COMPARATIVE STUDY OF ANTIMICROBIAL ACTIVITIES AND GC-MS ANALYSIS OF BIOACTIVE COMPOUNDS OF ESSENTIAL OIL OF SELECTED CITRUS PEELS

BY

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CERTIFICATION

This is to certify that this research work was carried out by AHMED, Olayinka Abdullahi with matric number **17/27/MMI002** in the Department of Microbiology, College of Pure and Applied Sciences. Kwara State University, Malete. The project has been read and approved as meeting the requirements for the award of Master of Science (M.Sc. Hons) degree in Microbiology.

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DEDICATION

I dedicate this work to Almighty Allah, and to my loving parents, Mr. and Mrs. AHMED.

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My in-depth gratitude go to Almighty Allah for His blessings and mercy. I am grateful for His endless love, protection, guidance, grace and showers of blessings upon me and my family.

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ABSTRACT

The present study was aimed to determine the chemical constituents and antimicrobial effects of essential oils extracted from peels of *Citrus aurantium, Citrus sinensis, Citrus paradisii, Citrus limon* and *Citrus tangerine* on some selected isolates. Fruits of citrus varieties were collected from Ipata market in Ilorin, Kwara State. Nigeria. The essential oil was extracted by steam distillation process. The essential oil compositions of the citrus varieties were analyzed by Gas chromatography – mass spectrometry (GC-MS). Antimicrobial screening was carried out by the agar-well diffusion method against test bacteria isolates (*Pseudomonas aeruginosa, Streptococcus agalactiae,* Methicillin Resistant *Staphylococcus aureus, Escherichia coli* and *Candida albicans*). *Citrus aurantium, Citrus paradisii,* and *Citrus tangerine* showed antimicrobial properties against most of the tested organisms when compared with standard antibiotics. The GC–MS analysis revealed that the essential oils from all Citrus fruit peel contain 9-oxabicyclo (6,1,0) nonane, 9-octadecanol and 11-(2-cyclopenten-1-yl) undecanoic acid. These might have accounted for the antimicrobial properties of the oil. Conclusively, it is recommended that *Citrus aurantium, Citrus paradisii,* and *Citrus tangerine* be further evaluated for pure antimicrobial agents.

CHAPTER ONE

INTRODUCTION

BACKGROUND OF THE STUDY

The emergence and re-emergence of multi-drug resistant organisms is of paramount concern to the world health system. Complications arising from the use/misuse of antibiotics and these complications can be attributed to either drug-drug interactions or constant evasion to these drugs by microbes through possession and modification of certain defense mechanisms against these antibiotics. Therefore, researches have been directed towards the alternative medicine which could stand the test of time with lesser side effects.

Medicinal plants are plants with pharmacological properties which have the potentials to render the activities and products of pathogenic microorganism inactive in the host system. (Joy *et al*., 1998) They have been found to be cheap and alternate source of medicine and the acclaimed success rate of their use in folk medicine have generated the interest of microbiologist and other scientists around the globe. Medicinal plants have formed the basis of health care throughout the world and have remained relevant in both developing and the developed nations of the world for various chemotherapeutics purposes. The use of plant derived natural compounds as part of herbal preparations for alternate source of medicaments continues to play major roles in chemotherapy especially in third world countries (Joy *et al*., 1998). Several studies carried out have shown that medicinal plants could provide better control than currently used conventional drugs. (Roja and Rao, 2000).

All over the world *Citrus* is one of the widespread genus due to its prominent production (Choi *et al*., 2000). *Citrus* essential oils are naturally occurring, volatile and odoriferous oils synthesized by non woody parts of plants such as seeds, buds, leaves, flowers, stems, fruits, twigs, roots and accumulated in secretary or epidermis cells and also sometimes in cavities (Ahmad, 2006). Essential oil from *Citrus* fruit peels is the fundamental product of genus *Citrus* and typically isolated by distillation or solvent extraction (Mondello *et al*., 2005). These are the complex mixtures of about 400 compounds of which 1-15 % are non-volatile whereas 85-99 % is the volatile constituents (Nannapaneni *et al*., 2009). Other organic compounds present in *Citrus* essential oils are aliphatic hydrocarbons, alcohols (linalool), aldehydes (citral), acids, esters and some aromatic compounds (Sharma and Tripathi, 2006).

Essential oils of *Citrus* peels are medicinally very important and show variety of biological effects because they are rich in flavonoids (flavone, flavonol and flavanone), terpenes, carotenes and coumarines which are responsible for antimicrobial activity (Tepe *et al*., 2005). Consequently, *Citrus* essential oils are extensively used in pharmaceutics as an antimicrobial, anti-diabetic, antioxidant, insect repellent, carminative, larvicidal, antiviral, anti-hepatotoxic and anti-mutagenic agent (Kanaze *et al*., 2008).

Citrus is grown widely all over the world for its numerous health benefits. Citrus fruits are consumed as a fresh fruit desert or used for making juice and jam. They are an excellent source of vitamins, especially vitamin C. Processing Citrus fruits results in a significant amount of waste (peels, seeds, and pulps), which accounts for 50 % of the fruit (Anwar *et al*., 2008). Citrus

waste is a valuable source of d-limonene, flavonoids, carotenoids, dietary fibers, soluble sugars, cellulose, hemicellulose, pectin, polyphenols, ascorbic acid, methane, and essential oils.

Citrus Essential oils (Eos) are broadly used as natural food additives in several food and beverage products (Ferhat *et al*., 2000) because they have been classified and generally recognized as safe (GRAS). Furthermore, Citrus Essential oil (EO) is used as natural preservatives due to their broad spectrum of biological activities including antimicrobial and antioxidant effects (Mitropoulou *et al*., 2017). The presence of terpenes, flavonoids, carotenes, and coumarins is thought to be responsible for the strong anti-oxidative and antimicrobial activities (Viuda-Martos *et al*., 2008). Due to their pleasant refreshing smell and rich aroma, Citrus EOs is also used in air-fresheners, household cleaning products, perfumes, cosmetics, and medicines. Because of their high economic importance, numerous studies have investigated the chemical composition of the peel, leaf, and flower of essential oils of different Citrus species. It is worth noting that there is a great variation in the chemical composition of Citrus oils due to differences in origin, genetic background, season, climate, age, ripening stage, method of extraction (Dosoky *et al*., 2014).

1.2 Statement of Problem

Bacteria and fungi infections have been a major health challenge to most health care systems in Nigeria. Their effective treatment options are expensive not only for the patients but also for the organizations. Several antibiotics are used for treatment of bacteria and fungi but the drugs are mostly obsolate and ineffective. Emergence of resistance and multi-drug resistant bacteria to these antibiotics has become a worldwide problem (Choi *et al*., 2000). This is attributed to

overuse and misuse of antibiotics. Resistance is also due to heavy metal pollution of water from industrial effluents, automobiles and agricultural practices. A long exposure of heavy metals to bacteria makes them resistant and clinically untreatable. Therefore, researches have been channel towards the use of medicinal plants, which are not as expensive, effective and could stand the test of time with lesser side effects.

1.3 Justification of the Study

Use of essential oil in controlling bacteria and fungi infections have continued despite advances in modern pharmaceutical products and dominance of synthetic drugs all over the world (Deresse, 2010). Sweet orange (*C. sinensis*), bitter orange (*C. aurantium*), Lemon (*C. limon*), tangerine (*C. tangerine*) and grape (*C. paradisi*) and their essential oils have been used in controlling bacterial and fungal infections though not clinically regulated due to lack of awareness and data to support the reported therapeutic claims (Rehman *et al*., 2011). There is need to develop new antimicrobial agents from known potential fruits to keep in check the growing resistance of bacteria to common conventional drugs (Wangari, 2008).

In Nigeria herbal medicine is gaining popularity because of its availability and affordability (Hatil, 2009). In spite of the presence of locally available foods that can be used to prevent or treat bacterial infections, minimum research has been done on them, leaving most people to rely on antibiotics that are relatively expensive and have enormous side effects (Linda *et al*., 2008). Essential oils have been previously investigated for their antibacterial properties against *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), Streptococcus, and *Candida albicans* (*C. albicans*) (Theresa, 2008). This study was

designed to assess the antimicrobial properties of the selected essential oil and determine their bioactive compounds using GC-MS.

1.4 Overall Objective

This study was designed to evaluate the antimicrobial activities of essential oils from citrus peels and also evaluate their bioactive compound.

1.5 Specific Objectives

The specific objectives of this study are to:

- Extract the essential oil from *citrus fruit* peel
- Determine antibiotic sensitivity profile of the tested organism
- Evaluate the antibacterial effectiveness of essential oil of citrus peels on standard organism
- Identify bioactive constituents of all the essential oils.

CHAPTER TWO

LITERATURE REVIEW

2.1 Antibacterial Compounds in Herbal Plants

The use and search for drugs and dietary supplements derived from plants have accelerated in recent years. Research carried out by Pavithra showed that presence of phytochemicals such as alkaloids, tannins, triterpenoids, steroids and glycosides in the extracts of *Delonix elata*, *Enicostemma axillare, Merremia tridentata, Mollugo cerviana* and *Solanum incanum* supports their traditional uses as medicinal plants for the treatment of various ailments in India (Pavithra *et al*., 2010). While 25 to 50% of current pharmaceuticals are derived from plants, they are not used as antimicrobials (Marjorie, 1999).

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Narayana *et al*., 2000). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment. Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state. Caffeic acid is effective against viruses (Wild, 1994) and bacteria (Brantner *et al*., 1996).

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive. These compounds, being colored, are responsible for the

browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin. In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al*.,1996), often leading to inactivation of the protein and loss of function. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism (Mohamoud *et al*., 2007).

In rational drug therapy, the concurrent administration of two or more drugs is often essential and sometimes mandatory in order to achieve the desired therapeutic goal or to treat co-existing diseases. However, the drug interaction may have different effects on the host as well as the infecting microorganism. The potential benefits of using combined antimicrobial therapy is that; it can be used for treatment of mixed infections, therapy of severe infections in which a specific causative organism is known, enhancement of antibacterial activity, reducing the needed time for long-term antimicrobial therapy and prevention of the emergence of resistant microorganisms (Muhammad and Ahsan, 2005). Antibacterial synergism between bioactive plant extracts is a novel concept and has been recently reported (Nascimento *et al*., 2000).

2.2 Antibiotics

Antibiotics are chemical substance that inhibits the growth of microorganism. They are also a type of antimicrobial drug used in the treatment and prevention of bacterial and fungal infections. They may either kill or inhibit the growth of bacteria or fungi. A limited number of antibiotics also possess antiprotozoal activity (WHO, 2014). Antibiotics are not effective against

viruses such as the common cold or influenza; drugs which inhibit viruses are termed antiviral drugs or antivirals rather than antibiotics (Cassir *et al.,* 2014). Sometimes, the term antibiotic (which means "opposing life") is used to refer to any substance used against microbes, synonymous with antimicrobial. Some sources distinguish between antibacterial and antibiotic; antibacterials are used in soaps and disinfectants, while antibiotics are used as medicine (Elsevier*,* 2013).

Antibiotics revolutionized medicine in the 20th century. Together with vaccination, antibiotics have led to the near eradication of diseases such as tuberculosis in the developed world. However, their effectiveness and easy access have also led to their overuse, prompting bacteria to develop resistance (Gualerzi *et al.,* 2013). This has led to widespread problems; World Health Organisation has declared antimicrobial resistance as a serious threat globally regardless of the country or age (WHO, 2014). Rising antibiotic resistance and the scarcity of new antimicrobials has long been acknowledged (Walsh and Toleman, 2012). A major challenge in global health care is the need for novel, effective and affordable medicines to treat microbial infections, especially in developing countries of the world, where up to one-half of deaths are due to infectious diseases (Awouafack *et al*., 2013).

The use of plant-based drugs world-wide is increasing. Through recent researches on herbal plants, there have been great developments in the pharmacological evaluation of various plants used in traditional systems of medicine (Gajendiran *et al* 2016). Herbal plant products are emerging all over the world due to the belief that many herbal medicines are free from health and

environmental effects. The widespread fear of the side-effect of synthetic drugs often accompanies their single or multiple health benefits (Al Sadhan *et al*., 1999).

2.3 Essential Oils

A concentrated hydrophobic liquid from plants having a volatile aroma is known as essential oil. These are ethereal oils also known as "oil of" the plant from which it is isolated, like oil of rosemary. The oil is called essential oil because it possesses a distinctive smell of the plant (Burt, 2004). These are usually known as "life force" of plants. Essential oils are the mixtures of fragrant and odorless substances. The fragrant substance is volatile and chemically pure substance. Due to fragrance and flavor, these oils are used in products like cosmetics, medicine and foods (WHO, 2014).

Essential oils are the products of secondary metabolism. These are extracted from plants by steam or hydro-distillation developed by Arabs in the middle Ages. The amount of oil in plants is in between 0.01 to 10 percent of the total. Essential oils are known for their various properties, i.e. antimicrobial, antioxidative, analgesic, sedative, spasmolytic, anti-inflammatory and locally anesthic properties.

Essential oils are volatile liquids which are rarely colored and have lower density than water. These are soluble in organic solvents. Out of 3000 essential oils known, only 300 are considered commercially important for various industries like, pharmaceutical, food, sanitary, perfume and agronomic. Some of the oils are known to have medicinal properties of curing organ dysfunction (Silva *et al*., 2011). Essential oils are intensively studied topic recently, still mechanism of action

of the oils is not so clear. These oils appear to be very good alternate to replace some of the synthetic compounds, without showing undesirable effects like that of synthetic products (kolli *et al*., 2016). Recent reports depict that benzoic acid used as additive changes to benzene in foods. Sorbic acid is decarboxylated to 1, 3-pentadiene which causes kerosene like odor by *Saccharomyces cerevisiae* and *Pichia anomala*.

2.3.1 Antimicrobial effects of Essential Oils (Eos): It has been shown in several reports that the bioactive components present in EOs might attach to the surface of the cell, and thereafter penetrate to the phospholipid bilayer of the cell membrane. The structural integrity of cell membrane is disturbed by their accumulation, which can detrimentally influence the cell metabolism causing cell death (Bajpai *et al*., 2013). *E. coli* treated with black pepper essential oil (BPEO) became deformed, pitted, shriveled, because BPEO led to the leakage, disorder and death by breaking cell membrane (Zhang *et al*., 2017). Zhang *et al*. determined the mechanism behind the antibacterial activity of cinnamon EO against *E. coli* and *S. aureus* and reported that the bacterial cell membrane was destroyed after addition of cinnamon EO at the MIC level, whereas addition of cinnamon EO at the MBC levels resulted in the killing of the bacterial cell (Zhang *et al*., 2016). In addition to this, cinnamon EO led to increase in the electric conductivity of samples at the first few hours due to leakage of small electrolytes rapidly, concentration of proteins and nucleic acids in cell suspension and 3–5 fold decreased bacterial metabolic activity as reflected by the results of membrane potential. EO from *Dipterocarpus gracilis* inhibited the growth of *Bacillus cereus* and *Proteus mirabilis* by acting on the cytoplasmic membrane as one of its targets. These activities could be exploited for food preservation in the food industry (Kolli *et al*., 2016). Further, it has been reported that action of EOs on the integrity of cell membrane

changes the membrane permeability which leads to loss of vital intracellular contents like proteins, reducing sugars, ATP and DNA, while inhibiting the energy (ATP) generation and related enzymes leading to the destruction of cell and leakage of electrolytes (Cui *et al*., 2015). Antimicrobial activity of EOs is therefore attributed to a cascade of reactions involving the entire bacterial cell (Macwan *et al*., 2016). As reported in a study, essential oil from mustard presented 10 times more bactericidal/bacteriostatic effect than cinnamon essential oil (Isabel *et al*., 2016).

