.33	AB1 and PT7	(Xu et al., 2022)
16	Glucopiericidin A	577.325	578.330	$[M+H]^+$	$C_{31}H_{47}NO_9$	9	3.80	AB1	(Shaaban et al., 2012)
17	Gombapyrone A	406.214	407.206	$[M+H]^+$	$C_{26}H_{30}O_4$	8	1.80	AB1 and PT7	(Helaly et al., 2009)
18	Xenocoumacin 2	406.210	407.218	$[M+H]^+$	$C_{21}H_{30}N_2O_6$	8	1.80	AB1	(Park et al., 2009)
19	7,3-di-(γ,γ-dimethylallyloxy)-5-hydroxy-4-methoxyflavone	436.188	437.199	$[M+H]^+$	$C_{26}H_{28}O_6$	13	2.96	AB1	(Ding et al., 2013)
20	Flavofungin	664.419	665.427	$[M+H]^+$	$C_{37}H_{60}O_{10}$	8	0.11	AB1	(URI & BEKESI, 1958)
21	Myxopyronin B	431.230	432.953	$[M+H]^+$	$C_{24}H_{33}NO_6$	11.5	3.47	AB1 and PT7	(Yakushiji et al., 2013)
22	5,7-Trihydroxy-3,4-dimethoxy isoflavone	314.079	353.042	$[M+H]^+$	$C_{17}H_{14}O_6$	5.0	2.3	AB1 and PT7	(Hosny & Rosazza, 1999)

Figure 2

Some Annotated Compounds in Extracts of Isolates PT7 and AB1 (actinomycetes)

Chemical Formula: $C_{29}H_{42}N_2O_{10}$
Exact Mass: 578.283
(4R)-4,5-dihydro-4-hydroxygeldanamycin

Chemical Formula: $C_{21}H_{30}N_2O_6$
Exact Mass: 406.210
Xenocoumacin 2

Chemical Formula: $C_{27}H_{24}N_2O_4$
Exact Mass: 440.173
SF2809-IV

Chemical Formula: $C_{26}H_{36}O_4$
Exact Mass: 412.261
Janthinopolyenemycin B

Chemical Formula: $C_{28}H_{46}O_9$
Exact Mass: 526.314
Butyrolactol A

Chemical Formula: $C_{17}H_{28}N_8O_6$
Exact Mass: 440.213
Blasticidin H

Chemical Formula: $C_{26}H_{32}O_5$
Exact Mass: 424.225
SF2415B2

Chemical Formula: $C_{29}H_{39}NO_2$
Exact Mass: 433.298
8-deoxyheronamide C

Chemical Formula: $C_{36}H_{60}O_{13}$
Exact Mass: 700.403
Aldgamycin L

Chemical Formula: $C_{26}H_{32}O_5$
Exact Mass: 424.225
Neomarinone

Chemical Formula: $C_{17}H_{14}O_6$
Exact Mass: 314.079
5,7-Dihydroxy-3',4'-dimethoxyisoflavone

Chemical Formula: $C_{26}H_{30}O_4$
Exact Mass: 406.214
Gombapyrone A

DISCUSSION

Actinomycetes are popular for being the flexible makers of bioactive metabolites that are antibacterial, anticancer, antifungal, etc. The primary technique for isolating novel antibacterials is traditional (el Karkouri et al., 2019). Soils are most prevalent for the production of bioactive bacteria (Demain & Fang, 2000). For the current investigation, actinomycetes were isolated using seven different soil samples that were taken at different altitudes in Nepal. The isolated soil microbes were identified based on

microscopic examination, biochemical analysis, both primary as well as secondary screening, and the analytical technique LC-HRMS. All the isolates exhibited varied morphological features and under microscopic observation by Gram staining; all the isolates were found to be Gram-positive. Based on microscopic observation, the isolates were further processed for antimicrobial screening, and only a few were found to be good inhibitors of the tested bacterial strains. BT5, BT13, BT33, BT36, BT39, PT7, and AB1, showed a good zone of inhibition for Gram-positive bacteria, and isolates PT7 and AB1 were further subjected to LC-HRMS for the annotation of the possible bioactive metabolites or antibiotics.

Furthermore, these seven samples showed good antimicrobial activity against *E. coli* ATCC 25922*, S. sonnei* ATCC 25931, *S. aureus* ATCC 43300, and *S. typhi* ATCC 14028. Most of the extracts showed an average zone of inhibition against the tested Gram-positive bacteria *S. aureus* ATCC 43300, whereas these extracts seemed less effective against *E. coli* ATCC 25922. Among them, BT36 showed the most effective against *E. coli* ATCC 25922, and *S. aureus* ATCC 43300. The MIC and MBC of all extracts were tested against *S. aureus* and *E. coli* and were shown to be more effective. Because of ongoing contamination and reviving conditions, it can be challenging to isolate actinomycetes from the soil. Thus, a few were carried out for antimicrobial activity and some for different other screening processes. Some of them did not show any growth during the subculture and were discarded. Gram-positive *S. aureus* is responsible for a wide range of clinical diseases. It is still challenging to treat the illness because of the increase in MDR strains like Methicillin-resistant *S. aureus* (MRSA) (Boucher & Corey, 2008). It may be a good idea to continue working with these isolates as the current investigation has shown a notable zone of inhibition against *S. aureus* and *E. coli*, which is further confirmed by the earlier study (Shrestha et al., 2019).

Various biochemical assays were conducted to characterize the biochemical state of Gram-positive bacteria. In addition to contributing to the identification of the microbes for phenotypic characterization, physiological and biochemical parameters also point to significant traits for biotechnological applications (Chater et al., 2010). All these bacterial extracts were negative for the indole test, and positive for the catalase test. A study by Sadiqi et al. (2022) reported the similar results of biochemical analysis. Additionally, LC-HRMS was used to analyze secondary metabolites and perform molecular annotation. This revealed the existence of a variety of bioactive chemicals, including immune suppressant, antibiotic, antifungal, anticancer, and antihelminthic substances. The crude extracts of PT7 and AB1 were found to consist of various types of antibiotics, okilactomycin A, epopromycin A, and aldgamycin L, along with other important bioactive compounds. Erythromycin A is an essential antibiotic that is commonly used in clinical medicine to treat infections brought on by Gram-positive bacteria, according to a variety of literature sources. It is also the primary treatment for many lung infections, including Legionnaire's disease (Kingston, 2000). The soil samples which are from higher altitudes belong to BT36 and BT13 and those of lower altitudes are PT7 and AB1. Furthermore, the antimicrobial efficacy of molecules isolated from BT36 is higher than PT7 and AB1. Thus, this study also suggested that the isolates obtained from higher altitude regions could be a potential source of potent molecules due to the ability acquired by the soil bacteria found in such higher places to deal with harsh conditions.

Finally, this investigation has suggested to isolate actinomycetes that can produce secondary metabolites from a variety of soil samples as they were taken from Nepal's hilly regions.

CONCLUSION

Many bioactive secondary metabolites with good activity against multiple pathogens have been demonstrated to be produced by actinomycetes. In this work, seven soil samples taken from Nepal's hilly regions yielded a considerable number of actinomycetes that were isolated. The potent extracts BT5, BT13, BT33, BT36, BT39, AB1, and PT7 exhibited a significant zone of inhibition against tested pathogenic bacteria even though some of them were multi-drug resistant bacteria and were subjected to LC-HRMS analysis. From LC-HRMS analysis, 22 different secondary metabolites were annotated from PT7, and AB1 extracts, which contain various bioactive metabolites. Here, antimicrobial activities were isolated, screened, and analyzed along with bioactive compounds (secondary metabolites). To sum up, this study found that the diverse altitudes and environmental conditions in Nepal provide favorable conditions for various actinomycetes. Therefore, soils from the hilly region of Nepal have significant potential for discovering novel actinomycetes that can produce potent secondary metabolites (antibiotics).

AUTHOR CONTRIBUTIONS

Conceptualization, N.P., and B.B.T.; methodology, B.B.T., S.S., N.S. and R.P.Y.; software, B.B.T., and S.S.; validation, N.P., K.R.S., and A.D.M.; formal analysis, S.S.; writing—original draft preparation, B.B.T., S.S., and N.S.; writing—review and editing, N.P., R.C.B. and K.R.S.; supervision, N.P.; project administration, N.P.; funding acquisition, N.P. All authors have read and agreed to the published version of the manuscript.

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APPENDIX

Table S1

List of Soil Samples Collected from Different Regions of Nepal

Samples	Places	Altitude	Coordinates	Source of Soils
BT5	Kaski (Pritam Deurali)	2370 m	28.2622° N, 84.0167° E	Forest
BT13	Kaski (Badal Dada)	3420 m	28.2096° N, 83.9856° E	Forest
BT33	Ramechhap (Tame)	2173 m	28.1901° N, 83.9592° E	Near waterfall
BT36	Tilicho	4919 m	28.6833° N, 83.8567° E	Near Lake
BT39	Rolpa	3234 m	28.3816° N, 82.6483° E	Barren land
PT7	Gulmi	2000 m	28.0889° N, 83.2934° E	Agricultural Land
AB1	Gorkha	1900 m	28.2964° N, 84.8568° E	Near Waterfall

Table S2

List of Chemicals Used in This Study

S.N.	Chemicals	Company/Brands
1	Mueller Hinton Agar (MHA)	HiMedia
2	Mueller Hinton Broth (MHB)	HiMedia
3	Tryptic Soya Broth (TSB)	HiMedia
4	Nutrient Agar (NA)	HiMedia
5	Agar-Agar	HiMedia
6	Bacto Agar	HiMedia
7	Ammonium Sulphate	Qualigens Fine Chemicals
8	Zinc Sulphate	Fischer Scientific
9	Ferrous Sulphate	Fischer Scientific
10	Sodium Chloride	Marck Life Science
11	Manganese Chloride	Fischer Scientific
12	Magnesium Sulphate	Marck Life Science
13	Dipotassium Phosphate	Fischer Scientific
14	Calcium Carbonate	Fischer Scientific
15	Soluble Starch	Marck Life Science
16	Neomycin	HiMedia
17	Resazurin	Loba Chemie
18	Dimethyl sulfoxide (DMSO)	Loba Chemie
19	Ethyl acetate	Marck Life Science
20	Cycloheximide	Loba Chemie
21	Nalidixic acid	Sigma-Aldrich
22	Gram's Iodine	Thermo Fisher Scientific

Table S3

List of Instruments Used in This Study

S.N.	Instruments	Chemicals/Brands
1	Biosafety Cabinet	Sanjeev Scientific Udyog
2	Incubator	BEING Scientific
3	Refrigerator	Whirlpool and Samsung
4	Shaking Incubator	Shivaki
5	Vortex	Swirlex
6	Centrifuging Machine	Thermo Scientific
7	Water Bath	Medico
8	Autoclave	Thermo Scientific

Table S4

MIC and MBC of Soil Microbes against E. coli and S. aureus

Extracts	*E. coli*		*S. aureus*	
	MIC Value	MBC Value	MIC Value	MBC Value
BT5	0.638 mg/mL	1.276 mg/mL	2.032mg/mL	4.064 mg/mL
BT13	2.472 mg/mL	4.945 mg/mL	1.573 mg/mL	3.144 mg/mL
BT33	3.141 mg/mL	6.281 mg/mL	1.958 mg/mL	3.917 mg/mL
BT36	1.952 mg/mL	3.905 mg/mL	1.202 mg/mL	2.405 mg/mL
BT39	1.653 mg/mL	3.307 mg/mL	1.115 mg/mL	2.231 mg/mL
PT7	0.012 mg/mL	1.213 mg/mL	1.017 mg/mL	1.240 mg/mL
AB1	0.029 mg/mL	1.312 mg/mL	1.213 mg/mL	1.452 mg/mL
Neomycin	0.625 µg/mL	1.253 µg/mL	0.783 µg/mL	1.562 µg/mL

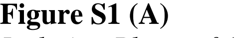


Figure S1 (A)

Isolation Plates of Actinomycetes

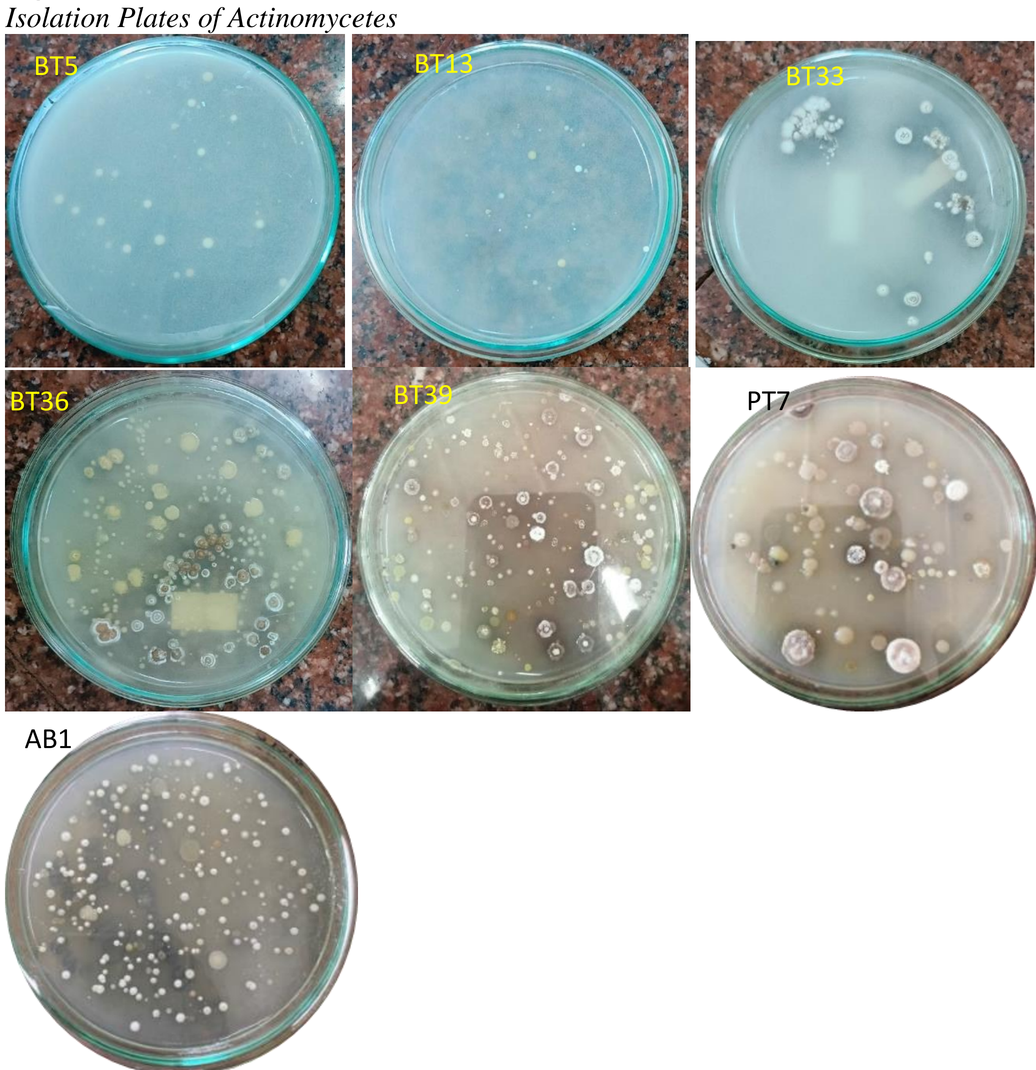


Figure S1 (B)

