ASSESSMENT OF ANTIBIOTIC PRODUCTION BY SOME MARINE *STREPTOMYCES* ISOLATED FROM THE NAHOON BEACH

BY

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DECLARATION

I, the undersigned, declared that this thesis submitted to the University of Fort Hare for the degree of Masters of Science in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirety for the award of any degree.

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DEDICATION

This dissertation

is dedicated in memory of my beloved Grandmother;

Mrs Edowaye Amadasun

who passed on while I was away for this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

ABSTRACT

Rapidly emerging strains of bacteria resistant to most advanced antibiotics have become issues of very important public health concern. Research currently directed towards marine actinomycetes presents a vast potential for new compounds that could be able to safely and effectively target resistant species. In this regard, ten putative *Streptomyces* strains isolated from the Nahoon beach were selected and assessed for antibiotic production and activity against a wide range of bacteria including reference strains, environmental strain and clinical isolates. The ethyl acetate extracts of the putative *Streptomyces* isolates showed activities against at least 6 and up to 26 of the 32 test bacteria. Inhibition zones were found to range between 9-32 mm diameters at a concentration of 10 mg/ml. The minimum inhibitory concentrations (MICs) of the crude extracts ranged from 0.039 - 10 mg/ml and the least minimum bactericidal concentration (MBC) demonstrated was 0.625 mg/ml against a reference strain *Staphylococcus aureus* ATCC 6538. Time kill kinetics of all extracts revealed bacteristatic and bactericidal activities. Average Log reductions in viable cell counts for all the extracts ranged from 0.86 Log_{10} and 3.99 Log_{10} cfu/ml after 3 h interaction and 0.01 Log_{10} and 4.86 Log_{10} after 6 h interaction at MIC, 2 × MIC, 3 × MIC and 4 × MIC concentrations. Most of the extracts were speedily bactericidal at 3 × MIC and 4 × MIC resulting in over 50 % elimination of most of the test bacteria within 3 h and 6 h interaction. The partial characterization of the crude extracts by IR spectral analysis revealed possibility of terpenoid, long chain fatty acids and secondary amine derivatives compounds in the extracts. It is therefore recommended that further investigation should address the relationship between the structure of the active component of the extracts and the broad spectrum activity, as well as a rapid method for large scale production and purification and whether this group of antibiotics has any application in managing human infectious disease.

CHAPTER ONE

1.0 Background of study

A few decades after the introduction of antibiotics into clinical practice, resistance by pathogenic bacteria has become a major health concern. Indeed, while in the mid 1970s infectious diseases were considered virtually conquered (Breithaupt, 1999); many Gram positive bacteria and Gram negative opportunistic pathogens were becoming resistant to virtually every clinically available drug (Greenberg, 2003). The use of antimicrobial drugs for prophylactic or therapeutic purposes in human and veterinary or for agricultural purposes, have provided the selective pressure favouring the survival and spread of resistant organisms. *Staphylococcus aureus,* for instance, a virulent pathogen that is responsible for a wide range of infections including pimples, pneumonia, osteomyelitis, endocarditis and bacteremia, has developed resistance to most classes of antibiotics.

Methicillin-resistant *S. aureus* (MRSA) strains appeared in the hospital environment after introduction of the semi-synthetic penicillin and methicillin, leaving vancomycin as the last chance for MRSA treatment (Enright, 2003). Vancomycin is the last tool for the treatment of the infections caused by the resistant Gram positive microorganisms including MRSA (Enright, 2003). Indeed, vancomycin resistance is difficult to acquire because it is a complex system involving up to 7 genes. However vancomycin-intermediately-sensitive *S. aureus* were first isolated in 1997 in Japan and later in other countries (Fridkin, 2001). In fact, vancomycin-resistant clinical isolates have been recently reported (Tenover *et al.,* 2004). Thus currently, no antibiotic class is effective against multi-resistant *S. aureus* infections and new antibiotics or alternative chemotherapeutic strategies are urgently needed.

Enterococci are responsible for urinary tract, wound, intra abdominal, and pelvic infections (Barsby *et al.,* 2001). The increase of vancomycin-resistant enterococci (VRE) as important agents of nosocomial infections is cause for great concern (Perl, 1999). It is thought that a selective pressure

favouring the survival and spread of VRE was the consequence of the use of antibiotics in food and agricultural practices (Bax, 2000). Actually, there are no effective antibiotics currently available for such organisms. Even worse, vancomycin-resistance is often associated with multiple-drug resistance (Perl, 1999).

Another cause of great concern is the Gram-negative antibiotic-resistant opportunistic pathogens. These bacteria, like *Pseudomonas aeruginosa*, are common environmental organisms, which act as opportunistic pathogens in clinical cases where the defense system for patient is compromised (Lyczak *et al.,* 2000). For instance, over 80 % of cystic fibrosis (CF) patients become chronically infected with *P. aeruginosa* (Lyczak *et al.,* 2000). In addition, other intrinsically antibiotic resistant organisms such as *Burkholderia cepacia* and *Stenotrophomonas maltophilia* (Saiman *et al.,* 2002), are emerging as opportunistic pathogens. Interestingly, changes in the bacterial phenotype have been observed concomitant with the appearance or increase of antibiotic resistance. Indeed, in CF infections, initially, strains are non mucoid, but over time a mucoid population showing slow growth phenotype with an increased capability to form biofilms, the small colony variants (SCV), develops (Haussler *et al.,* 1999). This ability is considered a major virulence trait because the bacteria are protected from adverse environmental conditions as well as from biological and chemical antibacterial agents (Haussler, 2004). Thus, new therapeutic drugs and/or approaches are needed to improve the management of these diseases and overcome these problems (Taylor *et al.,* 2002).

The appearance of multi resistant pathogenic strains has caused a therapeutic problem of enormous proportions. For instance, they cause substantial morbidity and mortality especially among the elderly and immunocompromised patients. In response, there is a renewed interest in discovering novel classes of antibiotics that have different mechanisms of action (Barsby *et al.,* 2001). With increasing misuses of antibiotics, the serious problems of antibiotic resistance are developing at an alarming rate. Hence, intensive search for new antibiotics has become imperative worldwide (Haque

et al., 1995; Oskay *et al.,* 2004; Parungao *et al.,* 2007) especially from new actinomycetes such as *Streptomyces* which is known as the greatest source of antibiotics.

The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals, for development of new therapeutic agents and for evaluation of the potential of lesser-known and/or new bacterial taxa (Lazzarini *et al.,* 2000). It has been estimated that approximately two-third of the thousands of naturally occurring antibiotics have been isolated from actinomycetes (Takizawa *et al.,* 1993). Indeed, the *Streptomyces* species produce about 75% of commercially and medically useful antibiotics (Miyadoh, 1993).

New microbial metabolites are permanently needed due not only to the increase in resistant pathogens, but also to the evolution of novel diseases and toxicity of currently used compounds (Demain, 1999). Therefore, several strategies have to be employed to find new bioactive drugs including the exploration for new compounds from well known and talented microorganisms, such as S*treptomycetes,* due to the fact that only a small range of their biosynthetic capacity is currently exploited (Zahner *et al.,* 1995).

1.1 Aim and Objectives of the study

This study aims at exploring the antibiotics production potentials of some indigenous marine *Streptomyces* isolated from the Nahoon beach in the Eastern Cape Province of South Africa. The specific objectives include:

1. To prepare crude ethyl acetate extracts of the *Streptomyces* fermentation products.
2. To screen the crude extracts for antibacterial activities using a panel of bacterial strains.
3. To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts.
4. To determine rate of kill of the crude ethyl acetate extracts on selected susceptible bacteria.
5. To characterize the ethyl acetate crude extracts of the *Streptomyces* fermentation products.

1.2 The rationale for the study (Justification)

The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals for the development of new therapeutic agents, and evaluation of the potential of lesser-known and/or new bacterial taxa (Bull *et al.*, 2000). There has been considerable progress on the detection and identification of marine microorganisms since they were reported to produce novel bioactive compounds (Fenical, 1993; Copp, 2003; Shin *et al.*, 2003). It is necessary to continue to screen for new metabolites and evaluate the potential of less known and new bacterial taxa so that new and improved compounds for future use against drug-resistant bacteria or for chemical modification purposes may be developed (Kurtboke, 2005).

One of the efficient ways of discovering novel bioactive metabolites is through isolating new microorganisms, especially actinomycetes which produce about 70% of the known bioactive metabolites (Miyadoh, 1993). Therefore, investigation of new ecosystems for isolation of actinomycetes is crucial for the discovery of novel actinomycetes and subsequently for natural product-based drug discovery. Recently, several studies reported the investigation of different habitats for isolation of novel actinomycetes as rich sources of bioactive compounds (Hozzein *et al.*, 2008). Watve *et al.* (2001), has estimated that only a fraction of the antibiotics produced by *Streptomyces* strains have been discovered, thus underestimating the actual potentials of this genus as sources of novel antibiotics.

Programmes aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have found applications in human medicine and agriculture. However, it soon became apparent during screening programmes that some microbial metabolites were discovered more frequently than others. As the number of described microbial metabolites increased, so did the probability of rediscovering known compounds (Busti *et al.*, 2006). Also to thwart resistance of

microorganism to already available antibiotics, there is need for episodic swap of existing antibiotics. To combat these needs there is a call to screen underexplored habitat.

The Eastern Cape Province of South Africa is richly endowed with indigenous aquatic actinomycetes of which *Streptomyces* are most abundant (Ogunmwonyi, 2008), and there is no evidence in literature that suggests that these indigenous actinomycetes of the aquatic environment of the Eastern Cape Province have been explored for their antibiotics production potentials. Yet, the sustainable exploitation of *Streptomyces* for antibiotic production holds considerable promise as a strategy to combat infectious diseases. It is interesting that *Streptomyces* strains continue to provide a larger number and wider variety of new antibiotics than any other actinomycete genus, suggesting that substantial numbers of *Streptomyces* species or strains with novel antibiotic productivity exist in nature (Okami and Hotta, 1988; Hwang *et al.*, 2001). Hence, I hypothesize that the marine *Streptomyces* diversity of Nahoon beach; a marine environment of the Eastern Cape Province of South Africa could be a potential source of novel antibacterial compounds. Extraction of these compounds, quantitative and qualitative screening of the bioactive compounds would lead to discovery of novel compound that can fight against organism pathogenic to humans. The discovery of this novel compound(s) would be useful to the pharmaceutical industry and medicine to compact prevailing human pathogens in South Africa and in the world at large.

CHAPTER TWO

Literature Review

2.0 Genus *Streptomyces*

The genus *Streptomyces* was proposed by Waksman and Henrici in (1943). They are well known as a rich source of antibiotics and bioactive molecules and are thus considered to be a rich biotechnological resource. The genus *Streptomyces* belong to the family *Streptomycetaceae,* Order *Actinomycetales,* Phylum *Actinobacteria,* Domain Bacteria, and includes the following species: *S. achromogenes, S. ambofaciens, S. aureofaciens, S. avermitilis, S. clavuligerus, S. coelicolor, S. felleus, S. ferralitis, S. filamentosus, S. griseus , S. hygroscopicus, S. iysosuperficus, S. lividans, S. noursei, S. scabies, S. somaliensis, S. thermoviolaceus, S. toxytricini, S. tsukubaensis, S. venezuelae, S. violaceoruber* plus ~500 additional species (Kampfer, 2006). *Streptomyces* species are well known by a linear chromosome, complex morphological differentiation, and an ability to produce many bioactive secondary metabolites, containing important compounds for pharmaceutical and agrochemical uses (Arakawa *et al.*, 2005; El-Gendy *et al.*, 2008b).

2.1 Marine Environments

Marine ecosystems contain several unique features that set them apart from other aquatic ecosystems, the main factor being the presence of dissolved compounds in seawater, particularly sodium chloride (NaCl). Salinity is expressed as the amount (in grams) of total dissolved salts present in 1 kg of water. Normal seawater with a salinity of 35 g/kg (or litre) of water is expressed as 35‰. Microorganisms, particularly fungi and yeasts are well known for their ability to grow in saline environments by adapting their osmoregulatory mechanisms that signal the production of osmolytes (e.g., polyols, amino acids) in conjunction with an increasing concentration of cytoplasmic ions

(Harris, 1981; Bloomberg and Adler, 1992; Roberts, 2005). For marine microorganisms, their cellular adaptation to moderate and high salt content is a fundamental biological process needed for survival and growth. It is postulated that marine microorganisms have different characteristics from those of their errestrial counterparts and, therefore, might produce different types of bioactive compounds (Mahyudin, 2008).

The definition of true marine *Streptomyces* is difficult but the ability of *Streptomyces* to grow in a marine environment has been demonstrated, notably in connection with their occurrence in salt-rich muds and sea areas close to the shore. Earlier studies showed that *Streptomyces* spp. can grow at 2 and 16% NaCl, while various species of *Streptomyces* and *Nocardia* grow well in the presence of 10% NaCl (Mahyudin, 2008). More recently, other studies have shown that various actinomycetes derived from marine sediments show good growth in the presence of NaCl (Jensen *et al.*, 1991; Mincer *et al.*, 2002; Kokare *et al.*, 2004; Magarvey *et al.*, 2004; Mahyudin, 2008).

During the past 30 years, a large number of new compounds with structures completely different from those isolated from terrestrial organisms were successfully discovered from marine sources (Elyakov *et al.*, 1994; El-Gendy *et al.*, 2008b). Marine environment is a relatively untapped ecosystem with regards to isolation of indigenous *Streptomyces*, although existence of cousins of terrestrial origin has been reported (Solanki *et al.*, 2008). The immense diversity of this habitat along with it's under exploitation is the fundamental reason for attracting researchers towards it for discovering novel metabolite producers.

Interest in the importance of marine organisms as a source of new substances is growing. With marine species comprising approximately half of the total global biodiversity, the sea offers an enormous resource for novel compounds (de Vries and Beart, 1995), and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity (Gerwick and Bernart, 1993). Marine and estuarine aquatic angiosperms, or sea grasses, produce antimicrobial compounds that may act to reduce or control microbial growth. Workers have described antibacterial

(Harrison and Chan, 1980; Bernard and Pesando, 1989; Devi *et al.*, 1997; Bhosale *et al.*, 2002), antialgal (Harrison, 1982), antifungal (Jensen *et al.*, 1998) and antiviral (Premnathan *et al.*, 1992) activities among marine organisms (Kumar *et al.*, 2008).

Searching for previously unknown microbial strains is an effective approach for obtaining new biologically active substances. It is known that the antimicrobial activity of the metabolic products of aquatic bacterial strains is not weaker than the corresponding activity of soil strains. Water, bottom sediments, as well as sponges and other aquatic organisms are potential sources of producers of these metabolites (Sponga *et al.*, 1999; Webster *et al.*, 2001; Terkina *et al.*, 2006). Over one half of the marine bacteria studied display antagonistic activity towards other pelagic bacteria (Long and Azam, 2001). Unilateral or bilateral antagonism, resulting from the effect of antibiotic substances, inhibits the growth of microorganisms or kills them. Antimicrobial interactions influence, first, the structure of the microbial community, i.e., the composition and diversity of bacterial species; second, the functioning of microbial cenoses; and overall, the nature and transformation rate of biogeochemical substances in water bodies (Long and Azam, 2001; Terkina *et al.*, 2006). Actinomycetes are the strongest antagonists among microorganisms. The antibiotic substances they produce display antibacterial, antifungal, antitumor, antiprotozoic, and antiviral properties. Of the ten thousand known antibiotics produced by microorganisms over a decade ago, about 70% are of actinomycete origin; of them, representatives of the genus *Streptomyces* account for two thirds (Miyadoh, 1993). Actinomycetes from the genus *Micromonospora* occupy the second place in this list: they produce over 300 broad-spectrum antibiotic substances (Vobis, 1992). Representatives of the genera *Micromonospora* and *Streptomyces* are widely abundant in aquatic ecosystems and frequently prevail over other groups of actinomycetes (Sponga *et al.*, 1999; Mincer *et al.*, 2002; Terkina *et al.*, 2006).

Within marine environments, taxonomically diverse bacterial groups exhibit unique physiological and structural characteristics that enable them to survive in extremes of pressure,

salinity and temperature, with the potential production of novel secondary metabolites not observed in terrestrial microorganisms (Cross, 1981; Gause *et al.*, 1981; Radajewski *et al.*, 2002). Much interest on the screening of marine and aquatic microorganisms is focused on screening sediment derived microorganisms (Bredholt *et al.*, 2008; Jensen *et al.*, 1991; Okazaki, 2003), and also on those that form highly specific symbiotic associations with marine plants and animals in response to the scarcity of nutrients in aquatic and marine environments, and thus produce compounds for defence and competition (Kurtböke, 2000; Eccleston *et al.*, 2008).

2.2 Secondary metabolites and their functions

Secondary metabolites, also known as natural products, are those products of metabolism that are not essential for normal growth, development or reproduction of an organism. In this sense they are "secondary". Contrary to primary metabolites these compounds are not ubiquitous in the living organisms that produce them; neither are they necessarily expressed continuously (Demain and Fang, 2000). Also, unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, fecundity, or aesthetics, or perhaps in no significant change at all (Demain, 1992). Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group. They can be classified by their chemical structure or physical properties into one or more of the following groups: alkaloids, terpenoids, polyketides, aliphatic, aromatic, and heteroaromatic organic acids, phenols, iridoids , steroids, saponins, peptides, ethereal oils, resins and balsams (Demain and Fang, 2000).

Microbial secondary metabolites are the low molecular mass products of secondary metabolism. They include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immuno-modulating agents, receptor antagonists and agonists, pesticides, antitumour agents and growth promoters of animals and plants. They have a major effect on the health, nutrition and economics of our society (Demain, 1992).