Ahmad *et al* 2006 showed that antifungal activity of *Coriaria nepalensis* essential oil (CNEO) against *Candida* isolates is due to the inhibition in the biosynthesis of ergosterol and disruption in the integrity of membrane. Similarly, another study described the utility in designing new formulations for candidosis treatment because of the antifungal activity of coriander essential oil on *Candida* spp., in which it was reported that the fungicidal effect of coriander essential oil is a result of damage in the membrane of cytoplasm and subsequent leakage of intracellular components such as DNA (Silva *et al*., 2011). Likewise, disruption of the fungal cell endomembrane system including the plasma membrane and mitochondria, i.e., the inhibition of ergosterol synthesis, malate dehydrogenase, mitochondrial ATPase, and succinate dehydrogenase activities was related to the antifungal activity of natural essential oil (EO) derived from turmeric (*Curcumalonga* L.) against *Aspergillus flavus* (Hua *et al*., 2017).

2.4 Antibiotics Mode of Action

The antimicrobial potency of most classes of antibiotic are directed at some unique feature of the bacterial structure or their metabolic processes.

2.4.1 Inhibition of cell wall synthesis

Most bacterial cells are encased by a rigid layer of peptidoglycan (PG), also called murein which protects the cells in the face of prevailing osmotic pressure. Peptidoglycan has a degree of cross-linking peptide bonds called β-(1-4) –N– acetyl Hexosamine (Bugg and Walsh, 1992). To stay alive, bacteria must synthesize peptidoglycan; they do this by the activity of PBPs which are transglycosylases and transpeptidases. These two enzymes play very pivotal roles by adding disaccharide pentapeptides to extend the glycan strands of existing peptidoglycan molecule and also cross-link strands of immature peptidoglycan units (Park and Uehara, 2008). Drugs like penicillins, carbapenems and cephalosporins are able to block the cross-linking of peptidoglycan units by inhibiting the peptide bond formation catalyzed by PBPs (Josephine *et al*., 2004). Most antibiotics belonging to the glycopeptide class of antibiotics (for example, vancomycin) are able to inhibit bacterial growth by inhibiting the synthesis of PG. They inhibit the synthesis of PG by binding themselves to PG units, as well as blocking transglycosylase and transpeptidase activity (Kahne *et al*., 2005).

2.4.2 Breakdown of the cell membrane structure or function

The classes of antibiotics that damage cell membranes of bacteria are specific in each microbial group based on the differences in the types of lipids in their cell membranes. For example, Daptomycin depolarizes calcium-dependent membrane, and that leads to the cessation of macromolecular synthesis and disruption of the cellular membrane in bacteria (Alborn *et al.,* 1991). The polymyxins cause disintegration of bacterial cell membrane by effectively binding to the lipid moiety of the lipopolysaccharide in the bacterial cell (Falagas *et al.,* 2010).

2.4.3 Inhibition of nucleic acid synthesis The metabolic pathways that result in synthesis of nucleic acids are very essential; disruption of nucleic acid synthesis is inimical to both the survival and posterity of bacterial cells. Antibiotics interfere with nucleic acid synthesis by blocking replication or stopping transcription. DNA replication involves the unwinding of the traditional double helix structure, a process facilitated by the helicase enzymes (Gale *et al*., 1981). The quinolones group of antibiotics, for example, do interfere with the functionality of the helicase enzyme thereby disrupts the enzyme from playing its function of unwinding DNA. This antibiotic action of the quinolones ultimately truncates the process of DNA replication and repair amongst susceptible bacteria (Chen *et al*., 1996). Antibiotics whose mode of action is inhibition of nucleic acid synthesis also target topoisomerase II and topoisomerase IV of bacteria. Disrupting the activities of these enzymes in bacteria adversely affects RNA polymerase which in turn prevents RNA synthesis. Quinolones that inhibit bacterial nucleic acid synthesis in this way do not interact with mammalian RNA polymerase, making them specifically antagonistic to Gram-positive bacteria and some Gram-negative bacteria.

2.4.4 Inhibition of protein synthesis

Living things including bacteria are defined by the amount and type of proteins they are composed of, and continually produce. Proteins are responsible for the structural composition, metabolic and physiological processes, and response to adverse conditions, amongst other roles. However, the type and amount of proteins produced by a bacterium at any given time is dependent on information contained in yet another very important biomolecule – Deoxyribonucleic acid (DNA). DNA determines the type of protein a bacterial cell produces

through certain information it harbors within itself. The information is a set of genetic codes called codons, handed down to an identical biomolecule – Ribonucleic acid (RNA), specifically messenger RNA (mRNA). Transfer RNA (tRNA), a similar biomolecule is also formed under the directive of DNA. This biomolecule together with mRNA travels to the ribosomes – the factory for protein synthesis in a living cell. The tRNA then deciphers the codons contained in the mRNA and facilitates the translation of the sequence of codons to a sequence of amino acids which are the building blocks of proteins (Etebu, 2013). The translation of mRNA into proteins occurs over three sequential phases (initiation, elongation and termination) involving the ribosome and a host of cytoplasmic accessory factors. Ribosomes are made up of RNA and proteins, and are generally called Ribonucleoproteins. The RNA component is what is referred to as Ribosomal RNA (rRNA), and comprises two subunits, one small subunit (SSU) and the other large subunit (LSU). These two subunits are usually described in terms of their sedimentation coefficients (that is, their rate of sedimentation is an ultracentrifuge), and are measured in Svedberg units (symbols) termed the 30S and 50S, respectively (Nissen *et al*., 2000). Bacterial possess 5S, 16S and 23S genes on their rRNA (Moore, 2001). The 16S rRNA gene resides as a single RNA gene in their SSU (16S) whilst the other two rRNA genes (23S and 5S) occur on the LSU of the bacterial ribosome. There is huge difference between prokaryotic and eukaryotic rRNA, and this feat has greatly enabled Scientists to develop antibiotics that would target rRNA of a wide spectrum of pathogenic bacteria (Hong *et al*., 2014). Given the importance of proteins in the metabolic and life processes of all living organisms, whatever disrupts the process of its synthesis in a bacterial cell would ultimately incapacitate the cell; inhibit its growth or even kill it completely. Drugs that inhibit protein synthesis are among the broadest classes of antibiotics

and can be divided into two subclasses: the 50S inhibitors and 30S inhibitors. Antibiotics such as erythromycin, clindamycin, lincomycin, chloramphenicol, linezolid etc. have been shown to be among the 50S ribosome inhibitors (Katz and Ashley, 2005).

In general terms, antibiotics that inhibit 50S ribosome do so by physically blocking either the initiation phase of protein translation or the elongation phase of protein synthesis where the incoming amino acid is linked up with the growing nascent peptide chain (Patel et al., 2001). Examples of antibiotic that block initiation of protein translation are members of Oxazolidinones (Patel *et al*., 2001) whilst macrolides such as lincosamide and streptogramin block protein synthesis by inhibiting the elongation phase of mRNA translation. These latter groups of antibiotics are therefore reportedly ineffective when elongation has progressed beyond a critical length (Tenson *et al*., 2003).

The 30S ribosome-inhibitors principally work by blocking the access of aminoacyl-tRNAs to the ribosome. Examples of antibiotics that function in this manner include the tetracycline, streptomycin, spectinomycin, etc. (Hong *et al*., 2014). It is worthy to note that some earlier works have shown that tetracycline also inhibits some proteins at the 50S ribosomes (Epe and Woolley, 1984). Among ribosome inhibitors, the naturally-derived aminoglycoside subclass is the only one that is broadly bactericidal. Macrolides, streptogramins, spectinomycin, tetracyclines and chloramphenicol are typically bacteriostatic. However, some of these ribosome inhibitory antibiotics that are typically bacteriostatic in action could be bactericidal under certain conditions relating to species- or treatment-specific fashion. For example, chloramphenicol known typically to be bacteriostatic has been shown to effectively kill *Streptococcus pneumoniae*

and *Neisseria meningitidis*, as well as *Heamophillus influenza*. This species-specific variability in ribosome inhibition or mediated cell death is potentially linked to sequence differences among bacterial species in the variable regions of the highly conserved ribosomal proteins and RNAs (Roberts *et al*., 2008).

2.4.5 Blockage of key metabolic pathways

Some antibiotics like sulphonamides and trimethoprim have been shown to mimic a substrate needed for cellular metabolism of bacteria. This deception cause bacterial enzymes to attach themselves to the antibiotic rather than the normal substrate. In particular, sulphonamides act like tetrahydrofolate which is required for the synthesis of folic acid in bacterial cells (Talaro and Chess, 2008). Folic acid is vital in the metabolism of nucleic acid and amino acids; For this reason, sulphonamides ultimately disrupt the production of nucleic acids (DNA and RNA) and amino acids, as they mimic substrates required for folic acid metabolism (Talaro and chess, 2008).

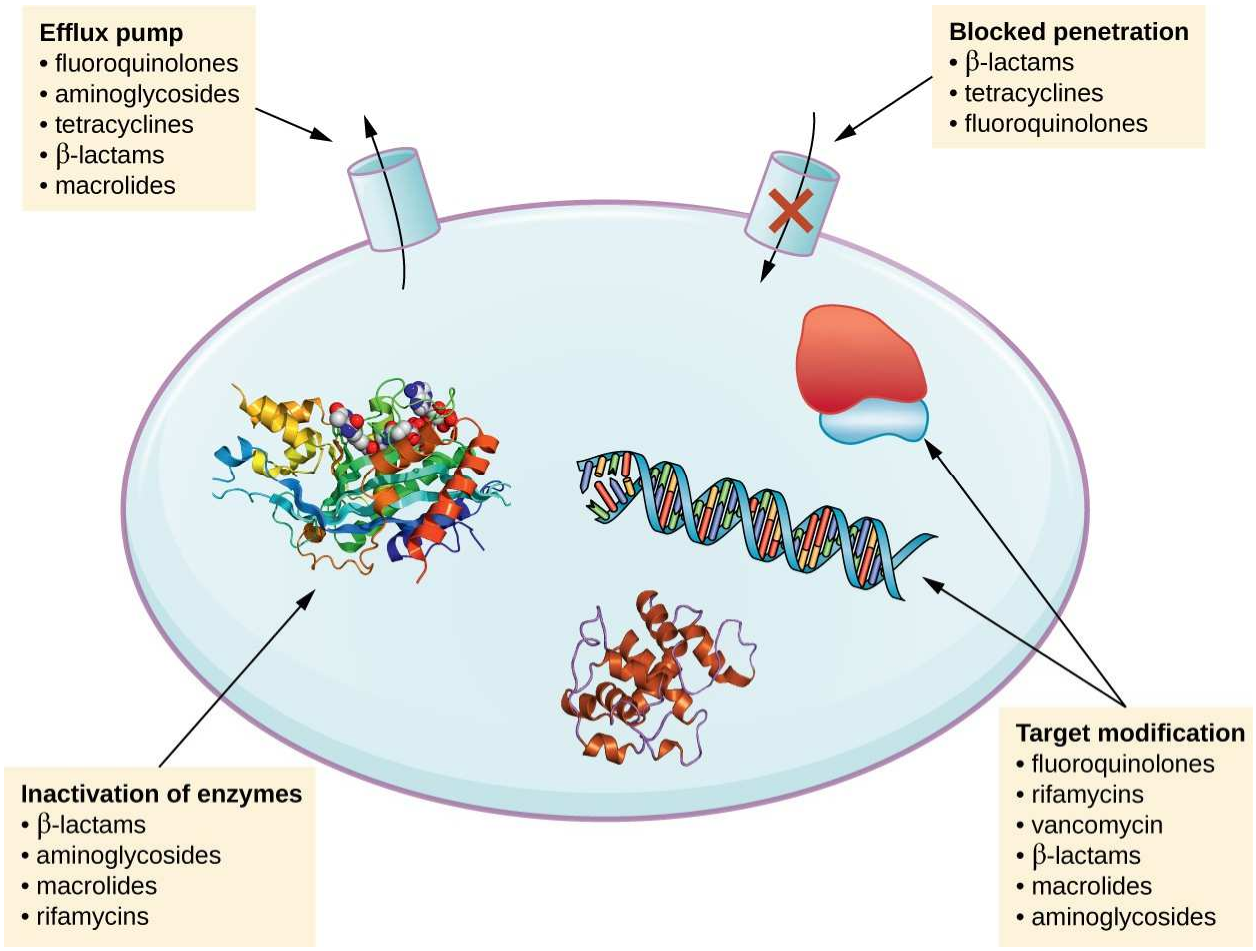

Figure 2.1: Mechanism of antimicrobial resistance

(Fong *et al*., 2002)

2.5 Mechanisms of Drug Resistance

There are several common mechanisms for drug resistance, which are summarized in Figure 1. These mechanisms include enzymatic modification of the drug, modification of the antimicrobial target, and prevention of drug penetration or accumulation.

2.5.1 Drug Modification or Inactivation

Resistance genes may code for enzymes that chemically modify an antimicrobial, thereby inactivating it, or destroy an antimicrobial through hydrolysis. Resistance to many types of antimicrobials occurs through this mechanism. For example, aminoglycoside resistance can occur through enzymatic transfer of chemical groups to the drug molecule, impairing the binding of the drug to its bacterial target. For β-lactams, bacterial resistance can involve the enzymatic hydrolysis of the β-lactam bond within the β-lactam ring of the drug molecule. Once the β-lactam bond is broken, the drug loses its antibacterial activity. This mechanism of resistance is mediated by β-lactamases, which are the most common mechanism of β-lactam resistance. Inactivation of rifampin commonly occurs through glycosylation**,** phosphorylation, or adenosine diphosphate (ADP) ribosylation, and resistance to macrolides and lincosamides can also occur due to enzymatic inactivation of the drug or modification.

