Potential of Endophytes and Rhizosphere Bacteria from Selected Indigenous Kenyan Plants around Juja as Sources of Antimicrobial Compounds

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature……..Date……...

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This thesis has been submitted with our approval as university supervisors.

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DEDICATION

This work is dedicated to my parents Mr. and Mrs. Kaaria, my brother and sisters. Thank you for all the support you have given me. Dad and mum you have laid in me a good foundation that has seen me through to this level of education. May God forever bless you and you are the very best.

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ABBREVIATIONS AND ACRONYMS

JKUAT	Jomo Kenyatta University of Agriculture and Technology
ICIPE	International Center of Insect Physiology and Ecology
BCED	Behavioral chemistry ecology department
MR-VP	Methyl red-Voges Proskauer
H_2O_2	Hydrogen peroxide
TSA	Tryptic soy agar
SIM	Sulphur indole motility
DNA	Deoxyribonucleic Acid
rDNA	ribosomal Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
NCBI	National center for biotechnology information
Rpm	Rotations per minute
TLC	Thin layer chromatography
UV	Ultra violet
GC-MS	Gas chromatography-Mass spectrophotometer
DCM	Dichloromethane
PTLC	Preparative thin layer chromatography
SNK	Student Newman Keuls test
OD	Optical density
TET	Tetracycline
GENT	Gentamycin

ATCC American Type Culture Collection

HIV-AIDS Human Immunodeficiency Virus-Acquired Immune Deficiency Syndrome

ABSTRACT

An increase in the number of people in the world having health problems caused by certain cancers, drug-resistant bacteria, parasitic protozoans, and fungi has caused alarm. Endophytes are a potential source of novel chemistry and biology products/compounds to assist in helping solve not only human health, but plant and animal health problems also. Rhizosphere microorganisms may produce a myriad of substances of potential use in modern medicine. By isolating the endophytes and rhizosphere bacteria and growing them in culture media, it is possible to harvest the bioactive compounds that they produce and these may contain potential novel compounds that may be effective candidates for treating emerging and re-emerging infectious human diseases.

The study was conducted with the aim of isolating endophytes and rhizosphere bacteria with antagonistic activity against pathogenic microbes. They were obtained from indigenous Kenyan plants around Juja that included; *Cleodendrum myricoides, Dombeya rotundifolia, Dalbergia menaloxylon, Lannea flavus, Dichrostachys cinerea, Gomphocarpus fruticosus, Balanites aegyptica, Schrebera alata, Jasminium floribundum* and *Hibiscus fuscus.* Characterization of the bacteria was done using morphological, physiological and molecular techniques while characterization of bioactive substances from culture filtrates was done using bioassay guided fractionation and spectroscopic methods. A total of fourty eight isolates (48) were obtained from both the endosphere and the rhizosphere regions. They were subjected to a cross streak antimicrobial screening against bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli*) and fungi (*Candida albicans*), to

determine their range of *in vitro* activity before extraction of the crude products. They showed a range of antagonistic activity against the test organism. Ten isolates (26%) were selected and investigated depending on their broad range of *in vitro* antimicrobial activity against the test bacteria and fungi and were further characterized. One isolate DM30 was subjected to molecular characterization. Phylogenetic analysis of amplified 16S rDNA sequence revealed that isolate DM30 belonged to the genus *Bacillus* and species *subtilis*. The crude products of the ten isolates were extracted (using ethyl acetate), yields determined and tested against the test bacteria and fungi. The products had different levels of activity and this was dependent on the test organism. There were significant differences ($P \leq 0.05$) in the antagonistic activity of the different crude products against the test organisms. Bioassay guided separation was carried out on the crude products after preparative thin layer chromatography. The products separated in different bands/fractions and the most active fraction (s) with an inhibition diameter of ≥7mm from each sample was further characterized to detect the secondary metabolites and the active components present with the aid of GC-MS machine. The identified compounds ranged from amines, acids, quinines, indole, steroids, azoles and many more. Compounds such as azoles have been known to be good antifungal agents. Toluene which was frequently detected in different fractions has been known to inhibit pathogenic microorganisms. The study demonstrated that a large number and range of secondary metabolites were present in the products. Further work might show whether these metabolites can be used to develop antimicrobial agents to replace the existing ones, once resistance builds up or for emerging pathogenic microorganisms.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Interest in novel products from biodiversity has varied greatly in the last decade, with a general decline in pharmaceutical bioprospecting by major companies, although resurgence is expected (Chapman, 2004). Based on the knowledge that many important drugs, such as aspirin, were derived from natural products (Jack, 1997) that are, generated in the tissues of native species, the industry has at various times invested heavily in the exploration of species-rich communities such as rain forests in search of commercially profitable pharmaceuticals (Ismail *et al.*, 1995; Bailey, 2001). Alarming levels of antibiotic resistance in many human pathogens is likely to provoke an increase in pharmaceutical bioprospecting, which remains a vital source of lead drug discovery (Wessjohann, 2000; McGeer and Low, 2003; Newman *et al.*, 2003). Malaria, one of the world's most deadly diseases, has been treated historically with drugs derived from natural products—quinine, chloroquine, mefloquine, and doxycycline—and today the artemisinins derived from the Chinese herb Qinghao (*Artemisia annua*) are at the forefront of the battle against this parasite. The probability that any single discovery of a drug, actually reaching the market place remains low. This is because the conventional process of drug discovery has several distinct and increasingly expensive stages: acquisition of the natural material; extraction of the active compounds; primary screening against a range of human disease organisms; isolation and chemical characterization of the active compounds; secondary screening assaying the compounds

in tissue cultures and experimental animals; structural chemistry and synthesis; pre-clinical development with a view to human trials; and clinical development, marketing, and distribution. The magnitude of the resource was illustrated by Henkel *et al*. (1999), who provided a summary of the wide range of organisms from which drugs have been derived, including bacteria and fungi (both terrestrial and marine), plants, algae, and a variety of invertebrates, including worms, insects and mollusks.

Natural products are still important sources of novel compounds for pharmaceuticals. An average of 62% of new, small molecule, non-synthetic chemical entities developed for cancer research over the period 1982–2002 were derived from natural products. In anti-hypersensitive drug research, 65% of drugs currently synthesized can be traced to natural structures. This emphasizes the important role of many natural products as blueprints rather than the actual end points. Newman *et al*. (2003), who assembled these data, noted that they had not been able to identify a *de novo* combinatorial compound approved as a drug during this time frame, despite massive investment in this technique by pharmaceutical companies.

Some of the most striking examples of recent drug development based on natural products are the drugs that inhibit cell division. The current assessment of bioprospecting by the large pharmaceutical companies is reflected in the focus of their research and development, where the major investment is in rational drug design and combinatorial chemistry (Hijfte *et al.,* 1999; Olsen *et al.,* 2002) rather than natural products. Such decisions have probably been based on three factors: recent advances in high throughput instrumentation, low ‘‘hit’’ rates from natural product exploration, and consequently the high risks of natural product investment. On the other hand, natural

product bioprospecting is the main activity of a variety of active small companies that sell their products to the larger ones that can afford the massive costs of drug development. Some contemporary researchers believe that natural product research is more likely to result in new lead discovery and that the great advantage of combinatorial chemistry is its capacity to take advantage of such leads.

Nature has provided a broad spectrum of structurally diverse secondary metabolites (Vandamme, 1994; Verpoorte, 1998; Maier *et al.*, 1999). Despite this great diversity, microbial secondary metabolites are synthesized from only a few precursors, in pathways with a relative small number of reactions, which branch from just a limited number of reactions of the primary metabolism (Demain and Fang, 2000). About 100,000 secondary metabolites of a molecular weight below than 2500 have been characterized, among them approximately 50,000 from microbial sources (Bezborodov, 1978). New microbial bioactive products continue to be discovered at an amazing pace: 200-300 per year in the late 70s, increasing to 500 per year by 1997 (Demain, 2000).

Antibiotics are perhaps the most widely studied type of secondary metabolites with 12000 antibiotics known up to 1995. Again, microbial cells are the most important source of this type of secondary metabolites. Indeed, from the known antibiotics 55% are produced by filamentous bacteria of the genus actinomyces, 11% from other actinomyces, 12% from non-filamentous bacteria and 22% from filamentous fungi (Janos, 2004).

Different alternatives for improving production of secondary metabolites with different activities for biotechnological applications have been extensively investigated.

Biotechnology industry is based on harnessing the metabolic activities of different organisms to produce a wide variety of diverse compounds, which are used by other industries. Two methods have been applied; optimization of fermentation process and improvement of strains (Parekh *et al.*, 2000). The microbial production of secondary metabolites is extremely sensitive to environmental factors or culture conditions (Bunch and Harris, 1986). For instance, the *in vitro* production of most antibiotics depends on the composition of the culture medium in which the producer organism is grown. For this reason, medium optimization has been the standard procedure for optimizing antibiotic production.

Historically, fungal and bacterial secondary metabolites have been an important source of lead structures for new drug compounds. It was the discovery of penicillin that led to later discoveries of potent antibiotics isolated from microbial broths. Despite the existence of potent antibiotics and antifungal agents available in the market, there is a continuous search for novel drug compounds as the numbers of drug resistant microorganisms are continuously increasing (Pinner *et al.*, 1996).

Over the last few years there has been increasing interest in the investigation of endophytic and rhizosphere bacteria, which live asymptomatically within plant tissues and on the root surface respectively, as a source of novel bioactive compounds. Although most early searches for bioactive compounds focused on soil fungi and bacteria, the rate of discovery of interesting new compounds from the soil has diminished.

Research has turned to exploration of niches that have not yet been explored (Bills, 1995; Pelaez *et al*., 1998) to find novel, pharmacologically active compounds in industrial screening programmes. One such niche is the healthy, green tissues of living plants, which are known to harbor a rich and diverse bacterial biota that is distinct from the soil mycobiota (Cannon and Simmons, 2002).

In this context, endophytic and rhizospheric bacteria isolated from medicinal plants are promising.

1.2 Endophytes

There are several definitions of endophytes, which in general describe fungi and bacteria including actinomycetes. Kado (1992) defined endophytes as: "bacteria that reside within living plant tissues without doing substantive harm or gaining benefit other than securing residency." However, in his definition, "substantive harm" is quite confusing because there are many cases in which even pathogenic bacteria do not always cause symptoms depending on the population density in their host plants. On the other hand, Quispel (1992) considered endophytes as only those bacteria that establish an endosymbiotic relationship with the plant, whereby the plant receives an ecological benefit from the presence of the symbionts, such as increased stress tolerance or plant growth promotion. His consideration is also confusing because non- or mildly pathogenic microbes are known to induce systemic or localized resistance of host plants. In such a case it is difficult to distinguish symbionts from pathogenic parasites. Hallmann *et al*. (1997) defined any bacterium as an endophyte if it does not visibly

harm the plant and it can be isolated from surface disinfected plant tissues or extracted from inside the plant.

Rosenblueth and Martinez-Romero (2006) proposed that criteria to recognize "true" endophytic bacteria require not only the isolation from surface-disinfected tissues but also microscopic evidence to visualize "tagged" bacteria inside plant tissues. True endophytes may also be recognized by their capacity to reinfect disinfected seedlings. They suggest that bacteria not validated microscopically are "putative" endophytes. According to their definition, most endophytes reported to date are "putative" endophytes, because microscopic evidence has not been obtained. Although their definition is logical in a strict sense, enough evidence has not been accumulated to discuss *in planta* localization and behavior of endophytic actinomycetes. Strobel and Daisy (2003) define endophytes as organisms that reside in the living tissues of the plant and do so in a variety of relationships, ranging from symbiotic to slightly pathogenic.

Endophytic microorganisms are microorganisms that grow in the intercellular spaces of higher plants and are recognized as one of the most chemically promising groups of microorganisms in terms of diversity and pharmaceutical potential (Wagenaar and Clardy, 2001). The endophytic microorganisms are not considered as saprophytes since they are associated with living tissues, and may in some way contribute to the well being of the plant. The plant is thought to provide nutrients to the microbe, while the microbe may produce factors that protect the host plant from attack by animals, insects or microbes (Yang *et al.,* 1994).

There are many reports demonstrating that many bioactive compounds could be produced by endophytic microorganisms (Huang *et al.*, 2001). Many endophytes are capable of synthesizing bio-active compounds that can be used by the plant for defense against pathogenic fungi and bacteria. Among the prominent natural products, which are produced not only by *Taxus sp.* but also by endophytes isolated from *Taxus brevifolia* and other plants (Strobel, 2002) is taxol (Figure 1), which is used as antitumor agent.

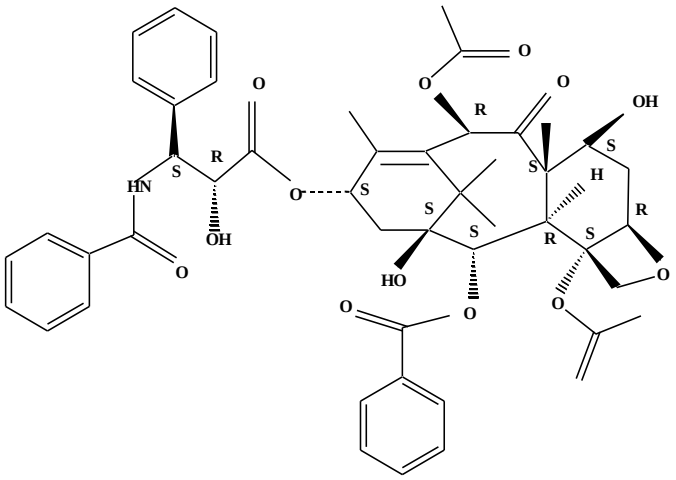

Figure 1: Structure of Taxol

Endophytic microbes including bacteria, actinomycetes and fungi are ubiquitous in most plant species, especially in field-grown plants (Fisher *et al.*, 1992). Although some of the endophytes are pathogenic to host plants and can locally or systemically colonize plant tissues, others latently reside in the internal tissues of non-symptomatic plants without causing any adverse effects to the plants. Consequently, intimate associations between endophytes and host plants can be formed without harming the plant. Endophytes have been demonstrated to improve and promote growth of host

plants as well as to reduce disease symptoms caused by plant pathogens and/or various environmental stresses. The low stress tolerance of axenic plants is commonly believed to result partly from the absence of endophytic microbes (Hallmann *et al.,* 1997).

1.3 Endophytes and biodiversity

Of the myriad of ecosystems on earth, those having the greatest biodiversity seem to be the ones also having endophytes with the greatest number of and most biodiverse microorganisms, for example tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land's surface, yet they harbor more than 60% of the world's terrestrial biodiversity (Mittermeier *et al.*, 1999).

As such, one would expect that areas of high plant endemicity also possess specific endophytes that may have evolved with the endemic plant species. Ultimately, biological diversity implies chemical diversity because of the constant chemical innovation that exists in ecosystems where the evolutionary race to survive is the most active.

Tropical rainforests are a remarkable example of this type of environment. Competition is great, resources are limited, and selection pressure is at its peak. This gives rise to a high probability that rainforests are a source of novel molecular structures and biologically active compounds (Redell and Gordon, 2000). Bills *et al.* (2002) describe a metabolic distinction between tropical and temperate endophytes through statistical data which compares the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin.

Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other tropical substrata (Bills *et al.*, 2002). This observation suggests the importance of the host plant in influencing the general metabolism of endophytic microbes.

Tan and Zou (2001) believe that the reason why some endophytes produce certain phytochemicals originally characteristic of the host might be related to a genetic recombination of the endophyte with the host that occurs in evolutionary time.

This is a concept that was originally proposed as a mechanism to explain why the endophytic fungus *Taxomyces andreanae* may be producing paclitaxel (Figure 2) (Stierle *et al.*, 1993).

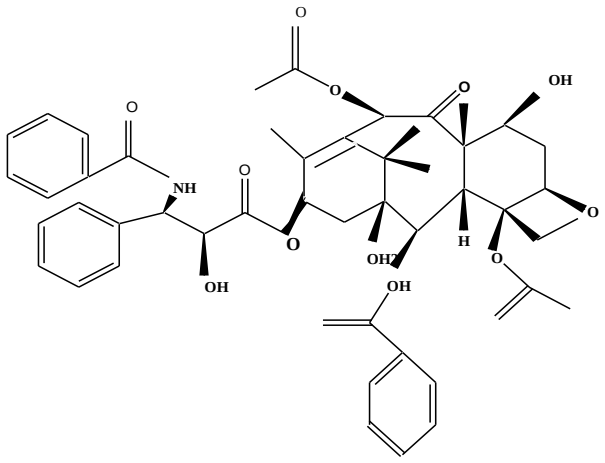

Figure 2: Structure of paclitaxel

Thus, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and

possibly rare plants but also preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a valued product may be easier and more economical to produce, effectively reducing its market price (Demain, 2000).

All aspects of the biology and interrelatedness of endophytes with their respective hosts is a vastly under-investigated and exciting field. Thus, more background information on a given plant species and its microorganismal biology would be exceedingly helpful in directing the search for bioactive products (Strobel and Daisy, 2003). Currently, no one is quite certain of the role of endophytes in nature and what appears to be their relationship to various host plant species.

Frequently, many endophytes (biotypes) of the same species are isolated from the same plant and only one of the endophytes will produce a highly biologically active compound in culture (Li *et al.*, 1996). A great deal of uncertainty also exists between what an endophyte produces in culture and what it may produce in nature. It does seem apparent that the production of certain bioactive compounds by the endophyte *in situ* may facilitate the domination of its biological niche within the plant or even provide protection to the plant from harmful invading pathogens (Strobel and Daisy, 2003). This may be especially true if the bioactive product of the endophyte is unique to it and is not produced by the host. Seemingly, this would more easily facilitate the study of the role of the endophyte and its role in the plant. Furthermore, little information exists relative to the biochemistry and physiology of the interactions of the endophyte with its host plant (Strobel and Daisy, 2003). It would seem that many factors changing in the host as related to the season and age, environment, and location may influence the biology of the endophyte.

Endophytic microorganisms are to be found in virtually every plant on earth (Strobel, 2003). Almost all vascular plant species are found to harbor endophytic bacteria and/or fungi (Sturz *et al.*, 2000). As a matter of fact, endophytes are important components of the microbial diversity (Clay, 1992).

Several to hundreds of endophytes species can be isolated from a single plant species, among them at least one species showing host specificity. The environmental condition under which the host is growing also affects the endophyte population (Hata *et al.*, 1998). Endophytes are presumably ubiquitous in the plant kingdom with the population being dependent on host species and location.

1.4 Natural products from endophytic microbes

Natural products are naturally derived metabolites and/or by-products from microorganisms, plants, or animals (Baker *et al.*, 2000). These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine.

Endophytes have been reported to be sources of antibiotics; low-molecular-weight organic natural products. These antibiotics are active at low concentration against other microorganisms (Demain, 1981). Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents including, but not limited to, phytopathogens, bacteria, fungi, viruses, and protozoan's that affect humans and animals (Strobel and Daisy, 2003).

There are a limited number of bacterial species known to be associated with plants, and one of the most common genera encountered is *Pseudomonas spp*. *Pseudomonas spp*. have representative biotypes and species that are epiphytic, endophytic, and pathogenic. Some of these species produce phytotoxic compounds as well as antibiotics (Strobel, 2002)

Ecomycins are produced by *Pseudomonas viridiflava*; a bacterium generally associated with the leaves of many grass species and is located on and within the tissues. Besides common amino acids; alanine, serine, threonine, and glycine, some unusual amino acids are incorporated into the structure of the ecomycins, including homoserine and beta-hydroxyaspartic acid tissues (Miller *et al*., 1998). The ecomycins are active against human pathogenic fungi such as *Cryptococcus neoformans* and *Candida albicans* (Marcia and Katia, 2004).

Apart from the bacterial endophytes, antimicrobials can also be prospected from the rhizosphere. The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. This zone is about 1 mm wide, but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root, and by microorganisms feeding on the compounds. The rhizosphere is frequently divided into the endorhizosphere, the rhizoplane and the ectorhizosphere. These respective compartments encompass the root tissues, the root surface and associated soil (Jose *et al.*, 2005).

The rhizosphere is a densely populated area in which the roots must compete with the invading root systems of neighboring plant species for space, water, and mineral

nutrients, and with soil-borne microorganisms, including bacteria, fungi, and insects feeding on an abundant source of organic material (Ryan and Delhaize, 2001).

Survival of any plant species in a particular rhizosphere environment depends primarily on the ability of the plant to perceive changes in the local environment that require an adaptive response. Upon encountering a challenge, roots typically respond by secreting certain small molecules and proteins (Stintzi and Browse, 2000; Stotz *et al.,* 2000). Local changes within the rhizosphere can include the growth and development of neighboring plant species and microorganisms.

High levels of moisture and nutrients in the rhizosphere attract much greater numbers of microorganisms than elsewhere in the soil. The composition and pattern of root exudates affect microbial activity and population numbers, which, in turn, affect other soil organisms that share this environment. Rhizosphere microorganisms produce vitamins, antibiotics, plant hormones and communication molecules that all encourage plant growth. Most soil microorganisms do not interact with plant roots, possibly due to the constant and diverse secretion of antimicrobial root exudates (Travis *et al.,* 2003). Observations have shown that the concentration of bacteria found around the roots of plants is generally much greater than in the surrounding soil and that the rhizosphere supports higher microbial growth rates and activities as compared to the bulk soil (Travis *et al.,* 2003). One of the main reasons for these higher growth rates is the increased availability of soluble organic compounds that results from plant root exudation. These are typically carbohydrates monomers, amino acids and sugars, but the composition and quantity of root exudates varies depending on the plant species (Smith, 1976) and abiotic conditions such as water content and temperature (Young,

1995). In turn, rhizosphere microorganisms increase root exudation through production of plant hormones or more directly by physically damaging the roots (Grayston *et al.*, 1996). In general, the nutrient-rich rhizosphere is naturally colonized by many beneficial or pathogenic bacteria and fungi which may have a considerable impact on plant growth, development and productivity.

Rhizosphere microbes may improve the uptake of nutrients by plants and/or produce plant growth promoting compounds. They also protect plant root surfaces from colonization by pathogenic microbes through direct competitive effects and production of antimicrobial agents (Foldes *et al.*, 2000).

1.5 Need for new medicines

There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and have a minor environmental impact. This search is driven by the development of resistance in infectious microorganisms (e.g., species of *Staphylococcus*, *Mycobacterium* and *Streptococcus*) to existing compounds and by the menacing presence of naturally resistant organisms. 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 (Antibiotic FAQ, 2008). Not only do diseases such as AIDS require drugs that target them specifically, but so do new therapies for treating ancillary infections which are a consequence of a weakened immune system. Furthermore, others who are immuno-compromised (e.g. cancer and organ transplant patients) are at risk for opportunistic pathogens, such as *Aspergillus*

spp., *Cryptococcus* spp., and *Candida* spp., that normally are not a major problem in the human population. In addition, more drugs are needed to efficiently treat parasitic protozoan and nematodal infections, such as malaria, leishmaniasis, trypanosomiasis, and filariasis. Malaria alone is more effective in claiming lives each year than any other single infectious agent with the exception of the HIV-AIDS virus and *Mycobacterium tuberculosis* (National Institutes of Health, 2001). Novel natural products and the organisms that make them offer opportunities for innovation in drug discovery. Exciting possibilities exist for those who are willing to venture into the wild and unexplored territories of the world to experience the excitement and thrill of engaging in the discovery of endophytes and rhizosphere microflora, their biology, and their potential usefulness (Strohl, 1997).

1.6 Natural products and traditional approaches in medicine

Today the largest users of traditional medicines are the Chinese, with more than 5,000 plants and plant products in their pharmacopoeia (Bensky and Gamble, 1993). In fact, the world's best known and most universally used medicinal is aspirin (Figure 3) (salicylic acid), which has its natural origins from the glycoside salicin which is found in many species of the plant genera *Salix* and *Populus*.

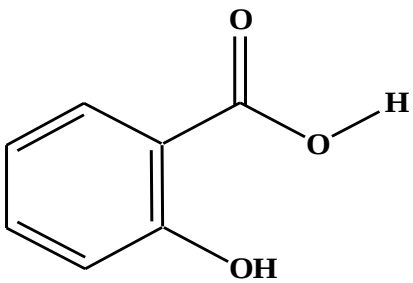

Figure 3: Structure of salicylic acid (Aspirin)

Examples abound of natural-product use, especially in small native populations in a myriad of remote locations on Earth. For instance, certain tribal groups in the Amazon basin, the highland peoples of Papua New Guinea, and the Aborigines of Australia each has identified certain plants to provide relief of symptoms varying from colds to massive wounds and intestinal ailments (Isaacs, 2002). History also shows that now-extinct civilizations had also discovered the benefits of medicinal plants. In fact, nearly 3,000 years ago, the Mayans used fungi grown on roasted green corn to treat intestinal ailments (Buss *et al.,* 2000). More recently, the Benedictine monks (800 AD) began to apply *Papaver somniferum* a pain reliever as the Greeks had done for years before (Grabley and Thiericke, 1999). Many people, in past times, realized that leaf, root, and stem concoctions had the potential to help them. These plant products, in general, enhanced the quality of life, reduced pain and suffering, and provided relief, even though an understanding of the chemical nature of bioactive compounds in these complex mixtures and how they functioned remained a mystery.

It was not until Pasteur discovered that fermentation is caused by living cells that people seriously began to investigate microbes as a source for bioactive natural products. Then, scientific serendipity and the power of observation provided the impetus to Fleming to usher in the antibiotic era via the discovery of penicillin from the fungus *Penicillium notatum* (Stainer *et al.,* 1986). Since then, people have been engaged in the discovery and application of microbial metabolites with activity against both plant and human pathogens. Furthermore, the discovery of a plethora of microbes for applications that span a broad spectrum of utility in medicine (e.g., anticancer and immunosuppressant functions), agriculture and industry is now practical because of the

development of novel and sophisticated screening processes in both medicine and agriculture. These processes use individual organisms, cells, enzymes, and site-directed techniques, many times in automated arrays, resulting in the rapid detection of promising leads for product development (Redell and Gordon, 2000).