Substrate Mycelium of Isolated Species

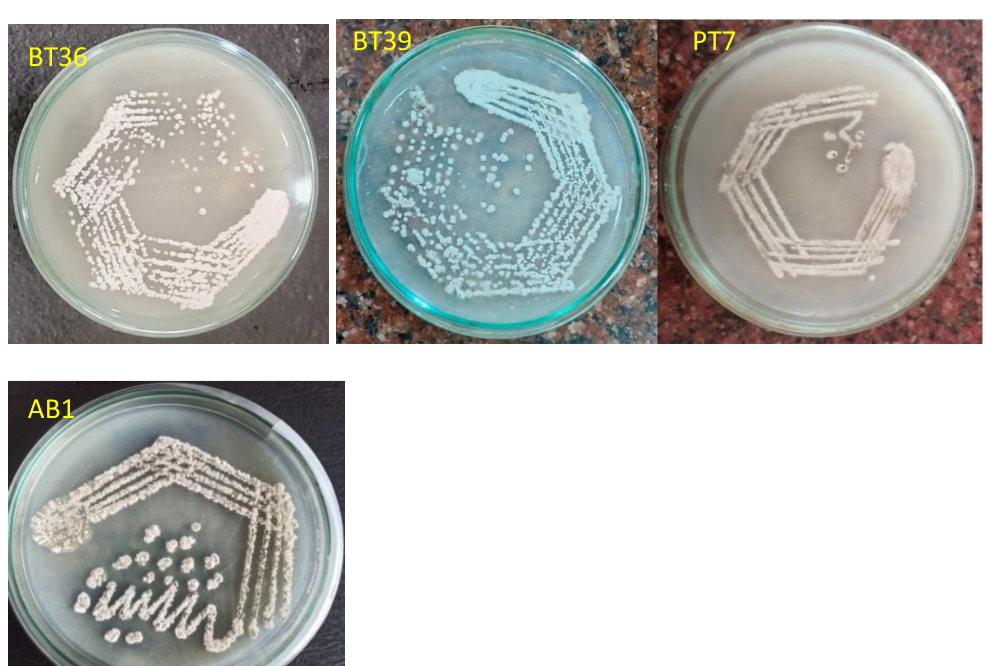


Figure S2

Filamentous Mycelia of Isolated Actinomycetes

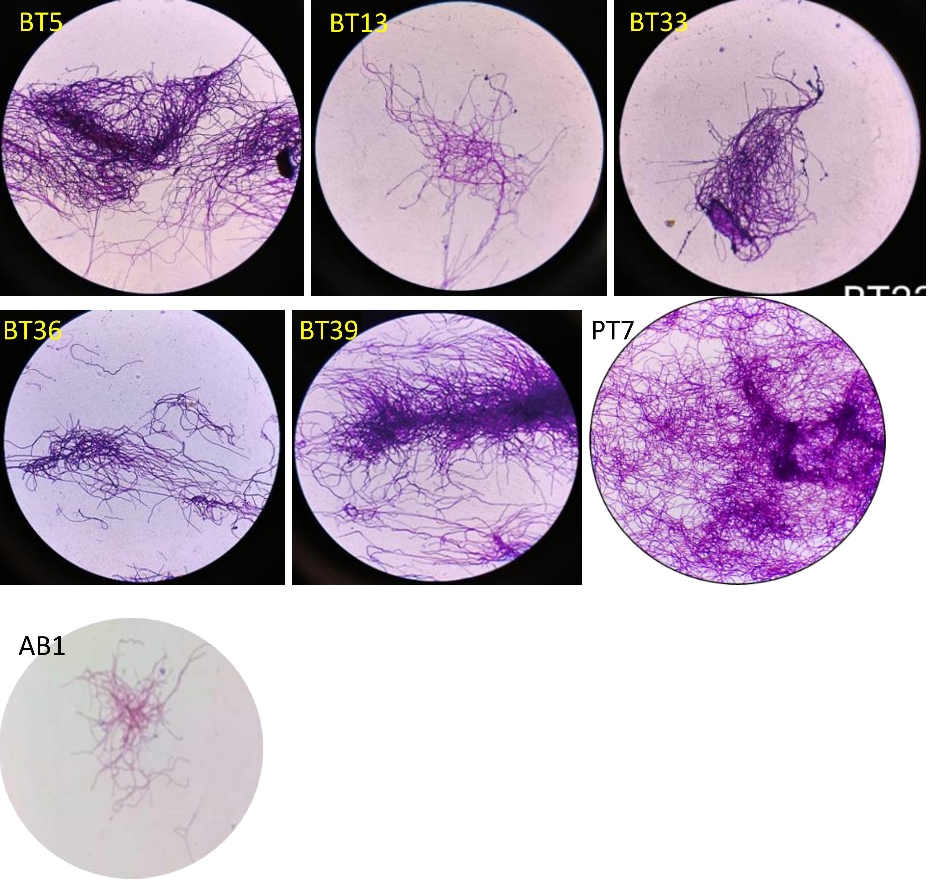


Figure S3

Zone of Inhibition Shown by Various Extracts

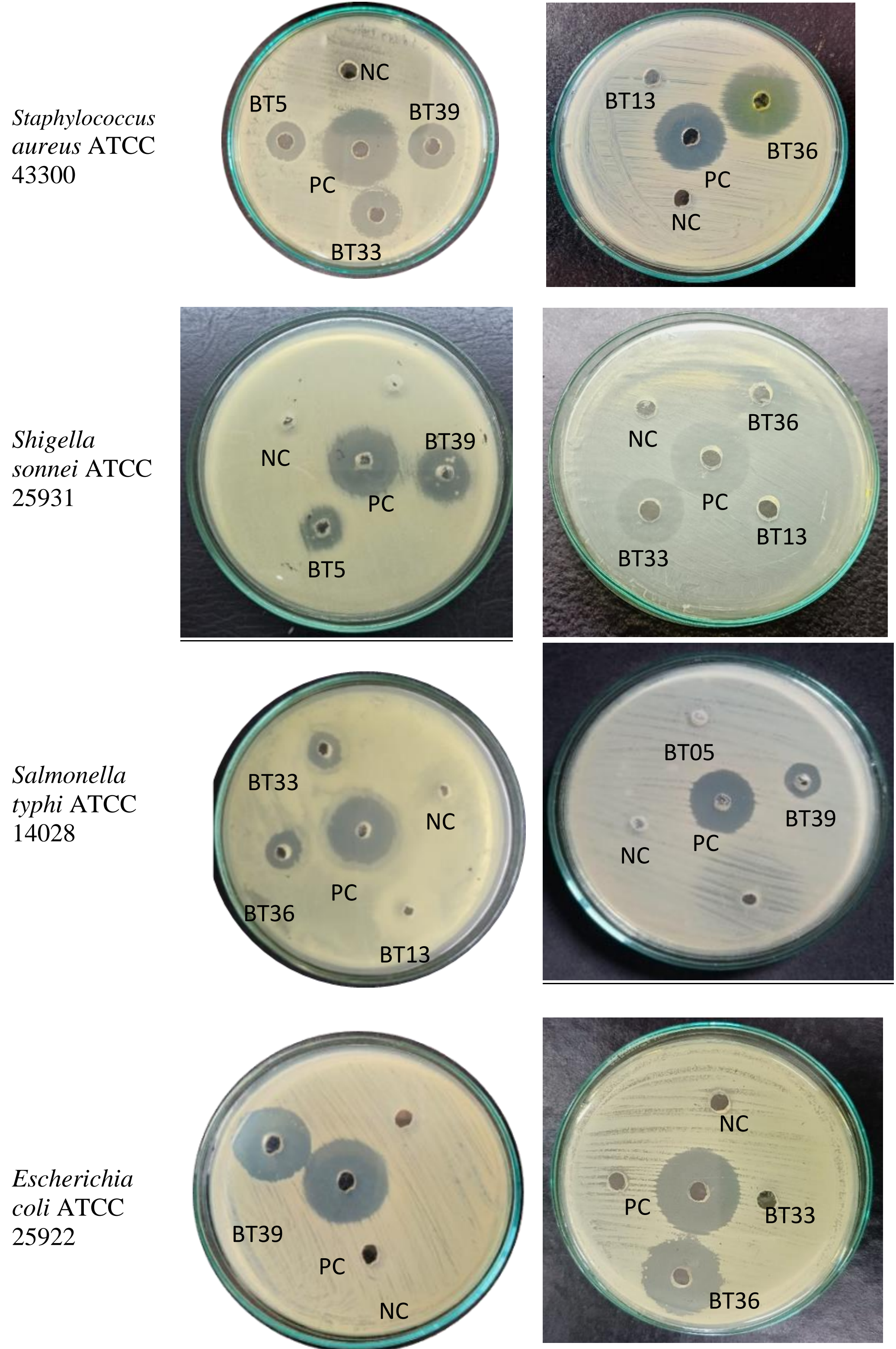
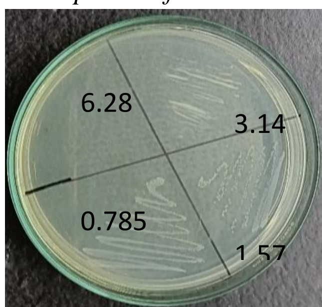
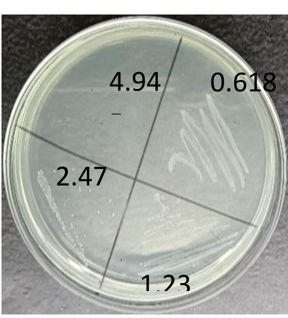


Figure S4

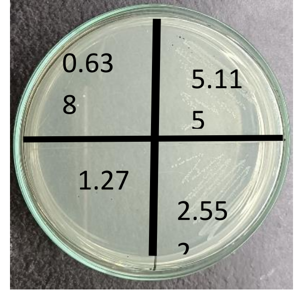
MBC plates of the extracts



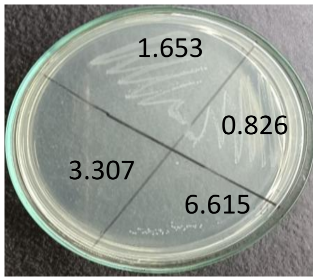
MBC of BT33 (6.281 mg/mL) in *E.coli*



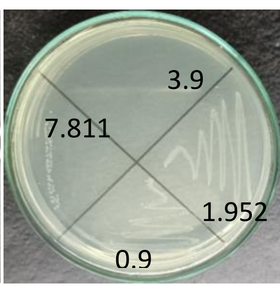
MBC of BT13 (4.945 mg/mL) in *E. coli*



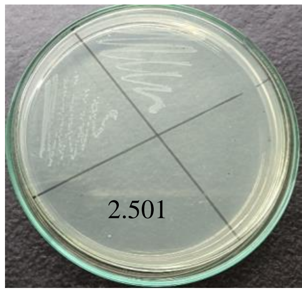
MBC of BT5 (1.276mg/mL) in *E. coli*



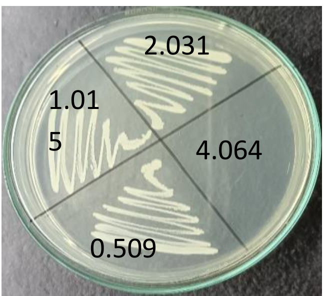
MBC of BT39 (3.307 mg/mL) in *E. coli*



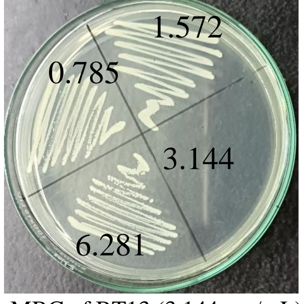
MBC of BT36 (3.905 mg/mL) in *E. coli*



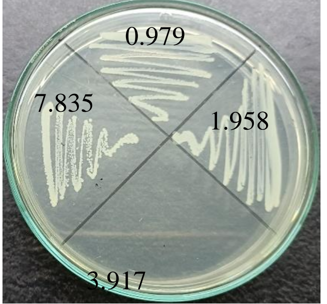
MBC of Neomycin (1.253 µg/mL) in *E. coli*



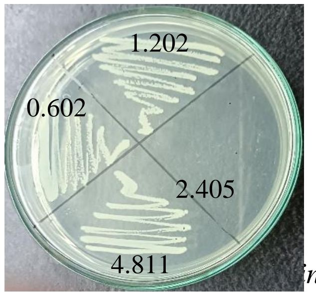
MBC of BT5 (4.064 g/mL) in *S. aureus*



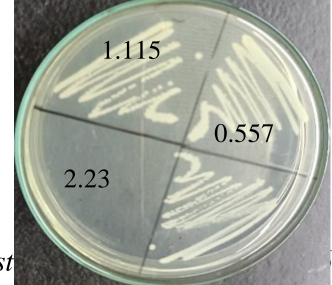
MBC of BT13 (3.144 mg/mL) in *S. aureus*



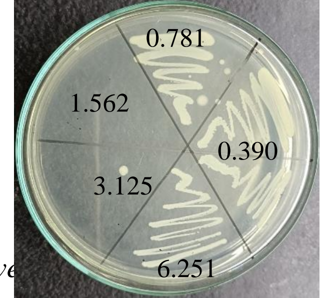
MBC of BT33 (3.917 mg/mL) in *S. aureus*



MBC of BT36 (2.405 mg/mL) in *S. aureus*



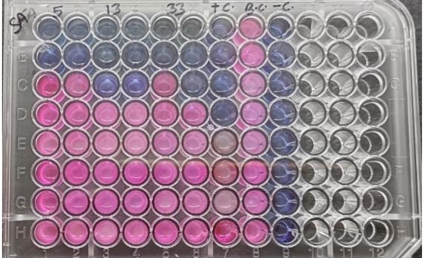
MBC of BT39 (2.231 mg/mL) in *S. aureus*



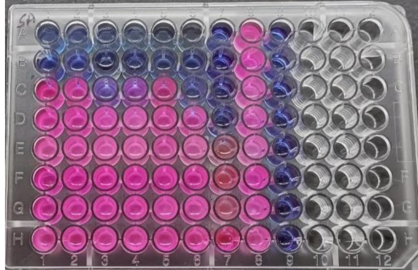
MBC of Neomycin (1.562 µg/mL) in *S. aureus*

Figure S5

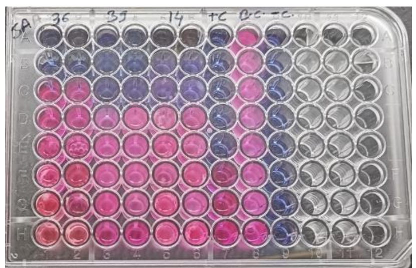
Showing 96 Well Plates with MIC of Each Extract against E. Coli and S. Aureus



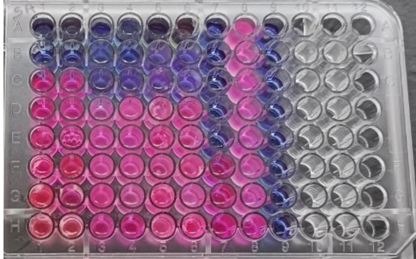
MIC in 96 well plate for BT05, BT13, BT33 and neomycin against *S.aureus* (Lid close)



MIC 96 well plate for BT05, BT13, BT33 and neomycin against *S.aureus* (Lid open)



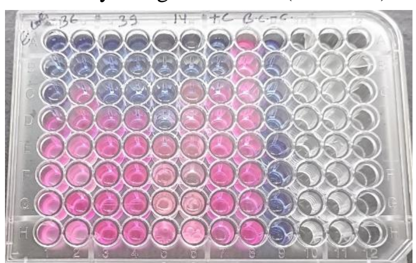
MIC in 96 well plate for BT36, BT39 and neomycin against *S.aureus* (Lid close)



MIC 96 Well plate for BT36, BT39 and neomycin against *S.aureus* (Lid open)

MIC in 96 Well plate for BT05, BT13, BT33 and neomycin against *E.coli* (Lid close)

MIC in 96 Well plate for BT05, BT13, BT33 and neomycin against *E.coli* (Lid open)



MIC in 96 well plate for BT36, BT39 and neomycin against *E.coli* (Lid close)

MIC in 96 well plate for BT36, BT39 and neomycin against *E.coli* (Lid open)

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Review

Technological Advancements for the Detection of Antibiotics in Food Products

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Abstract: Antibiotics, nowadays, are not only used for the treatment of human diseases but also used in animal and poultry farming to increase production. Overuse of antibiotics leads to their circulation in the food chain due to unmanaged discharge. These circulating antibiotics and their residues are a major cause of antimicrobial resistance (AMR), so comprehensive and multifaceted measures aligning with the One Health approach are crucial to curb the emergence and dissemination of antibiotic resistance through the food chain. Different chromatographic techniques and capillary electrophoresis (CE) are being widely used for the separation and detection of antibiotics and their residues from food samples. However, the matrix present in food samples interferes with the proper detection of the antibiotics, which are present in trace concentrations. This review is focused on the scientific literature published in the last decade devoted to the detection of antibiotics in food products. Various extraction methods are employed for the enrichment of antibiotics from a wide variety of food samples; however, solid-phase extraction (SPE) techniques are often used for the extraction of antibiotics from food products and biological samples. In addition, this review has scrutinized how changing instrumental composition, organization, and working parameters in the chromatography and CE can greatly impact the identification and quantification of antibiotic residues. This review also summarized recent advancements in other detection methods such as immunological assays, surface-enhanced Raman spectroscopy (SERS)-based assays, and biosensors which have emerged as rapid, sensitive, and selective tools for accurate detection and quantification of traces of antibiotics.

Keywords: antibiotics; solid-phase extraction; chromatography; capillary electrophoresis; surface-enhanced Raman scattering; biosensors

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1. Introduction

Antibiotics are life-saving drugs, which have made a remarkable revolution in the medical sector in the twentieth century, but many antibiotics are now "endangered species" due to the global emergence of antibiotic resistance. These miracle drugs have substantially improved the life expectancy and health of humans and animals by combating a wide range of infectious diseases [1]. Most of the antibiotics are natural products that are secreted primarily by *Streptomyces* spp. [2]. Most commonly prescribed antibiotics in clinics are glycopeptides, polyethers, sulfonamides, tetracyclines, aminoglycosides, β-lactams,

fluoroquinolones, and macrolides [3]. Among the several classes of antibiotics, most of them are classified based on their chemical structure, action mechanism, action spectrum, and route of administration [4]. Figure 1 depicts the various sources of how antibiotics reach up to our table [5,6]. Similarly, the structures of different classes of antibiotics are shown in Figure 2, and the examples of antibiotics belonging to the different classes are summarized in Table 1. Misuse and overuse of antibiotics in farming and subsequent contamination of the surrounding environment have been significantly linked with the emergence and spread of antimicrobial resistance (AMR). This increase also marks adverse effects on the food chain. A wide array of antibiotics, such as aminoglycosides, β-lactams, fluoroquinolones, and sulphonamides, etc., are reported as possible environmental pollutants by the World Health Organization; these are public health threats [7]. The emergence of antibiotic pollution has led to potential toxic effects on microorganisms, plants, animals, and ultimately humans [4]. When antibiotics are widely used, antibiotic-resistant bacteria evolved due to genetic or mutational alterations, which are also considered a new type of contaminant in the environment [7,8]. The uncontrolled use of antibiotics has made their presence almost everywhere in the environment, including water resources and soils. The wastewater and other biological wastes from farming soils, hospitals, and pharmaceutical industries may contain traces of antibiotics that could mix with water resources if discharged without proper treatment [9]. Hospital wastes are considered a breeding spot of antibiotic-resistant bacteria (ARB) [10]. ARB strains can survive and multiply even in harsh conditions compared to wild strains [11].

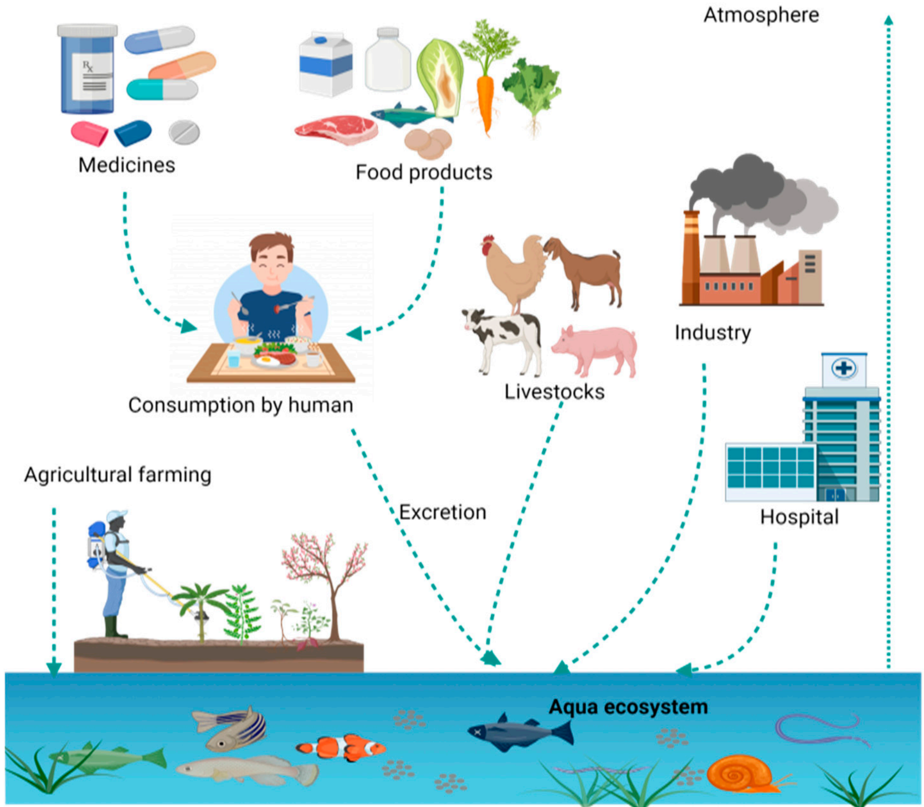

Figure 1. Pathways for antibiotic residues into the environment.

Enrofloxacin Clinafloxacin Ciprofloxacin Ofloxacin

Methacycline Tetracycline Doxycycline

Chlortetracycline Ampicillin Paromomycin

Netilmicin Difloxacin hydrochloride Clarithromycin

Monensin Danofloxacin Cefotaxime

Figure 2. *Cont.*

Figure 2. Structures of some antibiotics isolated from food products.

The presence of antibiotic residues in food products causes unfavorable effects on organ systems resulting in several health complications, such as immunopathological effects, skin allergy, mutagenicity, tuberculosis, liver problems, pneumonia, and even carcinogenicity in humans due to antibiotic-resistant bacteria [12,13]. To protect public health, maximum residue limits (MRLs) of antibiotics and other pharmacologically active substances in foodstuffs of animal origin were established by the European Commission [14,15]. In this connection, there have been significant efforts in the development of robust analytical methods with a combination of techniques such as High-Performance

Liquid Chromatography (HPLC) coupled with Mass Spectrometry (MS), and Liquid Chromatography coupled with tandem mass spectrometry (LC-MS/MS) to monitor antibiotic residues in food products. Antibiotic residues may be present in food products, and it is often difficult to detect because residues are found to be associated with matrices, which interfere with their analysis [16]. In essence, a small portion of the initial antibiotics is likely to be bioactive, which challenges extraction of antibiotic residues as well as detecting and quantifying them [17]. Old-fashioned screening techniques, such as paper-based devices as µPAD, are simple and affordable, but their sensitivity is not enough to capture antibiotic traces in food samples. Therefore, this need has led to the research and development of highly sensitive and selective analytical tools for the detection and quantification of antibiotic residues in complex matrices [18,19].

The food consumed comprises nutrients including, however not limited to, lipids, proteins, and carbohydrates that form a complex food matrix. This matrix may interfere with the signals obtained for analysis; thus, the preconcentration step is carried out to minimize matrix interferences and to increase the sensitivity of detection [20]. The sample pretreatment is a challenging process since many antibiotics are thermally unstable and have no chromophore (e.g., aminoglycosides) [21]. Before instrumental analysis, different preconcentration/extraction processes, such as liquid-liquid extraction (LLE), SPE, liquid-liquid microextraction (LLME), solid-phase microextraction (SPME), and magnetic solid-phase extraction (MSPE), are used to isolate antibiotic residues from different samples [22]. SPE is also widely used for preconcentration and cleaning up steps since it is safer, easily operated, and efficient [23,24].

Various analytical techniques such as chromatographic techniques (LC-MS/MS, UHPLC–MS/MS, HPLC-ELSD), CE, immunological methods, SERS, and biosensors were employed to detect antibiotic residues. Chromatographic techniques are commonly used for the detection of antibiotic residues in food samples for a long period. A technique such as LC-MS/MS has become a mainstream technique for detecting antibiotics accurately and simultaneously in different environmental mediums [25]. Other determination techniques, such as CE [26], Raman spectroscopy [27], and enzyme-linked immunosorbent assays, are also employed in the detection of antibiotic residues [28]. Additionally, biosensors have emerged as an alternative method for screening antibiotic residues in different environmental, food, and biological samples. The use of biosensors in the detection of antibiotic residues is rapidly increasing because biosensors are rapid, sensitive, specific, and require little sample preparation. Additionally, they are affordable, simple to install, and can be operated by personnel with minimal training [29,30].

Table 1. Major classes of antibiotics.