2.5.2 Prevention of Cellular Uptake or Efflux

Microbes may develop resistance mechanisms that involve inhibiting the accumulation of an antimicrobial drug, which then prevents the drug from reaching its cellular target. This strategy is common among gram-negative pathogens and can involve changes in outer membrane lipid

composition, porin channel selectivity, and/or porin channel concentrations. For example, a common mechanism of carbapenem resistance among *Pseudomonas aeruginosa* is to decrease the amount of its OprD porin, which is the primary portal of entry for carbapenems through the outer membrane of this pathogen. Additionally, many gram-positive and gram-negative pathogenic bacteria produce efflux pumps that actively transport an antimicrobial drug out of the cell and prevent the accumulation of drug to a level that would be antibacterial. For example, resistance to β-lactams, tetracyclines, and fluoroquinolones commonly occurs through active efflux out of the cell, and it is rather common for a single efflux pump to have the ability to translocate multiple types of antimicrobials.

2.5.3 Target Modification

Because antimicrobial drugs have very specific targets, structural changes to those targets can prevent drug binding, rendering the drug ineffective. Through spontaneous mutations in the genes encoding antibacterial drug targets, bacteria have an evolutionary advantage that allows them to develop resistance to drugs. This mechanism of resistance development is quite common. Genetic changes impacting the active site of penicillin-binding proteins (PBPs) can inhibit the binding of β-lactam drugs and provide resistance to multiple drugs within this class. This mechanism is very common among strains of *Streptococcus pneumoniae*, which alter their own PBPs through genetic mechanisms. In contrast, strains of *Staphylococcus aureus* develop resistance to methicillin (MRSA) through the acquisition of a new low-affinity PBP, rather than structurally alter their existing PBPs. Not only does this new low-affinity PBP provide resistance to methicillin but it provides resistance to virtually all β-lactam drugs, with the exception of the

newer fifth-generation cephalosporins designed specifically to kill MRSA. Other examples of this resistance strategy include alterations in

- ribosome subunits, providing resistance to macrolides, tetracyclines, and aminoglycosides;
- lipopolysaccharide (LPS) structure, providing resistance to polymyxins;
- RNA polymerase, providing resistance to rifampin;
- DNA gyrase, providing resistance to fluoroquinolones;
- metabolic enzymes, providing resistance to sulfa drugs**,** sulfones, and trimethoprim; and
- peptidoglycan subunit peptide chains, providing resistance to glycopeptides.

2.5.4 Target Overproduction or Enzymatic Bypass

When an antimicrobial drug functions as an antimetabolite, targeting a specific enzyme to inhibit its activity, there are additional ways that microbial resistance may occur. First, the microbe may overproduce the target enzyme such that there is a sufficient amount of antimicrobial-free enzyme to carry out the proper enzymatic reaction. Second, the bacterial cell may develop a bypass that circumvents the need for the functional target enzyme. Both of these strategies have been found as mechanisms of sulfonamide resistance. Vancomycin resistance among *S. aureus* has been shown to involve the decreased cross-linkage of peptide chains in the bacterial cell wall, which provides an increase in targets for vancomycin to bind to in the outer cell wall. Increased binding of vancomycin in the outer cell wall provides a blockage that prevents free drug molecules from penetrating to where they can block new cell wall synthesis.

2.5.5 Target Mimicry

A recently discovered mechanism of resistance called target mimicry involves the production of proteins that bind and sequester drugs, preventing the drugs from binding to their target. For example, *Mycobacterium tuberculosis* produces a protein with regular pentapeptide repeats that appears to mimic the structure of DNA. This protein binds fluoroquinolones, sequestering them and keeping them from binding to DNA, providing *M. tuberculosis* resistance to fluoroquinolones. Proteins that mimic the A-site of the bacterial ribosome have been found to contribute to aminoglycoside resistance as well.

2.6 Test Organisms and Antibiotics Used

The samples for this study are clinically isolated bacteria and fungi which include *methycillin resistance Staphylococcus aureus, Streptococcus agalactiae Escherichia coli, pseudomonas aeruginosa* and *Candida albicans*.

2.6.1 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive, round-shaped bacterium that is a member of the Firmicutes, and it is a member of the normal flora of the body, frequently found in the nose, respiratory tract, and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen (Masalha *et al*, 2001). Although *S. aureus* is not always pathogenic (and can commonly be found existing as a commensal), it is a common cause of skin infections including abscesses, respiratory infections such as sinusitis, and food poisoning. Pathogenic strains often promote infections by producing virulence factors such

as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies.

The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (*MRSA*) is a worldwide problem in clinical medicine. Despite much research and development there is no approved vaccine for *S. aureus*. *Staphylococcus* was first identified in 1880 in Aberdeen, Scotland, by surgeon Sir Alexander (1984) in pus from a surgical abscess in a knee joint. This name was later amended to *Staphylococcus aureus* by Friedrich Julius Rosenbach, who was credited by the official system of nomenclature at the time. An estimated 20% to 30% of the human population are long-term carriers of *S. aureus* which can be found as part of the normal skin flora, in the nostrils, and as a normal inhabitant of the lower reproductive tract of women (Tong, 2015).

S. aureus can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. It is still one of the five most common causes of hospital-acquired infections and is often the cause of wound infections following surgery. Each year, around 500,000 patients in hospitals of the United States contract a staphylococcal infection, chiefly by *S. aureus*. Up to 50,000 deaths each year in the USA are linked with *S. aureus* infections (Masalha *et al*, 2001).

2.6.2 *Pseudomonas aeruginosa*

Pseudomonas is a genus of Gram-negative, Gamma proteobacteria, belonging to the family *Pseudomonadaceae* and containing 191 validly described species. The members of the genus demonstrate a great deal of metabolic diversity and consequently are able to colonize a wide range of niches (Madigan and Martinko, 2005). Their ease of culture in vitro and availability of an increasing number of Pseudomonas strain genome sequences has made the genus an excellent focus for scientific research; the best studied species include *P. aeruginosa* in its role as an opportunistic human pathogen, the plant pathogen *P. syringae*, the soil bacterium *P. putida,* and the plant growth-promoting *P. fluorescens* (Lavigne *et al*., 2006). Because of their widespread occurrence in water and plant seeds such as dicots, the pseudomonads were observed early in the history of microbiology.

The generic name Pseudomonas created for these organisms was defined in rather vague terms by Walter Migula in 1894 and 1900 as a genus of Gram negative, rod-shaped and polar-flagellated bacteria with some sporulating species, the latter statement was later proved incorrect and was due to refractive granules of reserve materials (Palleroni, 2010). Despite the vague description, the type species, *Pseudomonas pyocyanea* (basonym of *Pseudomonas aeruginosa*), proved the best descriptor (Palleroni, 2010).

2.6.3 *Escherichia coli*

Escherichia coli (*E. coli*) is a gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-

blooded organisms (endotherms) (Tenaillon *et al.,* 2010). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and preventing colonization of the intestine with pathogenic bacteria, having a symbiotic relationship (Hudault *et al.,* 2001). *E. coli* is expelled into the environment within fecal matter. The bacterium grows massively in fresh fecal matter under aerobic conditions for 3 days, but its numbers decline slowly afterwards (Russell and Jarvis, 2001). *E. coli* and other facultative anaerobes constitute about 0.1% of gut flora, and fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease.

Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination. A growing body of research, though, has examined environmentally persistent *E. coli* which can survive for extended periods outside a host (Ishii and Sadowsky, 2008). The bacterium can be grown and cultured easily and inexpensively in a laboratory setting, and has been intensively investigated for over 60 years. *E. coli* is a chemoheterotroph whose chemically defined medium must include a source of carbon and energy. *E. coli* is the most widely studied prokaryotic model organism, and an important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority of work with recombinant DNA. Under favorable conditions, it takes up to 20 minutes to reproduce (Tortora, 2010). Most *E. coli* strains do not cause disease (Han and lee, 2006), but virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis, hemorrhagic colitis, and Crohn's disease. Common signs and symptoms

include severe abdominal cramps, diarrhea, hemorrhagic colitis, vomiting, and sometimes fever. In rare cases, virulent strains are also responsible for bowel necrosis (tissue death) and perforation without progressing to hemolyticuremic syndrome, peritonitis, mastitis, septicemia, and Gram-negative pneumonia. Very young children are more susceptible to develop severe illness, such as hemolytic uremic syndrome; however, healthy individuals of all ages are at risk to the severe consequences that may arise as a result of being infected with *E. coli*. (Lim *et al*., 2017)

2.6.4 *Candida albicans*

Many cases of urinogenitary tract infection *are* caused by *Candida albicans*, with 5 to 20% produced by *Candida glabrata* or *Candida tropicalis*. *C. vaginitis* is a common gynaecological finding among women worldwide (Nagai *et al.,* 2014).The disease caused by *C. albicans*is more common among sexually active women. Predisposing factors of this disease include abnormal use of antibiotics, which kill the good and beneficial bacteria, allowing yeast overgrowth, hormonal fluctuations as in pregnancy and use of oral contraceptives among others.

Ampicillin is an antibiotic use for prevention and treatment of a number of bacterial infections, It is active against many Gram-positive and Gram-negative bacteria. Ampicillin was the first broad spectrum' penicillin that is activity against Gram-(+) bacteria including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, some *Staphylococcus aureus*(but not penicillin-resistant or methicillin-resistant strains), and some *Enterococci*. Activity against Gram-(−) bacteria includes *Neisseria meningitidis*, some *Haemophilus influenzae*, and some Enterobacteriaceae (Hatil,

2009). Common side effects include rash, diarrhea, and nausea. In very rare cases it causes severe side effects such as angioedema, anaphylaxis and *Clostridium difficile* colitis.

Ciprofloxacin is an antibiotic used to treat a number of bacterial infections. This includes bone and joint infections, intra abdominal infections, certain type of infectious diarrhea, respiratory tract infections, skin infections, typhoid fever, and urinary tract infections, among others, Ciprofloxacin was introduced in 1987, and is among the listed essential drugs by World Health Organization's. it is effective against both Gram positive and Gram negative bacteria which includes Gram-negative (*Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*), and Gram-positive (methicillin-sensitive, but not methicillin-resistant *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Streptococcus pyogenes*) bacterial pathogens (Roberts *et al.,,* 2008)

Ofloxacin is a synthetic antibiotic of the fluoroquinolone drug class considered to be a second-generation fluoroquinolone. Ofloxacin is effective against aerobic Gram-positive microorganisms which include: *Staphylococcus aureus* (methicillin-susceptible strains), *Streptococcus pneumoniae* (penicillin-susceptible strains), *Streptococcus pyogenes* and aerobic Gram-negative microorganisms *Citrobacter koseri* (*Citrobacter diversus*) *Enterobacter aerogenes, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Neisseria gonorrhoeae, Proteus mirabilis, Pseudomonas aeruginosa* (Nissen *et al., 2000).*

Nystatin is an antifungal medication. It is used to treat *Candida* infections of the skin including diaper rash, thrush, esophageal candidiasis, and vaginal yeast infections. It may also be used to

prevent candidiasis in those who are at high risk. Nystatin may be used by mouth, in the vagina, or applied to the skin (A.S.H., 2016). Like amphotericin B and natamycin, nystatin binds to ergosterol, a major component of the fungal cell membrane. When present in sufficient concentrations, it forms pores in the membrane that lead to K^+ leakage, acidification, and death of the fungus.

Ketoconazole is an antifungal drug commonly used in the treatment of cutaneous and systemic infections; it is sold commercially as tablet for oral administration. Less toxic and generally more efficient triazole antifungal agent Fluconazole and thraconazole are usually preferred for systemic use. Ketoconazole has antifungal activity against many fungi that includes *Aspergillus, Candidas, coccidioides, Histoplasma* and *Blastomyces* (Rossi, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Sterile petri dishes, micropipettes, spreader, corkborer, peel of citrus fruits, antibiotic discs, clavenger apparatus, mortar and pestle, test tubes, nutrient agar, Muller Hintons agar, peptone water, separating funnel, ethanol, cotton wool, foil paper, swab stick, wire loop.

3.2 Sample Collection and Preparation

The fresh fully matured fruits of five species of Citrus: Sweet orange (*Citrus sinensis*), bitter orange (*Citrus aurantium*), lemon (*Citrus limon*), tangerine (*Citrus tangerine*) and grape (*Citrus paradisi*) were collected from Ipata market in Ilorin, Kwara State, Nigeria. The citrus varieties were identified at the Herbarium Unit of the Department of Plant Biology, University of Ilorin, Kwara State, Nigeria. The five citrus fruit species were washed with distilled water and then peeled off carefully with the help of a sharp razor blade to avoid any damage of oil glands. The peels were air-dried at constant temperature (30^0C) for 10 days, thereafter, the dried peels were blended using a blender and the powdered sample kept in a clean plastic container until required for extraction.

3.3 Collection and Maintenance of Test Isolates

Test isolates were obtained from the Department of Medical Microbiology and Parasitology, University of Ilorin Teaching Hospital, Kwara State, Nigeria. The organisms obtained were:

Methycillin Resistance *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. The organisms were sub cultured three times before their slant preparation and the prepared slants were refrigerated at 4ºC.

3.4 Preparation of Culture Media

3.4.1 Preparation of Potato Dextrose Agar

Preparation of potato dextrose agar (PDA) was done according to manufacturer's instruction. The composition of the media per litre was 4.0g of potato extract, 20.0g of dextrose and 15.0g of agar.

Potato Dextrose Agar was prepared according to manufacturer's prescription, 39 g of Potato Dextrose Agar powder was weighed using weighing balance and then transferred into a dry conical flask. Distilled/deionized water was added to make 1000ml and rocked gently to mix. The mouth of conical flask was plugged with cotton wool and covered with aluminium foil. The medium was heated in a water bath on hot plate to get homogenous mixture. The medium was sterilized in an autoclave for 121ºc for 15mins. It was then allowed to cool to around 45ºc before pouring it into Petri dishes. The plate was allowed to solidify before incubating.

3.4.2 Preparation of Mueller Hinton's Agar

Mueller Hinton Agar was prepared according to manufacturer's prescription, 38 g of Mueller Hinton Agar powder was weighed using weighing balance and then transferred into a dry conical flask. Distilled/deionized water was added to make 1000ml and rocked gently to mix. The mouth

of conical flask was plugged with cotton wool and covered with aluminium foil. The medium was heated in a water bath on hot plate to get homogenous mixture. The medium was sterilized in an autoclave for 121ºc for 15mins. It was then allowed to cool to around 45ºc before pouring it into Petri dishes. The plate was allowed to solidify before incubating.

3.4.3 Preparation of Peptone Water

Peptone water was prepared in 15g/litre. This was prepared by dissolving 15g of peptone water in 1000ml of distilled water. The mouth of conical flask was plugged with cotton wool and covered with aluminium foil. The medium was heated in a water bath on hot plate to get homogenous mixture and then pour in sterile test tubes. The medium was sterilized in an autoclave for 121ºc for 15mins. It was then allowed to cool before use.