Even with untold centuries of human experience behind us and a movement into a modern era of chemistry and automation, natural-product-based compounds have had an immense impact on modern medicine since about 40% of prescription drugs are based on them. Furthermore, 49% of the new chemical products registered by the U.S. Food and Drug Administration are natural products or derivatives thereof (Brewer, 2000). Excluding biologics, between 1989 and 1995, 60% of approved drugs and pre-new drug application candidates were of natural origin (Grabley and Thiericke, 1999). From 1983 to 1994, over 60% of all approved cancer drugs and cancer drugs at the pre-new drug application stage were of natural origin, as were 78% of all newly approved antibacterial agents (Concepcion *et al.,* 2001). In fact, the world's first billion-dollar anticancer drug, paclitaxel (Taxol), is a natural product derived from the yew tree (Wani *et al.,* 1971). Many other examples abound that illustrate the value and importance of natural products in modern civilizations. Natural products have been the traditional pathfinder compounds, offering an untold diversity of chemical structures unparalleled by even the largest combinatorial databases (Young et *al.,* 2006).

1.7 Drug discovery from terrestrial plants

Terrestrial plants, especially higher plants, have a long history of use in the treatment of human diseases. Several well-known species, including licorice (*Glycyrrhiza glabra*),

myrrh (*Commiphora* species), and poppy capsule latex (*Papaver somniferum*), were referred to by the first known written record on clay tablets from Mesopotamia in 2600 BC, and these plants are still in use today for the treatment of various diseases as ingredients of official drugs or herbal preparations used in systems of traditional medicine (Newman *et al.*, 2000). Furthermore, morphine, codeine, noscapine (narcotine), and papaverine isolated from *P. somniferum* were developed as single chemical drugs and are still clinically used. Hemisuccinate carbenoxolone sodium, a semi-synthetic derivative of glycyrrhetic acid found in licorice, is prescribed for the treatment of gastric and duodenal ulcers in various countries (Dewick, 2002).

Historical experiences with plants as therapeutic tools have helped to introduce single chemical entities in modern medicine. Plants, especially those with ethno pharmacological uses, have been the primary sources of medicines for early drug discovery. In fact, a recent analysis showed that the uses of 80% of 122 plant-derived drugs were related to their original ethno pharmacological purposes (Fabricant and Farnsworth, 2001). Current drug discovery from terrestrial plants has mainly relied on bioactivity-guided isolation methods, which, have led to discoveries of the important anticancer agents.

1.8 Drug discovery from terrestrial microorganisms

Until the development of penicillin in the early 1940s, most natural product-derived drugs were obtained from terrestrial plants. The success of penicillin in treating infection led to an expansion in the area of drug discovery from microorganisms. Terrestrial microorganisms are a plentiful source of structurally diverse bioactive

substances, and have provided important contributions to the discovery of antibacterial agents including penicillins, cephalosporins, aminoglycosides, tetracyclines, and polyketides (Dewick, 2002). Current therapeutic applications of metabolites from microorganisms have expanded into immunosuppressive agents (e.g. cyclosporins and rapamycin), cholesterol-lowering agents (e.g. lovastatin and mevastatin), antihelmintic agents (e.g., ivermectin), an antidiabetic agent (acarbose), and anticancer agents (e.g. pentostatin, peplomycin, and epirubicin) (Newman *et al.*, 2003; Butler, 2005; Sneader, 2005).

1.9 Sources of future antibiotics

Antibiotics are extremely important in medicine, but unfortunately bacteria are rapidly getting resistant to them and this has led to need to search for more new compounds.

Very few of today's classes of antibacterials are products of fungal fermentation. Thus from historical context, filamentous fungi may not make the best source of the next generation of antibacterial antibiotics (Van, 2006). Several approaches are being taken to address the issue of antibiotic-resistant bacteria, newly emerging infectious diseases, and related problems. There is obviously a critical need for new potent antibacterials to which resistance is not easily developed. Therefore there is a need to continue searching for new natural antibacterial products from any source through screening that discriminate between new functions and already discovered function (Redell and Gordon, 2000).

Recent studies have shown a great potential for synergism between known classes of antibiotics, and therefore by extension, to new potential antibiotics as well. Thus there

is a need for continued search and screening advancement (Baker *et al.*, 2000). Establishing the presence of individual populations of antagonistic microorganisms in soil and plants is an important step toward fully understanding the functional roles of the organisms in these natural environments (Young *et al.*, 2006). Additionally, the diversity within such indigenous populations of antagonistic microorganisms with a common biocontrol trait holds promise for further improvement of biological control, especially when the diversity reflects important interactions at the host –antagonistic level (Kelly, 2004).

1.10 Statement of the problem

Lives threatening disseminated diseases have risen dramatically over the past several years. New pathogens are evolving at an alarming rate and with the emergence of Human Immunodeficiency Virus-Acquired Immune Deficiency Syndrome, bacteria and fungal infections are on the increase and this has resulted in the emergence of multi-drug resistant pathogens, creating an urgent need for new drug development. Therefore intensive search for new and effective antimicrobial agents is needed. The search for newer drugs is in particular more important in view of the fact that so many diseases are developing immunity to some of the current treatments.

1.11 Justification

The vast majority of relevant antibiotics including antibacterials, antifungals have been either natural products or derived from natural products. Endophytes produce various useful bioactive molecules and this has encouraged a scientific effort to isolate and

study them for their unexplored antimicrobial compounds. An intensive search for newer and more effective agents to deal with diseases is now underway and endophytes and rhizospheric bacteria are a novel source of potentially useful medicinal compounds. Additionally the detection of an existing antimicrobial from a plant source and the detection of a microorganism that can produce the same antimicrobial will be a considerable contribution to the conservation of the environment and ease of production.

1.12 Hypothesis

Endophytic & rhizospheric bacterial isolates produce secondary metabolites with antimicrobial activity.

1.13 Objectives

1.13.1 General objective

The general objective of this study is to determine the potential of bacterial endophytes and rhizosphere bacteria as sources of antimicrobial compounds.

1.13.2 Specific objectives

1. To isolate endophytic and rhizospheric bacteria from selected indigenous plants.
2. To characterize the isolates and screen them for antimicrobial activity against *Staphylococcus aureus* (ATCC 22923), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 55732), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 90028).
3. To extract the isolates' natural products.

4. To carry out bioassay guided chromatographic separation of the isolates' natural products.
5. To characterize the natural products using Gas Chromatography-Mass Spectrophotometer (GC-MS).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study area/site

The plants were collected at Juja, located in lowland areas in the eastern parts of the Thika District, Central Province, Kenya. Juja lies between latitudes 3° 35" and 1°45" south of the equator and longitudes 36º 35" and 37º 25" east with an altitude of 1,060 metres above sea level.

The area is generally semi arid and receives low rainfall, 856mm bimodal distribution with a primary peak in April and secondary in November. There is a dry period of about 4 months from June to October and a relatively shorter one extending from December to February (Muchena *et al.*, 1978).

The mean annual temperature is 20ºC with the mean maximum temperature being 30ºC. Relative humidity ranges from 57% in February to 74% in July. Evaporation rate ranges from 2.6mm in July to 6.3mm in February (Muchena *et al.*, 1978).

2.2 Plant Material

The plant samples were collected in Juja (JKUAT Kengo forest) and the experiments were done in the Botany laboratory of Jomo Kenyatta University of Agriculture and Technology.

A broad range of medicinal indigenous plants were randomly selected, based on their ethno botanical use.

The plant root and soil rhizosphere samples were randomly collected from different areas in JKUAT Kengo forest. The plants; *Cleodendrum myricoides, Dombeya rotundifolia, Dalbergia menaloxylon, Lannea flavus, Dichrostachys cinerea, Gomphocarpus fruticosus, Balanites aegyptica, Schrebera alata, Jasminium floribundum* and *Hibiscus fuscus* were collected and used for the study.

2.3 Isolation of endophytic bacteria

Nutrient Agar (Oxoid) and Tryptic Soy Agar media (Oxoid) were used for isolation. They were prepared according to the manufacturers instructions, by weighing, dissolving in distilled water, boiling and autoclaving at 121°C for 15 minutes. After autoclaving they were allowed to cool to around 35° C and Nystatin added (50µg/ml) to inhibit fungal growth (William and Davis, 1965), and then poured in sterile plates.

Lateral roots from the indigenous plant species were used, and the plant materials were washed thoroughly in tap water to remove adhering soil debris, microscopic insects and other loosely bound microorganisms on the surface. A second wash was done using sterile distilled water. The samples were washed in 70% ethanol for 30 seconds and then treated with sodium hypochlorite (3-5% available chlorine) for 3 minutes and were then immersed in 70% ethanol for 3 minutes, followed by 10 minutes soaking in sodium hypochlorite (3-5%) with shaking. The plant materials were rinsed three times with sterile distilled water (5 minutes each), with shaking and then dried on sterile paper towels (Denise *et al.,* 2002).

The washed plant materials were then macerated in sterile distilled water in a flame sterilized pestle and mortar and homogenized.

50µl of homogenate was inoculated onto Nutrient Agar + Nystatin and Tryptic Soy Agar + Nystatin (3 plates per sample) and spread using a glass spreader. This was carried out aseptically on a clean bench in the fume hood.

Incubation was done in an inverted position at 25°C for 2-4 days. The bacteria growing on Nutrient Agar and Tryptic Soy Agar showing clear zones of inhibition were selected and sub cultured on Nutrient Agar to get pure cultures, which were further characterized.

2.4 Isolation of rhizosphere bacteria

Soil that surrounds the roots was used. Excess soil from the roots was removed, the rhizosphere soil was then washed using sterile distilled water into sterile beakers and shaken for 10 minutes to dissociate bacteria.

50µl aliquot was taken and inoculated onto Nutrient Agar and Tryptic Soy Agar and spread using a sterile glass spreader. 50µg/ml of Nystatin was added into the media to suppress fungal growth (William and Davis, 1965). Incubation was done in an inverted position at 25°C for 2-4 days. Bacteria growing on media showing zones of inhibition were isolated and purified.

2.5 Antimicrobial activity

To test the ability of each individual isolate to inhibit the growth of *B. subtilis* (ATCC 55732), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 22923) and fungi *C. albicans* (ATCC 90028), antimicrobial screening was carried out. Each bacterial isolate was cultured onto nutrient agar and incubated at 30°C for 18-24 hours

and the test organisms were cultured in nutrient broth and incubated at 37°C for 18-24 hours.

50µl of each test organism was placed on different Nutrient agar plates, spread evenly using a glass spreader and each bacterial isolate was streaked horizontally per plate and incubated at37°C for 18-24 hours (Cappuccino and Sherman, 2002). The isolates that inhibited the growth of test organisms after 24 hours were recorded as positive and were investigated further whereas isolates in plates where growth of the test organism was observed was recorded as negative and was not investigated further.

2.6 Characterization of endophytic and rhizosphere bacterial isolates

The bacterial isolates that showed broad spectrum activity on all the five test organisms were selected for further characterization. Preliminary characterization was based on morphological characteristics using a microscope (X 100).

Further characterization using physiological, biochemical and molecular studies was carried out to support the findings of the morphological characterization as detailed in sections 2.6.2, 2.6.3 and 2.6.4.

2.6.1 Morphological characterization

Morphological characteristics; the colony shape, color, elevation, texture and the bacteria Gram type were observed. The bacterial strains were subjected to Gram stain and observed under a microscope for shape and size. For bacteria Gram type 18-24 hour culture was used. A drop of water was placed on a clean slide. The bacterial colony was

then isolated with a sterile loop; bacteria were suspended in water and spread out on slide, allowed to dry and the cells heat fixed by passing through a flame 3-4 times.

For Gram staining, the slide (s) were dunked in crystal violet, removed, allowed to stand for 1 minute and then rinsed gently with water. The slide were dunked in Grams' iodine, removed, allowed to stand for 1 minute and rinsed gently with water. The slide were then dunked in decolorizer for approximately 10 seconds and rinsed gently with water and then finally dunked in safranin, removed and allowed to stand for 1 minute and rinsed gently with water. The slides were blot dried and observed under a microscope (Leica CME, model 1349522Y) with a 100X oil immersion lens. Bacterial isolate with a pink color were indicative of Gram negative while those with a purple color were indicative of Gram positive (Cappuccino and Sherman, 2002).

2.6.2 Physiological characterization

The effect of temperature, pH and salinity were carried out to determine their physiological effect on the growth of the bacteria, as described below.

2.6.2.1 Temperature

Growth at elevated temperatures was tested by growing the bacterial isolates in nutrient broth (20ml in universal bottles) and incubated at 25°C for 18-24 hours.100 µl of each bacteria broth was transferred into 10ml freshly prepared broth in universal bottles and incubated at 15°C, 25°C, 30°C, 37°C and 45°C for 18-24 hours. The absorbance of each isolate was taken at 600nm using a spectrophotometer (Pharmacia Biotechnology, Novaspec II) to determine the optimal temperature for growth (Cappuccino and Sherman, 2002).

2.6.2.2 Sodium chloride

Bacteria were grown in nutrient broth whose sodium chloride content was adjusted to the following concentrations; 0.1%, 0.5%, 1.0%, 1.5%, 2.0% and 3.0%. This was done by transferring 100µl of bacteria isolate into 10ml nutrient broth whose sodium chloride concentration had been adjusted as stated above. Incubation was done at the optimal temperature determined above, for 24 hours. This experiment was aimed at determining the tolerance of isolates to different sodium chloride levels and the optimal concentration for growth. The absorbance of each broth tube was taken at 600nm using a spectrophotometer (Pharmacia Biotechnology, Novaspec II) (Cappuccino and Sherman, 2002).

2.6.2.3 pH

Bacteria were grown in nutrient broth at pH 4, 5, 6, 7, 8 and 9 respectively and incubated at 30°C for 24 hours. This was done by transferring 100µl of bacteria isolate into 10ml nutrient broth whose pH had been adjusted as stated above. This experiment helped determine the optimal pH for growth of the bacteria isolates. The absorbance of each isolate was taken at 600nm using a spectrophotometer (Pharmacia Biotechnology, Novaspec II) to determine the optimal pH for growth (Cappuccino and Sherman, 2002).

2.6.3 Biochemical characterization.

Biochemical activities of microorganisms were studied for the purpose of identification as well as classification. Biochemical finger prints carried out were; catalase test, citrate utilization, sulphur indole motility test, triple sugar iron test, MR-VP test, urease test, gelatin hydrolysis test and nitrate reduction test.

2.6.3.1 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 indicated by formation of bubbles, indicative of catalase activity.

2.6.3.2 Citrate Utilization test

Simmon's citrate media was prepared as per the manufacturer's instruction into slants. The media was streaked with each bacteria isolate, incubated at 37°C for 24-48 hours after which the results were recorded. Positive results were indicated by a deep blue colour at the top of the media and negative results green (Cappuccino and Sherman, 2002).

2.6.3.3 SIM (Sulphur, Indole, Motility) test

Sulfur-Indole Motility (SIM) agar media was used for the SIM test. Using freshly prepared cultures (24 – 48 hour), inoculation was done by stabbing using a straight wire loop. The tubes were incubated at 37°C for 24-48 hours. 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 colouration in the media following incubation indicated the absence of hydrogen sulfide (Cappuccino and Sherman, 2002). Motility was observed by the migration of the inoculum from the stab line through the semisolid medium.

2.6.3.4 Triple Sugar Iron Agar (TSI)

Using a sterile loop, the media was stabbed deep into the butt with the bacteria isolate and then streaked along the slant for the TSI test (Cappuccino and Sherman, 2002). Incubation was done at 37°C for 18-24 hours. Acid production was indicated by change of colour from orange red to yellow and blackening of the media was indicative of hydrogen sulphide gas production.

2.6.3.5 Methyl Red test (MR) and Voges- Proskauer Test (VP)

MR-VP broth was prepared according to the manufacturer's directions. The tubes were then inoculated with bacterial isolates. They were incubated at 37°C for 24-48 hours and observed daily. The tubes were observed for growth, which was indicated by turbidity of the medium. For MR test, to each tube 5 drops of freshly prepared methyl red indicator was added and the results recorded immediately. Positive results were indicated by a bright red colour and a yellow colour was indicative of a negative result (Cappuccino and Sherman, 2002). For VP test, to each tube Barritt's reagent was added which comprises of a mixture of alcoholic alpha-naphthol and 40% potassium hydroxide solution. Colour change was recorded within 15 minutes. The results were recorded as positive when a deep rose colour developed in the media, and negative in the absence of the rose colour at the top of the media (Cappuccino and Sherman, 2002).

2.6.3.6 Urease Test

Urea broth was prepared as per the manufacturer's instructions. Using freshly prepared cultures, the tubes were inoculated and incubation was done at 37°C for 24-48 hours

and the colour changes indicated by phenol red noted daily. Positive results were indicated by a deep pink colour while a pale yellow colour indicated a negative result (Cappuccino and Sherman, 2002).

2.6.3.7 Gelatin liquefaction test

Nutrient Broth supplemented with 12% gelatin was used to demonstrate the hydrolytic activity of gelatinase (Harold, 2002). Cultures were inoculated and incubated at 25°C for 48 hours, after which they 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).

2.6.4 Molecular characterization

2.6.4.1 DNA extraction from bacterial cells

Pure subculture of the antagonistic isolate was inoculated in freshly prepared Luria Bertani broth and incubated for five days in a shaker incubator at 30°C and 200 rpm. Total genomic DNA was extracted using Ultra-Clean Microbial DNA Isolation kit (Mo Bio Laboratories, California USA) according to the manufacturer's specifications based on the method of Stach *et al.* (2003). 1.8 ml of bacterial culture was added into the centrifuge tube and centrifuged for 30 seconds at 10000 x g. The supernatant was discarded and the tube was spun one more time for 30 seconds at 10000 x g. The media supernatant was removed completely with a pipette tip. The cell pellet was then resuspended in 300 µl of MicroBead solution, vortexed gently to mix and then the resuspended cells were transferred to a Micro Bead tube. Fifty microlitres (50 µl) of

solution MD1 (Mo Bio Laboratories, California, USA) was added to the Micro Bead tube and heated in an incubator at 60°C for 10 minutes. The tube was secured horizontally on a flatbed vortex pad with tape and vortexed at 14000rpm for 10 minutes. The tube was centrifuged for 30 seconds at 10000 x g and the supernatant was transferred to clean micro centrifuge tubes. One hundred microlitres (100 µl) of solution MD2 (Mo Bio Laboratories, California, USA) was added to the supernatant, vortexed for 5 seconds and incubated at 4^{o} C for 5 minutes. The tube was centrifuged for 1 minute at 10000 x g and the entire volume of the supernatant transferred to a clean 2 ml tube. Nine hundred microlitres (900 µl) of solution MD3 (Mo Bio Laboratories, California, USA) was added to the supernatant and vortexed for 5 seconds. Seven hundred microlitres (700 µl) was loaded into the spin filter, centrifuged at 10000 x g for 30 seconds and the flow-through discarded. The remaining supernatant was added to the spin filter, centrifuged at the same conditions and all the flow-through discarded. Three hundred microlitres (300 µl) of solution MD4 (Mo Bio Laboratories, Calif. USA) was centrifuged at 10000-x g for 30 seconds and the flow-through discarded. The tubes were centrifuged again for 1 minute and the flow-through discarded. The spin filter was carefully placed in a new 2 ml tube, 50 µl of solution MD5 (Mo Bio Laboratories, Calif. USA) was added to the center of the white filter membrane and centrifuged for 30 seconds. The spin filter was discarded and the DNA in the tube was ready for application (Stach *et al.,* 2003). The DNA was semi quantified on a 1% agarose gel in 1xTAE buffer and visualized under UV by staining with ethidium bromide (Sambrook *et al*., 1989).

2.6.4.2 PCR amplification of bacterial 16S rDNA

The complete 1.6kb 16S rDNA region was amplified using the universal bacterial 16r DNA primer 8f (5'-AGR CTT TGA TCC TGG CTC AG-3') and 1492r (5'-CGG CTA CCT TGT TAC GAC TT-3').The PCR amplification of the target sequence was carried out in a total volume of 50µl of the following reaction mixture: 50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1%Triton X-100, 1.5mM Magnesium Chloride, 200µM dNTPs, 25 pmol of each primer, 25µl of the template and 2.5µl of Taq DNA polymerase. The PCR was performed in a Gene Amp PCR system using the following protocol: initial denaturation at 94°C for 5 minutes, annealing at 55°C for 50 seconds and an extension at 72°C for 1 minute 30seconds, followed by an additional extension at 72°C for 8 minutes. Amplification products (20 µl) were separated on a 1% agarose gel in 1XTAE buffer and visualized by ethidium bromide staining (Sambrook *et al.*, 1989).

2.6.4.3 Purification of PCR product

The PCR product was purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (Qiagen, Germany) was added to 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2 ml collection tube, the sample were applied to the QIAquick column to bind the DNA, and then centrifuged for 30-60 seconds at 13000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tube. To wash the DNA, 0.75 ml buffer PE was added to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded and the column centrifuged again for an additional 1 minute at 13000 rpm to remove

residual ethanol from buffer PE. The QIAquick column was placed in a 1.5 ml micro centrifuge and 30 µl of buffer EB (10mM Tris-Cl, pH 8.5) added to elute DNA. The tube was then centrifuged for 1-minute, the spin column removed and DNA stored at –20^{o}C for application (Sambrook *et al.,* 1989).

2.6.4.4 Phylogenetic data analysis

Consensus sequence was obtained by aligning sequences obtained using primers 8F and 1492R. One of the sequences was inverse complemented before the alignment. The consensus sequence was used to query the National Centre for Biotechnology Information (NCBI) database using the nucleotide Basic Alignement Search Tool (nucleotide BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to determine similarity to sequences in the Gene bank database (Altschul *et al*., 1990; Shayne *et al*., 2003). The megablast program which optimises for highly similar sequences was used for searching the non redundant (nr) nucleotide collection database. No organism limitation or search limitations were set during the query search. Multiple sequence alignment was done using Clustal X 2.0.10 using the Complete allignment option. Bootstrap N-J tree was generated in phylip format and the tree observed in TreeView 1.6.6 software (Saitou and Nei, 1987; Felsentein, 1989).

2.7 Extraction of the isolates' crude products

Each bacterial isolate was deposited in an Erlenmeyer flask containing 5 litres of Tryptic soy broth (Oxoid) whose sodium chloride and pH levels were adjusted to 2.0% and pH 8 respectively based on the optimum obtained from the physiological characterization (2.6.2.2 and 2.6.2.3). The fermentation flask was incubated at 110 rpm

on a rotary shaker at room temperature for 7 days. After fermentation the culture broth was filtered. Culture filtrates were extracted 3 times with ethyl acetate in a separatory funnel. The organic phase was passed through a pad of anhydrous sodium sulphate to remove any water and evaporated to dryness using a rotary vacuum evaporator. The yields of the extract were determined and recorded. 50% of the crude extract was used for biological activity tests and the other 50% was analyzed for bacterial secondary metabolites (Marcia and Katia, 2004).

2.7.1 Antimicrobial assays

Bacterial culture broths and crude extracts of broths were screened for their antibacterial and antifungal activity using the Kirby-Bauer disc diffusion method (Lorian, 1996) against potentially pathogenic bacteria; *B. subtilis* (55732), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 22923) and fungi *C. albicans* (ATCC 90028). 10µl/sample (Katia *et al.,* 2000) was pipetted to impregnate paper discs with the sample. The discs were allowed to dry in a fume chamber and placed on agar seeded with test organisms. Incubation was done at 37^{o}C for 24 hours. Diameter of zone of inhibition was then measured and recorded.

2.7.2 Isolation of the active components (Chemical screening)

The crude extracts from procedure 2.7 were analyzed by thin layer chromatography (TLC) and GC–MS to detect bacterial secondary metabolites and to determine the ratio of components present in the crude extract.

2.7.2.1 Thin Layer Chromatography (TLC)

Analytical TLC was performed on aluminium sheets precoated with silica gel 60 F_{254} (Merck) with a 0.25mm layer thickness. The plates were viewed under UV light hand lamp (Model UV GL-58 mineral light lamp, 254nm and 366nm).The plates were developed by spraying with vanillin reagent (Sulphuric aid solution – 1% vanillin in concentrated Sulphuric acid), and baking in oven at 110ºC for five minutes (Ronald, 2000).

Preparative TLC was performed on pre-coated silica gel glass plates (silica gel 60 F_{254}Merck, 0.25mm layer thickness). The bands were viewed under UV light (wavelength λ-254nm and λ-366nm) and respective bands boundaries marked (Qin and Judith, 1999). The number of bands in each sample was determined and recorded. Each band was then scrapped off onto a filter paper and the constituents eluted into a beaker using hexane: ethylactetate (3:2) and evaporated to dryness. Each sample was tested against potential pathogenic bacteria; (*E. coli, B. subtilis, P. aeruginosa*, and *S. aureus*) and fungi (*C.albicans).* Inhibitory activities were determined by means of Kirby-Bauer disc diffusion method and diameter of zone of inhibition for each sample recorded.

2.7.2.2 Gas chromatography coupled mass spectrometry (GC-MS)

The bands with inhibition activity of ≥7mm (from section 3.7.2.1) were selected for GC-MS analysis, for preliminary identification of the compounds present. The weights of the selected samples were determined and recorded.

The samples were analyzed by combined GC-MS (7890 a series GC Agilent technology), coupled to a 5975 C series mass spectrometer fitted with an 7683 B series auto sampler and a Triple Axis Detector. A HP5 MS 5% phenyl methyl silicone non-polar capillary column measuring 30 m x 0.25 mm (internal diameter) and 0.25 µm (film thickness) was employed for separation of chromatograms. The GC was coupled to a HP monitor (L1710) onto which chromatographic data were acquired and evaluated by Hp 3365 CHEMSTATION software. Oven temperature was programmed at 35ºC for 5 min followed by a rise at 10ºC per min up to 280ºC. The final temperature was maintained for 5 min. The injector and detector temperatures were set at 280ºC. Helium gas was used as the carrier gas at a constant flow rate of 1.2 ml min^{-1}. For electron impact (EI), the ionization voltage was 70 eV and temperature of the ion source and the interface were 230ºC and 150ºC, respectively. Matching mass spectra obtained within the John Wiley and NIST MS data libraries made tentative identification of the constituent compounds. In all cases, 1µl of the sample was injected into the split less mode with a 0.5 min delay before injection purging.

