ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF SECONDARY METABOLITES FROM BACTERIA ASSOCIATED WITH MARINE ALGAE OF THE KENYA COAST

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Antimicrobial and cytotoxic activities of secondary metabolites from bacteria associated with marine algae of the Kenya coast

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DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree award.

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DEDICATION

I dedicate this thesis to my husband, Peter Kithinji and daughters, Zaneta and Natania who have filled my days with love, joy and laughter.

Parents, Francis Kaaria and Dorothy Kaaria who have taught me how to be persistent in the pursuit of knowledge. Parents in-law Jasper Magiri and the late Eunice Magiri; sisters, Carol and Linda and brother, Mutwiri. Your continued prayers, support and encouragement have enabled me reach this far to produce this thesis.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
DA	Dihydroaustrasulfone alcohol
DAAD	Deutscher Akademischer Austauschdienst (German Academic Exchange Service)
DMS	Dimethyl Sulfide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EACC	East African Coastal Current
GC-MS	Gas Chromatography-Mass Spectrometry
Hep-2	Human Epithelial Type 2
IC_{50}	Initial Concentration at 50%
ILRI	International Livestock Research Institute
ITCZ	Inter Tropical Convergence Zone
JICA	Japan International Cooperation Agency
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KMFRI	Kenya Marine and Fisheries Research Institute
KEMRI-CMR	Kenya Medical Research Institute- Center for Microbiology Research
MEGA	Molecular Evolutionary Genetics Analysis

MEM	Minimum Essential Medium
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
mm	millimeter
MNP	Marine Natural Products
MRSA	Methicillin Resistant *Staphylococcus aureus*
MTT	3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide
m/z	Mass/charge number of ions
NA	Nutrient Agar
NACOSTI	National Commission for Science Technology and Innovation
NCBI	National Centre for Biotechnology Information
NDA	New Drug Application
NEM	Northeast Monsoon
NIST	National Institute of Standards and Technology
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
pmol	Picomoles
rDNA	ribosomal Deoxyribonucleic Acid
Rpm	Rotations per Minute
SEM	Southeast Monsoon
TAE	Tris-acetate-EDTA
TLC	Thin Layer Chromatography

TSA	Tryptic Soy Agar
µL	Microliter

ABSTRACT

The marine environment is a source of biologically active metabolites with great potential for the development of pharmaceuticals. Due to the rapidly increasing number of pathogenic microbes and tumorous cells that possess resistance towards established therapies causing a threat to public health, lead structures for the development of new drugs are on high demand. This study aimed to isolate bacteria associated with marine algae of the Kenyan coast, in an attempt to evaluate their antimicrobial and cytotoxic activity and identify the compounds responsible for bioactivity. Bacterial endophytes and epiphytes were isolated from 44 seaweed species of red, green and brown algal division, collected at three sites (Mkomani, Mtwapa, and Kibuyuni) along the Kenya coast. The obtained isolates were tested for their antimicrobial activity against eight human pathogenic strains of Gram-positive bacteria (*Staphylococcus aureus* (ATCC 25922) and Methicillin Resistant *Staphylococcus aureus* (MRSA), Gram-negative bacteria (*Escherichia coli* (ATCC 25923) and *Salmonella typhi*), fungi (*Candida albicans* (ATCC 90028), *Cryptococcus neoformans*, *Trichophyton metagrophyte* and *Microsporum gypseum).* Isolates that had a broad spectrum of inhibitory activity were further investigated for anticancer activity against human larynx Hep-2 cells. The active isolates were identified using the 16S ribosomal DNA gene sequence. Culture fermentation and bioassay guided fractionation was carried out on the active isolates. The compounds present in active factions were identified by GC-MS analysis. The study obtained 3493 bacterial isolates with bacterial epiphytes being the most abundant (54%) compared to bacterial endophytes (46%). Initial antimicrobial screening results revealed that 695 isolates (20%) inhibited the growth of at least one test organism, while further screening showed that 69 isolates (10%) had antimicrobial activity against three or more test pathogens. The results also showed that there was significant difference ($p=0.001$) in the mean susceptibility patterns of the Gram-negative and Gram-positive test strains, with Gram-positive (16.64±9.81) being more susceptible compared to Gram negative (12.37±6.94). The study showed there was a significant difference in the inhibitory activities among the three sampling sites, suggesting that the geographical location influences the production and bioactivity of secondary metabolites. A total of 33 isolates (48%) showed cytotoxity against Hep-2 cell line. The Phylogenetic analysis of 16S rDNA gene sequences showed they belong to the phyla Firmicutes (79%), Proteobacteria (12%) and Actinobacteria (9%). The active marine bacteria were assigned to the genera *Bacillus*, *Geobacillus*, *Desulfovibrio*, *Massilia* and *Streptomyces*. In addition, the metabolites produced significant cytotoxic activity against the tumorous Hep-2 cells compared to the normal cells ($p<0.05$). Cytotoxic profiles ranged from low IC_{50} value of $0.24 mg/ml^{-1}$ to a high of 50.01 mg/ ml^{-1}. Identification of the active metabolites showed the presence of several compounds such as phenolics, fatty acids, alkaloids, esters, indoles, alcohols, ketones, alkenes, alkanes, amines, nitriles, furan and azoles derivatives in the bioactive metabolites. These diverse ranges of compounds are known to have antimicrobial and cytotoxic activities.

In conclusion, the study demonstrates that seaweed associated microbes produce antimicrobial and cytotoxic compounds, which represents a promising and potential resource of natural product drugs, possessing a broad-spectrum activity.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Throughout the ages, humans have relied on nature for their basic needs and not least, their medicines. Nature has been instrumental as a source of medicinal agents for a number of years; a substantial number of modern drugs have been obtained from microorganisms (Cragg & Newman, 2013). However, though plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years, there is an increasing realization of the role played by microorganisms in the production of antibiotics and other drugs in the past century (Fenical, 1999).

The main emphasis in recent decades for pharmaceutical discovery from natural products has been on microbial sources, dating back to the discovery of penicillin from the moldy fungus *Penicillium notatum* in the first half of the twentieth century (Singh *et al.,* 2011). Microorganisms, including certain bacteria, fungi and algae, produce secondary metabolites which may have some amount of bioactivity, either against another microorganism or acting against certain physiological states of a diseased body thus making the microbial secondary metabolites an enormous source of pharmaceutical importance (Bhatnagar & Kim, 2010).

In the recent past, a large proportion of discovered marine secondary metabolites have been of microbial origin and not plant origin (Blunt *et al.,* 2009). For example, in 2007 there was a significant increase (38%), compared to the preceding year, in the number of new marine microbial derived compounds (Blunt *et al.,* 2009). In addition to structural variety, bioactives obtained from marine microorganisms are known for their broad range of biological effects, which include antimicrobial, antiprotozoan, antiparasitic, and antitumor activities (James *et al.,* 1996; Matz *et al.,* 2004; Fremlin *et al.,* 2009). Many of

these compounds are noted for their high potency, which could be related to the need to overcome the dilution of allelochemicals in the seawater (Haefner, 2003; Zhang *et al.*, 2005). Many bioactive producing marine microorganisms can be easily cultured and manipulated in bioreactors and therefore, represent the best renewable source of biologically active compounds (Sarkar *et al.*, 2008).

The marine resources have of late been widely studied due to several reasons, the main and most important being that the oceans cover more than 70% of the world surface and among 36 known living phyla, 34 are found in marine environments with more than 0.3 million known species of flora and fauna (Yan, 2004). This implies that the oceans offer unlimited potential for biological and chemical diversity. Secondly, it provides a vast habitat diversity, which means that a large population of useful microbes resides in the ocean. The continued exploitation of the marine environment has turned attention to microorganisms such as marine cyanobacteria, marine fungi and several other groups of marine bacteria, resulting in the production of metabolites with unmatched structures (Bhatnagar & Kim, 2010).

The basis of searching for drugs from marine environment stems from the fact that marine plants and animals have adapted to all sorts of marine environments and are constantly under tremendous selection pressure including competition for space, predation, surface fouling and reproduction, forcing them to produce bioactive compounds of medical and industrial importance (Armstrong *et al.*, 2001). A recent upsurge in scientific inventions in the search for inexpensive and reliable substances of economic value has opened up the possibilities of exploring the bountiful wealth of marine environment. Today, there is mounting interest in marine products for new and promising bioactive substances, as they are expected to provide a point of reference and serve as leading compounds for drug development or pharmacological tools.

The pharmaceutical market is growing rapidly and continuously hence; the demand for new drug discovery is on the rise. The reason behind this motivation could be the

appearance and escalating numbers of drug resistant infectious/diseases, the emerging of new pathogens including viral diseases and more upcoming disorders that are all serious problems claiming millions of lives on earth. The terrestrial resources have been greatly explored and thus academic and industry researchers are striving to get lead molecules from the inner space of oceans (Jirege & Chaudhari, 2010).

A major solution to the global crisis of antibiotic resistance is the discovery of novel antimicrobial compounds for clinical application. Compared to the terrestrial environment, which was the focus of the pharmaceutical industry for more than 50 years, marine habitats have remained virtually unexplored for their ability to yield pharmacological metabolites. In the last several decades, research has expanded from land to ocean in order to find new leads for drug candidates. Marine ecosystems comprise a continuous resource of immeasurable biological activities and vast chemical entities. Given such a background, the chemistry of marine natural products has been progressing at an unprecedented rate, resulting in a multitude of discoveries of carbon skeletons and molecules up and until now unseen on land. This diversity has provided a unique source of chemical compounds with potential bioactivities that could lead to potential new drugs candidates (Abad *et al.,* 2011). Hence, continued development of new antimicrobial compounds is important as an alternative source for the production of new antimicrobial agents. Though Kenya has a coastline, which is 600 km long with about 386 species of seaweeds, there have been limited studies on microbial and biotechnological potential of the Kenyan algal species.

This study aimed to isolate and identify bacteria associated with several algal species from the Kenya coast (Mtwapa, Mkomani and Kibuyuni) that may have the potential of producing compounds that possess antimicrobial and cytotoxic properties targeting pathogens of medical importance. The results presented form the basis of future efforts to embark on the development of antimicrobial drugs that can deal with the upsurging drug resistant pathogens and cancer cases leading to reduced mortality rates.

1.2 Statement of the problem

Infectious diseases caused by bacteria, fungi and viruses are still a major threat to public health despite the tremendous progress in human medicine. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance. Because of the continuous revolutionized therapy of infectious diseases caused by use of antimicrobial drugs, certain limitations occur due to changing patterns of resistance in pathogens and side effects they produce. These limitations demand for continued research for new antimicrobial compounds for development of drugs that are highly effective, possess low toxicity, and have minor environmental impact. Additionally, the increasing demand for biodiversity in the screening programs seeking therapeutic drugs from natural products has drawn a great interest in marine organisms. Besides, compounds have been derived from the marine environment, with a broad range of biological activities such as antibiotics, antivirals, antitumor and anti-inflammatory.

It has also become increasingly evident that the majority of compounds are actually produced by microorganisms associated with collected macroorganisms like algae (Newman & Cragg, 2014; Newman & Giddings, 2014). However, endophytes and epiphytes associated with marine algae have not been fully explored and documented, and are expected to be an important source for new natural bioactive agents. Moreover, bacterial endophytes and epiphytes from marine algae of Kenya have not been studied. Their bioactive compounds as well have not been documented compared with those from solvent extracted compounds of seaweeds. With this consideration, the present study aimed at isolating bacteria associated with seaweeds, assess the secondary metabolites for antimicrobial and cytotoxic properties and identify the compounds responsible, with a view to shedding light on the potential of microbes associated with Kenyan marine algae as a promising source for antimicrobial and anticancer agents.

1.3 Justification of the study

Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases and much of nature remains explored, particularly marine and microbial environments. Microorganisms have the ability to utilize various substrates because of the diversity of their biological and biochemical evolution. Metabolites produced by microbes associated with marine organisms are being recognized as a versatile resource of antimicrobial and anticancer agents. The continued development of new antimicrobial and anticancer compounds are most important in overcoming the difficulties related to the treatment of infections caused by resistant pathogens and adverse effects caused by cancer drugs and chemotherapy procedures respectively. The generation of new knowledge on the microbial diversity in the marine environment is equally important.

The increasing need for new drugs to control new illnesses, emerging and remerging diseases or resistant strains of microorganisms and the need for better cancer therapy has also stimulated research to look for unconventional new sources of bioactive natural products. This search is majorly driven by escalating clinical resistance, the acquired multi-drug resistance (MDR), the emergence of new pathogens, rising cancer cases etc., and all representing serious problems that cost millions of lives on earth.

Antimicrobial and anticancer drugs against human pathogens and cancerous cells are a continuing worldwide issue, newer and effective formulations for their treatment and control remain an important challenge. Additionally, the ingress to the human population of new diseases such as Acquired Immune Deficiency Syndrome (AIDS) and severe acute respiratory syndrome requires the discovery and development of new drugs to combat them. The immune-compromised (for example cancer and organ transplant patients) are at risk for opportunistic pathogens, such as *Aspergillus* sp., *Cryptococcus* sp. and *Candida* sp. (Fauci, 2001).

This study aimed to contribute significantly to the Kenyan pharmacological industry and Ministry of Health, by obtaining microbial bioactive compounds from the marine environment and providing chemical guidelines on the active group of compounds, with a view to setting the pace in getting drug compounds responsible to curb the menace of multidrug resistant pathogens and cancer, and obtain compounds possessing better efficacy and varying degrees of action.

1.4 Hypotheses

The Kenyan marine algae do not harbor bacterial microbes that produce secondary metabolites with antimicrobial and cytotoxic properties.

1.5 Objectives

1.5.1 General objective

To evaluate the antimicrobial and cytotoxic activities of secondary metabolites from bacteria associated with marine algae of the Kenya coast.

1.5.2 Specific objectives

a) To isolate endophytic and epiphytic bacteria from marine algae of the Kenya coast.
b) To screen bacterial colonies for antimicrobial and cytotoxic activities against eight multidrug resistant microorganisms and larynx Hep-2 carcinoma cells.
c) To identify the active endophytic and epiphytic bacterial colonies.
d) To extract and perform a bioassay guided fractionation of the secondary metabolites.
e) To screen fractionated secondary metabolites for antimicrobial and cytotoxic activities against eight multidrug resistant microorganisms and larynx Hep-2 carcinoma cells.
f) To identify fractionated compounds of secondary metabolites responsible for antimicrobial and cytotoxic activities.

CHAPTER TWO

LITERATURE REVIEW

2.1 Natural products

The use of natural products as medicines has been described throughout history in the form of traditional medicines, remedies, portions and oils with many of these bioactive natural products still being unidentified. Since the discovery of the first antibiotic in 1929 (Aminov *et al.,* 2010), which was the starting point for drug discovery from microbial sources, there has been an ever-increasing demand for substances to cope with medicinal problems caused by microbial pathogens. Research into the novel metabolites of natural products, and especially microorganisms was began to meet this demand, and continues to be a fascinating area of investigation. The dominant source of knowledge of natural product uses from medicinal plants is a result of man experimenting by trial and error for hundreds of centuries. Since, the story of antibiotics started more than 100 years ago, their usual definition in the widest sense is chemical compounds isolated or derived from nature, i.e., living organisms such as plants, animals and microorganisms. These compounds may be derived from secondary metabolism of these organisms (Bérdy, 2005). Chemistry of natural products involves the isolation, biosynthesis and structure elucidation of new products that lead to new medical and crop protection agents. Due to their chemical diversity and various activities against diseases, these natural products have been playing an important role in pharmaceutical and agricultural research (Biologie, 2006).

Natural products have led to excellent drugs for therapeutic purposes. In the period of 1989-1995, over 60% of the approved drugs and pre-NDA (New Drug Application) candidates were of natural origin (Cragg *et al.,*1997). Drugs of natural origin have been classified as original natural products, semi-synthetically from natural products or synthetic products based on natural product models. Thereafter natural products played an invaluable role in the drug discovery, particularly in the areas of cancer and infectious

diseases. Of the 211 pre- NDA anticancer drug candidates (in preclinical or clinical development for the period above), 61% were the original natural products and about 4% of the candidates were marine-derived (Cragg *et al.*,1997).

Additionally, since the early "golden age" (1930s through the 1950s) of microbial natural product screening, tens of millions of soil microorganisms have been isolated and screened (Baltz, 2005) and this effort provided the vast majority of microbial metabolites known today (Bérdy, 2012; Monciardini *et al.,* 2014). Similarly, numerous novel metabolites were explored from new natural sources (marine environment) together with semi-synthetic antibiotics from the pharmaceutical industry, which led to an exponential increase of new antibiotics (Siriwach, 2013). However, from the end of the golden age to the present time, the number of newly discovered compounds significantly decreased due to the limited ability to culture majority of the marine microbes and the lack of a systematic approach, which often resulted in the frequent re-discovery of known compounds. On the other hand, the number of resistant microbes has expanded, causing an imbalance in the ratio of new compounds to resistant pathogens. Based on this depressed status of compound discovery, focus is now shifting to ways of improving the isolation methodology, exploring new natural resources and tapping the chemical diversity of nature, for effective drug discovery.

About 10 million synthetic chemical compounds have been reported, mainly from the field of synthetic chemistry while the number of compounds from natural sources is only about one-tenth (Bérdy, 2012). The compounds from nature, especially those from microorganisms; tend to be much more bioactive than synthetic compounds. In addition, the natural products can be obtained with less stress to the environment, and are more structurally diverse. Hence, natural products are expected to serve as a powerful and promising resource of novel compounds (Bérdy, 2012). Consequently, marine organisms represent a promising source for natural products of the future due to the incredible diversity of chemical compounds that have been isolated.

2.2 Marine natural products

Though plants have proven to be a novel source for bioactive natural products, the marine environment has a clear record of accomplishment in offering novel structural entities. Given that, the oceans are massively complex and consist of diverse assemblages of life (Hagström *et al.,* 2002), the marine environment is an exceptional and unique reservoir of bioactive natural products, many of which demonstrate structural and/or chemical features not found in terrestrial natural products (Hussain *et al.,* 2012). Marine organisms have evolved biochemical and physiological mechanisms that include the production of bioactive compounds for such purposes as reproduction, communication, and protection against predation, infection and competition (Kijjoa & Sawangwong, 2004). Because of the physical and chemical conditions in the marine environment, almost every class of marine organism exhibits a variety of molecules with unique structural features.

The marine environment represents a largely unexplored source for isolation of new microbes (bacteria, fungi, actinomycetes, cyanobacteria and algae) that are potent producers of bioactive secondary metabolites. Extensive research has been done to unveil the bioactive potential of marine microbes (free living and symbiotic) and the results are amazingly diverse and productive. Some of these bioactive secondary metabolites of microbial origin with strong antibacterial and antifungal activities are being intensely used as antibiotics and may be effective against infectious diseases (Bhatnagar & Kim, 2010).

Since the marine environment harbors a rich source of microbial diversity, it has demonstrated tremendous abilities as producers of anticancer compounds, secondary metabolites that act against infectious diseases and inflammation. Blunt *et al*. (2007) listed that in marine environment, sponges (37%), coelenterates (21%) and microorganisms (18%), are major sources of biomedical compounds, followed by algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%) and bryozoans (1%). Apart from synthetic products, pharmaceutical industries in most of the developed and developing

countries are now concentrating on natural products derived from marine microorganisms, since they are prolific producers of bioactive substances and may serve as a means for novel drug discovery systems.

Exploration of the marine environment and organisms (algae, sponges, ascidians, tunicates and bryozoans), has resulted in the isolation of thousands of structurally unique bioactive marine natural products. Some examples include; Plitidepsin, a depsipeptide that was isolated from the Mediterranean tunicate *Aplidium albicans* (Urdiales *et al.,* 1996), is effective in treating various cancers, including melanoma, small cell and non-small cell lung, bladder as well as non-Hodgkin lymphoma and acute lymphoblastic leukemia (Mayer *et al.,* 2010); the brown alga, *Dictyota dichotoma* afforded diterpenes, 4-acetoxydictylolactone, dictyolides and nordictyolide which display antitumor activities (Faulkner, 1987; Ishitsuka *et al.,* 1988) and crenuladial, isolated from the brown alga *Dilophus ligatus* which displayed antimicrobial activity against *Staphylcoccus aureus*, *Micrococcus luteus* and *Aeromonas hydrophyla*.

As evidenced from past and ongoing research, microbial consortium has an excellent plethora of bioactivity. However, there is still a long way to go, although a diversified range of antibiotic, antifungal, cytotoxic, antiviral, antineoplastic and antiprotozoal activity are known. Research efforts are still needed to expand the marine microbes derived drug discovery to come up with new therapies to combat multi drug resistance and a serious threat of re-emerging infectious diseases, which is a growing concern in the medical fraternity.

2.3 Biosynthesis of marine secondary metabolites

Secondary metabolites are defined as small organic molecules that are derived from biosynthetic pathways, and not required for maintenance and growth of the respective organism (Jimenez-Garcia *et al.,* 2013). Besides their role in environmental adaptation, they often contribute to biological defense strategies and do not affect the immediate survival of the producing organism. They are also not essential for growth, respiration,

storage and reproduction but rather influence long-term survival by affecting the organism's interactions with its surrounding environment, hence termed secondary metabolites (Hanssen, 2014).

Marine organisms possess an inexhaustible source of useful chemical substances for the development of new drugs. Among these organisms, are marine algae capable of biosynthesizing a broad variety of secondary metabolites. On the other hand, bacteria and fungi that live in the oceans are also crucial organisms used in biotechnology in the discovery of new compounds from marine origin (Blunt *et al.,* 2011). Biosynthesis of secondary metabolites is initiated by a variety of environmental triggers. Many of these external factors are constantly changing, so is the expression of secondary metabolites under specific seasons, stress, nutrient availability, and developmental stages of the organism (Ramachandra & Ravishankar, 2002). These triggering factors commonly referred to as elicitors, can be of abiotic in nature e.g. high or low temperatures, pressure, light availability, drought, salinity and UV-stress (Ramakrishna & Ravishankar, 2011) or biotic (fungi, yeast, bacteria, predation) in origin (Johanningmeier & Fischer, 2010). The extreme conditions such as high salinity, low temperature, lightless and high pressure are the inducer of the prolific bioactive compounds of marine microorganisms (Debbab *et al.,* 2010). The microbes capacity to produce unique and unusual secondary metabolites are possibly because of adaptation to a very distinct set of environmental pressure (Jensen, 2002). It is also believed that the metabolites act as a chemical defense in competing for substrates (Gallo *et al.,* 2004).

It was previously perceived that secondary metabolites were biologically insignificant and served no particular function (Williams *et al.,* 1989). This perception is no longer valid, and secondary metabolites are now recognized as an important contributor for an organism's interaction with and response to its surroundings. These secondary metabolites produced or obtained from microorganisms, marine flora and fauna are very potent and biologically active. The potency of bioactive substances from marine life is mainly due to the intensive ecological pressure and from predators. Investigations in their

chemical ecology have also revealed that secondary metabolites not only play various roles in the metabolism of the producer but also in their survival strategies in the given environment. The study on marine chemical compounds produced by different organisms; shows the pharmaceutical potential applicable for human benefit (Munro *et al.,* 1999; Müller *et al.,* 2003). In order to fully understand the link between marine chemical warfare and human health, it is crucial to study chemical ecology in the oceans.

Chemical ecology of marine organisms relates very closely to biotechnology by exploring these secondary metabolites to develop drugs to treat various life threatening diseases. Since natural products released into the water are rapidly diluted, they need to be highly potent to have any effect. Therefore, since immense biological diversity exists in the ocean with biological activities, this could be useful in the quest of finding drugs with greater efficacy and specificity for the treatment of many human diseases (Mayer *et al.,* 2000; Proksch *et al.,* 2002).

2.4 Marine microbial diversity

Whereas in the last decades the focus in marine natural product research was mainly on macro organisms such as sponges, seaweeds, and others; nowadays, it is evident that microorganisms from the marine habitat are equally rich sources of novel constituents. These microorganisms are often the true metabolic producers of bioactive metabolites whose production was originally attributed to macro organisms (Haefner, 2003; Simmons *et al.,* 2008; Gerwick & Moore, 2012; Gerwick & Fenner, 2013).

Additionally, microorganisms are far more accessible through sustainable production by fermentation than many marine macro organisms that usually have to be collected from limited wild stocks (Proksch *et al.,* 2010). These two main factors reveal that microbes are truly the treasure troves of new marine pharmaceuticals. Consequently, as microorganisms occupy almost every niche on Earth, scientists speculate that each drop of water taken from the ocean will contain microbial species unknown to humans in a ratio 9:1 (Colwell, 2002).

To maximize the chemical diversity available from marine microbes, other sources like the deep- ocean and geothermal vents are becoming the focus of considerable interest from natural product research chemists (Bhatnagar & Kim, 2010; Pettit, 2011). In fact, diverse bioactive secondary metabolites have been reported from cultured extreme-tolerant microorganisms, extremophiles, and deep-sea microbes. Due to the extreme physical and chemical conditions at deep-sea hydrothermal vents, site conditions are constantly fluctuating, representing a nearly inexhaustible source of genomic innovation (Pettit, 2011). Although numerous natural products have been identified from marine microorganisms during the last decade, it is likely that a plethora of compounds still await discovery. This assumption results not only from the fact that only a small number of microorganisms have been cultivated and discovered yet, but also from recent genome sequencing projects (Brakhage & Schroeckh, 2011). Additionally, most of the earth's microbial diversity is found in the ocean. This ultimately translates to an enormous number of bioactive substances (Figure 2.1).

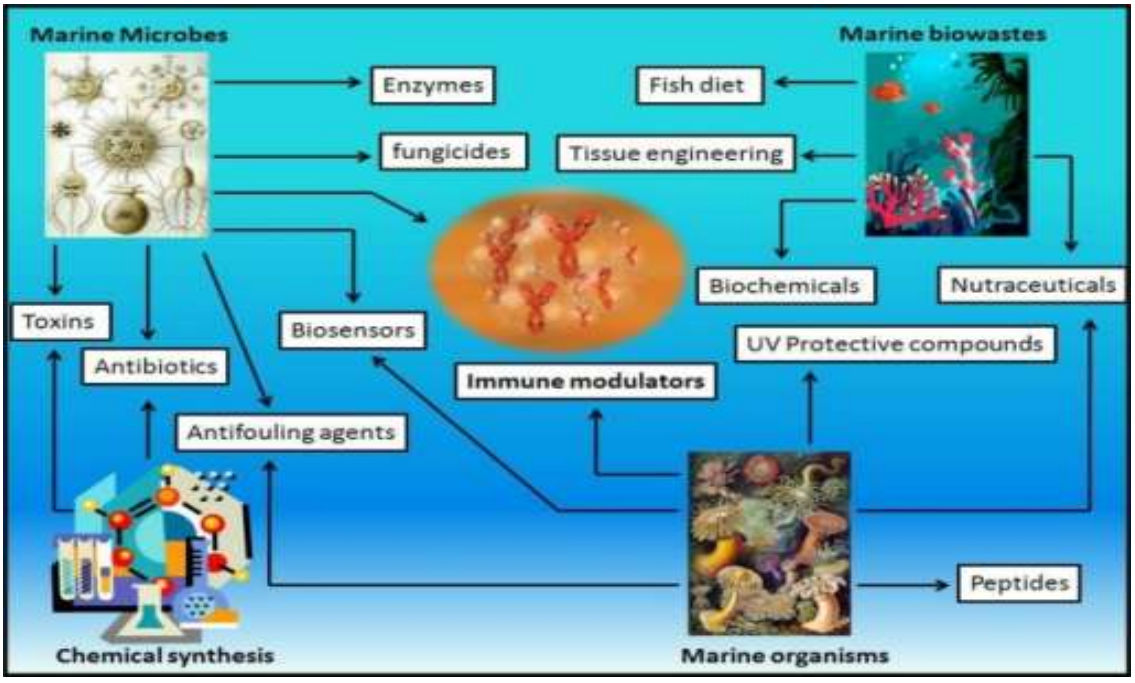

Figure 2.1: Diversity of bioactive substances produced in the marine environment (Bhatnagar & Kim, 2010).

Since the marine ambience is a complex ecosystem with an enormous plurality of forms of life, the most common associations found are between eukaryotic cells and microorganisms (Egan *et al.,* 2008). The surface of all the marine eukaryotic organism are covered by microbes that live adherent to diverse communities often immersed in a matrix or forming a bilayer (Pérez-Matos *et al.,* 2007). Marine macroalgae live in association with microbes (both inside and outside) and these microbes produce metabolites bioactive compounds of pharmaceutical significance.

2.4.1 Macroalgae

Marine macroalgae also referred to as seaweeds, grow almost exclusively in the shallow waters at the edge of the world's oceans. Macroalgae are classified into three classes: green algae (Chlorophyta), brown algae (Phaeophyta) and red algae (Rhodophyta). Marine macroalgae have been used as foods, especially in China and Japan, and as crude drugs for treatment of many diseases (Abad *et al.,* 2011).

In recent years, the bioactive properties of marine algae and marine microorganisms have been analyzed, and in both cases, positive results have been obtained. Many of the marine algae species often come accompanied by several bacterial and fungal strains which have been taken off the sea together with the algal cells, or have been the result of a contamination in the algal culture (Soria-mercado *et al.,* 2012). These mixed populations that are present in the culture and in the sea, show that the microorganism use organic substances secreted by living or dead algal cells. It has been observed that many types of seaweeds present a vigorous growth in the presence of bacteria and/or fungi than in their absence (Ramanan *et al.,* 2016). Some seaweed species need vitamins for their growth and possibly the microbes are partially responsible for the production of these substances; some of them produce antibiotics (Jasti *et al.,* 2005; Penesyan *et al.,* 2010).

Since seaweeds harbor a diversity of microbes (endophytes and epiphytes), they have been significantly evaluated for functional and chemical analyses, which have concluded that seaweed associated microbial communities, are highly diverse and rich sources of

bioactive compounds of exceptional molecular structure. However, there is need to increase extraction of bioactive compounds from these microbial communities, with a view of their future development as strong tools to discover novel drug targets. Additionally, though most studies have been carried out to understand bioactives produced mostly by bacterial and fungal sources associated with seaweeds as compared to other microorganism, this area still remains mostly unexplored and especially in Africa and more specifically Kenya.

2.4.2 Endophytes

Endophytes are microorganisms that reside in the internal tissues of living plants without causing any immediate overt negative effects (Strobel, 2006). Endophytes have been found in every plant species examined and are recognized as potential sources of novel natural products. These products have been exploited in medicine, agriculture, and industry with more and more bioactive natural products being isolated from the microorganisms (Bacon & White, 2000).

Endophytes being ubiquitous have a rich biodiversity, which have been found in every plant species examined to date. It is noteworthy that, for the nearly 300,000 plant species that exist on the earth, each individual plant is the host to one or more endophytes (Strobel & Daisy, 2003). In view of special colonization in certain hosts, it is estimated that there may be as many as one million different endophyte species. However, only a handful of them have been described (Petrini, 1991). This means, the opportunity to find new natural products from interesting endophytic microorganisms among myriads of plants in different niches and ecosystems is great.

Endophytes are the chemical synthesizers inside plants (Owen & Hundley, 2005). Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds have proven useful for novel drug discovery. However, few studies have investigated the endophytes of marine algae (Jones *et al.,* 2008). As a result, the overall body of research covering marine endophytes

from algae is small and requires more research, that may reveal new microbial species and new plant/host relationship. Marine endophytic fungus *Penicillium chrysogenum* isolated from the red algae genus Laurencia in China, was found to produce five secondary metabolites; penicitides A and B, 2-(2,4-dihydroxy-6-methylbenzoyl)-glycerol, 1-(2,4-dihydroxy-6-methylbenzoyl)-glycerol and penicimonoterpene. Both penicitides displayed potent activity against the pathogen *Alternaria brassicae*, and exhibited moderate cytotoxic activity against the human hepatocellular liver carcinoma cell line Gao *et al.* (2011). This means that algal associated endophytes are a potential source of drugs and drug leads.

2.4.3 Epiphytes

Surfaces of most plants are also characterized by an associated epiphytic microflora living in the phyllosphere (Hempel *et al.,* 2008). In aquatic ecosystems, bacteria occur often associated with surfaces, for example in biofilms or on lake (Costerton *et al.,* 1995). Biofilm associated bacteria are most abundant at intermediate nutrient availability while either low or high nutrient conditions favor planktonic growth of bacteria. Biofilms are not only formed on abiotic surfaces but also on living organisms such as aquatic plants and algae. Epiphytic microorganisms live on the surface tissues of their hosts. Studies indicate that complex interactions exist between the host and their epiphytic microorganisms, where the host provides organic nutrition and epiphytes act as chemical guards (Simon *et al.,* 2002). Therefore, compared with free-living marine microorganisms, the epiphytic marine microorganisms have drawn more interest from natural product chemists in the search for novel antimicrobial or other active compounds.

A study conducted by Ismail *et al.* (2016) in Tunisia, on the antimicrobial activities of bacteria associated with the brown algae, *Padina pavonica,* 18 epiphytic bacteria were isolated that produced inhibitory activities against pathogenic bacteria and fungi. They emphasized on potential use of *P. pavonica* associated antagonistic bacteria as producers of novel antibacterial compounds. Another study by Karthick *et al.* (2015), isolated a bacterial epiphyte *Serratia* sp. (KC 149511) from the coralline red algae, *Amphiroa anceps*. The crude extracts of the bacterium possessed antibacterial and antifungal

properties. Fractioned extract of the bacterial isolate was analyzed and presence of certain metabolites such as octadecanoic acid, phenol, 2, 4-bis (1, 1- dimethyl ethyl) and nonanoic acid- 9 oxo methyl ester were identified, which could be responsible for the antifungal and antibacterial activities. Therefore, seaweed associated epibionts are potential candidates for production of antimicrobial compounds which could be useful in drug discovery.

2.5 Bioactive natural products from algicolous microbes

Marine microbes having immense genetic and biochemical diversity look likely to become a rich source of novel effective drugs. The search for new bioactive chemicals from marine organisms has resulted in the isolation of about 10,000 metabolites (Kelecom, 2002), many of which are potential bio medicals. These agents show a broad spectrum of biological activities.

In the past decades, marine life forms have been the origin of a remarkable number of novel secondary metabolites. In fact, since the 1960s, more than 20,000 marine secondary metabolites have been characterized (Hu *et al.,* 2011). They have been isolated from macroorganisms like sponges, corals and other invertebrates, as well as from algae and microorganisms (Hu *et al.,* 2011).

The number of isolated compounds from marine sources has increased steadily, from an annual number of approximately 20 in 1984 (the total number of all novel natural products reported in 1985 was 3500 by Ramachandra and Ravishankar (2002), to an annual number of more than 1,000 in 2010 (Blunt *et al.,* 2011). As opposed to the terrestrial environment, where plants are considerably richer in secondary metabolites, marine invertebrates and bacteria have yielded substantially more bioactive natural products than marine plants.

Marine microorganisms are often taxonomically unique, which makes them interesting as potential sources of new drug leads. Marine bacteria are of considerable importance as

new sources of a huge number of biologically active compounds. For example, the non-pigmented strain of *Pseudoalteromonas issachenkonii* sp., isolated from the thallus of brown alga *Fucus evanescens* collected in the Kurile Islands of the Pacific Ocean, had bacteriolytic, proteolytic and haemolytic activities and degraded algal polysaccharides, synthesizing a number of glycoside hydrolases (fucoidanases, laminaranases, alginases, agarases, pullulanases, b-glucosidases, b-galactosidases, *b*-Nacetylglucosaminidases and *b*-xylosidases) (Hempel *et al.,* 2008). An antibacterial compound, magnesidin has been isolated from *Pseudomonas magnesiorubra* associated with green alga, *Caulerpa peltata*. This compound displayed strong activity against *Staphylococcus* and *Bacillus* strains (Isnansetyo & Kamei, 2003a). Ravisankar *et al.* (2013) identified an alkaloid from *Pseudomonas* sp. associated with brown alga *Padina tetrastromatica*. This compound inhibited growth of human pathogenic bacteria, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with 15 and 10 mm inhibitory zones, respectively at a concentration of 300 µg.