3.5 Isolation and Extractions of Essential Oils from Citrus Peels

The washed citrus fruit was peeled, dried at room temperature to constant weight before oil extraction (Fig 3.1) Essential oils were extracted from all the five species of Citrus using modified clavenger apparatus. Using a round-bottomed flask, 200g of each citrus powder was mixed with 1 liter of water and then steam distilled. A flask containing the homogenate was heated for three to four hours at 100ºC and the oil was separated from water using a separating funnel. The essential oils were put in amber colored vials, labeled and stored in refrigerator at 4° C before bioassay (Tassou *et al*., 1995).

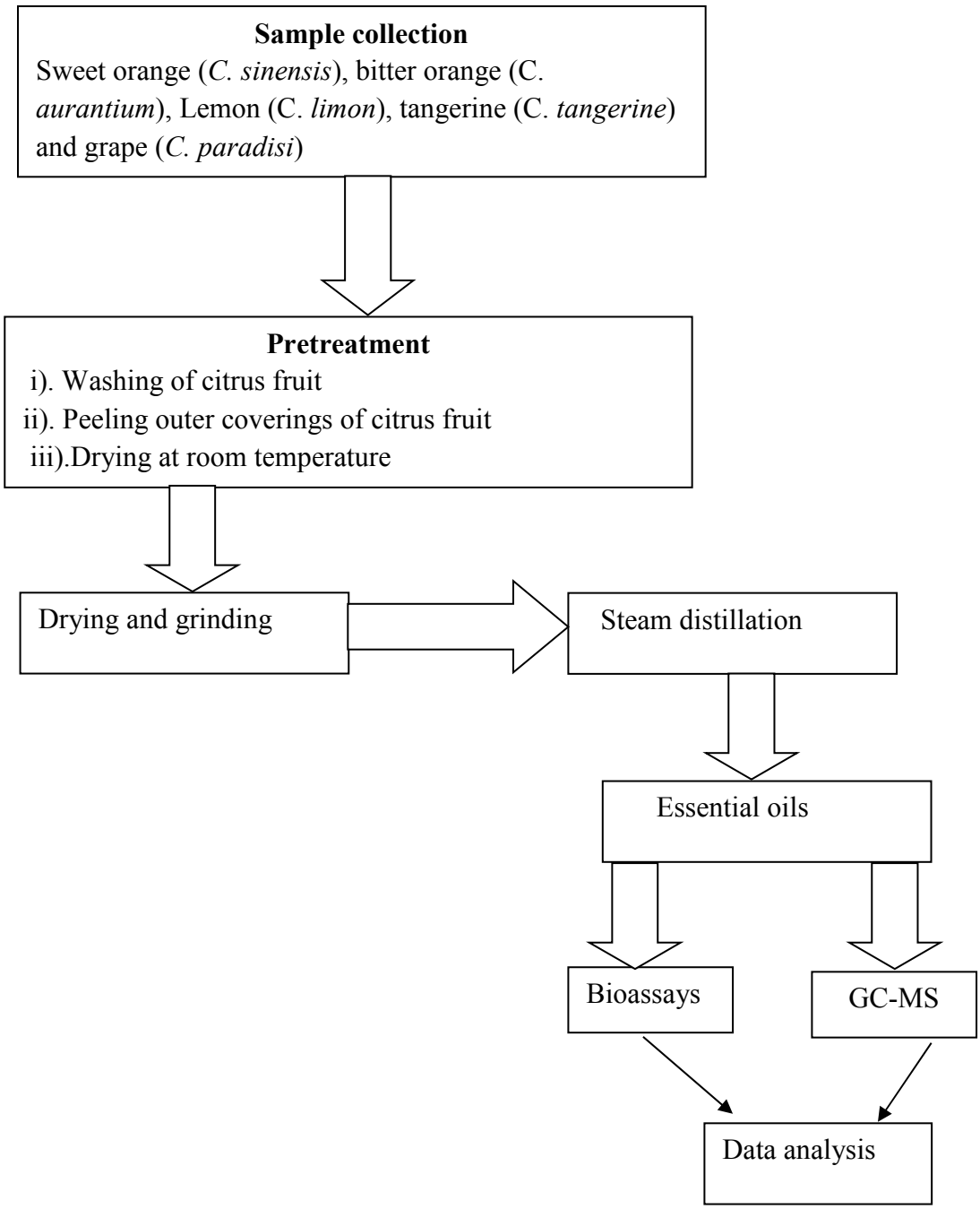

Figure 3.1: Flow chart showing citrus peel extract method adopted/employed

3.6 Preparation of Mcfarland Standard Solution

Half of a milliliter of 1% barium chloride was added to 99.5 ml of 1% tetraoxosulphate (VI) acid in the preparation of 0.5% McFarland and turbidity of resulting suspension was measured at 530 nM using spectrophotometer.

3.7 Standard Antibiotic Discs Used

Broad spectrum antibiotic discs were bought from Rapid Lab (Germany). The concentration of antibiotic discs used were as follow: Ciprofloxacin (CIP 5µg), Ampicillin (AMP 10µg), Ofloxacin (OFL 5µg), Ketoconazole (Ket 200mg) and Nystatin (3µg).

Zone size interpretative table accordance to CLSI for some antibiotics

Antimicrobial agents (AMAs)	Disc Conc.(µg)	Resistant (mm)	intermediate(mm)	Sensitivity(mm)
Ampicillin	30µg	≤ 13	14-16	≥ 17
Amikacin	30µg	≤ 14	15-16	≥ 17
Gentamicin	10µg	≤ 12	13-14	≥ 15
Norfloxacin	10µg	≤ 12	13-16	≥ 17
Ciprofloxacin	05µg	≤ 15	16-20	≥ 21
Cefuroxime	30µg	≤ 14	15-17	≥ 18
Ceftazidime	30µg	≤ 17	18-20	≥ 21
Cefpodoxime	30µg	≤ 17	18-20	≥ 21
Ceftriaxone	30µg	≤ 19	20-22	≥ 23
Cefepime	30µg	≤ 14	15-17	≥ 18
Cefoxitin	30µg	≤ 14	15-17	≥ 18
Amoxicillin/clavulanate	10µg	≤ 24	25-29	≥ 30
Ceftazidime/clavulanate	30/10µg	≤ 27	28-33	≥ 34
Piperacillin/tazobactam	75µg+10µg	≤ 17	18-20	≥ 21
Ticarcillin/clavulanate	75µg	≤ 14	15-19	≥ 20
Cefoperazone/sulbactum	75µg+30µg	≤ 27	28-32	≥ 33
Imipenam	10µg	≤ 19	20-22	≥ 23
Meropenam	10µg	≤ 19	20-22	≥ 23
Aztreonam	30µg	≤ 17	18-20	≥ 21
Nitrofurantoin	30µg	≤ 14	15-16	≥ 17
Co-trimoxazole	25µg	≤ 10	11-15	≥ 16
Imipenam / EDTA	10µg/750µg	≤ 25	26-30	≥ 31
Tetracycline	10µg	≤ 18	19-21	≥ 22
Penicillin G	10µg	≤ 28	-	≥29
Gentamicin	10µg	≤ 12	13-14	≥ 15
Vancomycin	30µg	-	-	≥ 15
Clindamycin	2µg	≤ 14	15-20	≥ 21
Erytromycin	15µg	≤ 13	14-22	≥ 23
Sulfametoxazole and Trimetoprim	15µg	≤ 10	11-15	≥ 16

3.8 Preparation of essential oil concentration

Varying concentrations (200, 250, 300, 350, 400, 450 and 500 µl/ml) of essential oil were prepared and dissolve in dimethyl sulfoxide (DMSO). To prepare a concentration of 200µl/ml, 200 µl of the extract was pipetted out in a test tube using a micropipette. Then it was diluted with 800 µl (making a total volume of 1 ml) of the solvent (DMSO). Similarly for 250µl/ml, Pipette out 250 µl of the extract and then dilute it with 750 µl of the DMSO and so on.

3.9 Antimicrobial Sensitivity Testing for Essential Oil

Prepared molten Mueller Hinton's agar for bacteria and potato dextrose agar for fungi were poured (20ml) into the Petri dishes and allowed to set. About 0.5 ml of standardized inoculums was drop onto the solidified agar surface and rocked so as to spread over the surface, cork borer of 6mm diameter was used to bore holes into the agar and 10 µl of prepared concentrations of extracts were poured into each hole and labeled. Plates were left to diffuse for 30minutes and incubated at 37^{o}C for 18hrs for bacteria and 28 ^{o}C for 72 hrs for fungi. The zones of inhibitions were measured in millimeter (mm) using transparent ruler. This was repeated in triplicates for each organism. (Bamidele *et al*., 2013).

3.10 Sensitivity Test for Standard Antibiotic Discs

Prepared molten Mueller Hinton's agar for bacteria and potato dextrose agar for fungi were poured into the Petri dishes and allowed to set. About 0.5 ml of standardized inoculums was drop onto the solidified agar surface and rocked so as to spread over the surface. The discs were placed on the surface of the agar with the aid of sterilized forceps. The plates were incubated at

37 ºC for 18 hrs for bacteria and 28 ºC for 72 hrs for fungi. The zones of inhibitions were measured in millimeter (mm) using transparent ruler. (Bamidele *et al*., 2013)

3.11 Determination of Minimum Inhibitory Concentration (MIC)

Prepared extracts were reconstituted, and three fold dilutions were made in accordance to antimicrobial sensitivity test i.e 200 µl/ml, 250 µl/ml, 300 µl/ml, 350 µl/ml, 400 µl/ml, 450 µl/ml and 500 µl/ml were made from the extract using dimethyl sulfoxide (DMSO). Two milliliter of sterile peptone water for bacteria and potato dextrose broth for fungi were inoculated with 0.5µl of organisms after addition of oil extract of each concentration, thus making three tubes of each of an extract for an organism. Broths free of organisms and extract with the other inoculated with organism were used as control. Test tubes containing nutrient broths were incubated at 37ºC for 24hrs while those with PDB were incubated at room temperature for 72hr. (Bamidele *et al*., 2013)

3.12 Determination of Minimum Bactericidal Concentration (MBC)

Sterile Mueller-Hinton agar plates were separately inoculated with bacterial inocula from each of the test tubes that showed no evidence of growth and swirled so as to spread over the surface. The plates were further incubated at 37ºC for 24 hours. The lowest dilution that yielded no bacterial growth was regarded as MBC (Orji *et al.,* 2012).

3.13 Determination of Minimum Fungicidal Concentration (MFC)

Sterile Potato dextrose agar plates were separately inoculated with sample from each of the test tubes that showed no evidence of growth and swirled so as to spread over the surface. The plates were further incubated at 27 ºC for 72hrs and observed the lowest dilution that yielded no fungi growth was regarded as MFC (Orji *et al.,* 2012).

3.14 Gas Chromatography (GC) Analysis

The composition of the fatty acids was determined using gas chromatography of the purified methylated sample (methylation was done using BF3 methanol). About 1µL was injected into a HP 5890 series II gas chromatograph fitted with a FID and an HP- FFAP capillary column. The helium carrier gas flow was 8ml/min. The detector temperature was 280ºC. The injector split 1:50 at 220ºC. A temperature programmer was used with an initial temperature of 160ºC held for 5 min, raised from 200 to 220 ºC at a rate of 2 ºC/ min and 220 ºC held for 30 min. The fatty acid methyl esters were identified by comparison with retention time of the known standards.

3.15 Data Analysis

The data of zones of inhibition obtained from essential oils were subjected to analysis of variance (ANOVA). Individual essential oil recorded activities which were less than 9 mm and their results were not subjected to ANOVA in accordance with CLSI guideline. The mean inhibition zones of various active essential oil against *P. aeruginosa*, *S. aureus, E. coli, streptococcus and Candida albicans* were compared. Treatment means showing significant difference ($p \leq 0.05$) were separated using Student-Newman-Keuls (SNK) at 5% significance level.

CHAPTER FOUR

RESULTS

4.1: Susceptibility of *Pseudomonas aeruginosa and Escherichia coli* to standard antibiotics

Table 1 shows antibiotic susceptibility testing against Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). The result showed that Ciprofloxacin (CPR), Gentamicin (GEN), Ofloxacin (OFL), Cefuroxime (CRX) Ceftazidime (CAZ) and Nitrofuratoin (NIT) had the zone of inhibitions of 26 mm, 26 mm, 24 mm, 22 mm, 20 mm and 16 mm respectively against *Pseudomonas aeruginosa* and Ampicillin (AMP) and Clavulanate/amoxicillin (AUG) had no zone of inhibition against *Pseudomonas aeruginosa.* Ciprofloxacin (CPR), Gentamicin (GEN), Ofloxacin (OFL), Ceftazidime (CAZ), Ampicillin (AMP), Nitrofuratoin (NIT) and Clavulanate/amoxicillin (AUG) had the zone of inhibitions of 30mm, 22mm, 30mm, 24mm, 18mm, 14mm and 18mm respectively against *Escherichia coli* and *Escherichia coli* had no zone of inhibition against Cefuroxime (CRX) which is in accordance with CLSI standard.

Table 1: Susceptibility of *Pseudomonas aeruginosa and Escherichia coli* to standard antibiotics

Standard Antibiotics	Zone of Inhibition (mm)	
	Pseudomonas aeruginosa	*Escherichia coli*
Ofloxacin	24.00±0.83 S	30.00±1.61 S
Ciprofloxacin	26.00±0.83 S	30.00±1.61 S
Gentamicin	26.00±0.83 S	22.00±0.82 S
Ceftriaxone	22.00±0.82 I	0.00±0.00 R
Ceftazidime	20.00±1.61 I	24.00±0.82 S
Ampicillin	0.00±0.00 R	18.00±1.41 S
Nitrofurantoin	16.00±1.41 I	14.00±1.41 R
Amoxycillin/Clavulanate	0.00±0.00 R	18.00±1.41 R

Data represent the mean± standard deviation of triplicate readings values

Key: S-Sensitive, I-Intermediate, R-Resistance

4.2: Susceptibility of *Streptococcus agalactiae* and Methycillin Resistance *Staphylococcus aureus* to standard antibiotics

Table 2 shows the result of the standard antibiotics against Gram positive bacteria (*Streptococcus agalactiae* and MRSA). The results showed that Ceftriaxone (CRX), had the highest zone of inhibition with 26 mm in diameter where Ceftazidime (CAZ), Gentamicin (GEN), Cefuroxime (CTR) Erythromycin (ERY), Cloxacillin (CXC), Ofloxacin (OFL) and Clavulanate/amoxicillin (AUG) had the zone of inhibitions of 18 mm, 20 mm, 20 mm and 24 mm respectively on *Streptococcus agalactiae* and *Streptococcus agalactiae* is resistance to Cefuroxime (CTR), Erythromycin (ERY) and Ofloxacin (OFL) which is in accordance with CLSI standard.