2.8 Data analysis

All statistical analyses were performed with SAS software package, version 9.1 (SAS Institute, 2003) using two way analysis of variance (ANOVA) for the replicated treatments. Student –Newman Keuls (SNK) test was used for the separation of means.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Bacterial isolation

A total of 48 pure isolates of endophytic and rhizospheric origin were obtained from the indigenous plants (Table 1). Bacterial growth was observed in all plates and those that showed zones of inhibition were selected for further characterization.

Table 1: Summary of indigenous plants collected and number of bacterial isolates obtained.

Host plant	**Code**	**Bacterial endophytes**	**Rhizosphere bacteria**
		Number	**Number**
Cleodendrum myricoides	C	5	3
Gomphocarpus fruticosus	G	7	1
Dichrostachys cinerea	DC	1	4
Jasminium floribundum	J	3	1
Hibiscus fuscus	H	1	1
Lannea flavus	L	3	1
Schrebera alata	S	-	6
Balanites aegyptica	B	1	4
Dalbergia menaloxylon	DM	-	1
Dombeya rotundifolia	DR	-	5
TOTAL		**21**	**27**

The isolated antagonistic endophytes and antagonistic rhizosphere bacteria prospected was twenty one and twenty seven respectively (Table 1). Antagonistic bacteria endophytes were obtained from the plant *G. fruticosus* (33%), *C. myricoides* (24%), *L.*

flavus and *J. floribundum* (14%) respectively, *D. cinerea* (5%), *H. fuscus* (5%), *B. aegyptica* (5%), but none was isolated from *S. alata, D. menaloxylon* and *D. rotundifolia.* In the case of rhizobacteria, antagonistic bacteria isolates were obtained from *S. alata* (22%), *D. rotundifolia* (19%), *D. cinerea* (15%), *B. aegyptica* (15%), *C. myricoides* (11%), *G. fruticosus* (4%), *H. fuscus* (4%), *L. flavus* (4%) and *D. menaloxylon* (4%).

A greater number of antagonistic bacteria were isolated from the rhizosphere as compared to the endophytes. This could be due to the high levels of moisture and nutrients in the rhizosphere that attracts great numbers of microorganisms (Foldes *et al*., 2000). The rhizosphere also acts as the highly used channel of entry by endophytes into plants, hence the high colonization of microorganisms at the rhizosphere (Probanza *et al.,* 1996). Reduced oxygen within the plant tissues compared to the rhizosphere, also explains the high population of microorganisms at the rhizosphere (Jeffrey, 1995; Flores and Hara, 2006).

A greater number of antagonistic bacteria were isolated from *C. myricoides* and *G. fruticosus* compared to the other plants. These results are in line with those of previous studies (Strobel and Daisy, 2003) showing that different plants are a host to one or more endophytes and their numbers vary from one plant to another. According to a study by Denise *et al.* (2002), the variations are also attributable to plant age and time of sampling.

3.2 Antimicrobial screening

The bacterial isolates were tested for their antagonistic activity against test bacteria and fungi, to determine the level of antagonism of each isolate against the individual test

organisms. The bacterial isolates showed varied levels of antimicrobial activity (Table 2).

Table 2: Mean inhibition diameters of the screened isolates

Isolate	Test organisms (Diameter in mm, n=2)				
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Escherichia coli*	*Candida albicans*
C25 Y	2.5 ± 2.5^{jkC}	27.5 ± 1.5^{bA}	19.0 ± 0.0^{cdB}	31.5 ± 1.5^{bA}	2.5 ± 2.5^{ghC}
C25 M	11.0 ± 1.0^{hgC}	49.0 ± 1.0^{aA}	22.0 ± 2.0^{cdB}	19.5 ± 2.5^{eB}	17.5 ± 0.5^{cB}
C15	23.0 ± 0.0^{cdeC}	18.5 ± 1.5^{defD}	38.5 ± 1.5^{aA}	23.5 ± 1.5^{cdC}	33.0 ± 0.0^{aB}
C17	21.0 ± 1.0^{defB}	11.5 ± 1.5^{fghiC}	24.0 ± 1.0^{cB}	33.0 ± 1.0^{bA}	0.0 ± 0.0^{hD}
C16	4.0 ± 2.0^{jkAB}	2.5 ± 1.5^{klmnAB}	6.5 ± 1.5^{fghA}	5.0 ± 0.0^{ghiA}	0.0 ± 0.0^{hB}
C24	25.0 ± 0.0^{bcdA}	15.5 ± 1.5^{fgB}	9.0 ± 1.0^{efgC}	26.0 ± 0.0^{cA}	0.0 ± 0.0^{hD}
C18	9.0 ± 1.0^{ihA}	0.0 ± 0.0^{nB}	1.0 ± 1.0^{ghB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
C19	40.5 ± 0.5^{aA}	22.5 ± 2.5^{cC}	33.0 ± 0.0^{bB}	43.5 ± 1.5^{aA}	0.0 ± 0.0^{hD}
G8T	0.0 ± 0.0^{kA}	4.0 ± 1.0^{klmnA}	2.5 ± 2.5^{ghA}	0.0 ± 0.0^{kA}	0.0 ± 0.0^{hA}
G8M	0.5 ± 0.5^{kB}	0.0 ± 0.0^{nB}	6.5 ± 1.5^{efghA}	5.0 ± 0.0^{hijA}	4.0 ± 1.0^{fghA}
G20	0.0 ± 0.0^{kB}	21.5 ± 1.5^{cdA}	18.5 ± 1.5^{cdA}	19.0 ± 1.0^{eA}	2.5 ± 2.5^{ghB}
G20C	20.0 ± 0.0^{defA}	0.0 ± 0.0^{nC}	13.5 ± 1.5^{deB}	22.0 ± 1.0^{cdA}	11.5 ± 0.5^{dB}
G21C	0.0 ± 0.0^{kB}	0.0 ± 0.0^{nB}	9.5 ± 2.5^{efA}	1.0 ± 1.0^{kB}	0.0 ± 0.0^{hB}
G43W	18.0 ± 1.5^{efA}	20.0 ± 0.0^{cdeA}	7.0 ± 2.0^{efghB}	8.5 ± 1.5^{ghB}	1.5 ± 1.5^{ghC}
G21W	0.0 ± 0.0^{kB}	0.0 ± 0.0^{nB}	6.0 ± 1.0^{fghA}	1.0 ± 1.0^{kB}	0.0 ± 0.0^{hB}
G9	0.0 ± 0.0^{kB}	10.0 ± 0.0^{ijA}	2.5 ± 2.5^{ghB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
DC1	24.0 ± 1.0^{bB}	12.0 ± 0.0^{ghiC}	13.5 ± 1.5^{deC}	23.5 ± 1.5^{cdB}	21.5 ± 1.5^{bB}
DC2	13.0 ± 0.0^{gA}	2.5 ± 2.5^{klmnB}	3.0 ± 3.0^{ghB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
DC3	19.0 ± 1.0^{defA}	5.5 ± 1.5^{klmB}	0.0 ± 0.0^{hC}	0.0 ± 0.0^{kC}	0.0 ± 0.0^{hC}
DC4	1.0 ± 1.0^{jkB}	10.0 ± 0.0^{ghijA}	0.0 ± 0.0^{hB}	0.5 ± 0.5^{ijB}	0.0 ± 0.0^{hB}
DC26	0.0 ± 0.0^{kA}	4.0 ± 1.0^{klmnA}	2.5 ± 2.5^{ghA}	0.0 ± 0.0^{jA}	0.0 ± 0.0^{hA}
J5	6.0 ± 1.0^{ijA}	0.0 ± 0.0^{nB}	2.5 ± 2.5^{ghAB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
J6	1.0 ± 1.0^{jkB}	0.0 ± 0.0^{nB}	0.0 ± 0.0^{hB}	4.5 ± 0.0^{ijA}	0.0 ± 0.0^{hB}
J7	2.0 ± 2.0^{jkAB}	5.0 ± 0.0^{klmnA}	1.5 ± 1.5^{ghAB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
J23	6.0 ± 0.0^{jklA}	0.0 ± 0.0^{nB}	1.0 ± 1.0^{ghB}	0.0 ± 0.0^{kB}	8.0 ± 1.0^{eA}
H10	6.0 ± 1.0^{ijA}	0.0 ± 0.0^{nB}	5.5 ± 0.5^{fghA}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
H11	18.0 ± 2.0^{fA}	0.0 ± 0.0^{nC}	6.5 ± 1.5^{efghB}	0.0 ± 0.0^{jC}	0.0 ± 0.0^{hC}
L12	0.0 ± 0.0^{kB}	0.0 ± 0.0^{nB}	8.0 ± 2.0^{efghA}	5.0 ± 0.0^{hijA}	0.0 ± 0.0^{hB}
L13	0.0 ± 0.0^{kB}	0.0 ± 0.0^{nB}	8.0 ± 2.0^{efghA}	6.0 ± 1.0^{hiA}	0.0 ± 0.0^{hB}
L22	0.0 ± 0.0^{kB}	0.0 ± 0.0^{nB}	1.5 ± 1.5^{ghAB}	0.0 ± 0.0^{kB}	3.0 ± 1.0^{ghA}
L14	0.0 ± 0.0^{kB}	6.0 ± 1.0^{klA}	2.5 ± 2.5^{ghAB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}

Table 2 continued: Mean inhibition diameters of the screened isolates

Isolate	Test organisms (Diameter in mm, n=2)				
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Escherichia coli*	*Candida albicans*
S27	0.0 ± 0.0^{kB}	5.5 ± 0.5^{klmA}	0.0 ± 0.0^{hB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
S28	5.0 ± 1.0^{ijkA}	0.0 ± 0.0^{nB}	1.0 ± 1.0^{ghB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
S33	32.5 ± 1.0^{bA}	16.5 ± 1.5^{efB}	1.5 ± 1.5^{ghC}	5.0 ± 0.0^{hijC}	1.0 ± 1.0^{hC}
S34	20.5 ± 0.5^{defA}	15.0 ± 0.0^{fghB}	0.0 ± 0.0^{hC}	0.0 ± 0.0^{kC}	0.0 ± 0.0^{hC}
S42L	19.5 ± 0.5^{defA}	4.0 ± 1.0^{klmnB}	0.0 ± 0.0^{hC}	5.0 ± 0.0^{hijB}	0.0 ± 0.0^{hC}
S42T	20.5 ± 1.5^{defA}	4.0 ± 1.0^{klmnA}	1.0 ± 1.0^{ghBC}	$.0\pm1.0^{ijkB}$	0.0 ± 0.0^{hC}
B29	0.0 ± 0.0^{kB}	0.0 ± 0.0^{nB}	6.5 ± 1.5^{efghA}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
B38	1.0 ± 1.0^{jkA}	1.5 ± 1.5^{lmnA}	0.0 ± 0.0^{hA}	0.0 ± 0.0^{kA}	0.0 ± 0.0^{hA}
B39	0.0 ± 0.0^{kB}	7.0 ± 0.0^{jkA}	2.0 ± 2.0^{fghB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
B40	0.0 ± 0.0^{kB}	11.5 ± 1.5^{hiA}	0.0 ± 0.0^{hB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
B41	1.0 ± 1.0^{jkB}	1.0 ± 1.0^{mnB}	2.5 ± 2.5^{fghB}	11.5 ± 1.5^{fA}	2.0 ± 2.0^{ghB}
DM30	2.5 ± 2.5^{jkB}	1.5 ± 1.5^{lmnB}	3.0 ± 3.0^{fghB}	1.5 ± 1.5^{jkB}	18.5 ± 1.5^{bA}
DR31	1.0 ± 1.0^{jkBC}	0.0 ± 0.0^{nC}	3.5 ± 1.5^{fghAB}	4.5 ± 0.5^{ijA}	0.0 ± 0.0^{hC}
DR32	5.5 ± 0.5^{ijkA}	0.0 ± 0.0^{nB}	0.0 ± 0.0^{hB}	0.0 ± 0.0^{kB}	0.0 ± 0.0^{hB}
DR35	2.5 ± 2.5^{jkB}	1.5 ± 1.5^{lmnB}	5.0 ± 0.0^{fghA}	7.0 ± 1.0^{hiA}	6.5 ± 1.5^{efA}
DR36	18.5 ± 1.5^{efA}	0.0 ± 0.0^{nC}	1.0 ± 1.0^{ghC}	10.5 ± 0.5^{fgB}	0.0 ± 0.0^{hC}
DR37	0.0 ± 0.0^{kC}	0.0 ± 0.0^{nC}	7.0 ± 1.0^{fghA}	6.0 ± 0.0^{hiAB}	4.5 ± 0.5^{fgB}
-ve control	0.0 ± 0.0^{kA}	0.0 ± 0.0^{nA}	0.0 ± 0.0^{hA}	0.0 ± 0.0^{kA}	0.0 ± 0.0^{hA}
TET	23.3 ± 0.7^{cdeB}	12.7 ± 0.9^{ghiC}	29.3 ± 0.7^{bB}	24.0 ± 0.6^{cdB}	11.3 ± 0.7^{dC}
GENT	21.0 ± 0.6^{defB}	18.7 ± 0.3^{defC}	20.7 ± 0.3^{cB}	22.3 ± 0.3^{dAB}	21.7 ± 0.3^{bAB}

Within a column, means compare inhibition diameters among the different isolates with the same test organism and mean values with the same lower case letter are not significantly different (P=0.05,SNK test). Within a row, means compare inhibition diameters among individual isolates with different test organisms and means with same uppercase letter not significantly different (P=0.05, SNK test).

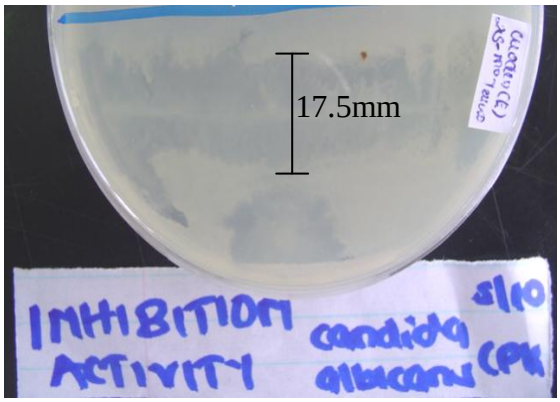

Plate 1a: Antagonistic activity of C25M on *C. albicans* (ATCC 90028).

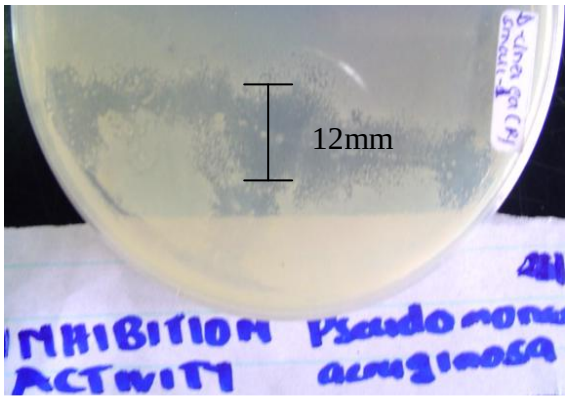

Plate 1b: Antagonistic activity of DC1 on *P. aeruginosa* (ATCC 27853).

Antimicrobial screening was to facilitate determination of the isolates with considerable inhibition activity against pathogenic bacteria and fungi. The isolates showed antagonistic activity that differed from one isolate to another. This could be due to the expected different modes of action and activity of the individual biochemical constituents of the respective isolates (Mao *et al.*, 2006).

Comparing inhibition activity among isolates from the same host plants, from the *C. myricoides* plant, C19 was the most active against *S. aureus* (40.5mm), C25M was the most active against *P. aeruginosa* with a diameter of 49mm, C15 was the most active against *B. subtilis* (38.5mm), and C19 had the higher inhibition zone against *E. coli* (43.5mm), while C15 was the most active against *C. albicans* with a diameter of 33mm (Table 2). For isolates obtained from *G. fruticosus* plant, isolate G20C was the most active against *S. aureus* (20mm), *E. coli* (22mm) and *C. albicans* (11.5mm). G20 was the most active against *P. aeruginosa* (21.5mm) and *B. subtilis* (18.5mm). From *D. cinerea* plant, isolate DC1 had the most notable inhibition activity against the five test organisms with diameters ranging from 12mm to 24mm (Table 2). Of the isolates from *J. floribundum* plant, J5 and J3 showed high activity against *S. aureus* (6mm), J7 had the most inhibition against *P. aeruginosa* (5mm), J5 and J7 had the most inhibition activity against *B. subtilis* with diameters of 2.5mm and 1.5mm respectively. J6 had the most inhibition against *E. coli* (4.5mm) and J23 had the most activity against *Candida albicans* (8mm). Two isolates obtained from *H. fuscus* plant, showed activity against *S. aureus* and *B. subtilis*. Isolate H11 had the most inhibition activity of 18 mm against *S. aureus* and 6.5mm against *B. subtilis.*

Low activity was recorded for isolates obtained from *L. flavus* plant. All isolates showed no inhibition activity against *S. aureus*. L14 was the most active against *P. aeruginosa* (6mm), L13 and L12 were the most active against *B. subtilis* (8mm), L13 was the most active against *E. coli* (6mm) and L22 had the most inhibition against *C. albicans* (3mm). Isolates from *S. alata* plant, S33 was the most active against *S. aureus* (32.5mm), *P. aeruginosa* (16.5mm) and *C. albicans* (1.0mm). S33 and S42T had the most inhibition against *B. subtilis* with diameters of 1.5mm and 1.0mm respectively. S33 and S42L were the most active against *E. coli* with diameters of 5.0mm. For isolates obtained from *B. aegyptica* plant, most isolates showed no inhibition activity against *C. albicans* except for B41 (2.0mm). Only B38 and B41 had inhibition activity against *S. aureus* (1mm), B40 was the most active against *P. aeruginosa* (11.5mm), B29 was the most active against *B. subtilis* (6.5mm) while B41 was the most active against *E. coli* with an inhibition diameter of 11.5mm. One isolate (DM30) was obtained from *D. menaloxylon* plant and it showed considerable activity against *C. albicans,* with an inhibition diameter of 18.5mm. Isolates from *D. rotundifolia* plant, showed no activity against *P. aeruginosa* except for isolate DR 35 (1.5mm). Isolate DR35 was the most active against *C.albicans* (6.5mm), DR36 was the most active against *S. aureus* (18.5mm) and *E. coli* (10.5mm), while DR 35 and DR 37 had the most inhibition activity against *B. subtilis* with diameters of 5.0mm and 7.0mm respectively.

Upon comparing the isolates' antagonistic activity with that of the standard drugs (Tetracycline and Gentamycin-positive controls), results showed that some isolates had a significant activity against the test organism compared to the drugs (Table 2). For

example, isolate C19 (40.5 mm), S33 (32.5mm) had the most significant activity against *S. aureus* compared to Tetracycline (23.3mm) and Gentamycin (21.0mm), C25M (49mm) had the most significant activity against *P. aeruginosa* compared to Tetracycline (12.7mm) and Gentamycin (18.7mm), C15 (38.5mm) had the most significant activity against *B. subtilis* compared to Tetracycline (29.3mm) and Gentamycin (20.7mm), C19 (43.5mm), C17 (33mm) had the most significant activity against *E. coli* compared to Tetracycline (24mm) and Gentamycin (22.3mm) and C15 had the most significant activity *C. albicans* compared to Tetracycline (11.3mm) and Gentamycin (21.7mm). In a study carried out by Ozgur *et al.* (2008), they emphasized the need for the development of new antimicrobial agents with activity against gram positive bacteria and gram negative antibiotic resistant opportunistic bacteria, to overcome certain undesirable side effects and the spread of pathogens with new antimicrobial resistance. From the antimicrobial screening results of this study, there is the potential of some isolates being used to develop broad spectrum therapeutic agents, because some of them showed activity against different microorganisms responsible for a wide range of infections and are important because some of the target disease causing organisms have developed resistance to most classes of antibiotics such as *S. aureus* (Enright, 2003). Therefore, these isolates should further be screened for their potential as a source of antibiotics, active against antibiotic resistant bacteria and fungi.

Least activity was demonstrated against *C. albicans* with a total number of 35 organisms (73%) having no activity against it. This low level of activity could be due to the fact that C*andida* infections are on the increase due to the emergence of HIV/AIDS epidemic and this has given rise to difficulties in coping with medical problems and

complications associated with fungal infections since they are greatly overwhelming the human population (Buss *et al.,* 2000), hence the increase in antimicrobial resistance as demonstrated in Table 2. *Candida spp* are also the common cause of opportunistic infections in hospitals and their resistance to antimicrobials is increasing at an alarming rate and has become problematic (Antibiotics FAQ, 2008). Least activity was also demonstrated against *S. aureus, E. coli, and P. aeruginosa,* with a total number of 25 (52%), 21 (44%) and 19 (40%) organisms respectively, having low or no inhibition activity at all against them. These results concur with a study carried out by the National Institutes of Health (2001), which indicated that the Gram negative bacteria e.g. *P. aeruginosa* and *E. coli,* have increased resistance to antimicrobials due to the continuous modification of their cell wall making it more complex and drug permeability a problem due to the barrier created. For the case of *S. aureus*, its resistance is on increase due to the dramatic emergence of multidrug- resistant strains which have developed resistance to most classes of antibiotics (Enright, 2003). The organism also has become a challenge in its treatment due to its resistance hence causing a high mortality rate. Most inhibition activity was demonstrated against *B. subtilis*. Its susceptibility to antimicrobials is due to the simplicity of its cell wall makeup, paving way to membrane permeability of drugs into the organisms' cells and this makes the drug mode of action highly effective (Foldes *et al.,* 2000).

A study carried out by Foldes *et al*. (2000) reported that, the difference in microbial sensitivity may also be attributed to the experimental conditions. They also stated that, the various forms of antagonism depend on the concentration and the amounts of the active substance(s) present in each individual bacterial isolate, causing a difference in

the levels of antagonism, which could be high in one and low in another. This could also be the probable reason as to why, the bacterial antimicrobial screening activity (Table 2), exhibited different levels of antagonistic activity against the different test organisms. Other contributing factors to the differences in antagonism, is the level of isolate inoculation. This is supported by a study by (Munimbazi and Bullerman, 1998), where they concluded that the higher the inoculation rate the higher the rate of antibacterial activity due to a high rate of production of antibacterial and antifungal compounds and the difference in the growth rate between the isolates and the test organisms.

3.3 Physiological characterization

Various physiological factors that have influence on the growth of the bacterial isolates were investigated, to determine the optimal growth conditions.

3.3.1 Temperature

All the fourty eight (48) bacterial isolates were subjected to different growth temperatures. The optimal temperature was found to be 30°C (Figure 4). There was growth at the different temperatures with the least growth recorded at 45°C.

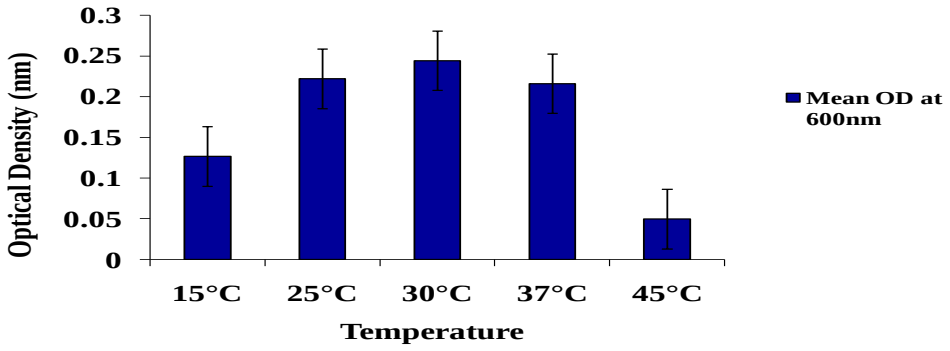

Figure 4: Effect of temperature on mean growth of all the isolates

Temperature is one of the most important environmental factors affecting the growth and survival of microorganisms. The bacterial isolates had considerable growth between 25ºC and 37ºC, with the optimum being 30ºC (Figure 4). A study carried out by Muchena *et al.* (1978) on the climatic conditions of Juja area, established that the mean minimum temperature was 20ºC and the mean maximum temperature was 37ºC. This explains the ability of the microorganisms to grow considerably well at the range of 25ºC to 37ºC. Most soil microorganisms have optima nearer the normal soil temperature of about 30ºC in the tropics, and certainly less than 37ºC which is common with human and other mammalian pathogens. There was reduced growth at 15ºC, compared to growth at 30ºC. This reduction in growth could be due to the fact that, below the organisms minimal growth temperature which is as low as 5ºC-15ºC, the cytoplasmic membrane no longer functions properly in nutrient transport or protein gradient formation hence causing an alteration in membrane lipid composition, leading to growth reduction, since the organisms are not adapted to low temperatures (Buford and Todd, 2004). As temperature rises, chemical and enzymatic reactions in the cells proceed at more rapid rates and growth becomes faster. However, as temperature rises proteins, nucleic acids and other cellular components may be irreversibly damaged leading to the death of the organisms. This explains the reduction of growth recorded at 45ºC, since the organisms are not adapted to growth at high temperatures (Rosso *et al.*, 1995). In relation to the isolates temperature optima, the isolates can be referred to as mesophiles (Michael *et al.*, 2000).

4.3.2 Sodium chloride

All the fourty eight (48) bacterial isolates were subjected to growth at different sodium chloride concentrations. The optimal sodium chloride concentration for growth was found to be 2.0%. There was considerable growth at all concentrations, with the least growth recorded at 0.10 % (Figure 5).