A novel macrolactin S has been reported (from a marine *Bacillus* sp.) and macrolactin W (from a marine *Bacillus* sp. 09ID194), that exhibited broad-spectrum antibacterial activity against Gram-positive and Gram-negative pathogenic bacteria (Lu *et al.,* 2008; Mondol *et al.,* 2011). Additionally, a novel polyketide family member 7-O-methyl-5′-hydroxy-3′-heptenoate-macrolactin has also been obtained from seaweed, *Anthophycus longifolius* associated with *Bacillus subtilis* MTCC 10403 strain (Chakraborty *et al.,* 2014). This particular compound showed 12–22 mm inhibitory zone against different species of *Vibrio* sp. Of interest is a bacteriocin (lichenicidin, a class of lentibiotics) that was also confirmed from seaweeds-associated *Bacillus licheniformis*. This suggests that seaweed-associated *Bacillus* sp. could be source for novel bacteriocin (Prieto *et al.,* 2012). Subsequently, an important antibacterial protein (30.7 kDa) has been obtained from *B. licheniformis* associated with brown seaweed, *Fucus serratus*. This novel protein showed activity against Methicillin Resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, and *Listeria monocytogenes* (Jamal *et al.,* 2006).

Recently, Tebben *et al.* (2014) identified 13 natural products from *Pseudoalteromonas strain* J010, which were isolated from surface of the crustose coralline alga, *Neogoniolithon fosliei*. Among the natural products, a newbromopyrrole, 4-((3,4,5-tribromo- 1H-pyrrol-2-yl) methyl) phenol and five new korormicins G–K were obtained and exhibited antibacterial activity. Interestingly, this strain also produced a coral larval metamorphosis inducer compound, tetrabromopyrrole which had a broad-spectrum activity against the tested bacteria, fungi, and protozoan (Tebben *et al.,* 2011; Tebben *et al.*, 2014). Two novel compounds (Violacein and YP1) were obtained from *Ulva australis* associated with *Pseudoalteromonas tunicata* (Franks *et al.,* 2006; Matz *et al.,* 2008). Violacein (an alkaloid) produced by *P. tunicata* and *P. ulvae* showed antiprotozoal activity against amoeba *Acanthamoeba castellanii* at nanomolar concentration (Matz *et al.,* 2008). It also demonstrated that violacein induces apoptosis-like cell death program in protozoan predators. It is also observed that violacein also induces apoptosis in mammalian cell lines (Kodach *et al.,* 2006) and therefore, could be a novel therapeutic agent to treat cancerous cells (Matz *et al.,* 2008).

Consequently, natural products derived from marine algae and its microbes are important ingredients in many products such as cosmetics and drugs for treating cancer. For instance, Villarreal-Gómez *et al.* (2010), isolated *Pseudoalteromonas* sp., *Bacillus* sp. and *Microbulbifer thermotolerans* from the seaweeds, *Centroceras clavulatum*, *Sargassum muticum* and *Endarachne binghamiae*, respectively, that produced unidentified anticancer compounds against HCT-116 colon cancer cells. Also, Cui *et al.* (2010) isolated *Aspergillus ochraceus* from the brown alga, *Sargassum kjellmanianum*, which yielded three previously unknown metabolites.

Two of these metabolites (7-Nor-ergosterolide & 3β,11α- dihydroxyergosta-8,24 (28)-dien-7-one) were found to possess activity against human tumor cell lines obtained from lung, liver, and pancreas with IC_{50} (inhibitory concentration) values of 5.0, 7.0 and 28.0 µg/ ml, respectively. In another study, Hawas *et al.* (2012) isolated *Aspergillus versicolor* from the inner tissue of green algae, *Halimeda opuntia* of the Red Sea. The ethyl acetate

extract had antibacterial, anticancer and antiviral activities. A new metabolite named isorhodoptilometrin-1-methyl ether along with the known compounds emodin, 1-methyl emodin, evariquinone, 7-hydroxyemodin 6, 8-methyl ether, siderin, arugosin C and variculanol were detected in the extract.

Such increasing evidence proves that the marine environment and organisms are potential sources of bioactive compounds, and the exploration of seaweed- associated microbes promises to deliver novel bioactives with potential pharmaceutical and therapeutical applications.

2.6 Need to explore the ocean as a source of bioactive compounds

With more than 70% of the earth's surface, the oceans represent the largest habitat of the earth and a prolific resource of organisms with high biological and chemical diversity. The marine environment is a different environment that results in a different transcriptome, proteosome and metabolome that allows an organism to survive. Therefore, it can be speculated that different secondary metabolites might be the result of special requirements to adapt to such an extreme environment (Firn & Jones, 2000). So, almost all forms of life in the marine environment have not been fully investigated for their natural products content, hence, represent a valuable source of novel compounds with great potential as pharmaceuticals, nutritional supplements, cosmetics, agrochemicals and enzymes, many of them with strong potential market value (Kijjoa & Sawangwong, 2004; Blunt *et al.*, 2013).

Bioactive marine natural products are biologically active products, including primary and secondary metabolites, from marine sources. Despite the increasing interest in marine primary metabolites such as lipids, enzymes and complex heteropolysaccharides, the focus and potential rely on the secondary metabolites. Over 22,000 structurally diverse marine metabolites have been isolated and characterized over the last fifty years (Blunt *et al.*, 2013; Hu *et al.*, 2011). Main areas of research on marine natural products are devoted to the discovery of new anticancer and antimicrobial drugs, somehow related to the

severe mortality and morbidity related to cancer, as well as the increasing drug resistant bacterial pathogens (Blunt *et al.,* 2013; Mayer *et al.,* 2013). The study of marine natural products so far has not only allowed the development of new drug leads, but also the identification of new molecular targets for therapeutic intervention (Amador *et al.,* 2003).

Moreover, the emerging resistance to antibiotics has raised serious concerns regarding the next source of new chemical entities that can meet the challenge of continually emerging drug resistance. Additionally, the number of reported chemotherapeutic drugs being used for cancer treatments clinically exhibit severe adverse side effects on the human body including bleeding, hair loss, diarrhea and immunosuppression (Kranz & Dobbelstein, 2012). Hence there is need to explore and unearth the oceans rich microbial diversity, for novel active metabolites to counteract and reverse the spread of antibiotic resistance pathogens and potent antitumor metabolites without any toxicity on normal cells. This remains an important challenge that could lead to big leap in the scientific community.

2.7 Kenyan marine algae as sources of natural products

Kenya has a coastline with a proliferation of habitats for different seaweed communities. A relatively large number of seaweed species have been documented and approximately 386 species have been identified (Bolton *et al.,* 2007). These seaweeds have also been well collected by indigenous phycologists. However, most studies in Kenya have focused on the ecology and taxonomy of the seaweeds, with diverse studies on the economic potential of harvesting seaweeds (Yarish & Wamukoya, 1990) and establishing seaweed farms (Wakibia *et al.,* 2006).

2.7.1 Marine algal resources

Seaweeds are known sources of thickening and gelling agents called phycocolloids. The most important phycocolloids are agar, carrageen and alginate. Kenya is a net importer of agar and alginate, although it may have the potential to be self-sufficient or even an exporter of these phycocolloids. The bulk of most low-grade agar import is comprised of

Gracilaria species, which are common and widely distributed throughout the Kenya coast. Significant populations of *Gelidium*, *Gelidiella*, *Gelidiopsis* and *Pterocladia* occur in Kenya and produce high quality bacteriological grade agar (Yarish & Wamukoya, 1990). The sources of carrageenan in Kenya are the genera Eucheuma, Kappaphycus, Halymenia and Hypnea, all of which produce high yields with a strong gel.

Seaweed farming is practiced in Kenya, where it commenced in 2004 with pilot experiments in south coast where growth rates of between 3.5 and 5.6% per day were recorded (Wakibia *et al.,* 2006). Commercial cultivation started in the year 2010, and seven coastal villages are practicing seaweed farming in Kenya using the off-bottom technique. Currently, substantial commercial production of seaweed is only practiced among three villages (Msuya *et al.*, 2014). The commonly cultivated seaweed species, *Eucheuma denticulatum* and *Kappaphycus alvarezii* along the Kenya coast are good carrageenan producers. However, the Kenya Coast Development Project funded by the World Bank is working on increasing seaweed production and spreading seaweed farming to the north coast in order to boost seaweed production in Kenya.

Phycocolloids are also applicable in the food and pharmaceutical industries. Asian countries such as Japan, China, Korea, Indonesia and Taiwan, commonly consume seaweeds as a source of human food (Dawes, 1998). Seaweeds are known to produce valuable sources of macronutrients such as proteins, fibre, carbohydrates and lipids and micronutrients such as minerals and vitamins as well as important bioactive compounds (Ortiz *et al.,* 2006). Thus, they have been recognized as being beneficial for human and animal health (Fleurence, 1999).

Seaweeds are relatively unimportant in the Kenyan diet since coastal people rarely consume them, as their beneficial value is not well documented. However, studies have shown the nutritional values of the Kenyan seaweeds (Mwalugha *et al.,* 2015; Muraguri *et al.,* 2016). Mwalugha *et al.* (2015) described the chemical composition of seaweeds in Kenya. They found them to be highly nutritious with various compounds present that

included, nitrogen free extracts (major component), crude ash, crude fibre, crude protein and crude fat (least component). This nutritional value of the Kenyan seaweeds means that, they are potential resource for seaweed based products for improved human and animal nutrition. Consequently, Muraguri *et al.* (2016) described the chemical and functional properties of five Kenyan seaweed species namely, *Hypnea musciformis, Eucheuma denticulatum, Laurencia intermedia, Sargassum oligocystum,* and *Ulva fasciata*, as potential fat replacer in chicken sausage processing. They found that nitrogen free extracts were the highest component while crude fat was the least. The highest mineral detected was calcium, while fatty acids were also detected with the saturated being the highest followed by the monounsaturated with the least being polyunsaturated. These findings demonstrated the potential of seaweeds in improving the chemical and functional characteristics of processed foods.

2.7.2 Antimicrobial and cytotoxic studies

The detection of antimicrobial activities is considered an indicator of the capacity of the seaweeds to synthesize bioactive secondary metabolites. Currently, there are no studies on the antimicrobial and cytotoxic potential of the microbes associated with the Kenyan seaweeds. However, in the continued interest in prospecting for novel pharmaceuticals from Kenyan marine resources, Dzeha *et al.* (2003), investigated the green algae, *Halimeda macrolaba* and its antimicrobial activity against the fungus, *Aspergillus niger* and the Gram-negative bacteria, *Escherichia coli*. The activity was attributed to a clionasterol, a triterpenoid, detected in the less polar extract (20% EtOAc in hexane) of the green algae.

In Eastern Africa, studies are also limited on the antimicrobial activities of seaweed associated microbes as well as antimicrobial activities of extracts from seaweeds. Mtolera and Semesi (1996) examined the antimicrobial activity of the extracts of six marine green algae from Tanzania against three bacterial species namely; *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS), *Escherichia coli* (EC), and a yeast, *Candida albicans*. A cytotoxic

study was also carried out against the *Artemia salina* larvae. Antimicrobial and cytotoxic activities of the seaweed extracts were observed. The study concluded that Tanzanian seaweeds are a useful source of antimicrobial substances. Since not much has been done and documented on the pharmaceutical potential of seaweed extracts and microbes associated with seaweeds in Eastern Africa, there is need for more research with special emphasis placed on Kenya.

2.8 Isolation and identification of bacteria from marine algae

Marine algae/seaweed surfaces offer a nutrient rich environment uniquely suited for microbial colonization (Egan *et al.,* 2008). Researchers are increasing the use of culture dependent and independent techniques, microbial metagenomics approach for characterization of microbes, to determine marine microbial diversity. Moreover, conventional culture- dependent methodologies have provided useful information for evaluating microbial diversity in various environments. Therefore, isolation of bacteria from seaweed surfaces can significantly increase the chances of isolating active metabolites of bacterial origin.

The standard procedure for the isolation of bioactive products of microbial origin includes several essential steps. The procedure starts with isolation of microorganisms from the sample such as seaweeds. After growing the microorganisms in the laboratory on nutritional media, the screening of individual isolates for biological activity is performed, followed by phylogenetic and phenotypic identification of the bioactive producing organism. In the search for endosymbiotic bacteria, elimination of ectosymbionts is a key point of attention. Commonly, the surface of the host (seaweed) itself is washed under running water, sterilized with 75% EtOH for one minute and household bleach (5% NaOCl) for three minutes, drained and immersed in 75% EtOH again for 30 seconds. The samples are finally rinsed with sterile ocean water and cut aseptically into one cm long segments, that are incubated in Marine agar (Ariffin *et al.,* 2011; De Felício *et al.,* 2015). The isolation of ectosymbiotic bacteria, involves washing

the seaweed surface with sterile ocean water to remove loosely bound epiphytic bacteria. The seaweed surface are scrapped with a sterile cotton swab and plated onto marine agar (Karthick *et al.,* 2015). However, in this study, a different approach was used by culturing in Nutrient agar and Tryptic soy agar medium, in an attempt to capture microbes that have not been cultivated in marine agar. The media was prepared using ocean water, in order to provide necessary series of compounds that are used as energy source and synthesizing the cellular constituents necessary for their survival of the seaweed bacteria (Soria-mercado *et al.,* 2012).

To study bacterial diversity, it is important to use molecular techniques that include the amplification of the 16S ribosomal gene (mostly used for bacterial identification) using the polymerase chain reaction technique (PCR) in order to isolate and characterize their genetic material (Prieto-davó *et al.,* 2008). Bacterial genera such as *Bacillus, Streptomyces, Vibrio, Pseudoalteromonas, Pseudomonas, Psychrobacter, Microbacterium, Shewanellae*, have been isolated and identified from several seaweed species in Indonesia, Japan, Tunisia and India (Kanagasabhapathy *et al.,* 2008; Susilowati *et al.,* 2015; Ismail *et al.,* 2016; Thilakan *et al.,* 2016). However, molecular identification of bioactive bacterial epiphytes and endophytes associated with Kenyan seaweeds is generally lacking. Therefore, this study aimed to identify bacterial species using molecular and phylogenetic analysis. An overview of bacterial isolation and identification methodology is shown in Figure 2.2.

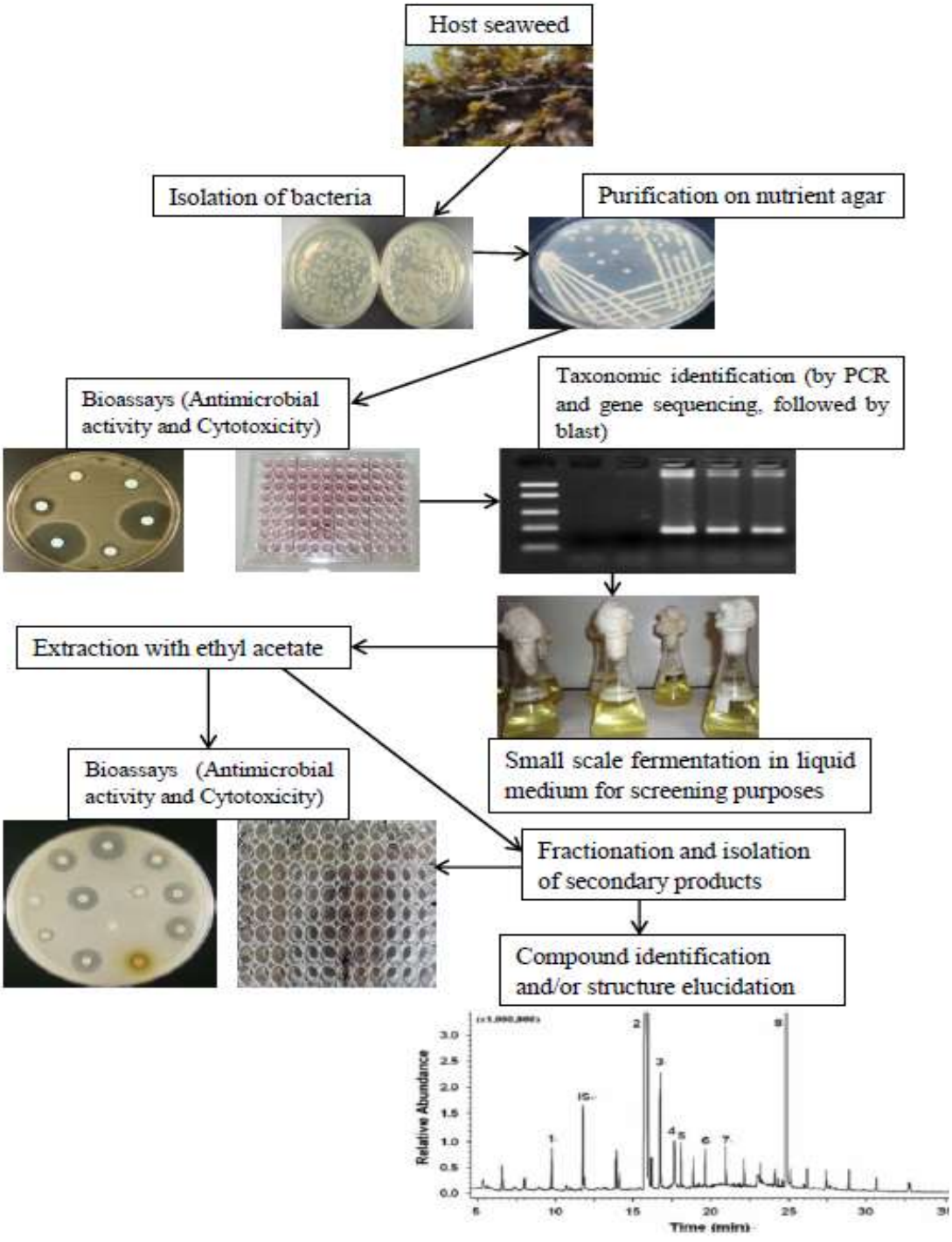

Figure 2.2: Schematic overview on steps involved in isolation of bacteria from seaweeds, extraction and identification of their secondary metabolites.

2.9 Extraction and chemical identification of bioactive secondary metabolites from marine algae bacteria

To obtain microbial bio-products from marine microorganisms, the microbes need to be cultivated and manipulated in bioreactors (Sarkar *et al.,* 2008). Optimum cultivation conditions are mandatory for microbial growth and production of specific metabolites. Growth parameters depend on the temperature, pH, pressure, incubation time, media composition and aeration (Pfefferle *et al.,* 2000). Usually this requires a producer strain to be cultured in the conditions optimal for the production of the active compound. According to the standard method, bacterial isolates grow at 30ºC for 1-3 days (Cappuccino & Sherman, 2014). Secondary metabolite compounds; extraction purification and characterization are important tasks of obtained compounds from marine bacteria. The bacterial filtrate is extracted using organic solvents such as ethyl acetate. The extraction process is repeated several times and extract concentrated in rotary evaporator and lyophilized (Oves *et al.,* 2016). This is followed by purification of the crude extract and structure elucidation of the active compounds present. However, purification and structure elucidation is considered a major bottleneck because of very limited sample material and the compounds of interest often represents less than 1% of the crude extract, of which in most cases is a mixture of hundreds of different compounds (Penesyan *et al.,* 2010).

However, the biologically active crude extract is purified using the preparative silica column chromatography. Variable ratios of the mobile phases are used for eluting the bioactive compounds. The different eluates are collected and concentrated by evaporation in a rotary evaporator. Initial characterization by thin liquid chromatography is performed by spotting on silica gel plates. Since, the crude organic extract usually contains complex mixtures of many different compounds, several sequential purification steps are necessary to obtain specific compounds (Oves *et al.,* 2016). A purified marine-natural compound is mandatory for structural characterization, and to identify its biological and chemical activities. The fractionation process is mainly conducted through the separation

of a crude mixture into several separate fractions. For instance, elute from chromatography column is divided into a feasible number of equal sized portions, and subsequently analyzed fractions in order to determine the possible compounds of interest (Cannell, 1998).

Chromatographic techniques such as Ultra High Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS), are employed for compound identification. Most of the characterization based on separation process by chromatography, which involves distribution of a compound between two phases: mobile phase and stationary phase. Recently, Karthick and Mohanraju (2018) characterized a number of bioactive products produced by epiphytic bacteria associated with eight different seaweeds collected from Little Andaman, India using GC-MS. They identified furan derivatives, 2-Pyrrolidinone, Phenol, 2, 4-bis (1, 1-dimethylethyl) and (1-Allylcyclopropyl) methanol. In this study, the bioactive bacterial compounds were characterized and identified, based on mass charge (m/z) and retention time of the ion spectra of GC-MS, and further accurately matched with standard data of library National Institute of Standards and Technology (NIST). The methodology for fermentation, extraction, and identification of bioactive compounds produced by the seaweed bacteria is as shown in Figure 2.2.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Three coastal sites; Mtwapa (Latitude 3° 57’ S and Longitude 39° 46’ E), Mkomani (Latitude 4° 4’ S and Longitude 39° 41’ E) and Kibuyuni (Latitude 4° 38’ S and Longitude 39° 20’ E) (Figure 3.1), along the Kenyan coast were surveyed for marine algae. The Kenya coast experiences two distinct monsoon seasons, the northeast monsoon (NEM) locally referred to as “*kaskazi”* and the southeast monsoon (SEM) locally referred to as “*kusi”*. The SEM runs from May to September while NEM from November to March. In between the NEM and SEM, there is one to two months of transition period characterized by variable and lower winds locally referred to as “*matlai”* (Church & Obura, 2004). These two seasons experience varying pressure, wind, humidity, cloud cover, rainfall, radiation, and evaporation, that influence local differences in the physical, chemical and biological oceanographic conditions of coastal waters (Smith & Codisporti, 1990).

Figure 3.1: Map of Kenya showing the three sampling sites (1-Mtwapa, 2-Mkomani, 3- Kibuyuni) for collection of marine algae. (Source: Google maps).

3.2 Sample collection

The following 44 marine algae were collected at the three sites: Red algae (*Gracilaria arcuata, Gracilaria salicornia, Gracilaria corticata, Acanthophora spicifera, Chrondrophycus papillosus, Hypnea musciformis, Hypnea pannosa, Hypnea hamulosa, Phacelocarpus tristichus, Sarconema filiforme, Laurencia intermedia, Laurencia* sp., *Solieria robusta, Halymenia durvillaei, Botryocladia leptopoda, Eucheuma denticulatum, Kappaphycus alvarezii* and *Neurymenia fraxinifolia*); Brown algae (*Padina tetrastromatica, Hormophysa cuneiformis, Cystoseira myrica, Cystoseira trinodis, Sargassum oligocystum, Sargassum cristaefolium, Sargassum* sp., *Turbinaria decurrens, Spatoglossum asperum, Dictyota cervicornis, Colpomenia sinuosa, Labophora variegata, Hydroclathrus clathratus, Dictyota bartayresiana* and *Stoechospermum polypodiodes*), and Green algae (*Ulva lactuca, Ulva reticulata, Ulva fasciata, Halimeda macrolaba, Avrainvillea erecta, Codium geppiorum, Caulerpa mexicana, Caulerpa sertulariodes, Caulerpa racemosa, Ulva* sp. and *Udotea* sp.). The algal species were collected purposefully (purposive sampling technique) based on: 1) their pharmaceutical potential as producers of antimicrobial and cytotoxic compounds and 2) their availability and abundance along the Kenya coast. Some of the algal species collected are shown in Plates, 3.1, 3.2 and 3.3, respectively.

Algal sampling was carried during the months of February, March, August and September of 2014. The algal species were collected from the intertidal zones at spring low tides at the three sites. The algal samples were handpicked and given a quick wash with seawater to remove the foreign particles, sand particles and epiphytes. The seaweed samples were put in labeled sterile zip lock plastic bags containing seawater (to avoid desiccation) placed in a cool box with ice blocks and transported to the Kenya Marine and Fisheries Research Institute (KMFRI) laboratory for sorting, processing and identification. The taxonomic identification of seaweed species was done by a marine botanist (Dr. J. Wakibia), using standard literature and taxonomic keys. Voucher specimens of all species collected are deposited in the herbarium at JKUAT.

Plate 3.1: Red algae from Kenyan coast (A: *Gracilaria salicornia*; B: *Acanthophora spicifera*). (Source: Photos by Purity Kaaria).

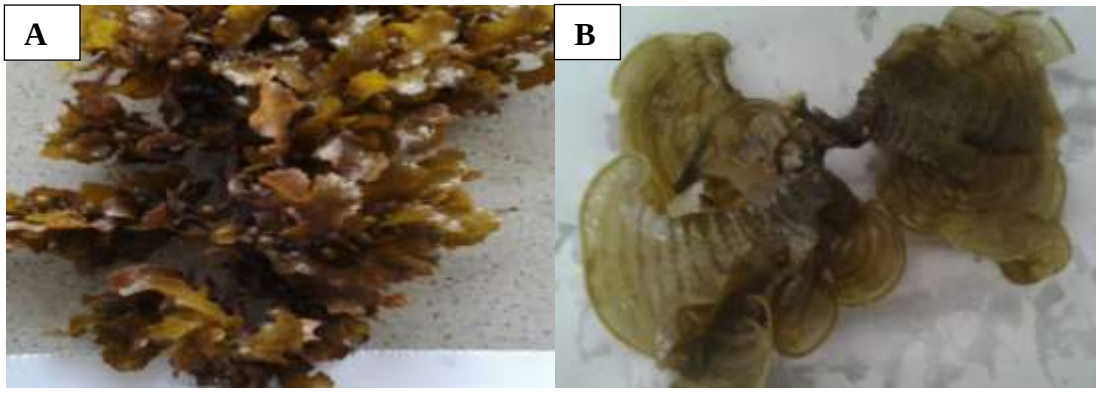

Plate 3.2: Brown algae from Kenyan coast (A: *Sargassum cristaefolium*; B: *Padina tetrastomatica*). (Source: Photos by Purity Kaaria).

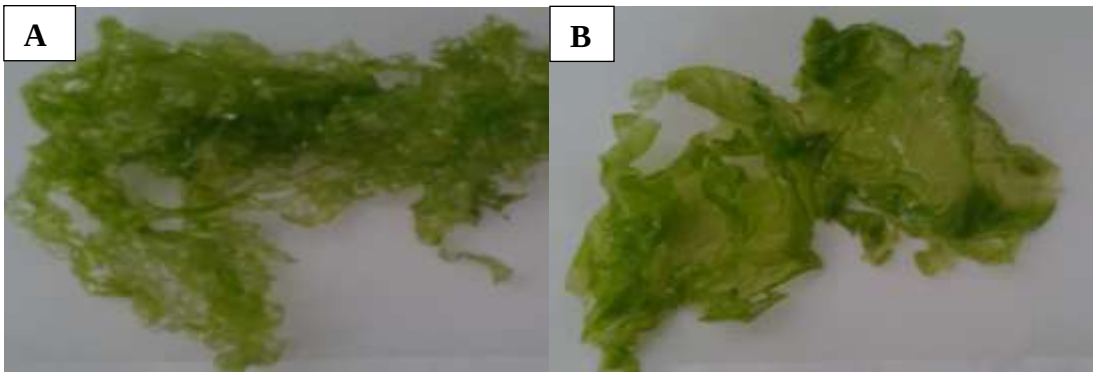

Plate 3.3: Green algae from Kenyan coast (A: *Ulva reticulata*; B: *Ulva lactuca*). (Source: Photos by Purity Kaaria).

3.3 Bacteria isolation

3.3.1 Isolation media

Bacteria isolation was done using nutrient agar (NA) and tryptic soy agar (TSA). The Media (Oxoid Ltd, England) were prepared using seawater. Sub culturing was done on NA (Williams & Davies, 1965).

3.3.2 Isolation of epiphytic bacteria from marine algae

The marine algae were thoroughly rinsed three times with sterile seawater to remove loosely attached microorganisms (Lemos *et al.,* 1985). This was followed by swabbing the surface of the marine algae using a sterile cotton swab. The swab was then used to inoculate on different media, as described by Kanagasabhapathy *et al.* (2008) and Villarreal-Gómez *et al.* (2010). Isolation of the epiphytic bacteria was done by spread plating on NA and TSA. The petri plates were incubated (aerobically) at temperatures of between 25ºC and 30ºC. Pure cultures were obtained by restreaking on agar plates, then cultured on NA slants and stored at 4ºC until further testing.

3.3.3 Isolation of endophytic bacteria from marine algae

For the pretreatment of the seaweed specimens, washing was first done with sterile seawater followed by two minutes wash in 70% ethanol and washing in 2% sodium hypochlorite for one minute. The seaweed samples were then rinsed with sterile seawater for five minutes with shaking and dried with sterile paper towels (Zinniel *et al.,* 2002). The seaweed samples were cut into sections of 2-3 cm long using a sterilized scalpel. The cut sections were then placed on the surface of different isolation media, to enhance the growth of the endophytes (Hung & Annapurna, 2004). The plates were incubated at different temperatures of between 25ºC and 30ºC. Pure cultures for endophytic bacteria were obtained by restreaking on agar plates and then cultured on NA slants and stored at 4ºC until tested.

To confirm success of the surface disinfestations process and to verify that no biological contamination from the surface of the samples were transmitted into the sample tissues, sterility checks were carried out for each sample. For these checks, parts of the cut sections were placed on petri plates of Tryptic soy agar (TSA) and Nutrient agar (NA). The absence of bacteria and fungi after six days of incubation in the sterility checks confirmed that microbes were purely endophytic.

3.4 Screening of bacterial isolates for antimicrobial and cytotoxic activity

3.4.1 Test microorganisms and tumor cells

The pathogenic bacteria strains used were; *Staphylococcus aureus* (ATCC 25922), *Escherichia coli* (ATCC 25923), Methicillin resistant *Staphylococcus aureus* (MRSA) and *Salmonella typhi*. The fungal strains were; *Candida albicans* (ATCC 90028), *Cryptococcus neoformans, Trichophyton metagrophyte* and *Microsporum gypseum* while the tumor cells were larynx Hep-2 cells. The microorganisms and tumor cells were obtained from the Kenya Medical Research Institute, Center of Microbiology Research, Mycology laboratory (American Type Culture Collection (ATCC) and Center for Traditional Medicine, Cell culture laboratory, respectively. First, the microorganisms were selected based on Gram-positive and Gram-negative nature of the bacteria, while fungi were based on the morphological differences on culture, i.e. yeasts and molds and secondly, the multidrug resistant nature of the microorganisms. The bacteria were maintained in Nutrient agar (NA) while Potato dextrose agar (PDA) slants were used for the maintenance of fungi.

3.4.2 Antimicrobial assay

In the first step of screening, determination of the antimicrobial activity of pure colonies was done by disc diffusion method (Balouiri *et al.,* 2016) on Mueller-Hinton Agar (MHA) using three multi drug resistant pathogenic microorganisms: *S. aureus* (ATCC 25922), *E. coli* (ATCC 25923) and *C. albicans* (ATCC 90028).

Pure stocked marine cultures were prepared by sub culturing on NA and MHA. The colonies were then cultured in 15 ml Mueller-Hinton broth by picking the distinct colonies from the Petri plates and inoculated in the broth and incubated (aerobically) at 25ºC for 24h. The broth cultures were centrifuged at 2000 rpm for two minutes, to separate cells and the supernatant. A volume of 20 µl of the cell free supernatant was aliquoted for each sample and loaded onto sterile paper discs, which were placed on ready prepared MHA plates, seeded and spread with 0.5 McFarland standard for the three test pathogenic organisms. Incubation was done at 37ºC for 24h.

Marine colonies that showed a clear halo surrounding each disc (zone of inhibition) against one or more pathogenic microorganism were recorded as active colonies. These active marine colonies were cultured for further screening against more pathogenic microorganisms that included; Methicillin resistant *S. aureus* (MRSA), *S. typhi* and fungi *C. neoformans, T. metagrophyte* and *M. gypseum*. This second step of the screening was repeated as described in the first step. Marine colonies, which showed a broad-spectrum activity against three or more test organisms, were recorded as highly active and were stocked for cytotoxicity assay in section 3.4.3. Antimicrobial activity was evaluated by measuring the inhibition zone (in mm) from the edge of the paper disc. All antimicrobial activities were performed in triplicates.

3.4.3 Cytotoxicity assay

The cytotoxicity assay measured the ability of the marine colonies to inhibit the growth of cancer cells *in vitro*. Larynx Hep-2 carcinoma cells and vero (normal) cells were cultured and maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) at 37ºC in 5% carbon dioxide for 24h. They were harvested by trypsinization (cell dissociation using trypsin, an enzyme which breaks down proteins, to dissociate adherent cells from the vessel in which they are being cultured) and pooled in a 50 ml culture vial. A volume of 100 µl of the cell suspension (2×10^5cells/ ml) was picked and added to each well in a 96 well microtiter plate. Each sample was replicated twice.

The active marine colonies from section 3.4.2 were prepared by sub culturing on MHA plates and incubated for 24h at 25ºC. A loop full of the active marine colonies were each put in 1.5 ml cryovials in 100 µl of MEM and enumerated using a neubar chamber, then standardized to a population of 1×10^4 cells/ ml.

In a 96 well plate, 100 µl of the Hep-2 cells and the vero cells were inoculated from a population of 2×10^5 cells per ml. Onto each plate well, 50 µl of the marine colonies were added from a population of 1×10^4 cells /ml (Mashjoor *et al.,* 2016). This was followed by incubation at 37ºC in 5% carbon dioxide for 48h. The cells in media without the marine colonies were used as controls. In each plate well, 10µl 3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added and the cells incubated for 2h at 25ºC, until a purple precipitate was visible under a light microscope (Betancur-Galvis *et al.*, 2002; Setoyama *et al.,* 2007). The medium together with MTT was aspirated off the cells. A volume of 100 µl of dimethyl sulfoxide was added in the plate wells and the plates shaken for five minutes. The absorbance for each well was measured at 540 nm in a microtiter plate reader to determine the optical density, which was a guide for inhibition of Hep-2 and Vero cell growth. The active marine colonies were cultured for the extraction and screening of anticancer substances according to Perry *et al.* (1999).

3.5 Characterization of active endophytic and epiphytic bacterial isolates

Based on broad-spectrum activity on all tested pathogens and Hep-2 cells inhibition, thirty-three (33) bacterial colonies were selected for characterization. Preliminary characterization was performed using morphological, biochemical and molecular methods.

3.5.1 Morphological characterization

For morphological characterization, the colonies were cultured and the following cultural characteristics were examined: color, form and colony appearance. Gram staining was also done for initial bacterium characterization.

3.5.2 Biochemical characterization

Various biochemical tests were performed for the characterization of the active bacteria colonies: fermentation of sugars (triple sugar iron), sulphur and indole production, methyl red, citrate utilization, urease test, mannitol hydrolysis, gelatin liquefaction, catalase test and oxidase test (Cappuccino & Sherman, 2002).

Triple sugar iron (TSI) test

This test was used to determine the ability of the bacteria colonies to ferment glucose, lactose or sucrose and form hydrogen sulphide and gas production.