Table 2: Susceptibility of Methycillin Resistant *Staphylococcus aureus* and *Streptococus agalactiae* to standard antibiotics

Standard Antibiotics	Zone of Inhibition (mm)	
	MR Staphylococcus aureus	*Streptococcus agalactiae*
Ceftazidime	0.00±0.00 R	18.00±0.83 I
Ceftriaxone	0.00±0.00 R	26.00±0.82 S
Gentamicin	0.00±0.00 R	20.00±0.83 S
Cefuroxime	0.00±0.00 R	0.00±0.00 R
Erythromycin	0.00±0.00 R	0.00±0.00 R
Cloxacillin	0.00±0.00 R	20.00±0.83 S
Ofloxacin	0.00±0.00 R	0.00±0.00 R
Amoxycillin/Clavulanate	0.00±0.00 R	24.00±1.41 R

Data represent the mean± standard deviation of triplicate readings values

Key: S-Sensitive, I-Intermediate, R-Resistance

4.3: Susceptibility of *Candida albicans* to standard antibiotics

Table 3 shows the result of the standard antibiotics against *Candida albican*. The results showed that Cefuroxime (CRX) had the highest zone of inhibition with 28 mm in diameter where Nitrofuratoin (NIT), Gentamicin (GEN), Ciprofloxacin (CPR), Ofloxacin (OFL), Clavulanate/amoxicillin (AUG) and Ceftazidime (CAZ) had the zone of inhibitions of 27 mm, 26 mm, 24 mm, 20 mm, 20 mm and 18 mm respectively. Ampicillin (AMP) had no zone of inhibition against *Candida albicans* and other used antibiotics were sensitive on *Candida albicans* which is in accordance with CLSI standard.

Table 3: Susceptibility of *Candida albicans* to standard antibiotics

Standard Antibiotics	Zone of Inhibition (mm) *Candida albicans*
Ofloxacin	20.00±1.41 S
Ciprofloxacin	24.00±1.61 S
Gentamicin	26.00±1.41 S
Ceftriaxone	28.00±1.41 S
Ceftazidime	18.00±0.82 I
Ampicillin	0.00±0.00 R
Nitrofurantoin	27.00±1.41 S
Amoxycillin/Clavulanate	20.00±1.41 R

Data represent the mean± standard deviation of triplicate readings values

Key: S-Sensitive, I-Intermediate, R-Resistance

All the essential oils of the citrus species, according to the agar well diffusion test results are presented in Table 4. According to the results of the study, *Citrus aurantium* oil against the tested bacteria showed strong antimicrobial activity on all strains except *MRSA*. It has showed the highest activity especially on *C. albicans* (22 mm). It showed a lower activity against the *P. aeuruginosa* with the diameter of inhibition of 13 mm. Other oils have demonstrated antimicrobial effects of different levels. Essential oils of *Citrus limon* had no zone of inhibition on *Pseudomonas aeuruginosa* and *S. agalactiae*. Essential oil of *Citrus paradisii* showed the lowest activity on the *E. coli and S. agalactiae*, it has demonstrated highest activity on the *C. albicans* (18 mm) which is in accordance with CLSI standard.

Citrus sinensis showed highest activity on the *C. albicans* (19 mm) while it showed the lowest activity on *S. agalactiae* (13 mm) and *E. coli* (13 mm). The essential oil of *Citrus limon* showed the lowest activity on *E. coli* (13 mm), whereas it has the highest activity against *C. albicans* with diameter of 17 mm inhibition. It showed no activity on *S. agalactiae, P. aeuruginosa* and *MRSA* while essential oil of C*itrus tangerine* showed the highest activity on *P. aeruginosa* (16 mm) which is in accordance with CLSI standard.

Table 4: Antimicrobial Activities of Different Essential Oils (Stock Concentration)

Test	**Zone of Inhibitions (mm)**				
Organisms	*C. sinensis*	*C. paradisii*	*C. limon*	*C. tangerine*	*C. aurantium*
C. albicans	19±0.82	18±0.82	17±1.41	12±1.41	22±1.83
E. coli	13±1.41	00±0.00	13±1.41	13±1.41	15±0.82
S. agalactiae	13±1.83	11±1.83	00±0.00	12±1.83	14±1.83
P. aeuruginosa	14±1.41	15±1.41	00±0.00	16±0.82	13±1.41
MRSA	00±0.00	00±0.00	00±0.00	00±0.00	00±0.00

Data represent the mean± standard deviation of triplicate readings values

Table 5a: Zones of inhibition (in mm) of varying concentration of essential oils against *Pseudomonas aeruginosa*

Essential oils	500µg/ml	450µg/ml	400µg/ml	350µg/ml	300µg/ml	250µg/ml	200µg/ml
Citrus sinensis	13.00	10.00	0.00	0.00	0.00	0.00	0.00
Citrus paradissi	13.00	11.00	11.00	10.00	8.00	0.00	0.00
Citrus lemon	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Citrus tangerine	14.00	13.00	10.00	10.00	8.50	0.00	0.00
Citrus aurantium	10.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 5b: Zones of inhibition (in mm) of varying concentration of essential oils against *Streptococcus agalactiae*

Citrus sinensis	12.00	11.00	9.00	0.00	0.00	0.00	0.00
Citrus paradissi	10.00	9.00	9.00	0.00	0.00	0.00	0.00
Citrus lemon	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Citrus tangerine	12.00	12.00	10.00	9.00	9.00	8.00	8.00
Citrus aurantium	13.00	10.00	0.00	0.00	0.00	0.00	0.00

Table 5c: Zones of inhibition (in mm) of varying concentration of essential oils against *Escherichia coli*

Citrus sinensis	13.00	13.00	9.00	0.00	0.00	0.00	0.00
Citrus paradissi	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Citrus lemon	12.00	10.00	10.00	9.00	9.00	8.50	0.00
Citrus tangerine	13.00	12.00	11.00	11.00	9.00	9.00	0.00
Citrus aurantium	14.00	14.00	14.00	10.00	0.00	0.00	0.00

Table 5d: Zones of inhibition (in mm) of varying concentration of essential oils against *Candida albicans*

Citrus sinensis	16.00	15.00	13.00	0.00	0.00	0.00	0.00
Citrus paradisii	16.00	16.00	14.00	13.00	11.00	0.00	0.00
Citrus Lemon	17.00	14.00	12.00	11.00	0.00	0.00	0.00
Citrus tangerine	12.00	10.00	10.00	9.00	9.00	0.00	0.00
Citrus aurantium	+	+	+	+	+	-	-

The antibacterial susceptibility test of varying concentration of citrus oil against *Escherichia coli* are shown in Table 6. The observed turbidity varied from one organism to another and from one citrus oil concentrate to another, the Bacteriostatic and Bacteriocidal concentrations of the citrus oils against *Escherichia coli* showed the MIC values ranged from 350 to 500 μg/ mL, while MBC values is ≥500 μl/mL for all the citrus oil studied. The results obtained showed that the MBC values for the citrus oil concentrates were higher than their MIC values. Thus, suggesting that the concentrates were bacteriocidal at higher concentrations, but bacteriostatic at lower concentrations.

Table 6: Minimum inhibitory and bactericidal concentrations of essential oils against *Escherichia coli*

Essential Oils	MIC (µl/ml)	MBC (µl/ml)
Citrus sinensis	**350**	**>500**
Citrus paradisii	**0.0**	**0.0**
Citrus limon	**500**	**>500**
Citrus tangerine	**500**	**>500**
Citrus aurantium	**400**	**500**

The antibacterial susceptibility test of varying concentration of citrus oil against *Pseudomonas aeruginosa* are shown in Table 7. The observed turbidity varied from one organism to another and from one citrus oil concentrate to another, the Bacteriostatic and Bacteriocidal concentrations of the citrus oils against *Pseudomonas aeruginosa* showed the MIC values ranged from 250 to 450 μl/ mL, while MBC values ranged from 250 to 500 μl/mL for all the citrus oil studied. The results obtained showed that the MBC values for the citrus oil concentrates were higher than their MIC values. Thus, suggesting that the concentrates were bacteriocidal at higher concentrations, but bacteriostatic at lower concentrations.

Table 7: Minimum inhibitory and bactericidal concentrations of essential oils against *Pseudomonas aeruginosa*

Essential oils	MIC (µl/ml)	MBC (µl/ml)
Citrus sinensis	**300**	**400**
Citrus paradisii	**450**	**450**
Citrus limon	**0.0**	**0.0**
Citrus tangerine	**450**	**>500**
Citrus aurantium	**250**	**250**

The antibacterial susceptibility test of varying concentration of citrus oil against *Streptococcus agalactiae* are shown in Table 8. The observed turbidity varied from one organism to another and from one citrus oil concentrate to another, The Bacteriostatic and Bacteriocidal concentrations of the citrus oils against *Streptococcus agalactiae* showed the MIC values ranged from 300 to 500 μl/ mL, while MBC values ranged from 350 to 500 μl/mL for all the citrus oil studied. The results obtained showed that the MBC values for the citrus oil concentrates were higher than their MIC values. Thus, suggesting that the concentrates were bacteriocidal at higher concentrations, but bacteriostatic at lower concentrations.

Table 8: Minimum inhibitory and bactericidal concentrations of essential oils against *Streptococcus agalactiae*

Essential Oils	MIC (µl/ml)	MBC (µl/ml)
Citrus sinensis	**350**	**500**
Citrus paradisii	**350**	**350**
Citrus limon	**0.0**	**0.0**
Citrus tangerine	**500**	**>500**
Citrus aurantium	**300**	**>500**

The antibacterial susceptibility test of varying concentration of citrus oil against *Candida albicans* are shown in Table 9. The observed turbidity varied from one organism to another and from one citrus oil concentrate to another, The Bacteriostatic and Bacteriocidal concentrations of the citrus oils against *Candida albicans* showed the MIC values ranged from 350 to 500 μg/ mL, while MBC values ranged from 450 to 500 μg/mL for all the citrus oil studied. The results obtained showed that the MBC values for the citrus oil concentrates were higher than their MIC values. Thus, suggesting that the concentrates were bacteriocidal at higher concentrations, but bacteriostatic at lower concentrations.

Table 9: Minimum inhibitory and bactericidal concentrations of essential oils against *Candida albicans*

Essential Oils	MIC (µl/ml)	MFC (µl/ml)
Citrus sinensis	**350**	**450**
Citrus paradisii	**450**	**>500**
Citrus limon	**400**	**450**
Citrus tangerine	**450**	**>500**
Citrus aurantium	**500**	**>500**

4.10: Comparison of the Common Bioactive Pytochemicals in Citrus

The chemical constituents of these oils were analysed using gas chromatography coupled with mass spectrometry are shown in Figure 4.1. It revealed the presence of 11-(2-Cyclopenten-1-yl) undecanoic acid in these percentages 6.35%, 1.67%, 1.67%, 16.15% and 8.69. 9-Oxabicyclo[6.1.0] nonane in these percentages 30.21%, 0.57% 20.58%, 3.08 and 30.63 and 9-Octadecenal in these percentages 2.22%, 0.88%, 1.21%, 0.40% and 1.05% for sweet orange, grape fruit, lemon, tangerine and bitter orange respectively.

Appendix I shows that Hexane and 1-butene are 6.36% and 7.34% in sweet orange respectively, 7.69% and 7.83% grapefruit respectively but absent in lemon. Hexanoic acid was present in sweet orange with 1.8% and lemon 1.28% but absent in grape fruit.

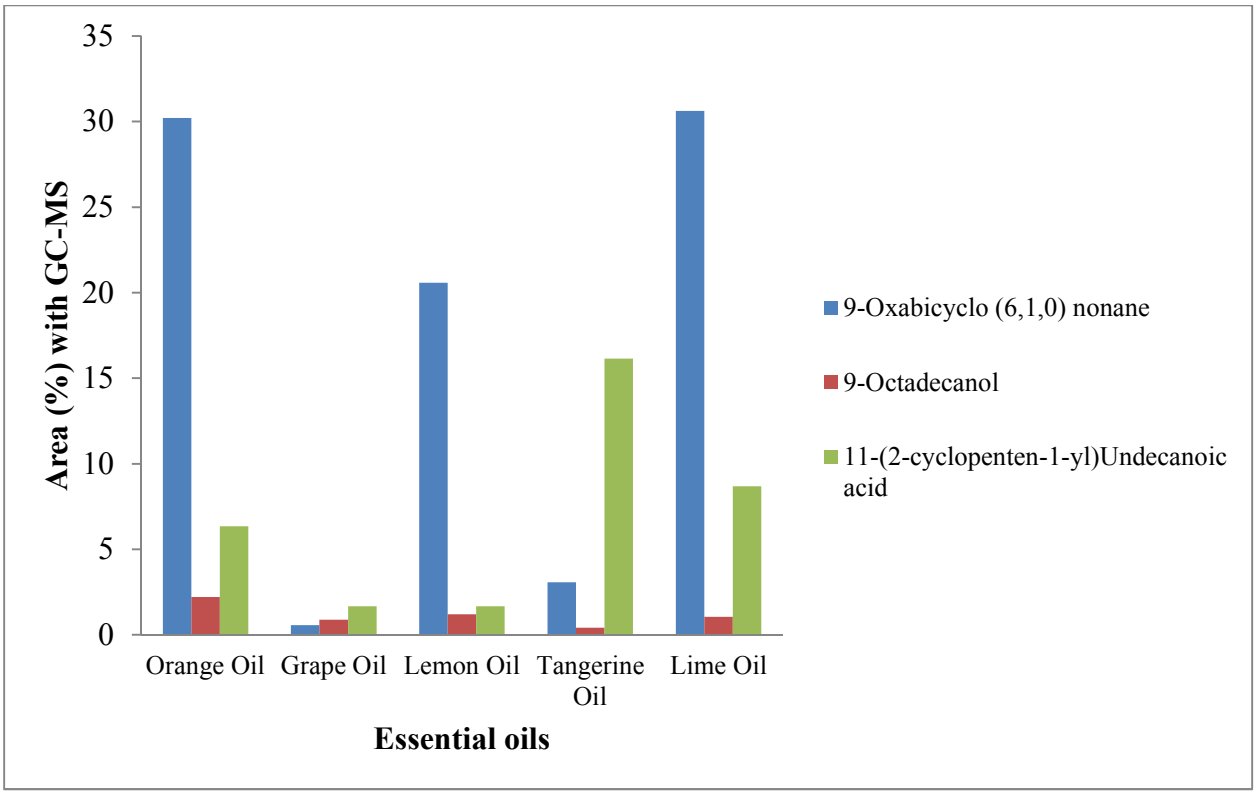

Figure 4.1: Comparison of the Common Bioactive Pytochemicals in Citrus

KEY: Orange oil- *Citrus sinensis*, Grape oil- *Citrus paradisii*, lemon oil- *Citrus lemon*, Tangerine oil- *Citrus tangerine*, Lime oil- *Citrus aurantium*

CHAPTER FIVE

DISCUSSION

Plants in general have the ability to synthesis compounds, while having no apparent function in primary metabolism. These are physiologically active against many organisms, so providing plants with one of their most important defense mechanisms. In recent years, there is increase in the use of herbal products as alternative to use of synthetic antibiotics and decrease in efficacy of antibiotics due to the development of resistance. These reports have fuelled the search among plants for potentially useful products to treat infections. The application of the oil on human at different part of the body had no significant detrimental effect on the human body Ezeonu *et al*., (2001).