Class	Examples	References
Glycopeptides	Vancomycin, Teicoplanin, Telavancin, Oritavancin, Dalbavancin	[31]
Sulfonamides	Sulfacetamide, Sulfadiazine, Sulfathiazole, Sulfapyridine, Sulfamerazine, Sulfamethazine, Sulfamethoxazole, Sulfasoxazole, Sulfachloropyridazine	[32]
Tetracyclines	Tetracycline, Oxytetracycline, Doxycycline, Chlorotetracycline, Methacycline	[32]
Aminoglycosides	Amikacin, Paramomycin, Dihydrostreptomycin, Hygromycin, Kanamycin, Netilmycin, Spectinomycin, Sisomycin, Streptomycin, Tobramycin, Gentamicin, Neomycin	[32]
B—Lactams	Amoxicillin, Ampicillin, Cloxacillin, Penicillin G	[32]
Macrolides	Erythromycin, Clarithromycin, Tylosin	[32]

Table 1. *Cont.*

Class	Examples	References
Fluoroquinolones	Lomefloxacin, Ciprofloxacin, Enroflaxacin, Danofloxacin, Difloxacin hydrochloride, Clinafloxacin	[31,32]
Polyethers	Lasalocid, Salinomycin, Monensin, Narasin, Nigericin	[33]

2. Extraction of Antibiotics from Food Samples

The selection of suitable pretreatment methods is important due to the complexity of matrices and the low concentration of antibiotic residues in food samples. Solid-phase extraction (SPE) is the most extensively used pretreatment approach for the isolation and preconcentration of trace contaminants in complex samples (Figure 3). This is due to its convenience of use, minimal organic solvent consumption, and high enrichment factor [34]. The activation of the sorbent, percolation/sorption of the analyte in the sample matrix into the sorbent, removal of matrix interferences, and elution and concentration of the analyte with an appropriate technique are the key steps for a simple SPE procedure. However, the mechanism of extraction can vary depending on the nature of the sorbent used, and choosing an appropriate method of SPE for each application is vital [35]. The analysis of numerous classes of compounds using SPE format has used cartridges, different columns as classical SPE sorbents; however, the use of novel sorbent-based materials, such as metal-organic frameworks (MOFs), carbon nanotubes (CNTs), graphene oxide (GO), and molecularly imprinted polymers (MIPs) have brought about very good extraction recoveries with smaller amounts of sorbents, which are discussed below.

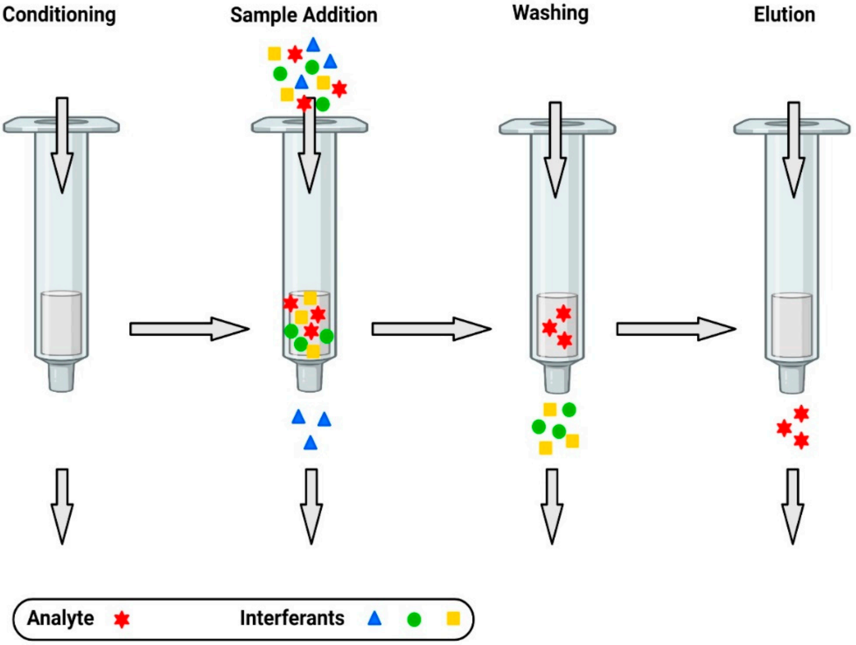

Figure 3. Typical four steps of SPE procedure.

2.1. Classical SPE Sorbents

During the analysis of antibiotics, the extraction and cleanup of target antibiotics from the matrix is an important step. From milk, antibiotics are most commonly isolated using liquid-liquid extraction (LLE) and SPE [36]. The isolation of low molecular mass basic compounds and neutral compounds can be accomplished using carboxy (CX) SPE cartridges and hydrophilic-lipophilic balance (HLB) SPE cartridges, respectively. A tandem SPE cleanup method using mixed cation exchange (MCX) and HLB cartridges compared with single cartridge methods provides significantly improved extraction efficiency with the recoveries of 94% vancomycin, 95% teicoplanin, 99% telavancin, 89% oritavancin, and 91%

dalbavancin, as shown by Deng et al. in their studies for the detection of five glycopeptide antibiotics in milk [37]. Similarly, the extraction of polyether antibiotics including lasalocid, salinomycin, monensin, narasin, and nigericin residues in milk, chicken, chicken livers, and egg samples were performed with acetonitrile and purified by ENVI-Carb SPE columns with an average recovery of the analytes fortified at three levels ranged from 68.2% to 114.3% and limit of quantification (LOQ) obtained for milk and chicken was 0.4 μg/kg, and for chicken livers and eggs, it was 1 μg/kg [33]. Among several cleanup cartridges, including HLB, ENVI-Carb, Silica, Neutral Alumina, and Florisil, ENVI-Carb was found to be the best SPE cartridge for cleaning up the extracts from milk, chicken, egg, and chicken liver. These cartridges were found to be efficient for removing background organic compounds and also reduced operation and sample preparation time [33]. Likewise, for the simultaneous determination of sulfonamides from fish, crab, and shrimp samples, ultrasonic extraction and liquid extraction of n-hexane was used, in which an online SPE cleanup equipped with a mixed cation exchange column (Oasis®MCX) connected with a hydrophilic-lipophilic balance column (Oasis®HLB) was used for sample pretreatment to remove interferences. As a result, the limit of detection (LOD) and LOQ obtained ranged from 1.46 to 15.5 ng/kg, and 4.90 to 51.6 ng/kg, respectively [38].

2.2. New SPE Sorbents

The sorbent is a critical component in determining the performance of SPE and several SPE sorbent materials, including metal-organic frameworks (MOFs), molecularly imprinted polymers, porous organic polymers (POPs), carbon nanotubes, electrospun nanofibers, magnetic nanocomposites, high internal phase emulsion polymers, and others were employed for the extraction of antibiotic residues from food samples [34,39,40]. Among POPs, porous covalent organonitridic frameworks (PCONFs) are an ideal sorbent for sulfonamide antibiotics as these sorbent materials possess high surface area and rich π-electron properties. This method, under optimized conditions, possesses the properties such as wide linear ranges (2.5–1000 ng$\cdot$L^{-1}) and low limits of detection (0.14–2.0 ng$\cdot$L^{-1}) [34]. The rapid extraction of sulfonamide residues (sulfacetamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine, sulfamethoxazole, and sulfisoxazole) in chicken meat and milk was carried out using PCONFs as SPE sorbents and methanol as an eluting solvent, demonstrating the suitability of these materials for the enrichment of polar sulfonamides [34]. Likewise, for the extraction of tetracyclines in chicken, electrospun graphene oxide-doped poly (acrylonitrile-co-maleic acid) nanofibers (E-spun-GO/PANCA-NFs) was fabricated as a novel adsorbent for SPE, which exhibited good stability, large extraction capacity, and excellent extraction efficiency. The result of which provided limits of detection from 20.4 to 44.8 μg/kg, and limits of quantification from 69.7 to 115.5 μg/kg [40]. Similarly, polyacrylonitrile@COFs (PAN@COF-SCU1) electrospun composite nanofibers were developed and used as an absorbent in pipette tip SPE (PT-SPE) for high-efficient extraction of tetracyclines in grass carp and duck samples in which LOD and LOQ were ranged from 0.6 to 3 ng mL^{-1} and 2 to 10 ng mL^{-1}, respectively [41].

Since the widely used SPE cartridges are weak cation-exchange and hydrophilic-lipophilic columns, molecularly imprinted polymers (MIPs) emerged recently as novel sorbents due to their selective extraction of target compounds as a result of strong interaction between MIPs and the target molecules [42]. Yang et al. proposed a method for simultaneous determination of 11 aminoglycoside residues, including amikacin, paromomycin, dihydrostreptomycin, gentamicin, hygromycin, kanamycin, netilmicin, spectinomycin, sisomicin, streptomycin, and tobramycin, in honey, milk, and pork samples using a Supel MIP SPE-Aminoglycoside cartridge, proving it to be powerful and selective material for the extraction and cleanup of antibiotics in complex food matrices. The method showed the LOD of 2–30 μg/kg and the LOQ of 7–100 μg/kg with the average recovery ranged from 78.2 to 94.8% [43]. Similarly, the extraction of polyether ionophore antibiotic residues in milk, eggs, and poultry meat and liver was performed by preparing carbon-nanotube magnetic nanoparticles (CNT-MNPs) applied to magnetic solid-phase

extraction (MSPE) [44]. It was demonstrated that these CNT-MNPs exhibited good extraction efficiencies for polyether ionophore antibiotics, and the method is green, low cost, and rapid [44]. Likewise, a variation to the MSPE method, namely, micro-solid-phase extraction (M-μ-SPE), was developed by using TMCNTs (thiol-functionalized magnetic carbon nanotubes) as a sorbent and used for the extraction of four sulfonamides in milk, eggs, and chicken meat samples [45]. The method under optimized TMCNTs-M-μ-SPE and HPLC-DAD conditions showed good linearity in the range of 0.1–500 μg L^{-1} ($r^2 \geq 0.9950$), low limits of detection (0.02–1.5 μg L^{-1}), good analytes recovery (80.7–116.2%), and acceptable RSDs (0.3–7.7%, n[M1] = 15). Since TMCNTs as a sorbent on M-μ-SPE enable analytes to be extracted and preconcentrated within 30 min, TMCNTs-M-μ-SPE can be regarded as a convenient method for identification of SAs in food samples [45].

On the other hand, besides SPE, the extraction of nine sulfonamides from milk samples by using dispersive liquid-liquid microextraction (DLLME) and modified Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) was reported; QuEChERS extraction was more reproducible while DLLME gave lower LOD values and higher recoveries of 90.8–104.7% as compared to QuEChERS recoveries of 83.6–104.8% [46]. The main steps of a typical QuEChERS procedure are shown in Figure 4. Moreover, the extraction of eight sulfonamides from butter samples was reported using an ionic liquid-magnetic bar-liquid-phase microextraction (IL-MB-LPME) [47]. A large number of antibiotic residues from different food samples such as milk products (cheese, butter), honey samples, chicken meat, etc., could be extracted using various extraction methods, such as SPE, online SPE, MSPE, LLE, mini-SAE, and many more. A summary of the extraction of different antibiotics from various food products is depicted in Table 2.

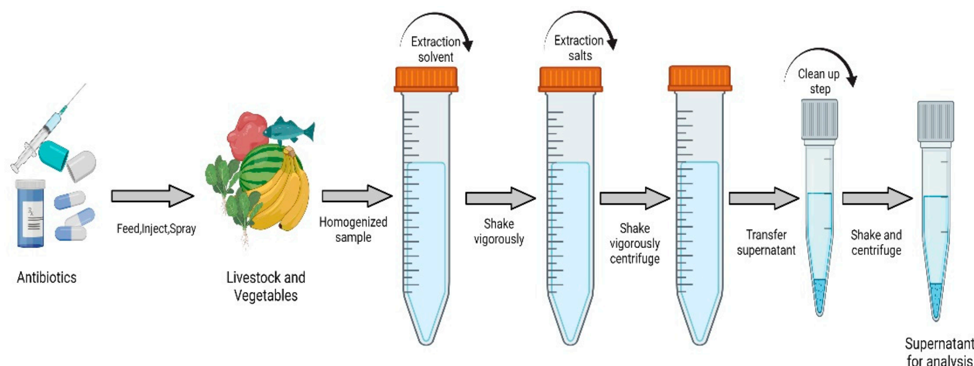

Figure 4. Schematic of a typical QuEChERS method.

Table 2. Extraction of antibiotics from different food samples.

Analytes	Amount	Antibiotics	Extraction Methods	Adsorbent	Elution	Separation and Detection	Year	References
Water, milk, pork, and fish	1 L, 250 g, 1000 g, and 500 g	Sulfonamide antibiotics	Magnetic SPE	Fe_3O_4 @MoS_2	Methanol containing 1% ammonium hydroxide	HPLC–MS/MS	2020	[48]
Water and food	5g	Quinolone antibiotics	SPE	TAPA-TFPB-COFs	Methanol and water	LC–MS	2020	[49]
Milk	5g	Amoxicillin, Ampicillin, and Cloxacillin	Micro-SPE	Starch-based polymer	Methanol	HPLC-UV	2019	[50]
Pork meat	1g	Macrolides	SPE	Molecularly imprinted polymer	10% acetic acid in methanol.	LC–MS/MS	2018	[51]
Chicken	5.0 mL	Tetracyclines	SPE	E-spun-GO-PANCMANFs	Ethanol/formic acid/dichloromethane, 40/20/40($v/v/v$)	HPLC-FLD	2019	[40]

Table 2. *Cont.*

Analytes	Amount	Antibiotics	Extraction Methods	Adsorbent	Elution	Separation and Detection	Year	References
Honey and milk	4 mg and 20 mg	Oxytetracycline, tetracycline, Doxycyclin, Cholrtetracycline, Methacycline	SPE	Multiwalled carbon nanotubes	Methanol and double-distilled water	UPLC-QTOF/MS	2016	[52]
Honey	100 mL	Tetracycline, cefotaxime	Micro-SPE	Electrospun graphene oxide doped polyethylene terephthalate nanofibers	Acetonitrile	HPLC-UV	2018	[53]
Raw milk	4.0 mL	Tetracyclines, Erythromycin, Chloramphenicol	SPE	Molecularly imprinted polymer	Ethanol, methanol, acetonitrile, and 0.05% ammonium acetate solution	HPLC-ELSD	2018	[54]
Milk	1.0 mL	Tetracyclines	SPE	Sep-Pak Vac C18 cartridges	Methanol	LVSS-CE	2018	[55]

Note: TAPA = Tris(4-aminophenyl)amine, TFPB = Tris(4-formylphenyl), COFs = Covalent organic frameworks, E-spun-GO-PANCMANFs = Electrospun graphene oxide-doped poly(acrylonitrile-co-maleic acid) nanofibers, SPE = Solid-phase extraction, HPLC = High Performance Liquid Chromatography, MS = Mass Spectrometry, UV = Ultraviolet Spectroscopy, LC = Liquid Chromatography, LVSS-CE = Large Volume Sample Stacking Capillary Electrophoresis, ELSD = Evaporative Light Scattering Detector, FLD-Fluorescence detection, UPLC-QTOF/MS-ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry.

3. Separation and Detection of Antibiotics

3.1. Chromatography

Previously, paper chromatography and thin-layer chromatography were fundamental tools for the separation, identification, and quality control of antibiotics. Later advancements of modern technology such as High-Performance Liquid Chromatography (HPLC), Liquid Chromatography-Mass Spectrometry(LC-MS), Ultra-High Performance Liquid Chromatography (UHPLC), and others have revolutionized the separation and determination of different antibiotics in food and other biological samples [38,49,56]. Liquid chromatography coupled with mass spectrometry is being widely used in the detection of antibiotics [48,51]. However, the high cost of a mass spectrometer limits its use in small laboratories for routine analysis. As a result of which, the LC system coupled with other detectors, mainly ultraviolet (UV), diode array detector (DAD), and fluorescence detector (FLD), are considered as potential alternatives [51,57–59]. For antibiotics lacking a chromophore, an evaporative light scattering detector (ELSD) is being used [60]. Such detectors are used in the detection of polypeptide antibiotics, which showed better separation, narrower peaks, and little peak tailing as compared to the traditional C18 column [61].

3.1.1. Column Selection

For optimal detection of desired antibiotics in the sample, the LC column must be selected such that the compounds of interest should be separated. Under optimum conditions, the Kinetex Biphenyl column was utilized in the separation of the polypeptide antibiotics [61]. New columns based on the core-shell particle technology were found to be effective in reducing the chromatographic run time and improving the resolution; Kinetex C18 core-shell separated sulfonamides in less than 8 min in HPLC-UV/DAD method [57]. The utilization of cheaper Hypersil BDS C18 columns has helped in the separation and retention of aminoglycosides through utilizing ion-pair reagents [62,63]. A Poroshell 120 SB-C18 column showed the most effective separation effect and the highest response for total analytes for the separation of polypeptide antibiotics [64]. Figure 5 shows the differ-

ent steps involved in the simultaneous determination of four polypeptide antibiotics in infant formula powder by using HPLC–MS/MS; the separation was carried out by using Poroshell 120 SC-C18 column.

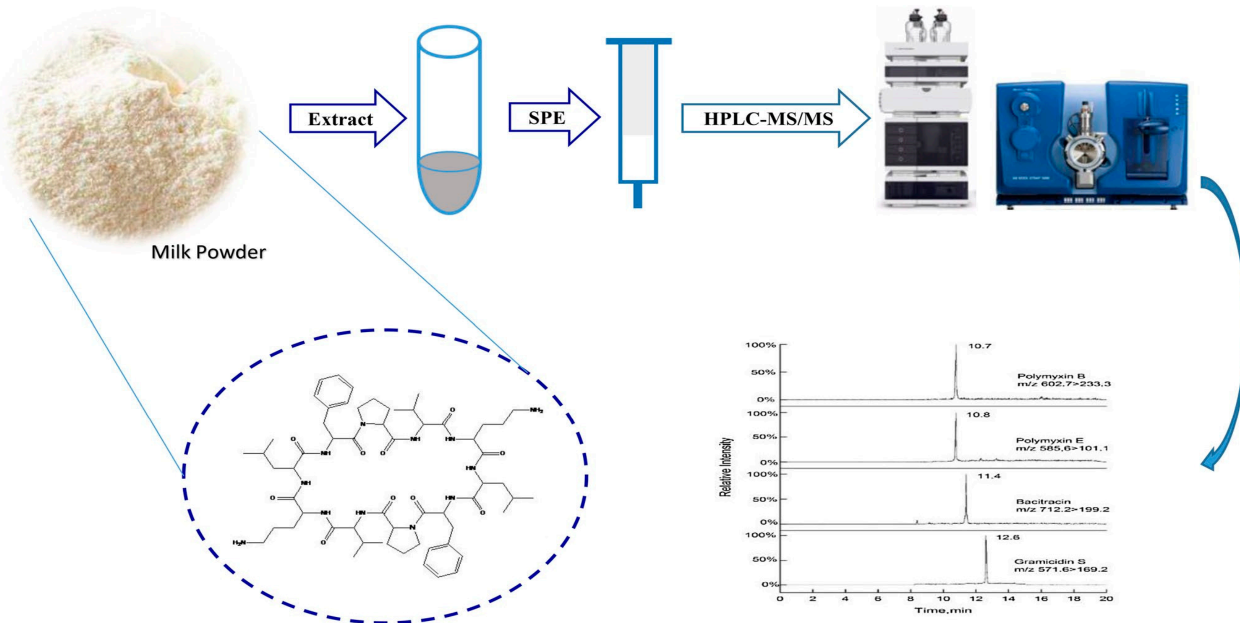

Figure 5. Simultaneous determination of four polypeptide antibiotics (polymyxin B, polymyxin E, bacitracin, and gramicidin S) in milk by HPLC–MS/MS (Reproduced with permission of the publisher) [64].

3.1.2. Mobile Phase Selection

The mobile phase used in the separation of antibiotics influences the detection in the chromatography. The additives and composition of the mobile phase were shown to influence the peak shape, resolution, and retention time of the compounds as well as the ionization efficiency of the compounds, which in turn, would affect the detection sensitivity of the analytes [65]. Methanol, acetonitrile (ACN), formic acid (FA), etc., are the most common mobile phases used in the separation of different antibiotics. Both methanol and ACN are a common choice as the mobile phase; methanol is cheaper and shows enhanced solubility of analytes which improves analytes detection, while acetonitrile shortened the analysis time and maintained the resolution of the entire components of the analytes [63,66]. Wang et al. used methanol or ACN with formic acid or acetic acid as an organic phase and water with formic acid as an aqueous phase for the separation of sulfonamides, quinolones, nitroimidazoles, pleuromutilins, and β- lactams. Additionally, it was demonstrated that the use of ACN provides better peak shapes, whereas the use of methanol provides better separation [65]. Similarly, the use of additives such as formic acid and ammonium acetate greatly improves the resolution for some classes of compounds, for example, between sulfonamides and impurities, due to which the quantification of antibiotics under optimized conditions is possible using HPLC-UV methods [58]. For the separation of polypeptide antibiotics, formic acid is used as an additive in the mobile phase, which improves the separation as formic acid not only increases the acidity but also provides protons to improve the ionization efficiency in HPLC–MS/MS [64]. Moreover, formic acid used in gradient elution mode for the separation of sulfonamides with similar polarity helps in obtaining chromatographic peaks with no interference in Ultra-High Performance Liquid Chromatography (UHPLC) [38].