The function or importance of these compounds to the organism's development is usually of ecological nature as they are used as defence against predators (herbivores, pathogens etc.), for interspecies competition, and to facilitate the reproductive processes (Demain and Fang, 2000). Secondary metabolites, including antibiotics, are produced in nature and serve survival functions for the organisms producing them. The antibiotics are a heterogeneous group, the functions of some being related to and others being unrelated to their antimicrobial activities. Secondary metabolites serve: (i) as competitive weapons used against other bacteria, fungi, amoebae, plants, insects, and large animals; (ii) as metal transporting agents; (iii) as agents of symbiosis between microbes and plants, nematodes, insects, and higher animals; (iv) as sexual hormones; and (v) as differentiation effectors (Demain and Fang, 2000).

Programmes aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have found applications in human medicine and agriculture. However, it soon became apparent during screening programmes that some microbial metabolites were discovered more frequently than others. As the number of described microbial metabolites increased, so did the probability of rediscovering known compounds. At the present time, with several thousands of described microbial metabolites, approach must be introduced into screening programmes to increase the chances of discovering novel compounds (Bull *et al.,* 2000; Lancini *et al.,* 1995, Busti *et al.,* 2006).

Secondary metabolites produced from marine actinomycetes have distinct chemical structures, which may form the basis for the synthesis of new drugs (Solanki *et al.,* 2008). Selective and enrichment media is capable of isolating *Streptomyces* from marine natural environment having the possibility to produce novel bioactive compounds. In order to provide a reasonable chance of yielding novel antibiotics, strains belonging to novel taxa must: (1) be able to produce secondary metabolites; (2) possess diversified pathways for secondary metabolism; (3) present significant

genetic diversity; (4) be retrievable in large numbers; and (5) be amenable to scale-up for large-volume fermentation (Busti *et al.*, 2006).

2.3 Genes for secondary metabolism

Genes encoding enzymes responsible for the synthesis of secondary metabolites are usually clustered on a contiguous piece of DNA (Solanki *et al.,* 2008). Genomic studies indicate that the genetic potential for producing secondary metabolites is not uniformly distributed within the bacterial world. In fact, most bacterial genomes lack any detectable gene cluster for secondary metabolism (Solanki *et al.,* 2008). On the other hand, *Streptomyces coelicolor* (Bentley *et al.*, 2002; Busti *et al.*, 2006) and *Streptomyces avermitilis* (Ikeda *et al.,* 2003; Omura *et al.,* 2001; Busti *et al.,* 2006), each possess more than 20 gene clusters devoted to the synthesis of secondary metabolites. Multiple gene clusters for secondary metabolism appear also to be a hallmark of several non-*Streptomyces* filamentous actinomycetes (Sosio *et al.,* 2000; Busti *et al.,* 2006) and of the myxobacteria (Gerth *et al.,* 2003; Busti *et al.,* 2005). It is interesting to point out that genomic data parallel the observed discovery events and indicate that *Streptomycetes*, *Pseudomonads* and *Bacilli* have yielded large numbers of metabolites since they can be easily isolated and have a relatively large genetic potential for producing secondary metabolites (Busti *et al.,* 2006). It is thus likely that most microbial strains that could be isolated in large numbers have actually been screened for bioactivities (Busti *et al.,* 2006).

A substantial fraction of the metabolites produced by *Streptomyces* and related actinomycetes consist of a polyketide or a polypeptide scaffold. Most peptide-derived metabolites are synthesized by NRPSs (Marahiel, 1997; Busti *et al.,* 2006), while two major routes exist for polyketide synthesis in actinomycetes (Hopwood, 1997; Busti *et al.*, 2006); one employs type I enzymes (PKS-I), also known as modular PKSs; and the other uses type II systems (PKS-II), mostly responsible for the synthesis of aromatic polyketides. Typical of actinomycetes is the presence, in a single strain, of genes encoding PKS-I, PKS-II and NRPSs. PKS-I and NRPS genes present a modular organization,

with a repetition of similar gene segments within a single gene cluster, so each amplified band may consist of different sequences originating from a single cluster (Busti *et al.,* 2006). In addition, a single actinomycetes strain normally contains more than one PKS-I or NRPS cluster. However, since KS and A domains present considerable sequence variation, primer pairs are seldom universal and can amplify only a fraction of the genes present. Consequently, only a fraction of the entire PKS-I or NRPS modules can be accessed through PCR (Busti *et al.,* 2006).

2.4 Marine *Streptomyces* producing secondary metabolite

Marine *Streptomyces* have proven to be efficient producers of new secondary metabolites (Solanki *et al.,* 2008), which show a range of biological activities such as antifungal, antitumor, antibacterial, immunosuppressive, insecticidal and enzyme inhibition, to name a few.

Secondary metabolites produced by marine *Streptomycetes* can be classified on the basis of their chemical structure as follows:

2.4.1 Terpenes and terpenoids

The most chemically diverse pool of secondary metabolites in nature is constituted by terpenes (Wendt and Schulz, 1998). In 1956, novobiocin was isolated as the first antibiotic with a terpenoid side chain from *Streptomyces niveus* (Hoeksema and Smith, 1961). Terpenes have been reported from the marine *Streptomyces* as evidenced by the following compounds:

a. Azamerone a meroterpenoid produced by a new marine bacterium related to the genus *Streptomyces* (Cho *et al.,* 2006a). It appears to be the first natural product with a phthalazione ring.
b. Three new pyrrolosesquiterpenes, glaciapyrroles A, B and C are produced by a *Streptomyces* strain (NPSOO 8187); and showed antibacterial activities (Macherla *et al.,* 2005).

c. Amorphane sesquiterpenes namely 10 α, 15-dihydroxyamorph-4-en-3-one, 10α, 11-dihydroxyamorph-4-ene and 5α,10 α,11-trihydroxyamorphan-3-one were produced by *Streptomyces* sp. M491 (Wu *et al.,* 2007). This is the first report of these sesquiterpenes from bacteria.

2.4.2 Polyketides

A marine inhabitant known as *Streptomyces psommoticus* produced antibiotic SBR-22 (Sujatha *et al.,* 2005) which showed *antibacterial* activity against methicillin-resistant *Staphylococcus aureus.*

a. Daryamides are cytotoxic polyketides isolated from culture broth of a *Streptomyces* strain, CNQ-085. These bioactive compounds show weak to moderate cytotoxicity against the human colon carcinoma cell line HCT-116 and very weak antifungal activities against *Candida albicans* (Asolkar *et al.,* 2006).
b. Actinofuranones A and B were isolated from the fermentation broth of a marine bacterium related to *Streptomyces* genus and show weak *in vitro* cytotoxicity against mouse splenocyte T-cells and macrophages (Cho *et al.,* 2006b).

2.4.3 Peptides

a. Cyclomarins A-C is cyclic peptides produced by a *Streptomyces* sp. that showed anti-inflammatory and antiviral activities (Renner *et al.,* 1999).
b. Piperazimycins are cytotoxic hexadepsipeptides isolated from the fermentation broth of a *Streptomyces* sp. Piperazimycin A exhibits potent *invitro* cytotoxicity against multiple tumor cell lines (Miller *et al.,* 2007).
c. Two cyclic peptides dehydroxynocardamine and desmethylenylnocardamine (Lee *et al.,* 2005); along with nocardamine was isolated from a *Streptomyces* sp. which was obtained from an unidentified marine sponge. These new compounds exhibit weak inhibition against the enzyme sortase B (Lee *et al.,* 2005).

d. Salinamides A and B are bicyclic depsipeptides produced by a *Streptomyces* sp., CNB-091, isolated from jelly fish *Cassiopeia xamachana*. These metabolites are useful as antibiotic and anti-inflammatory agents (Moore *et al.,* 1999).

2.4.4 Caprolactones

Two new caprolactones R-10-methyl-6-undecanolide and (6R, 10S)-10-methyl-6-dodeconolide are produced by a marine *Streptomyces* sp. These caprolactones show a moderate phytotoxicity and low cytotoxicity against cancer cells (Stritzke *et al.,* 2004).

2.4.5 Piericidins

Piericidins C7 and C8 produced by marine *Streptomyces* sp. showed selective cytotoxicity against rat glia cells transformed with the adenovirus EIA gene and neuro-2a mouse neuroblastoma cells (Hayakawa *et al.,* 2007).

2.4.6 Quinones

a. Resistomycin an antibiotic related to quinones, is produced by *Streptomyces corchorusii*. This is an inhibitor of HIV-1 protease (Shiono *et al.,* 2002).
b. Tetracenomycin D is an anthraquinone antibiotic produced by *Streptomyces corchorusii*. It shows cytotoxicity against cell line HMO2 (gastric adenocarcinoma) and HepG2 (hepatic carcinoma) and possesses weak antibacterial activities against gram-positive and gram-negative bacteria (Adinaryan *et al.,* 2006).
c. Resistoflavine is produced by *Streptomyces chibaensis*. It shows cytotoxicity against cell line HMO2 (gastric adenocarcinoma) and HepG2 (hepatic carcinoma) and possesses weak antibacterial activities against gram-positive and gram-negative bacteria (Kock *et al.,* 2005; Gorajana *et al.,* 2007).

d. Komodoquinone A is a neuritogenic anthracycline isolated from the fermentation broth of a marine *Streptomyces* sp. K53. It induces cell differentiation in the neuroblastoma cell line, Neuro2A and arrests cell cycle at the G1 phase (Itoh, 2003).

e. Himalomycins A and B are two new quinone antibiotics from a *Streptomyces* isolate, B6921. Himalomycins exhibit strong antibacterial activity against *Bacillus subtilis, Streptomyces viridochromogenes, Staphylococcus aureus* and *Escherichia coli* (Maskey *et al.,* 2003).

f. Helquinolines are new tetrahydroquinoline antibiotic isolated from culture broth of *Janibacter limosus*. Helquinoline shows moderate activity against *Bacillus subtilis, Streptomyces virdochromogenes* Tu57 *and Staphylococcus aureus* (Asolkar *et al.,* 2004).

g. CNQ-525 is a member of a new genus (tentatively called MAR4) within the family Streptomycetaceae, which produces three novel chlorinated dihydroquinones. These compounds possess new carbon skeletons but are related to several previously reported metabolites of the napyradiomycin class. The metabolites possess significant antibiotic properties and cytotoxicity against cancer cells (Mercado *et al.,* 2005).

2.4.7 Macrolides

A marine *Streptomyces* sp. M491 has been reported to produce a macrolide antibiotic named Chalcomycin A and also some terpenes (Wu *et al.,* 2007).

2.4.8 Esters

Bonactin is an antimicrobial ester produced by *Streptomyces* sp. BD21-2. Bonactin displayed antimicrobial activity against gram-positive and gram-negative bacteria as well as against several fungi (Schumacher *et al.,* 2003).

2.4.9 Chinikomycins

Chinikomycins A and B are chlorine containing aromatic manumycin derivatives. They exhibit antitumor activity against different human cancer cell lines, but are inactive as antiviral, antimicrobial and phytotoxic agents. These compounds are produced by *Streptomyces* sp. isolate MO45 (Li *et al.*, 2005).

2.4.10 Trioxacarcins

Trioxacarcins are complex compounds showing high antibacterial activity against gram-positive and gram-negative bacteria, and some of them show high antitumor and antimalarial activities as well. Trioxacarcin A also exhibits antifungal activities. Trioxacarcin A, B and C are obtained from *Streptomyces ochraceus* and *Streptomyces bottropensis* (Maskey *et al.*, 2004). Some of these compounds possess extremely high antiplasmodial activity, which is comparable to that shown by artemisinin, the most active compound against the pathogen of malaria (Maskey *et al.*, 2004). The producers of trioxacarcins also biosynthesize the related metabolite, gutingimycin (Maskey *et al.*, 2004).

2.4.11 Methyl pyridine

Streptokordin a new cytotoxic compound of the methylpyridine class was isolated from the cultural broth of *Streptomyces* sp. KORDI-3238. It exhibits significant cytotoxicity against several human cancer cell lines but showed no growth inhibition against various microorganisms, including bacteria and fungi (Jeong *et al.*, 2006).

2.4.12 Lactams

Aureoverticillactam, a novel 22-atom macrocyclic lactam was isolated from *Streptomyces aureoverticillaris*. It showed cytotoxicity against various tumor cell lines (Mitchell *et al.*, 2004).

Salinosporamide A and aureoverticillactam are lactams from marine actinomycetes. These are distinct from β-lactam compounds which contain a four-membered β-lactam ring. The structure of β-lactam second ring allows these compounds to be classified into penicillins, cephalosporins, clavams, carbapenes and monobactans (Liras and Martin, 2006). Most β-lactam compounds inhibit bacterial cell wall synthesis but others behave as β-lactamase inhibitors (e.g. cluvalanic acid) and even as antifungal agents (e.g. some clavams) (Liras and Martin, 2006), however salinosporamide A and aureoverticillactam showed cytotoxicity against cancer cells (Liras and Martin, 2006).

2.4.13 Enzyme inhibitors

Some enzymes inhibitors have been reported from marine environment and they include:

a. Alpha amylase inhibitor from *Streptomyces corchorusii* subsp. *rhodomarinus.* Subsp. nov (Imada and Simidu, 1988).

b. Pyrostatins A and B inhibitors of n-acetyl-beta-glucosaminidase, produced by *Streptomyces* sp. SA-3501 (Aoyama *et al.*, 2005).

c. Pyrizinostatin is an inhibitor of pyroglutamyl peptidase, isolated from culture of *Streptomyces* sp. SA-2289 (Aoyagi *et al.*, 1992; Solanki *et al.*, 2008).

2.5 Antibiotics

The first antibiotics were of natural origin, e.g. penicillins produced by fungi in the genus Penicillium, or Streptomycin from bacteria of the genus *Streptomyces*. Currently, antibiotics are obtained by chemical synthesis, such as the sulfa drugs (e.g. sulfamethoxazole), or by chemical modification of compounds of natural origin. Many antibiotics are relatively small molecules with a molecular weight of less than 1000 Da. The classical definition of an antibiotic is a compound produced by a microorganism which inhibits the growth of another microorganism (Mayer *et al.*, 1986). Antibiotics can be grouped by either their chemical structure or mechanism of action

(Kümmerer, 2009). They are a diverse group of chemicals that can be divided into different sub-groups such as β-lactams, quinolones, tetracyclines, macrolides, sulphonamides and others is shown in Table 2.1. They are often complex molecules which may possess different functionalities within the same molecule. Therefore, under different pH conditions antibiotics can be neutral, cationic, anionic, or zwitterionic. Because of the different functionalities within a single molecule, their physico-chemical and biological properties such as log Pow (Cunningham, 2008; Kümmerer, 2009) sorption behaviour, photo reactivity and antibiotic activity and toxicity may change with pH (Kümmerer, 2009).

Antibiotics are a chemically diverse group of compounds produced by microorganisms that have microstatic or microcidal activity. They function by a variety of mechanisms to disrupt microbial metabolism. The discovery and clinical use of antibiotics just over 50 years ago coupled with improvements in immunizations drastically reduced human suffering and deaths from infectious diseases (Nwosu, 2001). In addition to antibiotics, many semi-synthetic derivatives of antibiotics and synthetic antimicrobial agents are used in clinics and animal husbandry. What is now apparent from the medical and nonmedical use and abuse of antibiotics is a growing body of evidence of antibiotic resistance in microorganisms. Evidence of resistance came soon after clinical use of antibiotics and has been accelerating so rapidly that the threat of resurgence of infectious diseases as a major human threat is real (Nwosu, 2001).

Antimicrobial drugs have generally been classified into two categories; one includes the synthetic drugs, such as the sulfonamides and the quinolones, and the second, antibiotics, synthesized by microorganisms. In recent years, increasing numbers of semi-synthetic drugs have been developed which are chemical derivatives of antibiotics, thereby blurring the distinction between synthetic and natural antibiotics (Gangle, 2005).

Table 2.1: Summary of bioactive compounds produced by marine *Streptomyces*

Chemical group	Compound	Source	Activity
Meroterpenoid	Azamerone	*Streptomyces* sp.	None
Pyrrolosesquiterpenes	Glaciapyrroles A, B and C	*Streptomyces* sp. NPS008187	Antibacterial
Amorphane sesquiterpenes	10α, 15-dihydroxyamorph-4-en-3-one, 10α, 11-dihydroxyamorph-4-ene and 5α, 10α, 11-trihydroxyamorphan-3-one	*Streptomyces* sp. M491	None
Polyketide	SBR-22	*Steptomyces psommoticus* BT408	Antibacterial
Polyketide	Daryamides	*Streptomyces* sp.CNQ-085	Anticancer, antifungal
Polyketide	Actinofuranones A and B	*Streptomyces* sp.	Cytotoxic
Peptide	Piperazimycins	*Streptomyces* sp.	Anticancer
Peptide	Dehydroxynocardamine and desmethylenylnocardamine	*Streptomyces* sp.	Enzyme sortase B inhibitor
Peptide	Salinamides A and B	*Streptomyces* sp.	Antibacterial, anti-infl ammatory
Caprolactone	R-10-methyl-6-undecanolide (6R,10S)-10-methyl-6-dodeconolide	*Streptomyces* sp. B6007	Phytotoxic, anticancer
Piericidin	Piericidins C7 and C8	*Streptomyces*	Anticancer
Quinone	Resistomycin	*Streptomyces corchorusii* AUBN(1)/7	Antiviral
Quinone	Tetracenomycin D	*Streptomyces corchorusii* AUBN(1)/7	Anticancer, antibacterial
Quinone	Resistoflavine	*Streptomyces chibaensis* AUBN(1)/7	Anticancer, antibacterial
Quinone	Komodoquinone A	*Streptomyces* sp. K53	Neuritogenic activity
Quinone	Himalomycins A and B	*Streptomyces* sp. B6921	Antibacterial
Macrolide	Chalcomycin A	*Streptomyces* sp. M491	None

Manumycin derivatives	Chinikomycins A and B	*Streptomyces* sp. M045	Anticancer
Complex compounds	Trioxacarcins	*Streptomyces ochraceus* and *Streptomyces bottropensis*	Anticancer, antimalarial
Methylpyridine	Streptokordin	*Streptomyces* sp. KORDI-3238	Anticancer
Gamma lactam beta lactone	Salinosporamide A	*Salinispora tropica*	Anticancer
Macrocyclic lactam	Aureoverticillactam	*Streptomyces aureoverticillaris*	Anticancer
Enzyme inhibitor	Alpha-amylase inhibitor	*Streptomyces corchorusii* subsp. *rhodomarinus* subsp. nov	Enzyme Inhibition nov
Enzyme inhibitor	Pyrostatins A and B	*Streptomyces* sp. SA-3501	N-acetyl-betaglucosaminidase inhibition
Enzyme inhibitor	Pyrizinostatin	*Streptomyces* sp. SA-2289	Pyroglutamyl peptidase Inhibition

Source: Solanki *et al.* (2008).