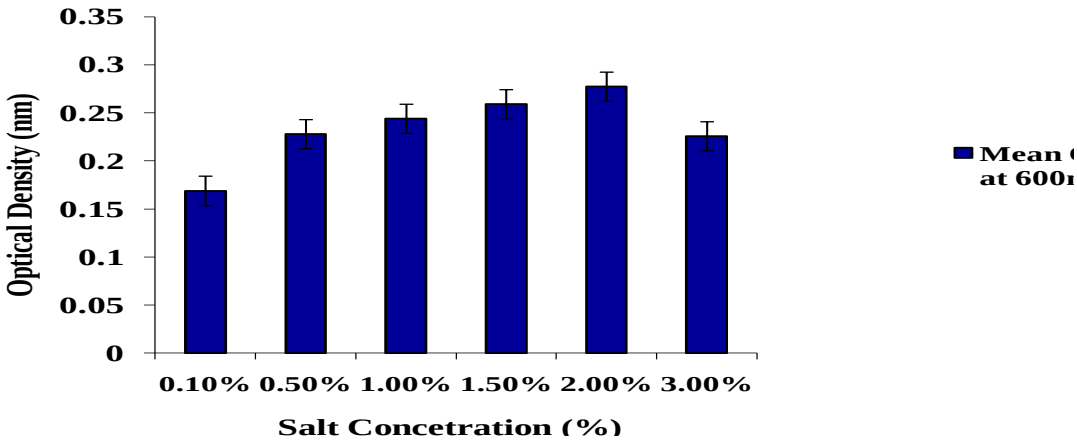

Figure 5: Effect of Sodium chloride (saline) concentration on mean growth of all the isolates

Water availability is an important factor affecting the growth of microorganisms in nature. Water availability is a function of solutes such as salts (sodium chloride) and other substances dissolved in water (Lederberg, 1992). The test results of growth under varying salinity conditions showed optimal growth at the 2.0% salt concentration (Figure 5). In relation to the isolates salt optima, the isolates can be referred to as mild halophiles (Michael *et al.*, 2000).

From the results in Figure 5, the isolates demonstrated their ability to tolerate salt. In a study carried out by Tan and Zou (2001), they established that the ability of microorganisms to grow inside the plants, affects their salt tolerance. This is due to the production of various phytochemicals by the plants, which changes the chemical

composition of the surrounding environment. As a result, the microorganisms perceive the changes of the local environment and they produce adaptive responses and this enhances their tolerance and continuous survival. They further established that the microorganisms produce these chemicals in a period of evolutionary time as a result of genetic recombination. This also contributes to the adaptability and tolerance of the microorganisms to their environments.

Microorganisms that reside on the plants root surface, also adapt to their environment as a result of production of chemical exudates via the roots. They do perceive the changes and produce an adaptive response (Ryan and Delhaize, 2001). This may explain the ability of the isolates in this study to exhibit salt tolerance at various concentrations (Figure 5).

3.3.3 pH

All the fourty eight (48) bacterial isolates were subjected to different pH growth requirements. The optimal pH for growth was found to be pH 8 with good growth also observed at pH 7. Most bacterial isolates grew poorly at pH 4, 5, 6 and 9 (Figure 6).

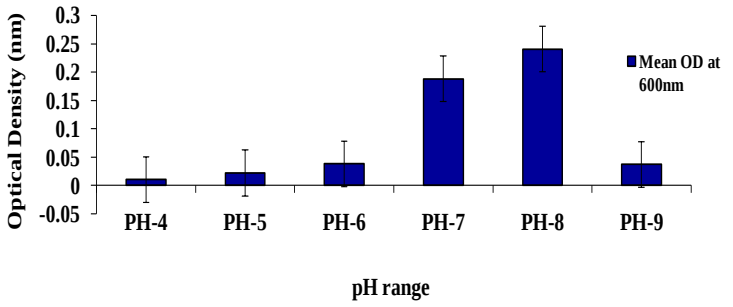

Figure 6: Effect of pH on mean growth of all the isolates

Growth and survival of microorganisms are greatly influenced by the pH of the extra cellular environment. Despite the pH requirements of a particular organism for a specific pH for growth, the optimal growth pH represents the pH of the extra cellular environment only; the intracellular pH must remain near neutrality in order to prevent the destruction of the acid or alkali–labile molecules in the cell. For organisms whose pH optimum is between 6 and 8, the cytoplasm remains neutral or very nearly so (Allsopp *et al.,* 1995). The pH range for majority of microorganisms is between 6 and 8 (Lederberg, 1992). This concurs with the test result (Figure 6), where by there was considerable growth at pH 7 and pH 8, with an optimal growth at pH 8. Therefore, in relation to the pH optima, the isolates can be referred as neutrophiles (Michael *et al.,* 2000).

3.4 Preliminary identification

Biochemical tests and Gram stain morphology were used for preliminary characterization of all the bacterial isolates as indicated in appendix 1 and 2 respectively. However, results for the isolates that showed potential antimicrobial activity are in Table 5 and 6 All the isolates demonstrated a negative reaction on sulphur, hydrogen sulphide gas and simmon citrate but had a positive reaction on hydrogen peroxide production. Gram staining reaction showed that a greater number of the isolates were Gram positives as compared to Gram negatives. Equal numbers of Gram positive and Gram negative endophytic bacteria were isolated. For the rhizobacteria, more Gram positives were isolated as compared to the Gram negatives.

3.5 Isolates selected for crude products

3.5.1 Host plants

A range of host plants from which the isolates were isolated were selected, in order to capture good performers from a wide range of plants (Table 3). The selection was based on; broad spectrum *in vitro* activity on the test bacteria, range of host plants, place of isolation, cell morphology and arrangement and biochemical differences.

Table 3: Summary of the ten selected isolates, host plant and place of isolation

Isolate code	Host plant	Place of isolation
C25M	*Cleodendrum myricoides*	Endosphere
G20C	*Gomphocarpus fruticosus*	Endosphere
G43W	*Gomphocarpus fruticosus*	Endosphere
DC1	*Dichrostachys cinerea*	Rhizosphere
H11	*Hibiscus fuscus*	Endosphere
L13	*Lannea flavus*	Endosphere
S33	*Schrebera alata*	Rhizosphere
B41	*Balanites aegyptica*	Rhizosphere
DM30	*Dalbergia menaloxylon*	Rhizosphere
DR35	*Dombeya rotundifolia*	Rhizosphere

3.5.2 Antimicrobial screening

The ten selected isolates antimicrobial screening activity is as shown in Table 4. This is indicative of the isolate initial *in vitro* activity against the test organisms. The ten isolates exhibited good antagonistic activity against the pathogenic test organisms.

Table 4: A summary of inhibition zone diameters of antimicrobial screening of the selected isolates

Isolate code	Test organisms (Diameter in mm, n=2)				
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Escherichia coli*	*Candida albicans*
C25M	11.0 ± 1.0^{hgC}	49.0 ± 1.0^{aA}	22.0 ± 2.0^{cdB}	19.5 ± 2.5^{eB}	17.5 ± 0.5^{cB}
G20C	20.0 ± 0.0^{defA}	0.0 ± 0.0^{nC}	13.5 ± 1.5^{deB}	22.0 ± 1.0^{cdA}	11.5 ± 0.5^{dB}
G43W	18.0 ± 1.5^{efA}	20.0 ± 0.0^{cdeA}	7.0 ± 2.0^{efghB}	8.5 ± 1.5^{ghB}	1.5 ± 1.5^{ghC}
DC1	24.0 ± 1.0^{bB}	12.0 ± 0.0^{ghiC}	13.5 ± 1.5^{deC}	23.5 ± 1.5^{cdB}	21.5 ± 1.5^{bB}
H11	18.0 ± 2.0^{fA}	0.0 ± 0.0^{nC}	6.5 ± 1.5^{efghB}	0.0 ± 0.0^{jC}	0.0 ± 0.0^{hC}
L13	0.0 ± 0.0^{kB}	0.0 ± 0.0^{nB}	8.0 ± 2.0^{efghA}	6.0 ± 1.0^{hiA}	0.0 ± 0.0^{hB}
S33	32.5 ± 1.0^{bA}	16.5 ± 1.5^{efB}	1.5 ± 1.5^{ghC}	5.0 ± 0.0^{hijC}	1.0 ± 1.0^{hC}
B41	1.0 ± 1.0^{jkB}	1.0 ± 1.0^{mnB}	2.5 ± 2.5^{fghB}	11.5 ± 1.5^{fA}	2.0 ± 2.0^{ghB}
DM30	2.5 ± 2.5^{jkB}	1.5 ± 1.5^{lmnB}	3.0 ± 3.0^{fghB}	1.5 ± 1.5^{jkB}	18.5 ± 1.5^{bA}
DR35	2.5 ± 2.5^{jkB}	1.5 ± 1.5^{lmnB}	5.0 ± 0.0^{fghA}	7.0 ± 1.0^{hiA}	6.5 ± 1.5^{efA}

Within a column, means compare inhibition diameters among the different isolates with the same test organism and mean values with the same lower case letter are not significantly different (P=0.05,SNK test). Within a row, means compare inhibition diameters among individual isolates with different test organisms and means with same uppercase letter not significantly different (P=0.05, SNK test).

3.5.3 Morphological characteristics, Gram reaction and cell morphology and arrangement

The ten selected isolates, morphological characteristics on nutrient agar media are indicated in Table 5, the Gram type of each isolate, the cell morphology and arrangement based on Gram staining.

Table 5: Morphological characteristics, Gram type and colony description of the ten antagonistic isolates

Isolate code	Morphological characteristics				Gram type	Cell morphology& arrangement
	Shape	**Elevation**	**Texture**	**Color**		
C25M	Round	Flat	Smooth	Yellow	Negative	Rods
G20C	Round	Flat	Smooth	Clear white	Positive	Cocci
G43W	Round	Flat	Smooth	White	Positive	Cocci (clusters)
DC1	Irregular	Flat	Smooth	Cream white	Positive	Rods
H11	Curled	Flat	Smooth and Glistening	Cream white	Positive	Rods
L13	Round	Flat	Smooth	Cream white	Positive	Rods
S33	Irregular	Flat	Glistening	Cream yellow	Positive	Rods
B41	Curled	Flat	Dull	Cream white	Positive	Rods
DM30	Irregular	Flat	Dull	White	Positive	Rods
DR35	Irregular	Flat	Dull	Cream white	Positive	Spindle shaped rods

Morphological studies characteristics showed that the isolates form various growth characteristics on nutrient agar culture media (Table 5). This difference is attributed to the genetic make up and the taxonomic grouping of the isolates (Cappuccino and Sherman, 2002).

3.5.4 Biochemical characteristics

The isolates were taken through a series of biochemical tests to determine their physiological characteristics i.e. their ability to excrete extra cellular enzymes. The ten antagonistic isolates selected showed different biochemical reactions .All the isolates were catalase positive. A few isolates for example S33, B41 and DM30 liquefied gelatin (Table 6).

Table 6: Biochemical test results for the ten isolates

	Biochemical tests												
Isolate code	**Triple Sugar Iron (TSI)**				**Sulphur (SIM)**	**Indole**	**Motility**						
	Butt	Slant	H_2S	Gas	sulphur	indole	motility	SC	MR	VP	Urea	gelatin	catalase
C25M	+	-	-	+	-	-	+	-	-	-	-	-	+
G20C	+	-	-	+	-	-	+	-	-	-	-	-	+
G43W	-	+	-	-	-	-	-	-	-	-	-	-	+
DC1	-	-	-	-	-	-	-	-	-	-	+	-	+
H11	+	-	-	+	-	-	+	-	-	-	-	-	+
L13	+	-	-	+	-	-	+	-	-	+	+	-	+
S33	-	-	-	-	-	-	-	-	-	-	-	+	+
B41	+	+	-	-	-	-	+	-	+	-	-	+	+
DM30	+	+	-	-	-	-	+	-	-	+	-	+	+
DR35	+	+	-	-	-	-	-	-	+	-	-	-	+

KEY:

+ A positive result for the reaction
- A negative test for the reaction

The growth and multiplication of bacteria is the consequence of active metabolism and this is a reflection of the ability to utilize certain substrates, presence or absence of specific enzymes and the production of specific end products. The biochemical fingerprints are properties controlled by the cells enzymatic activities. Hence this explains the reason to the varied biochemical reactions of the different isolates (Harold, 2002).

3.5.5 Molecular analysis of the 16S rDNA gene

Due to constrain of time and resources, only one of the isolates was subjected to molecular characterization

Genomic DNA was successfully extracted from isolate DM30. 16S rDNA amplification with bacterial based primers specific for this region of DNA yielded an amplification product of approximately 650bp, which was further sequenced and the products blasted against samples at NCBI database at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

3.5.5.1 Phylogenetic cluster analysis of sequences

The Phylogenetic position of isolate DM30 indicates that the isolate clusters with the genus *Bacillus.*

Figure 7: Phylogenetic tree showing position of isolate DM30. The scale bar indicates approximately 10% sequence difference.

The query subject(Isolate DM 30) had sequence identity with the 16S ribosomal DNA gene (partial sequence) of different strains of *Bacillus cereus, Bacillus sp., Bacillus thuringiensis, Acetobacter pasteurianus, Bacillus mycoides, Bacillaceae bacterium* and *Bacillus subtilis* at 99% sequence identity with an E value of 0.0. The organism DM30 can therefore be presumptively identified as a *Bacillus* species.

Sequences of the top eleven different strains/organisms in the blast results were obtained for phylogenetic analysis together with DM30 sequence.The sequence DM30 is on the same node with *B. subtilis* though distantly apart (Figure 7).

Bacillus represents a genus of gram positive bacteria, rod shaped and a member of the division Fimicutes, Class Bacilli, Order Bacillales and Family Bacillaceae (Michael *et al.,* 2000). Isolate DM30 adheres to all phenotypic traits of the genus *Bacillus,* including the fact that they are Gram positve, rod shaped (Table 5) and catalase positive (Table 6).These characteristics together with other biochemical properties distinguish isolate DM30 as a member of the genus *Bacillus*. *B. subtilis* strain has been found to produce either a broad spectrum antimicrobial compound or several compounds with different activities (Foldes *et al.,* 2000). The isolate DM30 identified as *B. subtilis* displayed antagonism against pathogenic bacteria and fungi owing to the production of antimicrobial compound(s).

3.6 Extraction of the isolates' crude products

The ten selected isolates were successfully fermented, their crude products extracted and the respective yields determined and recorded (Table 7).

Table 7: Yield (g/l) of the isolates crude products

Isolate	C25M	G20C	G43W	DC1	H11	L13	S33	B41	DM30	DR35
Yield (g/l)	0.76	0.13	0.16	0.56	0.32	0.19	0.25	0.20	0.24	0.30

Yields differed from one sample to another, with the greatest yield produced by C25M and the least produced by G20C (Table 7).

The yield difference could be attributed to the chemical composition (metabolites) and the individual genetic composition of the isolates. Different isolates are a host of different number of compounds/metabolites; these metabolites also have different molecular weights causing the difference in yields (Newman *et al.*, 2003). The growth medium also plays a role in the yields produced by an individual isolate i.e. maximum growth of different isolates is dependent on each isolates' ability to utilize nutrients in the medium. Hence, this also affects the production of the metabolites leading to a difference in yields (Lene, 1996).

3.7 Antimicrobial activity of the crude products

50% of the crude extract obtained (Section 3.6) for each isolate, was used to test antagonistic activity. Extracts of the isolates were tested for antagonistic effect on both bacteria (Gram positive and Gram negative) and fungi and antagonistic activity diameters recorded (Table 8). The positive controls used included Tetracycline and Gentamycin drugs while the negative control was a paper disc (6mm) without any content on it.

Table 8: Inhibition diameters (± SE) for the isolates' crude extracts against the test organisms

Isolate	Test organisms (Diameter in mm, n=3)				
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Escherichia coli*	*Candida albicans*
C25M	10.7 ± 0.3^{dB}	9.3 ± 0.3^{cdBC}	15.0 ± 0.0^{dA}	8.3 ± 0.3^{deC}	9.7 ± 0.9^{cdBC}
G20C	10.0 ± 0.0^{defA}	7.0 ± 0.0^{bcB}	9.7 ± 0.3^{efA}	9.0 ± 0.6^{deA}	9.7 ± 0.3^{cdA}
G43W	7.0 ± 0.0^{gA}	7.7 ± 0.3^{deA}	8.7 ± 1.2^{efA}	9.0 ± 1.0^{deA}	7.0 ± 0.0^{deA}
DC1	14.7 ± 1.3^{cA}	10.7 ± 0.7^{bcB}	12.0 ± 0.6^{deAB}	12.7 ± 0.3^{cAB}	11.3 ± 1.9^{bcAB}
H11	11.7 ± 0.9^{dAB}	7.0 ± 0.0^{deC}	10.3 ± 0.3^{efB}	9.7 ± 0.9^{dB}	13.7 ± 1.2^{bA}
L13	11.3 ± 0.7^{dB}	11.3 ± 0.7^{bcB}	17.7 ± 2.7^{cA}	10.3 ± 0.3^{dB}	12.3 ± 0.3^{bcB}
S33	8.0 ± 0.0^{efgBC}	11.7 ± 0.7^{bcA}	9.3 ± 0.7^{efB}	7.0 ± 0.0^{efC}	9.7 ± 0.7^{cdB}
B41	7.7 ± 0.3^{fgC}	10.7 ± 0.7^{bcB}	13.3 ± 0.9^{deA}	10.3 ± 0.3^{dB}	7.0 ± 0.0^{deC}
DM30	10.3 ± 0.3^{deAB}	9.3 ± 0.7^{cdAB}	9.0 ± 0.0^{efB}	9.0 ± 0.6^{deB}	10.7 ± 0.7^{bcA}
DR35	8.0 ± 0.3^{efgA}	7.3 ± 0.3^{deA}	9.3 ± 1.5^{efA}	7.3 ± 0.3^{efA}	7.3 ± 0.3^{deA}
-ve control	6.0 ± 0.0^{gA}	6.0 ± 0.0^{eA}	6.0 ± 0.0^{fA}	6.0 ± 0.0^{fA}	6.0 ± 0.0^{eA}
TET	23.3 ± 0.7^{aB}	12.7 ± 0.9^{bC}	29.3 ± 0.7^{aB}	24.0 ± 0.6^{aB}	11.3 ± 0.7^{bcC}
GENT	21.0 ± 0.6^{bB}	18.7 ± 0.3^{aC}	20.7 ± 0.3^{bB}	22.3 ± 0.3^{bAB}	21.7 ± 0.3^{aAB}

Within a column, means compare inhibition diameters among the isolates natural extracts with the same test organism and means with the same lower case letter are not significantly different (P=0.05, SNK test). Within a row, means compare inhibition diameters among the individual isolate crude extract with different test organisms and means values with same upper case letter are not significantly different (P=0.05, SNK test).

The bioassay procedure was performed as shown in sample plates 2, 3, 4a, 4b, 5a, 5b, 6a and 6b respectively.

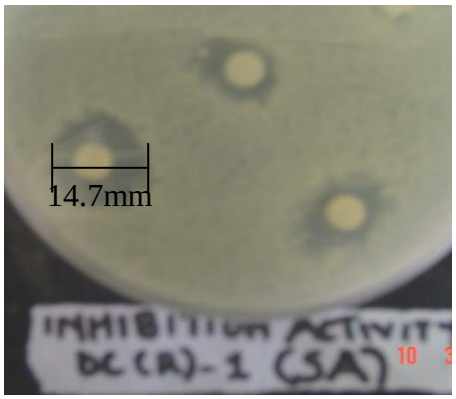

Plate 2: Antagonistic activity of DC1 crude extract on *S. aureus* (ATCC 22923). The zone of inhibition around the paper disc show antagonistic effect

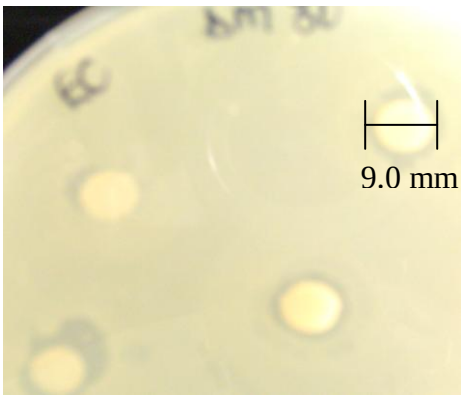

Plate 3: Antagonistic activity of DM30 crude extract on *E. coli* (ATCC 25922). The zone of inhibition around the paper disc show antagonistic effect

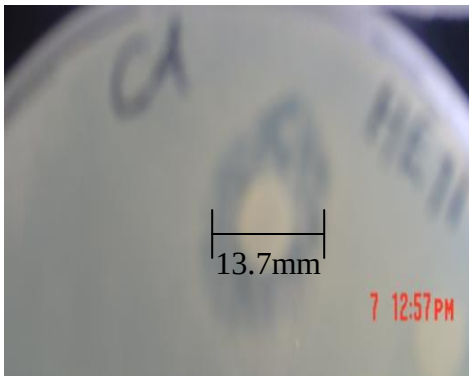

Plate 4a: Antagonistic activity of H11 crude extract on *C.albicans* (ATCC 90028). The zone of inhibition around the paper disc show antagonistic effect

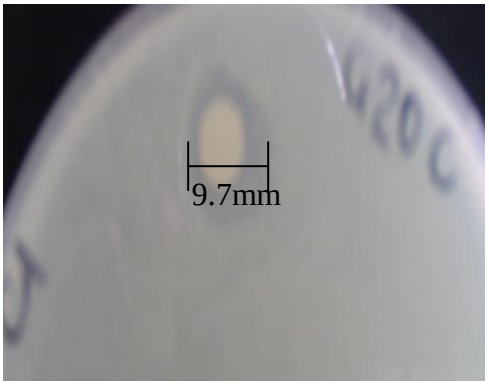

Plate 4b: Antagonistic activity of G20C crude extract on *C. albicans* (ATCC 90028). The zone of inhibition around the paper disc show antagonistic effect

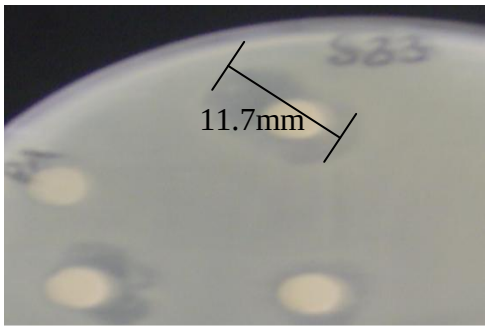

Plate 5a: Antagonistic activity of S33 crude extract on *P. aeruginosa* (ATCC 27853). The zone of inhibition around the paper disc show antagonistic effect

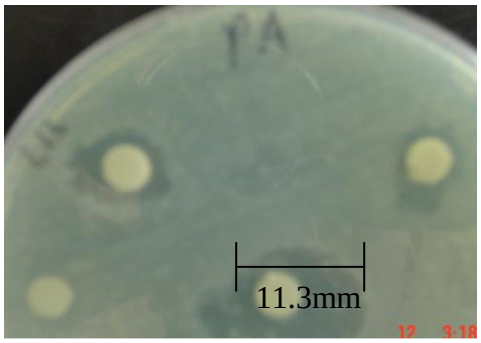

Plate 5b: Antagonistic activity of L13 natural crude on *P. aeruginosa* (ATCC 27853). The zone of inhibition around the paper disc show antagonistic effect

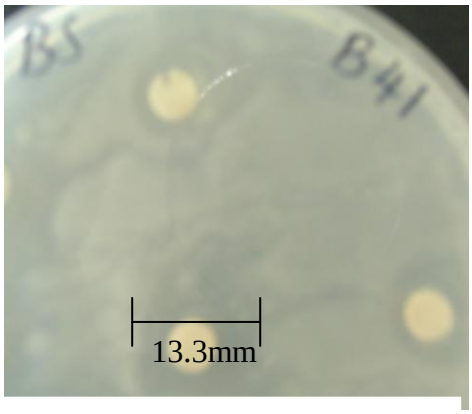

Plate 6a: Antagonistic activity of B41 crude extract on *B. subtilis* (55732). The zone of inhibition around the paper disc show antagonistic effect

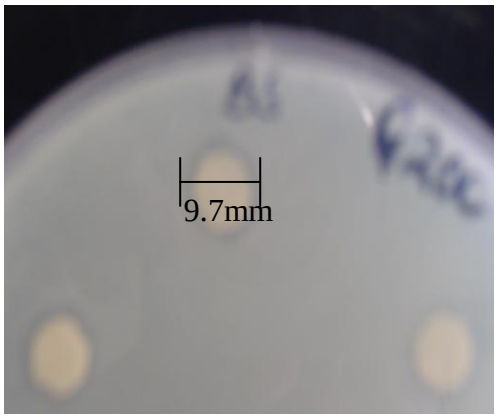

Plate 6b: Antagonistic activity of G20C crude extract on *B. subtilis* (55732).The zone of inhibition around the paper disc show antagonistic effect

3.8 Chemical screening (Analysis)

Antagonistic activity differed from one bacterial extract to another and from one test organism to another. Most of the bacterial extracts exhibited inhibitory activity against the five test organisms. Comparing antagonistic activity of the negative control and the bacterial extracts (Table 8), indicates that DC1 was the most active against *S. aureus* (14.7mm) (Plate3), DC1, L13, S33 and B41 were the most active against *P. aeruginosa* with diameters of 10.7mm, 11.3mm (Plate 6b), 11.7mm (Plate 6a) and 10.7mm respectively. L13 was the most active against *B. subtilis* (17.7mm), DC1 was the most active against *E. coli* (12.7mm), H11and L13 were the most active against *C. albicans* with diameters of 13.7mm (Plate 5a) and 12.3mm respectively. Comparing the isolates' natural extracts with the positive controls, the drugs were the most active against the test organisms except for the extract obtained from isolate H11 (13.7mm), which had a significant activity against *C. albicans*, compared to tetracycline (11.3mm). However, this does not disqualify the bacterial natural extracts from being used as potentials against bacterial and fungal infections because the difference in activity could be attributed to synergetic effects of compounds present in each extract, difference in the modes of action, concentration difference in the composition of compounds present in each extract and difference in the genetic composition/make up of the extract responsible for the activity i.e. different genes are responsible for the different levels of antagonistic activity since some of them give rise to chemical transformations of substrates and many others that do not (Stone and Williams, 1992). The difference in the *in vitro* activity among the various crude extracts could be due to the production of either a broad spectrum antimicrobial compound, or several compounds with different

activities (Foldes *et al.,* 2000). In a study conducted by Omura (1992), he concluded that the differences in levels of antagonism are dependent on concentration of the active substance(s).