Ciprofloxacin (CPX) and Ofloxacine (OFL) are the most active antibiotics against Gram negative bacteria. Ciprofloxacin and Gentamicin were specifically highly effective against *Pseudomonas aeruginosa* while Ciprofloxacin and Ofloxacin were found to be effective against *Escherichia coli.* Ampicillin (AMP) and Augmentin were not effective against the *Pseudomonas aeruginosa* and likewise cefuroxime against *Escherichia coli.* When compared with standard antibiotic (ciprofloxacin and gentamicin), *Pseudomonas aeruginosa*, was susceptible compare to *C. paradisii* and *C. tangerine* but not at the level of the standard antibiotics.

Cefuroxime (CRX) was shown in the study to be the most active antibiotic against *Streptococcus agalactiae* while methycillin resistance *Staphylococcus aureus* was resistant to all the antibiotic used and likewise applicable with the essential oils used. This is because it is a broad spectrum

antibiotic known for the purpose of treating infections caused by the Gram positive and Gram negative organisms from the result above. This is in line with the work of Evbuomwan *et al.* (2018).

Cefuroxime (CRX) was the most active antibiotic against *Candida albican,* yet not sensitive when compare with *C. aurantium*. This was similar with result of Anil *et al*. (2014) which reported *C. aurantium* and *C. aurantifolia* oils have showed potential antifungal activity against *C. albicans* from the result above.

The therapeutic use of plants especially as antimicrobials has been reported by González-Lamothe *et al.* (2009). Reports of antimicrobial activity of Sweet orange (*C. sinensis*), bitter orange (*C. aurantium*), Lemon (*C. limon*), tangerine (*C. tangerine*) and grape (*C. paradisi*) showed various levels of microbial growth inhibition against Gram positive, Gram negative bacteria and fungi. This indicates the broad spectrum nature of the plant extract. The present study shows that, *C. aurantium* is effective against *E. coli*. This was similar to work of Fullerton *et al.* (2011), as well as *Streptococcus agalactiae* and *Candida albicans* reported by Khalaphallah and Wagdi (2014). *Candida albicans* growth was however inhibited by the citrus oil. This present report is in agreement with the work of Rico-Molina *et al*. (2012). This essential oil (*C. aurantium*) is especially important in light of the increasing incidence of candidiasis infections as well as the emergence of resistant Candida species to existing antifungal drugs (Bardaweel *et al*., 2014). In the present result, *MRSA* showed high resistance to all the essential oils used and this is in agreement with the work of Mondello *et al*. (2005). Augmentin and Ampicillin were highly resistant to *P. aeruginosa* and this was in aggrement with work of Abu-

Shanab *et al*. (2004) which reported that infections caused by *P. aeruginosa* are among the most difficult to treat with conventional antibiotics. This was not similar when compare with essential oil derived from the peel of *C. tangerine* which tends not to have antimicrobial effect against *P. aeruginosa* when compare with standard antibiotic and CLSI standard.

The study shows that varying concentrations of tangerine, grape and sweet orange oil significantly inhibited the growth of *P. aeruginosa*. These essential oils may therefore hold promise in the management of *P. aeruginosa* infection. The grape, tangerine and lime oil showed good bacteriocidal activities against *C. albicans*. Lime oil showed good bacteriocidal activities against *P. aeruginosa* and *E. coli*. Tangerine oil showed good bacteriocidal activities against *P. aeruginosa, S. agalactiae*, *E. coli* and *C.albicans*. Sweet orange oil showed good bacteriocidal activities against *P. aeruginosa, S. agalactiae*, and *C.albicans*. Grape oil showed good bacteriocidal activities against *P. aeruginosa* and *S. agalactiae* and Lemon oil showed good bacteriocidal activities against *C. albicans* according to the result obtained in this study, though it was against the report of Narasimhan *et al*. (2006) which reported that essential oils in the 100% can only be effective against pathogenic organisms but in accordance with the report of Liu and Huan (2012) which reported that varying concentrations of *Cirus aureantium and Citrus limon* were effective against *Escherichia coli* and *Klebsiella Pneumoniae*.

Among the five plant peel used in this study (Sweet orange (*C. sinensis*), bitter orange (*C. aurantium*), Lemon (*C. limon*), tangerine (*C. tangerine*) and grape (*C. paradisi*)). *C. aurantium* was found to be most active against the tested organisms than other citrus essential oil of other

citrus plant peel extracts. It is of broad spectrum which was in accordance with the work reported by Narasimhan *et al*. (2006).

The results of this study showed that lemon oil was least effective as an antibacterial but effective as an antifungal agent. This is evidenced by the smaller zones of inhibition and larger MIC with no MBC values on bacteria which was in accordance with Kotan *et al*. (2007).

The chemical constituents of these oils were analysed using gas chromatography coupled with mass spectrometry. It revealed the presence of 11-(2-Cyclopenten-1-yl) undecanoic acid, 9-Oxabicyclo [6.1.0] nonane and 9-Octadecenal. *Citrus* species are known to contain chemicals that exhibit different properties (toxicity, deterrence) against microorganisms. The results, suggest that peels of *citrus aurantium* contain chemicals with antimicrobial properties and this is in accordance with the work of Kotan *et al*. (2007) as a result of the antagonistic and synergistic effect of combinations of compounds present in the oils that responsible for the antimicrobial activity. The observed variations in the activities of oils are due to the mixture of compounds, the proportion of the compounds and their quantities present in oils.

The bioactive compounds identified by GC–MS analysis from essential oils of selected Citrus oils have also been reported by many researchers as compounds possessing antimicrobial activity. The synergistic effect of combinations of compounds present in the oils may be responsible for the antimicrobial activity. The observed variations in the activities of oils may be due to the mixture of compounds and their quantities present in oils.

Many researchers across the globe have reported the various bioactive compounds present in the essential oils and plant extracts. Antimicrobial activity of 7-octenoic acid against Gram-positive, Gram-negative bacteria and fungi was reported by Omawunmi (1989) and Saddiq and Khayyat (2010). Siti Humeirah *et al*. (2010) reported the antimicrobial activity of oleic and Kotan *et al*. (2007) noted the antimicrobial activity of myristoleic. In the present study, *Citrus aurantium, Citrus sinensis, Citrus paradisii,* and *Citrus tangerine* oils have 7-octenoic acid, Oleic acid and 1-butene as their key constituents, and may be responsible for antimicrobial activity. Karlovic *et al*. (2000) proved antimicrobial activity of Butylamine group against *S. aureus*. In this study, *Citrus sinensis* and *Citrus paradisii* contain Butylamine group, suggesting that Butylamine group is a proved bioactive compound present in the essential oils. Loughlin *et al.* (2008), Mondello *et al.* (2006) and Lin *et al*. (1986) have reported the antifungal and antibacterial activity of Cyclopentanedecanoic acid, and in the present study it was observed that *Citrus aurantium, Citrus sinensis, Citrus paradisii, Citrus limon* and *Citrus tangerine* oil also contain Cyclopentanedecanoic acid, thus both oils may influence the antimicrobial properties. Siti-Humeirah *et al.* (2010), Kotan *et al*. (2007) and Lin *et al*. (1986) reported the antimicrobial activity of Pentanoic acid group; this study also revealed that *Citrus aurantium, Citrus sinensis, Citrus paradisii,* and *Citrus tangerine* and *Citrus tangerine* oils contain Pentanoic acid group, and this proved antimicrobial agent present in the oils may be responsible for antimicrobial activity.

Rico-Molina *et al*. (2012) reported that lactose in combination with galactopyranoside inhibited the growth of some human pathogenic bacteria. Huang *et al*. (2009) reported that trehalose and lactose alone has antibacterial and antifungal properties, and Eyambe (2011) reported the anti-

staphylococcal ability of galactopyranoside. In the present study, galactopyranoside was shown in *Citrus aurantium, Citrus limon, Citrus tangerine* and lactose was shown in *Citrus limon,* and *Citrus tangerine* oil: the presence of these compounds may make the oils antimicrobial.

Kotan *et al*. (2007) reported the antibacterial and antifungal properties of Oleic acid. In the present study, *Citrus aurantium, Citrus sinensis* and *Citrus paradisii* oil, having this compound, may suggest its antimicrobial properties. *Citrus aurantium* has both Myristoliec acid and Oleic acid which made it better has antimicrobial property better than *Citrus paradisii* which has on Myristoleic acid and *Citrus sinensis* that has only oleic acid.

Similar antimicrobial activity was observed with *Citrus aurantium, Citrus paradisii,* and *Citrus tangerine* with all test organisms. In support of this, GC–MS analysis revealed that 1-butene, Hexane and 7-Octenoic acid are commonly present in the essential oils of the citrus, but additionally *Citrus aurantium, Citrus paradisii,* and *Citrus sinensis* have Myristoleic acid and Oleic acid, and *Citrus tangerine* and *Citrus limon* have only pentose sugar (Trehalose and D-allose). This difference in the availability of additional compounds proved *Citrus aurantium, Citrus paradisii,* and *Citrus sinensis* oil as a better antimicrobial than *Citrus tangerine* and *Citrus limon*. This may be reason for very much lower antimicrobial activity against all the tested microbes. Generally, antimicrobial activities of essential oils are difficult to correlate with a specific compound due to their complexity and variability; nevertheless, some researchers stated that there is association between the chemical composition of the most predominant components in the essential oils and the antimicrobial activities.

5.2 Conclusion

This research work has shown that citrus plant has potential bioactive phytochemicals that may be responsible for its antibacterial activities. It has also proven that *citrus aurantium* and *citrus sinensis* contained more potent antibacterial substances than conventional antibiotics against *Pseudomonas aeruginosa*, *Escherichia coli, streptococcus agalactiae* and *candida albicans*. The results revealed that *citrus aurantium* showed higher inhibitory effects on most of the test organisms when compared with other extracts, hence appraising chromatography as a better purification method.

5.3 Recommendation

Based on this research the following are recommended;

The test plants used in this study are well known for their fruits having commercial value. Agriculture practices should be slightly changed in such a way that the leaves can be collected during non-productive seasons to extract essential oil and this will help the farmers with additional financial benefits. Further *in vivo* studies are essential to develop alternative and safe therapeutic for antibiotics by using proven antimicrobial compounds from essential oils.

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APPENDIX

Appendix I: GC-MS Analysis of Citrus peels

Appendix i: Chemical composition of oil extracted from peels sweet orange oil

Peak Number	RT	Compound Name	Area %
1	50.347	2,4-Dimethylhept-1-ene	7.19
2	56.135	9-Oxabicyclo[6.1.0]nonane	30.21
3	56.721	1,1'-Bicyclopropyl	6.72
4	57.088	1-Butene,	2.18
5	57.417	Hydrazine	1.13
6	57.527	Cyclobutane	0.84
7	58.516	1-Butene	0.93
8	60.165	Butyl amine	0.94
9	65.221	1-Propene	3.07
10	66.430	2-Butanamine	0.77
11	70.276	10-Triepoxydecane Hydrazine	0.88
12	71.082	11-(2-Cyclopenten-1-yl)undecanoic acid	6.35
13	74.233	o-Allylhydroxylamine	0.83

14	74.746	Isobutylamine	0.87
15	75.002	Pent-2-ynal 4-Cyclopentene-1	0.81
16	76.284	Propargyl alcohol	0.85
17	78.226	4-Pentenoic acid ethylester	1.03
18	78.666	2-Octyn-1-ol	0.84
19	80.351	7-Octenoic acid	0.82
20	80.607	1-Hexene	0.83
21	81.596	1-Butene	1.07
22	82.952	1,8-Nonadiene	1.04
23	83.978	7-Octenoic acid 9-Decen-1-ol	0.83
24	84.601	Hexane	0.89
25	84.747	9-Decen-1-ol	1.12
26	85.297	7-Octenoic acid	1.23
27	85.553	2-Pyridinemethanamine	0.80
28	85.810	*6-Bromohexanoic acid	1.64
29	85.993	*Trichloroacetic acid	1.01
30	86.103	*9-Octadecenal	2.22

31	86.469	Methyl 4,6-ethylidene-.alpha.-d-ga lactopyranoside	0.99
32	87.348	Methyl 4,6-ethylidene-.alpha.-d-ga lactopyranoside	0.92
33	88.191	7-Octenoic acid	1.01
34	88.484	11-(2-Cyclopenten-1-yl)undecanoic acid	0.76
35	88.814	*7-Octenoic acid	1.17
36	89.253	*Undecanoic acid	1.36
37	89.473	*7-Octenoic acid	1.07
38	90.132	9-Octadecenal	0.82
39	90.462	Trimethylsilyl-di(timethylsiloxy) silane	1.45
40	90.682	7-Octenoic acid	0.83
41	90.828	Oleic Acid	1.07
42	92.294	*Cyclononene 1,2-Epoxy-5	1.01
43	92.734	7-Octenoic acid	0.88
44	93.320	11-(2-Cyclopenten-1-yl)undecanoic acid	0.87

45	93.833	7-Octenoic acid	0.79
46	95.774	7-Octenoic acid	1.23
47	96.470	7-Octenoic acid	0.79
48	96.910	11-(2-Cyclopenten-1-yl)undecanoic acid	0.95
49	98.449	7-Octenoic acid	1.07

Appendix ii: Chemical composition of oil extracted from peels grape oil

Peak Number	RT	Compound Name	Area %
1	50.420	Heptanoic acid	6.05
2	54.304	Thiirane	0.68
3	54.743	2-Propenamide	0.93
4	56.245	cis-1-Methyl-2-(2'-propenyl)cyclop ropane	32.27
5	56.831	4-Aminocyclohexanone	7.14
6	57.234	*1,2:4,5:9,10-Triepoxydecane	1.43
7	57.344	2-Butynal	1.35
8	57.491	1-Butene	1.76
9	57.747	1,2:4,5:9,10-Triepoxydecane oxirane	1.35
10	57.967	1,2:4,5:9,10-Triepoxydecane	1.75
11	58.260	* 1,5-Hexadiene	1.46
12	58.590	11-(2-Cyclopenten-1-yl)undecanoic acid	1.30
13	58.883	1-Butene	2.77
14	59.213	1-Butene	0.96
15	59.616	1,5-Hexadiene	0.89