Twenty four-hour culture of the isolates were stabbed and streaked over a slant of TSI agar tube and incubated at 30ºC for 24-48h. Change in the color of butt or slant from red to yellow, indicated the type of sugar utilized by the organism. If butt changed from red to yellow, it showed the ability of the isolate to utilize glucose while change of color from red to yellow on the slant and butt indicated the ability of the isolate to glucose, sucrose and lactose. Cracks or bubbles on the medium indicate gas production. Presence of black coloration indicated production of hydrogen sulphide from the reaction (Cappuccino & Sherman, 2002).

Indole production and hydrogen sulfide production

Sulfur indole motility (SIM) agar media was used to test the production of tryptophanase enzyme and the ability to produce hydrogen sulfide from substrates such as sulfur containing amino acids and organic sulfur. Presence of indole was detected by addition of Kovac's reagent to 48-hour cultures of each isolates (Harold, 2002). Positive results were indicated by production of a cherry red layer. Absence of black coloration in the media following incubation indicated absence of hydrogen sulfide (Cappuccino & Sherman, 2002).

Methyl Red-Voges- Proskauer (MR-VP) test

The MR-VP test was used to determine the ability of the isolates to oxidize glucose with production and stabilization of high concentrations of acid end products according to Harold (2002). A MR-VP broth was inoculated with each of the isolates and incubated at 27°C for 48h. Methyl red indicator or Barrit's reagent was added to aliquots of each culture. For positive culture, methyl red appeared red and in VP positive culture gave a rose coloration (Cappuccino & Sherman, 2002).

Citrate utilization

Simmons' citrate agar slants were used to determine the capability of the isolates to use citrate as a carbon source for their energy (Harold, 2002). Bromothymol blue indicator incorporated in the media turned from green to prussian blue indicating positive tests (Cappuccino & Sherman, 2002).

Urease

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea broth media containing phenol red indicator according to the methods described Harold (2002). A positive reaction was indicated by development of deep pink color (Cappuccino & Sherman, 2002).

Mannitol hydrolysis

The mannitol hydrolysis determines the ability of colonies to ferment the carbohydrate (sugar) mannitol as a carbon source. Inocula from pure cultures were transferred aseptically to sterile tubes of phenol red mannitol broth. The medium is a nutrient broth to which 0.5-1.0% mannitol is added. The inoculated tubes were incubated at 35-37°C for 24h. A positive test consisted of a color change from red to yellow, indicating a pH change to acidic (Cappuccino & Sherman, 2002).

Gelatin liquefaction

Nutrient broth supplemented with 12% gelatin was used to demonstrate the hydrolytic activity of gelatinase (Harold, 2002). After incubation at 28ºC, the cultures were placed in the refrigerator at 4°C for 30 minutes. Cultures that remained liquefied were considered positive for gelatin hydrolysis (Cappuccino and Sherman, 2002).

Catalase test

Catalase production was determined by addition of 3% hydrogen peroxide to Tryptic Soy Agar (TSA) cultures of each isolate based on the methods outlined by Cappuccino and Sherman (2002). A positive reaction was shown by formation of bubbles, which indicated catalase activity.

Oxidase test

Oxidase production was determined using oxidase strips containing tetramethy-p-phenylenediamine dichloride. Blue purple coloration indicated a positive test (Cappuccino & Sherman, 2002; Harold, 2002).

3.5.3 Molecular characterization

The thirty-three (33) bacterial colonies that showed remarkable bioactivity against pathogenic microorganisms were further characterized based on PCR amplifications of the 16S rRNA genes sequence of nearly 1000 base pairs (bp).

DNA extraction

Pure subcultures of the antagonistic bacterial colonies were inoculated in freshly prepared Luria Bertani (LB) broth supplemented with 3.5% sodium chloride and incubated overnight (24h) at 37ºC in a shaker incubator at 222 rpm. The supernatant was discarded. Total genomic DNA was extracted using Invitrogen Kit for DNA extraction (Thermo scientific, Waltham, MA USA). A 380 µl of the lysozyme digestion buffer was added and

was briefly mixed by vortexing. Incubation was then done at 37ºC for 30 minutes. A 20 µl Proteinase K was added and mixed by brief vortexing. A 200 µl PureLink® Genomic Lysis/Binding Buffer was added and mixed well by brief vortexing. Incubation was done at 55°C for 30 minutes. A 200 µl 96–100% ethanol was added to the lysate and mixed well by vortexing for five seconds to yield a homogeneous solution. A 640 µl of lysate prepared with PureLink® Genomic Lysis/Binding Buffer and ethanol was added to the PureLink® Spin Column. The column was centrifuged at 10,000 × g for a minute at room temperature. The collection tube was discarded and the spin column placed into a clean PureLink® collection tube supplied with the kit. 500 µL Wash Buffer one prepared with ethanol was then added to the column. The column was centrifuged at room temperature for a minute at 10,000 × g. The collection tube was discarded and the spin column place into a clean PureLink® collection tube supplied with the kit. A 500 µl wash buffer two prepared with ethanol was then added to the column. The column was centrifuged at room temperature at maximum speed for three minutes.

The spin column was placed in a sterile 1.5 ml micro centrifuge tube. A 200 µl of PureLink® Genomic Elution Buffer was added to the column. Incubation was then done at room temperature for a minute. The column was removed and discarded. The tube containing purified genomic DNA was ready for application. To visualize the DNA, gel electrophoresis was done where 3 µl of DNA was loaded on 1.5% agarose gel stained with ethidium bromide and prepared with 1XTAE buffer. The DNA was stored at -20ºC.

Amplification of 16S rDNA gene

Purified total DNA from each bacterial isolate was used as a template for amplification of the 16S rDNA genes. This was done using the HotStar Taq Master Mix Kit (Qiagen, Hilden Germany). DNA extracts for 16S rRNA gene sequences were PCR-amplified using universal bacterial primer pair 27F (5'AGAGTTTGATCMTGGCTCAG-3’) and 1492R (5'TACGGTTAACCTTGTTACGACTT-3'). Amplification was performed using a model ABI Gen Amp 9800 Fast thermal cycler (Thermo scientific, Applied

Biosystems™, Waltham, MA USA). Amplification was carried out in a ten microliter mixture containing five microliter of HotStar Taq Master Mix, 0.1 µl (pmol) of 27F forward primer, 0.1 µl (pmol) of 1492R reverse primer, 1.5 µl of template DNA and 3.8 µl of water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 35 cycles: Initial activation of the enzyme at 95^{o}C for three minutes, denaturation at 95^{o}C for 30 seconds, primer annealing at 52^{o}C for a minute, chain extension at 72^{o}C for a minute and a final extension at 72^{o}C for 10 minutes (Roux, 2009). The 16S rDNA amplification products (10 µl) were separated on a 1.5% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining (Sambrook, 1989).

DNA sequencing

DNA sequencing was conducted at Macrogen Laboratory, Seoul Korea. Purification of polymerase chain reaction (PCR) amplification products was done using the QIAquick PCR purification KIT (QIAGEN) and for PCR, sequencing was performed by using Big Dye Terminator v.3.1 and capillary sequence analysis by using ABI 3130XL, Applied Biosystem. The sequences of amplified 16S rDNA genes were deposited in the Gene bank database the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The sequences were inserted to the advanced BLAST search program to identify the sequences of any closely related organisms.

Phylogenetic analysis

The result of DNA sequences were aligned with ClustalW Multiple Alignment program and the phylogenetic analyses were performed by using MEGA 5 program (Tamura *et al.,* 2011). Analysis of the 16S rDNA gene were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) in order to determine similarity to sequences in the Gene bank database (Altschul *et al.,* 1990) for preliminary species identification.

The 16S rDNA gene sequences with high similarities to those that were determined in the study were retrieved and added to the alignment based on BLAST results. The phylogenetic tree was constructed by neighbor-joining method (Saitou & Nei, 1987) using the Tamura and Nei model (Tamura & Nei, 1993).The resultant tree topology was evaluated by bootstrap analyses based on 1000 replications (Felsenstein, 1985).

3.6 Fermentation, extraction and bioassay guided fractionation of the microbial secondary metabolites

The 33 bacterial isolates that showed prominent and broad-spectrum activity against the test pathogenic microorganisms were taken for further mass scale fermentation and extraction of secondary metabolites.

3.6.1 Batch fermentation of potential bacterial isolates

Pure subcultures of the thirty-three (33) antagonistic active bacterial isolates were inoculated in universal bottles containing freshly prepared 20 ml Mueller-Hinton Broth (MHB) and incubated at 25^{o}C for 24h. The overnight cultures were dispensed into three litre sterile plastic containers containing two litres of Mueller-Hinton (Scharlau Chemie S.A, Spain) broth prepared using seawater and autoclaved at 121^{o}C for 40 min. To simulate natural conditions and production of the secondary metabolites, the cultures in the fermentation broth were incubated for 48hrs in an incubator shaker (New Brunswick Scientific Co. Inc, Edison, New Jersey, USA) at 30^{o}C with a rotary speed of 150 rpm.

3.6.2 Extraction of secondary metabolites

To extract the active secondary metabolites from the obtained fermentation broth in section 3.6.1, equal volume (*v/v*) of analytical grade ethyl acetate (Fisher Scientific, Loughborough, UK) was added. The mixture was shaken for four hours at 150 rpm then centrifuged at 4000 rpm for five minutes. The two immiscible layers were separated using separating funnel with ethyl acetate layer concentrated using rotor evaporator

(Laborota 4000 efficient, Heidolph, Germany) at 40^{o}C under vacuo to small volume (~20 ml). The resulting crude extracts were weighed and stored at -20^{o}C for further screening studies against pathogenic microorganisms and larynx Hep-2 cells.

3.6.3 Bioassay-guided fractionation

The 33 sample crude extracts were subjected to repeated separation through column chromatography using ethyl acetate- methanol solvent system. The active principles within the ethyl acetate extracts were fractionated on silica-packed column (Ø 15 mm by 300 mm). Silica gel (60-120 mesh size, FINAR®, Finar Ltd, Gujarat India) was suspended in ethyl acetate and conditioned for three hours before loading the sample extracts. Silica-adsorbed extracts were separately loaded onto the column and different fractions gradient eluted with ethyl acetate: methanol with a ratio of 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100 respectively as mobile phase. A volume of 100 ml of each eluent fraction was collected. Excess solvent was removed by rotary evaporation at < 40^{o}C and residual extract stored in universal bottles at -80˚C awaiting bioassay against pathogenic microorganisms and larynx Hep-2 cells.

3.7 Bioassay of fractionated secondary metabolites

3.7.1 Antimicrobial assay of fractionated products

Sample crude extracts and their fractions were tested for antimicrobial activity using the disc diffusion method as described in National Committee Clinical Laboratory Standards (NCCLS), standardized charts (Jorgensen, 1998) against eight pathogenic microorganisms (see section 3.4.1).

The disc diffusion assay was performed according to Kirby-Bauer method (Boyle *et al.*, 1973). For antimicrobial screening, 20 microliters of the test solution were applied to sterile filter-paper discs (6 mm diameter, Oxoid) in triplicates and allowed to dry. For positive controls, conventional antibiotics Chloramphenicol (10µg/ml, Oxoid) for bacteria and Clotrimazole (10µg/ml, Oxoid) for fungi were used. Mueller-Hinton Agar

(MHA, Oxoid) was seeded with McFarland 0.5 standard of the test strains. The impregnated discs were placed on agar plates seeded with the selected test organisms, along with discs containing normal saline as a negative control. The plates were incubated at 37°C (48h) for bacteria and 28°C (72h) for fungi. The antimicrobial activity produced by each fraction was recorded as the clear zone of inhibition surrounding the disc at which the diameter was measured in mm.

3.7.2 Cytotoxic assay of fractionated products

The sample crude extracts and their fractions were dried in a fume chamber to evaporate the solvent to dryness. Each fraction was weighed and the weights were recorded (mg). A volume of one ml of minimum essential medium (MEM) was added in each sample fraction and the original concentration determined, i.e. if a sample weighed 30 mg and 1ml of MEM was added it resulted to 30mg/ ml. A volume of 50 µl of MEM was transferred onto 96 well plate being dispensed in all wells except for column 3, 6, 9,12 (Figure 3.2), which acted as blanks (negative controls) without any cells or media. A volume of 50 µl of each compound fraction was added to the 96 well plates in well H (Figure 3.2). A serial dilution was performed upwards up to wells row C (Figure 3.2) by pipetting 50µl from the mixture. Rows A and B were left since they acted as positive controls (Figure 3.2). The cells were prepared as a cell suspension from the trypsinized cells from the original culture and counted using a neubar chamber up to 2×10^5 cells/ ml. A volume of 100 µl of the cell suspension was then added to the 96 well plates containing the sample fractions. The plates were incubated at 37°C and 5% CO_2 for 48h (Mashjoor *et al.*, 2016).

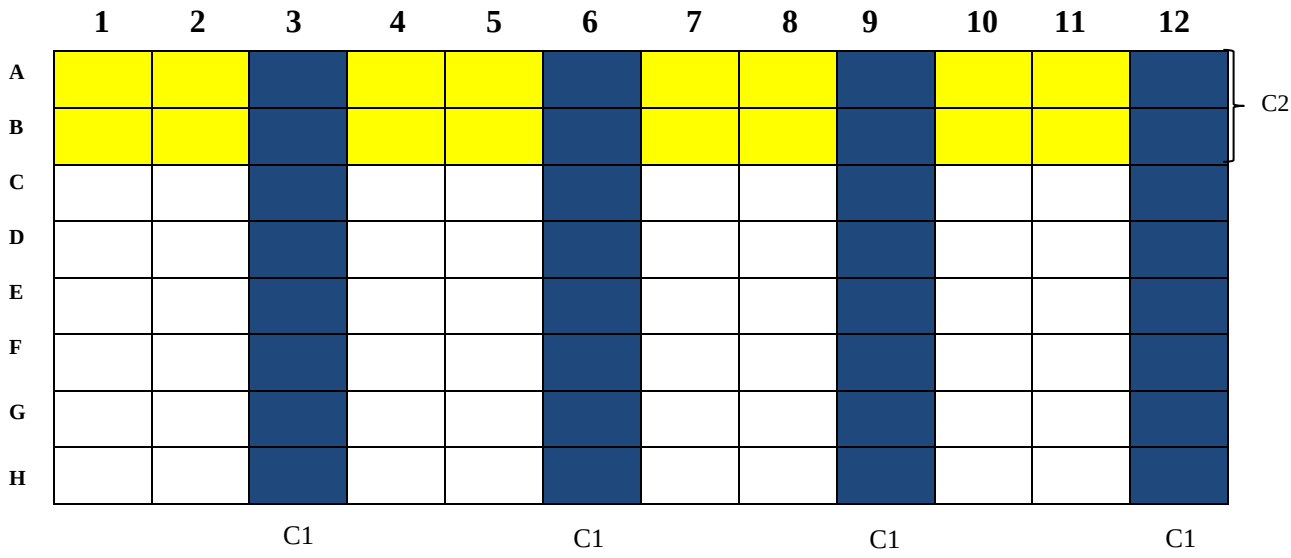

Figure 3.2: Microtiter 96 well plate for fractionated extracts cytotoxicity test. With blue: C1= negative control (Blank wells; No cells, No media), yellow: C2= positive controls (Cells and media) and white: sample wells (Cells, media and fractionated compounds).

After incubation, the medium was poured off and replaced with 10 µl of MTT 3-(4, 5-dimethylthiazole- 2-yl)-2, 5-diphenyltetrazolium bromide (concentration of 5mg/ ml), in all the wells. The plates were incubated between 2-4h in darkness until a purple precipitate was clearly visible. The precipitate (formazan) was diluted with 100 µl of DMSO and shaken for five minutes. The absorbance was read spectrophotometrically at 540 nm using a mean universal microplate reader (EL_x800 Bio-Tek Instruments Winooski, USA). Using the alamar blue assay programme, the IC_{50} values of the fractions were determined (Siti *et al.*, 2011). The IC_{50} values for both Hep-2 and vero cells were compared. A lower IC_{50} value for Hep-2 cells and a high IC_{50} value for vero cells were considered good fractions for further analysis.

The IC_{50} values of the extremely potent fractions was compared with the commercially available antitumor agent rapamycin 10 mg (Rap, Sigma) against cancer cells as positive

controls and DMSO was used as a negative control. The effects of the bacterial extracts (fractions) were expressed by IC_{50} value (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells). The IC_{50} values were determined using the Alamar Blue Assay Software (Rampersad, 2012). Fractions that showed a low IC_{50} value against the Hep-2 cells compared to the Vero cells were considered good fractions. This implied that the drug component present in the fractions perform better at a lower concentration on the cancerous cells.

3.8 Identification of active fractions of the secondary metabolites

3.8.1 Sample preparation

A volume of 1000 µl of ethyl acetate and 100 mg of Na_2SO_4 (drying agent) were added to each fractionated extract sample, vortexed for a minute, extracted by ultra-sonication in sonication bath (Branson 2510, Danbury, CT, USA) for 10 min, centrifuged at 13,000 rpm for five min at 5°C and the supernatant filtered by passing through glass wool each before analysis by gas chromatography- mass spectrometry (GC-MS).

3.8.2 Gas Chromatography Coupled Mass spectrometry (GC-MS) analysis

A GC-MS in full scan mode was used to detect and profile all the compounds present in the active fractionated extracts. The extracts were analyzed by GC-MS on a 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) linked to a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) by using the following conditions: inlet temperature 270°C, transfer line temperature of 280°C, and column oven temperature programmed from 40°C to 285°C with the initial temperature maintained for five minutes then 10°C/ min to 280°C held at this temperature for 10.5 min and finally 50°C/ min to 285°C and held at this temperature for 31.9 minutes. The GC was fitted with a HP-5 MS low bleed capillary column (30 mm× 0.25 mm i.e., 0.25 µm) (J&W, Folsom, CA, USA). Helium at a flow rate of 1.25 ml per minute served as the carrier gas. The mass selective detector was maintained at ion source temperature of

230°C and a quadruple temperature of 180°C. Electron impact (EI) mass spectra were obtained at the acceleration energy of 70 eV. A 1.0 µl aliquot of extract was injected in the split/split less mode using an auto sampler 7683 (Agilent Technologies, Inc., Beijing, China). Fragment ions were analyzed over 40–550 m/z mass range in the full scan mode. The filament delay time was set at five minutes. The compounds were identified by comparison of gas chromatographic retention time and fragmentation pattern with that of the authentic standards.

When there was a lack of corresponding reference compounds, the structures were proposed on the basis of their general fragmentation and using reference spectra published by library–MS databases: National Institute of Standards and Technology (NIST). A five-point serial dilution of authentic standard of indole (1-280 ng/µl) were also analyzed by GC-MS in full scan mode to generate linear calibration curves (peak area vs. concentration) which served as the basis for the external quantification.

3.9 Data analysis

All data were expressed as mean ± standard deviation (SD). The results for antimicrobial activity were compared using analysis of variance (ANOVA). Tukey's test was applied for comparison of means. The T-test was used to compare antimicrobial assay inhibition and cytotoxic assay absorbance and IC_{50} values. Statistical analyses were performed using SPSS for windows; version 22.0.

CHAPTER FOUR

RESULTS

4.1 Bacterial isolates from marine algae

A total of 3493 bacterial isolates were obtained from three sampling sites and from forty-four (44) seaweed species. Total epiphytes isolated were 1888 and endophytes were 1605. The growth of epiphytic colonies on plates after incubation is as shown in Plates 4.1 and 4.2, while Plate 4.3 indicates the growth of endophytic colonies.

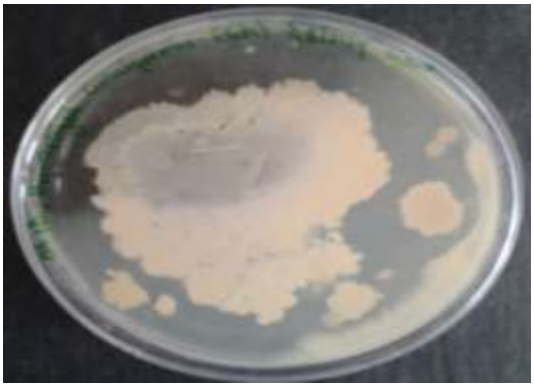

Plate 4. 1: **Nutrient agar plate showing the growth of epiphytic bacterial colonies isolated from brown algae (*Hormophysa cuneiformis*) after 2-3 days of incubation.**

Plate 4.2: Nutrient agar plate showing the growth of epiphytic bacterial colonies isolated from red algae (*Gracilaria salicornia*) after 2-3 days of incubation.

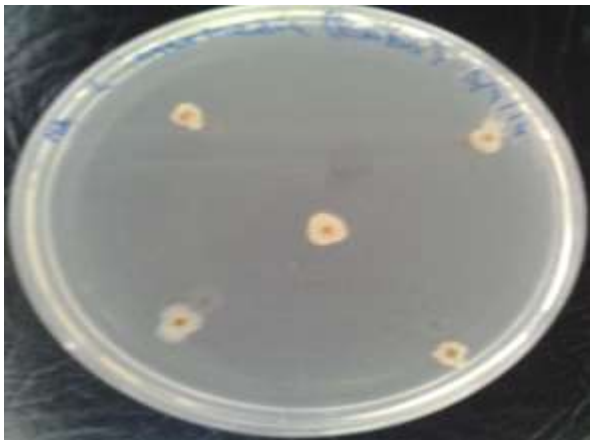

Plate 4.3: Nutrient agar plate showing the growth of endophytic bacterial colonies isolated from red algae (*Laurencia intermedia*) after 2-3 days of incubation.

Endophytic and epiphytic bacteria isolates

The endophytic and epiphytic bacterial isolates obtained from the different algal divisions and sites are shown in Table 4.1. Bacterial isolates varied, with frequent isolation of bacteria epiphytes (54%) compared to bacteria endophytes (46%).

Table 4.1: Total number of endophytic and epiphytic bacterial isolates obtained from three algal divisions (red, green, brown) and three sampling sites (Mtwapa, Mkomani, Kibuyuni) along the Kenya coast.

Bacterial isolate	**Algal division**	**Sites**		
		Mtwapa	**Mkomani**	**Kibuyuni**
Endophytes	Red	257	261	339
	Green	54	31	84
	Brown	304	174	101
Epiphytes	Red	246	293	316
	Green	163	180	88
	Brown	229	252	121
TOTAL		**1253**	**1191**	**1049**

4.2 Antimicrobial and cytotoxic screening

The total endophytic and epiphytic bacterial isolates (Table 4.2) were further screened for antimicrobial activity.

4.2.1 Antimicrobial screening

From the 3493 bacterial isolates obtained, 695 isolates (20%) produced antimicrobial activity against one or more of the test organisms as shown in Table 4.2. An isolate was considered active if it had an antagonistic activity against one or more of test organisms and was further screened against more test pathogens.

Table 4.2: Total bacterial isolates and active isolates from three algal divisions (red, green, brown) and three sampling sites (Mtwapa, Mkomani, Kibuyuni) along the Kenya coast.

Site	Algal division	Total isolates	Isolates with bioactivity	% of bioactive isolates
Mtwapa	Red	502	82	16
	Green	217	60	28
	Brown	533	115	22
Mkomani	Red	554	131	24
	Green	211	37	18
	Brown	426	105	25
Kibuyuni	Red	655	109	17
	Green	172	18	10
	Brown	222	38	17
TOTAL		**3493**	**695**	**20**

Among the 695 isolates that showed positive inhibition activity, 69 isolates (10%) were found to possess a broad-spectrum *in vitro* activity on three or more of the test pathogens (Table 4.3). The antagonistic activity of the isolates was indicated by the clear zone of inhibition around the paper disc (Plates 4.4 A, B and C).

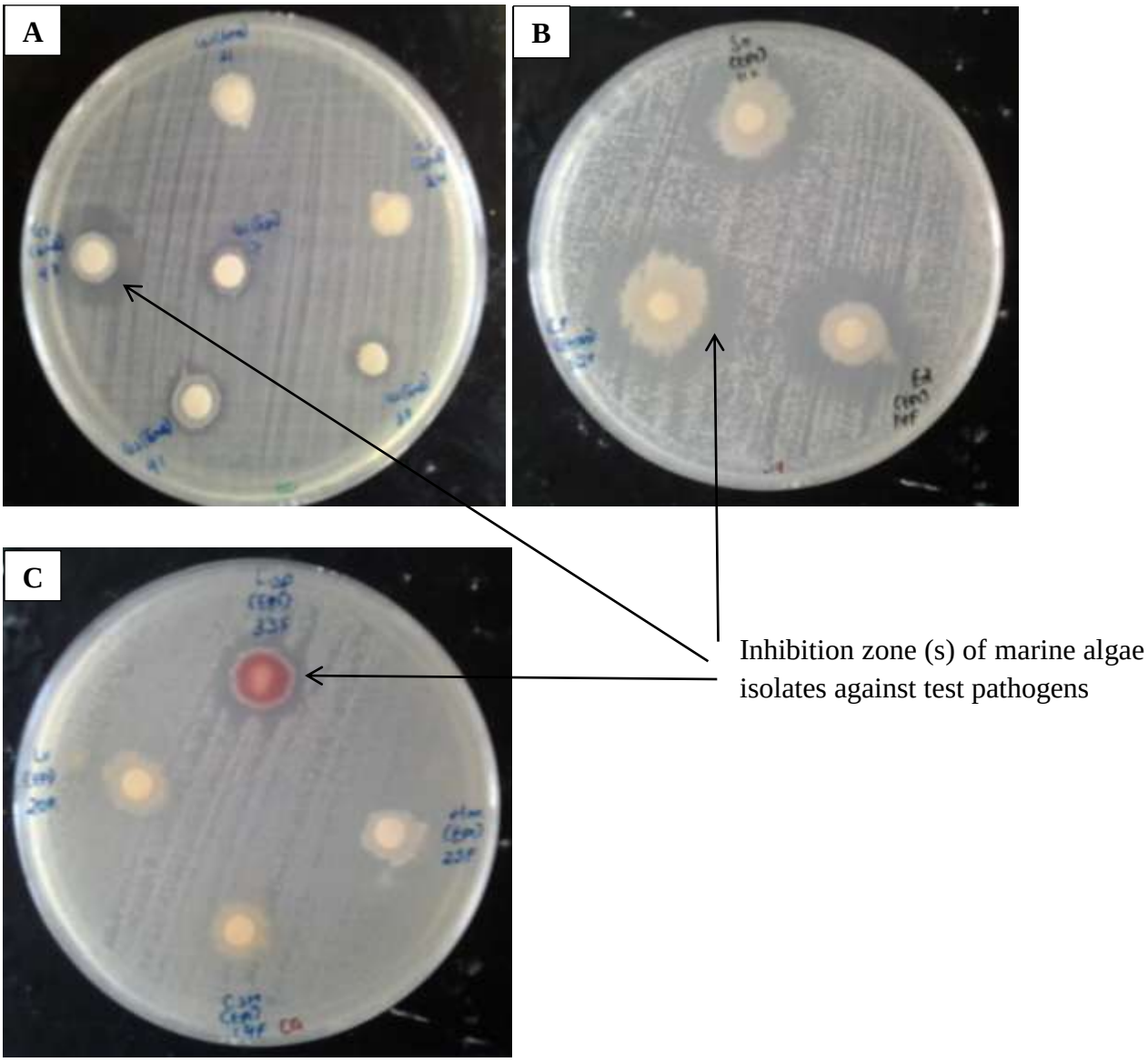

Plate 4.4: Antagonistic activity of isolates from marine algae against (A) *Escherichia coli,* (B) *Staphylococcus aureus* and (C) *Candida albicans*.

Table 4.3: Antimicrobial activity (inhibition zone: mm) of endophytic and epiphytic bacteria from three algal divisions collected at the three sites in Kenya, against eight pathogenic microorganisms (mean ± SD, n = 3).

Inhibition zones (mm)										
			Gram positive		Gram negative		Yeast		Molds	
Site	Algal division	Isolate code	*Staphylococcus aureus*	Methicillin Resistant *Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhi*	*Candida albicans*	*Cryptococcus neoformans*	*Trichophyton metagrophyte*	*Microsporum gypseum*
Mtwapa	Red	Gs(EPI)21	ND	ND	21.3±0.57^{b}	ND	ND	ND	45.6±0.57^{i}	21.3±0.57^{b}
		Gs(EPI)22	ND	21.6±1.15^{b}	21.0±0.00^{b}	ND	ND	ND	50.6±0.57^{jk}	24.0±1.732^{c}
		As(END)10	ND	26.0±0.00^{d}	21.3±0.57^{b}	17.3±0.57^{b}	18.0±0.00^{a}	ND	52.0±1.732^{k}	26.33±0.57^{c}
		As(END)11	19.0±0.00^{b}	26.3±0.57^{d}	21.0±0.00^{b}	16.6±0.57^{ab}	19.3±0.57^{a}	ND	37.6±0.57^{f}	28.3±0.57^{d}
		As(END)39	ND	21.0±0.00^{b}	ND	22.3±0.57^{c}	ND	ND	36.6±1.155^{f}	28.3±0.57^{d}
		As(EPI)40	ND	ND	21.3±0.57^{b}	16.6±0.57^{ab}	ND	20.0±0.00^{b}	41.0±0.00^{g}	28.6±1.155^{d}
		Cp(EPI)12	ND	ND	16.0±0.00^{a}	14.0±2.64^{a}	ND	ND	26.0±0.00^{b}	26.0±0.00^{c}
		Cp(EPI)14b	17.6±0.57^{a}	26.0±0.00^{d}	18.0±0.00^{a}	18.3±0.57^{b}	18.0±0.00^{a}	23.3±1.52^{c}	30.6±0.57^{d}	33.0±0.00^{f}
	Green	Ul(EPI) 4	ND	18.6±1.15^{a}	16.3±1.52^{a}	16.3±0.57^{a}	ND	ND	34.6±1.52^{e}	28.6±0.57^{d}
		Ul (EPI)7	ND	18.0±1.00^{a}	16.6±1.15^{a}	16.0±0.00^{a}	17.0±1.00^{a}	17.3±1.15^{a}	38.0±2.00^{f}	23.0±1.00^{b}
		Ur(EPI)15	21.3±0.57^{b}	36.0±0.00^{g}	21.0±0.00^{b}	19.3±0.57^{b}	18.0±0.00^{a}	27.0±1.00^{d}	28.0±0.00^{c}	26.0±0.00^{c}
		Ur(EPI)16	ND	31.0±0.00^{e}	ND	16.0±1.00^{a}	ND	16.0±0.00^{a}	ND	ND
		Ur(EPI)24	ND	36.0±0.00^{g}	ND	ND	ND	25.6±1.15^{d}	26.0±0.00^{b}	21.0±0.00^{b}
		Ur(EPI)22	26.0±0.00^{d}	21.0±0.00^{b}	19.6±1.52^{b}	17.6±1.15^{b}	ND	24.6±1.15^{cd}	41.0±0.00^{g}	26.6±1.15^{cd}
	Brown	Cmy(END)16	ND	21.0±0.00^{b}	16.6±1.15^{a}	18.0±0.00^{b}	18.6±1.15^{a}	18.0±0.00^{a}	36.0±0.00^{f}	33.3±.577^{f}
		Pt(EPI)15	21.0±0.00^{b}	31.6±1.15^{ef}	18.0±0.00^{a}	23.0±0.00^{d}	21.3±0.57^{b}	24.0±0.00^{c}	26.0±0.00^{b}	36.0±0.00^{g}
		Pt(EPI)25	25.3±0.57^{d}	31.0±0.00^{e}	22.0±1.00^{c}	21.0±.000^{c}	20.0±0.00^{b}	26.0±.000^{d}	26.3±1.52^{b}	36.0±0.00^{g}
		Pt(EPI)26	20.3±0.57^{b}	31.0±0.00^{e}	18.0±0.00^{a}	21.6±1.15^{c}	20.0±0.00^{b}	27.3±0.57^{d}	28.0±0.00^{c}	33.6±1.15^{f}
		Ct(END)2a	26.3±0.57^{d}	ND	ND	ND	ND	23.6±1.155^{c}	ND	22.6±1.52^{b}
		Ct(END)2b	24.6±1.52^{cd}	ND	ND	ND	ND	27.3±0.57^{d}	ND	21.6±1.15^{b}
		Ssp.(END)25	27.0±1.00^{d}	19.0±1.73^{a}	ND	ND	ND	22.3±0.57^{c}	ND	ND
		Ssp.(END)30a	26.3±0.57^{d}	ND	ND	ND	ND	21.3±0.57^{b}	21.6±1.15^{a}	ND
		Ssp.(END)30b	26.0±1.73^{d}	ND	19.0±1.00^{b}	18.0±0.00^{b}	ND	31.6±1.15^{f}	21.6±1.15^{a}	32.3±0.57^{e}
		Ssp.(END)32a	27.6±0.57^{de}	ND	ND	ND	ND	27.0±1.00^{d}	27.0±1.00^{c}	ND
		Ssp.(END)32b	22.6±1.15^{c}	ND	ND	ND	ND	21.6±1.15^{bc}	21.6±0.57^{a}	23.0±1.000^{b}
Mkomani	Red	Gs(END)6	ND	26.6±1.15^{d}	19.6±1.52^{b}	ND	ND	ND	45.6±.577^{i}	22.0±1.000^{b}
		Gs(END)37	ND	26.6±1.15^{d}	16.0±0.00^{a}	ND	ND	ND	36.3±.577^{f}	18.6±1.155^{a}
		Gs(END)41	21.6±1.15^{bc}	30.0±1.00^{e}	19.3±0.57^{b}	18.0±1.00^{b}	21.0±0.00^{b}	ND	34.3±0.57^{e}	22.6±1.15^{b}
		Gs(END)43	18.3±0.57^{a}	30.3±1.15^{e}	23.0±1.00^{c}	16.3±1.52^{a}	ND	ND	36.6±1.15^{f}	19.6±1.15^{a}
		Gs(EPI)7	ND	26.3±0.57^{d}	16.0±0.00^{a}	ND	ND	ND	34.3±0.57^{e}	21.3±0.57^{b}
		As(END)24	ND	17.0±0.00^{a}	ND	ND	ND	ND	31.3±0.57^{d}	19.0±0.00^{a}
		As(END)28	20.3±0.57^{b}	18.6±0.57^{a}	ND	ND	21.0±0.00^{b}	21.3±0.57^{b}	28.0±0.00^{c}	30.33±0.57^{e}