2.5.1 History of antibiotic development

Interest in antimicrobial chemotherapy was kindled as soon as microorganisms were understood to be agents of infectious disease. In earlier times, plant products were sometimes used successfully in the treatment of disease, but neither doctors nor patients knew the basis for the action of these therapeutic agents. Many early medicines were used to cure protozoan diseases, rather than bacterial diseases. As early as 1619, it was known that malaria could be treated with the extract of cinchona bark (quinine) and that amoebic dysentery could be treated with ipecacuanha root (emetine) (Garrod and O'Grady, 1971; Greenwood, 2000). Only a few antibacterials, such as mercury, which was used to treat syphilis, were in use when the era of true chemotherapy began (Gangle, 2005).

Paul Ehrlich first hypothesized that dyes could be used as antimicrobial drugs in the early 1900s, based on their differential affinities for various tissues. In 1904, Ehrlich and Shiga discovered that a red dye called trypanrot was effective against trypanosomes (Mølbak *et al.*, 1999). It was around this time that arsenicals drew Ehrlich's interest. Ehrlich, along with Sahachiro Hata, in 1909, found that arsphenamine (named Salvarsan) was active against spirochetes and, therefore, was an effective cure for syphilis (Greenwood, 2000; Gangle, 2005).

The first truly effective class of antimicrobial drugs was the sulfonamides, discovered by Gerhard Domagk in 1935 (Greenwood, 2000). In 1932, two scientists at the Bayer Company, Mietzsch and Klarer, synthesized Prontosil red, a red dye bound to a sulfonamide group (Garrod and O'Grady, 1971; Greenwood, 2000). Domagk showed, in 1935, that infections in mice caused by hemolytic *Streptococci* were cured by Prontosil red (Garrod and O'Grady, 1971; Greenwood, 2000).

Unfortunately for Bayer, Prontosil red was shown to have no antibacterial activity *in vitro*. This lack of activity was explained by Tréfouël and colleagues in 1935 when they showed that Prontosil red is split *in vivo* into its component dye and sulfanilamide, the active antibacterial agent and a previously described molecule that was already in the public domain (Garrod and O'Grady, 1971; Greenwood, 2000). From that point, sulfanilamide was manufactured by a number of

companies and work was begun to modify the molecule to enhance performance, leading to decreased side effects and a broader spectrum of action (Garrod and O'Grady, 1971; Greenwood, 2000).

Although penicillin was the first natural antibiotic to be discovered, the idea of using microorganisms therapeutically was not new. Fungi had been used in poultices for many years, and by 1899, a product called pyocyanase, which was an extract from *Pseudomonas aeruginosa*, was used in the treatment of wounds (Garrod and O'Grady, 1971). Penicillin was first isolated from *Penicillium notatum* in 1928 by Alexander Fleming in 1929 (Demian, 1999), but he was unable to isolate and purify enough drugs to be of any use. By 1941, Ernst Chain, Howard Florey, and Norman Heatley had shown the therapeutic value of penicillin (Chain *et al.*, 1940), but they were also unable to produce enough penicillin for commercial use. Collaboration with Andrew Moyer and Robert Coghill (Moyer and Coghill, 1946) at the USDA's Northern Regional Research Laboratory in Illinois led to much higher production yields of penicillin by 1943. After a worldwide search for *Penicillium* strains that could produce more penicillin, Raper and Fennel (Raper, 1946) found a strain of *Penicillium chrysogenum* on a moldy cantaloupe at a local market that was capable of even higher yields of penicillin (Demian, 1999).

A series of different antibiotics were quickly discovered after penicillin came into use. In 1940, Selman Waksman began searching for antibiotic compounds produced by soil microorganisms (Greenwood, 2000). In 1943, one of Waksman's students discovered streptomycin (Schatz, 1944), leading to a flood of researchers combing the world for new drugs. It was in this same period that Hotchkiss and Rene Dubos, (1941) discovered gramicidin, the first antibiotic active against gram-positive bacteria. Chlortetracycline, chloramphenicol, and others were discovered shortly thereafter (Garrod and O'Grady, 1971). Many discoveries were of drugs that were too toxic for human use, or that had already been discovered. Nevertheless, this work did lead to many new drugs and within only 10 years, drugs comprising the major classes of antibiotics were found (Greenwood, 2000). In

addition to soil, many of these drugs were discovered by isolating the producing microorganisms from interesting and unusual sources. (Garrod and O'Grady, 1971).

In 1962, one of the later discoveries was a synthetic drug, nalidixic acid, the first of the quinolones to be described, and although not therapeutically important by itself, modification of nalidixic acid led to the production of the highly effective fluoroquinolones. Members of this class, such as ciprofloxacin, norfloxacin, enrofloxacin, and ofloxacin, have become very important in the treatment of diseases in both humans and animals (Mitsuhashi, 1993). Since the 1960's, there have been few discoveries of new antibiotic drugs. The drugs developed since have mostly been chemical modifications of existing drugs. These modifications have been very useful in treating infectious diseases, leading to enhanced killing of pathogens, increased spectrum of action, reduced toxicity, and reduced side effects. Unfortunately, since the 1970's, only one new class of antibiotics has been introduced (Lipsitch, 2002) and a recent trend in antibiotic therapy has been to employ combinations of drugs with different mechanisms of action, in order to increase their effectiveness and to overcome the problem of drug resistance (Gangle, 2005).

2.6 Development of resistance to antimicrobial agent

Antibiotics have long been considered the “magic bullet” that would end infectious disease. Although they have improved the health of countless numbers of humans and animals, many antibiotics have also been losing their effectiveness since the beginning of the antibiotic era. Bacteria have adapted defenses against these antibiotics and continue to develop new resistances, even as we develop new antibiotics. In recent years, much attention has been given to the increase in antibiotic resistance. As more microbial species and strains become resistant, many diseases have become difficult to treat, a phenomenon frequently ascribed to both indiscriminate and inappropriate use of antibiotics in human medicine. However, the use of antibiotics and antimicrobials in raising food animals has also contributed significantly to the pool of antibiotic resistant organisms globally and

antibiotic resistant bacteria are now found in large numbers in virtually every ecosystem on earth. There is no doubt that the use of antibiotics provides selective pressure that result in antibiotic resistant bacteria and resistance genes (Gangle, 2005). While some resistant bacteria are found naturally in the environment, pathogens and nonpathogens are released into the environment in several ways, contributing to a web of resistance that includes human, animal, and the environment, essentially the biosphere (Gangle, 2005).

There is evidence that although resistant microorganisms existed in nature before the use of antibiotics, such microorganisms were mostly absent from human flora (Hughes and Datta, 1983). However, in the intervening years, antibiotic resistant microorganisms have become frighteningly common. Almost as soon as antibiotics were discovered, researchers began to find microorganisms resistant to the new drugs. Even by 1909, when Ehrlich first began to study dyes and arsenicals, he found drug resistant trypanosomes (Greenwood, 2000). Resistant strains of *Staphylococcus aureus* in hospitals grew from less than 1% incidence, when penicillin first came into use, to 14% in 1946, to 38% in 1947, to more than 90% today (Greenwood, 2000). Worldwide, ampicillin and penicillin resistance can be found together in more than 80% of *S. aureus* strains (O'Brien, 1987). After World War II, sulfonamides were widely used to treat *Shigella* infections in Japan, but by 1952, only 20% of isolates were susceptible. As the Japanese began to switch to tetracycline, chloramphenicol, and streptomycin, *Shigella* strains that were multiple-resistant quickly began to appear (Falkow, 1975). Within 30 years of their discovery, sulfonamides ceased to be an effective treatment for meningococcal disease (O'Brien, 1987; Gangle, 2005).

In general, as stated above, resistance has been found in many organisms, but some pathogens are of particular recent concern. These pathogenic organisms are becoming increasingly more common, especially with the greater frequency of travel worldwide and increase in the population of the elderly, both in the United States and in many of the developed countries

worldwide (Melhus and Tjernberg, 1996). Some specific examples of microbial species that have developed significant resistance are cited as follows *Enterococcus* spp.

The enterococci are a group of gram-positive cocci that are part of the normal resident flora of both humans and some animals (Melhus and Tjernberg, 1996). They are generally not considered virulent; however, their intrinsic resistance to many antibiotics (including cephalosporins, penicillin, and aminoglycosides) has made them important opportunistic pathogens and one of the most common causes of nosocomial infections (Gangle, 2005). Although the enterococci are opportunistic pathogens, they are a frequent cause of urinary tract infections, bacteremia, and endocarditis, all of which can be difficult to treat due to resistance (Melhus and Tjernberg, 1996). Mortality rates in enterococcal bacteremia can reach 70% (Melhus and Tjernberg, 1996; Gangle, 2005).

The traditional method of treatment has been a combination of an aminoglycoside and ampicillin or a glycopeptide. By the 1970's, only ampicillin and vancomycin (a glycopeptide) were effective treatment options in most cases (Frieden *et al.,* 1993). As of 2000, high level resistance to ampicillin and aminoglycosides was common, leaving vancomycin as the treatment of last resort (Jeljaszewicz *et al.*, 2000). In one study in New York City, Frieden *et al.* (1993) found that 98% of vancomycin resistant enterococci (VRE) infections were acquired nosocomially and 19% of these were resistant to all antibiotics. Although vancomycin has been used in humans for more than 40 years, VRE were not generally considered a clinical problem until recently. The first report of VRE in the lab was in 1969, but the first clinical cases were not seen until 1986 in England and in 1988 in New York City (Frieden *et al.,* 1993). Since then, the frequency of VRE infections has vastly increased, becoming a major health problem. In 1998, it was reported that approximately 20% to 40% of enterococcal nosocomial infections were vancomycin resistant (Khachatourians, 1998). A recently approved drug for the treatment of VRE is the streptogramin combination of quinupristin/dalfopristin; although only approved since 1999, there have already been reports of

sporadic resistance in human isolates and resistance is quite common on retail foods and in food animals (Gangle, 2005).

Antibiotic resistance is on the rise in *E. coli*. O'Brien *et al.* (1987) reported an incidence for sulfonamide resistance of between 21% and 85%, ampicillin resistance between 17% and 72%, tetracycline resistance between 24% and 60%, and trimethoprim resistance between 1% and 4%. Resistance to fluoroquinolones was rare for many years, but since the early 1990s, this has been on the rise; in one study in Spain, the incidence of resistance was shown to have reached 22% and more than a third of those isolates were resistant to three or more other antibiotics (Garau *et al.*, 1999).

2.7 Mechanisms of antibiotic action and resistance

There are several major classes of antibiotics; they can be categorized based on their mode of antibacterial action. In general, antibiotics can be defined as those that inhibit cell wall synthesis, those that inhibit protein synthesis, and those that inhibit nucleic acid synthesis. A summary of the major antibiotic classes is shown in Table 2.2. The selective toxicity of antibiotics lies in the differences in cellular structures between eukaryotic and prokaryotic cells. However, differences in cellular structure among bacterial species can lead to resistance to certain antibiotics (Gangle, 2005).

The definition of bacteria as resistant or susceptible is critical for clinicians. It is also very important to note the difference between intrinsic and acquired resistance to an antibiotic. Intrinsic resistance can best be described as resistance of an entire species to an antibiotic, based on inherent (and inherited) characteristics requiring no genetic alteration. This is usually due to the absence of a target for the action of a given antibiotic or the inability of a specific drug to reach its target. For example, mycoplasmas are always resistant to β-lactam antibiotics since they lack peptidoglycan (which the β-lactams act upon) (Gangle, 2005). Similarly, the outer membrane of gram negative cells can prevent an antibiotic from reaching its target. For example, *Pseudomonas aeruginosa* exhibits

high intrinsic resistance to many antibiotics due to its drug efflux pumps and restricted outer membrane permeability (Gangle, 2005).

Acquired resistance can arise either through mutation or horizontal gene transfer. Presence of the antibiotic in question leads to selection for resistant organisms, thereby shifting the population towards resistance. The major mechanisms of acquired resistance are the ability of the microorganisms to destroy or modify the drug, alter the drug target, reduce uptake or increase efflux of the drug, and replace the metabolic step targeted by the drug (Gangle, 2005).

Table 2.2: Major classes of antibiotics and some examples

Inhibitors of:					
Cell Wall Synthesis	**β-lactams** Penicillins	**Glycopeptides** Vancomycin			
	Cephalosporins	Avoparcin			
	Carbapenems				
Protein Synthesis	**Aminoglycosides** Streptomycin	**Chloramphenicol**	**Tetracyclines**	**Macrolides** Erythromycin	**Streptogramins** Virginiamycin
	Neomycin			Azythromycin	Quinupristin-Dalfopristin
	Kanamycin			Clarithromycin	Pristinamycin
	Gentamicin				
Nucleic Acid Synthesis	**Sulfonamides (diaminopyrimidines)** Sulfamethoxazole	**Quinolones** Ciprofloxacin			
	Trimethoprim	Norfloxacin			

Source: Gangle (2005).

2.7.1 Inhibitors of cell wall synthesis

There are two major groups of cell wall synthesis inhibitors, the β-lactams and the glycopeptides. As bacterial cell walls are wholly unlike the membranes of eukaryotes, they are an obvious target for selectively toxic antibiotics. The β-lactams include the penicillins, cephalosporins, and the carbapenems. These agents bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall (Greenwood, 2000). In gram negative cells, this leads to the formation of fragile spheroplasts that are easily ruptured; in Gram positive cells, autolysis is triggered by the release of lipoteichoic acid (Greenwood, 2000). The mechanism of β-lactam resistance is via the action of the β-lactamases. These enzymes catalyze hydrolysis of the β-lactam ring and, thereby, inactivating these antibiotics (Greenwood, 2000). Many bacteria contain chromosomally encoded β-lactamases necessary for cell wall production and it is only through over-production of these enzymes that resistance occurs (Greenwood, 2000). β-lactamases encoded on plasmids or other transmissible elements can lead to such overproduction and, therefore, to resistance (Normark and Normark, 2002). There are also some bacteria that possess altered PBP's that result in reduced penicillin binding (Greenwood, 2000).

Since the discovery of penicillin and resistant bacteria, various new versions of the β-lactams have been used that have different spectrums of activity and different susceptibility to β-lactamases. Since the 1970s, several compounds, such as clavulanic acid, have been discovered that have the ability to bind irreversibly to β-lactamases and, thereby, inhibit their action. Combinations of these compounds with β-lactam drugs have been very successful in treatment of diseases (Bryan, 1984). The glycopeptides are a group of antibiotics that include vancomycin, avoparcin, and others that bind to acyl-D-alanyl-D-alanine. Binding of this compound prevents the addition of new subunits to the growing peptidoglycan cell wall (Bryan, 1984). These drugs are large molecules that are excluded

from gram negative cells by the outer membrane, thus limiting their action to gram positive organisms (Bryan, 1984).

Glycopeptide resistance was long thought to be rare, but has recently been shown to be quite common (Bryan, 1984). Resistance in enterococci has developed through newly discovered enzymes that use D-alanyl-D-lactate in place of acyl-D-alanyl-Dalanine, allowing cell wall synthesis to continue (Greenwood, 2000). Other mechanisms of resistance involve the over-production of peptidoglycan precursors which overwhelm the drug (Greenwood, 2000).

2.7.2 Inhibitors of protein synthesis

There are many types of antibiotics that inhibit bacterial protein synthesis. These drugs take advantage of structural differences between bacterial ribosomes and eukaryotic ribosomes. The aminoglycoside antibiotics are a group whose mechanism of action is not completely understood. The three major groups of aminoglycosides are the streptomycins, neomycins, and kanamycins (Greenwood, 2000). These drugs enter bacterial cells by an active transport that involves quinones that are absent in anaerobes and streptococci, thus excluding these organisms from the spectrum of action.

Streptomycins act by binding to the 30S ribosomal subunit; kanamycins and neomycins bind to both the 50S subunit and to a site on the 30S subunit different from that of streptomycin (Greenwood, 2000). Activity involving initiation complexes and cell membrane proteins that contribute to cell death plays a role in the action of these antibiotics, but this is poorly understood (Bryan, 1984; Greenwood, 2000).

There are three mechanisms of aminoglycoside resistance that have been identified to date. The first involves only streptomycin. Since streptomycin; binds to one particular protein on the ribosome, alteration of this protein, even by a single amino acid in its structure, confers high-level

resistance to the drug (Bryan, 1984). The third mechanisms involve decreased uptake of the antibiotic and in one of these the cell membrane is altered, preventing active transport of the drug (Bryan, 1984). In the other, one of many enzymes alters the antibiotic as it enters the cell, causing a block in further active transport (Bryan, 1984).

Chloramphenicol is a broad-spectrum antibiotic that, although naturally occurring, is produced by chemical synthesis. Chloramphenicol inhibits peptide bond formation on 70S ribosomes (Bryan, 1984). This drug is especially useful in that it can penetrate eukaryotic cells and cerebrospinal fluid, making it a drug of choice for treatment of meningitis and intracellular bacterial infections such as those caused by Chlamydia (Greenwood, 2000). It is not in widespread use, however, because of potentially fatal side-effects, namely, aplastic anemia (Greenwood, 2000). Resistance to chloramphenicol is conferred by the enzyme chloramphenicol acetyl-transferase; a number of these enzymes have been discovered, each altering the chloramphenicol molecule to prevent binding to the bacterial ribosome (Greenwood, 2000). Chloramphenicol resistance in gram negative cells can also arise from alteration in outer membrane permeability that prevents the drug from entering the cell (Bryan, 1984).