Low activity was demonstrated against *P. aeruginosa* and *E. coli* by most natural extracts. The results obtained in this study are an indication that Gram positive bacteria (*B. subtilis* and *S. aureus*) are more susceptible to antibiotics than Gram negative bacteria (*P. aeruginosa* and *E. coli*) which are known to be more difficult to inhibit due to their cell wall morphology (Young *et al.,* 2006).

3.8 Chemical screening

3.8.1 Preparative Thin Layer Chromatography (PTLC)

From PTLC, several fractions were obtained from the respective bacterial crude extracts (Table 9).

Table 9: Number of compounds present in each sample after PTLC separation

Sample	C25M	G20C	G43W	DC1	H11	L13	S33	B41	DM30	DR35
No. of compounds present	10	13	9	13	9	11	14	12	12	11

Sample S33 had the most number of fractions (14) and the least were present in sample H11 (9) and G43W (9) respectively (Table 9). The crude extracts separated into different bands/fractions due to the mixture of compounds present in the extracts (Qin and Judith, 1999). Hence, the more the compounds present in the crude extract, the more the number of fractions present.

3.8.2 Antimicrobial activity of fractions / bands

Some of the fractions obtained from PTLC were subjected to antimicrobial activity against the test organisms and the respective activity values are represented in Table 10.

Table 10: Inhibition diameters (± SE) of bands/fractions against the test organisms

Isolate	Band/ fraction no.	Test organisms (Diameter in mm, n=3)				
		Staphylococcus aureus	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Escherichia coli*	*Candida albicans*
C25M	4	9.0 ± 0.6^{defAB}	7.0 ± 0.0^{efC}	9.3 ± 0.9^{defAB}	9.7 ± 0.3^{cdeA}	7.7 ± 0.7^{efghBC}
G20C	12	8.3 ± 0.9^{defA}	$8.3\pm0.31.2^{cdefA}$	10.0 ± 1.2^{defA}	7.7 ± 0.3^{deA}	7.7 ± 0.7^{efghA}
	13	7.3 ± 0.3^{efA}	8.3 ± 0.3^{cdefA}	10.0 ± 1.2^{defA}	9.0 ± 1.2^{lcdeA}	0.0 ± 0.0^{iB}
G43W	8	8.7 ± 0.7^{defB}	9.7 ± 0.3^{bcdeB}	14.3 ± 0.9^{cdA}	8.7 ± 0.3^{cdeB}	10.3 ± 0.3^{cdefB}
DC1	5	11.7 ± 0.9^{cdA}	10.3 ± 0.9^{bcdAB}	9.3 ± 0.9^{defB}	12.0 ± 1.2^{bcAB}	13.0 ± 1.0^{bcA}
	10	9.7 ± 0.9^{deBC}	7.3 ± 0.3^{defC}	12.3 ± 0.9^{cdeA}	11.3 ± 0.7^{cdAB}	12.0 ± 1.0^{cdAB}
H11	6	7.7 ± 0.7^{efAB}	7.7 ± 0.3^{cdefAB}	9.3 ± 0.3^{defA}	8.0 ± 1.0^{cdeAB}	7.0 ± 0.0^{ghB}
	7	8.3 ± 0.9^{defA}	7.7 ± 0.7^{cdefA}	10.3 ± 1.8^{defA}	8.3 ± 0.9^{cdeA}	7.3 ± 0.3^{fghA}
L13	8	14.3 ± 0.7^{bcA}	10.0 ± 1.2^{bcdeB}	12.0 ± 1.2^{cdeAB}	10.0 ± 1.7^{cdeB}	15.3 ± 0.7^{bA}
	11	15.3 ± 0.9^{bA}	18.0 ± 1.2^{aA}	15.7 ± 1.2^{bcA}	15.7 ± 0.7^{bA}	11.7 ± 0.0^{cdB}
S33	3	15.7 ± 0.7^{bA}	10.3 ± 0.3^{bcdB}	11.0 ± 0.6^{efB}	10.7 ± 0.7^{cdB}	10.3 ± 0.7^{cdefB}
	11	9.7 ± 0.9^{deAB}	10.7 ± 0.7^{bcA}	$8.7\pm0.^{efB}$	8.3 ± 0.3^{cdeB}	10.7 ± 0.3^{cdeA}
B41	7	8.3 ± 0.3^{defAB}	7.3 ± 0.3^{defB}	9.0 ± 0.6^{efA}	9.3 ± 0.7^{cdeA}	9.0 ± 0.6^{defghA}
	9	9.0 ± 0.6^{defB}	0.0 ± 0.0^{gD}	11.7 ± 0.3^{cdeA}	8.3 ± 0.3^{cdeBC}	7.3 ± 0.3^{fghC}
DM30	11	9.0 ± 0.3^{defAB}	9.3 ± 0.6^{cdeAB}	10.3 ± 0.9^{defA}	9.3 ± 0.9^{cdeAB}	7.7 ± 0.3^{efghB}
DR35	8	8.3 ± 0.3^{defA}	9.7 ± 0.3^{bcdeA}	9.3 ± 1.2^{defA}	7.7 ± 0.6^{deA}	9.7 ± 0.3^{defgA}
TET		23.3 ± 0.7^{aB}	12.7 ± 0.9^{bC}	29.3 ± 0.7^{aB}	24.0 ± 0.6^{aB}	11.3 ± 0.7^{cdC}
GENT		21.0 ± 0.6^{aB}	18.7 ± 0.3^{aC}	20.7 ± 0.3^{bB}	22.3 ± 0.3^{aAB}	21.7 ± 0.3^{aAB}
-Ve control		6.0 ± 0.0^{fA}	6.0 ± 0.0^{fA}	6.0 ± 0.0^{fA}	6.0 ± 0.0^{fA}	6.0 ± 0.0^{fA}

Within a column, means compare inhibition diameters among the isolates different fractions and means with the same lower case letter are not significantly different (P=0.05, SNK test).Within a row, means compare inhibition diameters among the individual isolate fraction with different test organisms and means with the same uppercase letter are not significantly (P=0.05, SNK test).

Comparing the different fraction activities against the test organisms, L13-fraction 11 and S33-fraction 3 were the most active against *S. aureus,* with inhibition diameters of 15.3mm and 15.7mm respectively. L13-fraction 11 was the most active against *P. aeruginosa, B. subtilis* and *E. coli* with inhibition diameters of 18mm, 15.7mm and 15.7mm respectively, while its fraction 8 exhibited the most *in vitro* activity against *C. albicans* (15.3 mm).

When bioactivity was compared with that due to the standard drugs (Gentamycin and Tetracycline), it was seen that there was no significant difference (P=0.05) in inhibition activity between L13 fraction 11 (18mm) and the commercial drug Gentamycin (18.7mm) against *P. aeruginosa*. L13 fraction 8, had a more significant activity against *C. albicans* (15.3mm) as compared to Tetracycline (11.3mm), but Gentamycin had the most significant activity compared to all fractions against *C. albicans* (21.7mm). Comparing the activity of the positive controls and the fractions against *S. aureus, B. subtilis* and *E. coli,* the positive controls had the most inhibition activity. The zones of inhibition of the isolates' fractions may be similar to the standard drugs, but if they have different modes of activity, this would still make them promising (Fatope, 1995). Consequently, the search for the secondary metabolites as lead compounds or templates in drug development is encouraged.

From the results, it's clear that G20C-fraction 13 and H11-fraction 6 had the least inhibition activity against S. *aureus*, with inhibition diameters of 7.3mm and 7.7mm respectively. B41-fraction 9 exhibited no inhibition activity at all against *P. aeruginosa* (0mm) and the same case applies to G20C-fraction 13 against *C. albicans.* S33-fraction

11 had the least activity against *B. subtilis* (8.7mm), while G20C-fraction 12 and DR 35-fraction 8 had the least activity against *E. coli* with diameters of 7.7 mm each.

The difference in activity among the fractions could be attributed to the degree of synergy of the compounds present in each fraction (Omura, 1992). This is due to the fact that different fractions have different compound composition (which could be unique to each fraction) and different compounds have different concentrations and modes of action. In the case of modes of action, some compounds do have a broad spectrum activity while others have a narrow spectrum activity which brings about the significant differences in the antagonistic activities (Ligon, 1999).

Comparing the results in Table 8 and Table 10, there are differences in the antagonistic activity of the natural/crude extracts and separated products respectively. The difference could be attributed to the synergetic effects of the compounds. Some compounds display good activity in a mixture while others work best when separated. Other compounds show no difference in activity when in a mixture or when separated and others show no activity at all when separated (Newman *et al.*, 2003).

From the results in Table 10, the fractions may produce or synthesize novel therapeutics, that would aid in the fighting of life threatening diseases once resistance builds up and since gram negative bacteria are among the notorious pathogens found in hospitals that have acquired resistance to several antibiotics in the past, the search for the secondary metabolites that could enable the synthesis of drugs should be encouraged.

3.8.3 Identification of chemical constituents

Identification of the compounds present in each sample fraction was done by analysis of the mass spectra and retention time.

3.8.3.1 Compounds from sample C25M fraction 4

Several compounds were identified by GC-MS data analyses (Table 11).

Table 11: Compounds identified from C25M, F4

Fraction	Compound name	Retention time	% of total
C25M-B4	=3-Penten-2-ol	3.311	0.090%
	=Toluene	5.170	3.427%
	=5-t-Butyl-4-methylimidazole	9.291	0.290%
	=decamethyl- Cyclopentasiloxane	13.681	0.048%
	=4-Pyridinamine	16.122	0.119%
	=2,6,10,14-tetramethyl- Hexadecane	16.861	0.070%
	=Tetradecane	17.175	0.142%
	=4-hydroxy- Benzene ethanol	17.555	1.799%
	=Decahydro-4,4,8,9,10-pentamethylnaphthalene	18.093	0.086%
	=Pentadecane	18.429	0.197%
	=Magnesium, bis(acetylacetonate)	19.280	0.036%
	=Hexadecane	19.638	0.354%
	=7,9-dimethyl- Hexadecane	20.198	0.605%
	=1,2-dimethyl-4-methylene-3-phenyl-=Cyclopentene	20.355	0.355%
	=3-Methyl-4-(methoxycarbonyl)hexa-2,4-dienoic acid	20.601	0.632%
	=1-Methyl-4-n-butylaminocytosine	21.587	1.221%
	=Anthracene	21.833	1.656%
	=Eicosane	21.945	2.022%
	=1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	22.617	2.251%
	=Bacchotricuneatin c	22.796	0.792%
	=1-Carbomethoxy-1,2,5,5-tetramethyl-cis-decalin(1R,2S,4as,8as)	23.155	0.615%
	=2-methyl- Phenanthrene	23.267	1.879%
	=N'-Pyridinecarbohydrazonamide	23.558	0.979%
	=2,3-dimethyl- Phenanthrene	24.095	1.011%
	=di-p-Tolylacetylene	24.431	1.136%
	=Pyrene	24.655	0.831%
	=Heneicosane	24.790	1.004%

KEY: =, **Represents an individual compound**

Table 11 continued: Compounds identified from C25M, F4

Fraction	Compound name	Retention time	% of total
C25M-B4	=1-methyl-7-(1-methylethyl)- Phenanthrene	26.044	0.995%
	1-iodo- Hexadecane	26.693	0.934%
	=1, 2, 3, 4,4a, 9, 10, 10a-octahydro-1, 4a-dimethyl-7-(1-methylethyl)-, methyl ester, [1R-(1.alpha, 4a.beta, 10a.alpha.)]- 1-Phenanthrenecarboxylic acid	27.074	1.016%
	=Tetracosane	27.365	2.806%
	=N-[3-[[(2,5-dioxo-1-pyrrolidinyl)ethyl]ethyl amino]phenyl]- Acetamide	27.746	0.845%
	=Benz[a]anthracene	28.037	0.915%
	=Octacosane	28.172	3.694%
	=Di-n-octyl phthalate	28.597	1.624%
	=Heneicosane	28.933	3.029%
	=2,6,10,14-tetramethyl- Hexadecane	29.381	1.295%
	=Coprostane	29.493	1.754%
	=Octacosane	30.478	2.961%
	=2, 3-epoxy-2-methyl-, (2.alpha, 3.alpha, 5.alpha.)- Cholestane	30.882	2.614%
	=5a, 8b, 9a, 14b, 17a, 20R-Cholestane	31.218	1.638%
	=Nonacosane	31.419	2.548%
	=Baccharane	31.979	0.719%
	=23,28-Bisnor-17.beta.(H)-hopane	32.203	0.969%
	=Stigmastane	32.875	1.020%
	=28-Nor-17.beta.(H)-hopane	33.211	0.548%
	=D:A-Friedooleanan-24-ol	33.413	0.559%
	=Furan-2-carboxamide, N-(2-fluorophenyl)-	34.600	0.153%
	=2,4,5,5,8a-Pentamethyl-6, 7,8,8a-tetrahydro-5H-chromene	36.772	0.128%

3.8.3.2 Compounds from sample G20C fraction 12 and 13

From sample G20C, two fractions were subjected to GC-MS analyses and the compounds identified are as indicated in table 12 and 13.

Table 12: Compounds identified from G20C, F12

Fraction	Compound name	Retention time	% of total
G20C-B12	=1,2-dimethyl-, trans- Cyclopentane	3.266	6.016%
	=Heptane	3.468	10.360%
	=Methyl- Cyclohexane	3.915	21.999%
	=Ethyl- Cyclopentane	4.162	2.529%
	=1,2,4-trimethyl- Cyclopentane	4.319	1.391%
	=Toluene	5.170	5.983%
	=3-methyl- Heptane	5.349	1.057%
	=1,1-dimethyl- Cyclohexane	5.685	0.325%
	=1-ethyl-2-methyl- Cyclopentane	5.886	0.397%
	=1,2-dimethyl-, trans- Cyclohexane	6.021	0.711%
	=1,2-dimethyl-, cis- Cyclohexane	6.939	0.083%
	=Ethyl- Cyclohexane	7.073	0.500%
	=1,1,3-trimethyl- Cyclohexane	7.185	0.271%
	=Ethyl benzene	7.880	0.054%
	=p-Xylene	8.081	0.197%
	=Nonane	8.865	0.027%
	=Pyrimidine-2,4,6(1H,3H,5H)-trione, 5-[1-(2-diethylaminoethylamino)propylidene]-	10.209	0.168%
	=Decane	10.993	0.035%
	=1-Piperidinecarboxaldehyde	13.434	0.011%
	=2,5-Dimethyl-1-pyrroline	13.994	0.173%
	=Thiophene, tetra hydro-, 1,1-dioxide	14.487	0.048%
	=Isothiocyanato- Cyclohexane	14.935	0.087%
	=Benzophenone	20.109	0.279%
	=Tributyl phosphate	20.221	0.192%
	=Oxalic acid, cyclohexylmethyl tetradecyl ester	20.915	0.664%
	=2,3-dihydro-1, 1,3-trimethyl-3-phenyl-1H-Indene	21.116	0.237%
	=(4-methylphenyl) phenyl- Methanone	21.520	0.132%
	=Indeno [2,1-c] pyridine, 1,4,6-trimethyl-	21.811	0.232%
	=2-(methylthio)- Benzothiazole	22.012	0.203%
	=Phthalic acid, isobutyl octyl ester	22.640	8.039%
	=2,4,7-trimethyl- Carbazole	22.863	0.293%
	=Hexadecanoic acid, methyl ester	23.132	1.271%
	=Dibutyl phthalate	23.558	9.013%
	=Isopropyl Palmitate	24.095	1.229%
	=9-Octadecenoic acid (Z)-, methyl ester	24.812	0.587%
	=2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	25.462	0.192%
	=2-Piperidinone, N-[4-bromo-n-butyl	26.402	0.143%
	=Tetracosane	27.365	0.497%
	=1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	28.597	4.907%

Table 13: Compounds identified from G20C, F13

Fraction	Compound name	Retention time	% of total
G20C-B13	=2-methyl-3-Buten-2-ol	3.311	0.334%
	=Heptane	3.490	0.462%
	=Methyl- Cyclohexane	3.938	0.620%
	=Toluene	5.170	0.292%
	=Octane	6.200	0.025%
	=Bacchotricuneatin c	17.175	0.031%
	=9-oxo-, methyl ester Nonanoic acid	17.623	0.237%
	=3-Chloro-benzalacetone	18.138	0.298%
	=Diethyl Phthalate	19.638	0.340%
	=4-(3-Hydroxy-2,6,6-trimethylcyclohex-1-enyl)pent-3-en-2-one	20.893	1.261%
	=1,5,7-trimethyl- Carbazole	21.811	2.327%
	=2-Imino-5-phenyl-1, 2-dihydro-3H-1, 3,4-benzotriazepine	22.595	0.597%
	=2,4,7-trimethyl- Carbazole	22.864	1.838%
	=Hexadecanoic acid, methyl ester	23.132	4.300%
	=2-methyl-3-phenyl-1H-Indole	23.536	0.755%
	=Isopropyl Palmitate	24.096	1.590%
	=Heptadecane	24.790	1.832%
	=Docosane	25.686	1.680%
	=Pentacosane	28.172	9.631%
	=1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	28.620	19.126%
	=Octacosane	30.479	4.726%
	=Nonacosane	31.419	3.289%
	=Eicosane	32.517	3.250%
	=Heneicosane	33.838	2.270%
	=Beta.-iso-Methyl ionone	34.824	0.238%

3.8.3.3 Compounds from sample G43WC fraction 8

From sampleG43W fraction 8, several compounds were identified (Table 14).

Table 14: Compounds identified from G43W, F8

Fraction	Compound name	Retention time	% of Total
G43W-B8	=Methyl- Cyclohexane	3.915	1.198%
	=Piperidine	4.923	0.705%
	=Toluene	5.170	0.436%
	=1,2-dimethyl- Cyclohexane	6.021	0.011%
	=N-butyl-1-Butanamine	10.209	3.052%
	=Acetophenone	12.225	0.032%
	=1-Piperidinecarboxaldehyde	13.434	0.017%
	=1-Tridecene	13.770	0.011%
	=N, N-Dibutyl- Formamide	15.898	0.033%
	=4-hydroxy-6-methyl-2 (1H)-Pyridinone	16.458	015%
	=Decahydro-2, 7-Imino-3, 6-methanonaphthalene	16.749	0.043%
	=Goitrin	17.175	0.141%
	=DL- Glutamic acid	17.958	0.025%
	=3-Methyl-4-phenyl-1H-pyrazol-5-amine	18.765	0.697%
	=Diethyl Phthalate	19.638	0.293%
	=Benzophenone	20.109	0.261%
	=Tributyl phosphate	20.243	0.251%
	=Hexadecane	20.781	0.417%
	=N-(2-thienylmethyl)- 1,2,4-Triazol-4-amine	20.937	6.781%
	=2,3-dihydro-1, 1,3-trimethyl-3-phenyl-1H-Indene	21.139	0.735%
	=2,4,6-trimethyl- Benzophenone	22.124	0.382%
	=l-Alanine, N-allyloxycarbonyl-, isobutyl ester	22.774	0.638%
	=Pentadecanoic acid, 14-methyl-, methyl ester	23.132	1.293%
	=N-butyl-1-Butanamine	23.379	0.854%
	=Dibutyl phthalate	23.580	5.488%
	=Hexadecanoic acid, ethyl ester	23.804	0.698%
	=Isopropyl Palmitate	24.095	0.743%
	=Methyl ester, (E)- 9-Octadecenoic acid	24.834	0.665%
	=Linoleic acid ethyl ester	25.394	5.743%
	=Octacosane	28.172	1.760%
	=1,2-Benzenedicarboxylic acid, diisooctyl ester	28.642	8.756%
	=Octadecane	28.933	0.845%

Table 14 continued: Compounds identified from G43W, F8

Fraction	Compound name	Retention time	% of Total
G43W-B8	=Triethylene glycol	29.314	5.408%
	=Heneicosane	29.672	0.097%
	=N2-(4-chlorobenzylidene)-4-methoxy-Thiophene-3-carbohydrazide	29.874	1.224%
	=Octacosane	30.479	0.600%
	=1-(7-oxo-2, 4,6-trimethylheptanoyl)- Pyrrolidine	30.904	0.718%
	=1-(benzob]-1,4-dioxane-6-yl)-3-(4fluorophenyl)-Propenone	31.800	3.087%
	=1-(3-hydrohy-3-Phenyl-1-triazenyl)-Antra-9, 10-quinone	33.838	0.089%
	=1,4,7,10,13-Pentaoxacyclohexadecane,15-(1,4,7,10-tetraoxacyclotridec-12-yl)-	34.891	0.216%
	=Di-n-Butyldithiocarbamic acid, zinc salt	42.304	18.091%
	=Zinc, bis (1-piperidinecarbodithioato-S, S')-, (T-4)-	44.432	2.467%

3.8.3.4 Compounds from sample DC1 fraction 5 and 10

From sample DC1, two fractions were subjected to GC-MS analyses and the compounds identified are as indicated in table 15 and 16.

Table 15: Compounds identified fromDC1, F5

Fraction	Compound name	Retention time	% of total
DC1-B5	=Methyl- Cyclohexane	3.893	0.025%
	=Piperidine	4.923	0.212%
	=N-sec-Butyl-n-propylamine	10.187	1.209%
	=Dodecane	14.375	0.083%
	=Isothiocyanato- Cyclohexane	14.957	2.257%
	=4-Methyl-dodec-3-en-1-ol	16.480	0.356%
	=Tetradecane	17.175	0.857%
	=Decahydro-4, 4,8,9,10-pentamethylnaphthalene	17.623	0.598%
	=Bacchotricuneatin c	18.457	0.963%
	=5-Butyl-5-ethyl-6(5H)-imino-2,4(1H,3H)-pyrimidinedione	18.765	0.345%
	=1,6,7-trimethyl- Naphthalene	18.944	0.625%
	=1H-Purine-2, 6-dione, 1,3-diethyl-3,9-dihydro-9-methyl- 123.0	19.302	0.311%
	=Hexadecane	19.661	1.160%

Table 15 continued: Compounds identified fromDC1, F5

Fraction	Compound name	Retention time	% of total
DC1-B5	=1,2,4-Triazol-4-amine, N- (2-thienylmethyl)-	20.960	3.398%
	=Anthracene	21.878	3.340%
	=1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	22.662	0.997%
	=2-methyl- Anthracene	23.311	0.950%
	=4b,8-Dimethyl-2-isopropylphenanthrene, 4b,5,6,7,8,8a,9,10-octahydro-	24.252	1.127%
	=2,5-dimethyl- Phenanthrene	24.431	0.440%
	=Heneicosane	24.834	2.743%
	=Pyrene	25.193	2.137%
	=N-phenyl-1-Naphthalenamine,	25.394	1.498%
	=Docosane	25.730	1.773%
	=1-methyl-7- (1-methylethyl)- Phenanthrene,	26.089	1.542%
	=1-(2-Nitrobenzyl) isoquinoline	30.971	0.889%
	=Nonacosane	31.531	1.758%
	=Baccharane	32.114	0.594%
	=28-Nor-17.beta. (H)-hopane	33.345	0.870%
	=Stigmastane	33.569	1.240%
	=1-(5-Hydroxy-5-pyridin-4-yl-3-trifluoromethyl-4,5-dihydropyrazol-1-yl)-2-methylpropan-1-one	33.972	1.240%
	=1-Penten-3-one,1-(2,6,6-trimethyl-1 cyclohexen-1-yl)-	35.025	3.625%
	=Lanost-8-en-11-one	36.055	0.097%
	= (16.beta, 18.alpha, 19.alpha.)- Urs-20-en-16-ol	36.705	1.009%
	=Beta.-iso-Methyl ionone	37.310	0.301%
	=2,4-dimethyl-10H-Indeno [1,2-g] quinoline	37.892	0.227%
	(5.alpha.)- Androstan-6-one	39.617	0.075%
	=Tridecanedial	40.109	0.122%
	=1-Penten-3-one,1-(2,6,6-trimethyl- cyclohexen-1-yl)-	40.490	0.369%
	=Beta. -Iso-Methyl ionone	41.072	0.173%
	=di-n-Butyldithiocarbamic acid, zinc salt	42.215	0.072%

Table 16: Compounds identified fromDC1, F10

Fraction	Compound name	Retention time	% of total
DC1-B10	=Methyl- Cyclohexane	3.916	0.032%
	=Toluene	5.170	0.009%
	=Thiophene, tetra hydro-, 1,1-dioxide	14.487	0.071%
	=4-methyl- Pyridine	14.666	0.010%
	=Benzothiazole	14.801	0.023%
	=2-hydroxy- Benzoic acid	15.853	0.974%
	=N-phenyl- Formamide	16.167	0.236%
	=Vanillin	17.219	0.134%
	=3-methyl- Thiophene	17.735	0.197%
	=2H-Indol-2-one, 1,3-dihydro-	18.138	0.164%
	=Butylated Hydroxytoluene	18.675	1.868%
	=1-Butanone, 1-(2,4,5-trihydroxyphenyl)-	19.190	0.248%
	=Hexadecane	19.638	2.388%
	=4-(1,1,3,3-tetramethylbutyl)- Phenol	19.773	0.765%
	=Tributyl phosphate	20.243	1.310%
	=2-hexyl- Thiophene	20.937	7.634%
	=m-Amino phenyl trifluoromethyl ether	21.251	0.463%
	=Tetradecanoic acid	21.453	0.899%
	=Octadecane	21.856	3.046%
	=Pentadecanoic acid	22.483	0.685%
	=Phthalic acid, cyclohexylmethyl butyl ester	22.640	3.002%
	=Hexadecenoic acid, Z-11-	23.356	4.008%
	=n-Hexadecanoic acid	23.580	9.384%
	=Eicosane	23.71	3.213%
	=Z-7-Pentadecenol	24.431	10.045%
	=Heneicosane	24.790	0.721%
	=6-Octadecenoic acid, (Z)-	25.305	13.505%
	=18-Nonadecenoic acid	26.178	0.683%
	=Docosane	26.559	2.374%
	=Tetracosane	27.388	4.730%
	=Pentacosane	28.172	2.942%
	=Nonacosane	31.419	1.886%
	=Cholest-5-en-3-ol (3.beta.)-	34.577	0.138%
	=2-octyl- Thiophene	35.070	0.254%

3.8.3.5 Compounds from sample H11 fraction 6 and 7

From sample H11, two fractions were subjected to GC-MS analyses and the compounds identified are as indicated in table 17 and 18.