		Cp(END)52F	31.6±1.15^{f}	ND	ND	ND	ND	31.0±0.00^{f}	21.6±1.15^{a}	ND
		Cp(EPI)15	ND	28.0±0.00^{d}	20.6±1.52^{b}	17.0±0.00^{b}	20.3±0.57^{b}	18.3±0.57^{a}	26.0±0.00^{b}	29.00±0.00^{d}
		Lsp.(EPI)33	26.6±1.15^{d}	25.6±0.57^{cd}	ND	ND	24.0±1.00^{c}	27.0±1.00^{d}	23.3±0.57^{a}	26.3±1.52^{c}
		Hmu(EPI) 9	ND	29.0±1.00^{e}	22.3±0.57^{c}	19.6±1.15^{bc}	ND	ND	41.6±1.15^{gh}	27.6±0.57^{d}
		Hmu(EPI) 13	25.3±0.57^{d}	27.0±1.73^{d}	28.0±0.00^{e}	26.0±1.73^{e}	26.3±0.57^{d}	24.3±0.57^{c}	46.6±1.15^{i}	28.3±0.57^{d}
		Hmu(EPI)16	ND	26.6±1.15^{d}	22.6±0.57^{c}	21.6±1.15^{c}	21.3±0.57^{b}	21.0±0.00^{b}	46.6±1.15^{i}	26.3±0.57^{c}
		Hmu(EPI)22	21.6±1.15^{bc}	26.6±1.15^{d}	20.3±3.78^{b}	19.6±1.15^{bc}	22.3±.577^{b}	20.3±0.57^{b}	41.6±1.15^{gh}	25.0±1.00^{c}
		Gc(EPI)12	ND	19.0±0.0^{a}	21.3±0.57^{b}	ND	ND	ND	36.6±1.15^{f}	21.6±0.57^{b}
		Hp(EPI)19	23.0±0.00^{c}	28.6±0.57^{de}	22.3±0.57^{c}	17.3±0.57^{b}	21.6±1.15^{b}	21.6±1.15^{bc}	44.6±1.52^{hi}	31.3±0.57^{e}
	Green	Cmex(END)8	ND	19.0±1.00^{a}	16.6±1.15^{a}	ND	ND	ND	38.6±1.15^{fg}	23.0±1.00^{b}
	Brown	Td(ENDO)26	ND	31.0±0.00^{e}	24.0±1.00^{c}	26.3±0.57^{e}	ND	ND	36.6±1.15^{f}	21.6±1.15^{b}
		Scr(ENDO)39	ND	17.6±0.57^{a}	19.3±0.57^{b}	ND	ND	ND	36.6±1.15^{f}	24.6±1.52^{c}
		Scr(EPI)14	ND	22.3±0.57^{b}	20.3±0.57^{b}	ND	ND	ND	27.3±1.15^{c}	19.0±1.00^{a}
		Scr(EPI)17	ND	26.3±0.57^{d}	22.0±1.00^{c}	ND	ND	ND	41.3±0.57^{g}	19.3±1.15^{a}
Kibuyuni	Red	Sr(END)7	ND	21.6±1.15^{b}	ND	ND	ND	ND	41.6±1.15^{gh}	32.0±1.00^{e}
		Sr(END)9	ND	24.3±0.57^{c}	ND	ND	ND	ND	49.3±0.57^{j}	36.6±1.15^{g}
		Sr(END)10	ND	23.3±0.57^{c}	ND	ND	ND	ND	46.6±1.15^{i}	36.3±0.57^{g}
		Gs(END)8	ND	ND	ND	ND	ND	18.3±0.57^{a}	31.0±0.00^{d}	21.6±1.15^{b}
		Gs(END)11	19.0±0.00^{b}	21.0±0.00^{b}	ND	ND	22.3±1.15^{b}	21.0±0.00^{b}	26.6±1.15^{bc}	22.6±0.57^{b}
		Gs(END)16	ND	ND	ND	ND	ND	21.0±0.00^{b}	31.6±1.15^{d}	25.0±0.00^{c}
		As(END)2	ND	23.6±1.52^{c}	ND	ND	ND	18.0±0.00^{a}	36.6±1.15^{f}	31.0±0.00^{e}
		As(END)10	ND	23.6±1.15^{c}	ND	ND	ND	16.0±0.00^{a}	36.3±0.57^{f}	26.0±0.00^{c}
		As(END)11	ND	22.6±1.15^{bc}	ND	ND	ND	21.0±0.00^{b}	22.6±0.57^{a}	22.3±1.52^{b}
		As(END)14	ND	22.0±0.00^{b}	ND	ND	ND	21.3±1.52^{b}	35.6±0.57^{ef}	27.0±1.00^{d}
		As(END)15	ND	19.0±0.00^{a}	ND	ND	ND	17.6±1.15^{a}	29.6±4.16^{cd}	26.3±0.57^{c}
		As(END)19	ND	ND	ND	ND	ND	17.0±0.00^{a}	27.0±1.00^{c}	21.6±1.15^{b}
		As(END)23	ND	ND	ND	ND	ND	18.0±0.00^{a}	26.0±0.00^{b}	27.0±0.00^{d}
		Cp(END)4	26.0±0.00^{d}	26.0±0.00^{d}	ND	ND	22.3±1.15^{b}	21.6±0.57^{bc}	26.3±0.57^{b}	29.6±0.57^{de}
		Cp(END)8a	24.0±0.00^{c}	ND	ND	ND	21.0±0.00^{b}	26.0±0.00^{d}	ND	ND
		Ka(EPI)13a	25.0±.1.00^{d}	ND	ND	ND	ND	27.0±1.00^{d}	36.0±.000^{f}	23.0±1.00^{b}
		Ka(EPI)13b	27.0±1.00^{d}	ND	ND	ND	ND	26.6±1.15^{d}	36.6±1.15^{fg}	26.6±1.15^{cd}
		Gc (EPI)1	21.0±0.00^{b}	30.6±0.57^{e}	19.0±0.00^{b}	18.0±1.00^{b}	21.0±0.00^{b}	22.0±1.00^{c}	31.0±0.00^{d}	26.3±0.57^{c}
		Gc (EPI)13	ND	ND	16.0±0.00^{a}	15.6±1.15^{a}	ND	ND	35.3±0.57^{e}	26.3±0.57^{c}
		Hp (EPI)1	ND	22.0±1.00^{b}	18.0±1.00^{a}	ND	ND	ND	41.6±1.15^{gh}	ND
		Ed(EPI)14	31.6±1.15^{f}	ND	ND	ND	ND	31.6±1.15^{f}	ND	20.0±1.00^{a}
	Brown	So (END)21	ND	ND	18.0±0.00^{a}	ND	ND	ND	43.3±0.57^{h}	26.3±0.57^{c}
		Hcl(EPI)14	19.0±0.00^{b}	31.3±0.57^{e}	16.3±1.52^{a}	17.3±1.52^{b}	18.6±0.57^{a}	19.0±1.73^{b}	45.6±0.57^{i}	26.3±1.52^{c}

Means with the same letters in each column are not significantly different at P<0.05, ND=not detectable.

Key for identifying the isolate codes: the alphabetical letters represent the genus and species of the seaweed where the bacteria isolate was obtained; Gc-*Gracilaria corticata*, So-*Sargassum oligocystum*, Hcl-*Hydroclathrus clathratus*, Gs-*Gracilaria salicornia*, As-*Acanthophora spicifera*, Cmex-*Caulerpa mexicana*, Td-*Turbinaria decurrens*, Scr-*Sargassum cristaefolium*, Hmu-*Hypnea musciformis*, Hp-*Hypnea pannosa*, Cp-*Chondrophycus papillosus*, Ul-*Ulva lactuca*, Ur-*Ulva reticulata*, Pt-*Padina tetrastromatica*, Sr-*Solieria robusta* and Ssp-*Sargassum* sp., Cmy- *Cystoseira myrica* , Ct-*Cystoseira trinodis* , Lsp- *Laurencia* sp., Ka-*Kappaphycus alvarezii*, Ed- *Eucheuma denticulatum.* END=Endophyte, EPI=Epiphytes. Numerals represent the colony number.

In the present study, the most active isolates that inhibited the eight test pathogens were ten and included; Cp(EPI)14b, Ur(EPI)15, Pt(EPI)15, Pt(EPI)25, Pt(EPI)26, Hmu(EPI)13, Hmu(EPI)26, Hp(EPI)8, Gc(EPI)1 and Hcl(EPI)14 (Table 4.3).

Among the 69 isolates, significantly higher antimicrobial activity was exhibited by the Gram-positive pathogenic bacteria (16.64±9.81), than the Gram-negative bacterial pathogens (12.37±6.94) ($P<0.001$). Meanwhile, the active isolates exhibited higher inhibition against fungal pathogens (20.96±11.87) than bacterial pathogens (14.50±8.75) ($P<0.001$). A higher significant antimicrobial activity was observed in epiphytic bacterial isolates (19.57±10.67) than that of endophytic isolates (16.57±10.93) ($P<0.005$).

A significant difference in inhibition of isolates against the test pathogens was observed among the three sites ($F_{2,\ 1653} = 10.04$, $P<0.001$), with Mtwapa having a mean of 18.13±10.58, Mkomani, 18.94±10.83 and Kibuyuni, 16.06±11.27. No difference was obtained among the three algal divisions. Among the fungal pathogens, *Trichophyton metagrophyte* was the most susceptible with a mean inhibition (32.26±11.21), while the most resistant was *Candida albicans* (22.00±10.63). Similarly, Methicillin Resistant *Staphylococcus aureus* (MRSA) was the most susceptible bacterial pathogen with a mean of 19.56±9.60, while *Salmonella typhi* was the most resistant (11.18±6.58). A significant interaction in antimicrobial activity of bacterial isolates against test pathogens between the sites and algal divisions was observed ($F_{3,\ 1648} = 4.29$, $P<0.005$).

The antagonistic activity of the isolates was indicated by the clear zone of inhibition around the paper disc as shown in Plates 4.5 A, B, C, D and E.

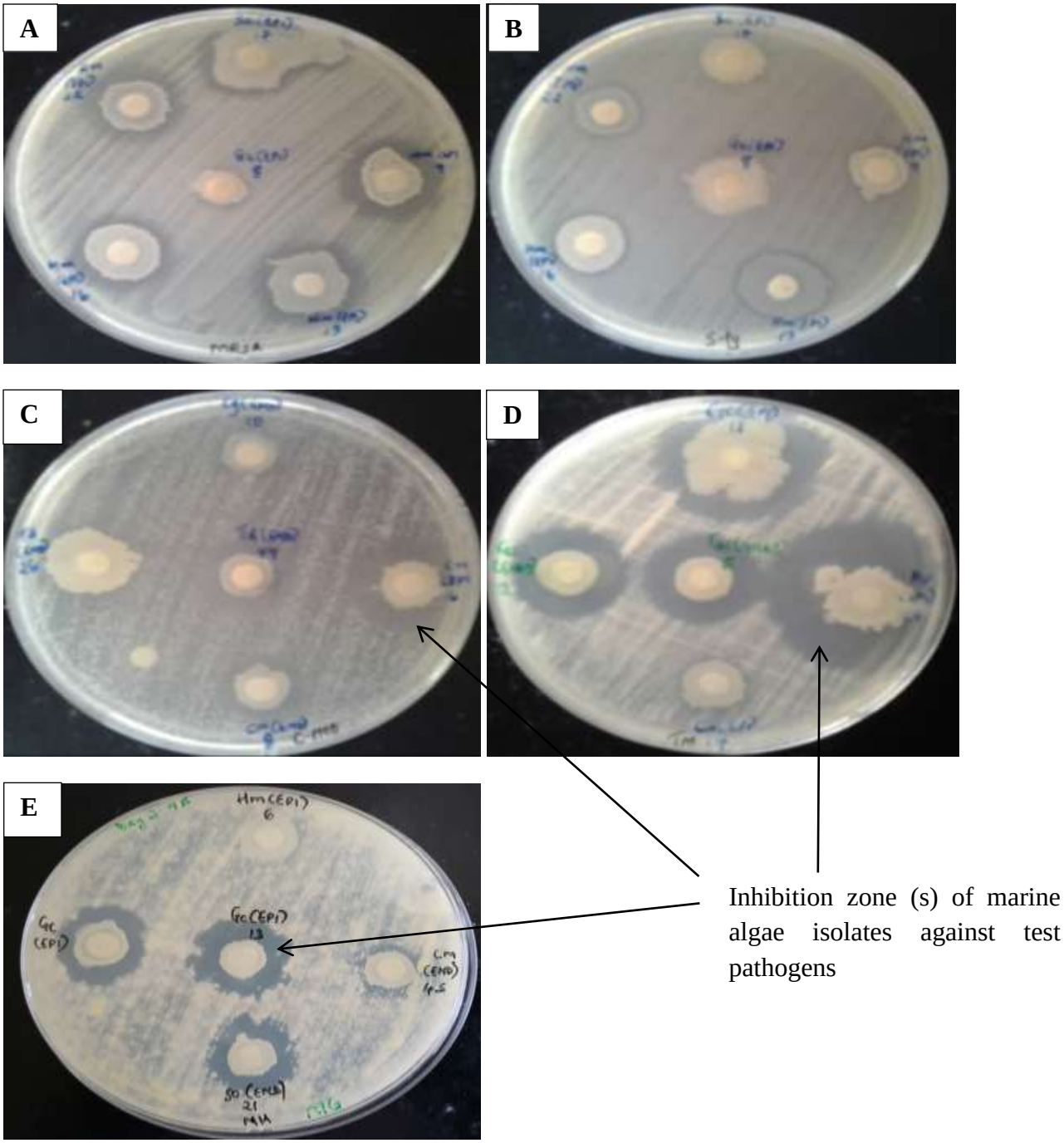

Plate 4.5: Antagonistic activity of isolates from marine algae against (A) Methicillin resistant *Staphylococcus aureus*, (B) *Salmonella typhi*, (C) *Cryptococcus neoformans*, (D) *Trichophyton metagrophyte* and (E) *Microsporum gypseum*.

4.2.2 Cytotoxic screening

The 69 bacterial isolates that had a broad spectrum antimicrobial activity (Table 4.3) were screened for cytotoxicity against larynx Hep-2 carcinoma cells and vero (normal) cells. Thirty-three (33) isolates (48%) exhibited considerable activity against the cancer cells. The 33 active bacterial isolates were chosen for more detailed study and to identify their phylogenetic position through 16S rDNA analysis. The cytotoxic activity of the bacterial isolates can be seen by the reaction of the purple and yellow precipitate as shown in Plates 4.6 A, B and C. The wells with the purple precipitate indicates that there was no inhibition (cytotoxic effect) by the bacterial isolates while the wells with the yellow precipitate is an indication of inhibition (cytotoxity) by the bacterial isolates.

4.3 Identification of the active bacterial isolates

The active bacterial isolates (above) were subjected to Gram staining and taken through a series of biochemical tests to determine their physiological characteristics. Molecular characterization was also performed to identify the active colonies to species level.

4.3.1 Morphological identification

Gram staining showed that 88% of the thirty-three active bacterial isolates were Gram positive while the rest were Gram negative (Table 4.4), with the majority (91%) being rod shaped. The colony color varied from white, cream white and medium white, with only one yellowish colony (Table 4.4). Growth forms also varied from rough, mucoid, smooth to dry while the varying margins were either irregular, rhizoid, filamentous, circular or punctiform (Table 4.4).

4.3.2 Biochemical identification

Biochemical tests of the thirty-three active bacterial isolates showed that all the isolates had the ability to ferment the sugar glucose as a source of energy in their environment while nineteen isolates were able to utilize citrate as an energy source (Table 4.5).

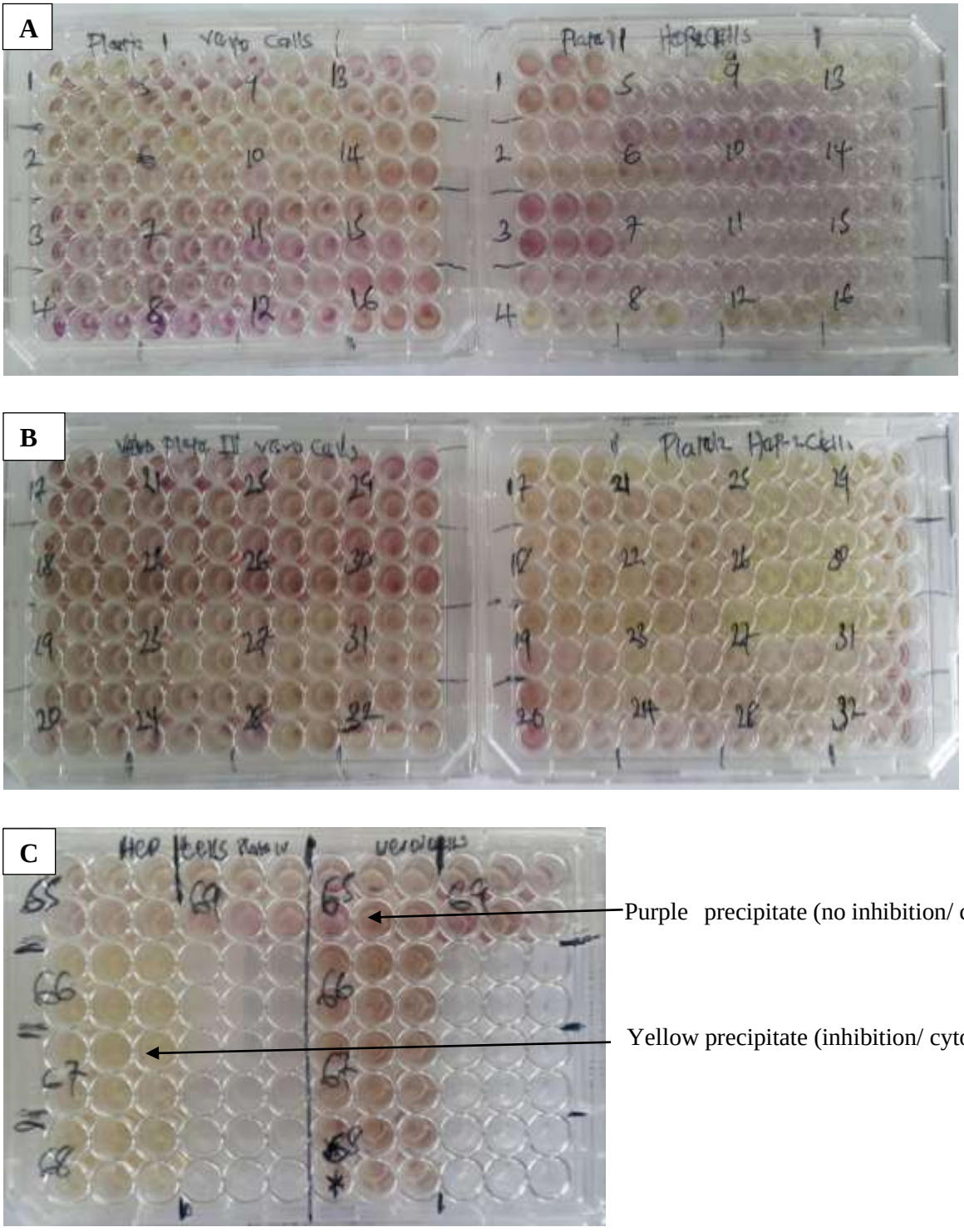

Plate 4.6: Cytotoxic effects against vero (normal) and larynx Hep-2 cells by bacterial isolates (A)1-16, (B) 17-32, (C) 65-69.

Table 4.4: Morphological characteristics of the 33 active bacterial isolates from Kenyan seaweeds.

Isolate	Colony color	Growth form	Shape	Gram reaction
Gc(EPI)1	Cream white	Mucoid, Irregular	Rods	+
So(END)21	Cream white	Mucoid, circular, smooth	Rods	+
Hcl(EPI)14	Cream white	Rough, Rhizoid	Rods	-
Gs(END)43	Cream white	Mucoid, Rhizoid	Rods	+
As(END)24	Cream white	Mucoid, Circular	Rods	+
As(END)28	Medium white	Smooth, punctiform	Rods	+
Cmex(END)8	White	Mucoid,wrinkled,Irregular	Rods	+
Td(END)26	White	Mucoid, Filamentous	Rods	+
Scri(EPI)17	Cream white	Mucoid, Circular	Rods	+
Hmu(EPI)9	Cream	Mucoid, Circular	Rods	+
Hmu(EPI)16	Cream white	Mucoid, Irregular	Rods	+
Hmu(EPI)22	White	Dry, Filamentous	Filamentous	+
Gc(EPI)12	Medium white	Smooth, Circular	Rods(chains)	+
Hp(EPI)19	White	Dry, Filamentous	Filamentous	+
Gs(EPI)21	Medium white	Smooth, Circular	Rods(chains)	+
Gs(EPI)22	Cream white	Mucoid, Circular	Rods	+
As(END)10	White	Mucoid, Filamentous	Rods	+
Cp(EPI)12	Medium white	Smooth, Circular	Rods(chains)	+
Ul(EPI)4	Medium white	Dry, Rhizoid	Rods(chains)	-
Ul(EPI)7	White	Mucoid, Filamentous	Rods	+
Ur(EPI)15	White	Dry, Filamentous	Filamentous	+
Ur(EPI)16	Medium white	Mucoid, Circular	Rods	+
Ur(EPI)24	White	Mucoid, irregular	Rods	+
Pt(EPI)15	White	Mucoid, Filamentous	Rods	+
Pt(EPI)25	White	Mucoid, Filamentous	Rods	+
Sr(END)7	Medium white	Smooth, Circular	Rods(chains)	+
Sr(END)9	Cream white	Mucoid, Irregular	Rods	+
Gs(END)8	Medium white	Smooth, Circular	Rods(chains)	+
As(END)2	Yellowish	Mucoid, Smooth , Circular	Rods	+
As(END)10	Cream white	Mucoid, Irregular	Rods	+
As(END)15	White	Mucoid, Filamentous	Rods	+
Ssp(END)30a	Medium white	Dry, Rhizoid	Rods	-
Ssp(END)32b	Medium white	Dry, Rhizoid	Rods	-

Key for identifying the isolate codes: the alphabetical letters represent the genus and species of the seaweed the isolate was obtained from; Gc-*Gracilaria corticata*, So-*Sargassum oligocystum*, Hcl-*Hydroclathrus clathratus*, Gs-*Gracilaria salicornia*, As-*Acanthophora spicifera*, Cmex-*Caulerpa mexicana*, Td-*Turbinaria decurrens*, Scri-*Sargassum cristaefolium*, Hmu-*Hypnea musciformis*, Hp-*Hypnea pannosa*, Cp-*Chondrophycus papillosus*, Ul-*Ulva lactuca*, Ur-*Ulva reticulata* Pt-*Padina tetrastromatica*, Sr-*Solieria robusta* and Ssp-*Sargassum* sp. END=Endophyte, EPI=Epiphytes. Numerals represent the colony number.

Table 4.5: Biochemical reactions of the 33 active bacterial isolates from Kenyan seaweeds.

	TSI (Triple Sugar Iron)					SIM (Sulphur, Indole, Motility)								
ISOLATE CODE	Butt	Slant	Gas	H_2S	SC	Sulphur	Indole	Motility	Urease	Gelatin hydrolysis	Catalase	Mannitol	Oxidase	MR
1.Gc(EPI)1	+	-	-	-	+	-	-	-	$+^w$	-	+	+	-	-
2.So(END)21	+	+	-	-	-	-	-	-	-	-	+	-	+	-
3.Hcl(EPI)14	+	-	-	-	-	-	-	+	-	-	+	-	+	+
4.Gs(END)43	+	-	-	-	-	-	-	-	$+^w$	+	+	+	-	-
5.As(END)24	+	+	-	-	+	-	-	-	-	-	+	+	-	-
6.As(END)28	+	-	-	-	+	-	-	-	$+^w$	+	+	+	+	$+^w$
7.Cmex(END)8	+	-	-	-	+	-	-	-	$+^w$	+	+	+	+	-
8.Td(END)26	+	-	-	-	-	-	-	-	$+^w$	+	+	+	+	-
9. Scri(EPI)17	+	+	-	-	+	-	-	-	$+^w$	-	+	-	-	-
10.Hmu(EPI)9	+	+	-	-	+	-	-	-	$+^w$	-	+	+	-	-
11.Hmu(EPI)16	+	-	-	-	+	-	-	-	$+^w$	+	+	+	-	-
12.Hmu(EPI)22	+	-	-	-	-	-	-	-	$+^w$	+	+	+	+	-
13.Gc(EPI)12	+	+	-	-	+	-	-	-	-	+	+	-	-	-
14.Hp(EPI)19	+	-	-	-	-	-	-	-	$+^w$	+	+	+	+	-
15.Gs(EPI)21	+	+	-	-	+	-	-	-	-	+	+	+	+	-
16.Gs(EPI)22	+	-	-	-	+	-	-	-	-	-	+	-	-	$+^w$
17.As(END)10	+	-	-	-	-	-	-	-	-	+	+	-	-	-
18.Cp(EPI)12	+	-	-	-	+	-	-	-	-	+	+	+	-	-
19.Ul(EPI)4	+	+	-	-	+	-	-	+	-	-	+	-	+	+
20.Ul(EPI)7	+	+	-	-	+	-	-	+	-	-	+	+	-	+
21.Ur(EPI)15	+	-	-	-	-	-	-	+	$+^w$	-	+	+	-	-
22.Ur(EPI)16	+	+	-	-	-	-	-	-	-	-	-	-	+	+
23.Ur(EPI)24	+	+	-	-	-	-	-	-	$+^w$	+	+	+	+	-
24.Pt(EPI)15	+	+	-	+	-	+	-	+	-	+	+	+	-	-
25.Pt(EPI)25	+	+	-	+	+	+	-	+	-	+	+	+	+	-
26.Sr(END)7	+	+	-	-	+	-	-	-	$+^w$	+	+	+	+	-
27.Sr(END)9	+	-	-	-	+	-	-	-	-	-	+	+	-	-
28.Gs(END)8	+	-	-	-	+	-	-	+	+	+	+	+	+	-
29.As(END)2	+	-	-	-	-	-	-	-	$+^w$	+	+	+	+	$+^w$
30.As(END)10	+	-	-	-	+	-	-	-	-	+	+	+	+	-
31.As(END)15	+	+	-	-	+	-	-	-	-	-	+	+	-	-
32Ssp(END)30a	+	+	-	-	-	-	+	+	-	-	+	-	+	+
33.Ssp(END)32b	+	+	-	-	-	-	-	+	-	-	+	-	+	+

Biochemical test results for the 33 isolates defined as (+) positive for the reaction, (-) negative for the reaction and ($+^{w}$) Weak positive. Key for identifying the isolate codes: the alphabetical letters represent the genus and species of the seaweed the isolate was obtained; Gc-*Gracilaria corticata*, So-*Sargassum oligocystum*, Hcl-*Hydroclathrus clathratus*, Gs-*Gracilaria salicornia*, As-*Acanthophora spicifera*, Cmex-*Caulerpa mexicana*, Td-*Turbinaria decurrens*, Scri-*Sargassum cristaefolium*, Hmu-*Hypnea musciformis*, Hp-*Hypnea pannosa*, Cp-*Chondrophycus papillosus*, Ul-*Ulva lactuca*, Ur-*Ulva reticulata* Pt-*Padina tetrastromatica*, Sr-*Solieria robusta* and Ssp-*Sargassum* sp. END=Endophyte, EPI=Epiphytes. Numerals represent the colony number. Biochemical media; TSI-Triple sugar iron, H_2S-Hydrogen sulfide, SC- Simmons' citrate, SIM- Sulfur indole motility and MR-Methyl red.

4.3.3 Molecular identification

PCR amplification of 16S rDNA gene from the isolates

Genomic DNA was successfully extracted from all the 33 active bacterial isolates. A 16S rDNA amplification with bacterial based primers specific for this region of DNA yielded an amplification product of approximately 1000 base pairs (bp) from all the 33 isolate samples as shown in Plate 4.7.

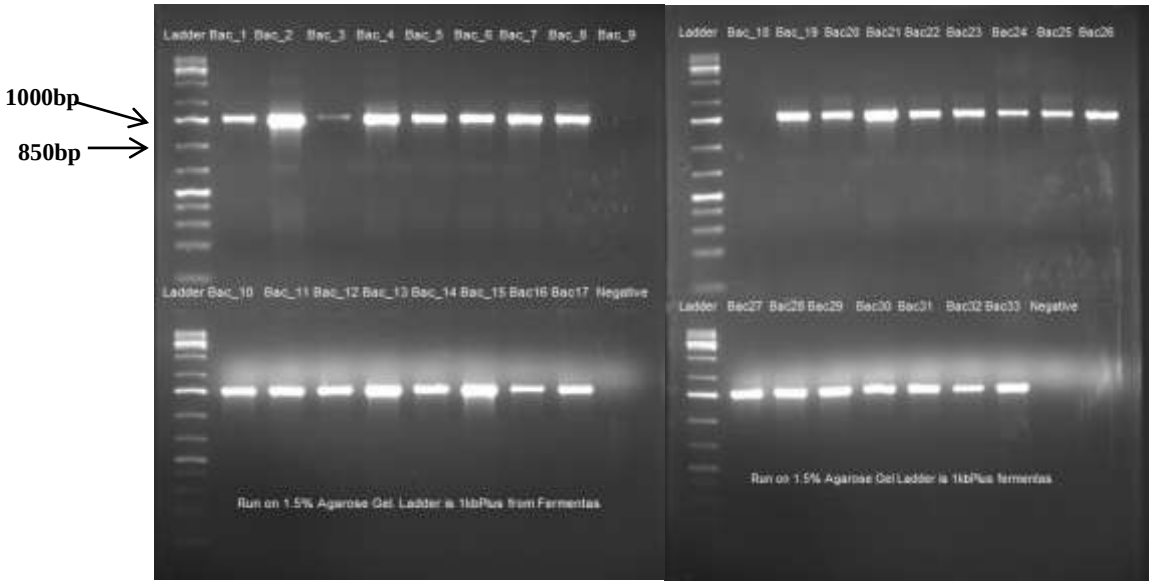

Plate 4.7: A 1.5% agarose gel showing PCR amplification of 16S rDNA of the isolates visualized after ethidium bromide staining. Bacteria sample 1-33 and negative, control respectively. DNA ladder of 1000bp plus used as a molecular marker.

Phylogenetic cluster analysis of sequences

The 16S rDNA amplified products from the thirty-three bacterial isolates were sequenced. Similarity searching using Basic Local Alignment Search Tool (BLAST) shows that they belong to the domain Bacteria with a high sequence similarity of between 85-99%. The phylogenetic tree showed three main phyla namely Firmicutes (79%), Proteobacteria (12%) and Actinobacteria (9%) (Figure 4.1).

The genus Bacillus was the most dominant (79%), while the least was the genus Massilia (3%). The genus Streptomyces and Desulfovibrio had nine percent (9%) dominance, respectively (Appendix A).

From *Methanoculleus thermophile* that was the out-group, the first cluster consisted of isolate HclEPI14, an epiphyte isolated from brown seaweed *Hydroclathrus clathratus,* clustered with the soil bacteria, *Massilia* sp. and this was supported with a bootstrap value of 100% and sequence identity of 85%.

In the second cluster, isolate UlEPI4, an epiphyte from green seaweed, *Ulva lactuca*, isolates SspEND30a and SspEND32b endophytes both from brown seaweed *Sargassum* sp. clustered closely with marine bacteria *Desulfovibrio vulgaris* with sequence identity of 99%, 98% and 96%, respectively. Isolate UrEPI16, an epiphyte from green seaweed *Ulva reticulata* clustered distantly with water bacterium *Bacillus cereus* with sequence identity of 97% and a bootstrap value of 100%.

The third cluster consisted of isolate PtEPI15 and PtEPI25 both epiphytes from brown seaweed *Padina tetrastromatica* whose close relative was a marine bacteria *Bacillus* sp. with a 98% identity and supported by bootstrap value of 72%.

Figure 4.1: Phylogenetic positions of the 33 bacterial isolates with broad-spectrum bioactivity. The scale bar indicates approximately 5% sequence difference. Bootstrap values are reported as percentages of 1000 bootstrap replications.

In the fourth cluster, most isolates clustered with the genus Bacillus with a bootstrap value of 78%. In this cluster, isolate UlEPI7, an epiphyte from the green seaweed *Ulva lactuca* was highly similar to *Bacillus* sp. with a sequence identity of 98% while isolate SrEND9, an endophyte from red seaweed *Solieria robusta* was similar to *Bacillus pumilus* at 99% identity. Isolate AsEND28, an endophyte from red seaweed, *Acanthophora spicifera*, GcEPI1 an epiphyte from red seaweed *Gracilaria corticata* and HmuEPI9, an epiphyte from red seaweed, *Hypnea musciformis* were similar to *Bacillus aerius* with a 99% sequence identity while isolate ScriEPI17, an epiphyte from brown seaweed *Sargassum cristaefolium* had a 99% identity with the marine sponge bacterium *Bacillus safensis*. Isolate AsEND15, an endophyte from red seaweed, *A. spicifera* clustered with *Bacillus* sp. from marine sediment with a 97% sequence identity; isolate GsEPI22 an epiphyte from red seaweed, *Gracilaria salicornia* was similar to *Bacillus safensis* with 98% similarity; isolate AsEND10(2), an endophyte from red seaweed, *Acanthophora spicifera* clustered with the soil bacterium *B. pumilus* at 98% sequence similarity. Isolate AsEND24 an endophyte from red seaweed, *A. spicifera* was similar to *B. safensis* which is a bacterium from the marine sediment with 98% sequence identity.

The fifth cluster consisted of *Bacillus* and *Streptomyces* strains at a bootstrap value of 98%. The cluster also comprised of isolate UrEPI24, an epiphyte from the green seaweed *Ulva reticulata* that clustered with *Bacillus methylotrophicus* at 97% sequence identity while isolate AsEND10, an endophyte from red seaweed *A. spicifera* clustered with *Bacillus* sp. associated with the soft corals at 99% sequence similarity. Isolate HmuEPI22, an epiphyte from the red seaweed *Hypnea musciformis* was similar to soil *Streptomyces* sp. at 98% sequence identity while isolate AsEND2, an endophyte from red seaweed *A. spicifera* clustered with *Bacillus tequilensis* an isolate from marine fish at 98% sequence identity. Isolate GsEND43, an endophyte from red seaweed *Gracilaria salicornia* was similar to *Bacillus amyloliquefaciens* at 99% sequence identity while isolate HpEPI19, an epiphyte from red seaweed *Hypnea pannosa* clustered with *Streptomyces* sp. with a 98% sequence similarity.

Isolate HmuEPI16, an epiphyte from red seaweed *H. musciformis* clustered with *Bacillus velezensis* at 97% sequence identity while isolate CmexEND8, an endophyte from green seaweed *Caulerpa mexicana* clustered with *Bacillus axarquiensis* at a 99% sequence similarity. Isolate UrEPI15, an epiphyte from green seaweed *U. reticulata* clustered with *Streptomyces* sp. at 98% sequence identity while isolate TdEND26, an endophyte from brown seaweed *Turbinaria decurrens* was similar to *Bacillus* sp. from mangrove soil at 98% sequence similarity. Lastly, isolates GsEND8, an endophyte from red seaweed *G. salicornia*, GsEPI21, an epiphyte from the red seaweed *G. salicornia*, CpEPI12, an epiphyte from red seaweed *Chondrophycus papillosus* and SrEND7, an endophyte from the brown seaweed *Solieria robusta* were similar to *Bacillus subtilis* at 99% sequence identity while isolate GcEPI12, an epiphyte from red seaweed *G. corticata* clustered with *B. subtilis* at 98% sequence identity and isolate SoEND21, an endophyte from brown seaweed *Sargassum oligocystum* was similar to *Geobacillus stearothermophilus* from mangrove sediments at 99% sequence identity (Figure 4.1). The key bacterial species identified that are uniquely associated to seaweeds include; Firmicutes (*Bacillus safensis*, *Bacillus axarquiensis*, *Bacillus velezensis*, *Bacillus methylotrophicus*, *Bacillus tequilensis* and *Geobacillus stearothermophilus)* and Proteobacteria *(Desulfovibrio vulgaris* and *Massilia* sp.).

4.4 Extraction and bioassay guided fractionation of secondary metabolites

4.4.1 Extraction

The secondary metabolites of the 33 bacterial culture fermentations were extracted using ethyl acetate. Different extracts yielded different quantities (weights) of the secondary metabolites (Table 4.6). The yields ranged from 5 g to 28 g.

Table 4.6: Weights (g) of extracts from 33 bacterial isolates of Kenyan seaweeds.