The tetracyclines are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis. They are brought into the cell by active transport and, once there, bind to the 30S subunit to prevent binding of aminoacyl tRNA (Roberts, 1996). Resistance to the tetracyclines occurs via three mechanisms. First, production of a membrane efflux pump removes the drug as rapidly as it enters and there are several genes encoding these pumps (Roberts, 1996). Second, several ribosome protection proteins act to prevent tetracycline from binding to the ribosome, thus conferring resistance (Roberts, 1996). Third, a protein found only in *Bacteroides* spp., enzymatically inactivates tetracycline (Roberts, 1996). Interestingly, efflux pump inhibitors have recently been discovered that

may allow combinations of these inhibitors and tetracyclines to be used against previously resistant strains (Chopra, 2002).

The macrolides are a group of antibiotics commonly used to treat gram positive and intracellular bacterial pathogens. Erythromycin was the first of these (Gaynor, 2003), and several other important macrolides have been discovered since, including clarithromycin and azithromycin. Azithromycin has a longer plasma half-life which allows treatment with a single dose for some pathogens or a once daily dose for others. Clarithromycin has enhanced absorption and causes less gastrointestinal discomfort (Gaynor, 2003). It was originally believed that erythromycin inhibited protein synthesis by competing with amino acids for ribosomal binding sites, but newer research show several mechanisms are involved (Garrod and O'Grady, 1971). The macrolides are now believed to promote dissociation of tRNA from the ribosome, inhibit peptide bond formation, inhibit ribosome assembly, and prevent amino acid chain elongation (Gaynor, 2003).

There are two major mechanisms of macrolide resistance. First, an efflux pump has been found that removes the drug from the cell (Gaynor, 2003). Second, modification of the ribosome can confer resistance; mutations at several sites of the ribosome can allosterically prevent macrolide binding and a common alteration is dimethylation of one nucleotide on the 23S rRNA. This dimethylation not only prevents macrolide binding, but also confers resistance to lincosamide and streptogramin antibiotics (Gaynor, 2003).

The streptogramins are another class of antibiotic that inhibits bacterial protein synthesis, mostly in gram positive organisms (due to decreased permeability of the gram negative outer membrane). These antibiotics are actually combinations of structurally different drugs; types A and B that act synergistically (Johnson *et al.,* 2002). These compounds bind to separate sites on the 50S subunit. Type A drugs block attachment of substrates at two sites on the 50S subunit, whereas type B drugs cause release of incomplete protein chains. The synergistic effect arises from a conformational

change induced by the binding of a type A drug which significantly increases affinity of type B drugs (Johnson *et al.,* 2002). Streptogramins currently in use include virginiamycin, pristinamycin, and quinupristin/dalfopristin (Johnson *et al.,* 2002; Gangle, 2005). Resistance to streptogramin antibiotics can be found in several forms. Efflux pumps for both type A and B streptogramins have been identified. Type A streptogramins can be inactivated by one of the virginiamycin acetyl-transferases, and several enzymes have been identified that can inactivate type B streptogramins (Johnson *et al.*, 2002; Gangle, 2005). Alteration of bacterial ribosomal proteins or RNA can also confer resistance. A common mutation is the dimethylation of one nucleotide on the 23S rRNA, mentioned previously, that gives rise to resistance to type B drugs, as well as macrolides and lincosamides (Johnson *et al.*, 2002; Gangle, 2005).

2.7.3 Inhibitors of nucleic acid synthesis

The sulfonamides and the diaminopyrimidines should be discussed together, in that both only indirectly inhibit nucleic acid synthesis by inhibiting folate synthesis. Folate is a coenzyme necessary for the synthesis of purines and pyrimidines. Although both types of drugs are useful on their own, they exhibit a synergistic effect when combined. Sulfonamides are currently not used commonly in medicine, but the combination drug trimethoprim-sulfamethoxazole is sometimes used in the treatment of urinary tract infections (Greenwood, 2000). Sulfonamides serve as an analog of *p*-aminobenzoic acid; therefore, they competitively inhibit an early step in folate synthesis (Greenwood, 2000). Diaminopyrimidines, of which trimethoprim is the most common, inhibit dihydrofolate reductase, the enzyme that catalyzes the final step in folate synthesis (Greenwood, 2000).

There are several resistance mechanisms microorganisms employed against each of the anti-folate drugs. For example, sulfonamides are rendered ineffective by over-production of *p*-

aminobenzoic acid or production of an altered dihydropteroate synthetase. The substrate for dihydropteroate synthetase is *p*-aminobenzoic acid, and the altered form has a much lower affinity for sulfonamides than for *p*-aminobenzoic acid (Then, 1982). Trimethoprim resistance can also result from several mechanisms, e.g., over-production of dihydrofolate reductase or production of an altered, drug-resistant form can lead to resistance (Bryan, 1984). In addition, both drugs can be enzymatically inactivated, resulting in resistance (Then, 1982).

The quinolones are a chemically varied class of broad-spectrum antibiotics widely used to treat many diseases, including gonorrhea and anthrax. Drugs in this class include nalidixic acid, norfloxacin, and ciprofloxacin. These drugs are commonly used and, worldwide, more ciprofloxacin is consumed than any other antibacterial agent (Acar, and Goldstein, 1997). Quinolones inhibit bacterial growth by acting on DNA gyrase and topoisomerase IV, which are necessary for correct functioning of supercoiled DNA (Greenwood, 2000). Although quinolones target both enzymes, in gram negative organisms the primary target is DNA gyrase and, in gram positive organisms, the primary target is topoisomerase IV (Ruiz, 2003).

There are three main mechanisms of resistance to quinolones. Resistance to some quinolones occurs with decreased expression of membrane porins (Normark and Normark, 2002). Cross-resistance to other drugs requiring these porins for activity also results from these changes. A second mechanism of resistance is expression of efflux pumps in both gram negative and gram positive organisms (Normark and Normark, 2002), and the third is alteration of the target enzymes. Several mutations have been described in both quinolone target proteins that result in reduced binding affinities (Ruiz, 2003). It is believed that high-level quinolone resistance is brought about by a series of successive mutations in the target genes, rather than a single mutation (Normark and Normark, 2002; Gangle, 2005).

2.8 Enzymatic aspect of antibiotic resistance

Antibiotic resistance is now well recognized as a major problem in the treatment of infections in hospitals and, with increasing and alarming frequency, in the community. Resistance can be active (i.e., the result of a specific evolutionary pressure to adapt a counterattack mechanism against an antibiotic or class of antibiotics) or passive (where resistance is a consequence of general adaptive processes that are not necessarily linked to a given class of antibiotic; e.g., the nonspecific barrier afforded by the outer membrane of Gram-negative bacteria). Bacteria achieve active drug resistance through three major mechanisms: (1) efflux of the antibiotic from the cell via a collection of membrane-associated pumping proteins; (2) modification of the antibiotic target (e.g., through mutation of key binding elements such as ribosomal RNA or even by reprogramming of biosynthetic pathways such as in resistance to the glycopeptide antibiotics); and (3) via the synthesis of modifying enzymes that selectively target and destroy the activity of antibiotics. All of these mechanisms require new genetic programming by the cell in response to the presence of antibiotics. In fact, in several cases, the antibiotics or their action actually genetically regulate the expression of resistance genes is shown in Table 2.3. Therefore, bacterial cells expend a considerable amount of energy and genetic space to actively resist antibiotics (Wright, 2005).

Table 2.3: Enzymatic strategies of antibiotic inactivation

Strategy	Type	Antibiotics Affected
Hydrolysis		β-Lactams
		Macrolides
Group transfer	Acyl	Aminoglycoside
		Chloramphenicol
		Type A streptogramin
	Phosphoryl	Aminoglycoside
		Macrolide
		Rifamycin
		Peptide
	Thiol	Fosfomycin
	Nucleotidyl	Aminoglycoside
		Lincosamide
	ADP-ribosyl	Rifamycin
	Glycosyl	Macrolide
		Rifamycin
Other	Redox	Tetracycline
		Rifamycin
		Type A streptogramin
	Lyase	Type B streptogramin

Source: Wright (2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Test bacteria

The test bacteria that were used in this study includ reference, environmental as well as clinical isolates. The typical reference strains were as follows:

Gram-positive: *Staphylococcus aureus* ATCC 6538, *Streptococcus faecalis* ATCC 29212, *Streptococcus pyogens* ATCC 10389, *Bacillus cereus* ATCC 10702, *Bacillus pumilus* ATCC 14884, *Acinetobacter calcaoceuticus* UP and *Acinetobacter calcaoceuticus* subsp *anitratus* CSIR.

Gram-negative: *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC7700, *Pseudomonas aeruginosa* ATCC 19582, *Proteus vulgaris* ATCC 6830, *Proteus vulgaris* CSIR 0030, *Enterobacter cloacae* ATCC 13047, *Serratia marscens* ATCC 9986, *Klebsiella pneumonia* ATCC 10131 and *Klebsiella pneumonia* ATCC 4352.

Environmental isolates: *Klebsiella pneumonia*, *Bacillus subtilis*, *Shigella flexineri*, *Salmonella* sp, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterococcus faecalis*, *Eschechia coli*, *Staphlococcus aureus* and *Bacillus stearothermophillus* were kindly provided by Dr. AO Olaniran of the University of KwaZulu-Natal.

Clinical isolates: Includes the following; staphylococcal strains isolated from septic wound abrasion: *Staphylococcus aureus* OKOH1, *Staphylococcus aureus* OKOH2A, *Staphylococcus aureus* OKOH3, and *Staphylococcus sciuri* OKOH2B with Gene Bank accession numbers Eu244633, Eu244634, Eu244635 and Eu244636 respectively.

3.2 Source of *Streptomyces*

The *Streptomyces* used in this study were isolated from the Nahoon beach (Ogunmwonyi, 2008) and stored in the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare. Eighty-four (84) Actinomycetes isolates were obtained and tentatively grouped into 15 generic morphotypes. Of these, 30 isolates belonging to the *Streptomyces* morphotypes were selected for preliminary screening for antibacterial activity and these were coded as NB003, NB008, NB009, NB012, NB013, NB017, NB018, NB022, NB023, NB025, NB031, NB033, NB035, NB037, NB039, NB041, NB042, NB043, NB046, NB050, NB051, NB052, NB056, NB059, NB061, NB063, NB070, NB073, NB078 and NB084.

3.3 Preparation of *Streptomyces* suspension

The test *Streptomyces* suspensions were prepared by suspending a loopful of pure *Streptomyces* colony in 2 ml sterile normal saline, vortexed to homogenize and stored at 4 ^{o}C until ready for use. This suspension was used as *Streptomyces* inoculants in all cultivations.

3.4 Preparation of test bacteria

All test bacteria were grown in Nutrient broth (biolab, Merck) and incubated at 37 ^{o}C for 24 hr and a total of 10 ml of the pure culture was centrifuged to pellet out the cells, washed twice with sterile physiological saline and the suspension adjusted to optical density 0.1 at 600nm which is equivalent to a cell population of about 10^{6} cells/ml on the McFarland standard. Bacteria suspension was stored in test tubes and refrigerated at 4 ^{o}C. These test bacteria were frequently checked for viability and re-prepared when appropriate.

3.5 *Streptomyces* cultivation media composition

Yeast Malt Broth (YMB) was used for the cultivation of the test Streptomyces and has the following composition: [Yeast extract 4 g/l (biolab, Merck), Malt extract broth10 g/l (Conda, Pronadisa), Glucose 4 g/l (Saarchem, Merck)]. Potato Dextrose Agar (PDA) [(Potato extract 4 g/l, Dextrose 20 g/l, Agar 15.0 g/l (biolab, Merck)]. Nutrient Agar (NA) [(Meat extract 1 g/l, Yeast extract 2 g/l, Peptone 5 g/l, Sodium chloride 8 g/l, Agar 15 g/l (biolab, Merck)]. Nutrient Broth (NB) [(Meat extract 1 g/l, Yeast extract 2 g/l, Peptone 5 g/l, Sodium chloride 8 g/l (biolab, Merck)]. Mueller-Hinton Agar (MHA) [(Meat infusion 5 g/l, Casein Hydrolysate 17.5 g/l, soluble starch 1.5 g/l, Agar 14 g/l (biolab, Merck)]. These media were prepared according to the manufacturer instructions.

3.6 Primary screening of actinomycetes for antibiotic production

3.6.1 Preparation of preliminary test organism

The test organisms used for preliminary screening were *Escherichia coli ATCC* 8739, *Bacillus cereus* ATCC 10702, *Bacillus subtilis* KZN and *Candida albicans.* Test bacteria were grown in 4.50 ml Nutrient broth for 18 hrs, and then standardized to McFarland standard of 0.1 at OD 600nm. *Candida albicans* was grown on Potato dextrose agar (PDA) plate for 48 hr, then re-suspended in Nutrient broth and standardized to McFarland standard of 0.1 at OD 600nm before use.

3.*6.2 Preliminary screening*

Yeast malt broth (YMB) was prepared and 50 ml dispensed into 250 ml capacity flask, autoclaved, allowed to cool and inoculated with 0.5 ml actinomycetes suspension. Flasks were incubated at 28 °C at 230 rpm for 8 days. Cultures were harvested by centrifugation at maximum speed for 15 min to obtain cell free extract which was used for preliminary screening for antibacterial activity. The preliminary screening was carried out by streaking method. Ten milliliter of the cell free extract was

incorporated into double strength 10 ml sterilized molten MHA at 50 °C, plates poured, allowed to set and then streaked upon by test bacteria and incubated at 37 °C for 24 hr. The same regimen was done with PDA for the test yeast, but incubation was at 28 ^{o}C for 24-48 hr. Control experiments were carried out using sterile nutrient broth in place of the cell free extracts.

3.7 Secondary screening of fermentation product for antibiotic production

3.7.1 Bulk fermentation and preparation of crude ethyl acetate extracts

Based on the results of preliminary screening, 10 putative *Streptomyces* isolate were selected for the fermentation and assessment of antibiotic production. The putative *Streptomyces* isolates included NB003, NB008, NB009, NB012, NB017, NB022, NB046, NB063, NB078 and NB084. Fermentation for production of antibiotic and subsequent extraction of the antibiotics was done as described by Ilic *et al.* (2007) with modification. Yeast malt extract broth (YMB) was prepared and 20 ml dispensed into 100 ml Erlenmeyer flask capacity, sterilized, allowed to cool and inoculated with 0.5 ml *Streptomyces* isolate suspension and incubated at 28 ^{o}C for 48 hr at 230 rpm. About 500 ml of YMB was prepared in 1L Erlenmeyer flask and inoculated with the 48 hr old pre-culture of *Streptomyces* isolate and incubated for 10 days at 28 °C and 230 rpm. At the end of the incubation period, the culture was harvested by centrifugation at maximum speed for 15 min. The culture supernatant was extracted twice with equal volumes of ethyl acetate (1:1 v/v) and vaporized to dryness in a rotary evaporator at 50 °C. The extract was re-constituted in 50 % filter sterilized methanol to obtain the desired concentration at every stage of screening.

3.7.2 Screening for antibacterial activity

The crude ethyl acetate extracts was screened for antibacterial activity using the cup well agar diffusion method as described by Pandey *et al.* (2004). For this purpose 20 ml of sterilized molten

Mueller Hinton agar (MHA) in McCartney bottles was seeded with 50 µl of standardized test bacteria swirl gently and aseptically poured into petri plate and allowed to solidify. Sterile cork borer (6 mm diameter) was used to bore holes in the plate. About 100 µl of the crude ethyl acetate extract at a concentration of 10 mg/ml was carefully dispensed into bored holes. This was done in duplicate. Extracts were allowed to diffuse for about 2 hr before incubating. Plates were incubated at 37 °C for 24 hr. The presence of a zone of inhibition around each well was indicative of antibacterial activity. Control experiment was carried out by loading 10 % methanol into control well against each test organism to ensure that it does not have activity against test bacteria.

3.8 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts

The MIC and MBC was determined using the broth micro dilution method as described by Eloff (1998) using test bacteria that have been standardized to optical density of 0.1 at 600 nm. Extracts were re-suspended in 10 % filter sterilized methanol to obtain a concentration of 40 mg/ml. Using a 96-well microtitre plate, 100 µl double strength nutrient broth was dispensed into every well and labeled appropriately. 50 µl of extract was added into well 3, mixed carefully using pipette and diluted serially along the row to row 11. Addition of the standardized test bacteria into the 96-well microtitre plate was incorporated. Plates were covered and incubated at 37 °C for 18-24 hr. Control wells were loaded with nutrient broth and extract with no bacteria added (wells 1 A-H), broth and bacteria with no extract (wells 12 A-H), 10 % MeOH, broth and bacteria (wells 2 A-H) to ensure that 10 % MeOH constitution solvent had no inhibitory effect on the test bacteria. Each extract was assayed in triplicate against test bacteria. Biolab micro plate reader (Model 680 S/N 19138) was used to quantify the optical density of the reactants in each well. A graph of optical densities of wells contents was plotted against extracts concentrations. From the graph the MICs of extracts against test

bacteria were estimated. Also, to complement this assay, bacterial growth was confirmed by adding 50 µl of 0.2 mg/ml solution of 2[4-iodophenyl]3[4-nitrophenyl]-5-phenyl-2H-tetrazolium chloride (INT) in each test well, and the plate was incubated further for at least 1hr at 37°C, to ensure adequate colour development. The lowest concentration in which there was a definite decrease in colour was taken as the MIC of that extract, for that particular organism.

The MBC of active extracts was determined by streaking on Mueller Hinton Agar plates from wells that showed growth inhibition. The concentration of extract in the wells where there was no growth on plate was considered as the MBC.