16	59.835	3-Hexen-1-ol Cyclohexane	1.12
17	60.275	*1,2:4,5:9,10-Triepoxydecane	1.63
18	60.788	1-Butene	0.56
19	61.228	1,3-Butadiene	0.86
20	64.012	1,2:4,5:9,10-Triepoxydecane	0.47
21	65.257	11-(2-Cyclopenten-1-yl)undecanoic acid	2.69
22	65.441	1,5-Pentanediol	0.62
23	66.027	*sec-Butylamine	0.64
24	66.393	1-Butene	0.65
25	68.408	Phosphonous dibromide	0.56
26	69.837	1-Butene	0.57
27	70.313	1,5-Pentanediol	0.74
28	71.119	Methyl-2-(2'-propenyl)cyclopropane	6.49
29	73.793	11-(2-Cyclopenten-1-yl)undecanoic acid	0.70
30	74.233	Hexane	1.03
31	77.713	*9-Oxabicyclo[6.1.0]nonane	0.57

32	79.069	11-(2-Cyclopenten-1-yl)undecanoic acid	1.05
33	79.508	3-Octen-1-ol	1.26
34	80.314	1-Trifluoroacetoxy-10-undecene	1.55
35	81.157	9-Oxononanoic acid	0.98
36	82.659	11-(2-Cyclopenten-1-yl)undecanoic acid	0.48
37	82.806	1,8-Nonadiene	0.48
38	83.245	Methyl 4,6-ethylidene-.alpha.-d-ga lactopyranoside	1.58
39	84.014	Dodecen-1-ol	1.08
40	84.381	1-Hexadecanol Dichloroacetic acid	0.56
41	84.967	7-Octenoic acid	0.68
42	85.223	*Z,Z-2,5-Pentadecadien-1-ol	1.40
43	85.956	Cyclopentaneundecanoic acid	1.23
44	86.066	Cyclopentaneundecanoic acid	1.39
45	86.322	9-Octadecenal Trichloroacetic acid	0.88
46	87.861	9-Decenoic acid	0.92

47	93.869	7-Octenoic acid	0.73
48	95.884	*.alpha.-D-Glucopyranose	0.75
49	98.009	9,12-Octadecadienoyl chloride	0.53
50	98.449	Myristoleic acid	0.51

Appendix iii: Chemical composition of oil extracted from peels lemon oil

Peak Number	**RT**	**Compound Name**	**Area %**
1	50.384	Hexanoic acid	3.47
2	56.245	9-Oxabicyclo[6.1.0]nonane	20.58
3	56.795	* 1,5-Hexadiene	3.10
4	65.221	11-(2-Cyclopenten-1-yl)undecanoic acid	2.49
5	66.430	2-Penten-1-ol	1.33
6	71.082	9-Oxabicyclo[6.1.0]nonane oxirane	7.21
7	80.388	1,5-Hexadiene	1.15
8	81.084	9-Oxabicyclo[6.1.0]nonane	1.03
9	81.340	9-Oxabicyclo[6.1.0]nonane	1.23
10	81.560	9-Octadecenal Chloroacetic acid	0.91
11	81.706	11-(2-Cyclopenten-1-yl)undecanoic acid	1.41
12	82.366	11-(2-Cyclopenten-1-	0.84

		yl)undecanoic acid	
13	82.659	9-Octadecenal	1.21
14	83.135	*Methyl 4,6-ethylidene-.alpha.-d-galactopyranoside	1.96
15	83.392	11-(2-Cyclopenten-1-yl)undecanoic acid	0.97
16	83.611	Methyl 4,6-ethylidene-.alpha.-d-ga lactopyranoside	1.65
17	83.868	*Cyclononene 9-Decen-1-ol	1.88
18	84.051	11-(2-Cyclopenten-1-yl)undecanoic acid	1.62
19	84.198	7-Octenoic acid	1.24
20	84.381	*Dodecanoic acid	2.00
21	84.711	Methyl 4,6-ethylidene-.alpha.-d-ga lactopyranoside	2.22
22	84.820	*Lactose	1.64
23	85.077	*9-Decen-1-ol	1.68
24	85.187	9-Oxabicyclo[6.1.0]nonane	1.34
25	85.370	Trimethylsilyl-di(timethylsiloxy) silane	1.35
26	85.553	Methyl d-glycero-.beta.-d-gulo-heptoside	1.87
27	85.663	*Cyclopentaneundecanoic acid	1.12
28	85.846	Methyl 4,6-ethylidene-.alpha.-d-galactopyranoside	1.67
29	85.993	*9,12-Octadecadienoyl	2.66

		chloride	
30	86.176	*Pentanoic acid	1.63
31	86.322	Pentanoic acid	1.18
32	86.579	*Trehalose	1.40
33	86.835	*Bicyclo[4.1.0]heptane,-3-cycloprop yl	1.61
34	87.238	*Trehalose	1.19
35	87.348	Methyl 4,6-ethylidene-.alpha.-d-ga lactopyranoside	1.41
36	87.751	7-Octenoic acid	2.46
37	87.934	trans-Traumatic acid	1.27
38	88.081	Methyl 4,6-ethylidene-.alpha.-d-galactopyranoside	1.91
39	88.447	Methyl 4,6-ethylidene-.alpha.-d-galactopyranoside	1.85
40	88.777	Methyl 4,6-ethylidene-.alpha.-d-galactopyranoside	1.23
41	88.960	1-Heptafluorobutyryloxy-10-undecen	1.14
42	89.070	Methyl 4,6-ethylidene-.alpha.-d-galactopyranoside	0.85
43	89.327	Oleic Acid	1.03
44	89.400	Methyl 4,6-ethylidene-.alpha.-d-galactopyranoside	0.83
45	89.620	4-Chloro-3-n-butyltetrahydropyran	1.04

46	89.949	7-Octenoic acid	1.14
47	90.133	Pentanoic acid	1.41
48	90.499	Methyl 4,6-ethylidene-.alpha.-d-galactopyranoside	0.86
49	90.975	9-Decenoic acid	0.78
50	91.891	9-Octadecenal	0.96

Appendix iv: Chemical composition of oil extracted from peels tangerine oil

Peak Number	RT	Compound Name	Area %
1	45.841	* 1H-Inden-1-one	21.39
2	46.280	* 1-Cyclohexylnonene	18.12
3	47.892	2-Cyclopenten-1-one	15.39
4	50.457	*D-Allose	3.85
5	56.282	11-(2-Cyclopenten-1-yl)undecanoic acid	16.15
6	56.831	1-Octyn-3-ol	3.57
7	57.381	1,2:4,5:9,10-Triepoxydecane	1.46
8	57.674	11-(2-Cyclopenten-1-yl)undecanoic acid	0.34
9	57.784	1,2:4,5:9,10-Triepoxydecane	0.79
10	58.004	1,6-Hexanediol	0.47
11	58.297	1,2:4,5:9,10-Triepoxydecane	0.90
12	58.516	1,2:4,5:9,10-Triepoxydecane	0.75
13	58.883	* Oxirane	0.49
14	58.993	3-Hexen-1-ol	0.75
15	59.359	1,5-Hexadiene	0.35
16	59.542	*1,2:4,5:9,10-Triepoxydecane	0.26
17	59.725	1,2:4,5:9,10-Triepoxydecane	0.32
18	59.982	1,2:4,5:9,10-Triepoxydecane	0.47
19	60.202	*4-Cyclopentene-1	0.80
20	60.788	*1,2:4,5:9,10-Triepoxydecane 1-Butene	0.21

21	61.521	Cyclopropane	0.41
22	61.887	*1-Butene	0.28
23	64.012	*1,5-Hexadiene	0.32
24	65.221	11-(2-Cyclopenten-1-yl)undecanoic acid	0.93
25	66.466	*4-Cyclopentene-1,3-diol	0.48
26	66.796	*1-Butene	0.25
27	70.313	11-(2-Cyclopenten-1-yl)undecanoic acid	0.36
28	71.082	9-Oxabicyclo[6.1.0]nonane	3.08
29	76.797	Z-1,9-Hexadecadiene	0.23
30	77.823	*3-Octen-1-ol	0.34
31	77.933	1,14-Tetradecanediol	0.24
32	78.519	*1-Hexene	0.48
33	80.314	9-Octadecenal	0.40
34	83.355	*cis-3-Nonen-1-ol	0.36
35	84.014	Cyclopentaneundecanoic acid	0.64
36	84.527	Cyclopentaneundecanoic acid	0.31
37	85.004	Cyclopentaneundecanoic acid	0.24
38	85.443	Cyclopentaneundecanoic acid	0.34
39	85.956	Cyclopentaneundecanoic acid	0.52
40	87.934	Cyclopentaneundecanoic acid	0.26
41	88.777	11-(2-Cyclopenten-1-yl)undecanoic acid	0.22
42	88.997	1-Octyn-3-ol	0.27
43	90.426	11-(2-Cyclopenten-1-	0.24

		yl)undecanoic acid	
44	90.902	Cyclopentaneundecanoic acid	0.22
45	92.111	Methyl 4,6-ethylidene-.alpha.-d-ga lactopyranoside	0.28
46	92.624	9-Octadecenal	0.23
47	93.503	Methyl d-glycero-.beta.-d-gulo-heptoside	0.35
48	94.419	Methyl d-glycero-.beta.-d-gulo-heptoside	0.36
49	96.324	Lactose	0.21
50	98.156	11-(2-Cyclopenten-1-yl)undecanoic acid	0.33

Appendix v: Chemical composition of oil extracted from peels bitter orange oil

Peak Number	**RT**	**Compound Name**	**Area %**
1	50.457 5.18	Cyclohexanebutanoic acid	5.18
2	56.282	9-Oxabicyclo[6.1.0]nonane	30.63
3	56.832	1-Hexene	3.00
4	58.883	* 5,10-Dioxatricyclo	0.64
5	60.275	1,6-Heptadiene	1.02
6	64.012	11-(2-Cyclopenten-1-yl)undecanoic acid	0.68
7	65.258	3-Octen-1-ol	4.45
8	69.361	* 2-Hexene	0.69
9	69.983	11-(2-Cyclopenten-1-yl)undecanoic acid	0.79
10	70.350	Phosphonous dibromide	1.15
11	71.119	11-(2-Cyclopenten-1-yl)undecanoic acid	8.69
12	71.852	9-Oxabicyclo[6.1.0]nonane	0.60
13	73.281	9-Oxabicyclo[6.1.0]nonane	0.58
14	74.306	9-Oxabicyclo[6.1.0]nonane	0.56
15	80.937	9-Oxabicyclo[6.1.0]nonane	0.93
16	81.267	Cyclopentaneundecanoic acid	0.72
17	81.743	*9-Decen-1-ol	0.85
18	82.000	Pentanoic acid	0.76
19	82.989	*2-Methyl-Z,Z-3	0.75
20	83.429	Undecanoic acid	2.31

21	83.538	Myristoleic acid	0.85
22	84.344	Cyclopentaneundecanoic acid	0.65
23	84.711	Cyclopentaneundecanoic acid	1.03
24	85.297	Cyclopentaneundecanoic acid	1.46
25	86.616	Cyclopentaneundecanoic acid	0.67
26	87.642	*9-Decen-1-ol	0.72
27	88.191	11-(2-Cyclopenten-1-yl)undecanoic acid	2.13
28	89.363	Cyclopentaneundecanoic acid	0.95
29	90.755	Oleic Acid	0.82
30	90.865	Cyclopentaneundecanoic acid	0.93
31	91.049	Paromomycin	0.65
32	91.305	Cyclopentaneundecanoic acid	0.77
33	92.001	Cyclopentaneundecanoic acid	0.68
34	92.551	Cyclopentaneundecanoic acid	0.61
35	92.990	Cyclopentaneundecanoic acid	1.16
36	93.173	11-(2-Cyclopenten-1-yl)undecanoic acid	0.64
37	93.503	9-Oxabicyclo[6.1.0]nonane	4.89
38	93.796	9-Oxabicyclo[6.1.0]nonane	1.82
39	94.016	Undecanoic acid	3.95
40	94.492	Cyclopentaneundecanoic acid	0.85
41	94.675	9-Oxabicyclo[6.1.0]nonane	1.46
42	96.031	9-Octadecenal	1.05
43	96.141	Cyclopentaneundecanoic acid	0.57
44	96.434	Cyclopentaneundecanoic acid	1.18

45	96.764	9-Oxabicyclo[6.1.0]nonane	0.59
46	97.240	Cyclopentaneundecanoic acid	0.76
47	97.606	11-(2-Cyclopenten-1-yl)undecanoic acid	0.71
48	97.936	Cyclopentaneundecanoic acid	0.95
49	98.192	Oleic Acid	0.77
50	99.035	Cyclopentaneundecanoic acid	0.76

Appendix 2: Plates of the MIC varying concentration of Antimicrobial analysis

Plate i: MIC analysis of varying concentration

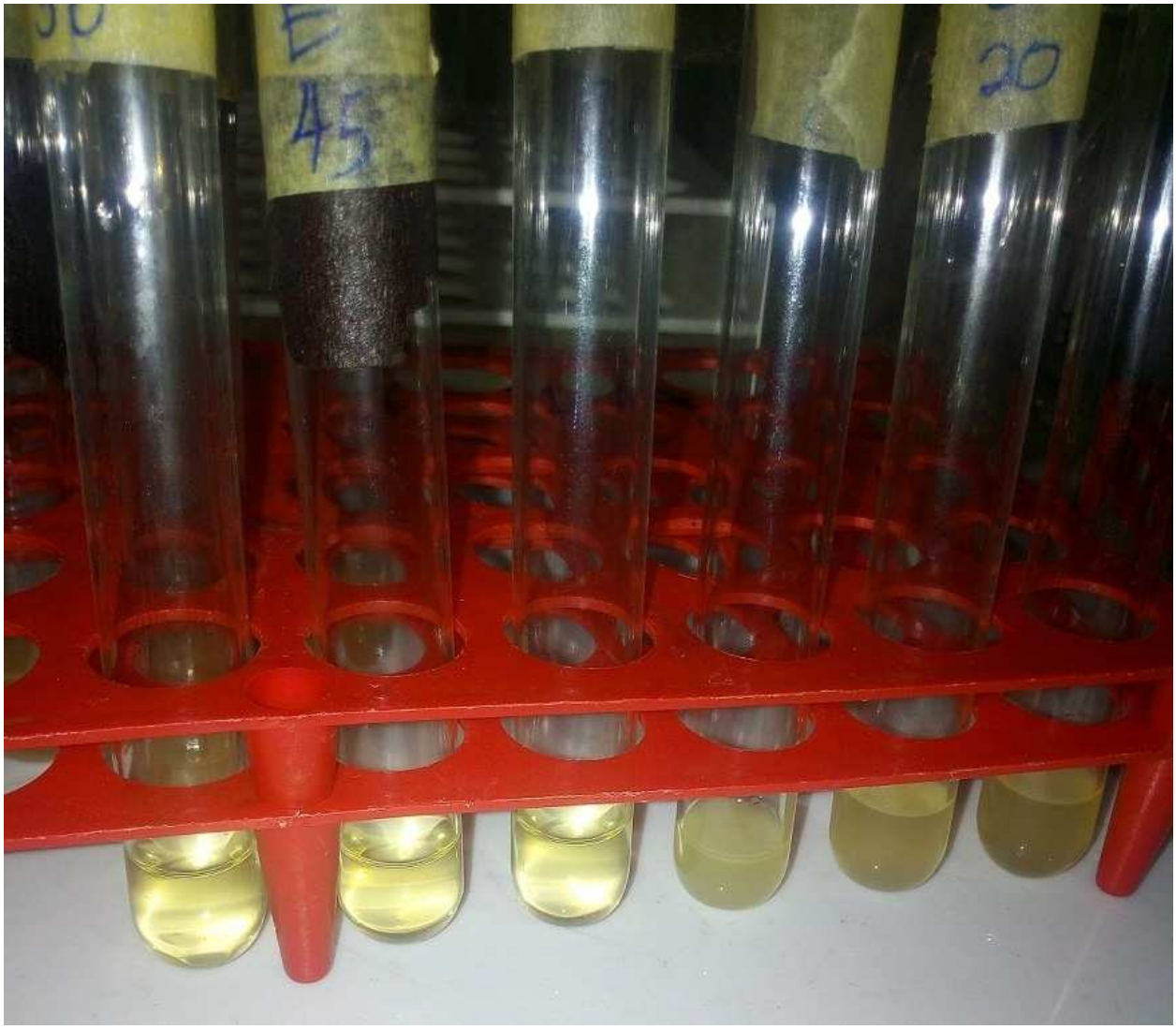

Plate ii: MIC analysis of varying concentration

Plate iii: Modified Clevenger-type apparatus used for extraction of essential oils