Sample No.	Isolate code	Strain	Weight(g)
1	Gc(EPI)1	*Bacillus aerius*	24
2	So(END)21	*Geobacillus stearothermophilus*	15
3	Hcl(EPI)14	*Massilia* sp.	27
4	Gs(END)43	*Bacillus amyloliquefaciens*	20
5	As(END)24	*Bacillus safensis*	20
6	As(END)28	*Bacillus aerius*	15
7	Cmex(END)8	*Bacillus axarquiensis*	16
8	Td(END)26	*Bacillus* sp.	18
9	Scri(EPI)17	*Bacillus safensis*	10
10	Hmu(EPI)9	*Bacillus aerius*	18
11	Hmu(EPI)16	*Bacillus velezensis*	20
12	Hmu(EPI)22	*Streptomyces* sp.	10
13	Gc(EPI)12	*Bacillus subtilis*	8
14	Hp(EPI)19	*Streptomyces* sp.	18
15	Gs(EPI)21	*B.subtilis*	8
16	Gs(EPI)22	*Bacillus safensis*	25
17	As(END)10	*Bacillus* sp.	12
18	Cp(EPI)12	*Bacillus subtilis*	20
19	Ul(EPI)4	*Desulfovibrio vulgaris*	10
20	Ul(EPI)7	*Bacillus* sp.	12
21	Ur(EPI)15	*Streptomyces* sp.	11
22	Ur(EPI)16	*Bacillus cereus*	10
23	Ur(EPI)24	*Bacillus methylotrophicus*	12
24	Pt(EPI)15	*Bacillus* sp.	11
25	Pt(EPI)25	*Bacillus* sp.	21
26	Sr(END)7	*Bacillus subtilis*	23
27	Sr(END)9	*Bacillus pumilus*	19
28	Gs(END)8	*Bacillus subtilis*	28
29	As(END)2	*Bacillus tequilensis*	20
30	As(END)10	*Bacillus pumilus*	21
31	As(END)15	*Bacillus* sp.	5
32	Ssp(END)30a	*Desulfovibrio vulgaris*	18
33	Ssp(END)32b	*Desulfovibrio vulgaris*	20

Key for identifying the isolate codes: the alphabetical letters represent the genus and species of the seaweed the isolate was obtained. END=Endophyte, EPI=Epiphytes. Numerals represent the colony number.

4.4.2 Bioassay guided fractionation

The bacterial crude extracts were purified using column chromatography with six fraction elutions for each of the thirty-three (33) bacterial extracts, to produce 198 fractions in total.

4.5 Antimicrobial and cytotoxic screening of fractionated secondary metabolites

The 198 fraction samples obtained in section 4.4 were screened for antimicrobial and antitumor activity against eight pathogenic microorganisms and larynx Hep-2 cancer cell (See section 3.4.1).

4.5.1 Antimicrobial assay

Thirteen sample fractions showed a broad-spectrum activity against three or more test pathogens as shown in Table 4.7. The most active fraction that inhibited most of test pathogens (six out of eight) were fraction one of Ul(EPI)7, Ur(EPI)24 and fraction two of Cmex(END)8 (Table 4.7).

Among the 13 bacterial fractions, significantly higher antimicrobial activity was exhibited by Gram-negative bacterial pathogens (13.32±6.09), than the Gram-positive bacterial pathogens (9.71±5.27) ($P<0.001$). No significant difference in inhibition was observed among the bacterial and fungal pathogens, epiphytic and endophytic fraction extracts. Among the fungal pathogens, *Candida albicans* and *Cryptococcus neoformans* were the most susceptible with mean inhibitions of 13.46±7.82 and 12.64±6.65 respectively, while the resistant were *Trichophyton metagrophyte* (11.87±7.20) and *Microsporum gypseum* (11.26±6.32). Similarly, *Escherichia coli* was the most susceptible bacterial pathogen with a mean of 16.05±4.83, while *Staphylococcus aureus* and *Salmonella typhi* were the most resistant with means of 8.56±4.81 and 10.33±4.98, respectively.

Comparing the positive control and the bacterial extracts against the test pathogens, the extracts produced potent activity against *Staphylococcus aureus, Salmonella typhi, Candida albicans* and *Cryptococcus neoformans.*

Table 4.7: Antimicrobial activity (inhibition zone: mm) of fractionated bacterial extracts from Kenyan seaweeds against eight pathogenic microorganisms (mean ± SD, n = 3).

		Inhibition zones(mm)							
Samples		**Gram positive**		**Gram negative**		**Yeast**		**Molds**	
Isolate code	Fraction Number	*Staphylococcus aureus*	Methicillin Resistant *Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhi*	*Candida albicans*	*Cryptococcus neoformans*	*Trichophyton metagrophyte*	*Microsporum gypseum*
As(END)28	1	ND	20±2.00^{b}	19.3±1.52^{c}	ND	15.00±2.00^{a}	ND	ND	ND
Hp(EPI)19	1	ND	ND	14±1.00^{a}	13.6±1.15^{a}	ND	17±1.00^{b}	ND	ND
Ul(EPI)7	1	ND	16.6±1.15^{a}	15.67±1.15^{ab}	15.0±1.00^{a}	ND	23±2.00^{d}	15.0±1.00^{a}	16.6±1.15^{b}
Ur(EPI)24	1	ND	ND	14.3±0.57^{a}	13.6±1.15^{a}	15.0±1.00^{a}	15.6±1.15^{a}	16.6±1.15^{a}	15.3±1.52^{a}
Ssp(END)30a	1	ND	17.0±1.00^{a}	17.6±1.52^{b}	ND	23.3±1.52^{d}	ND	ND	24.0±2.64^{d}
Ssp(END)32b	1	ND	ND	16.6±1.52^{b}	ND	18±2.00^{b}	18.0±2.64^{b}	ND	ND
As(END)10	2	ND	15.0±2.00^{a}	16.6±1.15^{b}	14.6±1.52^{a}	ND	22.0±1.73^{c}	ND	ND
Cmex(END)8	2	16.3±1.52^{a}	14.3±0.57^{a}	28.3±1.52^{e}	19.3±0.57^{c}	17.3±1.52^{b}	17.6±1.52^{b}	ND	ND
Hmu(EPI)22	2	ND	ND	15.0±1.00^{a}	ND	ND	ND	21.6±1.15^{c}	20±1.73^{c}
Pt(EPI)15	3	18.0±2.00^{a}	16.0±2.0^{a}	16.6±1.52^{b}	ND	ND	ND	16.3±1.52^{a}	13.3±0.57^{a}
Cp(EPI)12	4	ND	ND	13.67±1.15^{a}	ND	ND	ND	27.6±2.08^{e}	15.0±1.00^{a}
Gs(END)8	5	17.0±2.00^{a}	ND	14.6±1.52^{a}	ND	22.3±1.00^{c}	15.0±1.00^{a}	ND	ND
Pt(EPI)25:	5	ND	ND	ND	16.0±2.00^{b}	28.0±2.00^{e}	ND	15.0±1.00^{a}	ND
Antibiotic (Positive control)		**ND**	**30.3±2.51**	**31.3±3.21**	**ND**	**14.3±1.52**	**14.3±2.08**	**23.3±2.51**	**24±2.00**

Means with the same letters in each column are not significantly different at P<0.05, ND=not detectable.

4.5.2 Cytotoxic assay

Thirteen sample fractions showed cytotoxicity (anticancer activity) against Hep-2 larynx cells with lower IC_{50} values compared to vero cells and at different concentrations (Table 4.8). The fractions were more potent against the Hep-2 cell line compared to the normal (vero) cell line ($P<0.05$). Comparing Rapamycin (positive control) with the bacterial fractions (Table 4.8), concentration of the fractions influenced cytotoxicity activity on both the Hep-2 and vero cell lines. For example, Cp(EPI)12 fraction 4 had the same concentration (35mg/ ml) as Rapamycin had better cytotoxity on the Hep-2 cell line (14.48 mg/ ml^{-1}) (Table 4.8). Graphs on calculation of effective dilution (50%) of the fractionated extracts against Hep-2 cell line and vero cell line are shown in Appendix B.

Table 4.8: Cytotoxicity of fractionated bacterial extracts from Kenyan seaweeds against Hep-2 cell line and vero cell line.

Samples		Concentration	IC_{50} Values(mg/ml^{-1})	
Isolate code	**Fraction number**	**(mg/ml)**	**Hep-2 cells**	**Vero cells**
As(END)28	1	68	26.89	36.24
Hp(EPI)19	1	97	0.24	7.94
Ul(EPI)7	1	68	13.22	58.54
Ur(EPI)24	1	85	0.28	1.09
Ssp(END)30a	1	57	10.97	13.34
Ssp(END)32b	1	70	0.92	30.24
As(END)10	2	2	0.92	1.92
Cmex(END)8	2	25	1.21	15.91
Hmu(EPI)22	2	43	5.07	17.06
Pt(EPI)15	3	20	3.95	6.13
Cp(EPI)12	4	35	14.48	21.38
Gs(END)8	5	63	50.01	55.22
Pt(EPI)25	5	28	17.97	24.54
Rapamycin(Positive control)		**35**	**6.00**	**23.67**

Key for identifying the isolate codes: the alphabetical letters represent the genus and species of the seaweed the isolate was obtained. END=Endophyte, EPI=Epiphytes. Numerals represent the colony number. Fraction number represents the fraction used based on the solvent elution ratio (ethyl acetate: methanol), where 1= 50:50, 2=40:60, 3=30:70, 4=20:80 and 5=10:90.

4.6 Identification of the active secondary metabolites

Thirteen fractionated secondary metabolites responsible for antimicrobial and antitumor activities were analyzed and characterized by Gas Chromatography Mass Spectrometer (GC-MS). For GC-MS chromatograms, see Appendix C. Different compounds were detected in the different fractions associated with bacteria from Kenya seaweed as shown in Tables 4.9-4.21.

Fraction one

The most abundant compound produced by isolate As(END)28) that was similar to *Bacillus aerius* was propanoic acid, ethyl ester (29.79%), followed by alkaloids pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (20.93%) and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)- (18.27%) (Table 4.9). For isolate Hp(EPI)19) identified as *Streptomyces* sp., indole (43.58%) was the most abundant compound followed by alkaloid, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)- (23.99%) (Table 4.10). Compounds produced by isolate Ul(EPI)7 identified as *Bacillus* sp. showed that, phenol was the most abundant at 36.24%, followed by indole (35.28%) and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)- an alkaloid at 16.32% (Table 4.11). Indole was still the most abundant compound (80.43%) produced by isolate Ur(EPI)24 identified as *Bacillus methylotrophicus*, followed by alkaloid pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)- (12.60%) as shown in Table 4.12.

For isolates Ssp(END)30a and Ssp(END)32b both identified as *Desulfovibrio vulgaris*, the compound indole was the most abundant at 61.6% and 49.89%, respectively (Tables 4.13 and 4.14). Alkaloids pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)- were the second most abundant compounds at 14.70% (Table 4.13) and 10.66% (Table

4.14). The most notable compounds in fraction one, for the isolates were indole and pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)-.

Table 4.9: Compounds identified from isolate As(END)28 (*Bacillus aerius*) of Kenyan seaweeds: Fraction one.

Retention time (min)	Compound name	Compound derivative	% abundance
5.10	=Propanoic acid, ethyl ester	Ester	29.79
6.46	=Propanoic acid, 2-methyl-	Carboxylic acid	1.14
6.82	=1-Butanol, 2-methyl-, acetate	Carboxylate ester	0.35
7.09	=Ethyl butanoate	Ester	0.73
8.55	=Pentanoic acid, 3-methyl-	Carboxylic acid	3.29
8.84	=Hexanoic acid, 2-methyl-	Carboxylic acid	5.66
20.69	=Uracil	Pyrimidine	3.08
23.60	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Pyrrole, Alkaloid	20.93
25.08	=Cyclohexanone, 5-methyl-2-(1-methylethyl)-, cis-	Cyclic ketone	0.68
25.32	=Cyclohexanone, 5-methyl-2-(1-methylethyl)-, trans-	Cyclic ketone	0.36
25.75	=2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)-	Alkaloid	1.10
26.69	=2,5-Piperazinedione, 3-benzyl-6-isopropyl-	Alkaloid	4.39
27.70	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	Alkaloid (Pyrrole)	18.27
28.30	=2,2'-Diamino-5,5'-dimethoxy-biphenyl	Phenolic	0.44
29.22	=Hexacosane	Alkane	1.17
29.47	=Hexadecene<1->	Alkene	0.45
29.69	=Octadecane,3-methyl-	Alkane	0.34
29.78	=E-8-Methyl-7-dodecen-1-ol acetate	Ester	0.27
29.98	=Heneicosane	Alkane	1.29
30.83	=Tetracosane	Alkane	1.93
31.33	=2,5-Piperazinedione, 3,6-bis(phenylmethyl)-	Alkaloid	1.79
32.56	=2-Piperidinone, N-[4-bromo-n-butyl]-	Alkaloid	0.27
32.69	=Hexadecane, 1-(ethenyloxy)-	Ether	0.37
33.68	=Z-8-Methyl-9-tetradecenoic acid	Carboxylic acid	0.18
34.26	=2-Dodecen-1-yl(-)succinic anhydride	Ketone	0.14
34.46	=Octacosane	Alkane	0.57
35.22	=1-Nonadecene	Alkene	0.14
35.42	=Bicyclo[4.1.0]heptan-2-ol, 3,7,7-trimethyl-, (1.alpha.,2.alpha.,3.beta.,6.alpha.)-	Alcohol	0.12
35.60	=Cyclododecane, ethyl-	Alkane	0.10
35.74	=3-Octadecene, (E)-	Alkene	0.12

Table 4.10: Compounds identified from isolate Hp(EPI)19 (*Streptomyces* sp.) of Kenyan seaweeds: Fraction one.

Retention time (Min)	Compound name	Compound derivative	% abundance
4.96	=Acetic acid	Carboxylic acid	6.05
10.52	=Phenol	Phenolic	13.22
16.12	=Indole	Indole	43.58
16.41	=Benzeneacetonitrile	Benzyl nitrile	1.95
22.01	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	Alkaloid	2.87
23.51	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Alkaloid	8.30
27.81	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	Alkaloid	23.99

Table 4.11: Compounds identified from isolate Ul(EPI)7 (*Bacillus* sp.) of Kenyan seaweeds: Fraction one.

Retention time (Min)	Compound name	Compound derivative	% abundance
11.03	=Phenol	Phenolic	36.24
13.56	=Formic acid phenyl ester	Carboxylic acid	0.28
16.09	=Indole	Indole	35.38
17.46	=Benzene, 1-isocyano-2-methyl-	Phenolic	0.59
22.12	=4-Dodecene	Alkene	0.54
23.58	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Alkaloid	7.31
24.25	=2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4,4-dimethyl-	Phenolic	0.96
24.38	=1,2-Cyclopentanedione, 3,3,5,5-tetramethyl-	Ketone	1.05
27.74	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	Alkaloid	16.32
28.32	=Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.,10.alpha.)-	Alkaloid	1.28

Table 4.12: Compounds identified from isolate Ur(EPI)24 (*Bacillus methylotrophicus*) of Kenyan seaweeds: Fraction one.

Retention time (Min)	Compound name	Compound derivative	% abundance
4.36	=1-Pentanol	Alcohol	0.06
5.93	=Propane, 1,1-dimethoxy-2-methyl-	Alkane (methoxy)	0.09
7.72	=Propenal dimethylhydrazone	Hydrazine(Nitrogen compound)	0.01
10.59	=Dimethyl trisulfide	Sulfide (Sulfur compound)	0.20
11.08	=Phenol	Phenolic	0.16
12.20	=Creatinine	Alkaloid	0.20
13.52	=Disulfide, methyl (methylthio)methyl	Sulfide (Sulfur compound)	0.10
14.46	=4-Acetylbenzoic acid	Benzoic acid	0.26
16.12	=Indole	Indole (Nitrogen compound)	80.43
17.28	=Benzeneacetonitrile	Cyanide	0.07
17.39	=Benzonitrile, 4-methyl-	Cyanide	0.11
17.48	=Benzonitrile, 2-methyl-	Cyanide	0.07
19.23	=1H-Indole-1-carboxaldehyde, 2,3-dihydro-2-hydroxy-	Indole	0.08
19.88	=Benzoic acid, 4-(1H-1,2,3,4-tetrazol-1-yl)-, hydrazide	Azide	0.06
19.97	=Benzonitrile, 3-methyl-	Cyanide	0.05
20.08	=Benzene, 1-azido-2-bromo-	Azide	0.04
22.12	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	Alkaloid	0.28
22.46	=Phenol, 3,5-dimethoxy-	Phenolic	4.00
25.12	=Benzene, 1,4-diethenyl-	Aromatic compound	0.15
25.61	=5-Hydroxy-2,2,6,6-tetramethyl-4-cyclohexene-1,3-dione	Ketone	0.03
26.49	=2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone	Ketone	0.83
27.70	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	Alkaloid	12.60

Table 4.13: Compounds identified from isolate Ssp(END)30a (*Desulfovibrio vulgaris*) of Kenyan seaweeds: Fraction one.

Retention time (Min)	Compound name	Compound derivative	% abundance
4.25	=Pentanone<2->	Ketone	0.07
4.67	=Propanoic acid, ethyl ester	Carboxylic acid	2.96
5.30	=Disulfide, dimethyl	Sulfide (sulfur compound)	0.30
5.57	=2-Pentanone, 3-methyl-	Ketone	0.65
5.79	=sec-Butyl acetate	Ester	1.54
6.80	=Butanoic acid, ethyl ester	Carboxylic acid	0.05
7.11	=Butyl acetate	Ester	0.69
8.12	=Dimethyl Sulfoxide	Sulfoxide (Sulfur compound)	0.49
11.06	=Phenol	Phenolic	2.23
11.97	=6,6-Dimethylcycloocta-2,4-dienone	Ketone	0.23
12.98	=Phenylethyl Alcohol	Alcohol	0.66
14.80	=1-Pentanol	Alcohol	0.06
15.04	=Cyclopropane, 1,2-dimethyl-, trans-	Alkane	0.16
16.16	=Indole	Indole (Nitrogen compound)	61.6
17.39	=Benzene, 1-isocyano-3-methyl-	Cyano	0.14
17.62	=Benzonitrile, 2-methyl-	Nitrile	0.11
17.89	=Benzyl nitrile	Nitrile	0.04
18.02	=1H-Indole-1-carboxaldehyde, 2,3-dihydro-2-hydroxy-	Indole	0.07
18.13	=1-(3-Iodo-phenyl)-1H-tetrazole	Tetrazole (Alkaloid)	0.04
18.29	=Benzonitrile, 2-amino-5-nitro-	Amine (Nitrile)	0.03
18.40	=1,3-Isobenzofurandione, 4-methyl-	Furan, dione	0.03
18.89	=Propyl mercaptan	Thiol,sulfur compound	0.04
19.50	=Etiron	Thiourea (Sulfur compound)	0.05
19.79	=5-Methyl-2-N-methylethylamino-2-thiazoline	thiazoline	0.09
20.96	=Cyclopropane, 1-butyl-2-pentyl-, cis-	Alkane	0.06
21.36	=4-Piperidinecarboxamide, 1-methyl-	Piperidine (Nitrogen compound)	0.47
22.43	=5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one	Furan	3.84
23.64	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Alkaloid	14.70
24.00	=1,2-Cyclopentanedione, 3,3,5,5-tetramethyl-	Ketone	1.071
24.45	=3-Decen-5-one, 2-methyl-	Ketone	0.46
25.08	=2-Dodecene, (E)-	Alkene	0.07
25.28	=Methyl octadecanoate	Ester	0.19
26.13	=1,7-Dimethylene-2,3-dimethylindole	Indole	0.63
26.46	=2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone	Ketone	0.57
27.67	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	Alkaloid	5.33
29.47	=4,6-Dimethoxy-s-triazine-2(1H)-one	Triazines	0.05

Table 4.14: Compounds identified from isolate Ssp(END)32b (*Desulfovibrio vulgaris*) of Kenyan seaweeds: Fraction one.

Retention time (Min)	Compound name	Compound derivative	% abundance
4.45	=Propanoic acid, ethyl ester	Carboxylic acid	2.89
5.17	=1-Butanol, 3-methyl-	Alcohol	6.70
5.43	=3-Hexanone	Ketone	1.70
5.64	=sec-Butyl acetate	Ester	5.65
6.98	=Acetic acid, butyl ester	Carboxylic acid	3.65
8.86	=Dimethyl Sulfoxide	Sulphur compound	0.59
10.99	=Phenol	Phenolic	8.49
13.34	=Phenyl ethyl alcohol	Alcohol	1.89
14.30	=Benzene acetic acid, methyl ester	Carboxylic acid (Ester)	0.93
15.06	=trans-1-Butyl-2-methylcyclopropane	Alkane	0.52
16.14	=Indole	Indole (Nitrogen compound)	49.89
21.16	=4-Piperidinamine, N,1-dimethyl-	Alkaloid	0.13
22.43	=5-Isopropylidene-3,3-dimethyl-dihydrofuran-2-one	Furan	4.41
22.97	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Alkaloid	1.29
24.23	=3-Methyldec-3-ene	Alkene	0.42
24.67	=2-Propanamine, N-(1-methylethyl)-N-nitroso-	Amine	0.09
27.65	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	Alkaloid	10.66

Fraction two

Compounds produced by isolate As(END)10 identified as *Bacillus pumilus*, showed that indole was the most abundant at 46.05%, followed by an ester ethyl propanoate (15.41%) and dimethyl sulfoxide (3.84%) (Table 4.15). For isolate Cmex(END)8 similar to *Bacillus axarquiensis*, only dimethyl sulfoxide compound was detected and had 100% abundance (Table 4.16) while the same compound was the most abundant at 97.10% produced by isolate Hmu(EPI)22 identified as *Streptomyces* sp. (Table 4.17).

Table 4.15: Compounds identified from isolate As(END)10 (*Bacillus pumilus*) of Kenyan seaweeds: Fraction two.

Retention time (Min)	Compound name	Compound derivative	% abundance
4.25	=Isopropyl acetate	Ester	1.42
4.92	=Ethyl propanoate	Ester	15.41
5.17	=(3-Methyl-oxiran-2-yl)-methanol	Alcohol (epoxide)	0.24
5.34	=Ethyl Acetate	Ester	0.06
5.79	=2,3-Pentanedione	Ketone	1.21
6.02	=sec-Butyl acetate	Ester	1.63
7.36	=Acetic acid, butyl ester	Carboxylic acid	0.90
7.74	=Dimethyl Sulfoxide	Sulfoxide (Sulfur compound)	3.84
13.00	=Heptane, 2,4-dimethyl-	Alkane	2.45
15.06	=Heptane, 3,4-dimethyl-	Alkane	0.58
16.07	=Indole	Indole (Nitrogen compound)	46.05
17.28	=Benzene, (isocyanomethyl)-	Cyano methyl	0.14
17.64	=1H-Indazole, 6-nitro-	Indazole	0.11
17.80	=Benzoic acid, 3-(1H-1,2,3,4-tetrazol-1-yl)-, hydrazide	Alkaloid	0.07
17.98	=Benzene, 1-bromo-2-methyl-4-nitro-	Phenolic	0.08
18.29	=Propenamide, 2-cyano-3-(2-furyl)-N-(2-pyridyl)-	Pyridine	0.11
18.72	=Oxalic acid, cyclohexylmethyl nonyl ester	Carboxylic acid (Ester)	0.74
19.50	=Carbonic acid, decyl propyl ester	Carbonic acid (Ester)	0.25
19.88	=Oxalic acid, allyl dodecyl ester	Carboxylic acid (Ester)	0.45
19.97	=2-Thiopheneacetic acid, 4-tridecyl ester	Thiophene	0.36
21.16	=3-Cyclohexen-1-ol, 3-methyl-	Alcohol	1.57
21.38	=trans-1,2-Diethyl cyclopentane	Alkane	0.39
21.49	=Heptadecyl heptafluorobutyrate	Ester	0.22
21.60	=N,N'-Dibutylidene-hydrazine	Azine	0.23
21.78	=(S)-(+)-6-Methyl-1-octanol	Alcohol	0.18
22.10	=Tetradecane, 1-fluoro-	Fluoroalkane	0.15
22.19	=Tridecane	Alkene	0.13
22.39	=3-Dodecylcyclohexanone	ketone	0.08
22.81	=Bicyclo[3.1.0]hexan-2-ol	Alcohol	0.10
22.90	=3-Furanmethanol	Furan	0.23
23.13	=2-Undecene, 2,5-dimethyl-	Alkene	0.34
23.19	=Cyclohexane, 1,2,3-trimethyl-, (1.alpha.,2.alpha.,3.alpha.)-	Cycloalkane	0.23
23.40	=Methyl palmitate (Methyl hexadecanoate)	Ester	4.11
23.64	=Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	Alkaloid	2.66
24.00	=Sulfurous acid, cyclohexylmethyl hexadecyl ester	Ester(Sulfur compound)	1.95
24.07	=Sulfurous acid, cyclohexylmethyl octadecyl ester	Ester(Sulfur compound)	1.52
24.25	=1-Undecene, 9-methyl-	Alkene	0.53
24.70	=Isoheptadecanol	Alcohol	0.38
24.81	=2-Piperidinone, N-[4-bromo-n-butyl]-	Piperidine (Ketone)	0.41
25.08	=Oleic Acid	Fatty acid (Carboxylic acid)	0.52
25.30	=51.23 Methyl octadecanoate	Ester	5.30
25.64	=5-oxoheptanoic acid, 4,4-dimethyl-	Carboxylic acid	0.20
25.75	=1-Heptacosanol	Alcohol	0.10
25.93	=Cyclohexane, (1-hexyltetradecyl)-	Cyclic Alkane	0.12
26.06	=Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-	Cyclic alkane	0.07
26.80	=Oxalic acid, cyclobutyl pentadecyl ester	Ester	0.07
26.96	=2-Thiopheneacetic acid, 2-ethylhexyl ester	Thiophene (Ester)	0.06
27.94	=2-Furancarboxylic acid	Furan	1.42
28.41	=Ketone, vinyl -pyrrolidinyl-	Pyrrolidine	0.39

Table 4.16: Compounds identified from isolate Cmex(END)8 (*Bacillus axarquiensis*) of Kenyan seaweeds: Fraction two.

Retention time (Min)	Compound name	Compound derivative	% abundance
9.331	=Dimethyl Sulfoxide	Sulfoxide	100

Table 4.17: Compounds identified from isolate Hmu(EPI)22 (*Streptomyces* sp.) of Kenyan seaweeds: Fraction two.

Retention time(Min)	Compound name	Compound derivative	% abundance
4.65	=Ethyl propanoate	Ester	1.18
9.58	=Dimethyl Sulfoxide	Sulfoxide	97.10
15.80	=Dimethylsulfoxonium formylmethylide	Aldehyde (Sulfoxonium)	0.01
22.08	=n-Nonadecanol-1	Alcohol	0.08
23.44	=Pentadecanoic acid, 14-methyl-, methyl ester	Carboxylic acid	0.53
24.07	=Hexadecanol<n->	Alcohol	0.11
25.35	=Methyl octadecanoate	Ester	0.86
25.93	=C20-ol	Alcohol	0.10

Fraction three

In this fraction, only compounds produced by isolate Pt(EPI)15 similar to *Bacillus* sp. were identified and propanoic acid, ethyl ester was the most abundant at 26.35%, followed by dimethyl sulphoxide at 11.42% (Table 4.18).

Table 4.18: Compounds identified from isolate Pt(EPI)15 (*Bacillus* sp.): Fraction three.

Retention time (Min)	Compound name	Compound derivative	% abundance
4.18	=Isopropyl acetate	Ester	1.92
4.90	=Propanoic acid, ethyl ester	Carboxylic acid	26.35
6.08	=Toluene	Solvent, methylbenzene	0.33
6.73	=1-Butanol, 3-methyl-, acetate	Ester	0.36
7.00	=Butanoic acid, ethyl ester	Carboxylic acid	0.54
7.72	=Dimethyl Sulfoxide	Sulfur compound	11.42
16.43	=Indole	Indole	6.83
17.28	=1H-Pyrrole-3,4-dicarbonitrile	Nitrile (Nitrogen compound)	0.36
18.25	=Butyl pentyl carbonate	Carbonate	0.46
19.92	=Isobutyl tetradecyl carbonate	Carbonate	0.47
20.31	=Carbonic acid, butyl dodecyl ester	Carboxylic acid	0.18
20.91	=2-Thiopheneacetic acid, 3-tetradecyl ester	Carboxylic acid ester (Sulphur compound)	0.11
21.18	=Oxalic acid, cyclohexylmethyl tetradecyl ester	Ester	1.98
21.40	=1-Methylcyclohexylcarboxylic acid	Carboxylic acid	0.48
21.60	=Decane	Alkane	0.15
21.74	=1-Docosene	Alkene	0.11
22.12	=Heptadecane, 2,6-dimethyl-	Alkane	0.21
22.21	=Oxalic acid, 2-ethylhexyl hexyl ester	Ester	0.23
22.72	=2-Cyclohexen-1-ol	Alcohol	0.60
22.93	=3,5,5-Trimethylhexyl acetate	Ester	0.33
23.42	=Methyl hexadecanoate	Ester	8.97
24.02	=3-Decen-5-one, 2-methyl-	Ketone	3.98
24.27	=Cyclopropane, 1-heptyl-2-methyl-	Alkane	2.13
24.38	=1,3-Cyclohexanedione, 5-isopropyl-	Ketone	0.90
24.83	=2-Piperidinone, N-[4-bromo-n-butyl]-	Ketone	0.73
25.32	=Methyl octadecanoate	Ester	11.85
25.95	=Octadecane, 1-[2 (hexadecyloxy)ethoxy]-	Alkane	0.34
26.26	=17-Pentatriacontene	Alkene	0.30
27.23	=Bis-(3,4-dimethoxyphenyl)-hydroxyacetic acid, 1-methyl ester	Phenolic	1.13

27.29	=Heptadecanoic acid, heptadecyl ester	Ester (Carboxylic acid)	0.39
27.65	=Tetratetracontane	Alkane	1.99
27.85	=3-Hexene, 2,2-dimethyl-, (Z)-	Alkene	1.21
27.97	=1,2-Cyclopentanedione, 3-methyl-	Ketone	2.21
28.44	=Sulfurous acid, 2-propyl tetradecyl ester	Ester	6.31
29.85	=2,4-Hexadiene, 1,1-diethoxy-	Alkene (methoxy)	3.95

Fraction four

In this fraction, compounds were identified from isolate Cp(EPI)12 similar to *Bacillus subtilis*, that had Heptadecanoic acid, 16-methyl-, methyl ester a carboxylic acid as the most abundant compound at 27.72%, followed by 1-Undecanol, an alcohol at 17.69% (Table 4.19).

Table 4.19: Compounds identified from isolate Cp(EPI)12) (*Bacillus subtilis*) of Kenyan seaweeds: Fraction four.

Retention time (Min)	Compound name	Compound derivative	% abundance
5.34	=1-Pentanol	Alcohol	10.26
14.71	=Carbonic acid, propyl tridecyl ester	Ester	5.94
17.46	=3-Octadecene, (E)-	Alkene	16.73
19.88	=1-Undecanol	Alcohol	17.69
21.18	=Cyclohexane, 1-methyl-2-pentyl-	Cyclic alkane	8.70
22.10	=trans-1,2-Diethyl cyclopentane	Cyclic alkane	2.85
23.84	=Pyrollidine, 2,5-bis(imino)-	Alkaloid	2.07
24.49	=Sulfurous acid, cyclohexylmethyl octadecyl ester	Ester	8.01
25.37	=Heptadecanoic acid, 16-methyl-, methyl ester	Carboxylic acid	27.72

Fraction five

From this fraction, isolate Gs(EPI)8 identified as *Bacillus subtilis* produced propanoic acid, ethyl ester a carboxylic acid as the most abundant compound at 32.54. This was followed by esters methyl octadecenoate and methyl hexadecanoate at 19.26% and 19.23% respectively (Table 4.20). In isolate Pt(EPI)25 similar to *Bacillus* sp., ethyl

octadecenoate (ester) was the most abundant at 44.69% and was followed closely by methyl palmitate (Methyl hexadecanoate) at 31.99% (Table 4.21). In this fraction, the notable compounds are methyl octadecenoate and methyl hexadecanoate.

Table 4.20: Compounds identified from isolate Gs(EPI)8) (*Bacillus subtilis*) of Kenyan seaweeds: Fraction five.

Retention time (Min)	Compound name	Compound derivative	% abundance
4.87	=Propanoic acid, ethyl ester	Carboxylic acid	32.54
19.12	=1-Triacontanol	Alcohol	0.97
19.23	=Ethanol, 2-(tetradecyloxy)-	Alcohol	1.47
19.36	=6H-Purine-6-thione, 1,7-dihydro-8-methyl-	Purine (Nitrogen compound)	1.35
19.99	=2-Thiopheneacetic acid, 4-tridecyl ester	Acid (Sulphur compound)	1.51
20.44	=5-Octen-4-one, 7-methyl-	Ketone	3.22
20.64	=1-Methyl-4-piperidinyl 3-ethylphenylcarbamate tms	Carbamate	2.18
20.96	=2-Thiopheneacetic acid, 3-tetradecyl ester	Thiophene (Sulphur compound)	0.68
21.16	=Sulfurous acid, cyclohexylmethyl undecyl ester	Sulphur compound	2.59
21.43	=3-Heptene, 4-propyl-	Alkene	2.17
21.78	=1-Nonadecene	Alkene	1.22
22.21	=2-Thiopheneacetic acid, 3-tridecyl ester	Thiophene (Sulphur compound)	0.27
22.61	=6-Methyl-1,5-diazabicyclo[3.1.0]hexane	Alkane (Nitrogen compound)	0.28
22.95	=Cyclohexane, 1,3-dimethyl-, trans-	Alkane	0.27
23.04	=Nonahexacontanoic acid	Carboxylic acid	0.47
23.22	=9-Octadecenoic acid (Z)-, methyl ester	Carboxylic acid	3.78
23.40	=Methyl hexadecanoate	Ester	19.23
23.82	=Cycloheptane, methyl-	Alkane	0.57
24.27	=2-Dodecenal	Aldehyde	0.48

24.56	=(S)-(+)-6-Methyl-1-octanol	Alcohol	0.40
24.81	=2-Ethylbutyric acid, 3,7-dimethyloctyl ester	Ester	0.60
24.90	=3-(4-Methylpent-3-enyl)thiophene	Thiophene (Sulphur compound)	0.53
25.10	=trans-13-Octadecenoic acid, methyl ester	Carboxylic acid	2.86
25.32	=Methyl octadecanoate	Ester	19.26
25.61	=1-Heptadecene	Alkene	0.26
25.93	=1-Hexacosene	Alkene	0.32
26.51	=1-Pentene, 2,3-dimethyl-	Alkene	0.38

Table 4.21: Compounds identified from isolate Pt(EPI)25 (*Bacillus* sp.) of Kenyan seaweeds: Fraction five.