3.9 Determination of rate of kill

The method of Spangler *et al.* (1997) was adopted for the time-kill assay against some selected susceptible bacteria. Viable counts of the test bacteria were first determined. Initial inocula of 10^5 to 10^6 cfu/ml were prepared from 100 µl aliquots of test bacteria in normal saline and this was verified by performing colony counts. Eighty microliter (80 µl) volume of suspension of known cell density of selected test bacteria was added to 10 ml of Nutrient broth in McCartney bottles of known concentration (relative to MIC) of each of the extracts. Time kill assay of each extract against the selected test bacteria were determined using the following extract concentrations: MIC, 2 × MIC, 3 × MIC and 4 × MIC and the reactants bottles were incubated in a rotary incubator at 37 °C. The time kill kinetics were determined at 0 hr, 3 hr and 6 hr. Exactly 0.5 ml volume of the reaction mixture was withdrawn at the appropriate time and transferred to 4.5 ml of nutrient broth recovery medium containing 3 % sodiumthioglycolate or 3 % "Tween-80" to neutralize the effects of the extracts carry-overs from the test suspensions and diluted serially in sterile nutrient broth. Approximately 100 µl aliquots of each dilution were plated out for viable counts by pour plate technique. Each experiment was done in duplicate, and the mean of two almost identical results was calculated. Only

plates yielding 30 to 300 colonies were selected for counting. Data were analyzed by expressing growth as the $\log_{10}$ colony forming unit per milliliter (cfu/ml). Bacteriostatic activity was defined as a reduction of 0 to 3 $\log_{10}$ cfu/ml, and bactericidal activity was defined as a reduction of ≥ 3 $\log_{10}$ cfu/ml at 3 hr and 6 hr compared to that at 0 hr (Spangler *et al.*, 1997). McCartney bottles containing broth and test organism without extract was used as growth control in each experiment.

3.10 Characterization of crude extracts

3.10.1 Infrared spectroscopy

The Infrared (IR) spectra of the crude extracts were measured (as KBr discs) between 400-4000cm^{-1}on Perkin Elmer 2000 FT-IR spectrophotometer. The important IR bands, such as υ (C-N), υ (O-H), υ (C-H), υ (C=C), υ (N-H), υ (C-O) and (C-H) symmetric and asymmetric stretching, and stretching frequencies were studied to determine the presence of functional group in the ethyl acetate crude extracts.

CHAPTER FOUR

RESULTS

4.1 Preliminary screening of tentative isolates of *Streptomyces*

The result of preliminary screening is as shown in Table 4.1. Of the thirty putative *Streptomyces* screened, twelve showed varying levels of antimicrobial activities against the four test organisms including *Escherichia coli* ATCC 8739, *Bacillus cereus* ATCC 10702, *Bacillus pumilus* ATCC 14884 and *Candida albicans.* NB025 and NB042 showed activity against *Candida albicans* only, while NB003, NB008, NB009, NB012, NB017, NB046, NB063 and NB078, showed broad spectrum activity against the tested bacteria. Isolate NB022 showed only a narrow spectrum activity against the test Gram positive, and isolate NB084 was active against all test bacteria and *Candida albicans*. Eighteen of the isolate viz. NB013, NB018, NB023, NB031, NB033, NB035, NB037, NB039, NB041, NB043, NB050, NB051, NB052, NB056, NB059, NB061, NB070 and NB073 did not show any activity against the test organisms.

Table 4.1: Results of preliminary screening of antimicrobial activities of the *Streptomyces* isolates.

Test	**Antimicrobial activity**			
Streptomyces	*E. coli*	*B. cereus*	*B. subtilis*	*C. albicans*
NB003	+	+	+	_
NB008	+	+	+	_
NB009	+	+	+	_

NB012	+	+	+	_
NB013	_	_	_	_
NB017	+	+	+	_
NB018	_	_	_	_
NB022	_	+	+	_
NB023	_	_	_	_
NB025	_	_	_	+
NB031	_	_	_	_
NB033	_	_	_	_
NB035	_	_	_	_
NB037	_	_	_	_
NB039	_	_	_	_
NB041	_	_	_	_
NB042	_	_	_	+
NB043	_	_	_	_
NB046	+	+	+	_
NB050	_	_	_	_
NB051	_	_	_	_
NB052	_	_	_	_
NB056	_	_	_	_
NB059	_	_	_	_
NB061	_	_	_	_
NB063	+	+	+	_
NB070	_	_	_	_

NB073	_	_	_	_
NB078	+	+	+	_
NB084	+	+	+	+

Legend: + = extract active against test organism; - = extract inactive against test organism.

4.2 Screening of fermentation products for antibacterial activity

The results of the screening of the ethyl acetate crude extracts of the selected ten putative *Streptomyces* for antibacterial activities are presented in Table 4.2. These extracts showed activities against a minimum of 6 test bacteria and maximum of 26 among the 32 test bacteria amounting to approximately 18.8 to 81.3 % antibacterial activities (Table 4.2).

NB003 extracts showed activity against twenty-six test bacteria with zones of inhibition ranging from 10 mm to 22 mm, while NB008 and NB009 extracts showed activity against 25 and 15 test bacteria respectively (Table 4.2). Isolates NB012, NB017, NB022, NB046, NB063, NB078 and NB084 extracts were active against 19, 6, 17, 19, 18, 20 and 12 test bacteria respectively with zones of inhibition ranging from 9-32 mm diameter (Table 4.2).

Table 4.2: Antibacterial activities of fermentation products of the selected putative *Streptomyces* isolates

Test bacteria	Gram reaction	Antibacterial activity (zone of inhibition in mm) (Mean ± SD)									
		NB003	**NB008**	**NB009**	**NB012**	**NB017**	**NB022**	**NB046**	**NB063**	**NB078**	**NB084**
Escherichia coli ATCC 8739	-	12 ± 0.2	0 ± 0	15 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Escherichia coli ATCC 25922	-	13 ± 0.1	12 ±0.4	0 ± 0	17 ± 1.2	10 ± 0.6	11 ± 1.2	0 ± 0	13 ± 1.5	0 ± 0	0 ± 0
Pseudomonas aeruginosa ATCC 19582	-	10 ± 0.2	13 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18 ± 0.5	18 ± 0.1	0 ± 0	0 ± 0
Staphylococcus aureus ATCC6538	+	10 ± 1.2	0 ± 0	0 ± 0	22 ± 0.2	0 ± 0	13 ± 0.4	10 ± 1.2	0 ± 0	0 ± 0	10 ± 1.0
Streptococcus faecalis ATCC 29212	+	0 ± 0	12 ± 1.1	0 ± 0	17 ± 1.5	0 ± 0	0 ± 0	16 ± 1.4	16 ± 0.5	0 ± 0	14 ± 1.0
Bacillus cereus ATCC 10702	+	13 ± 0.2	14 ± 0.4	18 ± 1.5	15 ± 1.4	12 ± 0.1	14 ± 0.1	15 ± 1.0	15 ± 0.2	12 ± 0.5	12 ± 1.0
Bacillus pumilus ATCC 14884	+	14 ± 1.2	11 ± 0.8	18 ± 0.5	19 ± 2.1	0 ± 0	14 ± 1.0	15 ± 1.2	15 ± 0.5	13 ± 0.2	16 ± 0.1
Pseudomonas aeruginosa ATCC 7700	-	15 ± 2.1	12 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	10 ± 0.4	16 ± 0.8	0 ± 0	0 ± 0
Enterobacter cloacae ATCC 13047	-	14 ± 0.9	11 ± 0.5	0 ± 0	17 ± 1.4	0 ± 0	15 ± 1.5	0 ± 0	16 ± 1.1	13 ± 0.5	0 ± 0
Klebsiella pneumonia ATCC 10031	-	12 ± 0.8	13 ± 1.1	15 ± 1.5	18 ± 2.0	12 ± 0.1	13 ± 0.5	0 ± 0	17 ± 1.5	12 ± 0.6	16 ± 0.8
Klebsiella pneumonia ATCC 4352	-	12 ± 0.2	13 ± 0.4	15 ± 0.5	18 ± 0.9	12 ± 0.5	10 ± 0.4	18 ± 1.2	0 ± 0	12 ± 1.1	17 ± 1.5
Proteus vulgaris ATCC 6830	-	14 ± 0.9	12 ± 0.8	13 ± 0.7	17 ± 0.3	0 ± 0	0 ± 0	17 ± 0.4	0 ± 0	14 ± 0.7	0 ± 0
Proteus vulgaris CSIR 0030	-	0 ± 0	12 ± 0.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	12 ± 1.2	12 ± 0.9	11 ± 0.5	0 ± 0

Serratia marscens ATCC 9986	-	10 ± 0.5	12 ± 0.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	15 ± 0.4	15 ± 0.1	13 ± 0.5	9 ± 0.1
Acinetobacter calcaoceuticus UP	+	15 ± 0.2	12 ± 0.1	16 ± 0.5	17 ± 1.1	0 ± 0	10 ± 0.5	14 ± 0.8	0 ± 0	13 ± 0.6	14 ± 0.2
Acinetobacter calcaoceuticus subsp *anitratus* CSIR	+	15 ± 1.1	13 ± 1.2	0 ± 0	18 ± 1.4	0 ± 0	0 ± 0	15 ± 1.0	15 ± 1.2	13 ± 0.5	10 ± 0.2
Klebsiella pneumonia KZN	-	12 ± 0.4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	12 ±0.2	12 ±0.5	20 ± 2.1	0 ± 0
Bacillus subtilis KZN	+	15 ± 1.0	12 ± 0.5	14 ± 0.5	22 ± 2.0	0 ± 0	12 ± 0.5	0 ± 0	10 ± 0.5	15 ± 0.4	0 ± 0
Shigella flexineri KZN	-	0 ± 0	0 ± 0	11 ± 0.7	11 ± 0.8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Salmonella sp KZN	-	12 ± 0.5	12 ± 0.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0	12 ± 0.1	0 ± 0	15 ± 0.5	0 ± 0
Staphylococcus epiderdemis KZN	-	10 ± 0.8	0 ± 0	0 ± 0	20 ± 1.5	0 ± 0	12 ± 0.9	0 ± 0	17 ± 0.9	0 ± 0	0 ± 0
Pseudomonas aeruginosa KZN	-	0 ± 0	12 ± 0.6	10 ± 0.1	10 ± 0.5	0 ± 0	0 ± 0	0 ± 0	17 ± 0.7	11 ± 1.2	0 ± 0
Proteus vulgaris KZN	-	14 ± 0.5	15 ± 1.0	12 ± 1.0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	12 ± 0.9	0 ± 0
Enterococcus Faecalis KZN	-	0 ± 0	0 ± 0	10 ± 0.9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Escherichia coli KZN	-	15 ± 0.1	14 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Staphylococcus aureus KZN	+	17 ± 0.2	9 ± 0	0 ± 0	22 ± 0.5	0 ± 0	20 ± 0.8	16 ± 0.4	0 ± 0	0 ± 0	0 ± 0
Staphylococcus aureus OKOH1	+	22 ± 0	12 ± 0.5	0 ± 0	0 ± 0	10 ± 0.1	22 ± 0	0 ± 0	0 ± 0	10 ± 0.5	13 ± 0.9
Staphylococcus aureus OKOH 2A	+	0 ± 0	0 ± 0	0 ± 0	29 ± 2.0	0 ± 0	12 ± 0.5	18 ±0.9	32 ± 1.5	0 ± 0	0 ± 0

Staphylococcus sciuri OKOH 2B	+	12 ± 0.8	10 ± 0.5	11 ±0.9	0 ± 0	0 ± 0	13 ±0.4	17 ±0.6	17 ±0.2	0 ± 0	0 ± 0
Staphylococcus aureus OKOH 3	+	20 ± 1.2	17 ± 0.5	10 ± 0.2	14 ± 1.0	14 ± 0.8	27 ± 1.5	12 ± 0.5	0 ± 0	12 ± 0.9	15 ±0.1
Micrococcus kristinae	+	19 ± 0.9	15 ± 0.8	12 ± 0.5	0 ± 0	0 ± 0	25 ± 1.5	16 ± 0.9	12 ± 0.2	11 ± 1.0	12 ± 1.2
Micrococcus luteus	+	14 ± 0.8	10 ± 0.5	0 ± 0	27 ± 1.2	0 ± 0	10 ±0.5	0 ± 0	0 ± 0	13 ± 0.1	0 ± 0
% of susceptible* bacteria		**81.3**	**78.1**	**46.9**	**59.4**	**18.8**	**53.1**	**59.4**	**56.3**	**62.5**	**37.5**

*Diameter of zone of inhibition reported on susceptibility was based on the recommended guidelines by Clinical and Laboratory Standards Institute (2005).

4.3 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The results of minimum inhibitory concentration (MIC) of the crude extracts against the susceptible bacteria are presented in Table 4.3. Extract of NB003 exhibited the MIC in the range of 0.1562 mg/ml to 5 mg/ml, while NB008 had MIC varying between 0.1562 mg/ml and 2.5 mg/ml. The MIC of NB009 extract was revealed to range between 0.039 mg/ml and 1.25 mg/ml and that of NB012 ranged from 0.1562 mg/ml to 1.25 mg/ml. For NB017, NB022, NB046, NB063 and NB078, and NB084 the MICs ranged between 0.039 mg/ml and 10 mg/ml. The MBCs of the extracts ranged between 0.625 and >10 mg/ml, with most of the extracts having MBCs of >10 mg/ml (Table 4.4).

Table 4.3: Minimum inhibitory concentrations (MICs) of the crude extracts

Test bacteria	Gram reaction	MIC (mg/ml)									
		NB003	**NB008**	**NB009**	**NB012**	**NB017**	**NB022**	**NB046**	**NB063**	**NB078**	**NB084**
Escherichia coli ATCC 8739	-	0.625	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Escherichia coli ATCC 25922	-	2.5	0.156	0.1562	0.625	1.25	0.625	N/A	0.625	N/A	N/A
Pseudomonas aeruginosa ATCC 19582	-	2.5	1.25	N/A	N/A	N/A	N/A	0.625	0.1562	N/A	N/A
Staphylococcus aureus ATCC6538	+	2.5	N/A	N/A	0.3125	N/A	0.625	1.25	0.1562	N/A	1.25
Streptococcus faecalis ATCC 29212	+	N/A	0.625	N/A	1.25	N/A	N/A	0.625	ND	N/A	0.3125
Bacillus cereus ATCC 10702	+	2.5	0.625	0.078	0.3125	1.25	0.625	0.078	N/A	0.078	0.3125
Bacillus pumilus ATCC 14884	+	1.25	0.625	0.625	0.625	N/A	0.3125	1.25	0.3125	0.625	1.25
Pseudomonas aeruginosa ATCC 7700	-	2.5	2.5	N/A	N/A	N/A	N/A	0.625	0.3125	N/A	N/A
Enterobacter cloacae ATCC 13047	-	5	2.5	N/A	1.25	N/A	0.3125	N/A	0.625	1.25	N/A
Klebsiella pneumonia ATCC 10031	-	0.3125	2.5	0.3125	0.3125	2.5	0.625	N/A	0.1562	0.625	0.3125
Klebsiella pneumonia ATCC 4352	-	0.625	2.5	0.1562	0.3125	2.5	0.625	0.3125	N/A	0.3125	0.3125
Proteus vulgaris ATCC 6830	-	2.5	1.25	0.625	0.625	N/A	N/A	1.25	N/A	0.078	N/A
Proteus vulgaris CSIR 0030	-	N/A	2.5	N/A	N/A	N/A	N/A	0.625	0.078	0.3125	N/A

Serratia marscens ATCC 9986	-	0.625	1.25	N/A	N/A	N/A	N/A	0.625	0.078	0.625	N/A
Acinetobacter calcaoceuticus UP	+	0.625	1.25	0.625	0.1562	N/A	2.5	0.625	N/A	0.078	0.625
Acinetobactercalcaoceuticus subsp *anitratus* CSIR	+	0.625	0.3125	N/A	0.3125	N/A	N/A	0.039	0.3125	0.3125	0.1562
Klebsiella pneumonia KZN	-	5	N/A	N/A	N/A	N/A	N/A	0.625	0.3125	0.625	N/A
Bacillus subtilis KZN	+	1.25	0.625	1.25	0.625	N/A	1.25	N/A	0.1562	0.1562	N/A
Shigella flexineri KZN	-	N/A	N/A	0.625	1.25	N/A	N/A	N/A	N/A	N/A	N/A
Salmonella sp KZN	-	0.1562	0.078	N/A	N/A	N/A	N/A	1.25	N/A	10	N/A
Staphylococcus epidermides KZN	-	1.25	N/A	N/A	0.625	N/A	5	N/A	ND	N/A	N/A
Pseudomonas aeruginosa KZN	-	N/A	1.25	0.3125	2.5	N/A	N/A	N/A	0.3125	5	N/A
Proteus vulgaris KZN	-	0.3125	1.25	0.625	N/A	N/A	N/A	N/A	N/A	0.3125	N/A
Enterococcus faecalis KZN	-	N/A	N/A	0.078	N/A	N/A	N/A	N/A	N/A	N/A	2.5
Escherichia coli KZN	-	2.5	1.25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Staphylococcus aureus KZN	+	0.3125	2.5	N/A	0.3125	N/A	1.25	0.625	N/A	1.25	N/A
Staphylococcus aureus OKOH1	+	5	1.25	N/A	N/A	ND	2.5	N/A	N/A	ND	0.625
Staphylococcus aureus OKOH 2A	+	N/A	N/A	N/A	0.3125	N/A	5	1.25	0.625	N/A	N/A

Staphylococcus sciuri OKOH 2B	+	1.25	0.039	1.25	N/A	N/A	0.625	0.3125	N/A	N/A	N/A
Staphylococcus aureus OKOH 3	+	2.5	0.3125	1.25	0.3125	1.25	0.3125	0.039	N/A	0.625	0.625
Micrococcus kristinae	+	0.3125	0.625	0.039	0.625	N/A	1.25	0.3125	ND	0.3125	0.625
Micrococcus luteus	+	0.1562	0.625	N/A	N/A	N/A	0.3125	N/A	N/A	0.625	N/A

Legend: ND- not determined; N/A – Not applicable.