Along with this, different buffers (ammonium acetate and ammonium formate) are used for maintaining acidic pH, which not only generates high chromatographic resolution but also results in improved ionization efficiency and signal intensity in LC-MS/MS [62]. In reversed-phase liquid chromatography, tetracyclines were found to give broad and

unsymmetrical peaks due to ionic interactions of these analytes with ionic free silanol groups and metal ions present in the silica-based stationary columns, and these frequently observed limitations were solved by using methanesulfonic acid as an eluent additive; the acid neutralizes the anionic silanol sites and electrostatic attractions with analytes [66]. Accordingly, heptafluorobutyric acid (HFBA) was used as an ion-pair reagent in the mobile phase to form an ion-pair complex with aminoglycosides to retain well in the reverse phase chromatographic column in High-Performance Liquid Chromatography-Evaporative Light Scattering Diode (HPLC-ELSD) [63]. The major challenges during the detection of antibiotics using the chromatographic technique are matrix interferences, polar differences, and the low concentration of antibiotics in food samples [38,66,67]. Therefore, to assess the effectiveness of the proposed method, the right analytical technique must be selected. A summary of the detection of antibiotics using chromatographic techniques is shown in Table 3.

Table 3. Detection of antibiotics using different chromatographic methods.

Analytes	Antibiotics	Detection Methods	Extraction Methods	Mobile Phase	Stationary Phase	LOD	Year	Recovery (%)	References
Fish, shrimp, and crab	Sulfonamides	UHPLC-MS/MS	On-line SPE	(1) 0.1% formic acid in water	C18 pentafluorophenyl (F5 or PFP) column	0.00146–0.0155 ng/mL	2020	71.5–102	[38]
				(2) 0.1%formic acid in ACN		0.00490–0.0516 ng/mL			
Eggs	Sulfonamides, quinolones, tetracyclines, macrolides, lincosamide, nitrofurans, β-lactams, nitromidazoles, and cloram-phenicols	LC–MS/MS	HILIC-SPE	(1)H2O and ACN,	Poroshell 120 EC-C18 column	0.005−2.00 ng/mL	2017	70.8–116.1	[68]
				(2)H_2O and ACN containing0.1% formic		0.015−6.00 ng/mL			
Animal feed	Cyclopolypeptide	HPLC-ELSD	On-line SPE	Methanol and ammonium acetate aqueous solution containing formic acid (B)	Kinetex Biphenyl column	2–5 µg/mL	2018	72.0–105.4	[61]
Eggs	Sulfonamides, quinolones, pleuromutilins,β-lactams	UHPLC–MS/MS	Dispersive SPE	0.1% FA and MeOH:ACN,2:8, *v*/*v*, containing 0.1% FA	BEH C18 column	0.1–1 ng/mL	2021	70.5–119.2	[65]
Animal feeds	Aminoglycosides	HPLC-ELSD	Dispersive SPE	Acetonitrile and water	Hypersil BDS C18	0.2–0.7 µg/mL	2017	61.2–104.0	[63]
Foods of animal origin	Sulfonamides	HPLC-UV	Centrifugation	Mixtures of acetonitrile, water, formic acid, and ammonium	Inertsil ODS-3	6.5–11.0 ng/mL	2018	85–95	[58]
Milk	Sulfonamides	HPLC/UV-DAD	Centrifugation	Acetate buffer solution at pH 4.50 and a mixture of methanol acetonitrile 50:50 (*v*/*v*)	C18 column	2.7–15 ng/mL	2018	55–86	[57]

Note: UHPLC = Ultra-High Performace Liquid Chromatography, MS = Mass Spectrometry, LC = Liquid Chromatography, HILIC-SPE = Hydrophilic Solid-Phase Extraction, ELSD = Evaporative Light Scattering Detection, ESI = Electro Spray Ionization, UV-DAD = UV-Diode Array Detection.

3.2. Capillary Electrophoresis

The chromatographic techniques require excess solvent, more time for sample preparation, and different types of stationary phases, which make the techniques more cumbersome in laboratories with limited resources [67,69]. Capillary electrophoresis (CE) is cost-effective, is simple to operate, consumes fewer reagents, and provides high separation efficiency; this technique was widely employed in the separation and detection of antibiotics in a wide range of food samples. The performance of CE can be improved by various means, such as changing buffer type, pH, voltage, mode of the CE, etc. [70,71]. Similarly, various preconcentration techniques were employed to enhance the sensitivity in CE. Apart from these, injection techniques were modified to optimize the CE [72].

CE optimization can be achieved through maintaining buffer type, pH, and voltage [73]. Buffer composition not only enhances the separation but also improves the electrophoretic characteristics of the target analytes; when electrolytes are mixed with phosphate and borate, complex formation between borate anions (tetrahydroxyborate) and tetracyclines occurs, resulting in the modification of electrophoretic characteristics and thereby separation of tetracyclines [74]. With an increase in the buffer concentration under the combined influence of the electro-osmotic flow and the electrophoretic force, the separation of the sulfonamides in the milk sample was improved. However, with the increase in the buffer concentration above optimum concentration, friction between the buffer and the inner capillary wall increased. As a result, the temperature of the column increased, resulting in the broadening of the peaks and reduction in the capillary lifetime [74,75]. It was found that the migration rate and chemiluminescence signals of sulfonamides were affected by the concentration of sodium borate buffer, and the signal intensity was found to be inversely proportional to the buffer concentration [76]. Figure 6 depicts the synthesis of PEG@MoS2 and its application in the determination of eight sulfonamides in milk samples by dispersive solid-phase extraction (DSPE)-Capillary zone electrophoresis (CZE).

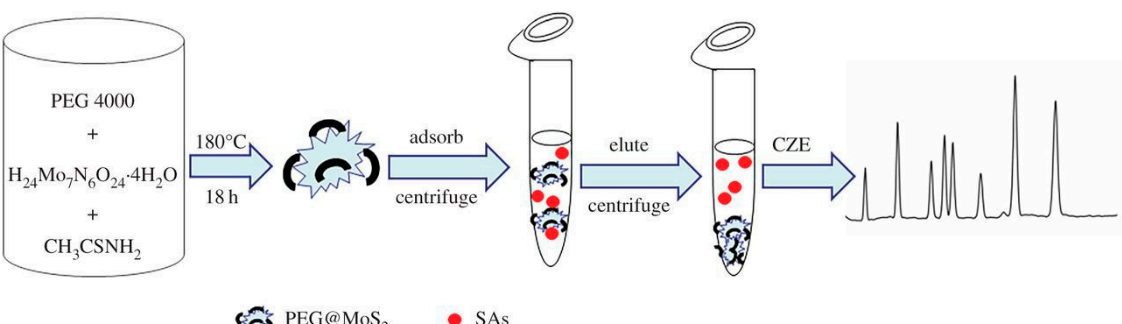

Figure 6. Schematic illustration of detection of eight sulfonamides by using DSPE-CZE [75] (Reproduced with permission of the publisher).

In CE, the pH value of the running buffer plays an important role in the separation of analytes. In the separation of tetracyclines and quinolones, a basic condition was maintained using ammonium acetate and ammonium carbonate buffers at pH 9.0 as the basic condition was found to be suitable for effective separation of these antibiotics [73]. It was also demonstrated that with an increase in pH, the separation between sulfadiazine and sulfachlorpyridazine first increased and then decreased, with the optimum pH for separation being 7.26 [75]. Similarly, the pH of the running buffer at about 9.5 was found to provide good separation of sulfamethazine, sulfadiazine, and sulfathiazole [76].

The effect of pH was significant in the separation of five macrolides from a milk sample; when the pH is less than 6.5, erythromycin and clarithromycin somewhat overlap. However, when the pH is greater than 8.0, tylosin almost completely overlaps the negative solvent peak, making it difficult to detect [77]. The baseline separation of macrolides was accomplished when the pH was between 6.5 and 7.5; in addition, the shortest migration time and the best peak shape were achieved at pH 7 [77]. Similarly, the applied voltage is

another factor that affects the separation of analytes in CE; for example, adequate separation of fluoroquinolones was observed at an applied voltage of 20 kV [78]. It should be noted that the voltage applied on a certain range is better for the separation because higher voltage increases Joule heating, which represses the capillary efficiency and viscosity of the running buffer [78].

Detectors are a key component in CE, and an efficient combination of the separation capillary and a detector is required for the optimal detection of separated analytes. For effective detection of analytes using CE, the capillary was combined with various detectors such as a mass spectrometer, UV detector, light-emitting diode, etc. [73,75]. The CZE coupled with ion trap mass analyzer (for MS/MS analysis) was developed as an alternative to LC-MS/MS for the separation and detection of twelve benzimidazoles in meat samples [79]. Employing MS/MS as a detection system improves the selectivity and sensitivity of CE analysis [79]. Similarly, graphene quantum dots (GQDs) were used to enhance the sensitivity of CE with fluorescence detectors for the quantitative determination of ofloxacin in milk samples [78]. Sensitivity was determined in the presence and absence of GQDs, and a significant enhancement in sensitivity was observed when GQDs are injected into the capillary before sample loading [78]. Interestingly, it was observed that the GQDs enhance the photoluminescence of hydrophobic antibiotics, such as lomefloxacin, norfloxacin, and ofloxacin, an aqueous medium [78]. Moreover, a low-cost microchip CE system containing an electrophoresis chip and fluorescence detector was constructed for the determination of ciprofloxacin in milk samples [80]. Apart from these, CZE using online chemiluminescence (CL) detector and CE-UV detector were used for the detection of the sulfonamides and fluoroquinolones, respectively, in food samples [76,81]. Figure 7 depicts the schematic representation of CZE with an online chemiluminescence detector.

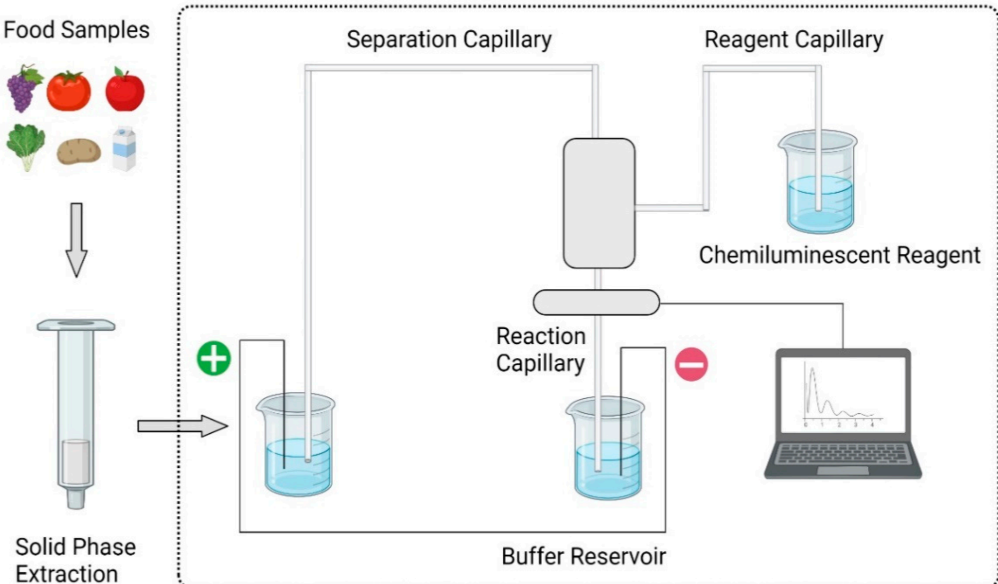

Figure 7. Schematic representation of CZE with chemiluminescence detection.

CE was used in various modes as per the need for samples to improve the separation and detection of antibiotics. Among various techniques, field amplified sample injection-capillary zone electrophoresis (FASI-CZE) is an operation mode in which the sample is preconcentrated in a capillary as an effect of amplified electricity between a sample and a running buffer [74]. While performing preconcentration of samples, it was found that the greater the contrast between the buffer conductivity and the sample, the better the preconcentration of the analytes of interest [74]. Similarly, electrokinetic injection (EKI) and pressure-assisted electrokinetic injection (PAKEI) were employed as online preconcentration techniques in CE for the effective stacking of anionic analytes. In the case of PAKEI, during injection, the velocity of electro-osmotic flow (EOF) is counterbalanced

by the external pressure, thereby creating a stationary boundary between the sample zone and background electrolyte (BGE) at the capillary inlet due to which a large number of analytes are introduced into the capillary [70]. The different parameters and analytical performance of the CE-based methods for the analysis of different antibiotics in food samples are summarized in Table 4.

Table 4. Detection of different antibiotics in foods using capillary electrophoresis methods.

Analytes	Antibiotics	Extraction Methods	Buffer	pH	Limits of Detection	Voltage (kV)	Recovery (%)	Year	Reference
Milk	Oxytetracycline	SPME	Sodium phosphate + EDTA disodium salt +5% 2-propanol	12	0.07 μg/mL	14	-	2019	[72]
Milk	Tetracyclinesand Quinolones	SPE	Mcllvaine's buffer	9.0	0.5–2.9 ng/mL	25	72.6–105.8	2017	[73]
Milk	Sulfonamides	DSPE	Phosphate buffer	7.26	0.03–0.20 μg/mL	18	60.52–110.91	2018	[75]
Milk, pork, and chicken	Sulfonamide	SPE	Sodium borate buffer	9.5	0.65–3.14 μg/mL	18	79.5–112.4	2017	[76]
Water	Fluoroquinolones and sulfonamides	DLLME	Borate buffer	10	1.96 ng/mL 4.06 ng/mL	15	83.3–98.7	2019	[70]
Milk	Macrolides	Ultrasonic and centrifugation	Phosphate+ sodium cholate+ cetyltrimethylammonium bromide	7.0	0.002–0.004 μg/mL 2 ng/mL 4 ng/mL	10	72.8–93.7	2018	[77]
Milk	Ofloxacin	MEP	sodium tetraborate+SDS+ 10% (v/v) methanol	7.5	1.07 ng/mL	20	_	2019	[78]
Meat	Benzimidazoles	DLLME	Formic acid	2.2	>0.003 μg/mL	20	70.1–95.5	2017	[79]

Note: HPLC = High Performance Liquid Chromatography, SPE = Solid-Phase Extraction, MS = Mass Spectrometry, MSPE = Magnetic Solid-Phase Extraction, DLLME = Dispersive Liquid–Liquid Micro Extraction, PLE = Pressurized Liquid Extraction, LC = Liquid Chromatography, PMME = Polymer Monolith Micro Extraction, SPME = Solid-phase micro-extraction, MEP = Microextraction by packed solvent, DSPE = Dispersive solid-phase extraction.

3.3. Immunological Methods

Chromatographic and CE-based methods are specific, accurate, and can be used for simultaneous determination of multiple antibiotics; however, these methods are limited by high instrumental cost and long and complicated procedures due to which on-site detection of antibiotics is not possible. Immunological assays, on the other hand, are simple, highly selective, rapid, and cost-effective; thus, they can be used for the on-site detection of antibiotics [81]. Enzyme-linked immunosorbent assay (ELISA), indirect complete ELISA (ic-ELISA), fluorescence polarization immunoassay (FPIA), immunochromatographic assay (ICA), etc., are various immunological techniques currently in use [82–85]. The principle of the immunoassays for the detection of antibiotics is shown in Figure 8.

The antibody is the most important factor for the development of efficient immunological assays. Immunoglobulin G (IgG)-derived antibodies from rabbit and mouse was traditionally used for the development of immunoassays. Recently, there were several

studies in the use of immunoglobulin Y (IgY), a chicken egg yolk antibody, as a superior alternative to IgG. Liang et al. compared under parallel conditions to know the sensitivity, specificity, and matrix effects in the detection of sulfamethazine in milk samples and found that IgY can be an alternative to IgG for the detection of antibiotic residues in food products [86]. Similarly, Li et al. evaluated immunoglobulin Y (IgY) using FPIA and ic-ELISA to detect gentamicins/kanamycin and found that the LOD and IC_{50} values for the ic-ELISA are better than FPIA, which indicated the suitability of ic-ELISA over FPIA for detecting antibiotics in animal-derived samples [83].

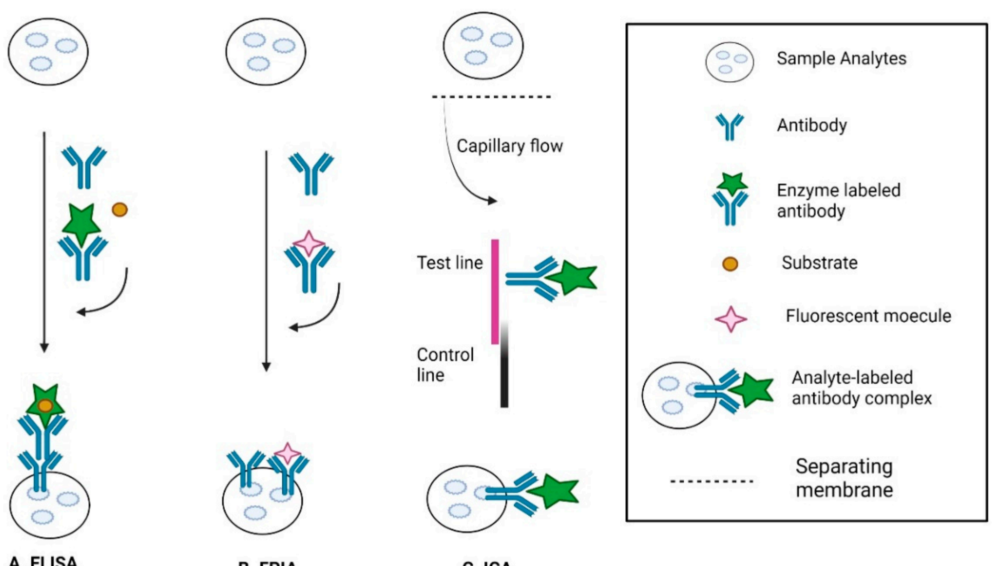

Figure 8. Schematic representation for detection of antibiotics by immunoassay methods.

Moreover, the use of monoclonal antibodies (mAbs) has widened the scope of the immunological assays; Li et al. developed an ultra-high sensitive ic-ELISA based on the broad-specificity mAbs for simultaneous detection of five antibacterial synergists (trimethoprim, diaveridine, brodimoprim, ormetoprim, and baquiloprim) in chicken and milk samples [83]. The traditional ELISA requires a time-consuming multi-step separation process, and to overcome this limitation, fluorescence polarization immunoassay (FPIA) was developed, which obviates the need for a separation step [87].

A simple fluoroimmunoassay was developed to detect tetracyclines, in which the receptor of tetracyclines, Tet repressor protein (TetR), was directionally mutated to produce two mutants that exhibited high affinity and high sensitivity for the detection of tetracyclines; the mutants were combined with fluorescence-labeled tracers for simultaneous detection of nine tetracyclines in egg samples [88]. The assay process of the Tet repressor protein (TetR) mutant-based fluoroimmunoassay for the detection of antibiotics is shown in Figure 9.

Similarly, a magneto immunofluorescence assay was developed to improve the selectivity and sensitivity of immunoassays; the use of magnetic beads (MB) combined with antigens or antibodies provides more surface area for efficient capture of target analytes [82]. Kergaravat et al. found a competitive step between free quinolones in samples and immobilized quinolones on MB for anti-quinone antibody (Ab1), as a result of which non-specific adsorption was reduced, thereby minimizing matrix effects [82]. The structure of a tracer could greatly influence the performance of a fluorescence polarization immunoassay; a heterologous tracer is recognized weakly by an antibody than a homologous tracer so that a heterologous tracer provides higher sensitivity than a homologous tracer. Considering this is a highly sensitive fluorescence polarization immunoassay, it was designed for the detection of clinafloxacin in goat milk [89]. Moreover, immunochromatographic assay (ICA) was developed employing time-resolved fluorescent nanobeads (TRFN) as a label for ultrasensitive detection of sulfamethazine in egg, honey, and pork samples; the accuracy

of this assay was confirmed by the use of HPLC–MS/MS [85]. For the detection of fluoroquinolone, anti-pefloxacin (PEF)-monoclonal antibody(mAb), ic-ELISA, and lateral flow test strips were developed, and it was demonstrated that the methods based on ic-ELISA and lateral flow test strips are capable of sensitive and simultaneous detection of nine fluoroquinolones in chicken muscle samples [90]. Various immunological methods and their analytical performance for the detection of antibiotics in food samples are summarized in Table 5.