Retention time (Min)	Compound name	Compound derivative	% abundance
5.59	=1,6-Diazabicyclo[4.1.0]heptane	Nitrogen compound	0.91
5.93	=2-Pentanone, 3-methyl-	Ketone	2.20
15.60	=Carbonic acid, heptadecyl propyl ester	Ester	0.51
18.56	=5-Amino-3-methylpyrazole	Amine	1.18
21.18	=Sulfurous acid, cyclohexylmethyl undecyl ester	Acid ester (Sulphur compound)	3.45
23.15	=Oxalic acid, cyclohexylmethyl octyl ester	Ester	2.58
23.26	=Cyclohexane, 1,4-dimethyl-, cis-	Alkane	1.75
23.42	=Methyl palmitate (Methyl hexadecanoate)	Ester	31.99
23.84	=Oxalic acid, cyclohexylmethyl nonyl ester	Ester	1.09
23.96	=Cyclohexane, 1,1-dimethyl-	Alkane	1.46
24.02	=Sulfurous acid, cyclohexylmethyl ethyl ester	Ester (Sulphur compound)	6.10
24.72	=2-Cyclohexen-1-ol, 1-methyl-	Alcohol	0.68
24.85	=Cyclohexane, 1,2-dimethyl-, trans-	Alkane	0.89
25.32	=Methyl octadecanoate	Ester	44.69
26.29	=2,4-Dimethyl-1,5-diazabicyclo[3.1.0]hexane (trans)	Cycloalkane (Nitrogen compound)	0.46

CHAPTER FIVE

DISCUSSION

5.1 Bacterial isolates from marine algae

The present study shows that diverse seaweed species are a host to numerous bacterial isolates. A large population of bacteria (3493) was obtained from 44 seaweed species. This implies that bacteria are ubiquitous on seaweed surface. Other studies have shown that seaweeds are a rich source of surface associated bacteria. For instance, Karthick and Mohanraju (2018), enumerated 77 epiphytic bacteria from eight different algae collected from Little Andaman, India, while Janakidevi et al. (2013), isolated 126 bacteria from five different seaweeds in Gulf of Mannar, South east coast of India and Ismail-Ben et al. (2012), obtained 19 bacterial isolates from one algal species in the northern coast of Tunisia, southern Mediterranean Sea. This shows that the Kenyan coastal environment is a habitat for numerous seaweeds, which harbor large bacterial communities. Additionally, studies have also shown that, seaweeds surface provide suitable substratum for settlement of microorganisms and secretes various organic substances that function as nutrients for multiplication of bacteria (Staufenberger *et al.,* 2008; Singh *et al.,* 2013). These bacteria also produce chemical compounds to sustain competition from other microbes, hence increasing their chances of survival and position on the host (Shanmugaraju *et al.*, 2013), leading to their high isolation frequency.

The present study also revealed that bacterial epiphytes thrived more compared to the endophytes in marine algae. Burke *et al.* (2011) and Lachnit *et al.* (2011), documented notable population in epibacterial communities associated with different seaweeds. This suggests that colonization patterns are host-specific and strongly influenced by the seaweed, because of physicochemical constraints (Popper *et al.,* 2011), such as cell wall component diversity and/or active defense mechanisms (Potin *et al.,* 2002; Cosse *et al.,* 2008). However, no study has documented interaction of both endophytes and epiphytes with the seaweeds to bring about the disparity in colonization. This study is to the best of

my knowledge the first to report on the isolation of both endophytes and epiphytes from different algal divisions and seaweed species. The current study has revealed seaweeds thrive in association with microbes, which enhance their survival in their ecosystem.

5.2 Antimicrobial and cytotoxic screening

Seaweeds are known to be part of a highly productive ecosystem with a rich diversity of associated microorganisms compared to other multicellular organisms. They are a host of numerous bioactive compound-producing microbes. Inhibitory activities among seaweed-associated microbes are of great interest to search for antimicrobial substances. Several studies have reported the antimicrobial potential of bacteria associated with seaweeds (Kanagasabhapathy *et al.,* 2008; Villarreal-Gómez *et al.,* 2010; Singh *et al.,* 2014). The results of the current study showed that 20% of the isolated marine bacteria showed antimicrobial activity against one or more test microorganisms while further antimicrobial screening revealed that 10% of the bacteria had abroad spectrum inhibitory activity. This infers that marine bacteria isolates produced active antibacterial and antifungal compounds.

Several studies have revealed evidences that have proven that marine associated bacteria produce valuable bioactive compounds. For instance, Wiese *et al.* (2009) found that 50% of bacteria strains associated with the brown algae *Laminaria saccharina* inhibited the growth of Gram-negative or Gram-positive bacteria as well as yeast. In addition, Dhanya *et al.* (2016), assessed the antimicrobial activity of the green algae *Ulva reticulata* and its endophytes and found they were active against the human pathogens: S*taphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Bacillus subtilis*. Similarly, Karthick and Mohanraju (2018), isolated 77 epiphytic bacteria from eight different algae and found that six of the isolates showed significant antimicrobial activity. Hence, the production of these bioactive natural compounds by the seaweed-associated bacteria may be attributed to the competition of space and nutrients, in the marine environment, which enables these bacteria inhibit the settlement of potential

competitors (Armstrong *et al.,* 2001; Goecke *et al.,* 2010). Therefore, it is necessary to study the chemical interactions of algae and bacteria for a better understanding of the production process of bioactive secondary metabolites, since these compounds are known to possess antimicrobial, anticancer and antifouling properties.

The antimicrobial susceptibility patterns of the present study further revealed that, the Gram-negative bacteria pathogens were more resistant to the seaweed microbes compared the Gram-positive bacteria pathogens. This is owed to the phenomenon of the Gram-negative bacteria possessing the multidrug efflux system, which confers on them the ability to limit the antimicrobial access of the antimicrobial agents to the targets (Kohler & Peche, 1999). Additionally, the Gram-negative bacteria species have a thicker outer membrane and murein layer acting as a barrier to many environmental substances and inhibitors and Gram-positive bacteria lacking these features are susceptible to bioactive compounds. This therefore contributes to the intrinsic resistance of Gram-negative bacteria to a wide range of antibiotics (Kohler & Peche, 1999). These findings are supported by most surveys documented in literature, on the antimicrobial activities on both Gram-negative and Gram-positive bacteria pathogens, where the Gram-positive bacteria are reported to be more susceptible (Ibtissam *et al.,* 2009; Ghanthikumar *et al.,* 2012; Mishra, 2018).

It was also fascinating to note that in the current study, fungal test pathogens were more susceptible to the seaweeds microbes compared to the bacterial test pathogens. This is not usually the case as fungi are considered to be more resistant compared to bacteria. This is ascribed to the nature of fungal cell wall that is composed of glucosamine polymer chitin, which is relatively resistant, including cases of microbial decomposition (Mashjoor *et al.,* 2016). In contrast, these seaweed microbes are thought to impart antimicrobial effects possibly via the induction of changes in the cell membranes of the targeted fungal pathogen, with alterations in the cell envelope causing impaired regulation of osmolality and ultimately cell death.

Since seaweed surfaces provide a suitable substratum for the settlement of microorganisms that are highly diverse, over a long evolutionary period, the marine organisms sharing a common environment have established associations; where microbes present outside or inside the algal cells protect the host against pathogens by producing bioactive compounds (Singh *et al.,* 2014). From this current study, sixty-nine (69) seaweed-isolated microbes screened for antimicrobial activity showed that, epiphytic bacterial isolates produced pronounced bioactivity against the test pathogens compared to the endophytic ones. It is not however clear why the epiphytes showed higher antimicrobial activity than endophytes, since both microbial communities play a key role in the survival of the seaweeds by secreting, secondary metabolites that protect the algal surface against settlement of pathogenic microbes and biofouling microbes (Egan *et al.,* 2013). Additionally, the endophytic and epiphytic microbes form a beneficial symbiotic association with the host algae, where the microbes produce compounds that are thought to protect the seaweeds from pathogens and other competing microorganisms (Janakidevi *et al.,* 2013). Evidence also indicates that many bioactive compounds found in marine animals and plants are in fact produced or metabolized by the associated microorganisms (Proksch *et al.,* 2002). Moreover, investigations have been conducted to study the antimicrobial activities of seaweed-associated epiphytic and endophytic microbes, and results have shown that these microbes produce secondary metabolites that have the capacity to inhibit bacteria and fungi as well as tumorous cells. This makes them interesting for natural products screening programs.

Various natural factors such as light, temperature, salinity, reproductive state and age of the seaweed may influence the production of antimicrobial compounds therefore influencing the chemical composition of the seaweed associated microbe (Pérez *et al.,* 2016). Nonetheless, inhibitory activities of the isolates among the algal divisions were not significant, implying that the algal division did not influence antimicrobial activity. This is in contrast with a study conducted by Alghazeer *et al.* (2013) and Lavanya *et al.* (2011), who observed that microbes associated with the red and brown algae have high inhibitory activities than the green algae. However, Vallinayagam *et al.* (2009), noted a

higher antimicrobial activity of brown algae extracts compared to the red algae. In regards to the algal division, more microbes from different seaweed species and algal divisions need to be isolated and screened for their antimicrobial activities in order to ascertain whether the algal division influences production of bioactive compounds.

Comparisons of the inhibitory activities of the different isolates and the three sites of sampling showed there was a significant difference among the sites. Isolates from the north coast sites (Mtwapa and Mkomani) were more active compared to isolates from the south coast site (Kibuyuni). This shows that the geographic location of sampling influenced antimicrobial activity. This is consistent with the known phenomenon that, seaweeds are exposed to variable environmental factors such as light intensity, food availability, salinity and pollution concentrations, as well as to biotic pressures such as predation and spatial and trophic competition. All these factors influence the host and associated microbes physiology, that have direct effects on the production of secondary metabolites producing variations in antibacterial and antifungal activity among the different geographical locations (Vidyavath & Sridhar, 1991; Martí *et al.*, 2004; Alghazeer *et al.*, 2013). In this case therefore, the bioactivity differences could also be attributed to the habitat differences i.e. difference in coverage of water of the reefs along the Kenya coast with parts completely uncovered in Kibuyuni, parts with shallow water in Mkomani and parts with larger and smaller pools in Mtwapa during the low spring tide. This varied ecological and geographical aspect could also produce the bioactivity variation among the sites.

Marine microorganisms are often considered taxonomically unique, which makes them interesting as potential sources of new drug leads. One of the major areas of research on marine natural products is devoted to the discovery of new anti- cancer drugs. Based on the broad-spectrum level of antimicrobial activity in this study, 48% of the seaweed-associated microbes displayed significant cytotoxic activity. This means that the seaweed microbes also produce biologically active compounds, that have potential as

chemotherapeutic agents by decreasing tumor cell proliferation and inducing a specific cytotoxicity effect (Ariffin *et al.,* 2011).

This is in line with several studies that have proven the cytotoxic potential of microbes associated with marine algae against various cell lines (Villarreal-Gómez *et al.,* 2010; Ariffin *et al.,* 2011; Soria-mercado *et al.*, 2012; Pandey & Chalamala, 2013).

5.3 Identification of the active endophytic and epiphytic isolates

The Gram classification is an interesting tool to differentiate between the structure and chemical composition of the two main groups of bacteria i.e. Gram-positive and Gram-negative. Gram staining showed that more of the seaweed isolates were Gram-positive as compared to the Gram-negative. Previous studies on bacterioplankton showed that majority of marine bacteria are Gram-negative. However, in recent studies on marine sediments, evidence has shown that most bacteria from this environment seem to be Gram positive (Gontang *et al.,* 2007), just as depicted in this current study. This implies that the Gram-positive bacteria are also successful colonizers of seaweed surfaces just like their Gram-negative counterparts. Tentative identification using biochemical tests was confirmed by molecular analysis without any discrepancy.

The enormous majority of studies related to the micro biome of macroalgae have to date focused on bacteria. Of interest to note is that the historical focus on bacteria is in agreement with recent metagenome and transcriptome analysis, which indicates that bacteria indeed dominate these communities (Burke *et al.,* 2011; Oliveira *et al.,* 2012), and a high population of bacteria was noted in the study. Nonetheless, molecular phylogenetic analysis demonstrated bacterial isolates with numerous antimicrobial activity belonged to the phylum Firmicutes, with the least being Actinobacteria, though they are considered excellent producers of bioactive secondary metabolites (Jensen *et al.,* 2005). Additionally, macroalgae represent niches with unique and selective properties hence, experience symbiotic interactions with a diverse community of microbes and especially bacteria, since bacteria are the primary colonizers of submerged surfaces. The

abundance trend of specific bacterial groups in the marine algae is linked to various chemical interactions. The present study revealed that the bioactive bacterial isolates belonged to the phyla Firmicutes, Proteobacteria and Actinobacteria, and this is consistent with studies of previous work, that have found the same phyla i.e., Firmicutes (Bacillaceae), Proteobacteria (Oxalobacteraceae) and Actinobacteria (Streptomycetaceae), to mostly colonize the seaweeds at times having a tendency to colonize specific algae groups (Villarreal-Gómez *et al.,* 2010). Similarly, a study conducted by Janakidevi *et al.* (2013), isolated 126 marine bacteria from different seaweed surfaces and found that more than 50% of the bacteria belonged to the phyla Firmicutes and Proteobacteria, which have been known to be common and dominant in aquatic environments.

In the present study, the phylum Firmicutes had the highest number of active isolates, which belong to the genus Bacillus. This agrees with several studies, that found Bacillus being the most dominant genus proliferating excellently on marine algae (Burgess *et al.*, 2003; Kanagasabhapathy *et al.,* 2008; Villarreal-Gómez *et al.,* 2010; Singh *et al.,* 2014; Susilowati *et al.,* 2015). These authors demonstrated that *Bacillus* genus had a high antibacterial activity against fouling bacteria and has been recognized as a prolific producer of antibiotics (Radjasa *et al.,* 2013), hence the broad spectrum of antimicrobial activity. This capacity to produce antibiotics by the *Bacillus* species contributes to its survival in its natural habitat (Stein *et al.,* 2005), thus leading to the high frequency of its isolation. Contrary to this current study, Soria-mercado *et al.* (2012), reported that the Proteobacteria phylum predominate the marine algae and are the most prolific producers of antimicrobial compounds. Similar results were reported by Ismail-Ben Ali *et al.* (2012), who focused on heterotrophic aerobic bacteria species associated with coralline red alga *Jania rubens* and their inhibition against several microbial marine and terrestrial species. They identified 19 isolates responsible for the antimicrobial activity, of which 14 strains belonged to the phyla Proteobacteria, Bacteroidetes (four strains) and Firmicutes (one strain). On the other hand, since the present study has demonstrated that the seaweed bacteria species belonging to the Bacillus genera are mainly responsible for inhibitory

activity against pathogenic bacteria, they are highly promising candidates for pharmaceutical applications.

Based on the genetic results, this study reveals that different *Bacillus* species were isolated from the various algal groups. However, some isolates were closely related to each other or even identical. Although the most abundant strains were *Bacillus* sp. and *Bacillus subtilis*, the abundance trends of other *Bacillus* strains varied depending on the species. In the study, however, all isolates were treated as distinct strains since they differed in their individual inhibition ability and/or growth patterns. Based on previous research, bacteria belonging to the Bacillaceae family are widespread in the marine environment (Burke *et al.*, 2011; Bour *et al.*, 2013), hence the high frequency of isolation depicted in the current study. This family is particularly abundant on the surface of numerous marine macro organisms such as seawater, marine sediment, corals, seaweeds, seagrass and sponges, where they form commensal, symbiotic, or pathogenic associations (Singh & Reddy, 2014).

Studies have also shown that among the Firmicutes group, *Bacillus* species are dominantly present on the surface of diverse seaweeds (Lachnit *et al.*, 2009; Tujula *et al.*, 2010), with the most common species isolated being *Bacillus cereus, Bacillus pumilus, Bacillus* sp. and *Bacillus subtilis*. However, other *Bacillus* strains such as *Bacillus clausii, Bacillus anthracis* and *Bacillus alcoliinulinus* have been isolated from red, brown and green marine algae (Zheng *et al.*, 2005; Kanagasabhapathy *et al.*, 2006; Kanagasabhapathy *et al.*, 2008; Villarreal-Gómez *et al.*, 2010; Susilowati *et al.*, 2015). Additionally, *Bacillus amyloliquefaciens* has been isolated from red and brown algae collected in the Gulf of Mannar on the southeast coast of India (Thilakan *et al.*, 2016). The present study identified bacteria of the genus Bacillus such as, *Bacillus safensis, Bacillus axarquiensis, Bacillus velezensis, Bacillus methylotrophicus* and *Bacillus tequilensis* that have never been isolated from seaweeds as per my knowledge. Another strain i.e. *Geobacillus stearothermophilus* belonging to the family Bacillaceae was also

identified and similarly, no study has previously documented its association with seaweeds.

The phylum Proteobacteria has also been reported to be commonly associated with marine algae and seawaters (Burke *et al.,* 2011). Studies have shown that the strains mostly encountered are the *Pseudomonas* sp. and *Vibrio* sp., that produce bioactive compounds which contribute to their abundance in surface-associated communities (Kanagasabhapathy *et al.,* 2008; Hamid *et al.,* 2013). This is contrary to what was observed in this study, which identified Proteobacteria strains; *Desulfovibrio vulgaris* to be associated with a brown and green algae, *Sargassum* sp. and *Ulva lactuca,* respectively and *Massilia* sp. associated with brown algae, *Hydroclathrus clathratus.* The association of seaweeds with these two bacterial species has never been reported. This outcome denotes that, this study is the first to document their association with seaweeds and their pharmaceutical potential.

Among the phylum of marine Actinobacteria, the genus Streptomyces is represented in nature by the largest number of species and varieties, which differ greatly in their morphology, physiology, and biochemical activities. Studies have shown that *Streptomyces* species are dominant in the terrestrial environment but, reports have also linked it to the marine environment (Singh *et al.,* 2014). Marine Streptomyces occur in different biological sources such as fishes, molluscs, sponges, seaweeds and mangroves, besides seawater and sediments (Dharmaraj, 2010). In the present study, *Streptomyces* sp. was isolated and identified from *Hypnea musciformis* and *Hypnea pannosa* (red algae) and *Ulva reticulata* (green algae). This is in line with previous studies that showed that Streptomyces was isolated from different intertidal brown, red and green algae (Braña *et al.,* 2015). A study by Penesyan *et al.* (2009), also documented the presence of *Streptomyces* sp. associated with a red algae *Delisea pulchra* and a green algae *Ulva australis*. In addition, Zheng *et al.* (2000) also found that a high population of *Streptomyces* sp. was associated with surface, epidermis and intestines of sea plants and

animals collected from Taiwan Strait, China. This infers that the marine *Streptomyces* is widely distributed in various biological sources.

The diverse bacteria-seaweed associations are an indication that surface colonization in the marine environment is ubiquitous. Consequently, studies on culturing and microscopy have also clearly indicated differences between the microbial composition associated with macro algae and that of the surrounding seawater, between different algal species, across different seasons as well as between different sections of a macro algal thallus (Egan *et al.,* 2013; Florez, *et al.,* 2017). These observations of host specificity as well as temporal and spatial variation were further refined by a number of culture-independent studies (Cundell *et al.,* 1977; Bolinches *et al.,* 1988). Where, host specificity refers to the occurrence of a specific set of bacterial epiphytes and endophytes on one type of alga that are absent (or only found in very low numbers) on other algal species.

In addition, macro algal communities also experience spatial and temporal shifts, which may be a reflection of the changing local conditions, host physiology, or chemical and physical parameters. For instance, Lachnit *et al.* (2011) found reproducible seasonal shifts in the bacterial communities of three different co-occurring seaweed hosts, with a specific winter and summer bacterial community composition recurring over consecutive years. However, the observed variations and similarities can also be impacted by methodological limitations. These notwithstanding, given the diversity of macro algal hosts and the variability of the environment in which they live as in the present study, it is likely that macro algal–bacterial interactions will be equally diverse and range from specialist to generalist. It is therefore, important to gain an understanding of the biological, physical, and chemical factors that influence the epiphytic and endophytic community on individual macro algal species (Egan *et al.,* 2013).

5.4 Extraction and bioassay guided fractionation of the secondary metabolites

The batch fermentation and extraction process yielded different quantities (weights) of the secondary metabolites. This could be attributed to several factors including; the

different bacterial strains, the strains adaptability to the culture conditions enhancing their metabolite production and the solubility of the compounds formed, implying that some compounds formed or produced are more soluble in the solvents employed. This observation agrees with a study done by Patterson and Bolis (1997) who stated that yield differences may be caused by the chemical interactions between the different species of bacteria which in turn affects the production and secretion of antimicrobial secondary metabolites.

In addition, some bacteria that could not previously produce such metabolites, when exposed to other culture conditions or pressures, may increase or decrease their ability to produce the metabolites and this directly influences the yields produced and composition thereof. In the current study, ethyl acetate and methanol were used at varying ratios and since they possess different polarities, where ethyl acetate is less polar than methanol, extracts less polar to medium polar compounds, while methanol extracts highly polar compounds. Therefore, this may have contributed to the yield differences. In line with this, Salem *et al.* (2011), used both solvents for the extraction of antimicrobial compounds from some marine algae of Egypt and found that the yields and bioactivity of the compounds was dependent on the solvent used. Furthermore, since extraction yields is impacted by the solvent of choice, which in turn affects the efficacy of the extracts, Rajauria *et al.* (2013) reported a different combination of methanol and water (20%-80%), to have significant effect on the yield of antimicrobial and antioxidant polyphenolic compounds from the Irish brown seaweed *Himanthalia elongate.* The highest yield (6.8%) was achieved using 60% methanol compared to the lowest yield (1.2%) using 100% methanol. This implies that the choice of solvent and its polarity influences the quantities of secondary metabolites in a particular sample. The fractionation process is also important in that, it helps in the purification of the compounds in order to identify the compounds that constitute the metabolites. This enables commercial processing of the active compounds, towards designing active drug agents against pathogens (Malviya & Malviya, 2017).

5.5 Antimicrobial and cytotoxic screening of fractionated secondary metabolites

Seaweeds and their associated microbial communities form a complex and a vastly dynamic ecosystem. This complex interaction leads to the production of specific bioactive compounds of interest. Numerous studies have previously reported on antimicrobial compounds of seaweed origin (Goecke *et al.,* 2010). Similarly, studies have also documented anticancer potential of seaweed associated microbes (Soria-mercado *et al.,* 2012). As there is a growing interest in the usage of microbial secondary metabolites in medical applications, various pathogenic strains were included in the present study. Therefore, the extraction of the secondary metabolites, the display of their antimicrobial and antitumor activities was considered an indication of the capability of the microbes associated with the seaweeds to synthesize bioactive secondary metabolites.

The present study showed that antimicrobial susceptibility patterns of Gram-negative bacteria pathogens tested were higher for the fractionated secondary metabolites compared to the Gram-positive bacteria pathogens. This contradicts most studies that have shown Gram-negative bacteria are least susceptible/more resistant compared to Gram-positive bacteria (Ibtissam *et al.,* 2009; Ghanthikumar *et al.,* 2012). The difference in the inhibitory action on the bacterial strains could be due to differences in the cell membrane of Gram positive and negative bacteria. However, in the initial screening process with the bacterial broth, Gram-negative bacteria were resistant. Since fractionation is often considered a process of compound purification, it postulated that several bioactivity inhibitors were removed during the fractionation process, contributing to an effectiveness of the fractionated secondary metabolites against the Gram-negative bacterial pathogens. The susceptibility of the Gram-negative pathogens in this case may be attributed to the compounds present in the solvent fractionated metabolites and their varying polarities (Shannon & Abu-Ghannam, 2016).

The present study also revealed that susceptibility patterns of both the bacterial and fungal pathogens, to the fractionated secondary metabolites were similar. The similarity

in the bacterial and fungal pathogens susceptibility patterns may be contributed by similar pharmacological modes of action of the compounds present in the bacterial fractionated metabolites (Shannon & Abu-Ghannam, 2016).

In addition, the biological phenomenon of allelopathy in the marine habitats contributes to the production of potentially antibacterial and antifungal compounds that exert detrimental effects on competing organisms, the result of which offers a competitive advantage to the alga and associated microbes, contributing to the similar inhibitory action against bacteria and fungi pathogens.

However, this disagrees with a study by Padmakumar and Ayyakkannu (1997), who screened 80 seaweed species against bacterial and fungal pathogens and found that, of the algae, 70% exhibited antibacterial activity but only 27.5% showed antifungal activity.

From the current study, secondary metabolites produced by endophytic and epiphytic bacteria had no significant difference in their inhibitory activities against the test pathogens. This corroborates with findings from several studies (Kanagasabhapathy *et al.*, 2006; Kanagasabhapathy *et al.*, 2008; Susilowati *et al.*, 2015; Ismail *et al.*, 2016), that have shown the potential of seaweed associated endophytic and epiphytic bacterial extracts, to inhibit panels of pathogenic microbes. However, on the other hand, endophytic bacterial communities have not been well investigated, but are expected to be an important source for natural bioactive agents (Zheng *et al.*, 2005; Newman & Hill, 2006). Nevertheless, Mayer and Gustafson, (2008) studied endophytes and established that they possess both antimicrobial and antitumor agents.

In the search for cytotoxic compounds from seaweeds associated bacteria, the assay results of this study showed that the fractionated microbial secondary metabolites displayed significant cytotoxic activity against Hep-2 cells (Human Laryngeal Carcinoma). Cytotoxicity which was measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay, targets the activity of succinate dehydrogenase in mitochondria, which in turn reduces the tetrazolium salt into formazan crystals (Corneal

et al., 1996). The intensity of the color of formazan dye correlates to the number of viable cells. The microbial secondary metabolites showed growth inhibition of the Hep-2 cells lines with varied IC_{50} values and at different concentrations. This agrees with a study in Todos Santos Bay by Villarreal-Gómez *et al*. (2010), who found that bacteria associated to the surface of seaweeds produce compounds capable of inhibiting the growth of HCT-116 colorectal cancer cells. Likewise, Zheng *et al.* (2000), in a study done in the southwest coast of the Taiwan Strait, China, where the marine actinomycetes were isolated from the surface, epidermis and intestines of seaweeds (*Ulva lactuca*, *Enteromorpha*, *Gracilaria verrucosa*) and animals (*Aplysia dactylomela*, sea anemone, *Actiniaria*), found that the bacterial genus *Streptomyces* displayed antitumor activities against murine leukemia P388 cells and three human cancer cell lines, KB cells, HLF cells and CNE cell. Therefore, the fractionated bacterial secondary metabolites used in this study were selective towards the cancer cell lines when compared to the normal cells. Thus, it might be reasonable to presume that these metabolites have potential as chemotherapeutic agents by decreasing tumor cell proliferation and inducing cytotoxic effect. This is usually the required criterion for effective chemotherapeutic drugs (Johnstone *et al.,* 2002). Also, the different IC_{50} values (translating to specific activity) of the extracts against the particular cell lines, indicates a selective mechanism of action and maybe attributed to the presence of different compounds polarity.

Solvent polarity and solvent ratio influenced bioactivity of the secondary metabolites as depicted in this study, where the most active metabolites were present in fraction one, which was composed of intermediaries of polar and non-polar compounds. This means that solvent fractionation using both non-polar and polar solvent produces maximum antimicrobial and antitumor activities compared to only using the polar solvent methanol. As for the effectiveness of the solvents used in extraction, some studies have shown that methanol yields higher bioactivity compared to ethyl acetate or other solvents (Val *et al.,* 2001; Lavanya *et al.,* 2011;) whereas in others, ethyl acetate was better than methanol (Salem *et al.,* 2011). It is clear that the use of organic solvents provides efficiency in extracting bioactive compounds. Ethyl acetate and methanol where selected in this study

based on documented reports about the effectiveness of both solvents in extraction. The differences in the fraction bioassay results could have been brought about by several other factors that include; the difference in strain species, solvent ratios used that causes a difference in the recovery of the active metabolites and susceptibility of the target strains. Additionally, the presence of different bioactive substances in the solvent extracts from the tested metabolites may also be the reason for the variation of the antimicrobial and cytotoxic activities as reported by El Shafay *et al*. (2016).

5.6 Identification of active secondary metabolites

Different kinds of compounds have been detected from seaweed associated microorganisms and other marine microorganisms. In the present study, various compounds present in the fractionated metabolites of the seaweed-associated bacteria, that were responsible for the antimicrobial and cytotoxic activities were identified and included various phenolics, alkaloids, carboxylic acids, alkanes, alkenes, esters, alcohols, indoles, esters, ketones and amines. Similarly, Blunt *et al.* (2013) reported the same range of bioactive compounds in marine natural products. The production of these compounds by the seaweed associated bacteria, may be brought about by the fact that, seaweeds are part of highly productive and competitive ecosystems and are therefore habitats of bacterial communities that produce plant growth-promoting substances, quorum sensing signaling molecules, bioactive compounds and other effective molecules responsible for normal morphology, development and growth of seaweeds (Singh & Reddy, 2014).

From the present study, several compounds such as indole, pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)-, ethyl propanoate, dimethyl sulphoxide, propanoic acid-ethyl ester, heptadecanoic acid, 16-methyl-methyl ester, 1-Undecanol, methyl octadecenoate and methyl hexadecanoate were frequently detected and in high percentages of the different fractions and could be responsible for antimicrobial and cytotoxic activities among the fractionated secondary metabolites. For instance, indole and an alkaloid pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenyl methyl)- were

the most abundant in most of the fractions. This is because indole and alkaloids are known to constitute the largest number of antimicrobial compounds reported from marine species (Choudhary *et al.,* 2017), and are therefore considered lead compounds for the discovery of new drugs in medicinal chemistry. This also concurs with a study conducted by Trischman *et al.* (2004), who isolated thirteen bacterial strains from the seaweed *Ulva californica* and found that two of the 13 isolates produced Indole and cyclo (Phe-Pro) that had antimicrobial activity against different Bacillus target stains. In addition, the alkaloid, pyrrole (1, 2, a) pyrazine 1, 4, dione, hexahydro 3-(2-methyl propyl) (PPDHMP) was produced by a marine bacterium isolated from the deep sea sediments of Bay of Bengal India and exhibited antimicrobial activity against human pathogenic bacteria and *in vitro* anticancer potential against lung (A549) and cervical (HeLa) cancer in a dose-dependent manner with IC_{50} concentration of 19.94±1.23 and 16.73±1.78 µg ml^{-1} respectively (Lalitha *et al.,* 2016). This implies that the indole and alkaloid compound derivatives play a role in the antimicrobial and antitumor activities produced by the seaweed bacterial metabolites.

Other compounds such as carboxylic acids (Propanoic acid, ethyl ester, Heptadecanoic acid, 16-methyl-, methyl ester), esters (ethyl propanoate, methyl octadecenoate, methyl hexadecanoate), , alcohol (1-Undecanol) and sulphoxide (dimethyl sulphoxide), were also frequently detected and abundant in the diverse bacterial metabolites as shown in the present study. Most of these classes of compounds have been reported to have certain antimicrobial and antitumor activities, though not the specific compounds identified in the present study. For instance, Phenazine-1-Carboxylic acid, produced by marine bacterium *Pseudomonas aeruginosa* PA31x inhibited the growth of bacteria pathogen *Vibrio anguillarum* and caused cancer cell death (Zhang *et al.,* 2017). 1,2-Benzene Dicarboxylic acid, Mono 2-Ethylhexyl Ester extracted from marine derived *Streptomyces* sp. VITSJK8,exhibited cytotoxic activity against HepG2 (human hepatocellular liver carcinoma) and MCF- 7 (human breast adenocarcinoma) cancer cell lines with IC_{50} values of 42 µg/ ml and 100 µg/ ml respectively (Krishnan *et al.,* 2014).

The present study also revealed the presence of esters that could be responsible for the antimicrobial and cytotoxic activities of the seaweed-associated bacteria. However, the ester, methyl octadecanoate was frequently detected in the fractionated metabolites. In other studies, an antimicrobial ester (Bonactin) has been isolated from the *Streptomyces sp.* BD21-2 obtained from marine sediment sample collected at Hawaii India. It displayed antibacterial activity against both Gram-positive and Gram-negative bacteria as well as antifungal activity (Schumacher *et al.,* 2003). Similarly, Mathan *et al.* (2011) extracted and detected several ester compounds from the marine fungus *Aspergillus protuberus* SP1, isolated from marine sediments of South Indian Coast. The fungal isolate showed antibacterial activity towards various Gram-positive and Gram-negative human pathogens and potent cytotoxicity against Hep-2 cells.

Marine microorganisms also produce biologically active alcohols as depicted in the current study. Several alcohols were detected for instance, 1-Pentanol produced by *Bacillus methylotrophicus*, 1-Heptacosanol detected in *Bacillus pumilus.* The alcohols 1-Penatol and 1-Undecanol, identified in fraction four of *Bacillus subtilis* isolated from the red algae *Chondrophycus papillosus,* were the most abundant. This could probably mean they influenced bioactivity. Other studies have also shown that, alcohols derived from the marine environment possess antimicrobial, anticancer and anti-inflammatory properties. An example is an active compound Dihydroaustrasulfone alcohol (DA), that was firstly isolated from marine corals, exhibited anti-cancer and anti-inflammatory activities (Li *et al.,* 2015). In an attempt to study Marine Actinobacteria of East Coast of Andhra Pradesh, India, Kavitha and Savithri (2017) isolated an array of volatile antimicrobial compounds such as 1,2-benzene dicarboxylic acid, 2-piperidinone, pyrrol[1,2-a]pyrazine-1,4-dion, propionic acid and phenyl ethyl alcohol. These compounds were reported to show strong antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and *Candida albicans.* Thus, the detection of alcohols from marine microbes derived metabolites in the present study, intimates the biological potentiality of the alcohol derivatives of marine algae associated bacteria from the Kenyan coast.

The sulfur compound, dimethyl sulfoxide (DMSO) is an abundant form of methylated sulfur in marine systems and is known to be produced from dimethyl sulfide (DMS). Bacteria capable of reducing DMSO to DMS have been isolated from seawater (Gonzalez *et al.,* 1999). In the present study, DMSO was identified in most of the bacterial metabolites and with a high abundance in some metabolites. This sulfur compound has not been detected and/or identified in seaweed-associated bacteria and according to my knowledge; this study was the first to reveal the antimicrobial and cytotoxic potential of DMSO. Thus, its presence suggest that, it could be involved in the antagonistic activities against other microbes and may contribute immensely to the pharmaceutical industry.

Another compound frequently identified in the fractionated metabolites in the present study was phenol. Phenol was detected in fraction one of several isolates such as; *Streptomyces sp.*, *Bacillus* sp., *Bacillus methylotrophicus* and *Desulfovibrio vulgaris.* Phenols are known to constitute the largest group of plant secondary metabolites and widespread in nature, though they are not directly involved in plant primary processes. Moreover, they are found in most classes of natural compounds having aromatic moieties, which range from simple structures with one aromatic ring to highly complex polymeric substances. In addition, phenolic compounds occasionally incorporating halogen occur frequently in the marine environment (Pérez *et al.,* 2016). Thus, the detection of phenols in the present study contributed to the inhibitory activities displayed by the bacterial secondary metabolites. This is supported by a study that identified 4,4,6-tribromo-2,2'-biphenol a phenolic compound, from the marine bacterium *Pseudoalteromonas* sp., which displayed significant antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (Fehér *et al.,* 2010). Similarly, Isnansetyo and Kamei (2003b), identified another phenolic compound, 2,2',3- tribromo-biphenyl-4,4'- dicarboxylic acid, produced by another marine bacterium *Pseudoalteromonas phenolica.* This compound showed highly effective antibacterial properties towards methicillin-resistant *Staphylococcus aureus, Bacillus subtilis* and *Enterococcus*

serolicida. These results demonstrated that phenol compounds have high *in vitro* activity and might be useful as a lead compound in developing antimicrobial substances.

Therefore, the diverse assortment of the compounds detected in the fractionated metabolites could have a key role in the antimicrobial and cytotoxic potential of these metabolites. This is because, synergy can occur between two or more compounds with pharmacological activity, which can produce a negative effect in the form of contraindication, where active substances exert a more potent effect when combined than they would if used individually (Shannon & Abu-Ghannam, 2016). Synergy between marine algal bacteria derivatives could be used to develop an innovative approach to combat antibiotic-resistant bacteria as well as tumorous cells.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In the search for biologically active natural products, the present study deals with the isolation and identification of bacteria associated with marine algae, screening strategies for bioactive production, isolation and identification of the bioactive compounds.