Table 17: Compounds identified fromH11, F6

Isolate	Compound name	Retention time	% of total
H11-B6	=1,2-dimethyl- Cyclopentane	3.311	0.636%
	=Heptane	3.490	1.056%
	=Methyl- Cyclohexane	3.938	2.839%
	=Ethyl- Cyclopentane	4.184	0.350%
	=Piperidine	4.923	2.036%
	=Toluene	5.170	1.192%
	=3-methyl- Heptane	5.371	0.281%
	=Octane	6.200	0.155%
	=Dibutylsulfamic acid	10.209	6.879%
	=Isocyanato- Cyclohexane	10.948	0.193%
	=Dimethyl phthalate	17.936	0.128%
	=1-(Piperidin-2-ylmethyl) Piperidine	18.742	0.443%
	=Diethyl Phthalate	19.638	1.704%
	=Tributyl phosphate	20.243	0.176%
	=Heptadecane	20.758	0.547%
	=Oxalic acid, cyclohexylmethyl tetradecyl ester	20.915	6.926%
	=1,6-dimethyl-4-(1-methylethyl)- Naphthalene	21.116	0.125%
	=Pentadecanoic acid	22.483	1.027%
	=2-Butenedioic acid (Z)-, monododecyl ester	22.774	4.498%
	=1,1-diphenyl-, (Z)- 1,3-Pentadiene	23.177	0.680%
	=N-butyl-1-Butanamine	23.379	1.784%
	=n-Hexadecanoic acid	23.513	4.904%
	=Hexadecanoic acid, ethyl ester	23.804	0.737%
	=Isopropyl Palmitate	24.095	0.979%
	=Cyclic octaatomic sulfur	24.476	1.407%
	=Docosane	25.685	1.374%
	=N,N'-dicyclohexyl- Thiourea	26.917	0.495%
	=Hexagol	27.052	2.455%
	Tetracosane	27.365	1.003%
	=3-[(2-Methyl-5-nitro-phenylimino)-methyl]-phenol	28.216	0.769%
	=1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	28.597	0.472%
	=Heneicosane	28.933	0.609%
	=Heptacosane	29.672	1.350%
	=2,6-Difluorobenzoic acid, 4-chlorophenyl ester	29.851	3.024%
	=Hexamethyl- Cyclotrisiloxane	30.478	2.159%
	=6-Azathymine	30.882	2.224%
	=Propenone, 1-(benzo [b]-1,4-dioxane-6-yl)-3-(4-fluorophenyl)-	31.777	3.067%
	=Cholest-5-en-3-ol (3.beta.)-	34.465	0.264%
	=1,4-Diamino-2-(hydroxymethyl) anthraquinone	39.393	0.152%
	=di-n-Butyldithiocarbamic acid, zinc salt	42.327	8.567%

Table 18: Compounds identified fromH11, F7

Fraction	Compound name	Retention time	% of total
H11-B7	=Methyl- Cyclohexane	3.196	0.028%
	=Toluene	5.170	0.007%
	=Cyclohexane, isocyanato-	10.926	0.021%
	=N, 4-dimethyl- Benzenamine	14.196	0.004%
	=Lenthionine	14.375	0.011%
	=Benzothiazole	14.801	0.055%
	=n-Decanoic acid	15.361	0.010%
	=2-ethyl-6-methyl- Benzenamine	15.943	0.022%
	=Bacchotricuneatin c	16.861	0.027%
	=Tetradecane	17.175	0.124%
	=Nonanoic acid, 9-oxo-, methyl ester	17.623	0.057%
	=Cyclohexane, 1-(cyclohexylmethyl)-2-ethyl-, trans-	18.205	0.175%
	=Pentadecane	18.429	0.312%
	=Sulfur	18.631	0.038%
	=Dodecanoic acid	19.191	0.282%
	=8-Methyloctahydrocoumarin	20.243	0.813%
	=2-Benzothiazolamine, N-ethyl-	20.333	0.061%
	=2(3H)-Benzothiazolone	20.512	0.502%
	=Heptadecane	20.781	0.333%
	=2-(1-phenylethyl)- Phenol	21.117	1.063%
	=Tetradecanoic acid	21.475	1.359%
	=Octadecane	21.856	1.125%
	=Pentadecanoic acid	22.528	1.252%
	=Z-11- Hexadecenoic acid	23.401	2.668%
	=Eicosane	23.872	1.483%
	=2-hydroxy- Cyclopentadecanone	24.431	1.567%
	=Cyclic octaatomic sulfur	24.566	4.683%
	=Heneicosane	24.812	2.056%
	=Octadec-9-enoic acid	25.327	4.899%
	=Tetracosane	25.708	0.350%
	=Tricosane	26.582	4.263%
	=2,4-bis (1-phenylethyl)- Phenol	27.769	2.232%
	=Nonacosane	28.956	4.366%
	=1-iodo- Octadecane	29.404	0.690%
	=Heptacosane	29.695	2.988%
	=2,3,9,10-Tetracyanodibenzo (5,5a, 6:11,11a, 12)-1,4,7,10-tetraazanaphthacene	33.211	1.143%
	=5,5', 8,8'-Tetrahydroxy-3, 3'-dimethyl-2, 2'-binaphthalene-1, 1', 4,4'-tetrone	33.435	1.408%
	=28-Nor-17.alpha.(H)-hopane	33.637	0.433%
	=3-Indolethanamine, N-acetyl-5-fluoro-6-methoxy-	36.571	0.223%
	=4-(3H-Imidazo[4,5-b] pyridin-2-ylsulfanylmethyl)-6-=piperidin-1-yl-[1,3,5]triazin-2-ylamine 191.0	37.153	0.152%
	=Beta. -Iso-Methyl ionone	40.358	0.071%
	=Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	45.933	2.824%

3.8.3.6 Compounds from sample L13 fraction 8 and 11

From sample L13, two fractions were subjected to GC-MS analysis and the compounds identified are as indicated in table 19 and 20.

Table 19: Compounds identified fromL13, F8

Fraction	Compound name	Retention time	% of total
L13-B8	=Heptane	3.490	0.209%
	=Methyl- Cyclohexane	3.938	0.631%
	=Toluene	5.170	0.513%
	=1H-Pyrazole, 4,5-dihydro-3, 4,5-trimethyl-	5.483	0.103%
	=o-Xylene	8.081	0.047%
	=1,2,4-trimethyl- Benzene	10.859	0.027%
	=3-butyl- Pyridine	13.725	0.035%
	=Octanoic Acid	13.927	0.021%
	=Thiophene, tetra hydro-, 1,1-dioxide	14.487	0.450%
	=Indole	15.808	1.841%
	=n-Decanoic acid	16.727	0.594%
	=Vanillin	17.219	0.331%
	=Undecanoic acid	17.533	0.259%
	=Dimethyl phthalate	17.936	0.135%
	=But-2-enylidene-[1-methyl-1-(4-methyl-cyclohex-3-enyl)-ethyl]-amine	18.294	0.111%
	=6-hydroxy-2 (1H)-Pyridinone	18.474	0.194%
	=Butylated Hydroxytoluene	18.675	0.164%
	=4-Isopropenylcyclohexanone	19.011	1.208%
	=Dodecanoic acid	19.235	2.982%
	=Tridecanoic acid	19.952	1.558%
	=2-hydroxy- Cyclopentadecanone	20.221	0.057%
	=Tetradecanoic acid	21.027	0.631%
	=N-butyl- Benzene sulfonamide	21.788	0.749%
	=Pentadecanoic acid	22.214	4.305%
	=Bacchotricuneatin c	22.863	0.259%
	=Z-11- Hexadecenoic acid	23.423	21.864%
	=n-Hexadecanoic acid	23.603	21.323%
	=Isopropyl Palmitate	24.095	1.552%
	=Oleic Acid	25.215	2.609%
	=Tetracosane	27.365	0.391%
	=1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	28.597	1.717%
	=Heptacosane	2.672	0.857%
	=Tetracosane	30.478	0.900%
	=7H-1, 4-Dioxino [2,3-H] 1,4-benzodiazepin-7-one, 2,3,6,8-tetrahydro-10-ethyl-	30.859	6.109%
	=Eicosane	31.397	0.695%
	=2-methyl-4,6-diphenyl- Pyridine,	31.979	0.141%
	=4-octyl-N- (4-octylphenyl)- Benzenamine	32.113	0.192%
	=N, N-diphenyl- Benzenamine	32.315	3.382%
	=Naphth [2,3-b] azet-2 (1H)-one, 1-phenyl-	32.69	0.484%
	=1,3,5-Triazin-2-amine, 4-(2-furyl)-6-(1-piperidyl)-	39.271	2.036%
	=N- (1-pyrenyl)- Formamide	41.498	1.884%

Table 20: Compounds identified fromL13, F11

Fraction	Compound name	Retention time	% of total
L13-B11	=Isopropylcyclobutane	3.266	1.116%
	=Heptane	3.468	3.097%
	=Methyl- Cyclohexane	3.916	8.191%
	=Cycloheptane	4.184	2.197%
	=1,2,4-trimethyl- Cyclopentane	4.341	1.505%
	=1, 2, 3-trimethyl-, (1.alpha, 2.alpha, 3.beta.)-Cyclopentane	5.543	1.462%
	=Toluene	5.215	6.215%
	=1,3-dimethyl-, trans- Cyclohexane	5.506	7.996%
	=1,1-dimethyl- Cyclohexane	5.707	0.853%
	=1-ethyl-2-methyl-, cis- Cyclopentane	5.887	0.830%
	=Octane	6.245	4.026%
	=Ethyl- Cyclohexane	7.096	2.119%
	=1-isopropyl-5-methyl-2-Pyrazoline	7.589	0.186%
	=p-Xylene	8.082	2.652%
	=1-Ethyl-4-methylcyclohexane	8.507	0.438%
	=(1-methylethyl)- Cyclohexane	9.269	0.034%
	=Propyl- Cyclohexane	9.515	0.460%
	=3-ethyl-2-methyl- Heptane	9.784	0.614%
	=1,2,3-trimethyl- Benzene	10.859	0.345%
	=4-methyl- Decane	11.419	0.407%
	=Undecane	12.785	0.367%
	=1,2,4,5-tetramethyl- Benzene	13.076	0.170%
	=1-ethyl-4-(1-methylethyl)- Benzene	13.524	0.030%
	=2-methyl- Undecane	13.815	0.244%
	=Thiophene, tetrahydro-, 1,1-dioxide	14.487	0.342%
	=Hexyl- Cyclohexane	14.980	0.018%
	=2,6-dimethyl- Octane	15.428	0.043%
	=Indole	15.809	0.202%
	=N-phenyl- Formamide	16.100	0.031%
	=Triacetin	16.480	0.011%
	=2-methyl- Tridecane	16.682	0.033%
	=2,6,10,14-tetramethyl- Hexadecane	16.861	0.040%
	=Vanillin	17.242	0.583%
	=2,5-Cyclohexadiene-1, 4-dione, 2,6-bis (1,1-dimethylethyl)-	18.138	0.177%
	=Bacchotricuneatin c	18.429	0.473%
	=Butylated Hydroxytoluene	18.675	0.184%
	=Hexadecane	19.638	0.264%
	=2-(methylthio)- Benzothiazole	19.795	0.190%
	=Dodecanoic acid, 1-methylethyl ester	19.952	0.767%
	=Heptadecane	20.781	1.361%

Table 20 continued: Compounds identified fromL13, F11

Fraction	Compound name	Retention time	% of total
L13-B11	=8-methyl- Heptadecane	21.251	0.964%
	=Pyrrol [(4-chloroanilino)(imino) methyl] aminomorphomethanimidamide	22.393	0.387%
	=Dibutyl phthalate	22.617	0.576%
	=Hexadecanoic acid, methyl ester	23.155	0.568%
	=10-methyl- Eicosane	23.289	0.251%
	=1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	23.580	1.219%
	=3-[(2-Methyl-5-nitro-phenylimino)-methyl]-phenol	24.118	1.183%
	=2,6,10,15-tetramethyl- Heptadecane	25.171	0.460%
	=1-iodo- Hexadecane	25.730	0.873%
	=Hexacosane	26.604	3.701%
	=1,4'-Bipiperidine	27.612	2.370%
	=Octacosane	28.239	2.967%
	=Tetracosane	28.485	0.339%
	=Heptacosane	29.740	3.271%
	=O-ethyl- Atheroline	30.299	0.263%
	=11-decyl- Heneicosane	30.546	3.033%
	=2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	30.837	0.682%
	=Nonacosane	31.487	2.647%
	=1-Hexacosene	31.823	0.340%
	=Triacontane	32.584	2.103%
	=2-methyl- Azetidine	33.502	0.026%
	=Dotriacontane	35.518	1.273%
	=1-bromo- Triacontane	42.506	0.504%
	=Hexatriacontane	45.597	0.305%

3.8.3.7 Compounds from sample S33 fraction 3 and 11

From sample S33, two fractions were subjected to GC-MS analyses and the compounds identified are as indicated in table 21 and 22.

Table 21: Compounds identified fromS33, F3

Fraction	Compound name	Retention time	% of total
S33-B3	=Heptane	3.467	0.290%
	=Methyl- Cyclohexane	3.915	0.946%
	=1,2,4-trimethyl- Cyclopentane	4.341	0.020%
	=2-Octanol	4.543	0.162%
	=Toluene	5.170	0.187%
	=1,3-dimethyl-, cis- Cyclohexane	5.461	0.049%
	=1,2-dimethyl-, trans- Cyclohexane	6.043	0.012%
	=Benzo [h] quinoline, 2,4-dimethyl-	7.006	0.005%
	=Ethyl benzene	7.880	0.003%
	=p-Xylene	8.081	0.011%
	=Undecane	12.785	0.019%
	=N,N-dimethyl- Octanamide	13.121	0.135%
	=2,5-Pyrrolidinedione	13.479	0.027%
	=Decamethyl- Cyclopentasiloxane	13.681	0.007%
	=2,4-dimethyl- Oxazole	13.994	0.267%
	=Dodecane	14.375	0.035%
	=Benzene acetic acid	15.428	7.219%
	=Tridecane	15.831	0.086%
	=Tetradecane	17.175	0.152%
	=4-hydroxy- Benzene ethanol	17.578	0.182%
	=Decane, 5,6-bis (2,2-dimethylpropylidene)-, (E, Z)-	17.824	0.027%
	=Eicosane	17.958	0.046%
	=Tetra ethylene glycol monododecyl ether	18.541	0.021%
	=2,4-Imidazolidinedione, 5-(2-methylpropyl)-(S)-	19.011	0.133%
	=Sulfur	19.190	0.111%
	=9-Octadecene, (E)-	19.549	0.139%
	=Diethyl Phthalate	19.638	0.536%
	=Benzene acetamide	20.333	0.185%
	=2(3H)-Benzothiazolone	20.467	0.133%
	=2-pentyl- Thiophene,	20.601	0.069%
	=3,8-dimethyl- Decane	21.452	0.219%
	=2,5-dinitro- Benzoic acid	21.945	0.474%
	=1H-Indole-3-carboxaldehyde	22.057	0.646%
	=n-Hexadecanoic acid	23.513	0.834%
	=N,N-Dimethyldecanamide	26.201	1.256%
	=Bis (dimethylthiocarbamyl) sulfide	27.567	0.367%
	=Thiophene, 2-pentyl-	28.284	0.678%
	=Zinc, bis (dimethylcarbamodithioato-S, S')-, (T-4)-	31.038	2.467%

Table 22: Compounds identified fromS33, F11

Fraction	Compound name	Retention time	% of total
S33-B11	=1,2-dimethyl- Cyclopentane	3.288	0.013%
	=1,2,4-trimethyl- Cyclopentane	4.341	0.011%
	=Piperidine	4.901	1.777%
	=Toluene	5.170	0.297%
	=1,3-dimethyl-, cis- Cyclohexane	5.461	0.032%
	=p-Xylene	8.081	0.012%
	=N-butyl-1-Butanamine	10.254	6.170%
	=Benzyl Alcohol	11.665	0.090%
	=Benzene methanol, alpha, alpha.-dimethyl-	12.561	0.020%
	=Phenyl ethyl Alcohol	13.054	0.439%
	=Isothiocyanato- Cyclohexane,	14.980	4.042%
	=Indole	15.808	0.043%
	=N-phenyl- Formamide	16.144	0.172%
	=Benzenamine, N, N, 3,5-tetramethyl-	16.906	0.345%
	=Flucytosine	17.175	0.053%
	=3-Methyl-4-phenyl-1H-pyrazol-5-amine	18.765	0.290%
	=Diethyl Phthalate	19.638	0.304%
	=1,1'-carbonylbis- Piperidine,	20.310	0.023%
	=1H-Indole-2,3-dione	20.915	0.310%
	=1,2,4-Triazol-4-amine, N- (2-thienylmethyl)-	20.960	10.265%
	=Tetradecanoic acid	21.475	0.365%
	=Pentaethylene glycol	21.744	1.157%
	=Lactose	22.012	0.395%
	=Pentadecanoic acid	22.505	0.115%
	=1-Piperidinecarboxaldehyde, 2-(1-formyl-2-pyrrolidinyl)-	22.796	1.484%
	=7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6, 9-diene-2, 8-dione	23.177	0.446%
	=Ethanamine, N, N-diethyl-2- [2-(2-methoxyethoxy) ethoxy]-	23.401	1.256%
	=n-Hexadecanoic acid	23.647	13.342%
	=N,N'-Trimethylenebis[s-3-aminopropylthiosulfuric acid]	24.521	14.556%
	=Hexagol	24.700	1.457%
	=Octadecanoic acid, methyl ester	25.058	0.154%
	=7-Hydroxy-4-trifluoromethylcoumarin	25.148	0.082%
	=Octadec-9-enoic acid	25.282	2.390%
	=Octadecanoic acid	25.439	1.218%
	=Octadecanoic acid, ethyl ester	25.663	0.280%
	=2-Benzothiazolamine, N-cyclohexyl-	25.798	0.137%
	=18-Nonadecenoic acid	26.268	4.776%

Table 22 continued: Compounds identified fromS33, F11

Fraction	Compound name	Retention time	% of total
S33-B11	=2(3H)-Furanone, dihydro-5-tetradecyl-	26.761	0.073%
	=Sulfurous acid, cyclohexylmethyl ethyl ester	28.351	0.143%
	=4-ethyl-2-propyl- Thiazole	29.180	1.057%
	=Octadecane	29.672	0.157%
	=2,6-Difluorobenzoic acid, 4-chlorophenyl ester	29.874	1.939%
	=5-Methylthiopyridin-2-ol	30.680	0.301%
	=Pyrrolidine, 1-(7-oxo-2, 4,6 trimethylheptanoyl)-	30.904	1.000%
	=5-Acetoxy-3-(3,4-diacetoxyphenyl)-7-methoxy-4H-chromen-4-one	31.106	0.062%
	=Dicyclohexano-24-crown-8	31.464	0.313%
	=Propenone, 1-(benzo [b]-1,4-dioxane -6-yl)-3-(4-fluorophenyl)-	31.800	1.678%
	=7-Amino-2-trifluoromethylphenothiazine	32.920	0.370%
	=1,4-phenylenebis [trimethyl- Silane	34.868	0.284%
	=Hexamethyl- Cyclotrisiloxane	35.451	0.061%
	=S22, 23-dihydro-stigmasterol	38.116	0.05%
	=di-n-Butyldithiocarbamic acid, zinc salt	42.327	14.568%
	=Zinc, bis (1-piperidinecarbodithioato-S, S')-, (T-4)-	44.365	0.619%

3.8.3.8 Compounds from sample B41 fraction 7 and 9

From sample B41, two fractions were subjected to GC-MS analyses and the compounds identified as indicated in table 23 and 24.

Table 23: Compounds identified from B41, F7

Fraction	Compound name	Retention time	% of total
B41-B7	=1,2-dimethyl- Cyclopentane	3.311	8.696%
	=Methyl- Cyclohexane	3.960	33.258%
	=Ethyl- Cyclopentane	4.207	1.949%
	=Toluene	5.192	7.663%
	=1,3-dimethyl-, cis- Cyclohexane,	5.483	3.015%
	=1,2-dimethyl- Cyclohexane,	6.043	0.371%
	=Ethyl- Cyclohexane,	7.096	0.213%
	=p-Xylene	8.081	0.168
	=N-sec-Butyl-n-propylamine	10.232	0.016%
	=2-(2-Chloroacetamido)-5-methyl-1,3,4-thiadiazole	15.450	0.037%
	=Indolizine	15.808	0.152%
	=N-phenyl- Formamide,	16.100	0.859%
	=N-phenyl- Benzamide	17.197	0.052%
	=Nonanoic acid, 9-oxo-, methyl ester	17.623	0.043%
	=4'-hydroxy- Acetophenone	17.712	0.107%
	=2H-Indol-2-one, 1,3-dihydro-	18.138	0.057%
	=2-Acetyl-3-methylthiophene	18.877	0.213%
	=3-(2-Phenylethyl) pyridazine	19.302	0.379%
	=Diethyl Phthalate	19.638	0.295%
	=Tributyl phosphate	20.243	0.443%
	=1H-Indole-2, 3-dione	20.848	0.062%
	=Oxalic acid, cyclohexylmethyl undecyl ester	20.915	0.607%
	=Phthalic acid, 6-ethyl-3-octyl isobutyl ester	22.617	0.174%
	=Tetradecane	22.864	0.299%
	=Hexadecanoic acid, methyl ester	23.132	0.454%
	=n-Hexadecanoic acid	23.468	1.051%
	=Isopropyl Palmitate	24.095	5.195%
	=Cyclic octaatomic sulfur	24.476	0.450%
	=2,6,10,14-tetramethyl- Hexadecane	24.790	0.818%
	=Octadecanoic acid	25.350	0.448%
	=Docosane	25.686	0.530%
	=Tetracosane	27.365	1.064%
	=1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	28.597	3.570%
	=Octadecane	28.933	0.786%
	=Bacchotricuneatin c	29.672	1.141%
	=Squalene	30.792	0.457%
	=Nonacosane	31.397	0.725%
	=Heneicosane	32.494	0.328%

Table 24: Compounds identified from B41, F9

Fraction	Compound name	Retention time	% of total
B41-B9	=Isopropylcyclobutane	3.288	2.181%
	=Heptane	3.467	3.272%
	=Methyl- Cyclohexane	3.915	8.809%
	=1,2,4-trimethyl- Cyclopentane	4.341	0.415%
	=1, 2, 3-trimethyl-, (1.alpha, 2.alpha, 3.beta.)- Cyclopentane	4.543	0.333%
	=Toluene	5.170	2.047%
	=3-methyl- Heptane	5.371	0.392%
	=Cyclooctane	5.886	0.121%
	=1,3-dimethyl-, trans- Cyclohexane	6.043	0.232%
	=Octane	6.200	0.459%
	=Ethyl- Cyclohexane	7.073	0.160%
	=4-Amino-6-hydroxypyrimidine	7.185	0.079%
	=p-Xylene	8.081	0.089%
	=Nonane	8.865	0.012%
	=1,2-Dipentylcyclopropene	13.994	0.053%
	=3,5-dimethyl- Benzenamine	14.375	0.035%
	=N-phenyl- Formamide	14.666	0.042%
	=Indole	15.808	0.064%
	=2,4,6-trimethyl- Benzenamine	15.920	0.044%
	=N-(.alpha.-methyl-4-methoxymethylbenzylidene)- Methanamine	16.906	0.187%
	=3-ethyl- Quinoline	18.026	0.022%
	=2,3-dihydro-4-methyl-1H-Indole	18.138	0.108%
	=5,6,7,7a-tetrahydro-4, 4,7a-trimethyl-2(4H)-Benzofuranone	18.966	0.059%
	=Dodecanoic acid	19.190	0.430%
	=Diethyl Phthalate	19.638	0.171%
	=Tridecanoic acid	20.019	0.658%
	=5,6-dimethyl-2-Benzothiazolamine	20.333	0.052%
	=2(3H)-Benzothiazolone	20.489	0.255%
	=Oxalic acid, cyclohexylmethyl tetradecyl ester	20.915	0.817%
	=Pentadecanoic acid	22.147	4.291%
	=7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6, 9-diene-2, 8-dione	23.177	0.566%
	=Z-11- Hexadecenoic acid	23.379	6.734%
	=n-Hexadecanoic acid	23.670	37.861%
	=Oleic Acid	24.386	1.240%
	=Eicosane	24.790	0.273%
	=Octadec-9-enoic acid	25.282	8.095%
	=Octadecanoic acid	25.417	1.759%
	=Heptadecane	25.685	0.325%
	=9-octyl- Heptadecane	27.365	0.484%
	=Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	27.656	0.397%
	=Octadecane	28.3172	0.566%
	=1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	28.597	0.663%
	=Heneicosane	28.933	0.580%
	=Heptacosane	29.672	0.578%
	=Tetracosane	30.478	0.535%
	=Octadecane	32.494	0.278%
	=Estra-1, 3,5(10), 15-tetraen-17-one, 3-methoxy-	32.875	0.615%
	=Tetracosane	33.838	0.181%
	=Octacosane	35.451	0.138%

3.8.3.9 Compounds from sample DM 30 fraction 11

From sample DM30 fraction 11, several compounds were identified (Table 25).