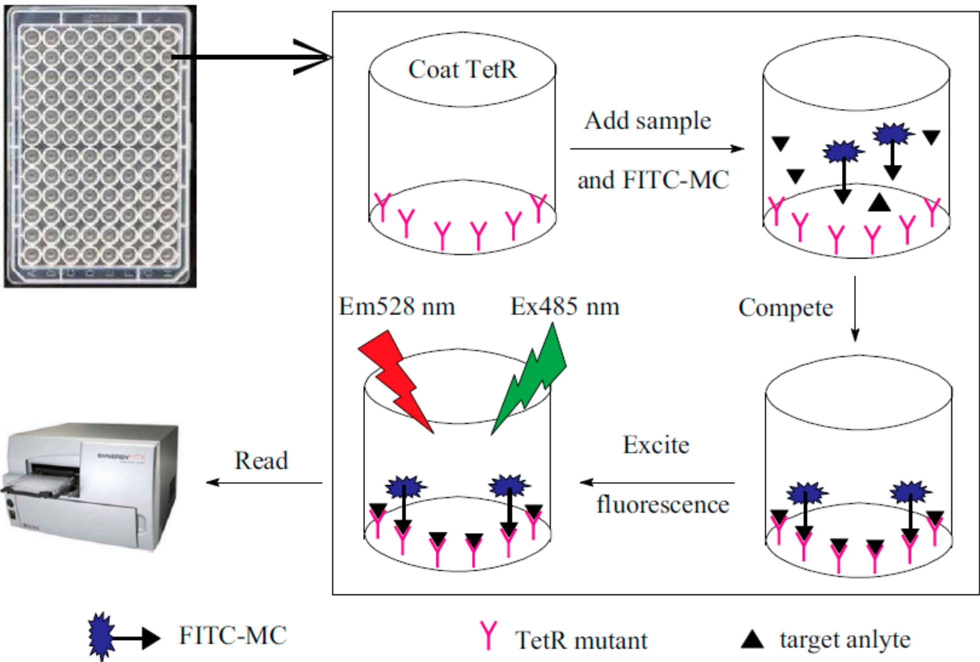

Figure 9. Schematic of the assay process of the Tet repressor protein (TetR) mutant based fluoroimmunoassay for the detection of antibiotics (Reproduced with permission of the publisher) [88].

Table 5. Detection of antibiotics by immunological methods.

Analytes	Antibiotics	Detection	Extraction	IC_{50}	LOD	Recovery (%)	Year	References
Milk	Sulfamethazine	ic-ELISA FPIA	Centrifugation and dilution	6.70 ng/mL 4.76 ng/mL 1.66 ng/mL	----	86.1–131.8	2018	[86]
				25.29 ng/mL 23.92 ng/mL 10.60 ng/mL		81.8–120.2		
Animal-derived food	Gentamicins	FPIA ic-ELISA	PEG precipitation	7.70 ± 0.6 µg/mL	0.17 µg/mL	----	2017	[83]
				0.32 ± 0.06 µg/mL	0.001 µg/mL			
	Kanamycin	FPIA ic-ELISA		7.97 ± 0.9 µg/mL	0.007 µg/mL			
				0.15 ± 0.01 µg/mL	0.001 µg/mL			
Egg yolk	Gentamicins	ic-ELISA	Centrifugation	2.69 ng/mL	0.01 ng/mL	----	2016	[91]
Pork and chicken	Enrofloxacin	FPIA	Centrifugation	21.49 ng/mL	1.68 ng/mL	91.3–112.9	2019	[87]
Egg	Tetracyclines	Fluoroimmunoassay	Centrifugation	3.1–17.2 ng/mL	0.3–5.8 ng/mL	----	2019	[88]
Goat milk	Clinafloxacin	FPIA	Centrifugation	29.3 µg/L	4.1 µg/L	86.8–104.5	2015	[89]

Table 5. *Cont.*

Analytes	Antibiotics	Detection	Extraction	IC_{50}	LOD	Recovery (%)	Year	References
Egg, honey, and pork	Sulfamethazine	Immunochromatographic assay	Centrifugation	- - - -	0.016 ng/mL	90.5–113.9	2020	[85]
					0.049 ng/mL	82.4–112.0		
					0.029 ng/mL	79.8–93.4		
Chicken muscle	Fluoroquinolones	ic-ELISA Lateral Flow Test Strip	Centrifugation	0.2 ng/mL	0.082 ng/mL	- - - -	2017	[90]

Note: ic-ELISA = Indirect complete enzyme-linked immunosorbent assay, FPIA = Fluorescence polarization immunoassay, PEG Precipitation = Polyethylene Glycol Precipitation.

3.4. Surface-Enhanced Raman Spectroscopy (SERS)-Based Methods

SERS is a new technique developed based on Raman spectroscopy; this technique has benefits of high detection speed, low detection cost, and simple operation, and the high statistical binding of target analytes to active sites or “hot spots” of noble metal nanostructures is crucial for enhanced detection sensitivity [92]. The core components of a SERS-based method are a target molecule, a metal nanostructure, and electromagnetic radiation [93]. Previously, metal or metal oxide film layers were deposited on the surface to enhance the Raman signal to detect antibiotics. Recently, the majority of SERS-based sensor substrates were modified with metal nanoparticles [94], primarily gold or silver colloids. For instance, ciprofloxacin was physically adsorbed on a self-assembling gold nanofilm for quantitative detection, and SERS was utilized to detect it in fish [95]. A schematic of the pretreatment of fish samples followed by SERS detection on the gold nanofilm is shown in Figure 10. Jiang et al. assembled silver nanoparticles on a glass substrate to prepare SERS substrate to detect benzylpenicillin sodium [96].

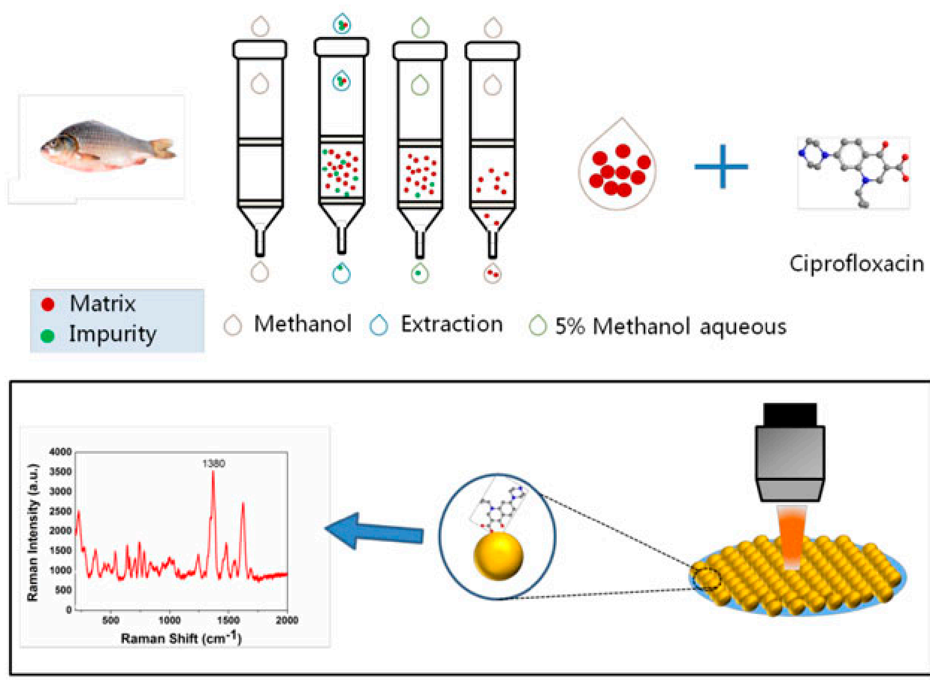

Figure 10. Schematic of the pretreatment of fish samples with SERS detection of ciprofloxacin on a self-assembled gold nanofilm (Reproduced with permission of the publisher) [95].

Recently, a unique methodology based on SERS combined with gold nanoparticles was devised for rapid detection of amoxicillin residues in duck flesh [97]. Similarly, another

method was devised that uses gold nanoparticles/porous silicon (AuNPs/PSi) as a SERS active substrate for rapid and precise detection of ultra-low concentrations of penicillin G [98]; porous silicon surface modification enhances the density of hot spots and specific surface area of nanoparticles, resulting in improved performance and reproducibility [99]. Likewise, Ali et al. used Psi/AuNPs SERS sensors with a mud-like structure to detect ultra-low concentrations of amoxicillin, and enhanced SERS activity were obtained due to a higher density of small sizes of hot spot regions [100]. SERS substrates made of nanoparticles are extensively utilized for signal enhancement; however, inconsistencies of SERS signals were observed in some cases due to random distribution of nanoparticles, difficulties in controlling interparticle gap, and difficulties in fabrication of metal nanoparticles with high uniformity [101,102].

In recent years, a combination method between SERS and lateral flow assay (LFA) has been used. Shi et al. used gold nanoparticles (AuNPs) conjugated to the Raman active molecule 4-amino thiophenol (PATP) and to monoclonal antibodies against neomycin and norfloxacin to develop multiple immuno-nanoprobes based on SERS for ultrasensitive detection of neomycin and quinolones via LFA, and these exhibited high detection sensitivity and satisfactory recovery from spiked milk samples [103]. In addition, Fan et al. employed a SERS-based lateral flow immunosensor method for simultaneous determination of tetracycline and penicillin residues in milk with the use of synthesized Au@Ag nanoparticles labeled with Raman molecules as a SERS substrate [104]. Besides this, with the emergence of novel materials such as carbon nanotubes and graphene oxide (GO), the adsorption capacity and sensitivity of SERS sensors can be boosted by incorporating these materials into sensing devices [93]. For the enrichment and SERS detection of antibiotics from water samples, silver nanoparticles (AgNPs) and carbon nanotube-intercalated graphene oxide laminar membranes (Ag NPs/CNT-GO membranes) were successfully fabricated [105]. The produced AgNPs/CNT-GO membranes displayed a high enrichment ability due to the π-π- stacking and electrostatic interactions of GO with antibiotic molecules, which increased the sensitivity of SERS measurements allowing the detection of antibiotics at sub-nM concentration levels [105].

Likewise, a method of integrating SPME and SERS by co-deposition of reduced graphene oxide (RGO) and silver on silver–copper (Ag–Cu) alloy fibers was developed for detection of sulfadiazine and sulfamethoxazole in spiked tissue mimics, and such a hybrid coating exhibits a high SERS enhancement factor [106]. On the other hand, newly developed semiconductor SERS-active substrates (TiO_2, CuTe, etc.) attracted increased interest [107], and such semiconductor SERS substrates have an ultra-sensitive detection potential under low concentrations and remarkable stability with a high SERS effect due to charge transfer (CT) phenomena as compared to the electromagnetic enhancement of metal SERS substrates [108]. For instance, Wang et al. developed a SERS strategy based on a semiconducting Ag–TiO_2 substrate for ultrasensitive detection of five quinolones (difloxacin hydrochloride, ciprofloxacin, enoxacin, enrofloxacin, and danofloxacin) in water samples [109]. Moreover, Ag–TiO_2 nanoparticles can also act as an efficient photocatalyst for antibiotic residues degradation, thereby showing excellent potential for simultaneous detection and degradation of antibiotic residues in the real environment [109].

For further improving sensing performance, a transparent SERS substrate was developed. Muhammad et al. fabricated and optimized a transparent SERS substrate composed of highly ordered AgNP arrays through anodic aluminum oxide (AAO) template-assisted electrochemical deposition approach for highly sensitive detection of tetracycline and dicyandiamide residues in milk samples [101]. A schematic of the experimental procedure of using transparent SERS substrate for the determination of antibiotics in milk samples is shown in Figure 11.

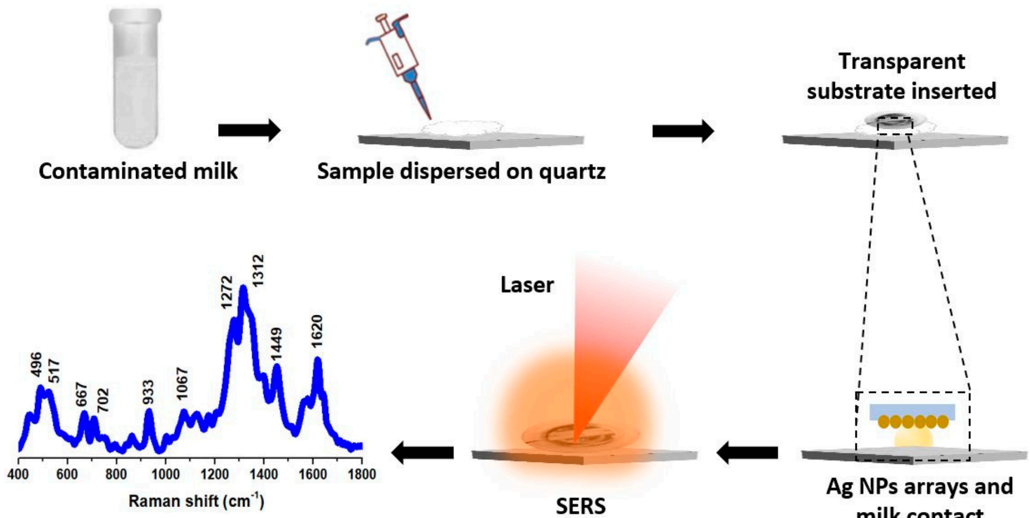

Figure 11. Schematic of the experimental procedure of using transparent SERS substrate for the determination of antibiotics in milk samples. (Reproduced with permission of the publisher) [101].

Xie et al. used a combination of molecularly imprinted polymers with Au as a SERS substrate to detect chloramphenicol in milk samples [110]. Likewise, Ashley et al. employed magnetic molecularly imprinted polymer (MMIP) microspheres for selective extraction, and vertical gold (Au) capped silicon nanopillars as a SERS active substrate to detect cloxacillin in pig plasma [111]. Additionally, hybrid nanomaterials were prepared using magnetite nanoparticles (MNPs) and by decorating them with gold nanostars (AuNSs), and these hybrid nanostructures were found to serve as colloidal nano sorbent for SERS-based detection of tetracyclines in water samples [112]. A schematic of the procedure for the magnetic separation and SERS detection of antibiotics by using MNP-AuNS is illustrated in Figure 12. Thus, the key of SERS-based detection of antibiotics is primarily focused on enhancing SERS signals, repeatability, and stability of SERS measurements [94]. Different SERS-based methods, along with their analytical performance for the detection of antibiotics in different food samples, are summarized in Table 6.

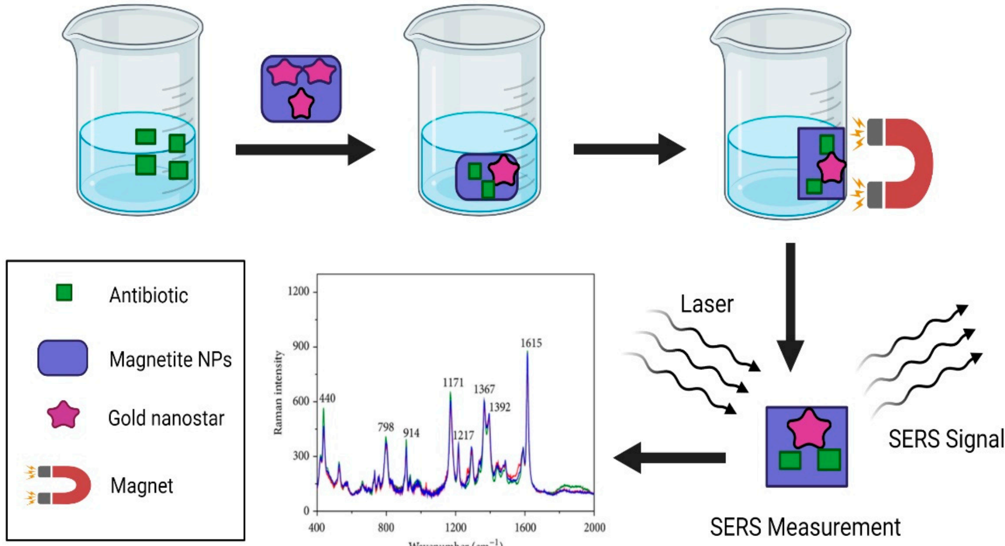

Figure 12. Schematic representation of SERS-based detection of antibiotics using Magnetite NPS-AuNSs.

Table 6. The surface-enhanced Raman spectra (SERS)-based methods for antibiotics detection.

Analytes	SERs Activenanomaterial	Target Antibiotics	Extraction	LOD	Recoveries (%)	Year	References
Milk	Ag–AgNPs	Penicillin G	Centrifugation and Acetonitrile extraction	0.85 µg/kg	76–97	2017	[113]
Fish	Gold nanofilm	Ciprofloxacin	Centrifugation and SPE	0.19 µg/mL	84.6–103.8	2019	[95]
Duck	AuNPs	Amoxicillin	Centrifugation	0.2 mg/L	96–139	2017	[97]
Milk	Au@AgNPs	Tetracyclines Penicillins	- -	0.015 ng/mL 0.010 ng/mL	88.8–111.3	2020	[104]
Water	Ag–TiO_2	Danofloxacin	Centrifugation	3.16×10^{-11} mol/L	>80.8	2019	[109]
Animal tissue mimics	RGO/Ag	Sulfonamide Sulfamethoxazole	- -	1.9 ng/mL 4.4 ng/mL	- - - -	2018	[106]
Chicken and water	Au–Ag composites	Ciprofloxacin	Centrifugation	2×10^{-7} M 8×10^{-8} M	91–105	2017	[114]

Note: Ag–AgNPs = Silver–Silver nanoparticles, SPE = Solid-phase extraction, AuNPs = Gold nanoparticles, Ag–TiO_2 = Silver–Titanium dioxide, RGO/Ag = Reduced graphene oxide

3.5. Biosensors

A biosensor is a functional integrated device that combines a biological recognition element or bioreceptor for generating signals that vary as a function of the concentration of analytes present in a sample [115]. Biosensors emerged as an innovative alternative tool for rapid, sensitive, and on-site screening of antibiotic residues in food products [19]. Currently, fluorescent, electrochemical, colorimetric, surface plasmon resonance (SPR), and quartz crystal microbalance (QCM) biosensor techniques are most commonly used for the screening of antibiotic residues [30,116]. Fluorescent biosensors are powerful analytical tools for the detection of antibiotics present in different matrices due to their inherent advantages, such as high sensitivity, high selectivity, operation convenience, rapid hybridization kinetics, and ease of automation [117]. Fluorescent biosensors based on GO and AuNPs nanomaterial-based quenchers are used for antibiotics detection. Tan et al. designed a GO hydrogel-based fluorescence aptasensor with ssDNA as the recognition element for oxytetracycline detection, and the limit of quantitation (LOQ) of 25 µg/L was achieved [118]. In other studies, an aptamer-based fluorescent biosensor was developed to detect chloramphenicol using MNPs and UCNPs [29,119]. Similarly, Yue et al. reported sensitive fluorescent biosensors based on UCNPs for the detection of kanamycin [120]. The schematic representation of biosensor-based detection of antibiotics is shown in Figure 13.

Likewise, DNA and nanoparticle colorimetric biosensors have gained significant interest in antibiotic detection because of the inherent optical properties of nanomaterials, and the signal can be detected using smartphones and UV-Vis spectrophotometers [121,122]. Several colorimetric reagents, including metallic nanoparticles, visible dyes, enzymes, and metal ions, were employed to fabricate sensor arrays for the analysis of different types of antibiotics in various samples [123]. A chemiluminescent (CL)-based biosensor was developed for simultaneous detection of three antibiotics, including oxytetracycline, tetracyclines, and kanamycin in milk samples [124]. Emrani et al. developed a colorimetric aptasensor, based on aqueous gold nanoparticles and double-stranded DNA (dsDNA), for the detection of streptomycin in milk and serum samples [125]. In the absence of streptomycin, there is a salt-induced aggregation of gold nanoparticles resulting in blue color, while in the presence of streptomycin, the gold nanoparticles are dispersed, showing a wine-red color [125].