Table 4.4: Minimum bactericidal concentrations (MBCs) of the crude extracts

Test bacteria	Gram reaction	MBC (mg/ml)									
		NB003	**NB008**	**NB009**	**NB012**	**NB017**	**NB022**	**NB046**	**NB063**	**NB078**	**NB084**
Escherichia coli ATCC 8739	-	>10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Escherichia coli ATCC 25922	-	>10	>10	>10	>10	>10	>10	N/A	>10	N/A	N/A
Pseudomonas aeruginosa ATCC 19582	-	10	10	N/A	N/A	N/A	N/A	5	2.5	N/A	N/A
Staphylococcus aureus ATCC6538	+	10	N/A	N/A	0.625	N/A	10	5	N/A	N/A	>10
Streptococcus faecalis ATCC 29212	+	N/A	10	N/A	>10	N/A	N/A	2.5	ND	N/A	>10
Bacillus cereus ATCC 10702	+	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10
Bacillus pumilus ATCC 14884	+	>10	>10	>10	>10	N/A	>10	>10	>10	>10	>10
Pseudomonas aeruginosa ATCC 7700	-	10	5	N/A	N/A	N/A	N/A	2.5	1.25	N/A	N/A
Enterobacter cloacae ATCC 13047	-	>10	>10	N/A	>10	N/A	>10	N/A	>10	>10	N/A
Klebsiella pneumonia ATCC 10031	-	>10	>10	>10	>10	>10	>10	N/A	>10	>10	>10
Klebsiella pneumonia ATCC 4352	-	>10	>10	>10	>10	>10	>10	>10	N/A	>10	>10
Proteus vulgaris ATCC 6830	-	>10	>10	>10	>10	N/A	N/A	>10	N/A	>10	N/A
Proteus vulgaris CSIR 0030	-	N/A	>10	N/A	N/A	N/A	N/A	>10	>10	2.5	N/A

Serratia marscens ATCC 9986	-	>10	10	N/A	N/A	N/A	N/A	5	5	>10	>10
Acinetobacter calcaoceuticus UP	+	5	10	5	>10	N/A	5	2.5	N/A	>10	2.5
Acinetobactercalcaoceuticus subsp *anitratus* CSIR	+	5	2.5	N/A	10	N/A	N/A	2.5	>10	>10	1.25
Klebsiella pneumonia KZN	-	10	N/A	N/A	N/A	N/A	N/A	1.25	2.5	2.5	N/A
Bacillus subtilis KZN	+	10	>10	2.5	>10	N/A	>10	N/A	>10	>10	N/A
Shigella flexineri KZN	-	N/A	N/A	2.5	2.5	N/A	N/A	N/A	N/A	N/A	N/A
Salmonella sp KZN	-	10	5	N/A	N/A	N/A	N/A	5	N/A	>10	N/A
Staphylococcus epidermides KZN	-	10	N/A	N/A	>10	N/A	>10	N/A	ND	0	N/A
Pseudomonas aeruginosa KZN	-	N/A	2.5	5	5	N/A	N/A	N/A	1.25	>10	N/A
Proteus vulgaris KZN	-	10	2.5	2.5	N/A	N/A	N/A	N/A	N/A	10	N/A
Enterococcus Faecalis KZN	-	N/A	N/A	5	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Escherichia coli KZN	-	10	2.5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Staphylococcus aureus KZN	+	10	5	N/A	5	N/A	5	2.5	N/A	>10	N/A
Staphylococcus aureus OKOH1	+	10	2.5	N/A	N/A	ND	10	N/A	N/A	ND	>10
Staphylococcus aureus OKOH 2A	+	N/A	N/A	N/A	5	N/A	>10	2.5	2.5	N/A	N/A

Staphylococcus sciuri	OKOH 2B	+	5	2.5	5	N/A	N/A	5	2.5	N/A	N/A	N/A
Staphylococcus aureus	OKOH 3	+	5	2.5	2.5	10	>10	>10	5	N/A	5	10
Micrococcus kristinae		+	1.25	2.5	2.5	2.5	N/A	10	1.25	ND	2.5	>10
Micrococcus luteus		+	2.5	2.5	N/A	N/A	N/A	5	N/A	N/A	10	N/A

Legend: ND- not determined; N/A – Not applicable.

4.4 Time kill assay

The time-kill profile of the crude extracts of the test actinomycetes are as shown in Table 4.5. All extracts exhibited both varying degrees of bactericidal and bacteristatic activities depending on the test bacteria. A significant decrease in mean viable count of isolates was observed at each time interval. Results are presented as Log_{10} cfu/ml change in the viable colony number. Mean Log reduction in viable cell count for NB003 range between 2.18 $Log_{10,}$ 2.84 Log_{10}, 2.61 Log_{10} and 3.09 Log_{10} cfu/ml after 3 hr interaction and between 2.36 $Log_{10,}$ 2.56 Log_{10}, 2.80 Log_{10} and 3.15 Log_{10} cfu/ml after 6 hr interaction in MIC, 2 × MIC, 3 × MIC and 4 × MIC respectively. For NB008 Log reduction in viable cell count varied from 1.7 Log_{10}, 2.48 Log_{10}, 2.67 Log_{10} and 3.05 Log_{10} cfu/ml after 3 hr of interaction at MIC, 2 × MIC, 3 × MIC and 4 × MIC respectively while at 6 hr interaction it was revealed as 0.62 Log_{10}, 2.53 Log_{10}, 2.69 Log_{10} and 3.15 Log_{10} at MIC, 2 × MIC, 3 × MIC and 4 × MIC respectively. For NB009, NB012, NB022, NB046, NB063, NB078 and NB084 extracts, the Log reduction in viable cell densities ranged between -0.51 Log_{10} and 4.86 Log_{10}. The utmost reductions in cell densities were achieved by NB063, NB022 and NB046 for *Staphylococcus aureus* OKOH 2A (clinical isolate), *Micrococcus luteus* and *Salmonella* sp. KZN (environmental isolates) with the average reduction in viable cell of 4.86 Log_{10}, 4.38 Log_{10} and 3.74 Log_{10} respectively.

4.5 Characterization of crude extract

The Infrared (IR) spectral of the crude ethyl acetate extracts of putative *Streptomyces* isolates are presented in Appendix A to J. The presence of some functional group as revealed by IR spectral is presented in Table 4.6.

4.5.1 Infrared (IR) analysis

The FTIR Spectral of extracts NB003, NB008, NB009**,** NB012, NB046, NB063, NB078 and NB084 show certain common absorption bands between 1700 and 1730 cm^{-1} characteristics of a C=O

stretching vibration of a carboxylate functional group. The peaks between 3390 and 3420 cm^{-1} are characteristics of hydroxyl υ (O-H) and υ (N-H) vibrational frequency which are interchangeable. A common 2 bands Vibrational peak between 2855 and 2979 cm^{-1} are characteristic of a υ (C-H) symmetrical vibration of saturated hydrocarbon.

The Vibrational frequency υ (C-O) was observed in the spectra of all the extracts around 1100cm^{-1}. Deviation from this region to a higher wave number was observed in the spectra of NB008, NB012 and NB084 which could be ascribed to a υ(C-N) vibrational stretch of amide. Corresponding N-H symmetrical vibration was observed around 3418 cm^{-1} in the extracts of these three isolates indicative of a secondary amide. These peaks were sharper than the υ (O-H) peaks due to reduction in hydrogen bonds which increases with electro negativity.

In extract NB084, the absence of vibrational peaks between 1600 cm^{-1} and 1640 cm^{-1} gave indicates for the absence of unsaturation. Vibrational peaks between 1620 cm^{-1} and 1680 cm^{-1} in extract NB022 and NB078 signify possibility of an aromatic compound. Based on the physical state (oily) of the extracts and the characteristic features of the infra-red Vibrational peaks in the spectra, terpenoids, long chain fatty acids and secondary amine derivatives are possible compounds in the extracts.

Table 4.5: Antibacterial Time-kill profile of the crude extracts of the fermentation products

Extracts	Test bacteria	MIC (Log_{10} kill)		2 × MIC (Log_{10} kill)		3 × MIC (Log_{10} kill)		4 × MIC (Log_{10} kill)	
		3 hr	6 hr	3 hr	6 hr	3 hr	6 hr	3 hr	6 hr
	Proteus vulgaris KZN	2.18	2.36	2.48	2.56	2.61	2.80	3.09	3.15
NB003	*Staphylococcus sciriu* OKOH 2B	1.76	1.06	1.97	1.43	2.16	2.57	2.55	2.80
	Klebsiella pneumonia ATCC 4352	1.10	-0.97	1.25	-0.12	1.38	1.92	1.41	2.24
	Escherichia coli ATCC 25922	1.38	-1.0	2.01	0.36	2.65	2.65	3.04	3.08
NB008	*Salmonella* sp. KZN	1.55	-0.89	1.16	1.14	1.65	1.97	1.78	2.63
	Streptococcus faecalis ATCC 29212	1.70	0.62	2.48	2.53	2.67	2.69	3.05	3.15
	Staphylococcus sciriu OKOH 2B	2.64	0.19	2.71	0.72	3.42	1.06	3.58	1.37
NB009	*Bacillus cereus* ATCC 10702	1.39	-2.81	1.47	-0.74	3.01	1.27	3.46	2.64
	Pseudomonas aeruginosa KZN	1.73	1.20	2.26	2.04	2.38	2.11	2.58	2.58
	Staphylococcus aureus ATCC6538	2.03	2.11	2.05	2.17	2.18	2.25	2.50	3.39
NB012	*Klebsiella pneumonia* ATCC 10031	1.39	1.87	1.39	2.18	1.56	2.36	1.59	2.39
	Bacillus pumilus ATCC 14884	1.52	2.10	1.97	2.39	2.40	3.04	3.69	3.79

NB022	*Staphylococcus aureus* ATCC 6538	1.52	2.10	1.97	2.39	2.40	3.06	3.69	3.79
	Micrococcus luteus	1.5	1.49	1.72	1.72	3.44	2.55	3.99	4.38
NB046	*Streptococcus faecalis* ATCC 29212	1.53	-0.83	1.53	0	2.19	0.26	2.31	0.60
	Salmonella sp. KZN	1.14	-2.4	1.50	0.55	2.59	2.70	3.07	3.74
NB063	*Staphylococcus aureus* OKOH 2A	1.44	1.26	1.65	2.14	1.81	2.47	3.92	4.86
	Pseudomonas aeruginosa ATCC 19582	2.71	2.63	3.01	3.03	3.04	3.21	3.59	3.78
	Serratia marscens ATCC 9986	1.32	-1.51	2.59	-0.51	2.63	-0.35	2.75	-0.22
	Klebsiella pneumonia ATCC 4352	1.39	-0.81	1.73	0.19	2.42	1.97	2.72	2.8
NB078	*Proteus vulgaris* CSIR 0030	0.86	-2.16	1.10	-0.89	1.30	-0.79	1.30	0.01
	Staphylococcus aureus OKOH 3	2.10	-2.10	2.67	-1.83	3.02	1.47	3.04	1.01
NB084	*Streptococcus faecalis* ATCC 29212	1.26	-1.32	2.30	0.183	2.34	2.61	3.41	3.45
	Acinetobacter calcaoceuticus subsp *anitratus* CSIR	2.35	0.51	2.49	0.63	2.71	1.05	3.25	1.27

Table 4.6: IR spectral of functional groups present in crude ethyl acetate extracts of putative *Streptomyces* isolate

Extracts	υ (N-H) (cm^{-1})	υ (C=O) (cm^{-1})	υ(C-O) (cm^{-1})	υ (O-H) (cm^{-1})	υ (C-N) (cm^{-1})	υ (C=C) (cm^{-1})	υ (C-H) (cm^{-1})
NB003		1709, 1665	1090, 1016	3392			2975
NB008	3415	1702			1270		2961
NB009		1712	1121		3411		2902
NB012	3420	1724	1052			1638	2924
NB017			1100	3435		1648	2959
NB022		1726		3417		1662	2926
NB046		1664	1077	3400			2962
NB063		1712	1091	3392			2975
NB078		1709	1091	3397		1655	2979
NB084	3415	1709			1217		2964

Legend: υ(N-H) Vibrational frequency of Nitrogen-Hydrogen bond; υ(C=O) Vibrational frequency of Carbon oxygen double bond; υ(C-O) Vibrational frequency of Carbon oxygen single bond; υ(O-H) Vibrational frequency of hydroxyl group; υ(C-N) Vibrational frequency of carbon nitrogen bond; υ(C=C) Vibrational frequency of carbon- carbon unsaturated bond; υ(C-H) Vibrational frequency of saturated Carbon hydrogen bond, (sp^3 hybridised).

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 Discussion

The incidence of multi drug resistant organisms is increasing and is a problem of global concern. Despite the broad range of activities, the most studied bioactive metabolites through history has been the antibiotics. Currently, appearance of multiple antibiotic resistances in pathogenic bacteria is increasing, compromising the clinical treatment of a growing number of infectious diseases. There is thus an urgent need for new drugs effective against current antibiotic resistant pathogens and opportunistic pathogens. Interestingly marine actinomycetes produce many potential pharmacological compounds with antibiotic and antitumor properties Suthindhiran and Kannabira, 2009. Streptomyces have been proven as a potential source of bioactive compounds and richest source of secondary metabolites (Suthindhiran and Kannabira, 2009). The search for active secondary metabolites produced by environmental isolates, using poorly explored microorganisms, could provide a new source for discovering novel bioactive compounds.

Significant attention is currently being paid to the isolation and characterization of *Streptomyces* from poorly researched habitats given the premise that screening such organisms raises the prospect of discovering new natural products that can be developed as a resource for biotechnology (Bredholt *et al.,* 2008; Eccleston *et al.,* 2008). This reasoning appears to be sound as novel *Streptomyces* isolated from unexplored marine habitats are proving to be a valuable source of new bioactive metabolites (Bull and Stach, 2007; Fiedler *et al.*, 2005). It seems timely, therefore, to extend this approach to another poorly studied environment, such as the Nahoon beach ecosystem.

It was not intended in the present investigation to assemble a detailed record of the kinds of *Streptomyces* found in the Nahoon beach environment or to isolate and characterize novel bioactive

compounds, but the study was designed to prepare the ground for such studies by isolating, partially characterizing and screening a diverse range of *Streptomyces*.

Many of the strains evaluated in the present study showed bioactivity against one or more of the screening systems, thereby underpinning and extending results from previous studies which showed Nahoon beach to be a source of bioactive *Streptomyces*. It is becoming increasingly evident that the taxonomic and metabolic diversity encompassed by *Streptomycetes* is remarkable, as new and putatively novel *Streptomyces* species are being continuously isolated from under-researched habitats and shown to be valuable sources of new bioactive compounds.

The antimicrobial activities of the extracts were tested against a wide range of microorganisms (Table 4.2). These extracts showed activities against a minimum of 6 test bacteria and maximum of 26 among the 32 test bacteria amounting to approximately 18.8 to 81.3 % antibacterial activities (Table 4.2). This percentage is higher than those described by Barakate *et al.* (2002) studying the activity of Moroccan soil *Streptomyces*. These results were also different from those of other authors showing 16% in soil of Turkey (Oskay *et al.,* 2004); 53 – 61% in Algerian soil (Sabaou *et al.,* 1998) and 44.5% in soils of South-Eastern Serbia (Illic *et al.,* 2005; 2007). On the other hand, similar inhibition pattern was demonstrated by the ethyl acetate extracts of marine *Streptomyces* RM17 and RM42 (Remya and Vijayakumar, 2008); marine *Streptomyces* isolates from the Andaman Coast of the Bay of Bengal (Peela *et al.,* 2005); and marine *Streptomyces* strain Merv 1996 and Merv 7409 (El-Gendy *et al.,* 2008b); although more bacteria species were screened in this study. The antibacterial spectrum exhibited by all extracts highlights their potentials and suggests that they could be important candidates for antibiotics in this regard. Further studies on the bioactive metabolites produced by these extracts which exhibits broad spectrum activity is under progress. Overall the study gives the first hand information on the antimicrobial activity of putative indigenous *Streptomyces* isolated from the Nahoon beach environment.

The differences in the susceptibilities of Gram positive and Gram negative bacteria to the study *Streptomyces* extracts have been observed by previous workers (Ilic *et al.*, 2007). Gram negative bacteria are inherently more resistant to antimicrobials than Gram positive organisms and this has been ascribed to the combined exclusion of antimicrobial compounds by double membrane barrier and transmembrance efflux present in this group of organisms (Zgurskaya and Nikaido, 2000).

The biological activities (MIC and MBC) of the crude extracts emphasizes that the extracts are active against Gram-positive and Gram-negative bacteria. The MICs values exhibited by all extracts in this study ranged between 0.039 mg/ml and 10 mg/ml. This was relatively higher than the MIC values obtained from marine *Streptomyces* strain Merv 1996 (El-Gendy *et al.,* 2008b) against *B. subtilis* ATCC 6051, *S. aureus* ATCC 6538 and *M. luteus* with MICs of 0.0036 mg/ml, 0.0008 mg/ml and 0.002 mg/ml respectively; *Streptomyces* sp. AZ-NIOFD1 isolated from River Nile water, Egypt (Atta *et al.,* 2009) with MIC range of 0.0117 mg/ml - 0.03125 mg/ml; and marine *Streptomyces* sp. Merv 8102 (El-Gendy *et al.,* 2008a). However, Pandey *et al.* (2004) mentioned that the MIC is not a constant for a given agent, since it is influenced by a number of factors. These factors include the nature of the test organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration.

