Chapter 1: Introduction and Objectives

1.1 Introduction

Microorganisms have gained significant attention from scientists over the years due to their essential role in numerous biological processes, particularly in the production of valuable compounds such as vitamins, antibiotics, and other pharmaceuticals (Rani et al., 2021). In fact, nearly 70% of the FDA-approved drugs are either natural products or a derivative of a natural product and the majority of these compounds are produced by Actinobacteria, especially by members of the genus *Streptomyces* (Alam et al., 2022; Ma & Karthik, 2022). *Streptomyces* is the largest genus of phylum Actinobacteria and holds paramount significance for the pharmaceutical industry, with about 50% of cytotoxic compounds approved for cancer therapy originating from actinobacteria (Laskaris & Karagouni, 2021).

Streptomyces are ubiquitous, aerobic, neutrophilic, facultative, gram-positive, and filamentous soil-dwelling bacteria, with very high GC content (70%) and low pathogenicity (Beroigui & Errachidi, 2023; Swiercz et al., 2008). They belong to the Streptomycetaceae family and order Streptomycetales (Donald et al., 2022). *Streptomyces* has a linear and moderately large genome (8-10 Mb) with multiple biosynthesis gene clusters (BGCs) on the genome, which exhibit potential for the synthesis of several bioactive compounds (Stulberg et al., 2016). *Streptomyces* possess both eukaryotic signalling and prokaryotic two-component regulatory systems, which are ideal for developing kinase inhibitor screening assays for eukaryotic pathways. *Streptomyces 85E* is a suitable strain for testing kinase inhibitors by the agar diffusion method due to its growth characteristics on solid media and ability to reveal cytotoxicity of tested compounds (Tiwari et al., 2017; Watersa et al., 2002).

Streptomyces clavuligerus and *Streptomyces fragilis* are gram-positive, filamentous actinobacteria that comprise aerial mycelium with branched hyphae and spores (Bascarán et al., 1990; Nithya et al., 2017). *Streptomyces clavuligerus* produces β-lactam antibiotics which include clavulanic acid, cephamycin C, penicillin N, O-carbamoyl derivative deacetylcephalosporin C, and deacetoxycephalosporin C (Nabais & da Fonseca, 1995). Clavulanic acid is a novel β-lactamase inhibitor (broad-spectrum antibiotic) effective against gram-positive and gram-negative bacteria (Saudagar et al., 2008). It also retains the ability to produce anticancer metabolites, including holomycin with a pyrrothine structure, Clavulactones, and Tunicamycins (Martínez-Burgo et al., 2019). Tunicamycins exert their anticancer effects by inhibiting protein glycosylation, a process crucial for the proper functioning of proteins involved in cell growth and survival (Hiss et al., 2007; Y. Wang, Zhang, et al., 2020). By disrupting glycosylation, tunicamycins induce stress responses within tumour cells and lead to apoptosis, or programmed cell death (Yun et al., 2023). Additionally, *Streptomyces clavuligerus* produces kinase and phosphatase inhibitors, which can be lead molecules in the target-based cancer treatment (López-Agudelo et al., 2021).

Streptomyces fragilis, a lesser-known species within the *Streptomyces* genus, remains underexplored despite its potential (Law et al., 2019). This species produces Azaserine, a tumor-inhibiting antibiotic that targets nucleic acids and is effective against gram-positive and gram-negative bacteria, protozoa, and fungi (Pittillo & Hunt, 1967; Van Cura et al., 2023). Further research on *Streptomyces fragilis* could reveal new bioactive compounds, novel anticancer compounds, and other valuable biochemical products, making it a promising candidate for future scientific and industrial applications.

The genus *Streptomyces* produces several antitumor drugs, including glycopeptides (bleomycin and actinomycin D), anthracyclines (daunomycin, doxorubicin, and

aclarubicin), enediynes (neocarzinostatin), aureolic acids (mithramycin), antimetabolites (pentostatin), carzinophilin, mitomycins, and others (Abdelghani et al., 2021; Olano, Mendez, et al., 2009). Bleomycin binds to DNA and induces single-strand breaks. Anthracyclines block topoisomerase II, thus preventing DNA replication. Mithramycin hinders RNA synthesis. Mitomycins and azinomycin B create crosslinks between complementary DNA strands and block replication (Czaja et al., 2022; Marinello et al., 2018; Miller et al., 1987; Semlow et al., 2016). Enediyne binds to DNA and causes oxidative damage, while Pentostatin inhibits adenosine deaminase, which is necessary for DNA precursor synthesis (Nicolaou et al., 1993; H. Zhang et al., 2022). Among them, anthracyclines are a potent chemotherapy drug. They are included in the World Health Organization's Model List of Essential Medicines for cancer treatment. (McGowan et al., 2017). Anthracyclines are given to 32% of breast cancer patients, 57-70% of elderly lymphoma patients, and 50-60% of childhood cancer survivors as part of their treatment (P. H. Nguyen et al., 2020).