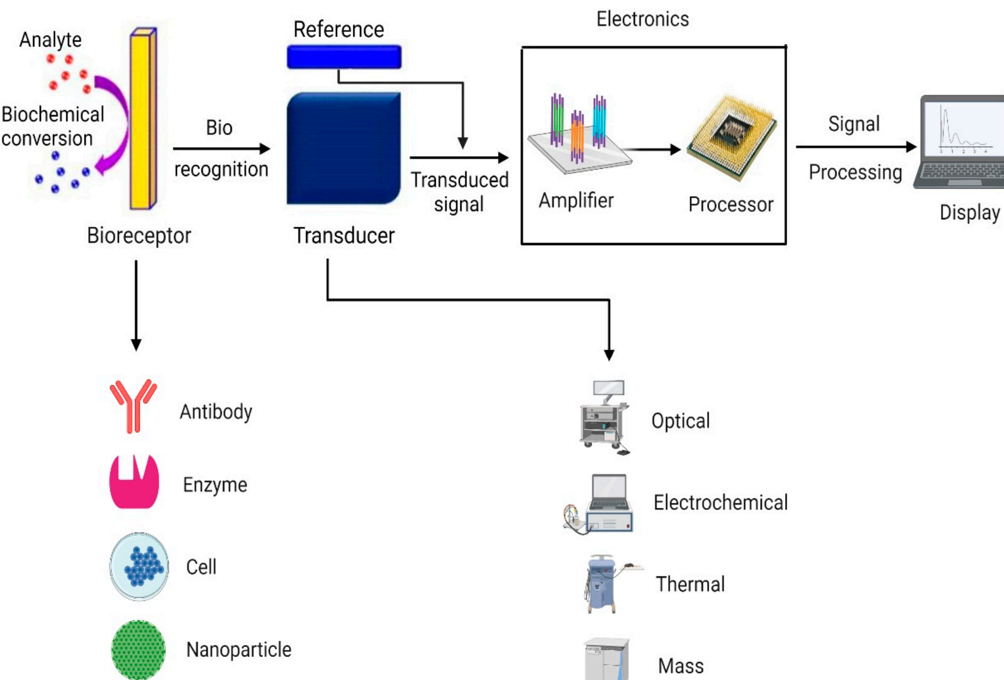

Figure 13. Biosensor-based detection of antibiotics.

Additionally, electrochemiluminescent biosensors (ECL) are very sensitive tools for the detection of antibiotics in different samples; ECL is based on the principle of production of light from an electrochemical reaction [126]. Yang et al. developed a chemiluminescence aptasensor for sensitive and selective detection of sulfamethazine in milk samples by employing a supernormal aptamer [127]. The aptamers were developed based on in vitro selection and were further analyzed by molecular docking [127]. The detection limit obtained was 0.92 ng/mL [127]. Similarly, surface plasmon resonance (SPR) biosensors belong to a powerful optical technology whose working principle is based on oscillation at the interface between two materials that can be generated by photons and electrons [128]. A label-free SPR aptasensor was developed by immobilizing a specific aptamer on a gold surface, and the aptasensor was used for real-time detection of ampicillin with a linear range of 2.5–1000 μmol L^{-1} and a limit of detection of 1 μmol L^{-1} [129].

High Fundamental Frequency Quartz Crystal Microbalance (HFF-QCMD) has the potential for simultaneous detection of several samples in a single analysis by integrating several sensors, thereby saving time and minimizing sample consumption. Sulfathiazole in honey was detected using a piezoelectric immunosensor based on HFF-QCMD technology with a LOD that was 40–50 times lower than those reported by other techniques [130]. The HFF-QCMD immunosensor was envisioned to be a feasible alternative to current techniques for the highly sensitive and rapid determination of sulfathiazole in honey with minimum sample preparation [130]. Electrochemical biosensor (ECB) is one of the most remarkable devices, and the working principle of ECB is based on chemical reactions occurring between immobilized biomolecules and target analytes that convert chemical information into measurable electrical signals [131]. A highly sensitive and specific homogeneous electrochemical aptasensor for ampicillin detection was developed by Wang et al. using target-induced and T7 exonuclease-assisted dual recycling signal amplification strategy, and the detection limit obtained was 4.0 ppm [132]. The principle of the assay and the differential pulse voltammetric (DPV) response as a function of ampicillin concentration are shown in Figure 14. A brief description and the analytical performance of various biosensors are outlined in Table 7.

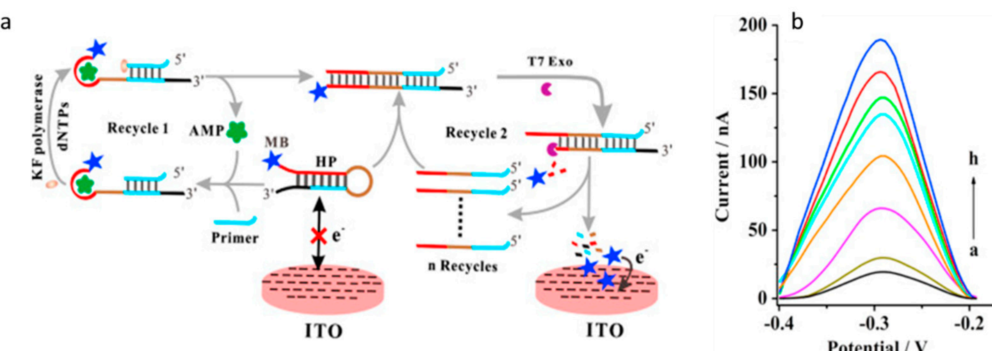

Figure 14. (**a**) Schematic showing the principle of homogeneous electrochemical strategy for highly sensitive detection of ampicillin and (**b**) differential pulse voltammetric response as a function of ampicillin concentration (reproduced with permission of the publisher) [132].

Table 7. Different types of biosensors for the detection of antibiotics.

Biosensor	Detection Method	Target Antibiotics	Bioreceptor	Sample	LOD	Year	References
Electrochemical biosensor	Amperometry	Sulfapyridine	Antibody	Milk	2.4 ng/mL	2018	[133]
	Electrochemical impedance spectroscopy (EIS)	Tetracyclines	Antibody	Water	12.4 ng/mL	2020	[134]
	Differential pulse voltammetry (DPV)	Ampicillin	Aptamer	Milk	3.8×10^{-4} ng/mL	2015	[135]
	Amperometry	Chloramphenicol	Antibody	Pork, chicken, beef	0.045 ng/mL	2016	[136]
Optical biosensor	Colorimetric	Tobramycin	Aptamer	Milk, chicken, egg	10.89 ng/mL	2018	[122]
	Chemi-luminescent (CL)	Sulfamethazine	Aptamer	Milk	0.92 ng/mL	2019	[127]
	Surface plasmon resonance (SPR)	Enrofloxacin	Antibody	Milk	0.07 ng/mL	2017	[137]
Mass sensitive biosensors	Piezoelectric quartz crystal microbalance (QCM)	Streptomycin	Antibody	Milk	0.3 ng/mL	2015	[138]
	Piezoelectric surface acoustic wave (SAW)	Penicillin G	Antibody	Milk	2.2 ng/mL	2016	[139]
	Cantilever	Oxytetracycline	Aptamer	Meat, egg	0.85 ng/mL	2015	[140]

4. Conclusions and Future Perspectives

The use of antibiotics in animals can result in antibiotic residues in food products such as milk, meat, and eggs. These antibiotic residues can have adverse health effects, and to protect public health, MRLs of antibiotics in food products were established. A wide range of analytical methods was therefore developed for highly sensitive and selective detection

and quantification of antibiotic residues in food samples. The LOD and LOQ values are found to depend on the antibiotics and the method employed for their detection, and these values are found to be lower than their MRLs. Mass spectrometry is a highly sensitive and selective technique, but the instrument is expensive, bulky, and consumes high power; thus, the recent research is more focused on developing an alternative. The optimization of the mass spectrometry has provided a better response for the quantification of antibiotic residues. To achieve good analytical results, sample pretreatment is an essential step where SPE is extensively used as a pretreatment approach in antibiotics detection. The development of highly selective SPE sorbents, such as magnetic sorbents, molecularly imprinted sorbents, etc., will enhance the performance of the chromatography and CE-based methods in the future towards detection of antibiotic residues. The selection of column and mobile phase is crucial while separating antibiotics using chromatography for the effective separation of specific antibiotics from a complex mixture. Similarly, in the case of CE, buffer type, pH, and voltage need to be adjusted properly such that targeted antibiotics could be separated from interfering species. Future development of online or automatic extraction and detection will make chromatography and CE more applicable in the places such as animal farms where antibiotics need to be detected quickly.

Recently, advancements were made in immunological assays, SERS, and biosensors for the detection of antibiotics. These techniques use monoclonal antibodies, nanoparticles, DNA, etc., and are found to be comparatively sensitive, selective, and accurate. Immunological methods can also be used as on-the-spot tools for the detection of antibiotics. In the future, research should be directed towards the production of directionally mutated proteins or antibodies that can specifically detect particular antibiotics. Further research should be focused on fabricating more uniform nanoparticles so that consistent SERS signals can be observed during analysis. Moreover, another avenue for research could be the development of more effective biosensors, which are promising tools for the detection and quantification of antibiotics in food products. The analytical performance of these biosensors can be enhanced by improving electrode materials, transducers, and biorecognition elements.

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Article

Characterization of *Streptomyces* Species and Validation of Antimicrobial Activity of Their Metabolites through Molecular Docking

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Abstract: Finding new antibacterial agents from natural products is urgently necessary to address the growing cases of antibiotic-resistant pathogens. Actinomycetes are regarded as an excellent source of therapeutically important secondary metabolites including antibiotics. However, they have not yet been characterized and explored in great detail for their utility in developing countries such as Nepal. In silico molecular docking in addition to antimicrobial assays have been used to examine the efficacy of chemical scaffolds biosynthesized by actinomycetes. This paper depicts the characterization of actinomycetes based on their morphology, biochemical tests, and partial molecular sequencing. Furthermore, antimicrobial assays and mass spectrometry-based metabolic profiling of isolates were studied. Seventeen actinomycete-like colonies were isolated from ten soil samples, of which three isolates showed significant antimicrobial activities. Those isolates were subsequently identified to be *Streptomyces* species by partial 16S rRNA gene sequencing. The most potent *Streptomyces* species_SB10 has exhibited an MIC and MBC of 1.22 μg/mL and 2.44 μg/mL, respectively, against each *Staphylococcus aureus* and *Shigella sonnei*. The extract of *S.* species_SB10 showed the presence of important metabolites such as albumycin. Ten annotated bioactive metabolites (essramycin, maculosin, brevianamide F, cyclo (L-Phe-L-Ala), cyclo (L-Val-L-Phe), cyclo (L-Leu-L-Pro), cyclo (D-Ala-L-Pro), N6, N6-dimethyladenosine, albumycin, and cyclo (L-Tyr-L-Leu)) were molecularly docked against seven antimicrobial target proteins. Studies on binding energy, docking viability, and protein-ligand molecular interactions showed that those metabolites are responsible for conferring antimicrobial properties. These findings indicate that continuous research on the isolation of the *Streptomyces* species from Nepal could lead to the discovery of novel and therapeutically relevant antimicrobial agents in the future.

Keywords: *Streptomyces*; antimicrobial; molecular docking

1. Introduction

Antibiotics have been considered powerful tools to fight against microbial infections and have revolutionized the healthcare system globally. However, the misuse and overuse of antibiotics have accelerated the development of antibiotic resistance. Such resistance is one of the most serious problems confronting healthcare facilities in the twenty-first century [1]. Several bacterial resistance mechanisms, such as the multidrug efflux system [2], the production of β-lactamases [3], and aminoglycoside-modifying enzymes [4] have emerged in the last decades. The microbial world has shown incredible resilience in

prevalent antibiotics, and the development of resistance is virtually impossible to avoid. The relentless development of resistance can render valuable antibiotics useless. This scenario demands an urgent need for the development of new antibiotics that have effective therapeutic activities against infectious diseases [5,6].

Actinomycetes have proven particularly beneficial to the pharmaceutical industry due to their seemingly limitless ability to make secondary metabolites with diverse chemical structures and biological functions. Among actinomycetes, *Streptomyces* is the most prolific genus used in therapeutic applications and pharmaceutical industries [7,8]. Approximately 60% of antibiotics used today are derived from the genus *Streptomyces* [9]. *Streptomyces* are filamentous, Gram-positive, and sporulating bacteria with high GC content in a range of 55–75% [10]. *Streptomyces* species are saprophytic organisms that spend the majority of their life cycles as dormant spores [11]. Though *Streptomyces* are valuable prokaryotes of clinical and medicinal importance, novel antibiotics have not been discovered in the last few decades. This is because of the repeated rediscovery of similar secondary metabolites from bacteria of similar ecological niches [12]. We believe the continuous screening of actinomycetes from different habitats could lead to the discovery of alternative antibiotics.

The process of new drug discovery is an arduous task; however, the use of computer-aided tools in drug discovery is now gaining more popularity and appreciation [13]. To find medications from natural sources, a computational platform has emerged as a key area of research. The computer-aided drug discovery (CADD) approach entails the discovery of potential drug targets, high-throughput screening, optimization of lead compounds, and examination of potential side effects and toxicity [14]. Molecular docking can be used to model the interaction between a small molecule and a target protein, thereby allowing the characterization of the behavior of small molecules at the binding site of target proteins [15].

Nepal, being a country with a wide range of geographies, altitudes, and vegetation along with many unexplored and untouched ecosystems, has a high probability of isolating actinomycetes producing new and potent secondary metabolites. Soil is the habitat for a large number of *Streptomyces* species, and a majority of soils have 10^4 to 10^7 *Streptomyces* colony-forming units per gram of soil, accounting for 1 to 20% or even more of the total viable counts [16]. Nevertheless, Nepalese ecosystems have not been extensively explored in the search for actinomycetes.

In the present paper, an attempt was made to isolate, characterize, and validate the antimicrobial properties of *Streptomyces* species isolated from soil collected from untouched habitats of Nepal covering higher altitudes to lowlands. The focus was on characterizing the *Streptomyces* species based on morphology, biochemical tests, molecular sequencing, and molecular annotation of secondary metabolites in some potent isolates. Finally, those metabolites were used to dock against some target proteins of several bacteria to understand the mechanism of action of these metabolites for the exploration and validation of antimicrobial properties.

2. Materials and Methods

2.1. Collection of Samples and Isolation of Streptomyces

Ten soil samples (SB1 to SB10) were collected from different geographical locations in Nepal covering different altitudes and untouched habitats, as shown in Table 1.

2.2. Isolation of Streptomyces

One gram of soil was taken and suspended in 10 mL of autoclaved water by vortexing. The vegetative cells in the soil sample were killed by heat treatment at 80 °C for 30 min. The suspension was further diluted up to 1000-fold in sterilized water and 100 µL of suspension was spread into individual ISP4 (International Streptomyces Project 4) plates and incubated at 28 °C for 7 to 14 days. The actinomycetes were isolated on the ISP4 medium with the addition of nalidixic acid (20 mg/mL) and cycloheximide (50 mg/mL). The growth of Gram-negative bacteria was inhibited by nalidixic acid, and the growth of the fungus was controlled by cycloheximide. Based on morphological characterization, the colonies were

sub-cultured and the glycerol stocks were prepared for further use. The details of the soil sample collection and isolation of *Streptomyces* species were carried out as described in the literature [17,18].

Table 1. Description of the soil samples collected from untouched habitats in Nepal.

Soil Samples	Location	Habitats	Altitude (m)	Geographical Coordinates
SB1	Halesi, Khotang	Bare land	3100	27.1846° N, 86.5938° E
SB2	Muchchok, Gorkha	Forest	1300	28.1371° N, 84.6584° E
SB3	Shigash, Baitadi	Forest	2800	29.5174° N, 80.5938° E
SB4	Pame, Kaski	Agriculture land	822	28.2256° N, 83.9466° E
SB5	Nagarkot, Bhaktapur	Forest	2175	27.7107° N, 85.5023° E
SB6	Simbhanjyang, Makawanpur	Rhizosphere	2310	27.5921° N, 85.0855° E
SB7	Tatopani, Myagdi	Hot spring	2180	28.4949° N, 83.6194° E
SB8	Swargadawari, Pyuthan	Rhizosphere	2100	28.1214° N, 82.6744° E
SB9	Betini, Okhaldhunga	Forest	1500	27.2866° N, 86.4733° E
SB10	Muktinath, Mustang	Bare land	3710	28.8190° N, 83.8716° E

2.3. Morphological Characterization

Macroscopic and microscopic studies were implemented for the morphological characterization of the isolated colonies. The isolates were preliminarily identified by macroscopic characterization in which aerial and substrate mycelium, color, shape, pigmentation, and appearance of the colonies were observed [19]. The appearance of the colonies was noted based on dryness, roughness, toughness, and elevation. Microscopic identification was done by Gram staining and observing under 100× magnification [20]. Likewise, several biochemical tests including lipase, gelatin, amylase, sulfur test, indole, nitrate reduction, motility, urease, catalase, and MRVP tests were performed (Tables S3 and S4) [21].

2.4. Molecular Characterization

The isolates were cultured in a tryptic soy broth (TSB) medium in an Erlenmeyer flask at 28 °C and 180 rpm for 4–5 days with glass beads. Then, 15 mL of the culture was taken in a falcon tube and the cells were separated by centrifugation at 8000 rpm for 10 min. Genomic DNA was isolated using the phenol-chloroform method [22]. Universal primers 27F: AGAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACTT were used for the amplification of 16S rDNA. The PCR amplification was performed in 29 cycles in a 50 µL reaction mixture containing 10 µM oligonucleotides, 5× premix having *Taq* polymerase, and 2 µL (100 ng) genomic DNA. A cycle was run at 95 °C initial denaturation, 51.4 °C annealing, and 95 °C extensions. The resulting PCR products were then purified using Monarch® PCR and a DNA Cleanup kit (New England Biolabs Inc., Ipswich, MA, USA). The unidirectional sequencing of 16S rDNA was carried out in Macrogen, Seoul, South Korea. The BLAST search tool was used for the annotation of 16S rDNA partial sequences in the database [23].

2.5. Shake Flask Fermentation

Bacterial isolates were cultured in TSB broth (30 g/L) for 3 to 4 days at 140 rpm, 28 °C in a shaking incubator. After the full growth of the bacteria, its 1 mL suspension was transferred to freshly prepared TSB broth (100 mL) for fermentation and kept for 5 to 7 days (until bacterial growth meets stationary phase) at 28 °C with constant shaking at 140 rpm [24]. The suspension was mixed with an equal volume of ethyl acetate for the extraction of secondary metabolites. Finally, the organic phase was further used for antimicrobial assays.

2.6. Antimicrobial Assays

The antimicrobial activity of 17 *Streptomyces* isolates was initially tested by the primary screening method. The pathogens examined were *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 2591, *Kleibsella pneumonia* ATCC 700603, *Salmonella typhi* ATCC 14028, *Shigella sonnei* ATCC 25931, and *Acinetobacter baumanii* ATCC 19606. The antimicrobial assays of potent bacterial isolates in perpendicular screenings were performed by using the agar well diffusion method. The assays were carried out in triplicate with 5 µg of *Streptomyces* extract in each well along with 1 mg/mL neomycin and 50% DMSO as a positive and negative control, respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the most potent bacterial isolates were determined using broth microdilution [25].

2.7. Mass Spectrometry

The mass spectrometry of the ethyl acetate extracts of *S.* species_SB1 and *S.* species_SB3 was performed in the Sophisticated Analytical Instrument Facility (SAIF), Lucknow, India using an Agilent 6520 Q-TOF mass spectrometer. The data were acquired using positive electron spray ionization (ESI) mode. The mobile phase consisted of 5 to 80% gradient acetonitrile and 5 mM acetate buffer [25]. For SB10, low resolution (LR)-LCMS was obtained by using an HPLC system (1100 Series, Agilent Technologies, Waldbronn, Germany) coupled with an MS (ABSCIEx 3200 Q Trap, Darmstadt, Germany). Likewise, the extract was also subjected to HR-LC-ESI-MS/MS (Bruker TOF-MS MaXis impact ESI-HR-MS) profiling [18]. The positive mode ESI spectrum was deposited in the Global Natural Products Social (GNPS) Molecular Networking Platform (https://gnps.ucsd.edu) (accessed on 1 October 2022).

2.8. Molecular Annotation

The molecular profiling of the extracts from isolates *S.* species_SB1 and *S.* species_SB3 was executed by the previously described procedure [26]. The raw data of LC-HRMS obtained from extracts of isolates *S.* species_SB1 and *S.* species_SB3 were analyzed using MestreNova 12.0 software (Santiago de Compostela, SPAIN) for peak detection, alignment, and annotation. They were then compared to the database library, literature, dictionary of natural products, and METLIN metabolite searching cloud. The molecular formula for *m/z* and MS/MS fragmentation of isolate *S.* species_SB10 was read using Bruker Compass Data Analysis 4.4 and the molecule annotation was carried out in SIRIUS 4.9.12 through CSI: FingerID user interface by exporting a .mgf file [18].