In general, some extracts of these putative *Streptomyces* had antibacterial activities against Gram positive bacteria, especially staphylococci. Staphylococci are among the most commonly encountered pathogens in clinical practice. *S. aureus* is a major cause of nosocomial infections, food poisoning, osteomyelitis, pyoarthritis, endocarditis, toxic shock syndrome, and a broad spectrum of other disorders (Todd, 1998; Hajjeh *et al.,* 1999; Rubin *et al.,* 1999). In recent years, there has been an alarming increase in nosocomial staphylococcal infections by strains with multiple drug resistance (Al-Masaudi *et al.,* 1991; Kloos and Bannerman, 1995; Hiramatsu *et al.,* 1997). At present, this situation is leading to the evaluation of staphylococcal pathogens potentially resistant to any available

antibiotic (Noble *et al.,* 1992; Huycke *et al.,* 1998; Rubin *et al.,* 1999). Therefore, the result of this study may suggest that the extracts of the species possess compounds with antimicrobial properties which can be used as antimicrobial agents in new drugs for therapy of infectious diseases in human.

Time-kill studies have been used to investigate numerous antimicrobial agents. They are also often used as the basis for *in vitro* investigations into pharmacodynamic drug interactions. They provide descriptive (qualitative) information on the pharmacodynamics of antimicrobial agents. They are also used to provide clinically relevant assay of the activity of antimicrobial agents. The time kill assay of the crude extracts of the test S*treptomyces* isolates gave variable kinetics among susceptible bacteria tested (Table 4.5). Both bactericidal and bacteristatic activities were demonstrated by the extracts. The extracts exhibited a partially concentration-dependent killing profile. Although literatures on time kill kinetics of marine *Streptomyces* is scarce, time kill kinetics of a marine bacterium against clinical methicillin resistant *Staphylococcus aureus* (MRSA) isolate has been reported by Isnansetyo and Kamei (2003). In their study, bactericidal activity has demonstrated to be much higher than vacomycin therefore, time kill studies of these marine *Streptomyces* proposes their possible outcome in *in vivo* studies.

Extracts of NB008 was bactericidal with 4 × MIC against *Streptococcus faecalis* ATCC 29212 at 3 hr and a slight increase in activity at 6 hr interaction. However, a bacteristatic activity was observed at lower concentrations. Watanakunakorn and Tisonel (1982), reported time kill kinetics of the combination of N-Formimidoyl' thienamycin (MK0787) a stable derivative of thienamycin, derived from *Streptomyces cattleya* with gentamicin or with tobramycin against enterococci. In their study a combination of N-Formimidoyl' thienamycin with gentamicin or with tobramycin showed over 95% bactericidal activity against forty-seven strains of *Streptococcus faecalis* tested. Therefore Extracts of NB008 compound or its derivative either single or in combinational therapy with other antibiotics suggest potential use against enterococcal infections. NB008 showed bacteristatic action against *Salmonella* sp at all concentrations tested. However, stronger bacteristatic activity was

observed with higher concentrations (3 × MIC and 4 × MIC) and longer time. It can be assumed that at higher concentration of this extract, aggressive bactericidal activity against *Salmonella* sp would be achieved.

NB063 exhibited bacteristatic activity with MIC at 3 hr and 6 hr against *Pseudomonas aeruginosa* ATCC 19582. Furthermore, bactericidal activity was demonstrated with 2 × MIC, 3 × MIC and 4 × MIC at 3 hr and 6 hr interactions. *P. aeruginosa* is an important pathogen associated with serious nosocomial infections such as pneumonia and sepsis (Tam *et al.,* 2005). It is also associated with multiple mechanisms of resistance to various antimicrobial agents (Tam *et al.,* 2005). Treatment of pseudomonal infections often presents a challenge to clinicians and combination therapy is commonly used to prevent the emergence of resistance (Tam *et al.,* 2005). However, from the Time-kill profile among all extracts and susceptible bacteria tested in this study, NB063 showed the best time kill profile against *Pseudomonas aeruginosa* ATCC 19582 in the sense that bactericidal activity was observed with 2 × MIC with a stepwise increase in bactericidal activity with concentration and time. Although NB009 exhibited a strong bacteristatic activity against an environmental strain of *Pseudomonas aeruginosa* KZN, NB063 is the only extract that demonstrated bactericidal activity with 2 × MIC concentration. NB063 proposes to be an important agent against pseudomonal infections.

Anti-pneumococcal activity of two strains of pneumococcal bacteria - *Klebsiella pneumonia* ATCC 10031 and *Klebsiella pneumonia* ATCC 4352 was demonstrated by NB012 and NB078 respectively. Time kill profile of anti-pneumococcal activities of some antibiotics has been reported (Pankuch *et al.,* 1996) but the anti-pneumococcal time kill profile of marine *Streptomyces* is limited, this appear to be the first report of anti-pneumococcal time kill profile of extracts of marine *Streptomyces* (Table 4.5). These extract (NB012 and NB078) promises to be important drug candidates for the development of anti-pneumococcal antibiotics. The re-growth phenomenon

exhibited by some extracts against certain bacteria could be attributed to instability of the crude extracts in the growth medium.

IR spectra of crude extracts showed some similarities in their content due to presence of some functional groups, but the different vibrational peaks of these functional groups in these extracts depicts that the extracts were different hence the diverse activity they exhibited against test organisms especially during the susceptibility screening and MIC assessment. The IR spectrum of an antifungal compound: 4' phenyl -1-napthyl –phenyl acetamide from *Streptomyces* sp. DPTB16 indicates that the compound had NH_2 and -OH group (Dhanasekaran *et al.,* 2008). Similar functional group and spectra profiles were exhibited by these extracts suggesting similarity with these extracts and this could be attributed to antifungal activity demonstrated by some of the extracts during preliminary screening (Table 4.1). The distribution of the antibiotic inhibition phenotype of *Streptomycetes* with great antibacterial and antifungal activity which gave a similar spectral profile has also been reported (Illic *et al.,* 2007). Nevertheless, further investigation is needed in order to purify and determine the structure of the active components in the extracts.

5.2 Conclusion

The present finding highlights the importance for further investigation towards the goal of obtaining novel antimicrobial agent from the putative *Streptomyces* from this untapped habitat. The Nahoon beach habitat appears to be an unexplored area in this environment, with unique ecological niches and rich in biodiversity (Unpublished data). The microbiology of the Nahoon beach environment has to be further explored in order to get benefit out of the precious biowealth, more so as it has great demand from the perspective of health care especially as regards combating existing and emerging drug resistant pathogens. The emergence and dissemination of antibacterial resistance is well documented as a serious problem worldwide. It is anticipated that due to the antibacterial profile and

characterization of the crude extracts, putative marine Streptomyces isolated from Nahoon Beach promises to be useful in the discovery of novel antibiotics.

It is therefore recommended that further investigation should address the relationship between the structure of the active component of the extracts and the broad spectrum activity, as well as a rapid method for large scale production and purification and whether this group of antibiotics has any application in managing human infectious disease.

5.3 Limitation of the study

There was limit using Mass spectrum, nuclear magnetic resonance (NMR) spectrum and High performance liquid chromatography (HPLC) spectrum to elucidate the possible structure of the metabolites (compound) isolated from the *Streptomyces* strains due to time and resource constrains and lack of elemental analytical instruments in my institution (University of Fort Hare).

5.4 Recommendation

Based on the findings of this study the following recommendations are suggested:

- There is need for further studies to optimize the production conditions of the bioactive compounds isolated from the marine *Streptomyces*.
- In the future elemental analytical instrumentation should be employed in the identification and purification of the isolated bioactive compounds; possibly as a doctoral study.
- There may be need to test the anticancer (cytotoxicity studies), antifungal, antiviral, and anti-inflammatory activities of the extracted biomolecule in the future.

5.5 Potential for future development of the study

The marine environment is becoming increasingly appreciated as a rich and untapped reservoir of novel natural products. Bioactive compounds are frequently associated with marine organism. In the current study, *Streptomyces* strains (marine origin) showed good potentials as source of novel secondary metabolites. Although time-kill kinetics was used to monitor the rate of kill of the extracted biomolecules against individual bacteria, there is limited information in the literature on imaging of time-kill kinetics at the cellular level. Thus future studies may focus on this area to broaden our knowledge and understanding of the mechanisms involved in this process. Information from such research may add to our current knowledge of the pathogen-host relationship and will help to determine the target site of the cell organelles that these biomolecules attack or have affinity for. This could also help to tackle resistance problem.

The focus of the current study was on the antimicrobial activity of extracted biomolecule from *Streptomyces* against a panel of bacteria; in the future biological activity of the biomolecule could be expanded to include other biological assay such as anticancer (cytotoxicity studies), antifungal, antiviral, and anti-inflammatory activities. Such investigations could give an excellent perspective to the metabolites activity. Other assays that may be considered in future studies include: chemical characterization of bioactive metabolites using chromatographic methods (e.g. HPLC) in order to isolate pure metabolites. Structural elucidation using mainly one dimensional (1D) or two dimensional (2D) NMR techniques. This will greatly enhance the identification of the active components in the metabolites (culture extracts). The discovery of novel metabolites in marine microorganisms has become more difficult due to the enormous number of known compounds already described in the literature.

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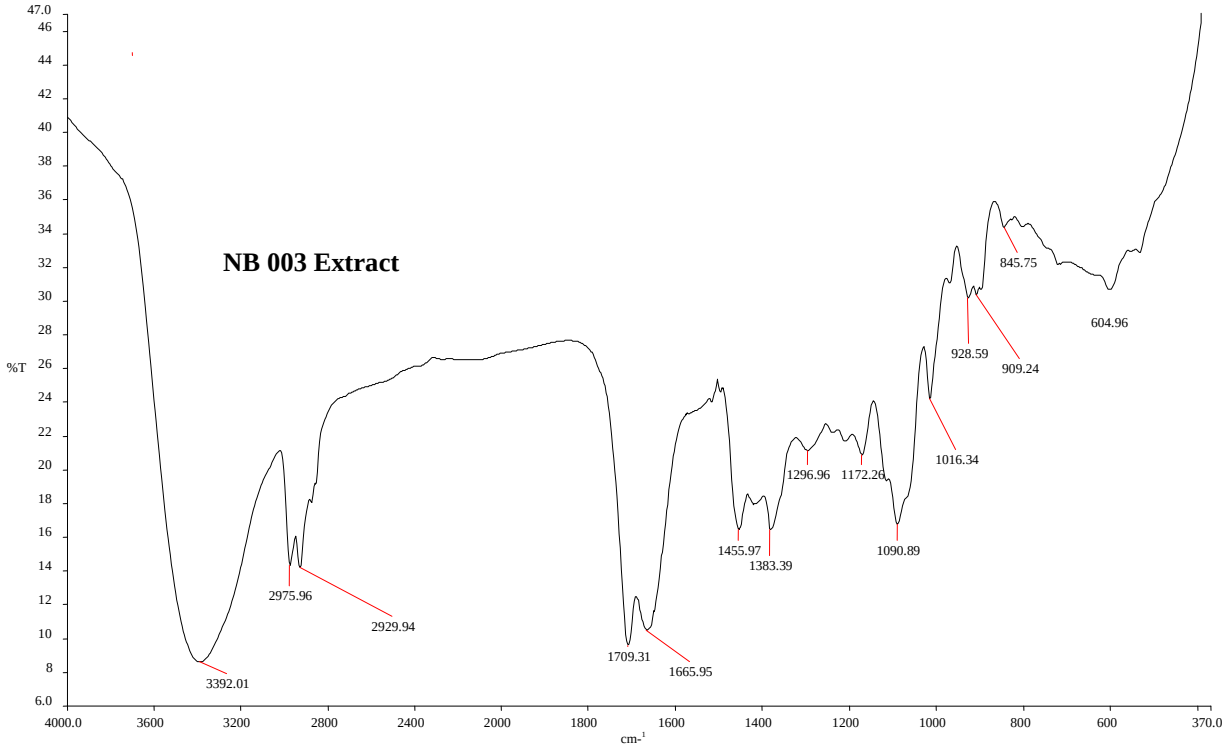
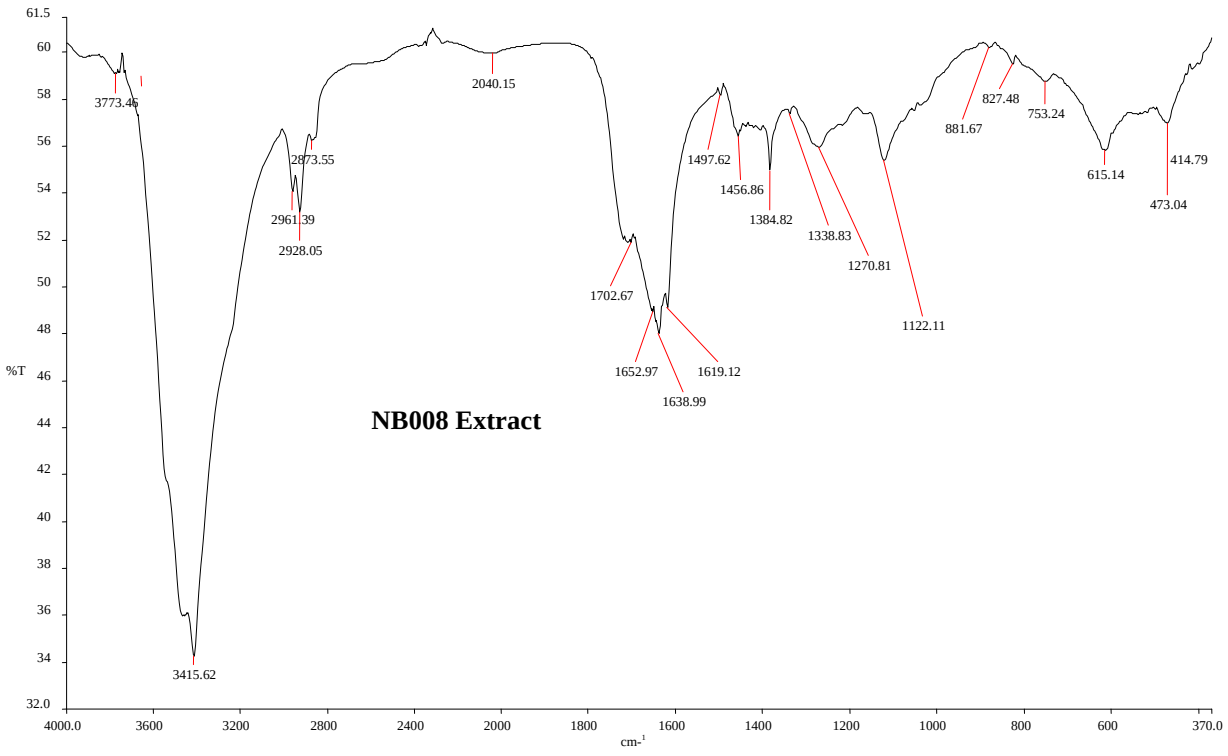
APPENDIX A