In this study, 3493 bacterial isolates were isolated from forty-four seaweed species, with epiphytic bacteria frequently isolated compared to endophytic bacteria. This shows that seaweed-microbe interaction is diverse and abundant and that bacteria form stable associations with the host. Seaweeds sampled from Mtwapa site had the highest population of associated bacterial isolates compared to Mkomani and Kibuyuni sites. This probably depicts that the site/geographical location influenced bacteria population.

Thirty-three bacterial isolates showed considerable antimicrobial and cytotoxic activity against human pathogenic microbes and human larynx Hep-2 cell line, respectively. These isolates were closely related to the genus Bacillus, Geobacillus, Massilia, Streptomyces and Desulfovibrio.

The study also profiled the 16S rDNA gene analysis of the active bacterial isolates associated with the seaweeds found along the Kenya coast. The isolates belonged to three phyla namely: Firmicutes, Proteobacteria and Actinobacteria, with phylum Firmicutes being the most dominant. Key bacterial species identified were *Bacillus safensis*, *Bacillus axarquiensis*, *Bacillus velezensis*, *Bacillus methylotrophicus*, *Bacillus tequilensis Geobacillus stearothermophilus*, *Desulfovibrio vulgaris* and *Massili*a sp., and the present work is that first to report of their association with seaweeds surfaces.

Bioassay-guided fractionation led to the production of 198 fractionated extracts. Thirteen fractionated metabolites produced broad-spectrum bioactivity against the human pathogenic strains of microbes and low IC_{50} values against the human larynx Hep-2 cell lines. The most active fractions belonged to isolate Ul(EPI)7, closely related to *Bacillus* sp., Ur(EPI)24, closely related to *Bacillus methylotrophicus*, both of which had solvent elution ratios of 50:50 (ethyl acetate: methanol) and isolate Cmex(END)8, closely related to *Bacillus axarquiensis*, that had a solvent elution ratio of 40:60 (ethyl acetate: methanol). This supports the view that marine natural products might be promising sources for antimicrobial and anticancer agents.

Identification of the bioactive fractionated metabolites showed the presence of phenolics, alkaloids, carboxylic acids, alkanes, alkenes, esters, alcohols, indoles, esters, ketones and amines in the thirteen active fractions. The GC-MS profiles showed that indole alkaloids, carboxylic acids, esters, alcohols, dimethyl sulfoxide and phenols were abundant in most fractionated metabolites. Therefore, these compounds may be candidates for development of antibacterial, antifungal and anticancer drugs.

In summary, the null hypothesis that guided the study is not supported (rejected) by the results and isolation of seaweed-derived bacteria accompanied by the extraction, identification of bioactive compounds provides a multitude of unexplored abundant resources available in the Kenyan coast in respect to drug discovery.

6.2 Recommendations

Based on the results of this study, the following recommendations are advanced:

1. Since seaweed microbes are efficient producers of bioactive compounds, more studies need to be conducted on the isolation of seaweed-associated microbes using various culture dependent and culture independent techniques. This will assist in capturing a broader range of microbes capable of producing a wider range of bioactive substances that will aid in meeting the growing demand for novel bioactive compounds.

2. The most promising bacteria isolated from this study are, isolate Ul(EPI)7, closely related to *Bacillus* sp., isolate Ur(EPI)24, closely related to *Bacillus methylotrophicus* and isolate Cmex(END)8, closely related to *Bacillus axarquiensis*. These isolates can be used to produce pharmaceuticals that can deal with malignant cells and diseases caused by bacterial and fungal pathogens.
3. Marine algae host bacteria that have shown to produce bioactive compounds. Marine algae are fishery resources, that are not incorporated in the current Fisheries Act (GoK, 2016). For sustainable utilization of seaweeds, the government should formulate laws and regulations to protect and promote marine algae resources along the Kenya coast.

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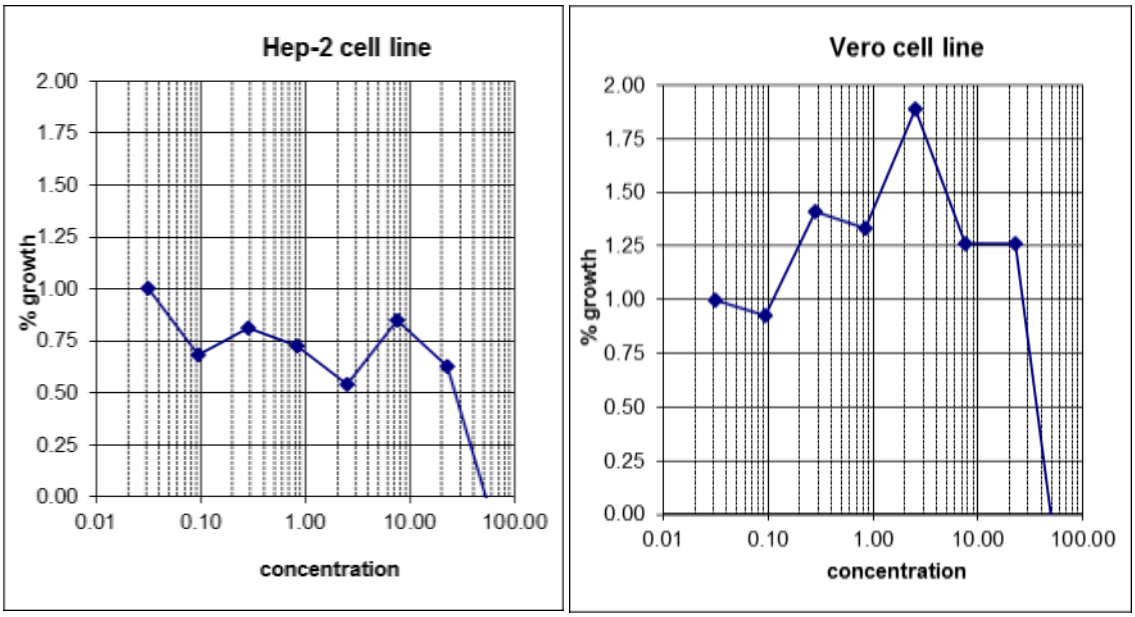
APPENDICES

Appendix A: Blast analysis of partial sequences of the 16S rDNA of the active bacterial isolates from the Kenya seaweeds and identity of the isolates based on the data base percentage sequence similarity.

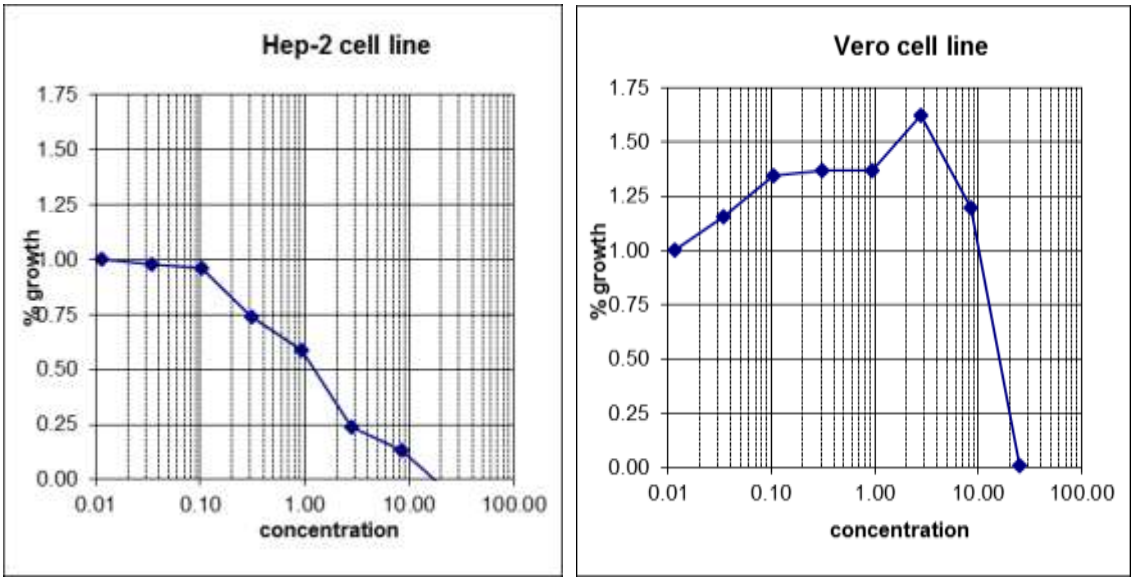
Isolate code	Accession number	% similarity	Taxa	Next neighbor
Gc(EPI)1	KY818962.1	99	Firmicutes	*Bacillus aerius*
So(END)21	KU529690.1	99	Firmicutes	*Geobacillus stearothermophilus*
Hcl(EPI)14	KF591403.1	85	Proteobacteria	*Massilia* sp.
Gs(END)43	KX462881.1	99	Firmicutes	*Bacillus amyloliquefaciens*
As(END)24	LT891939.1	98	Firmicutes	*Bacillus safensis*
As(END)28	KY818962.1	99	Firmicutes	*Bacillus aerius*
Cmex(END)8	KX018267.1	99	Firmicutes	*Bacillus axarquiensis*
Td(END)26	KU556315.1	98	Firmicutes	*Bacillus* sp.
Scri(EPI)17	JN128238.1	99	Firmicutes	*Bacillus safensis*
Hmu(EPI)9	KY818962.1	99	Firmicutes	*Bacillus aerius*
Hmu(EPI)16	KY769954.1	97	Firmicutes	*Bacillus velezensis*
Hmu(EPI)22	GU358071.1	98	Actinobacteria	*Streptomyces* sp.
Gc(EPI)12	MF144586.1	98	Firmicutes	*Bacillus subtilis*
Hp(EPI)19	GU358071.1	98	Actinobacteria	*Streptomyces* sp.
Gs(EPI)21	KX344013.1	99	Firmicutes	*Bacillus subtilis*
Gs(EPI)22	KC844810.1	98	Firmicutes	*Bacillus safensis*
As(END)10	KT583403.1	99	Firmicutes	*Bacillus* sp.
Cp(EPI)12	KX344013.1	99	Firmicutes	*Bacillus subtilis*
Ul(EPI)4	KC462187.1	99	Proteobacteria	*Desulfovibrio vulgaris*
Ul(EPI)7	MF045086.1	98	Firmicutes	*Bacillus* sp.
Ur(EPI)15	GU358071.1	98	Actinobacteria	*Streptomyces* sp.
Ur(EPI)16	KM974759.1	97	Firmicutes	*Bacillus cereus*
Ur(EPI)24	KT719463.1	97	Firmicutes	*Bacillus methylotrophicus*
Pt(EPI)15	AB490789.1	98	Firmicutes	*Bacillus* sp.
Pt(EPI)25	AB490789.1	98	Firmicutes	*Bacillus* sp.
Sr(END)7	KU862331.1	99	Firmicutes	*Bacillus subtilis*
Sr(END)9	KX453927.1	99	Firmicutes	*Bacillus pumilus*
Gs(END)8	KX344013.1	99	Firmicutes	*Bacillus subtilis*
As(END)2	KF746588.1	98	Firmicutes	*Bacillus tequilensis*
As(END)10	KY698015.1	98	Firmicutes	*Bacillus pumilus*
As(END)15	LT220204.1	97	Firmicutes	*Bacillus* sp.
Ssp(END)30a	KC462187.1	98	Proteobacteria	*Desulfovibrio vulgaris*
Ssp(END)32b	KC462187.1	96	Proteobacteria	*Desulfovibrio vulgaris*

Appendix B: Graphs on calculation of Effective Dilution 50% (ED_{50}) of the isolate fractionated secondary metabolites against Hep-2 cell line and vero cell line.

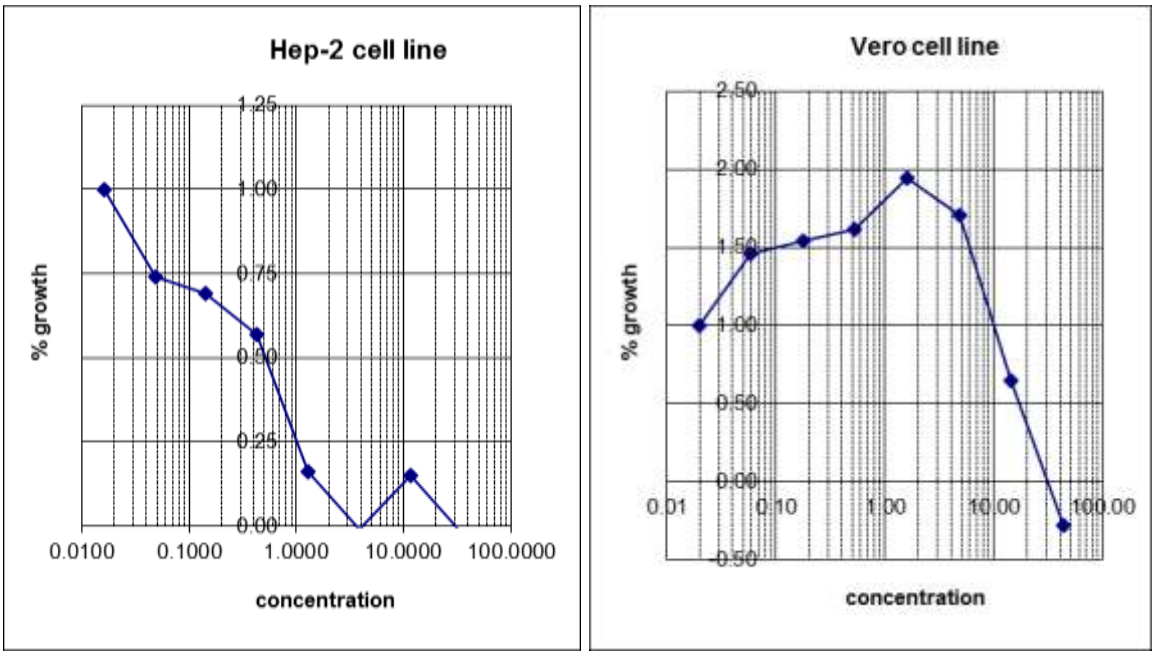
1. ED_{50} graph of *Bacillus aerius* (Isolate As(END)28): F1



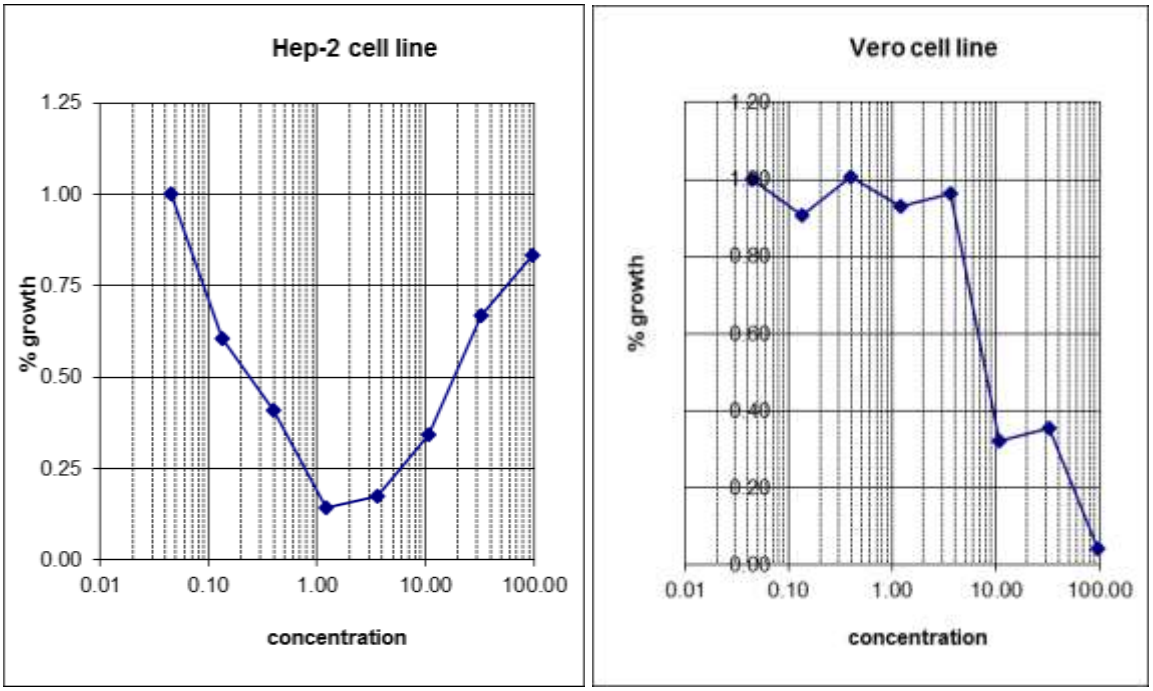
2. ED_{50} graph of *Bacillus axarquiensis* (Isolate C.mex(END)8): F2



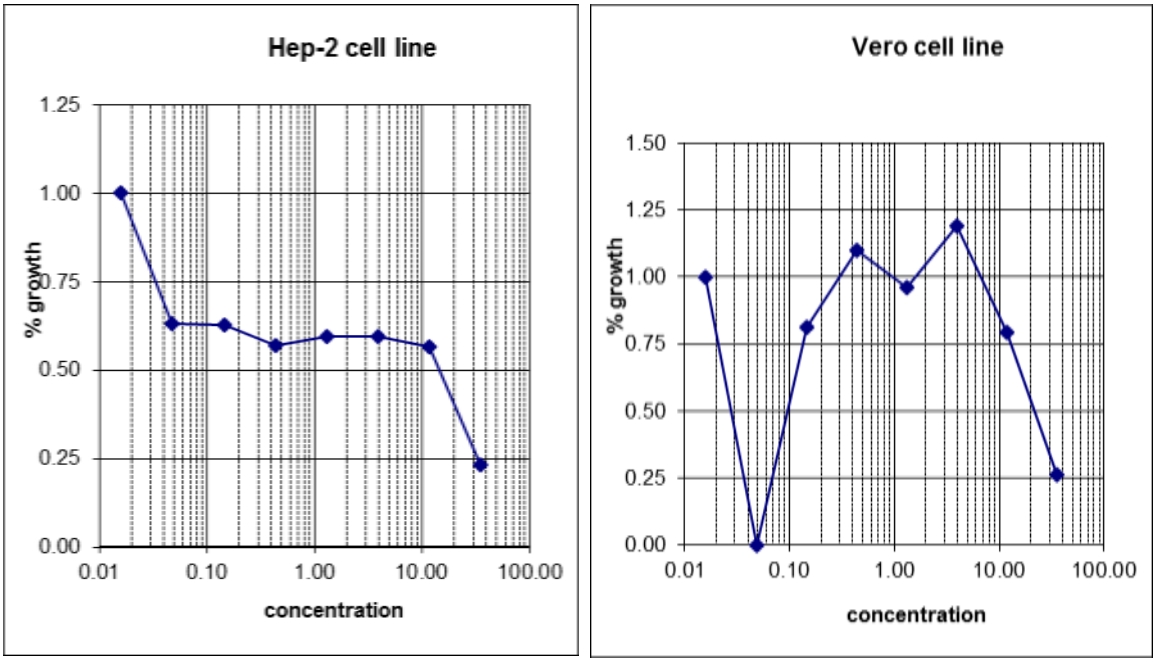
3. ED_{50} graph of *Streptomyces sp.* (Isolate H.mu(EPI)22): F2



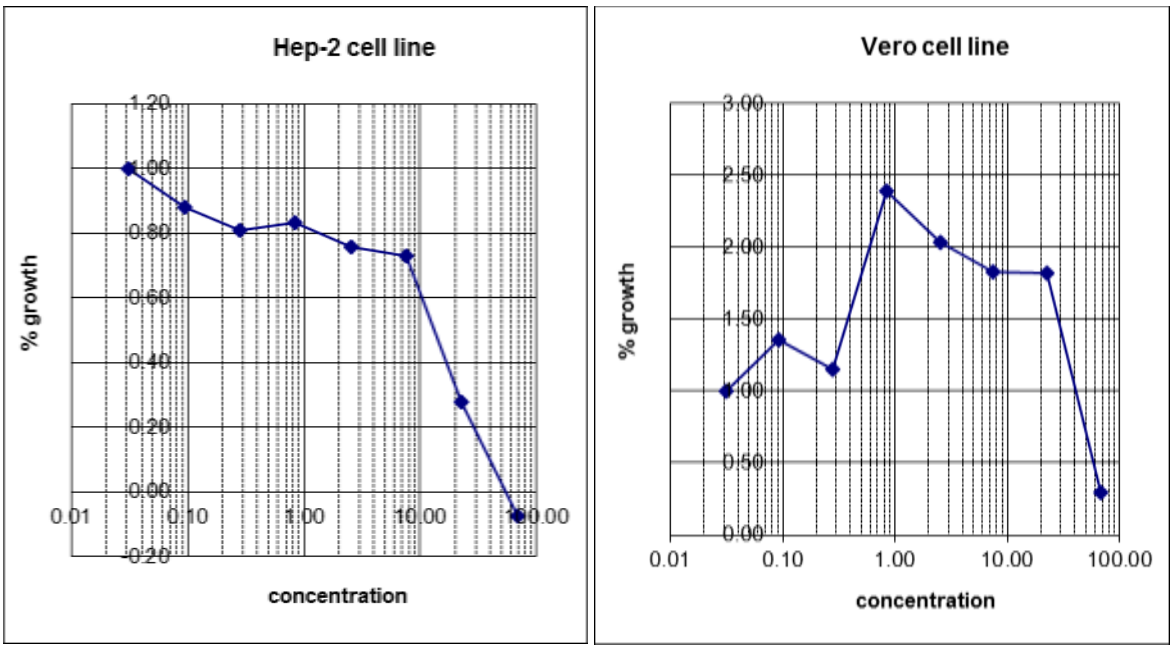
4. ED_{50} graph of *Streptomyces sp.* (Isolate Hp(EPI)19): F1



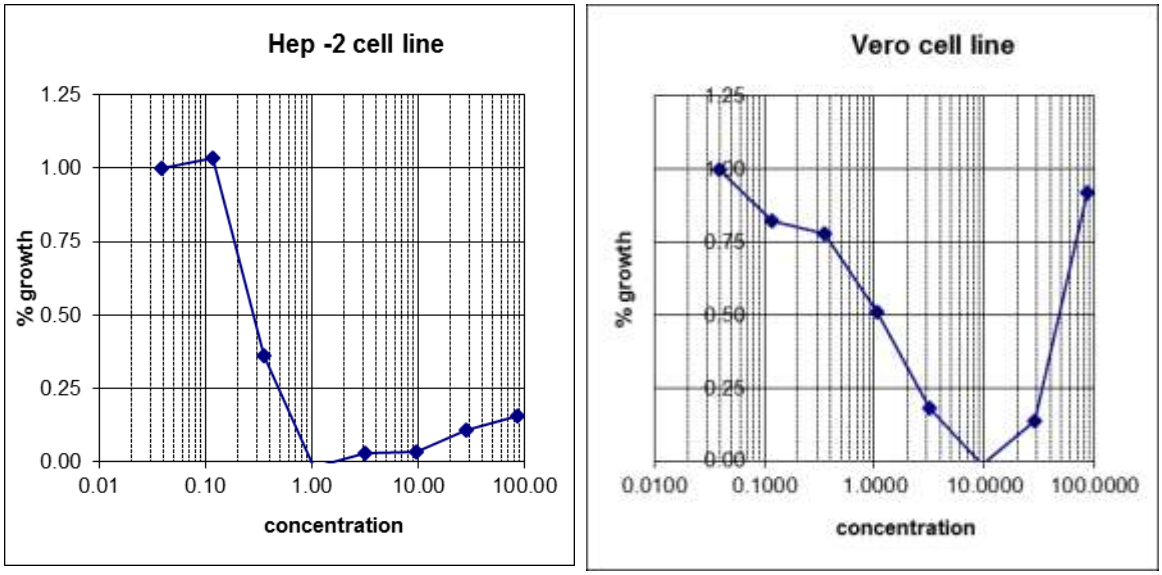
5. ED_{50} graph of *Bacillus subtilis* (Isolate Cp(EPI)12): F4



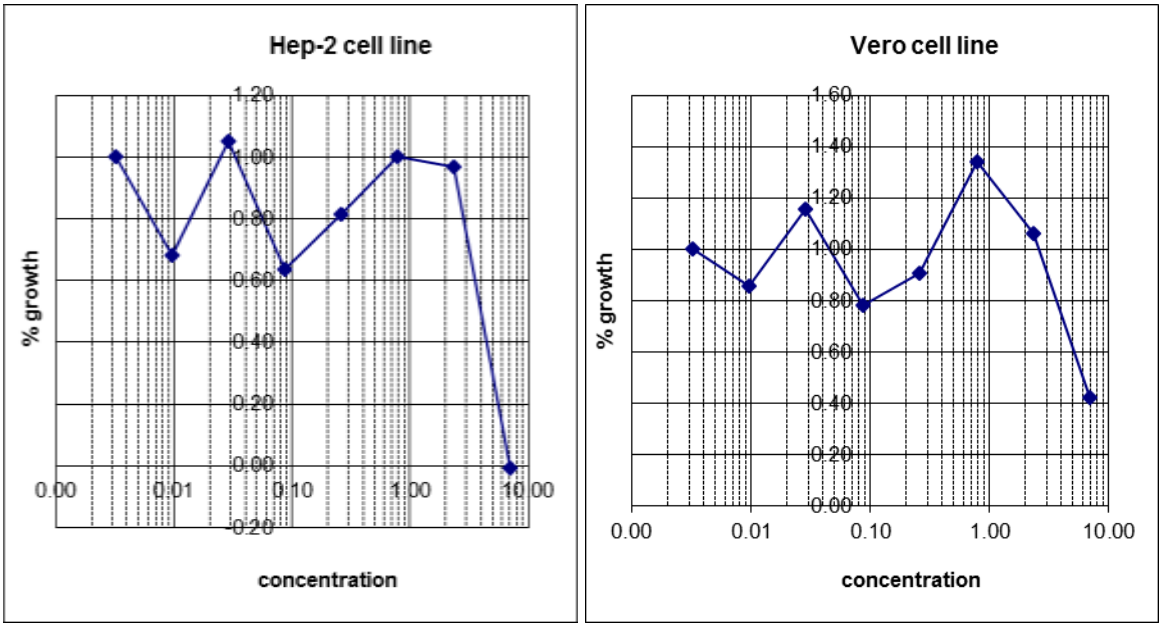
6. ED_{50} graph of *Bacillus subtilis* (Isolate Ul(EPI)7): F1



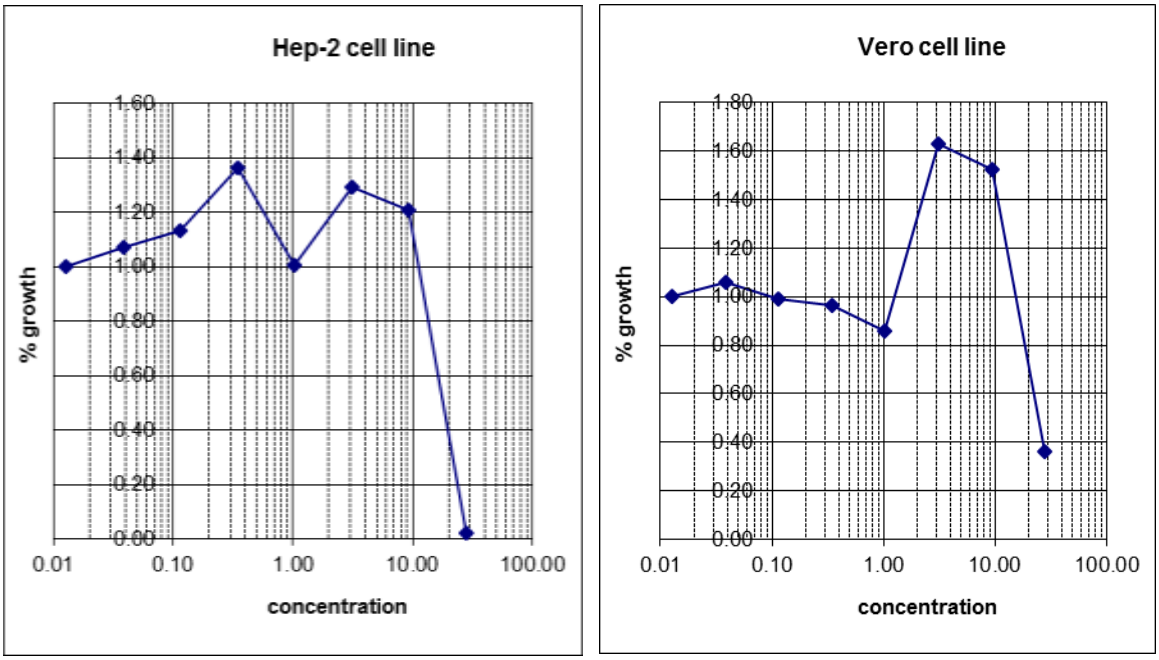
7. ED_{50} graph of *Bacillus methylotrophicus* (Isolate Ur(EPI)24): F1



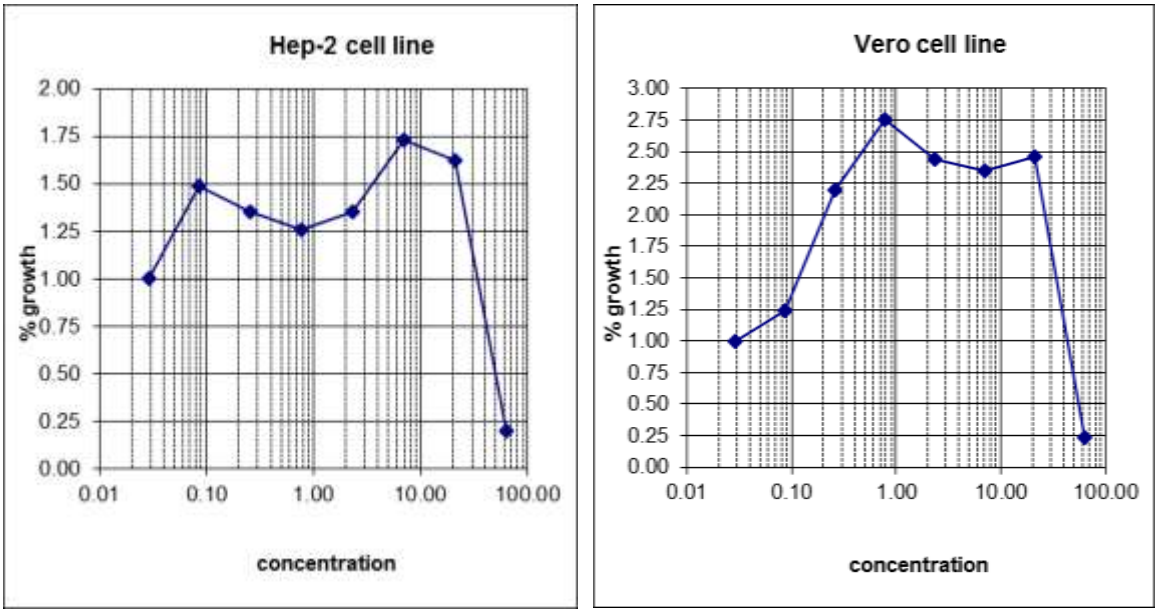
8. ED_{50} graph of *Bacillus subtilis* (Isolate Pt(EPI)15): F3



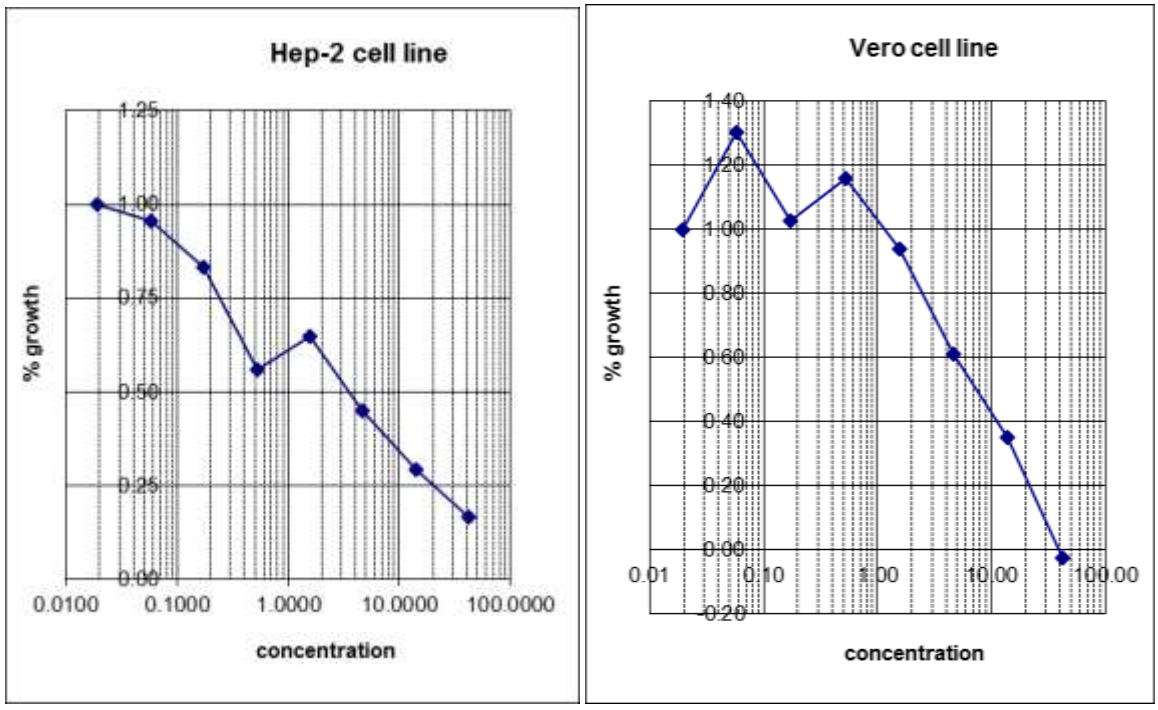
9. ED_{50} graph of *Bacillus sp.* (Isolate Pt(EPI)25): F5



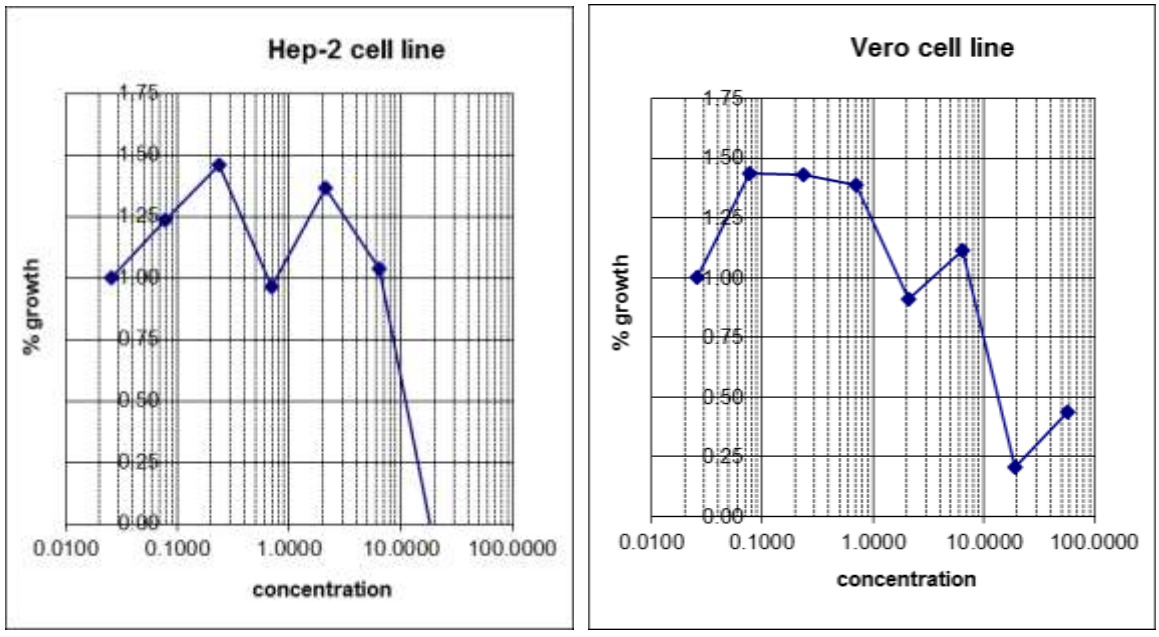
10. ED_{50} graph of *Bacillus subtilis* (Isolate Gs(END)8): F5