Table 25: Compounds identified from DM30, F11

Fraction	Compound name	Retention time	% of total
DM30-B11	=Heptane	3.445	1.169
	=Methyl-Cyclohexane	3.901	5.709
	=Ethyl- Cyclohexane	4.162	0.232
	=Piperidine	4.901	1.543
	=Toluene	5.147	1.514
	=3-ethyl-thiophene	5.439	0.271
	=p-Xylene	8.059	0.061
	=N-sec-butyl-n-propylamine	10.187	4.991
	=Isothiocyanato-Cyclohexane	14.957	8.552
	=Indole	15.786	0.123
	=N-phenyl-formamide	16.100	0.219
	=6-methyl-2-pyridinecarbaldehyde	17.309	0.242
	=2(1H)-Quinolinone, 4,6-dimethyl-	18.765	0.089
	=Diethyl phthalate	19.638	0.554
	=Cyclohexylmethyl -tridecyl ester Oxalic acid	20.915	9.378
	=Pentaethylene glycol	21.721	1.246
	=7H-Purine, 7-methyl-6- (methylthio)-	21.945	0.868
	=Hexadecanoic acid, methyl ester	23.132	1.092
	=N-butyl-1-butanamine	23.379	1.254
	=Isopropyl Palmitate	24.095	0.872
	=Benzo [1,2,5] oxadiazole-4, 6-diamine, 5,7-dinitro-1-oxy-	24.476	1.210
	=Hexagol	24.566	2.410
	=Chrysene, 1,2,3,4,4a, 7,8,9,10,11,12,12a-dodecahydro-6-octyl-	28.194	2.921
	=Heptacosane	28.933	2.424
	=Heptaethylene glycol	29.992	1.561
	=Tetracosane	29.695	2.170
	=N-Acetyl-4-fluoro-.alpha.-carboethoxyhistidine ethyl ester	29.896	1.561
	=Octacosane	30.479	1.596
	=2,4(1H, 3H)-Pyrimidinedione, 5-amino-	30.904	0.590
	=Nonacosane	31.419	1.068
	=2,6-Difluorobenzoic acid, 2-naphthyl ester	31.778	0.370
	=Heptadecane	32.517	0.432
	=di-n-Butyldithiocarbamic acid, zinc salt	42.170	10.269
	=Zinc, bis (1-piperidinecarbodithioato-S, S'0)-, (T-4)-	44.320	2.935

3.8.3.10 Compounds from sample DR 35 fraction 8

From sample DR35 fraction 11, several compounds were identified (Table 26).

Table 26: Compounds identified from DR35, F8

Fraction	Compound name	Retention time	% of total
DR35-B8	=3-Penten-2-ol	3.288	1.499%
	=Heptane	3.445	1.226%
	=Toluene	5.170	0.407%
	=Tetradecanoic acid	21.430	2.080%
	=Z-11- Hexadecenoic acid	23.334	23.407%
	=n-Hexadecanoic acid	23.513	60.773%
	=Z-7-Pentadecenol	24.513	3.531%
	=Oleic Acid	25.215	2.416%
	=Bendazol	45.104	2.213%

From the GC-MS analysis of all the samples, the constituent compounds were a mixture of alkanes, their derivatives with functional groups, carbonyls and esters. It would be of interest to find out which functional group is responsible for the bioactivity. This will aid in gaining insight of synergism among the different functional groups. Perhaps if the functional groups are separated, efficacy would decrease and likewise when these functional groups are in a mix, there is synergy that enhances activity. Future research will provide an answer to these questions.

Endophytes colonize the inside of the plant tissues, and usually get nutrition and protection from the host plant. In return they confer enhanced fitness to the host plant by producing certain functional secondary metabolites (Strobel, 2003). Toluene, which has frequently been obtained from both rhizosphere and endophytic bacteria (Safigh *et al*., 2005), was observed in most isolates in this study and has been of use in the pharmaceutical industry. According to a study carried out by Rogers and Heslop (1948),

on the inhibitory action of toluene on coliform bacilli and routine cultures, they found out that toluene appeared to inhibit the growth of pathogenic organisms (Gram negative or Gram positive) if it is left long enough in contact with them, the inhibiting action being accelerated by increased temperature.

Different classes of secondary metabolites were obtained. Identified amines included: 4-pyridinamine, N-butyl-1-butanamine, 1-Dibenzofuranamine, N-ethyl-2-benzothiazolamine, benzeneacetamide and Piperidine. Amides obtained were: N, N-Dibutyl-formamide, N-buyl-benzesulfonamide, Benzeneacetamide and N-phenyl-formamide. Isolated acids included 9-oxo-methyyl ester Nonanoic acid, Tetradecanoic acid, oleic acids and Octadecanoic acid. Pyrrolizidines included, 1-(7-oxo-2, 4, 6-trimethylheptanoyl)-Pyrrolidine, 1-isopropyl-5-metyl-2-pyrazoline, 2 and 5-dimetyl-1-pyrroline. Indole derivatives included, indole, Indolizine, 2, 3-dihydro-4-methyl-1H-indole and 1H-indole-2, 3-Dione. Quinones included, Benzophenone, Beta –iso-methyl ionone, Acetophenone, 1-(3-hydrohy-3-phenyl-1-triazenyl)-antra-9, 10-quinone, Lanost-8-en-11-one1, 4-diamino-2-(hydroxymethyl) anthraquinone and 2(3H)-Benzothiazolone. Steroids included, 1, 1-diphenyl-(Z)-1, 3-pentadiene and S22-23-dihydro-stigmasterol. All these compounds detected from endophytic and rhizosphere bacteria have been documented by studies carried out by Hua *et al*. (2006) and Tan *et al.* (2001). Apart from the common or documented secondary metabolites obtained from endophytic and rhizosphere bacteria, other unique groups of compounds were detected such as alcohols which included: 3-peten-2-ol, 4-methyl-dodec-3-en-1-ol, 2-actanol and Z-7-pentadcenol. Hydrocarbons included methylcyclohexane, Nonacosane, heneicosane, Docosane, Tetradecane and hexatriacontane. Azoles which are known to

be good antifungal agents (Mohr *et al.,* 2008) were also identified and they do inhibit the enzyme 14-alpha-demethylase which produces ergosterol an important component of the fungal plasma membrane (Andes *et al.,* 2008). Some of them included 5-t-Butyl-4-methyllimidazole, 2-(methylthio)-Benzothiazole, 4, 5-dihydro-3, 4, 5-trimethyl-1 H pyrazole and 2, 4-dimethyl-oxazole. Goitrin was also identified, which has been used in the treatment of goitre. Vanillin was detected and it has been used as a chemical intermediate in the production of pharmaceuticals (Hocking *et al*., 1997).

CHAPTER FOUR

CONCLUSIONS

The study has demonstrated that indigenous Kenyan plants around Juja are a host of various bacteria endophytes and rhizosphere bacteria.

A total of fourty eight (48) bacterial isolates were obtained, that had different morphological and biochemical characteristics. Molecular characterization to determine the final identity was not done for all the bacterial isolates except for the isolate (DM 30), which showed that the isolate was a *Bacillus subtilis*. The study has also demonstrated that, endophytes and rhizosphere bacteria from indigenous plants are a potential source of antimicrobial compounds, since they produce secondary metabolites that are active against spoilage and pathogenic bacteria and yeast strains. The antimicrobial screening results indicate that some isolate exhibited better antimicrobial activity compared to the drugs Tetracycline and Gentamycin. The isolates included; C19 effective against *S. aureus*, C25M effective against *P. aeruginosa*, C15 effective against *B. subtilis*, C17 effective against *E. coli* and C15 effective against *C. albicans*. Hence, these isolates' can be further used to develop effective antimicrobial agents.

In addition, this study shows that the crude extracts obtained from isolate H11 had a significant antagonistic activity against *C. albicans* as compared to Tetracycline. From all the crude extracts antimicrobial activity, it is a clear indication that they can be used against spoilage and pathogenic bacterial and fungal strains and can be used to develop

therapeutic agents of treatment, despite the different levels of antagonistic activity of the crude extracts compared to Tetracycline and Gentamycin.

The chromatographic separation indicated that, different number of fractions/ compounds were present in the crude extracts that differed from one sample to another. From the fractions antimicrobial assay, L13 fraction 11 and S33 fraction 3 were the most active against *S. aureus*, L13 fraction 11 was the most active against *P. aeruginosa* and *B. subtilis* while L13 fraction 8 was the most active against *C. albicans*. The antimicrobial activity of the fractions may be similar to Tetracycline and Gentamycin but this still makes them promising in the development of therapeutic agents since they may have different modes of activity/action and concentrations. Different classes of compounds were obtained from the bioactive fractions by GC-MS, ranging from azoles, alkaloids, phenolic acids etc.

Bacterial endophytes and rhizosphere bacteria have been documented to produce various classes of secondary metabolites that have antimicrobial properties that differ from one isolate to another. From this study it's clear that the antagonistic bacteria endophytes and rhizosphere bacteria were successfully isolated and exhibited different levels of antimicrobial activity against the pathogenic test organisms due to the production of the active secondary metabolites with different levels of antagonistic activity. Therefore endophytes and rhizosphere bacteria isolated from the indigenous plants around Juja are a potential source of antimicrobial compounds and hence form a great foundation to the development of therapeutic agents that can be used once resistance builds up.

CHAPTER FIVE

RECOMMENDATIONS

Endophytic bacteria and rhizobacteria are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural and industrial arenas, I recommend that more research should be carried out to exploit these untapped promising groups of microorganisms. In addition, mechanisms through which endophytes exist and respond to their surrounding must be understood in order to be more predictive about which higher plant(s) to seek, study and spend time isolating micro floral components. This may facilitate the product discovery process.

A range of culture media should be used in order to capture missed isolates in the initial isolation process, since only nutrient agar and Tryptic soy agar were used in this study. Consequently, different culture media and different cultural conditions (e.g. Temperature, pH etc) should be used to optimize the production of compounds and/or metabolites with potential antimicrobial activity.

In order to gain more insights into the bioactivity from endophytes and rhizosphere bacteria, all isolates obtained showing antagonistic effects against any test organisms need to be investigated further such as isolates C19, C15, G20 B40 etc.

Further molecular work should be carried on the remaining nine isolates in order to characterize them, to fully determine their identity since their crude products were identified.

The isolated metabolites should be further tested for their antagonistic activity against other pathogenic microorganisms of great medical importance especially those showing multi-drug resistance such as *Mycobacterium tuberculosis*, *Salmonella typhi*, *Vibrio cholera*, *Shigella* , Methicillin resistant *Staphylococcus aureus* etc. This will be important in the discovery of broad spectrum therapeutic agents, against these pathogens which are becoming increasingly resistant to existing antimicrobials.

The rest of the isolates, whose crude products were not extracted and had showed antagonistic activity against one or more test organisms, should be followed up as they could have potential in the development of therapeutic agents and could also be effective against one or several dangerous pathogens.

Tracking the compounds respective activity should be done, in order to find out which compound was responsible for the bioactivity i.e. the active ingredient(s) and whether they have a novel mode of action, as this could lead to the development of therapeutic agents against harmful pathogens.

I suggest that, further work should be carried out to optimize the effective doses and blends of the active compounds. It would also be important to determine toxicity of the active compounds with a view of developing commercial products.

Different commercial drugs other than Tetracycline and Gentamycin that have been used in the eradication of the test organisms used in this study should be incorporated

as positive controls during further research, inorder to determine their levels of antagonistic activity compared with that of the isolates' crude extracts. This will help determine the level effectiveness of the bacterial crude extracts compared to the commercial drugs and vice versa.

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7.0 APPENDICES

1. Table of biochemical tests of all the antagonistic isolates

Isolate	**Triple sugar iron**				**Sulphur Indole Motility**								
	Butt	Slant	H_2S	Gas	sulphur	indole	motility	SC	MR	VP	Urea	gelatin	catalase
C25Y	+	-	-	-	-	-	-	-	-	-	-	-	+
C25M	+	-	-	+	-	-	+	-	-	-	-	-	+
C15	+	-	-	+	-	+	+	-	-	-	+	+	+
C17	+	-	-	+	-	+	+	-	-	-	+	+	+
C16	+	-	+	+	-	+	+	-	-	+	+	+	+
C24	+	-	-	+	-	+	+	-	-	+	+	+	+
C18	+	-	-	+	-	-	+	-	-	-	-	+	+
C19	+	-	-	+	-	-	+	-	-	-	+	+	+
G8T	+	-	-	+	-	-	+	-	-	-	-	-	+
G8M	+	-	-	+	-	-	+	-	-	+	-	-	+
G20	+	-	-	+	-	-	+	-	-	+	-	-	+
G20C	+	-	-	+	-	-	+	-	-	-	-	-	+
G43W	-	+	-	-	-	-	-	-	-	-	-	-	+
G9	+	-	-	+	-	-	+	-	-	-	+	-	+
DC4	+	+	-	-	-	-	+	-	-	-	-	-	+
DC1	-	-	-	-	-	-	-	-	-	-	+	-	+
DC2	-	-	-	-	-	-	-	-	-	-	-	+	+

DC3	+	-	-	-	-	-	-	-	-	-	-	-	+
J23	+	-	-	+	-	-	+	-	-	+	-	-	+
H11	+	-	-	+	-	-	+	-	-	-	-	-	+
H10	+	-	-	+	-	-	-	-	-	-	+	-	+
L12	+	-	-	+	-	-	+	-	-	-	+	+	+
L13	+	-	-	+	-	-	+	-	-	+	+	-	+
L14	-	-	-	-	-	-	-	-	-	+	-	+	+
S27	-	-	-	-	-	-	-	-	-	-	-	-	+
S28	-	-	-	-	-	-	-	-	-	-	-	+	+
S33	-	-	-	-	-	-	-	-	-	-	-	+	+
S34	-	-	-	-	-	-	-	-	+	-	-	-	+
S42L	+	-	-	-	-	-	+	-	-	+	+	-	+
S42T	-	-	-	-	-	-	-	-	-	-	-	-	+
B39	-	-	-	-	-	-	-	-	-	-	-	-	+
B40	+	+	-	-	-	-	-	-	+	-	-	-	+
B41	+	+	-	-	-	-	+	-	+	-	-	+	+
DM30	+	+	-	-	-	-	+	-	-	+	-	+	+
DR31	+	+	-	+	-	-	+	-	+	-	+	-	+
DR35	+	+	-	-	-	-	-	-	+	-	-	-	+
DR36	+	+	-	-	-	-	+	-	+	-	-	-	+
DR37	+	-	-	-	-	+	+	-	+	-	+	-	+

KEY:

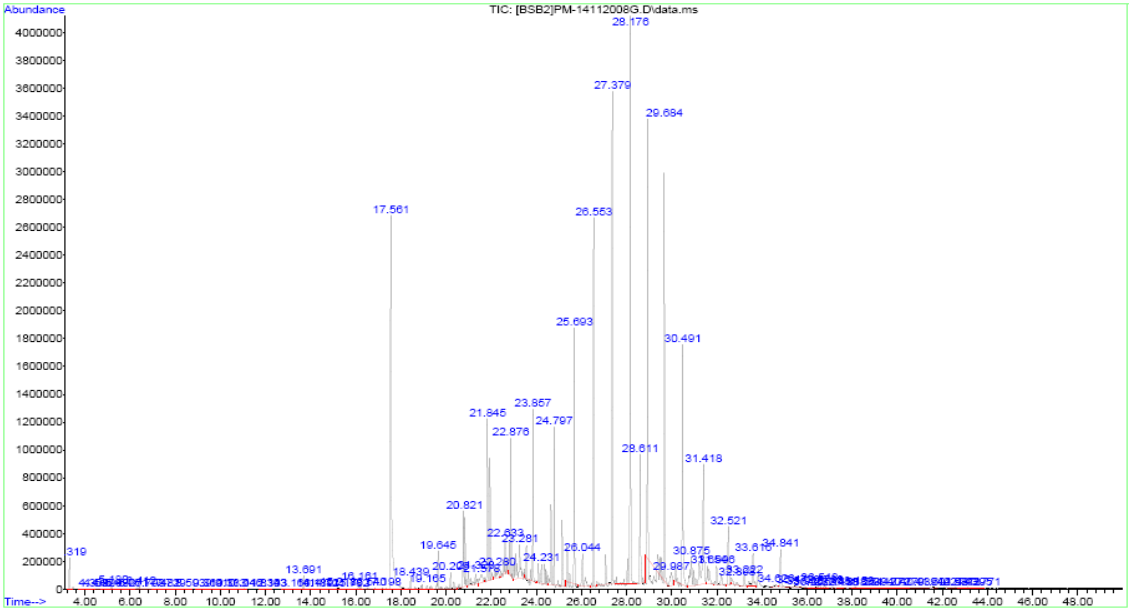
+ A positive result for the reaction, - A negative test for the reaction

2. Table of gram reaction and colony description

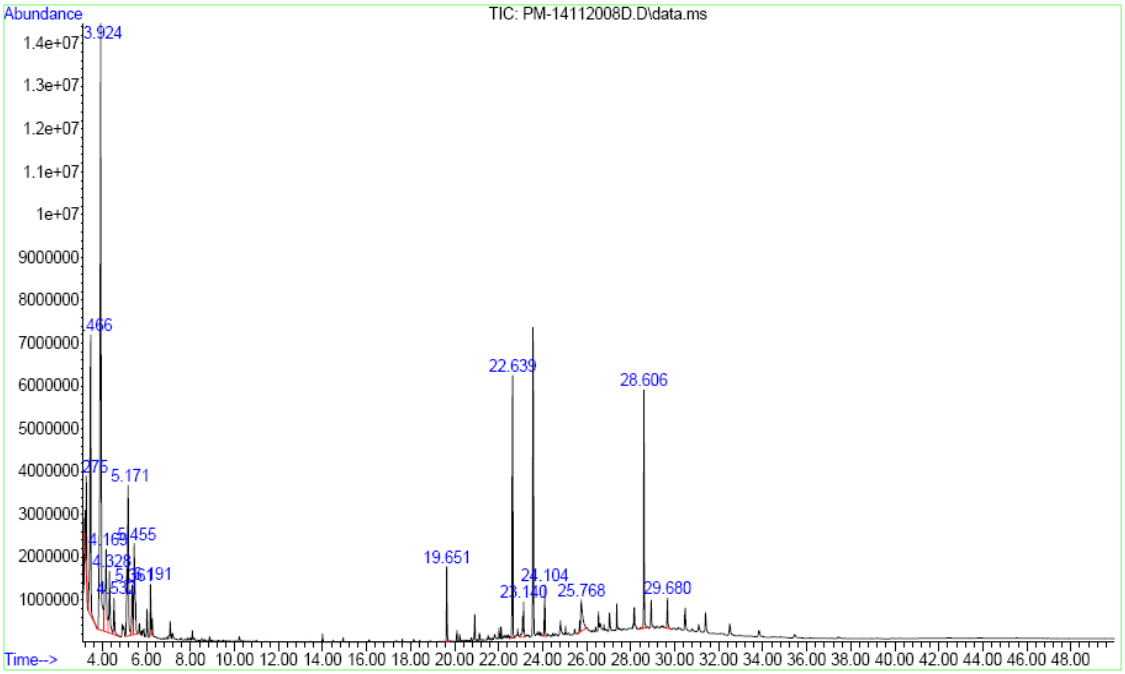
Isolate code	Gram reaction	Colony description	Isolate code	Gram reaction	Cell morphology &arrangement	Isolate code	Gram reaction	Cell morphology & arrangement
C25Y	negative	rods	**G9**	positive	cocci (clusters)	**S33**	positive	rods
C25M	negative	rods	**DC4**	positive	rods	**S34**	positive	chains
C15	negative	rods	**DC1**	positive	rods	**S42L**	negative	rods
C17	negative	rods	**DC2**	positive	chains	**S42T**	negative	rods
C16	negative	rods	**DC3**	negative	rods	**B39**	positive	chains
C24	negative	rods	**J23**	negative	rods	**B40**	positive	chains
C18	negative	rods	**H11**	positive	rods	**B41**	positive	chains
C19	negative	rods	**H10**	positive	rods	**DM30**	positive	rods
G8T	negative	rods	**L12**	positive	cocci	**DR31**	positive	rods spindle shaped
G8M	negative	rods	**L13**	positive	rods	**DR35**	positive	rods
G20	positive	cocci	**L14**	negative	rods	**DR36**	positive	rods spindle shaped
G20C	positive	rods	**S27**	negative	rods	**DR37**	positive	rods
G43W	positive	cocci(clusters)	**S28**	negative	rods			