IR spectral of crude ethyl acetate extract of isolate NB003



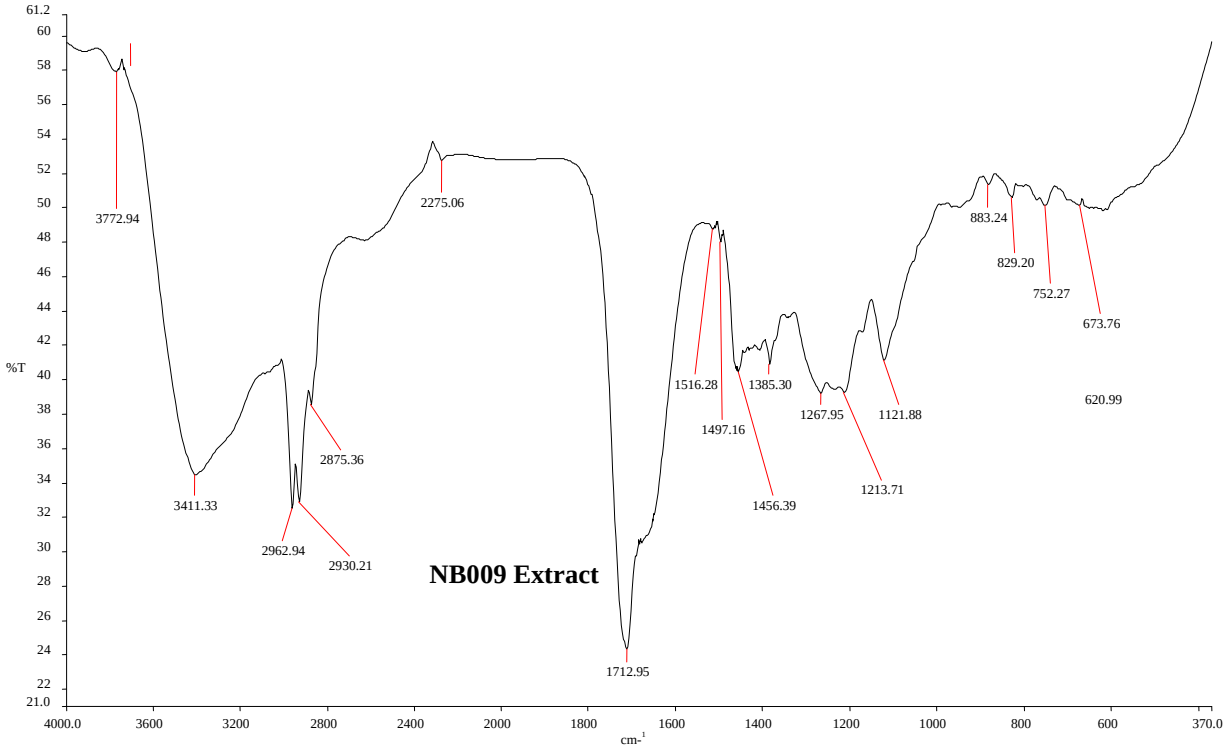
APPENDIX B

IR spectral of crude ethyl acetate extract of isolate NB008



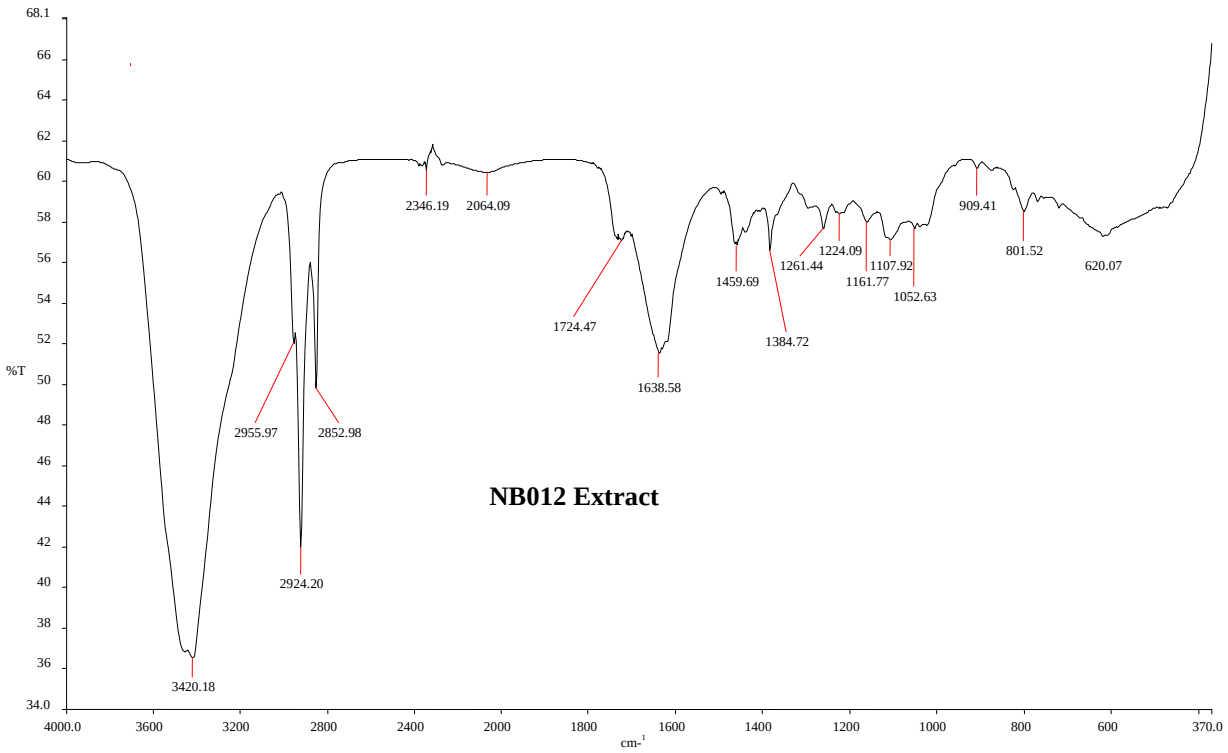
APPENDIX C

IR spectral of crude ethyl acetate extract of isolate NB009



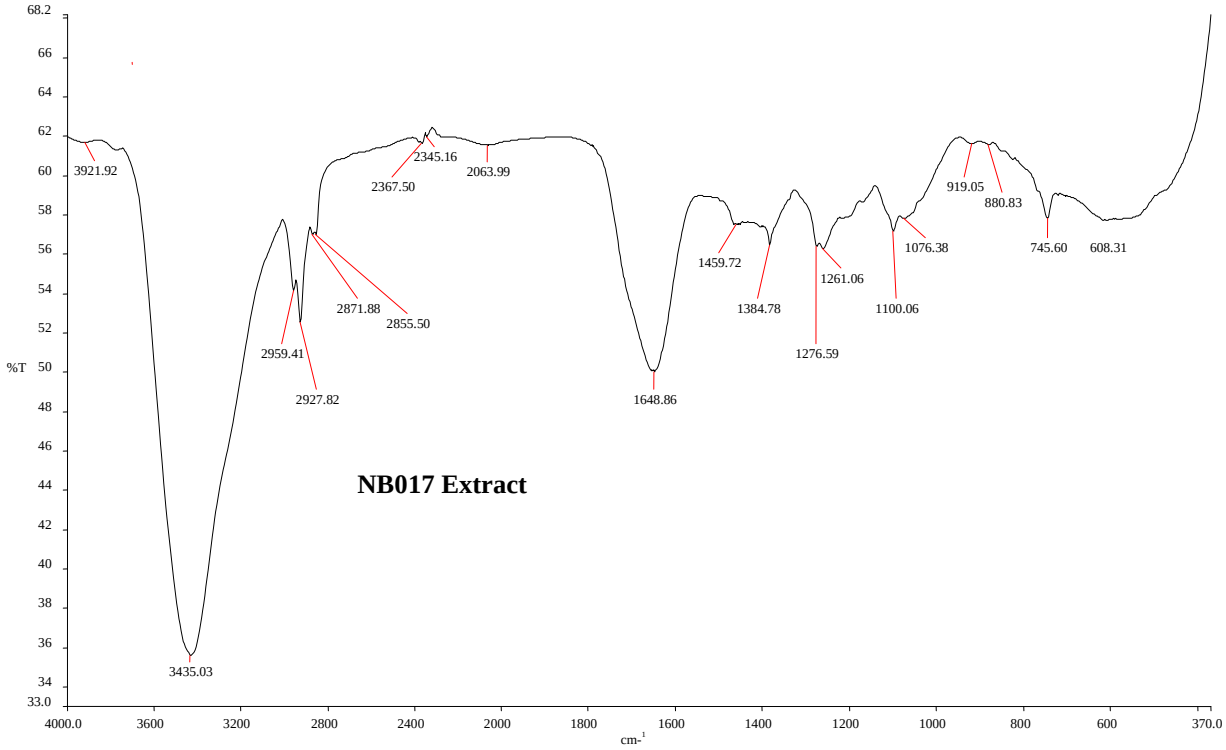
APPENDIX D

IR spectral of crude ethyl acetate extract of isolate NB012



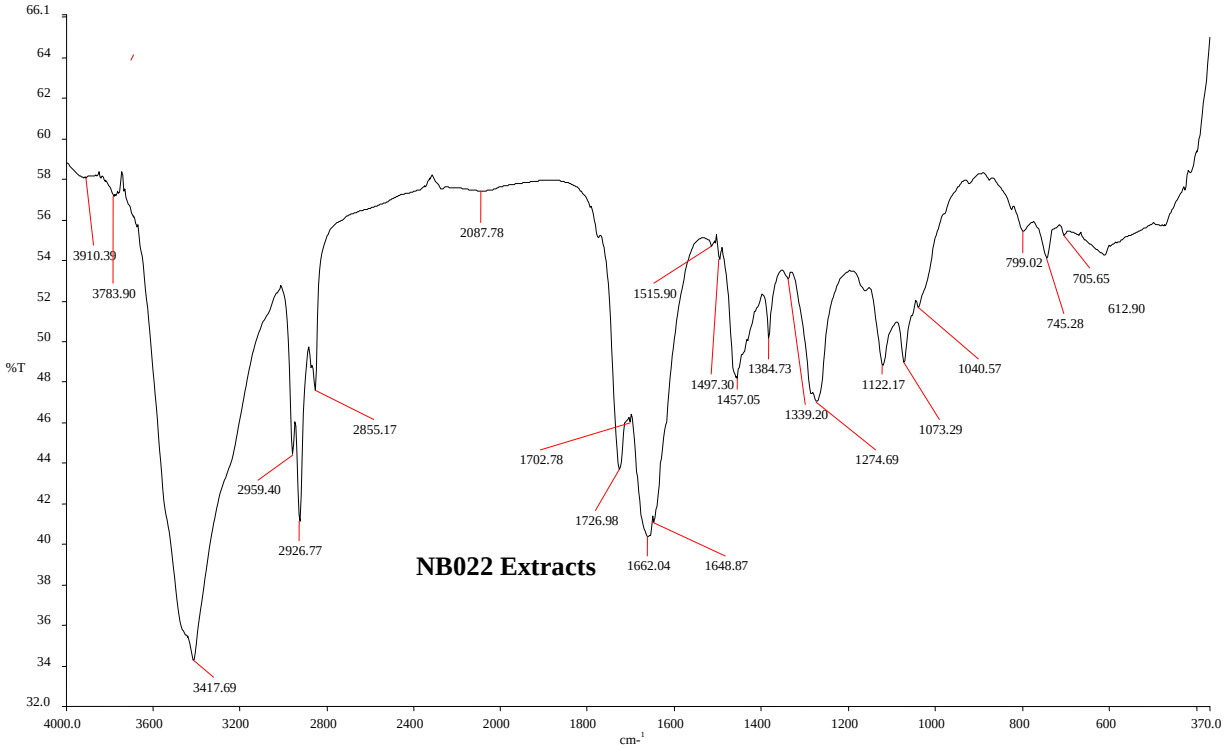
APPENDIX E

IR spectral of crude ethyl acetate extract of isolate NB017



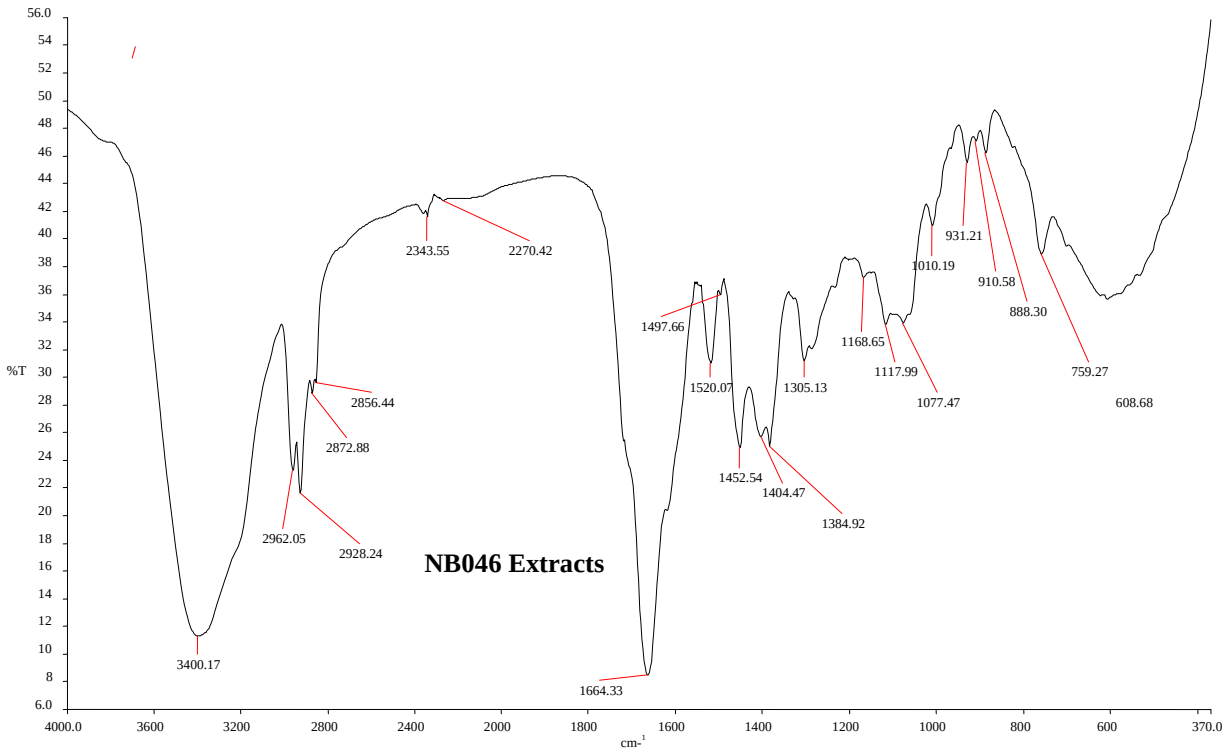
APPENDIX F

IR spectral of crude ethyl acetate extract of isolate NB022



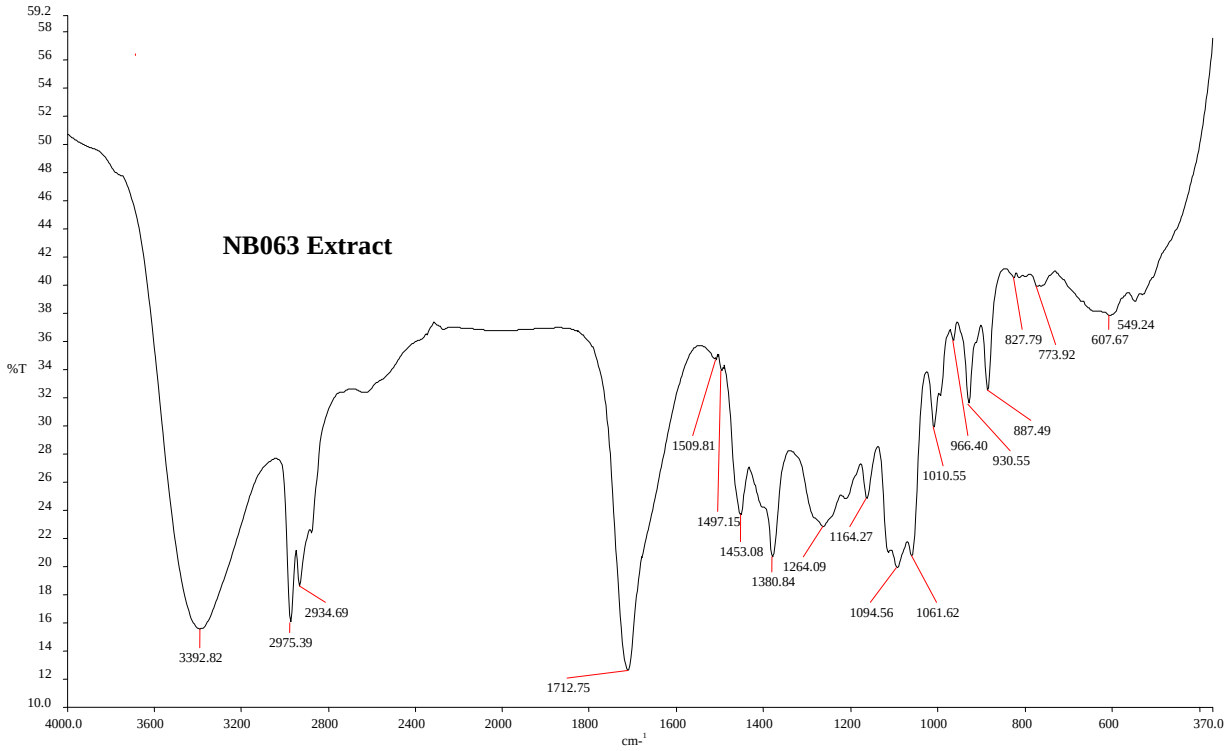
APPENDIX G

IR spectral of crude ethyl acetate extract of isolate NB046



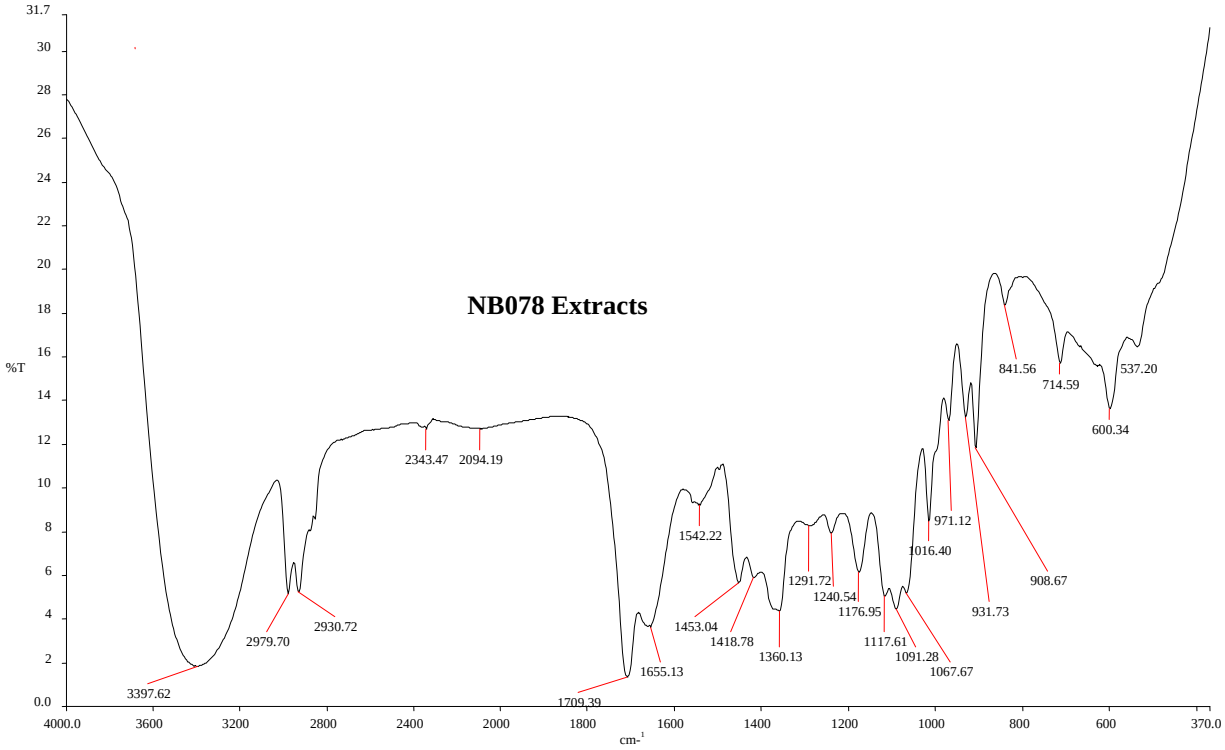
APPENDIX H

IR spectral of crude ethyl acetate extract of isolate NB063



APPENDIX I

IR spectral of crude ethyl acetate extract of isolate NB078



APPENDIX J

IR spectral of crude ethyl acetate extract of isolate NB084