The anthracyclines naturally produced by *Streptomyces* belong to the family of aromatic polyketides that are synthesized by type II polyketide synthases (PKSs). The biosynthesis begins with the formation of a primary poly-β-ketone by PKS, followed by the reduction of carbon-9 (C-9). This intermediate then undergoes cyclizations and dehydrations by cyclase enzymes, leading to the oxidation of the second ring and the formation of the anthraquinone structure, which is characteristic of all anthracyclines. The final step includes the hydrolytic release of the anthraquinone and the creation of a primary anthracycline core, which contains a C-4 hydroxyl group in the D-ring. This core is then altered by various enzymes to produce different types of anthracyclines, with the C-4 hydroxyl group being the fundamental feature of most anthracyclines (Risdian et al., 2019; Z. Zhang et al., 2017)

The most important anthracycline is daunorubicin and doxorubicin isolated from *Streptomyces peucetius,* which is widely used and a benchmark for the discovery of several other analogs. These drugs got FDA approval for cancer therapy in the 1960s (Bayles et al., 2023; van der Zanden et al., 2021). Daunorubicin is clinically approved in the treatment of acute lymphoblastic or myeloblastic lymphoma and doxorubicin is used in breast cancer, solid tumors in children, soft tissue sarcomas, and aggressive lymphomas (Mattioli et al., 2023). Beyond these two compounds, numerous anthracycline analogs have been structurally modified or synthesized via semi-synthesis or total synthesis.

Doxorubicin is associated with several side effects, including gastrointestinal and hepatic toxicity, cardiotoxicity, bone marrow toxicity, myelosuppression, nausea, vomiting, mouth ulcers, alopecia, and local aggressiveness (Mauldin et al., 1992).

Therefore, it is important to focus on less toxic anthracyclines for chemotherapy, and epirubicin is known for its reduced cardiotoxicity compared to Doxorubicin and represents a promising alternative. It is a 4' epimer of doxorubicin, widely used as a chemotherapeutic agent for its efficacy and tolerability in the treatment of various malignancies (Lagunes & Pezo, 2021). Epirubicin-based adjuvant therapy has been a focus of research, showing positive outcomes in the treatment of breast cancer. The use of epirubicin in combination with other agents like cyclophosphamide and 5-fluorouracil has become a preferred regimen for treating breast cancer due to its efficacy and manageable toxicity profile (Ackland et al., 2001). By producing Epirubicin from *Streptomyces* and optimizing the fermentation conditions, and harnessing the biosynthetic capabilities of *Streptomyces*, the yield and purity of Epirubicin can potentially be enhanced. This approach not only improves patient outcomes by reducing adverse effects but also advances the field of antibiotic production through biotechnological innovation.

Genus *Streptomyces* contains both eukaryotic and prokaryotic signalling systems, which is ideal for screening kinase inhibitors in eukaryotic pathways. The *Streptomyces 85E* strain is a suitable strain for conducting kinase inhibitory testing due to its growth on solid media and ability to reveal cytotoxicity through agar diffusion (Watersa et al., 2002). The kinase inhibitors like tyrphostin and genistein inhibit hyphae formation in the *Streptomyces 85E* strain, resulting in "bald" colonies (Shanbhag et al., 2015a). The Streptomyces 85E assay identifies kinase inhibitors with three phenotypes: no zone (inactive drug), clear zone (cytotoxic), and turbid grey zone (inhibits sporulation and hyphae). This is a cost-effective method for identifying eukaryotic protein kinase inhibitors (Batool et al., 2023).

A protein kinase is an enzyme that regulates cellular functions like cell signalling, growth, and apoptosis by phosphorylating specific proteins and modulating their activity (Ardito et al., 2017). Receptor tyrosine kinases (RTKs) are a subclass of protein kinases located on the cell surface. They are involved in critical physiological processes, and their dysregulation is often linked to diseases, especially cancers. Targeting RTKs with specific inhibitors is a common therapeutic strategy in oncology (Shabani & Hojjat-Farsangi, 2016).

The aim of this research was to address the protein kinase inhibitory potential of both strains, Streptomyces clavuligerus and Streptomyces fragilis, by the *Streptomyces 85E* assay. Additionally, a computational study was done to demonstrate the inhibitory potential of secondary metabolites of both strains against several receptor tyrosine kinase proteins. This research focuses on the production of bioactive compounds by submerged fermentation from *Streptomyces fragilis* by utilizing low-cost nutrient sources, particularly natural carbon sources instead of expensive synthetic carbon sources with the aim to lower down the production costs of metabolites. An optimization process, such as

response surface methodology (RSM) and artificial neural networks (ANN), was conducted to enhance the production of bioactive compounds. Additionally, the production of epirubicin by the underexplored species *Streptomyces fragilis* was achieved through submerged fermentation without any genetic manipulation. Epirubicin underwent thorough purification and characterization to ensure its purity and structural integrity. The purified epirubicin was then subjected to transcriptomic analysis on the A549 human lung cancer cell line to study changes in gene expression.

1.2 Objectives

→ Computational study and protein kinase inhibitory assay of secondary metabolites of *Streptomyces clavuligerus* and *Streptomyces fragilis*.

→ Optimization of culture conditions and different nutrient sources for the production of therapeutic compounds by *Streptomyces fragilis* through submerged fermentation.

→ Production, purification, and characterization of therapeutic compounds, epirubicin.

→ Evaluation of the anticancer potential of purified epirubicin against human lung cancer cells.