3 GC-MS profiles of the identified compounds present in the different sample fractions

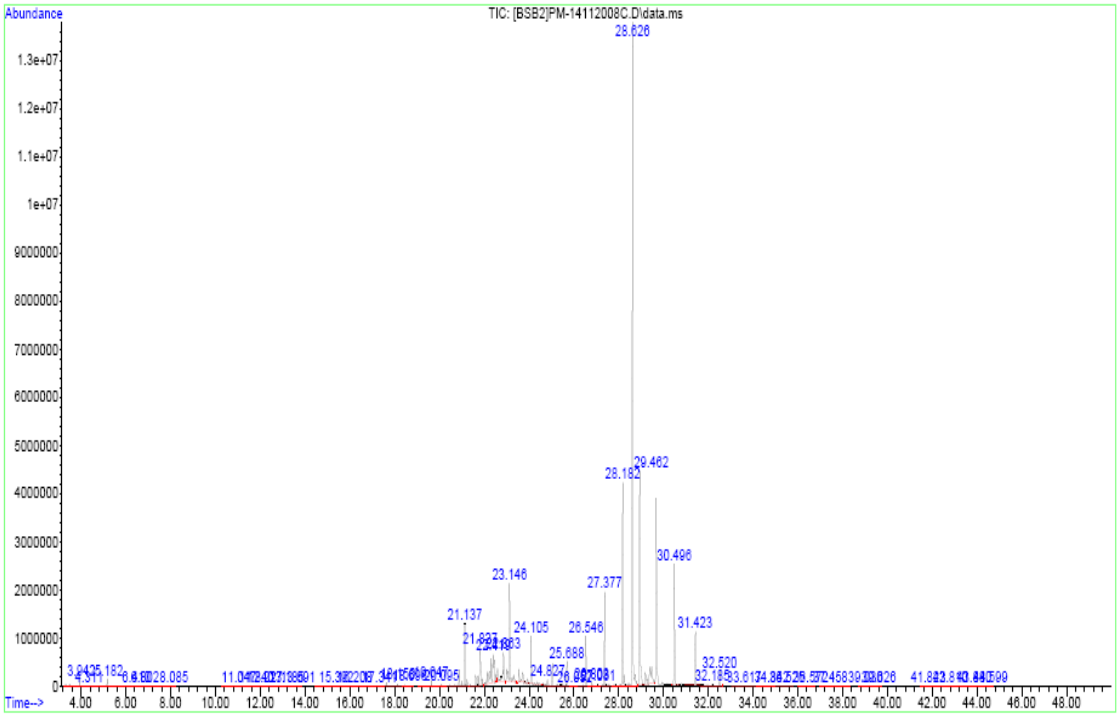
3.1 GC –MS profile of C25M, F 4



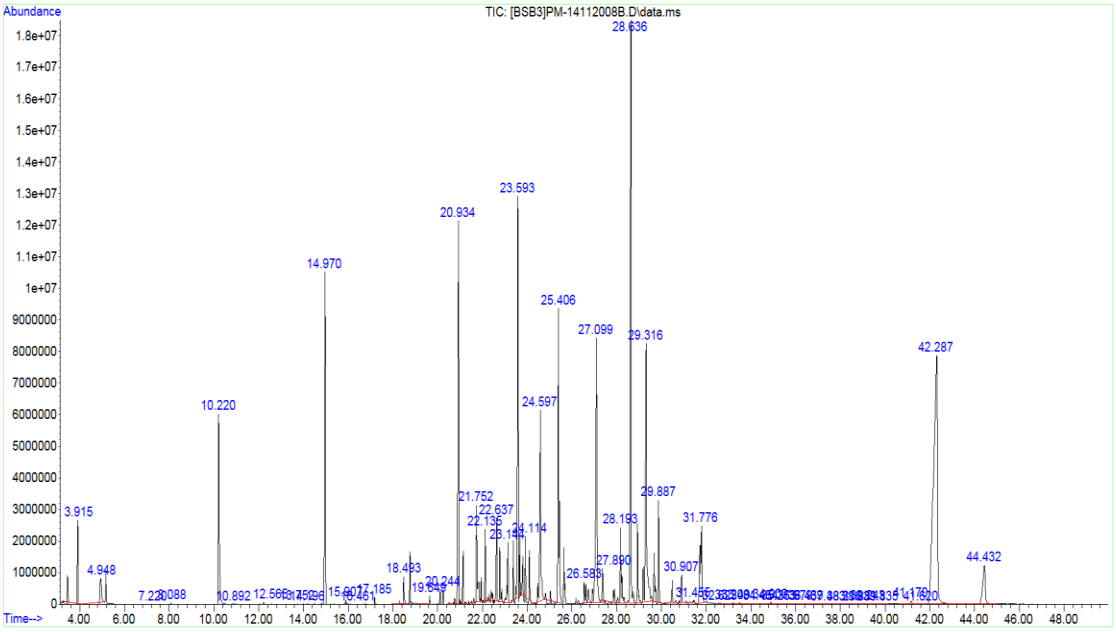
3.2 GC-MS profile of G20C, F12



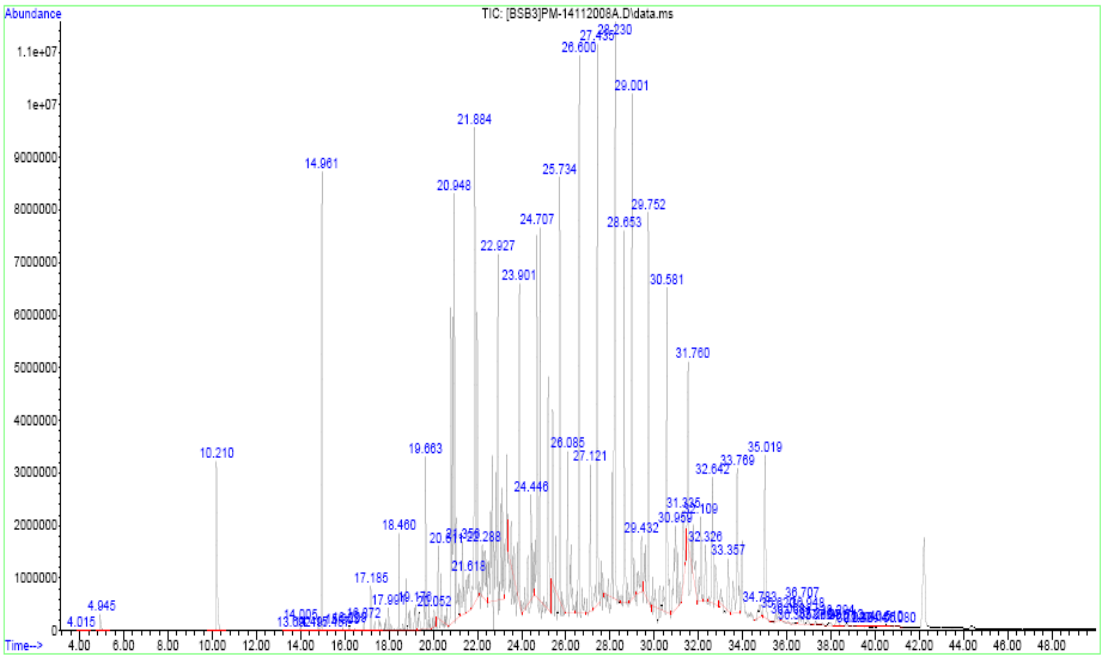
3.3 GC-MS profile of G20C, F13



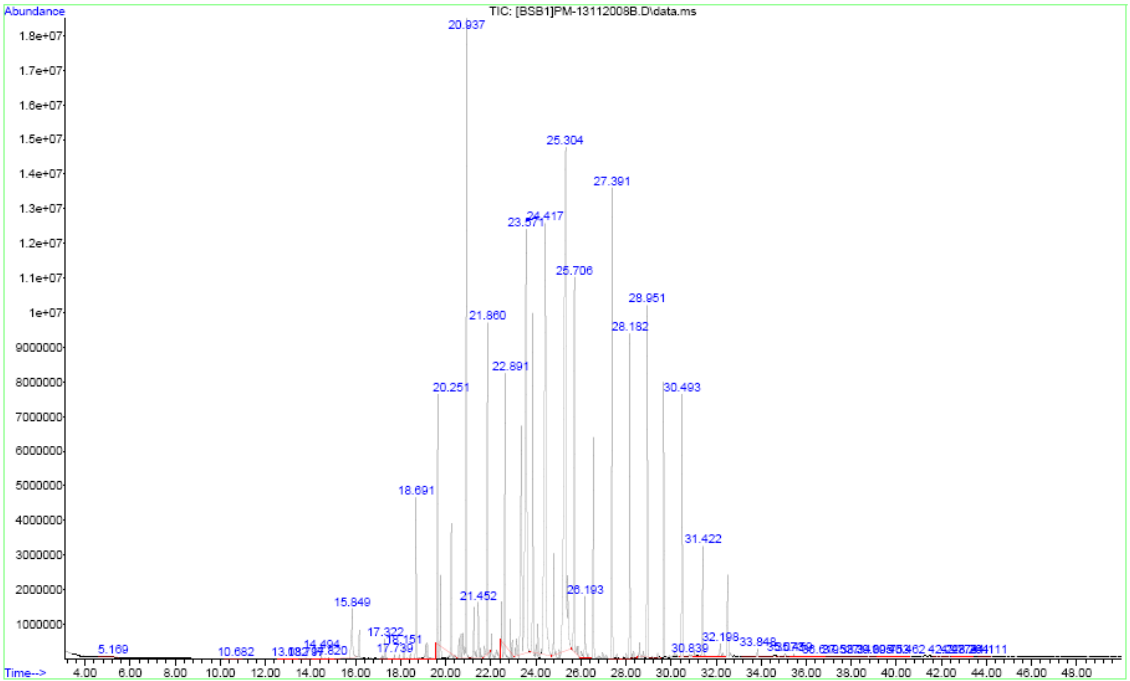
3.4 GC-MS profile of G43W, F8



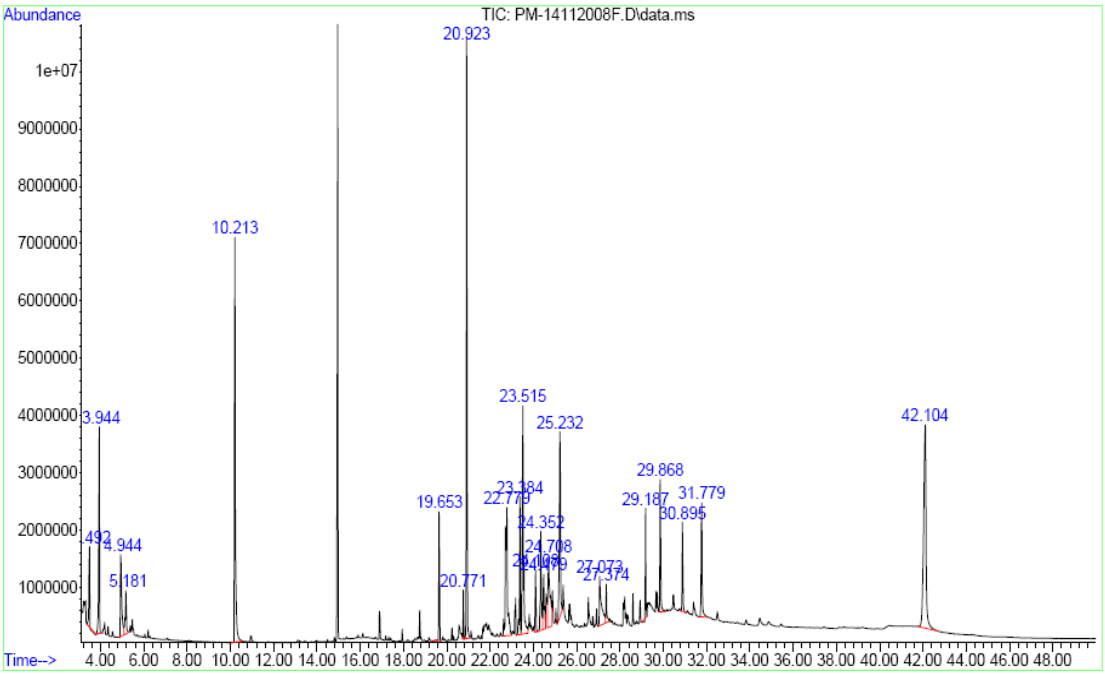
3.5 GC –MS profile of DC1, F5



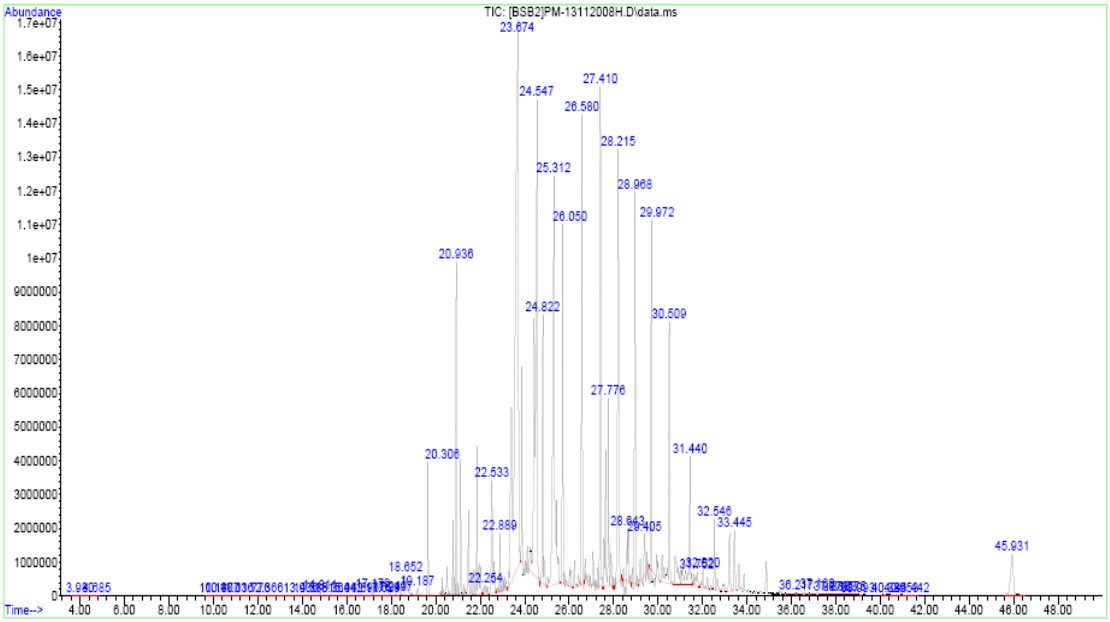
3.6 GC-MS profile of DC1, F10



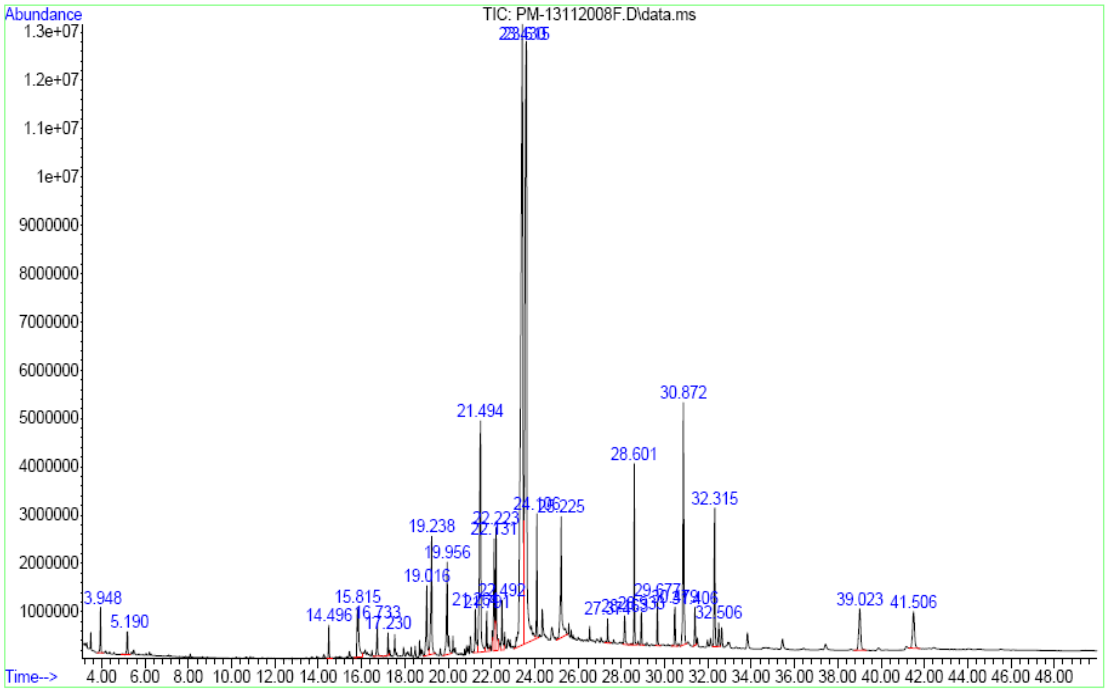
3.7 GC-MS profile of H11, F6



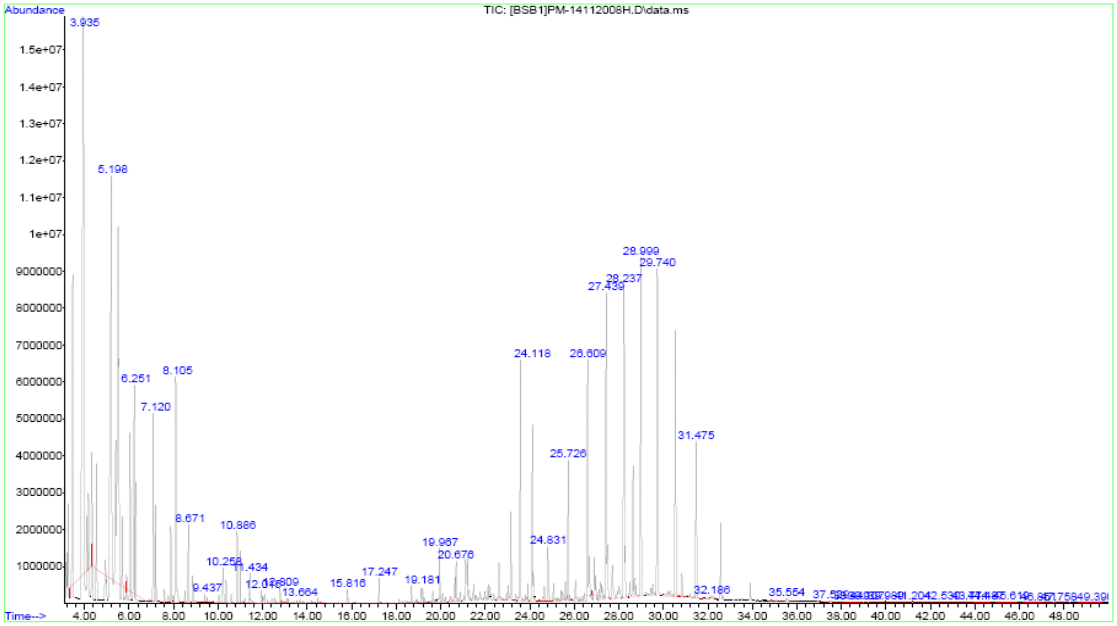
3.8 GC –MS profile of H11, F7



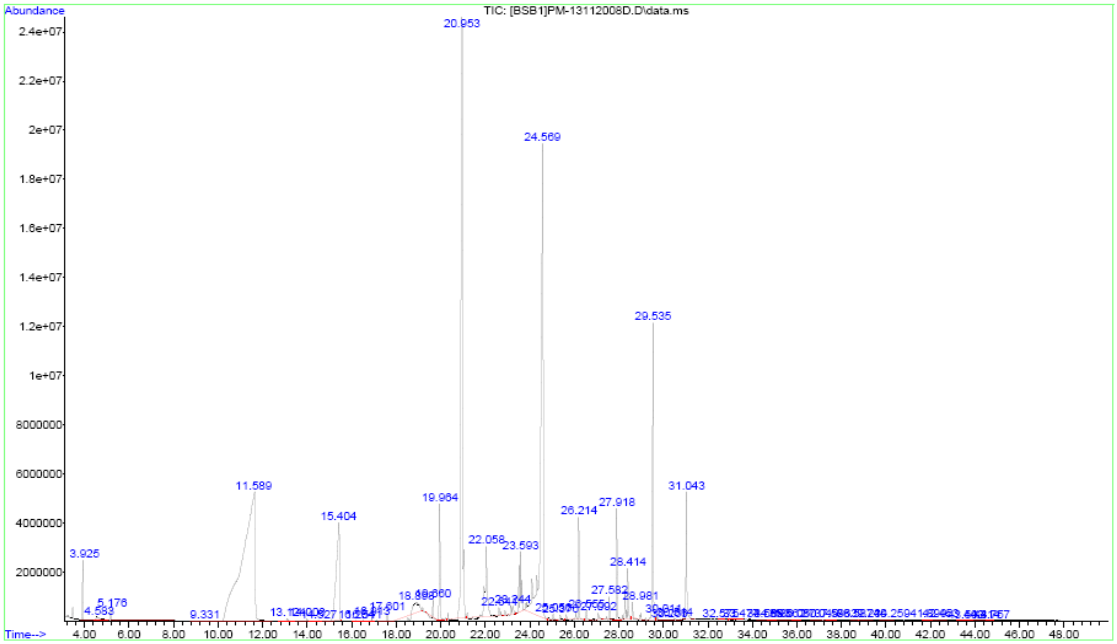
3.9 GC-MS profile of L13, F8



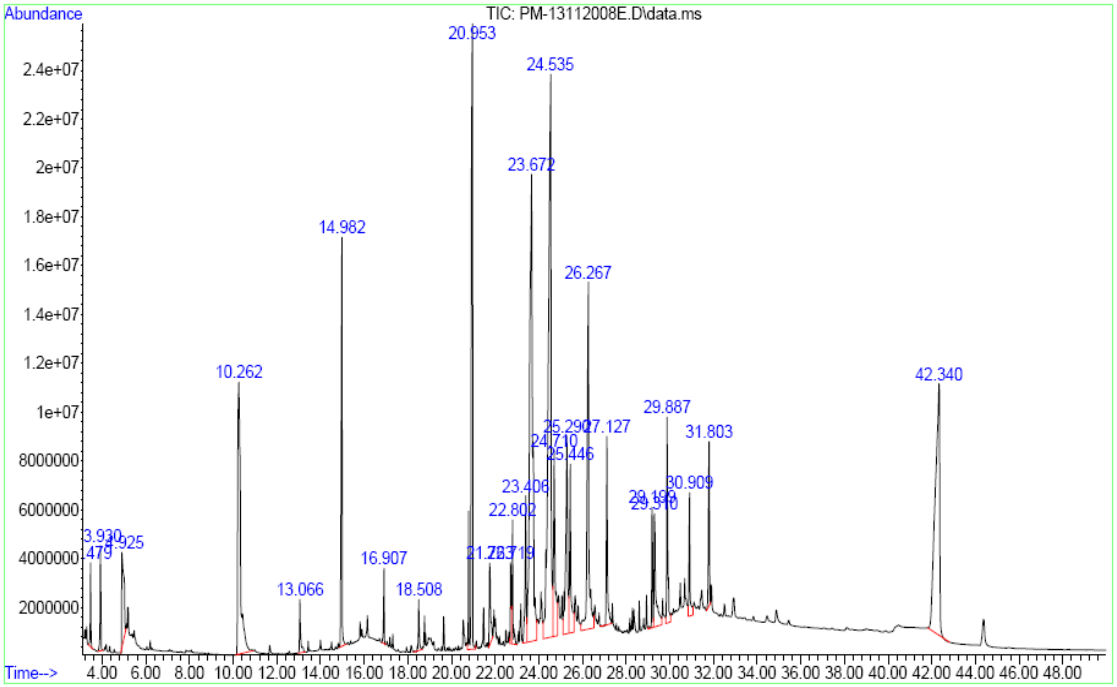
3.10 GC-MS profile of L13, F11



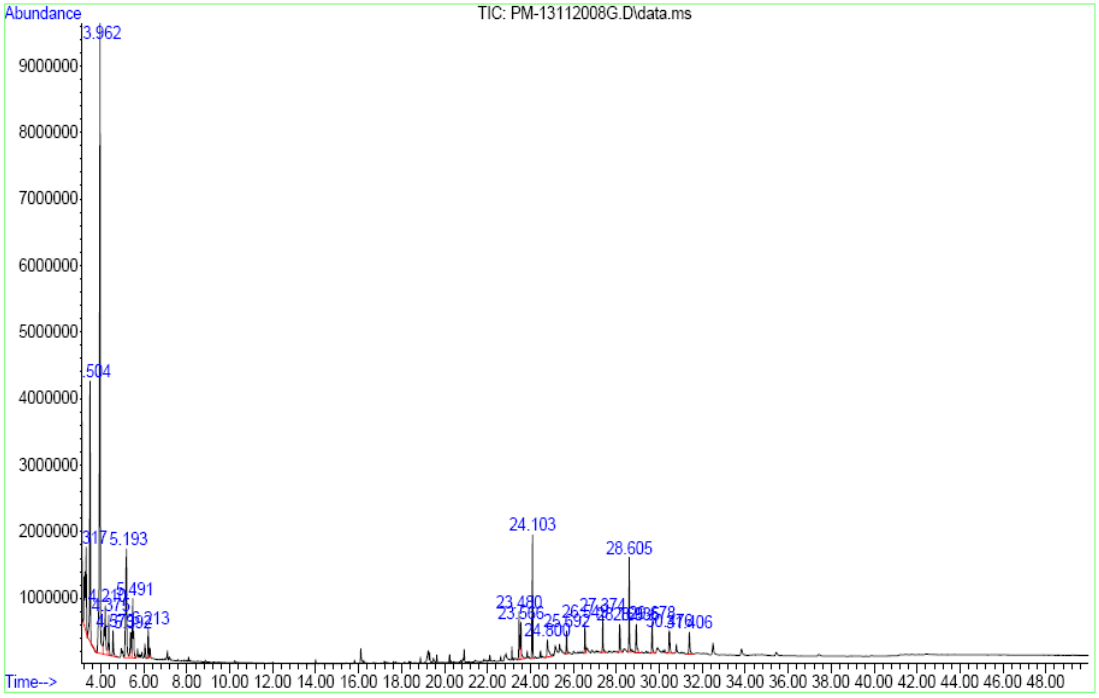
3.11 GC-MS profile of S33, F3



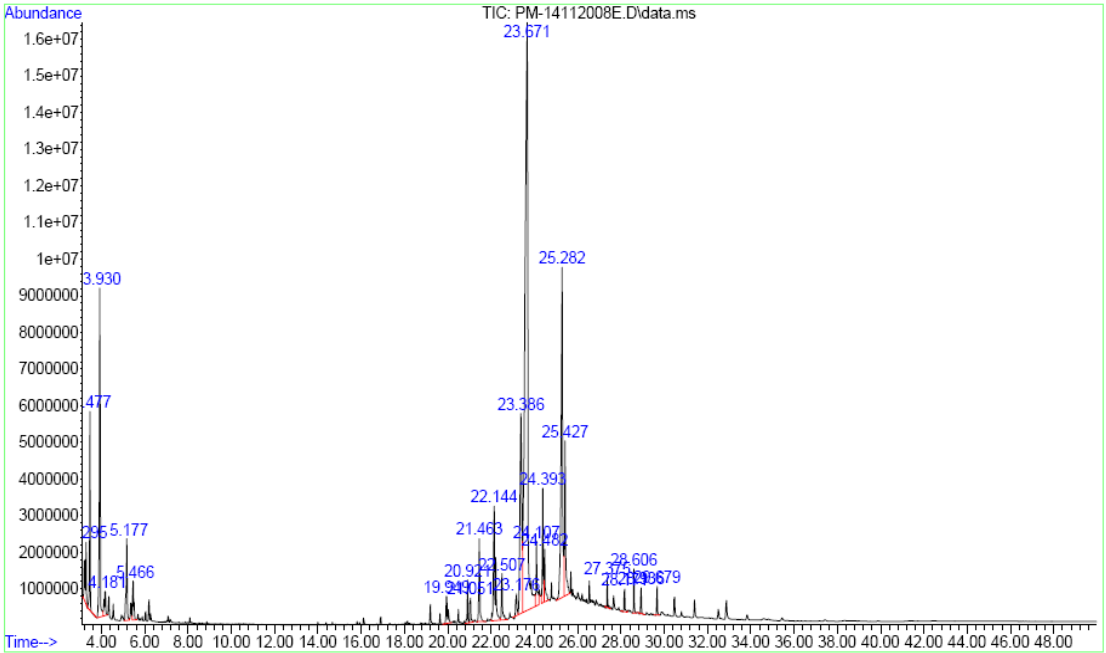
3.12 GC profile of S33, F11



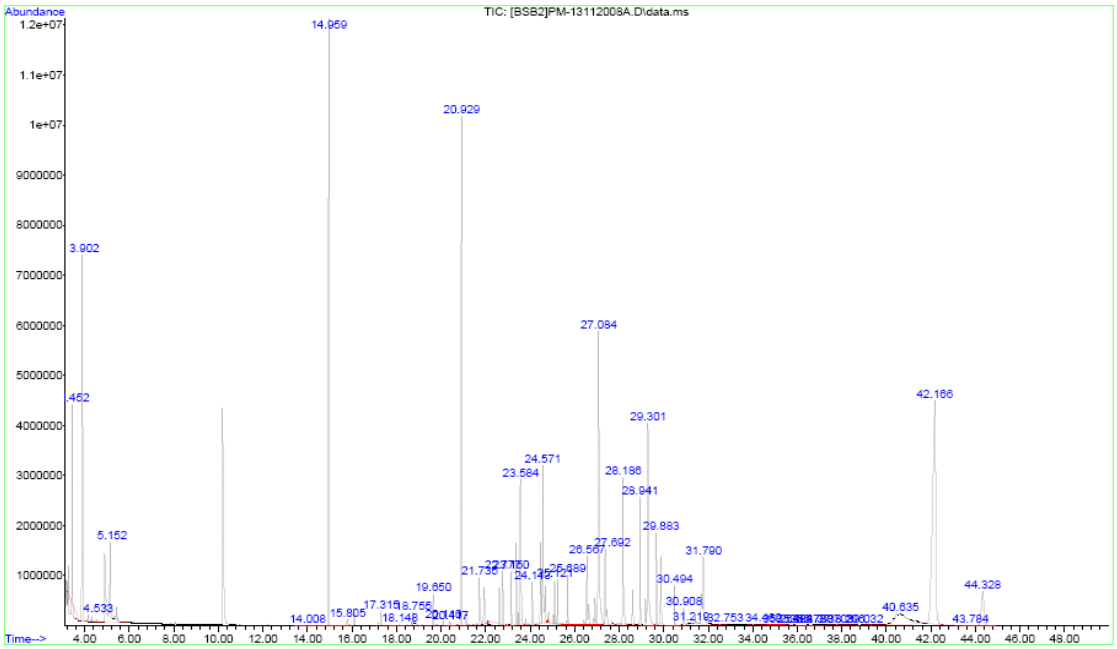
3.13 GC-MS profile of B41, F7



3.14 GC-MS profile of B41, F9



3.15 GC-MS profile of DM30, F11



3.16 GC –MS profile of DR35, F8