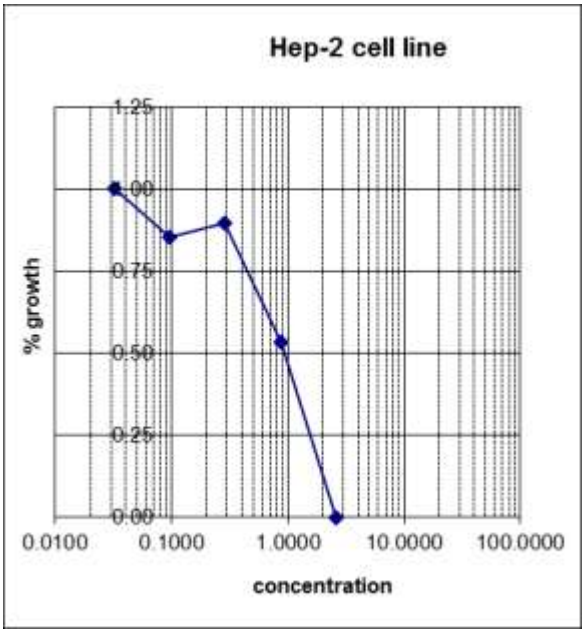
11. ED_{50} graph of *Bacillus pumilus* (Isolate As(END)10): F2

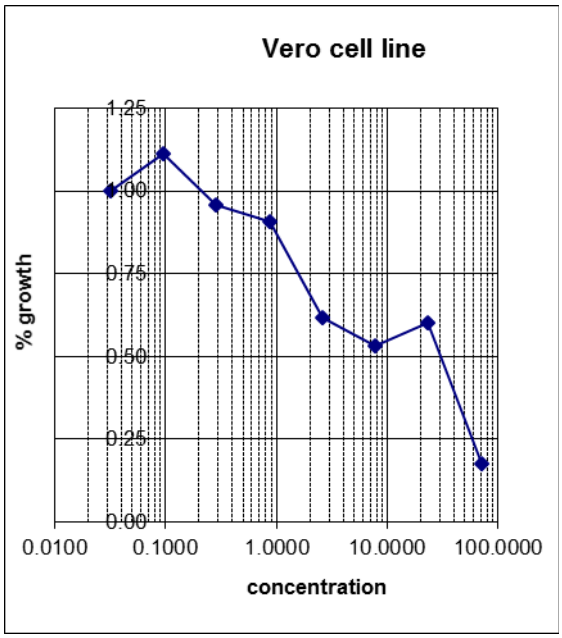


12. ED_{50} graph of *Desulfovibrio vulgaris* (Isolate S.sp(END)30a):F1

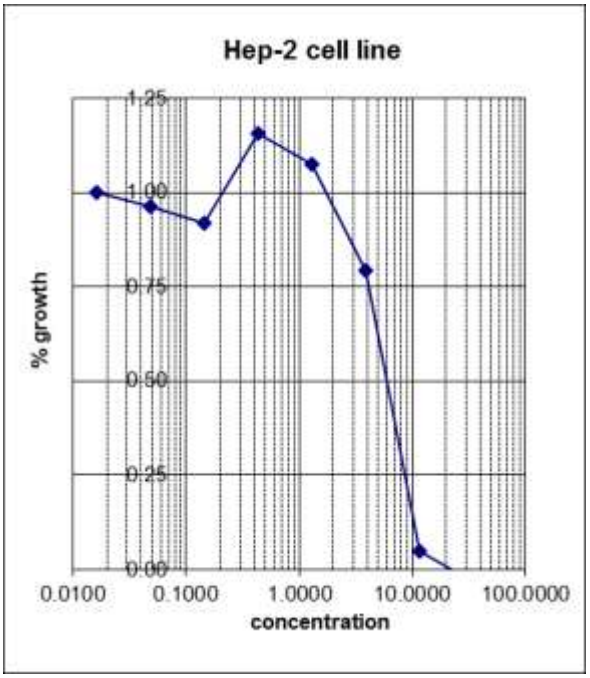


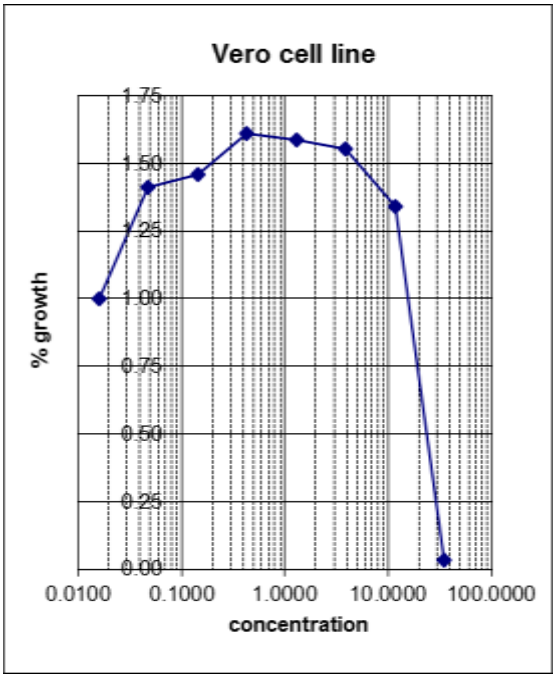
13. ED_{50} graph of *Desulfovibrio vulgaris* (Isolate S.sp(END)32b): F1





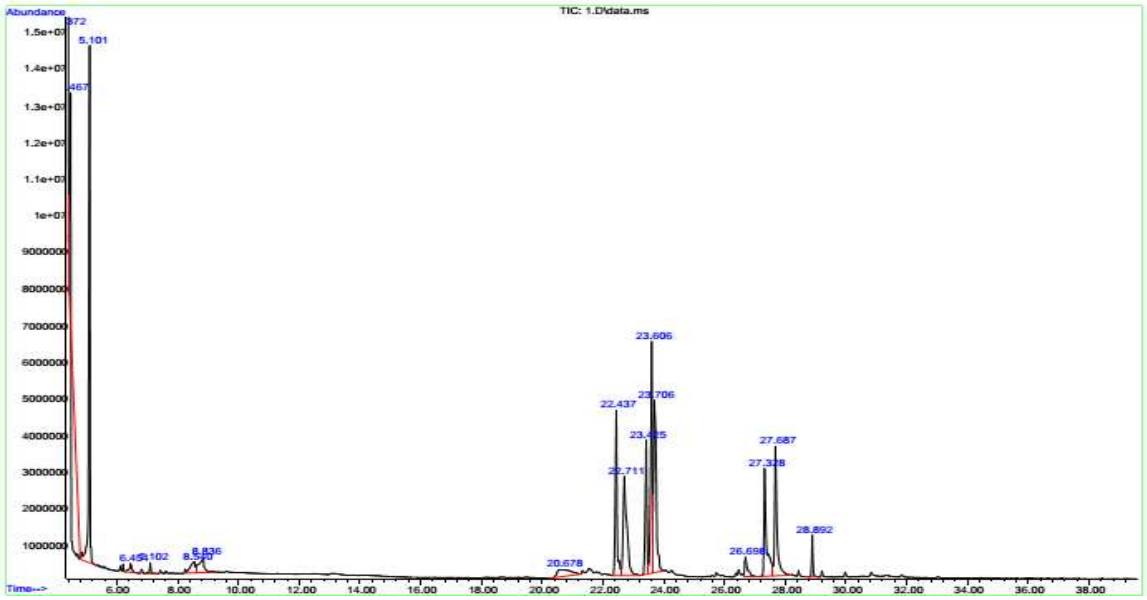
14. ED_{50} graph of Rapamycin drug (Positive control)



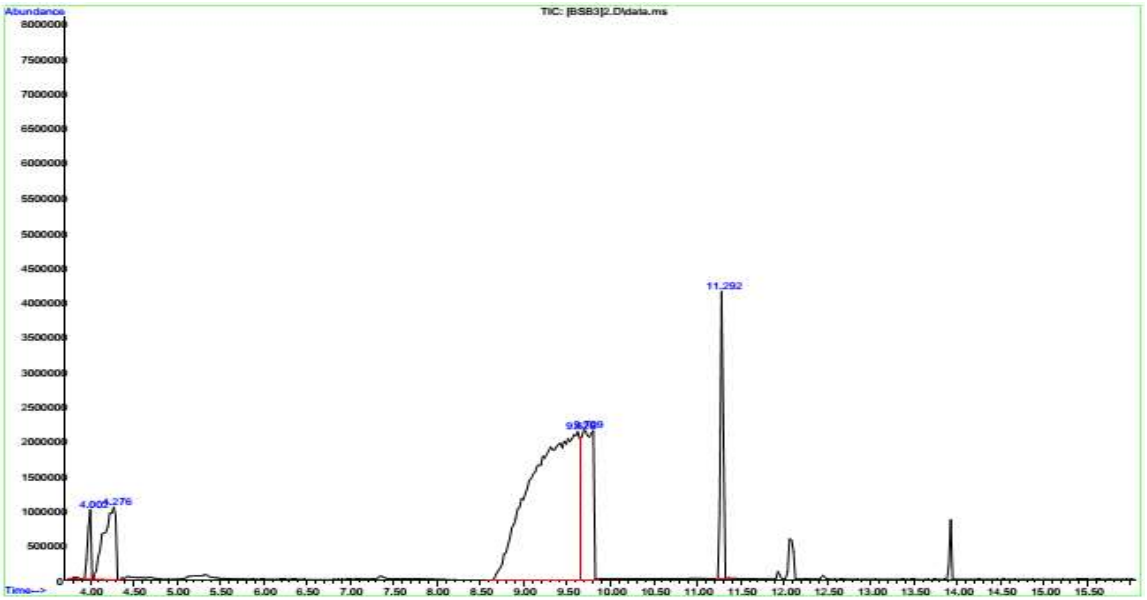


Appendix C: GC-MS spectra of characterized compounds.

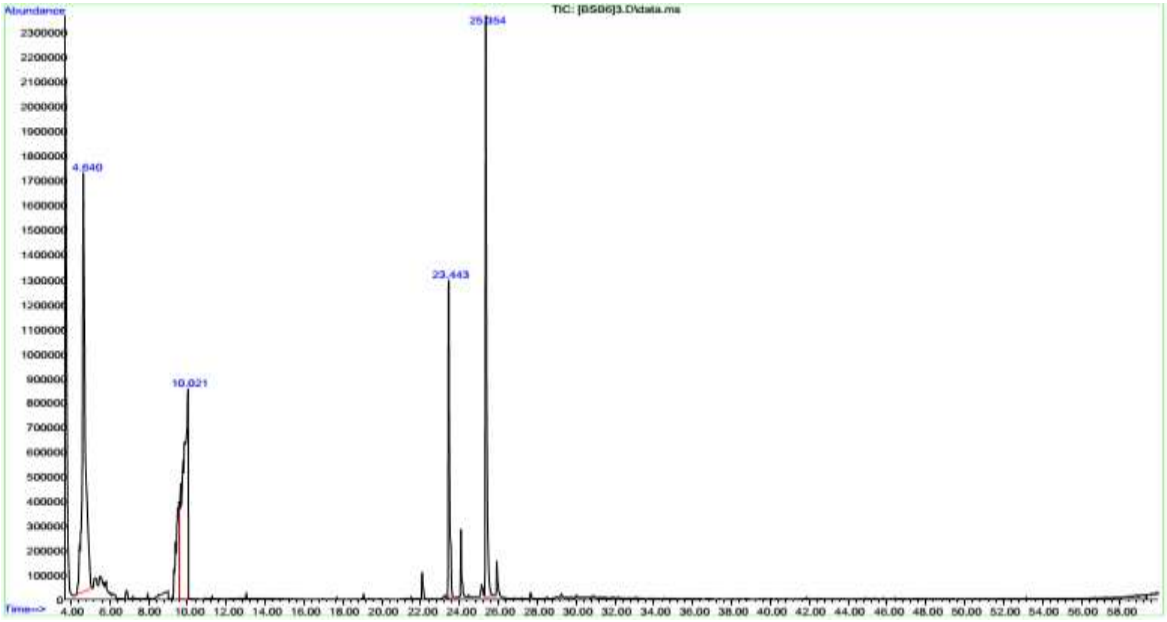
1. GC-MS chromatogram of 50:50 Ethyl acetate: methanol F1 of *Bacillus aerius* (Isolate As(END)28)



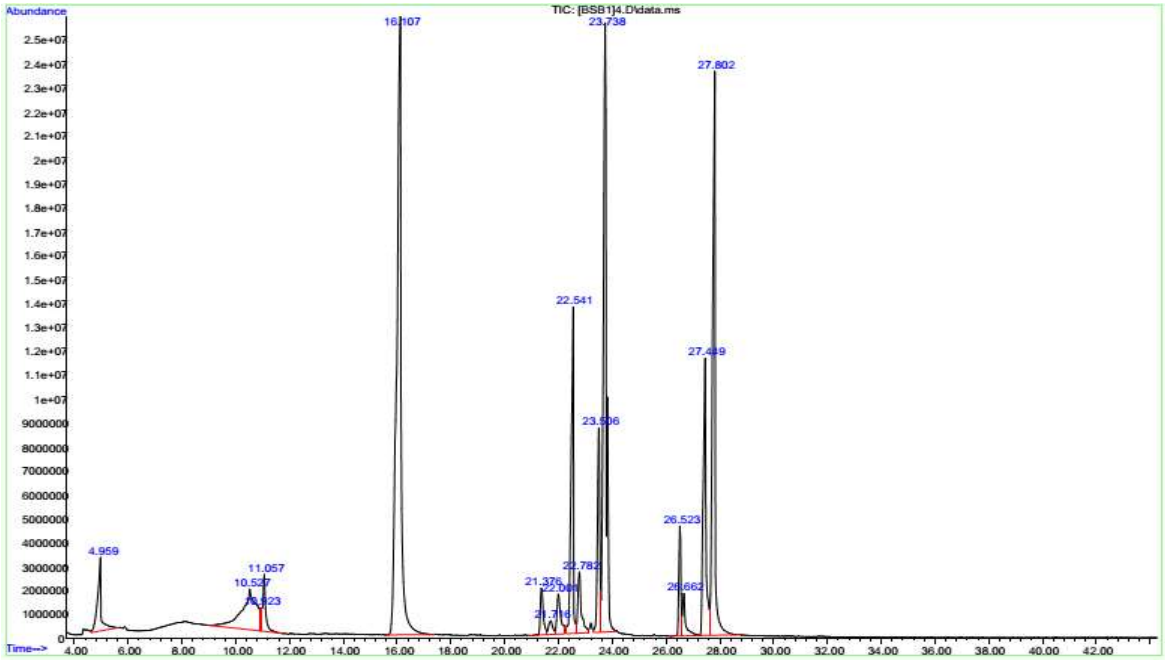
2. GC-MS chromatogram of 40:60 Ethyl acetate: methanol F2 of *Bacillus axarquiensis* (Isolate C.mex(END)8)



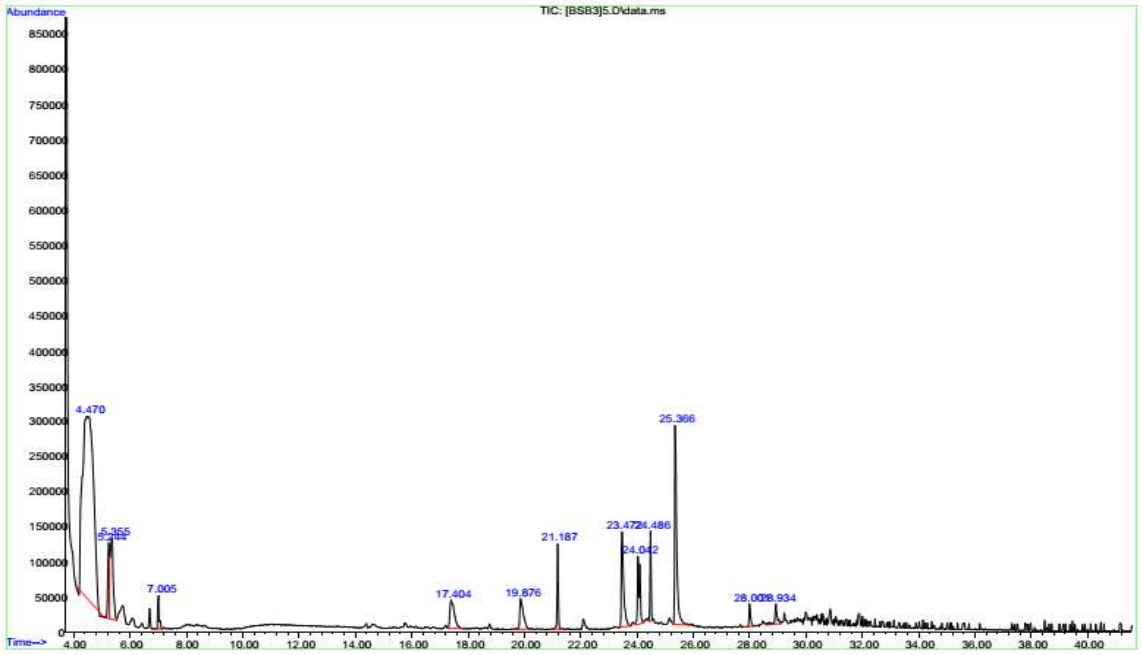
3. GC-MS chromatogram of 40:60 Ethyl acetate: methanol F2 of *Streptomyces sp*. (Isolate H.mu(EPI)22)



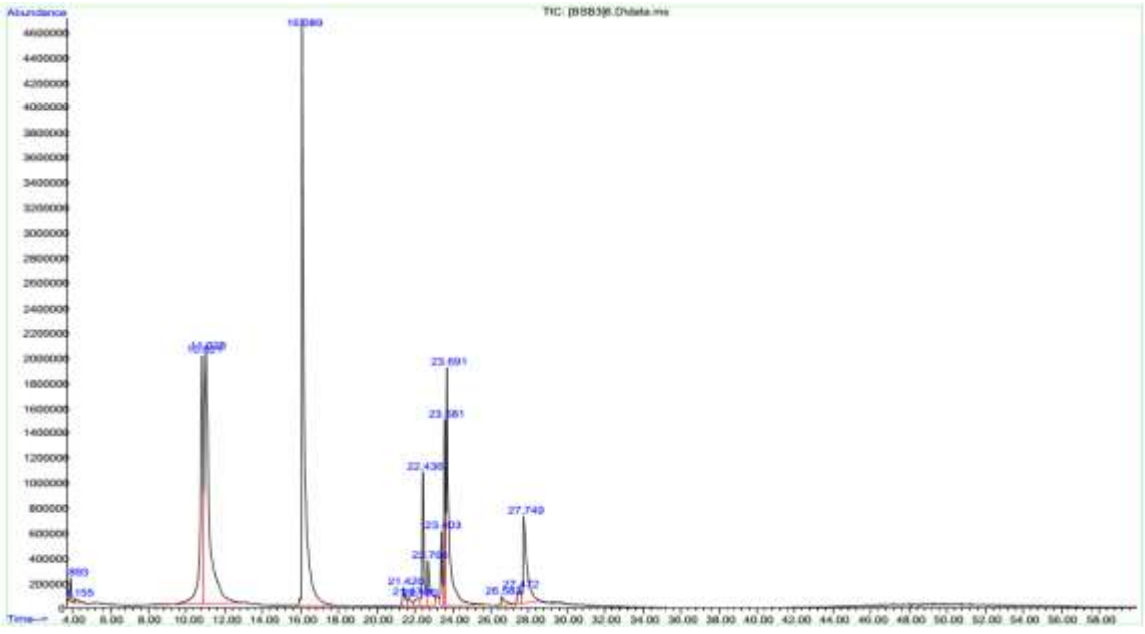
4. GC-MS chromatogram of 50:50 Ethyl acetate: methanol F1 of *Streptomyces sp*. (Isolate Hp(EPI)19)



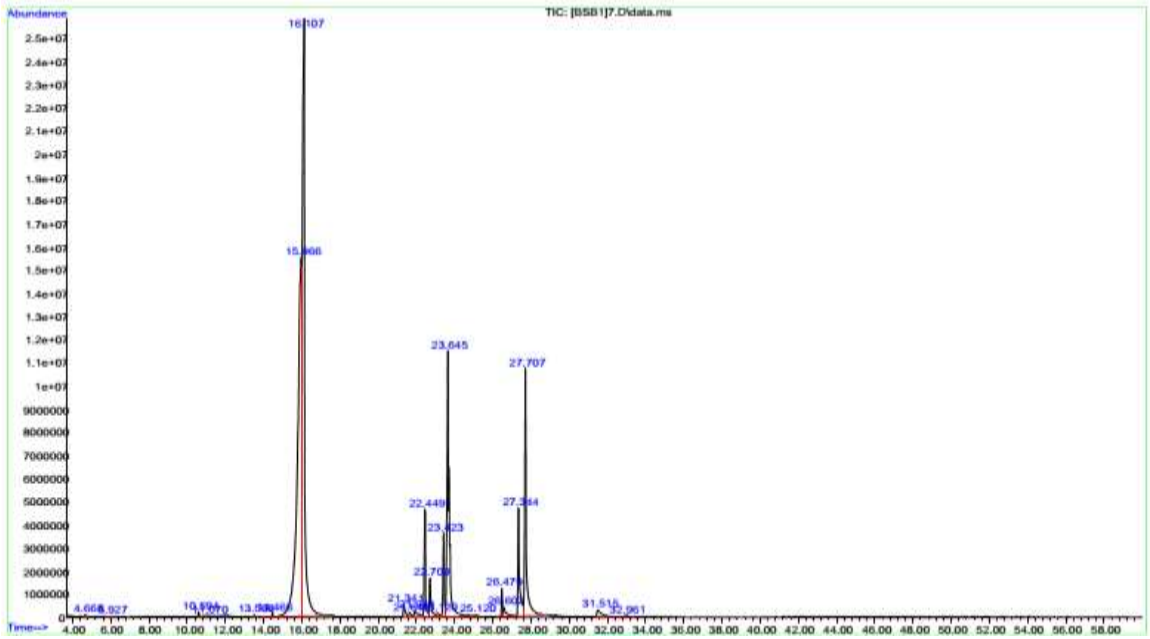
5. GC-MS chromatogram of 20:80 Ethyl acetate: methanol F4 of *Bacillus subtilis* (Isolate Cp(EPI)12)



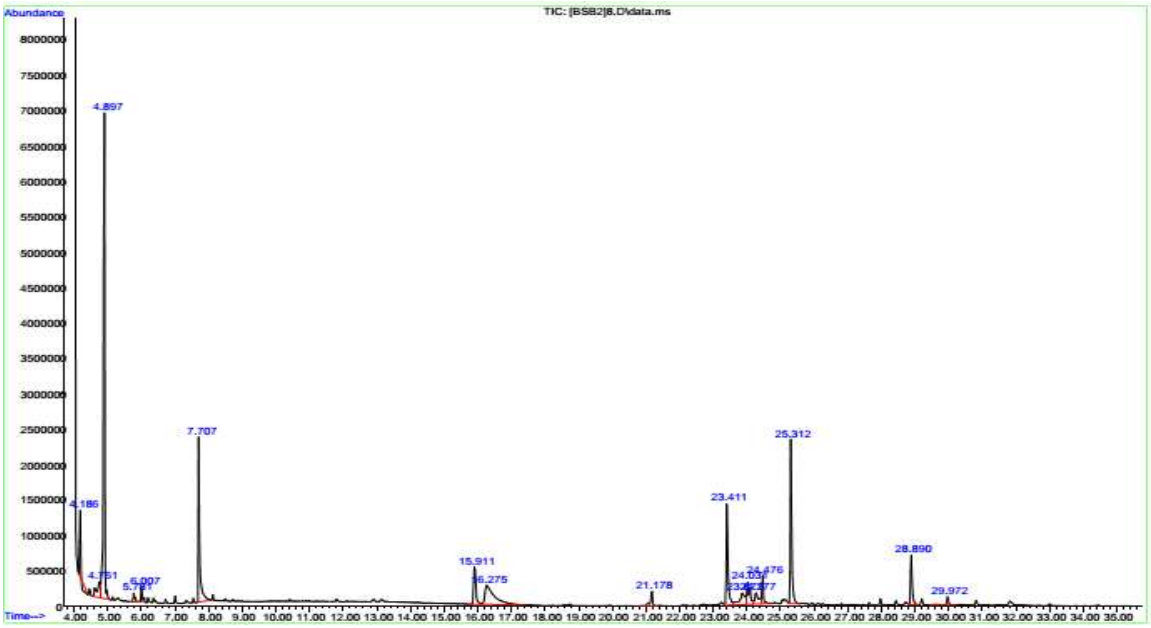
6. GC-MS chromatogram of 50:50 Ethyl acetate: methanol F1 of *Bacillus sp.* (Isolate Ul(EPI)7)



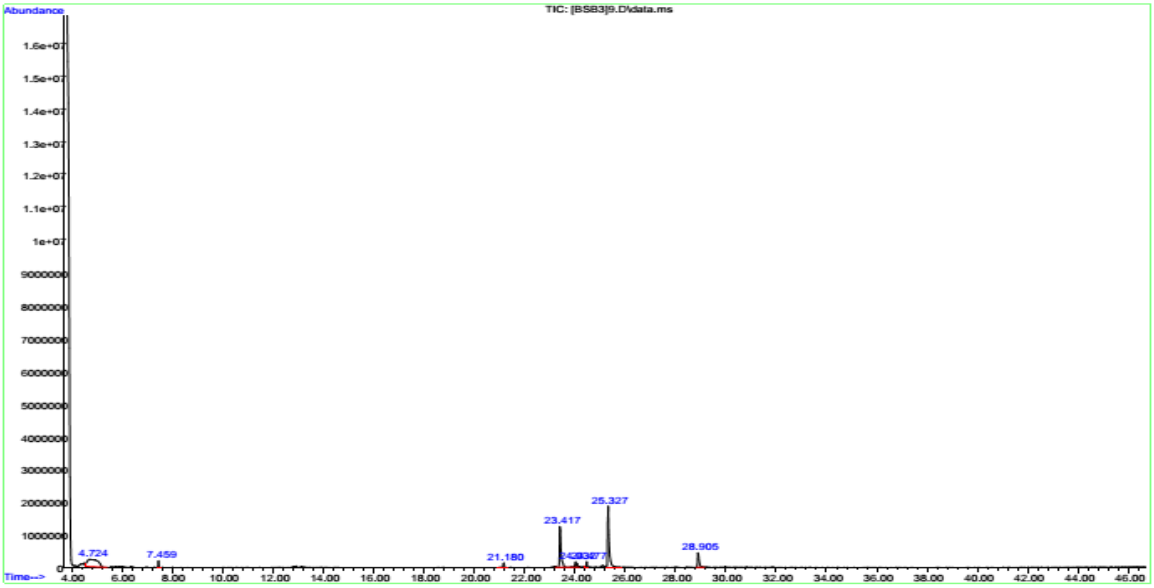
7. GC-MS chromatogram of 50:50 Ethyl acetate: methanol F1 of *Bacillus methylotrophicus* (Isolate Ur(EPI)24)



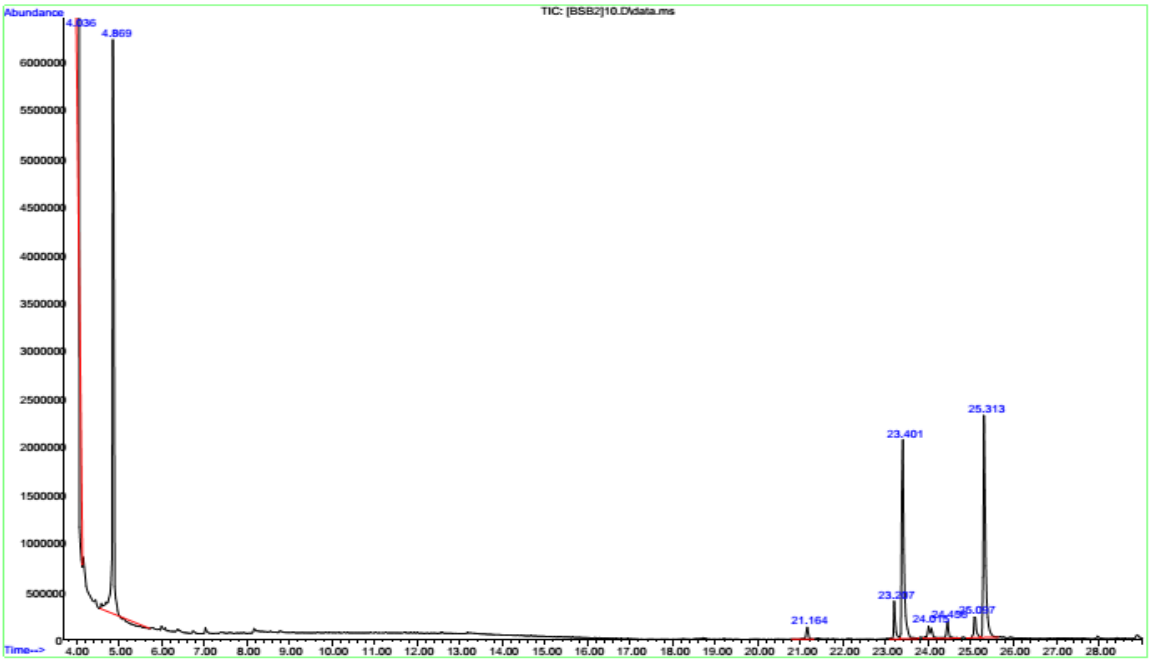
8. GC-MS chromatogram of 30:70 Ethyl acetate: methanol F3 of *Bacillus sp.* (Isolate Pt(EPI)25)



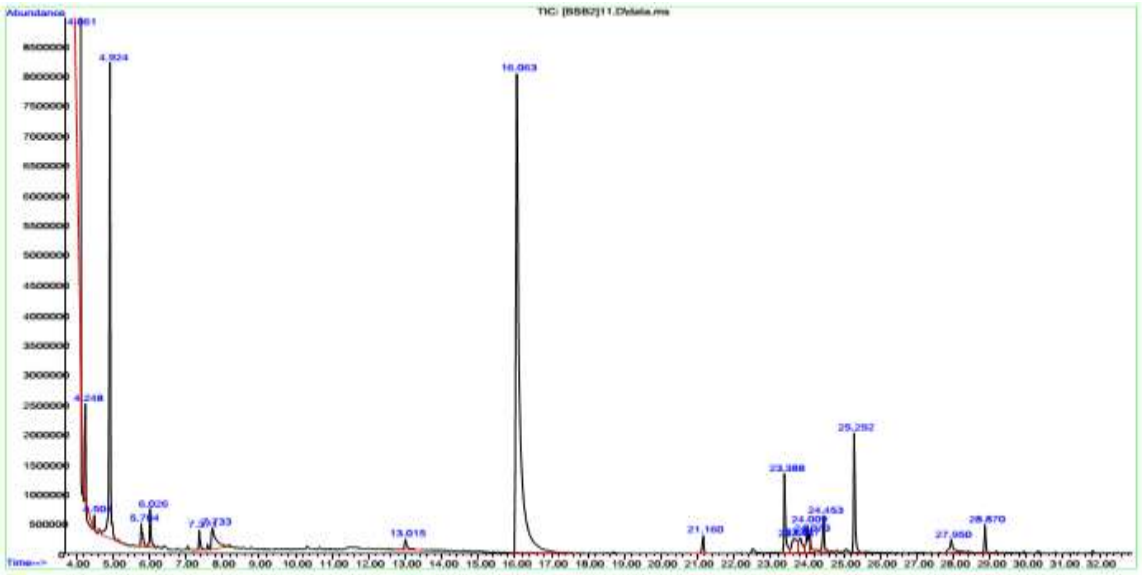
9. GC-MS chromatogram of 10:90 Ethyl acetate: methanol F5 of *Bacillus sp.* (Isolate Pt(EPI)26)



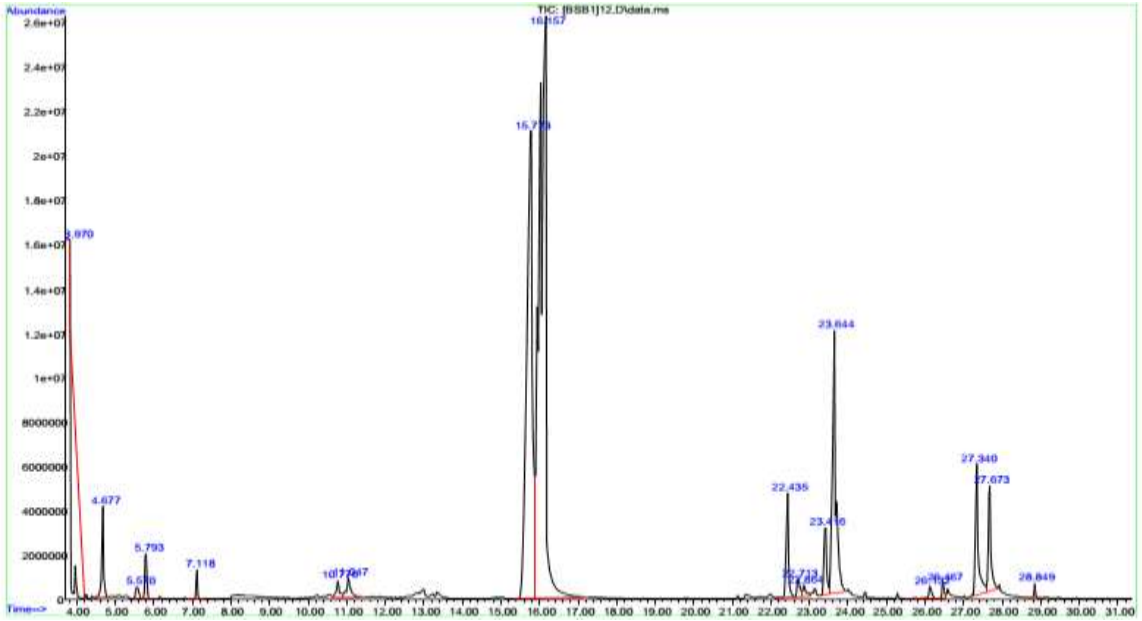
10. GC-MS chromatogram of 10:90 Ethyl acetate: methanol F5 of Bacillus subtilis (Isolate Gs(END)8)



11. GC-MS chromatogram of 40:60 Ethyl acetate: methanol F2 of *Bacillus pumilus* (Isolate As(END)10)



12. GC-MS chromatogram of 50:50 Ethyl acetate: methanol F1 of *Desulfovibrio vulgaris* (Isolate S.sp(END)30a)



13. GC-MS chromatogram of 50:50 Ethyl acetate: methanol F1 of *Desulfovibrio vulgaris* (Isolate S.sp(END)32b)