2.9. Molecular Docking for the Connection with Antimicrobial Assays

2.9.1. Binding Site Prediction

The binding site residues of the target proteins were chosen in this investigation based on previous findings as well as through the co-crystallized ligand in the retrieved protein [27,28]. After discovering binding sites, the coordinates of the binding pocket for each target protein were generated using the define and edit binding site tool of BIOVIA Discovery Studio.

2.9.2. Ligand Preparation

The annotated compounds were downloaded in .sdf format through PubChem [29,30] and translated to .pdb format via Pymol [31,32]. For docking purposes, polar hydrogens and Gasteiger charges were added, and the compounds were saved in PDBQT format using the AutoDock application.

2.9.3. Receptor Preparation

The protein targets, 1JIJ (crystal structure of *S. aureus* TyrRS in complex with SB-239629); 6J33 (crystal structure of ligand-free PulA from *Klebsiella pneumoniae*); 3TTZ (crystal structure of a topoisomerase ATPase inhibitor); 3SRW (*S.aureus* Dihydrofolate Reductase

complexed with novel 7-aryl-2,4-diaminoquinazolines); 4UMW (crystal structure of zinc-transporting PIB-type ATPase in E2.PI state); 3UDI (crystal structure of *Acinetobacter baumannii* PBP1a in complex with penicillin G); and 7KRK (putative FabG from *Acinetobacter baumannii*) were attained from the RCSB PDB database. The proteins were prepared in BIOVIA Discovery Studio by detaching water components and other associated ligands before the docking experiments. The structures were saved in PDBQT format after optimizing with Kollmann charges and adding polar hydrogen in AutoDock tools [33]. A three-dimensional affinity grid box was generated around the binding sites of the target proteins with the size of 40 × 40 × 40 Angstrom and centered on the important residues of all target proteins where potent metabolites would bind.

2.9.4. Molecular Docking and Validation

AutoDock tools version 1.5.6 was used to dock the annotated compounds into the binding site of the target proteins. The outcome is a list of poses that are arranged according to ΔG, the predicted binding energy in kcal/mol. Using the superimposition technique, the root-mean-square deviation (RMSD) values of the first and second docked low-energy protein-ligand complex postures were determined. If the RMSD values are under 2 Å, the validation is said to have been successful. The poses of compounds in target proteins were analyzed using BIOVIA Discovery studio, and the best pose was chosen based on hydrogen bonding, hydrophobic, and π–π interactions with the binding residues.

3. Results

3.1. Morphological Characterization

A total of 17 isolates showing the characteristics of *Streptomyces* were isolated from 10 different soil samples. The detail and morphological features of all the isolates are presented in Supplementary Tables S1 and S2. The aerial and substrate mycelium of *S.* species_SB10 were grayish and white, respectively, along with no diffusible pigments (Figure 1).

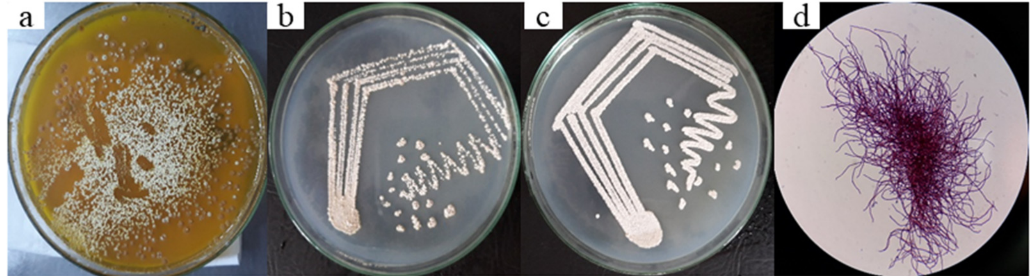


Figure 1. Morphological characterization; (**a**) Isolation plate; (**b**) Substrate mycelium; (**c**) Aerial mycelium; and (**d**) Gram staining of *Streptomyces* species SB10.

3.2. Molecular Characterization of Isolates

Universal primers were used to amplify the 16S rRNA. The 16S rRNA gene sequencing of SB1, SB3, and SB10 showed that the isolates resembled the genus *Streptomyces* on the BLAST sequence search generated from NCBI. A DNA sequence analysis showed that SB1, SB3, and SB10 were closely related to *Streptomyces bungoensis*, *Streptomyces aureus*, and *Streptomyces griseoplanus* with percentage similarities of 84.31, 95.79, and 95.92, respectively. Thus, according to morphological and molecular characterization, it was concluded that isolates from the soil samples SB1, SB3, and SB10 were members of the genus *Streptomyces*.

3.3. Antimicrobial Assays

Out of 17 isolates, *S.* species_SB1, *S.* species_SB3, and *S.* species_SB10 showed potential antimicrobial activities in the primary screening and hence were considered for further studies. The antimicrobial assays performed on the three *Streptomyces* isolates

showed moderate to strong activity against Gram-positive (*S. aureus*) and Gram-negative (*S. typhi*, *K. pneumoniae*, *S. sonnei*, *A. baumannii*, and *E. coli*) bacteria. The zones of inhibition (ZoIs) measured were recorded as shown in Table 2 and Supplementary Figure S1. The antibacterial activity of *S.* species_SB10 was the highest against *S. aureus* and *S. sonnei* with a ZoI of 30 mm for each bacterium. The results showed that the extract of *S.* species_SB10 was more potent than the extracts of *S.* species_SB1 and *S.* species_SB3. Therefore, only the MIC and MBC of the *S.* species_SB10 were determined. The MIC of *S.* species_SB10 against *S. aureus* and *S. sonnei* was found to be 1.22 µg/mL, while the MBC was 2.44 µg/mL (Figure S2). On the other hand, the MIC of neomycin (positive control) against *S. aureus* and *S. sonnei* was 1.56 µg/mL, and the MBC was 12.5 and 6.25 µg/mL, respectively.

Table 2. Zones of inhibition exhibited by *Streptomyces* isolates.

Organisms	Zone of Inhibition (mm)			
	Streptomyces Species_SB1	*Streptomyces* Species_SB3	*Streptomyces* Species_SB10	Neomycin (Control)
S. aureus	11	19	30	22
E. coli	-	-	18	18
K. pneumoniae	-	10	-	16
S. typhi	-	-	-	15
S. sonnei	10	17	30	23
A. baumannii	10	10	18	20

3.4. Liquid Chromatography–Mass Spectrometry Analysis

Streptomyces species showing good antimicrobial properties (*S.* species_SB1, *S.* species_SB3, and *S.* species_SB10) were further processed for mass analysis in ethyl acetate extracts. The annotated metabolites are shown in Tables 3 and 4. The metabolites were found to contain multiple chemical scaffolds. Extracts of *S.* species_SB1 and *S.* species_SB3 primarily consisted of diketopiperazines (DKPs). The extract of *S.* species_SB10 showed the presence of the valuable metabolite albumycin and aminocoumarin antibiotics. At least 20 different secondary metabolites were identified from these three isolates.

Table 3. Some annotated compounds from the ethyl acetate extracts of *S.* species SB1 and *S.* species SB3.

S.N.	Annotated Compounds	Calculated Mass	Observed Mass	Molecular Formula	Double Bond Equivalence	Absolute Error (ppm)	Retention Time (min)	Source	Reference
1.	Cyclo-(L-Pro-4-OH-L-Leu)	227.13	226.13	$C_{11}H_{18}N_2O_3$	4.0	4.87	5.63	SB1/SB3	[34]
2.	cyclo-(L-Pro-L-Val)	196.12	197.12	$C_{10}H_{16}N_2O_2$	4	3.76	5.39	SB1/SB3	[35]
3.	cyclo (Tyr-Pro)/Maculosin	260.11	261.12	$C_{14}H_{16}N_2O_3$	8	1.17	6.09	SB1/SB3	[36]
4.	Cyclo-(L-Leu-L-Pro)	210.13	211.14	$C_{11}H_{18}N_2O_2$	4	1.92	6.71	SB1/SB3	[37]
5.	(3R,8aS)-3-Methyl-1,2,3,4,6,7,8, 8a-octahydropyrrolo [1,2-a]pyrazine1,4-dione	168.08	169.09	$C_8H_{12}N_2O_2$	4	3.79	3.24	SB3	[38]
6.	Essramycin	268.09	268.103	$C_{14}H_{12}N_4O_2$	11	3.38	4.82	SB3	[39]
7.	2-Piperidinone	99.06	100.07	C_5H_9NO	2	0.47	3.66	SB1/SB3	[40]
8.	cyclo-[Pro-Phe]	244.12	245.12	$C_{14}H_{16}N_2O_2$	8	0.62	7.65	SB1/SB3	[41]
9.	cyclo-(L-Pro-L-Val)	196.12	197.12	$C_{10}H_{16}N_2O_2$	4	3.76	5.42	SB1/SB3	[35]

3.5. Molecular Docking Analysis

The major ten annotated compounds in mass spectrometry were docked through AutoDock tools with the target proteins mentioned earlier. The binding energies of ligands (docked compounds) with proteins are displayed in Table S5. The results of the docking analysis suggested that brevianamide F, essramycin, cyclo (L-Phe-L-Ala), and cyclo (L-Val-L-Phe) were the potential candidates that could inhibit the target proteins of various antimicrobials. These compounds exhibited suitable binding affinities with acceptable binding interactions along with one or more hydrogen-bonding interactions with binding residues of proteins. Most of the interacting residues fell under binding sites

of co-crystallized ligands on target proteins, which are in bold in Table 5. The binding interactions of essramycin and Brevianamide F complexed with target proteins, *S. aureus* TyrRS (PDB ID: 1JIJ*); S. aureus* dihydrofolate reductase (3SRW); and *S. sonnei* Zinc transporting PIB-type ATPase in E2.PI state (4UMW), respectively, are shown in Figure 2 and the other interactions of potent complexes were displayed in supplementary Figures S3–S6.

Table 4. Annotated compounds from the ethyl acetate extract of *S.* species SB10.

S.N.	Annotated Compound	Calculated Mass	Observed Mass	Adduct Type	Molecular Formula	Retention Time (Min)	RDB	Error ppm	Spectral Match (Sirius Score)	Reference
1.	(3S,6S)-3-benzyl-6-isopropylpiperazine-2,5-dione [Cyclo (L-Val-L-Phe)]	246.14	247.14	$[M + H]^+$	$C_{14}H_{18}N_2O_2$	16.0	7.0	−1.1	32.59%	[42]
2.	Nonactic acid-trihomononactic acid dilactone	410.27	411.27	$[M + H]^+$	$C_{23}H_{38}O_6$	35.1	5.0	3.1	50.26%	[43]
3.	Albaflavenol	220.18	221.18	$[M + H]^+$	$C_{15}H_{24}O$	16.5	4.0	−2.4	56.10%	[44]
4.	Succinilene D	338.25	339.25	$[M + H]^+$	$C_{20}H_{34}O_4$	33.7	4.0	4.1	45.32%	[45]
5.	Benzyl acetate	150.07	151.07	$[M + H]^+$	$C_9H_{10}O_2$	13.2	5.0	−1.2	27.38%	[46]
6.	(6S,3S)-6-benzyl-3-methyl-2,5-diketopiperazine [Cyclo(L-Phe-L-Ala)]	218.11	219.11	$[M + H]^+$	$C_{12}H_{14}N_2O_2$	12.7	7.0	−1.5	66.22%	[47]
7.	P-hydroxyphenylacetaldoxime	151.06	152.06	$[M + H]^+$	$C_8H_9NO_2$	10.1	5.0	−1.0	46.36%	[48]
8.	N6,N6-dimethyladenosine	295.23	296.23	$[M + H]^+$	$C_{12}H_{17}N_5O_4$	9.3	7.0	−1.2	75.56%	[49]
9.	Cyclo(leucylprolyl)	210.14	211.14	$[M + H]^+$	$C_{11}H_{18}N_2O_2$	13.0	4.0	−3.3	49.77%	[37]
10	Albumycin	190.07	191.07	$[M + H]^+$	$C_{10}H_{10}N_2O_2$	8.8	7.0	4.1	18.59%	[50]
11.	Cyclo (L-Tyr-L-Leu)	276.15	277.15	$[M + H]^+$	$C_{15}H_{20}N_2O_3$	12.8	7.0	−1.9	69.25%	[51]
12.	Brevianamide F[Cyclo (L-Trp-L-Leu)]	283.13	284.13	$[M + H]^+$	$C_{16}H_{17}N_3O_2$	15.6	10.0	−1.2	81.80%	[52]
13.	Maculosin[cyclo (Tyr-Pro)]	260.11	261.12	$[M + H]^+$	$C_{14}H_{16}N_2O_3$	14..4	8.0	−2.5	-	[40]
14.	Cyclo-(L-Pro-4-OH-L-Leu)	226.13	227.13	$[M + H]^+$	$C_{11}H_{18}N_2O_3$	5.6	4.0	4.87	-	[34]
15.	cyclo-(L-Pro-L-Val)	196.12	197.12	$[M + H]^+$	$C_{10}H_{16}N_2O_2$	5.3	4.0	3.76	-	[35]
16.	Cyclo-(L-Leu-L-Pro)	210.13	211.14	$[M + H]^+$	$C_{11}H_{18}N_2O_2$	6.0	4.0	1.92	-	[37]
17.	cyclo-[Pro-Phe]	244.12	245.12	$[M + H]^+$	$C_{14}H_{16}N_2O_2$	7.6	8.0	0.6	-	[41]

Note: Metabolites 13–17 are common DKPs detected in *S.* species SB1, SB3, and SB10.

Table 5. The binding energies and interacting residues (important residues are represented in bold) of target proteins with potent compounds.

Target Proteins (PDB ID)	Binding Energy (kcal/mol)				Interacting Residues			
	Brevianamide F	Essramycin	Cyclo(L-Phe-L-Ala)	Cyclo (L-Val-L-Phe)	Brevianamide F	Essramycin	Cyclo(L-Phe-L-Ala)	Cyclo (L-Val-L-Phe)
S. aureus TyrRS (1JIJ)	−9.0	−9.1	−8.0	−7.8	**Gly 38** **Asp 40** **Tyr 170** **Gly 193** Gln 196 **His 50** Pro 53	**Asp 40** **Tyr 170** **Gln 174** Gln 196 Ala 39 **His 50** Leu 70 **Asp 195**	**Gly 38** **Gln 174** Leu 70	**Gly 38** **Asp 80** **Tyr 170,** **Gln 174** Gln 196 Lys 84
K. pneumonia PulA (6J33)	−8.7	−7.6	−7.3	−7.0	His 607 Leu 678 Asp 560 Tyr 892	Asp 834 Glu 706 Asn 835 Arg 675 Tyr 559 Trp 557 Trp 708 Pro 745	Glu 706 His 833 Arg 675 Tyr 559 Cys 643	Glu 706 His 833 Arg 675 Asp 834 Tyr 559 Cys 643 Trp 708 Leu 678
S. aureus topoisomerase ATPase inhibitor (3TTZ)	−8.7	−8.5	−6.2	−6.3	**Asp 81** Ser 55 Glu 58 **Ile 86** **Ile 51** **Ile 175** Arg 84 Pro 87 Leu 103	**Asp 81** Ser 55 Gly 85 Asn 54 **Ile 86** **Pro 87** **Ile 51** **Ile 175** Leu 103	**Asp 81** Ser 55 Glu 58	Gln 66 His143 Lys 170
S. aureus dihydrofolate reductase (3SRW)	−9.1	−8.7	−7.5	−8.0	**Leu 21** Ile 15 Thr 47 Lys 46	Gly 95 Thr 47 Lys 46 Leu63	**Leu 21** Phe 93 Gly 95	Ala 8 Ile 15 Thr 47 Phe 93 **Leu 21** **Val 32**

Table 5. *Cont.*

Target Proteins (PDB ID)	Binding Energy (kcal/mol)				Interacting Residues			
	Brevianamide F	Essramycin	Cyclo(L-Phe-L-Ala)	Cyclo (L-Val-L-Phe)	Brevianamide F	Essramycin	Cyclo(L-Phe-L-Ala)	Cyclo (L-Val-L-Phe)
*S. sonnei*Zinc transporting p-type ATPase (4UMW)	−8.1	−7.0	−7.3	−5.5	Arg 345 Glu 184 **Phe 210** Leu211 Pro 166 Glu214 Val397	Arg 345 Leu 396 Ile 347 Pro 401 Val 397 Glu 184	Arg 345 Glu 184 **Phe 210** Ile 167 Pro 166 Glu 214	Gly 694 **Lys 693** Leu 69 Tyr 354 Phe 350 Leu 701 Val 361 Leu 697
A. baumannii PBP1a (3UDI)	−7.5	−7.7	−6.2	−6.6	**Ser 434** Gly 708, **Tyr 707** **Thr 672**	**Ser 434** Gly 708 **Tyr 707**	**Ser 434** **Thr 672** Leu 486 **Asn 489**	**Ser 434** Thr 672 Leu 48 **Asn 489** **Tyr 707**
A. baumannii FabG (7KRK)	−7.1	−7.4	−6.6	−6.5	Ile 19 Asn 88 Gly 90 Ala 89 Gly18 Ser 15 Leu 39 Val 111	Asp 38 Gly 90 Ala 89 Leu 39 Val 111 Val 62	Gly 12 Ile 19 Gly 20 Asn 88 Gly 90 Gly 18 Ala 89	Ile 19 Gly 20 Asn 88 Gly 90 Ala 89

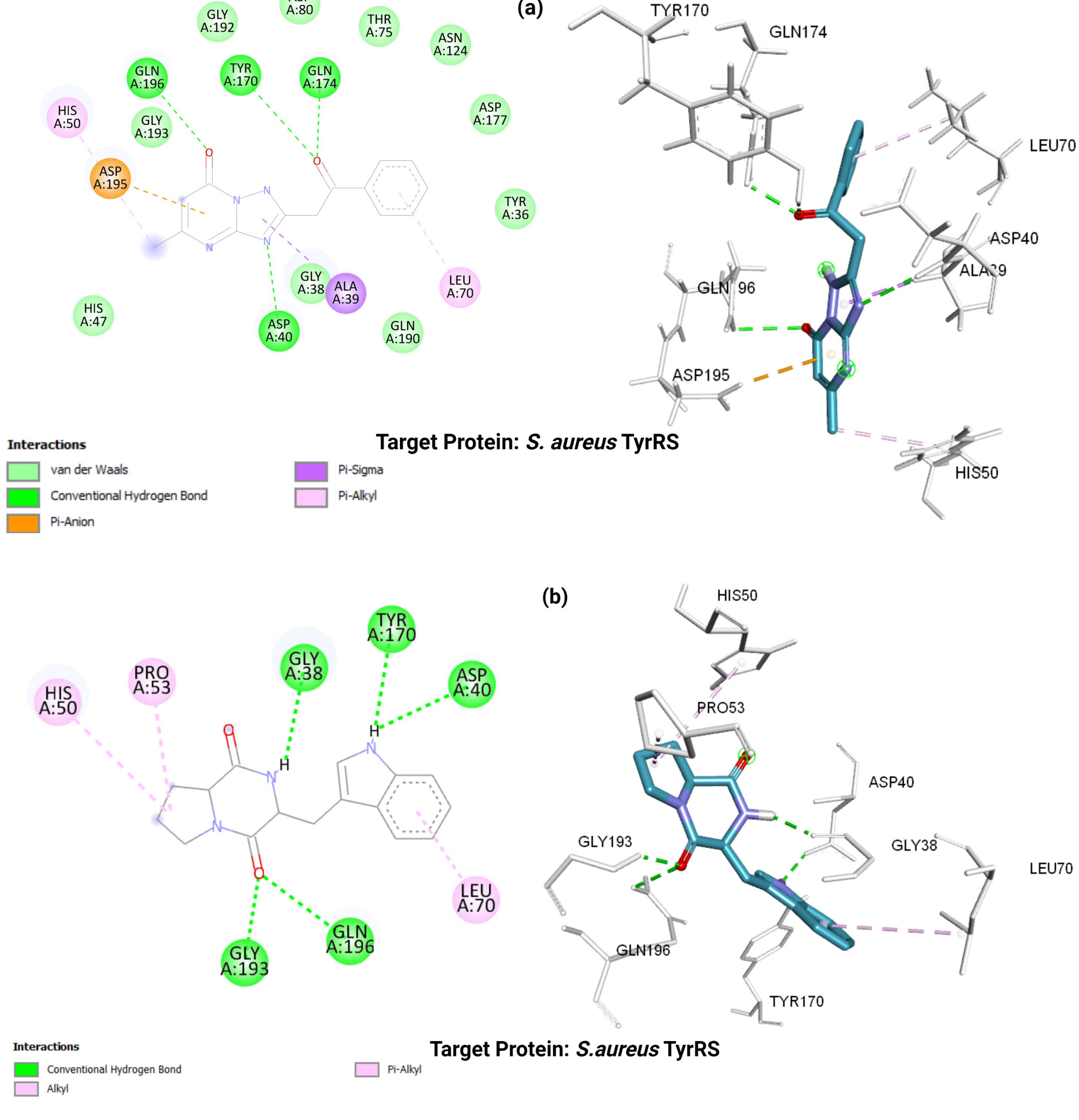
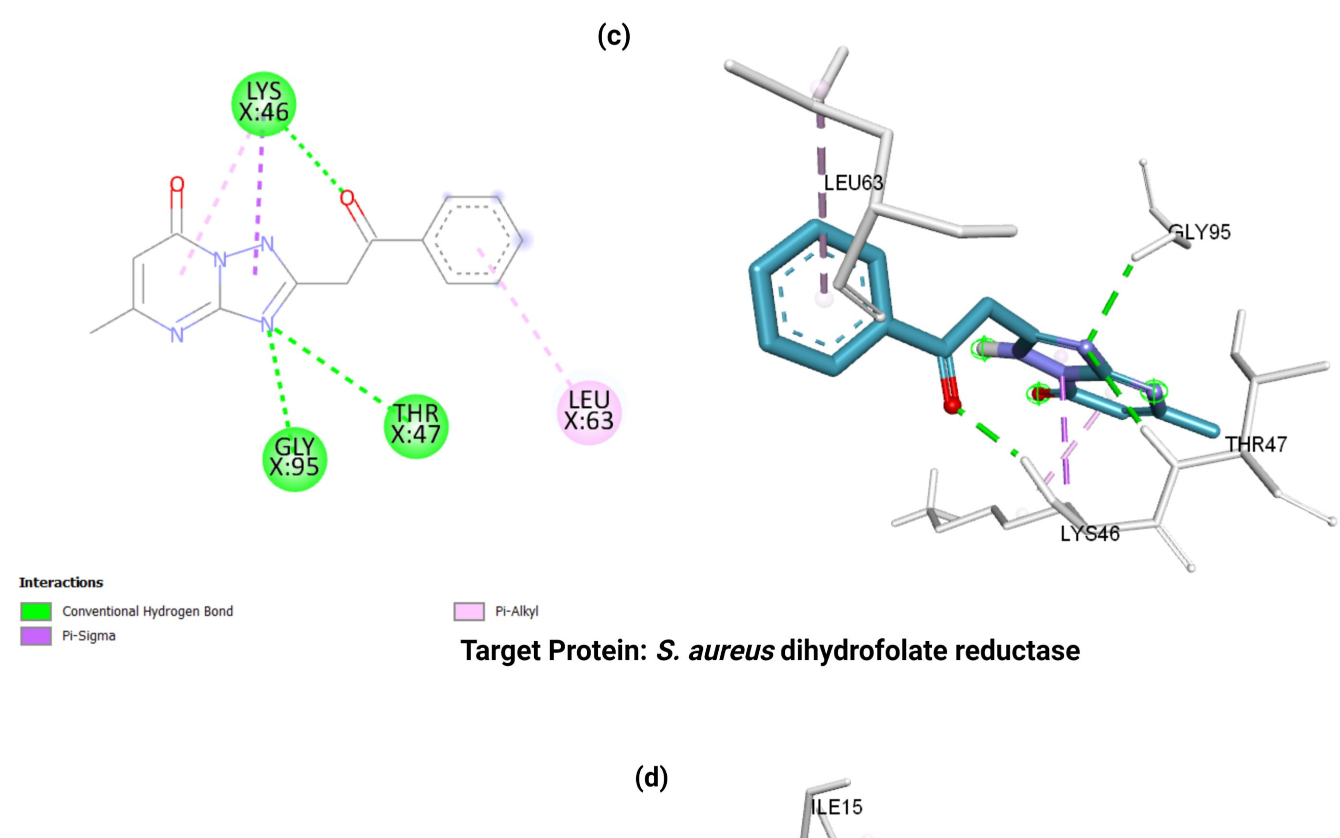


Figure 2. *Cont.*



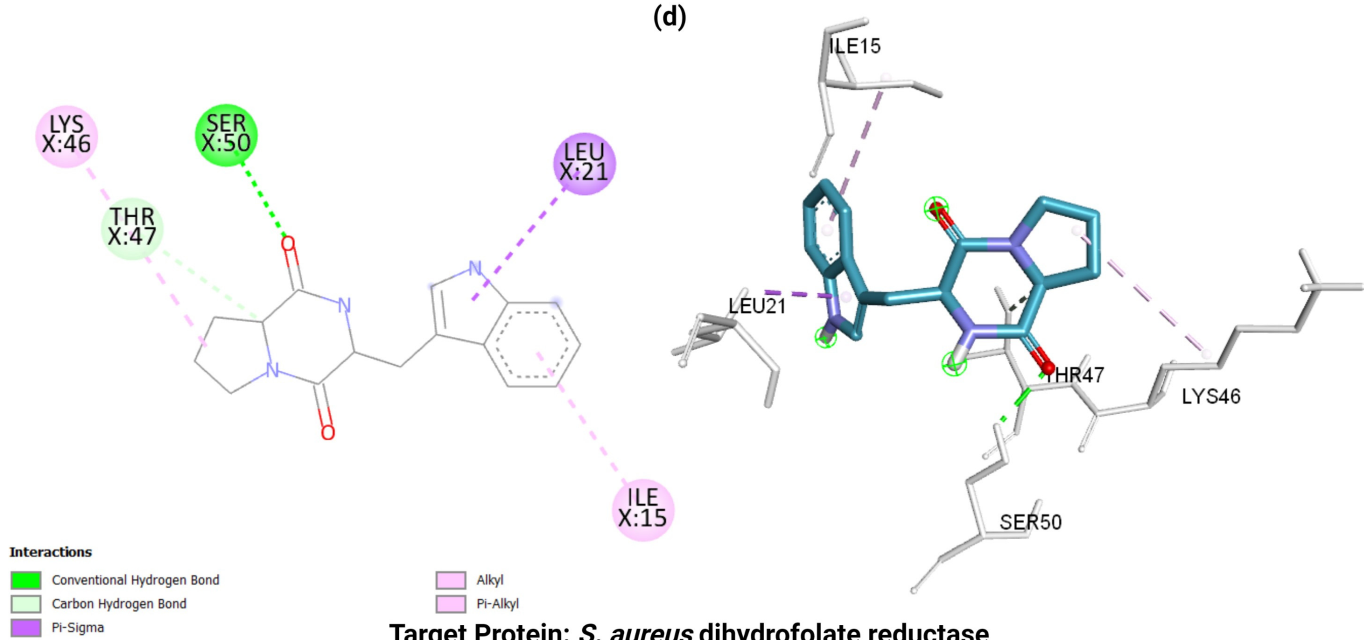


Figure 2. *Cont.*

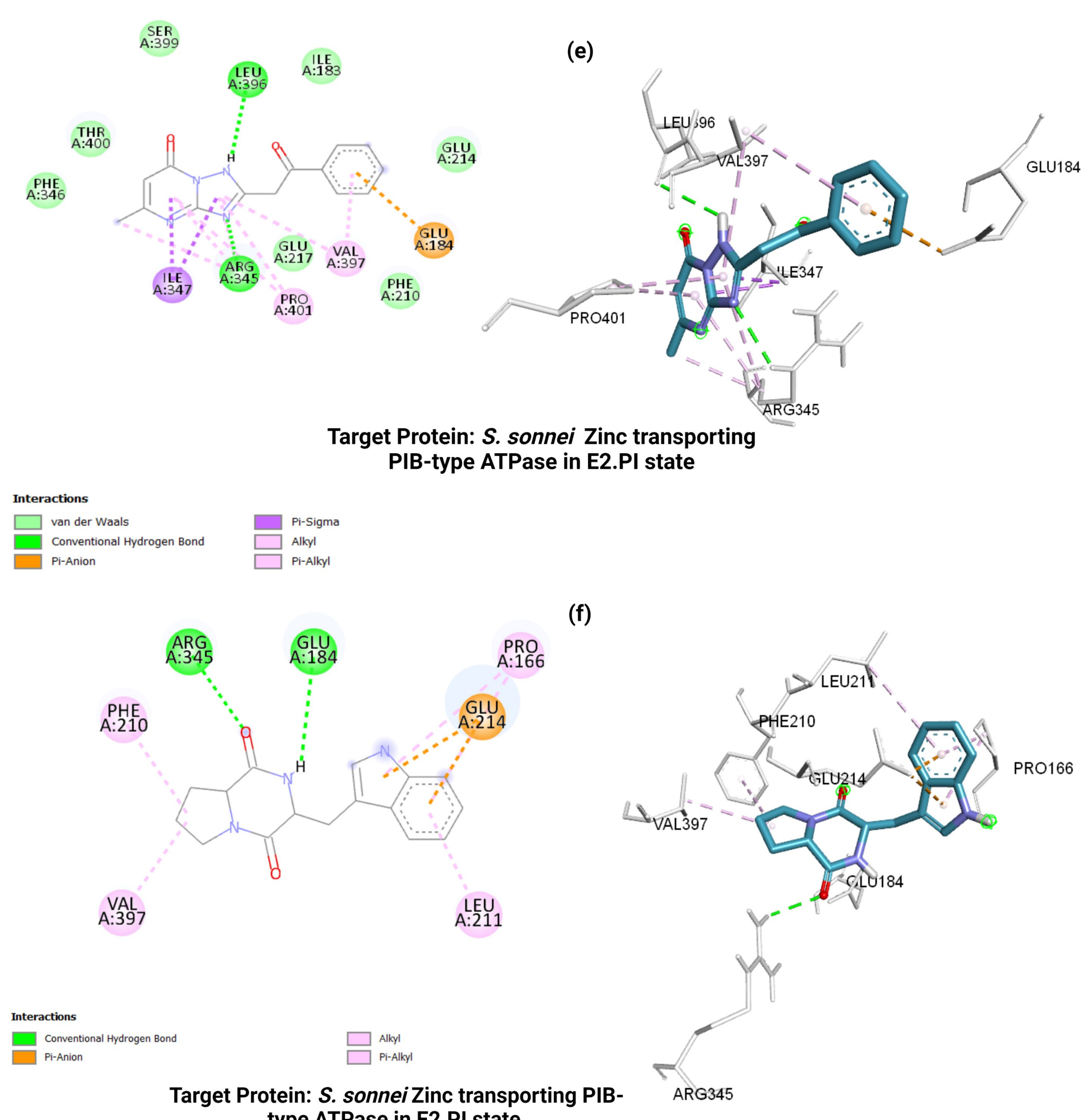


Figure 2. (**a**,**b**) represent binding interactions of essramycin and brevianamide F complexes with target protein *S. aureus* TyrRS, respectively; (**c**,**d**) represent binding interactions of essramycin and brevianamide F complexes with the target protein *S. aureus* dihydrofolate reductase; (**e**,**f**) represent binding interactions of essramycin and brevianamide F complexes with target protein *S. sonnei* Zinc-transporting PIB-type ATPase in E2.PI state.

4. Discussion

Microbial species are the source of secondary metabolites with undeniable biological activities. Most importantly, actinomycetes are given particular attention for their role in the production of various bioactive secondary metabolites. *Streptomyces* species are continually used to discover novel compounds with various biological functions [53]. Antibiotics are the primary and most crucial output of *Streptomyces* species [54]. So, in this work, we

have investigated the antibiotic-producing *Streptomyces* species from untouched habitats in Nepal.

Despite the development of antibiotics and the success of their use, infectious diseases continue to be the second biggest cause of death globally. Bacterial infections account for over 17 million fatalities each year, primarily affecting the elderly and young [54]. In addition to this, multi-drug resistance (MDR) has posed a serious issue in treating infectious diseases. This situation highlights the urgent need to investigate new antimicrobial agents to tackle MDR. The main contender for antibiotic discovery is still soil microorganisms [55]. Hence, this study is focused on the isolation and characterization of *Streptomyces* species and the validation of their antimicrobial activities using the metabolites present in their extracts through a computational approach.

We have isolated 17 different strains of actinomycetes from varying locations. Among these, three *Streptomyces* species, namely, *S.* species_SB1, *S.* species_SB3, and *S.* species_SB10 were determined to possess potential antibacterial properties. *S.* species_SB10 showed an interesting antibacterial effect against *S. aureus* and *S. sonnei* with ZoIs even higher than neomycin (1 mg/mL). This isolate showed an MIC and MBC of 1.22 µg/mL and 2.44 µg/mL, respectively, against *S. aureus* and *S. sonnei*. The potential of *S.* species_SB10 as well as *S.* species_SB1 and *S.* species_SB3 to inhibit the pathogens establishes them as effective candidates for antibiotic production that can combat MDR.

These three potent *Streptomyces* strains were found to contain DKPs such as maculosin, cyclo (L-Phe-L-Ala), cyclo (L-Val-L-Phe), cyclo (L-Leu-L-Pro), cyclo (D-Ala-L-Pro), cyclo (L-Tyr-L-Leu), and brevianamide F. DKPs are known for their rich source of new biologically active compounds. They show a wide spectrum of biological activities, suggesting them as potential therapeutic candidates [56]. They are known to function as antibacterial [57], antiviral [58], antifungal [59], anti-hyperglycemic [60], and antitumor agents [61]. These 2,5 DKPs are also involved as enzyme modulators and biochemical mediators [62]. The availability of donor and acceptor atoms for hydrogen bonding, rigid conformation, and resistance towards proteolysis favor interaction with biological targets [62]. Likewise, Brevianamide F is used as a medicament for cardiovascular dysfunction and cognitive enhancement [62]. The presence of these chemical scaffolds might be the reason for the effective antibacterial activity shown by the extracts. Furthermore, the isolation and characterization of these compounds are needed for other bioactivity-related studies. Moreover, to obtain the full potential of isolated strains, different media cultivation might be required in the future.

Albumycin was previously noted to have antibacterial activities [50]. Similarly, aminocoumarins were also described to show potential antibacterial effects against *Staphylococci*, including a methicillin-resistant *Staphylococcus aureus* strain [63]. Reports show that 3-aminocoumarin has been used for the synthesis of metal complexes possessing antimicrobial and antioxidant activities [64]. The presence of these chemical scaffolds increases the likelihood of *S.* species_SB1, SB3, and SB10 being employed as therapeutic drugs, particularly as antimicrobial agents. For further validation of antimicrobial activity, molecular docking was performed.

Molecular docking helps minimize the binding energy by optimizing the conformation of both the receptor and ligand and the orientation between the ligand and receptor. Although activity assays are still required to verify the activities of the ligands, molecular docking is a theoretical and reliable approach to anticipating the interactions between the receptor and ligand [65]. To find possible inhibitors for target proteins, we employed molecular docking analysis to predict binding sites and the potential activity of the top-scoring compounds [66]. The results were evaluated according to the docking poses and the protein-ligand interactions [65]. Furthermore, the lower the binding energy, the higher the stability of the complex [67]. The present study suggests brevianamide F, essramycin, cyclo (L-Phe-L-Ala), and cyclo (L-Val-L-Phe), as potent inhibitors for target proteins with significant binding energy and appropriate interactions.

The compounds brevianamide F and essramycin have comparatively lower binding energies with the target proteins, and most of the interacted residues fall into binding sites on comparison with the interacted residues of co-crystallized ligands of retrieved protein from RCSB and also with the important residues of target proteins, according to the literature [68,69]. The orientation of ligands in the target proteins within the binding site was analyzed through hydrogen-bonding, hydrophobic, and π–π interactions. According to earlier in silico and in vitro studies, natural compounds such as acacetin and chrysin can prevent the growth of *S. aureus* by inhibiting tyrosyl-tRNA synthetases (TyrRSs) [70]. In addition, through the molecular docking method too, ciprofloxacin analogs were identified as potential candidates against *S. aureus* DNA gyrase [66]. Furthermore, a molecular docking study revealed acetylated abietane quinone as a suitable inhibitor of *S. aureus* clumping factor A (ClfA) with good binding interactions and binding energy [71]. Hence, molecular docking methods can be taken into account to corroborate the potent metabolites.

The secondary metabolites from *Streptomyces* are significant products that can be utilized to combat various MDR pathogens. The production and commercial viability of these secondary metabolites are not yet fully established, but shortly, their application may accelerate, specifically in the manufacturing of antibiotics. It has been estimated that only 10% of discovered antibiotics are from screened bacterial strains and only 1% from all microbes [72]. Actinomycetes found in terrestrial soil comprise around two-thirds of all natural antibiotics. According to recent estimates, to find the next new antibiotics class, 107 actinomycetes strains would need to be screened [73]. Hence, for the utilization of the soil actinomycetes for antibiotics production, the pace of screening and validation should be intensified. Considering this fact, the use of computational techniques may be extended into validating newer antibiotic-potent products showing a broad spectrum of activity.

5. Conclusions

The present study depicts that based on the variations of altitude and soil types and their contents, there is a huge probability of obtaining a varying diversity of antimicrobial-producing *Streptomyces*. In this study, a total of 17 distinct strains were isolated based on the morphological characteristics of 10 different soil samples collected from different habitats. Three samples were selected for further study based on the antimicrobial screening test. The three *Streptomyces* species SB1, SB3, and SB10 exhibited significant antibacterial activity against *S. aureus*, *S. sonnei*, and *A. baumanii*. At least 24 different secondary metabolites were identified from these three isolates, and these metabolites included different chemical scaffolds such as DKPs and aminocoumarin antibiotics. These metabolites were further subjected to docking to validate their antibacterial properties. Docking positively signified brevianamide F, essramycin, cyclo(L-Phe-L-Ala), and cyclo(L-Val-L-Phe) as potent inhibitors for target proteins, thereby showing the potential of isolated *Streptomyces* species for use in the production of antibiotics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10102149/s1, Table S1: Soil profile, total isolated actinomycetes and characterization method of 10 samples; Table S2: Culture characteristic and Gram staining of samples SB1–SB10; Table S3: Biochemical test of microbial strains; Table S4: Cultural and physiological characteristics of microbial strains; Table S5: The binding energies of target proteins with potent compounds; Figure S1: Zone of inhibition exhibited by SB1, SB3, and SB10 against *S. aureus* and *S. sonnei*; Figure S2: MIC and MBC of the extract SB10 against *S. sonnei* and *S. aureus*; Figure S3: (A–D) represents 2D structures of essramycin with target proteins: PulA from *Klebsiella pneumonia* (6J33), Topoisomerase ATPase inhibitor (3TTZ), *A. baumannii* PBP1a (3UDI), and Putative FabG from *A. baumannii* (7KRK), respectively; Figure S4: (A–D) represent 2D structures of brevianamide F with target proteins: PulA from *Klebsiella pneumonia* (6J33), Topoisomerase ATPase inhibitor (3TTZ), *A. baumannii* PBP1a (3UDI), and Putative FabG from *A. baumannii* (7KRK), respectively; Figure S5: (A–G) represent 2D structures of cyclo(L-Val-L-Phe) with target proteins: *S. aureus* TyrRS (1JIJ), PulA from *Klebsiella pneumonia* (6J33), Topoisomerase ATPase inhibitor (3TTZ), *S.aureus* Dihydrofolate Reductase (3SRW), Zinc transporting PIB-type ATPase in E2.PI state (4UMW), *A. baumannii* PBP1a

(3UDI), and Putative FabG from *A. baumannii* (7KRK), respectively; Figure S6: (A–E) represent 2D structures of cyclo(L-Phe-L-Ala) with target proteins: PulA from Klebsiella pneumonia (6J33), Topoisomerase ATPase inhibitor (3TTZ), *S.aureus* Dihydrofolate Reductase (3SRW), *A. baumannii* PBP1a (3UDI), and Putative FabG from *A. baumannii* (7KRK), respectively.

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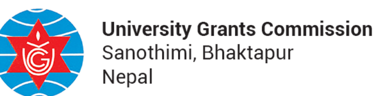

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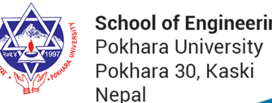

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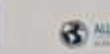

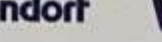

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