Appendix 3: Figure of the chromatogram showing the peak of each of the citrus peels

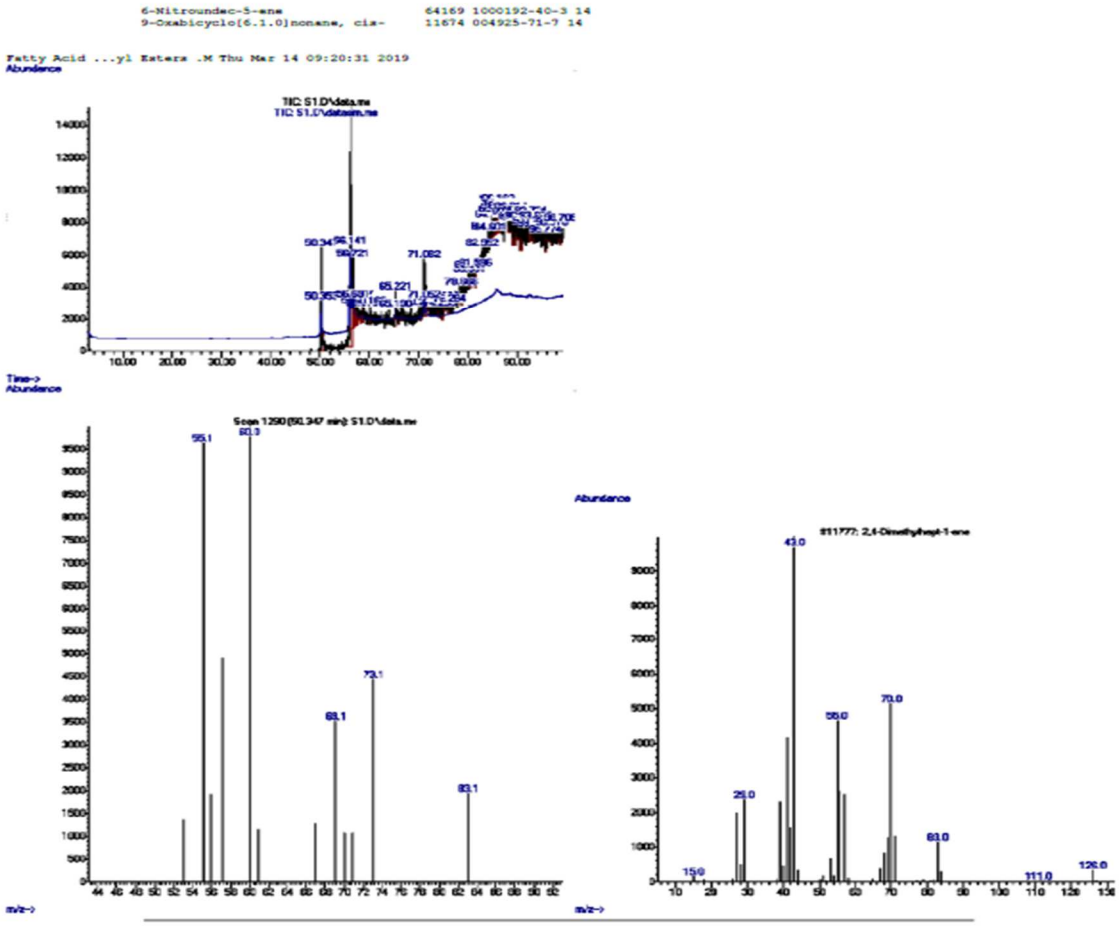

Figure 5: Chromatogram of *Citrus sinensis* using GC-MS

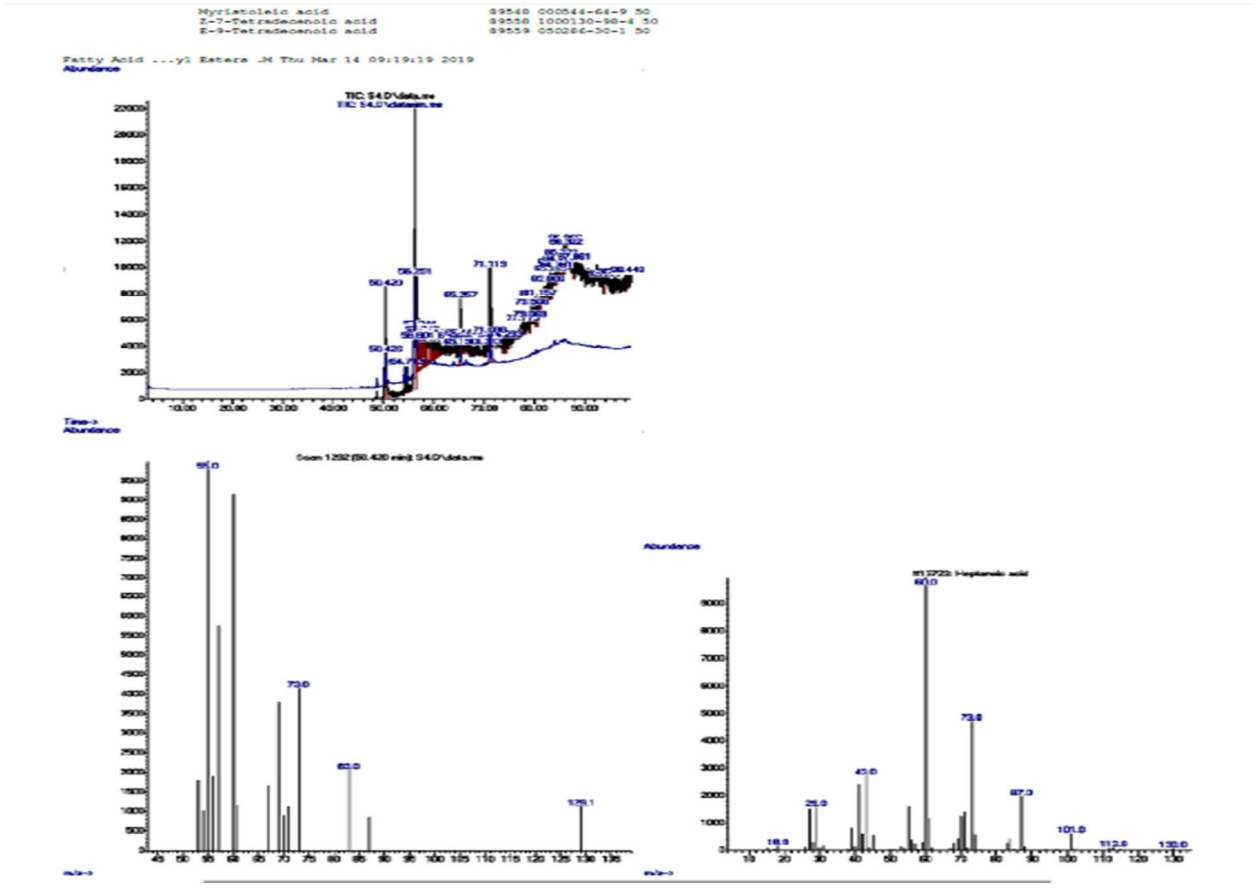

Figure 6: Chromatogram of *Citrus paradisii* using GC-MS

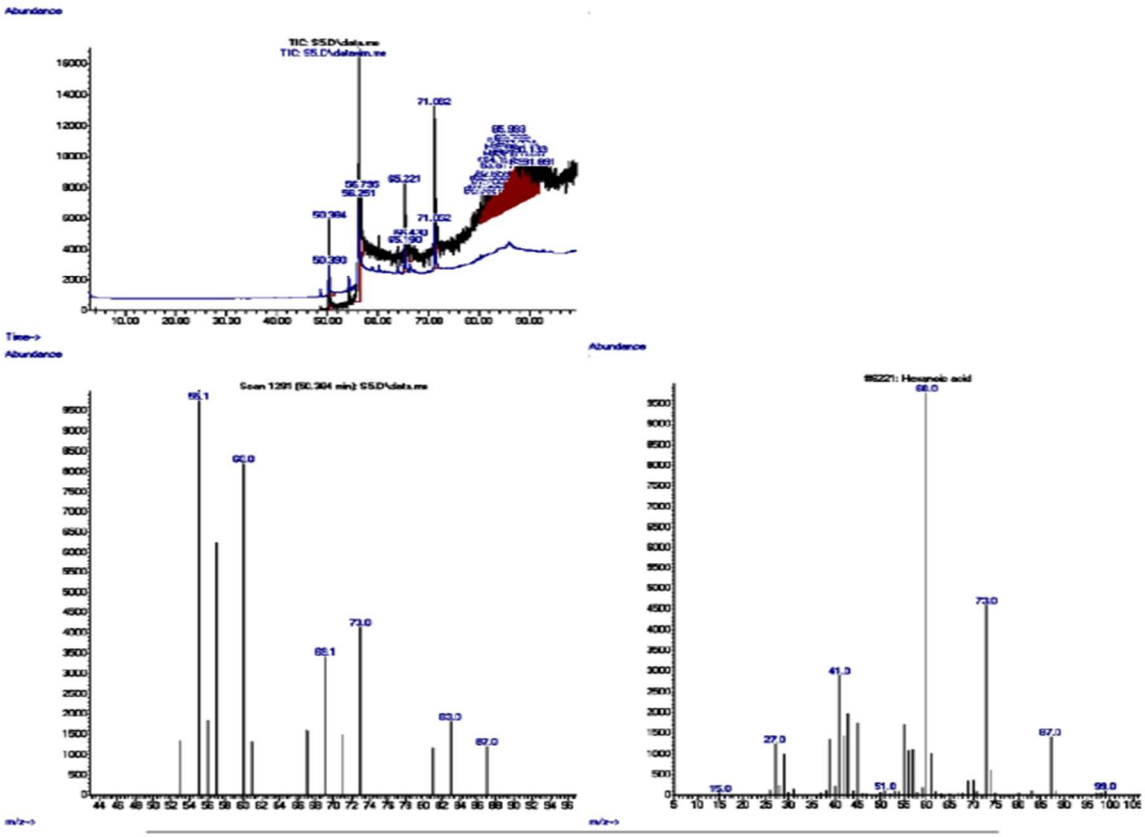

Figure 7: Chromatogram of *Citrus lemon* using GC-MS

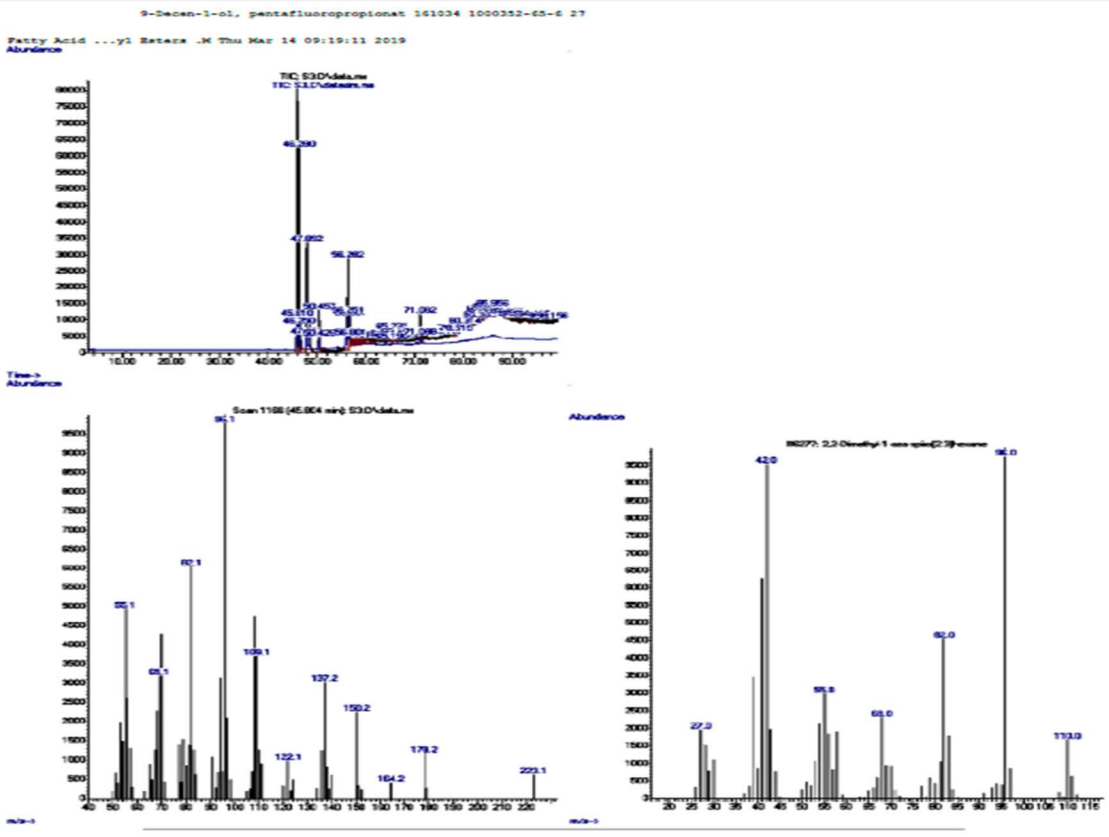

Figure 8: Chromatogram of *Citrus tangerine* using GC-MS

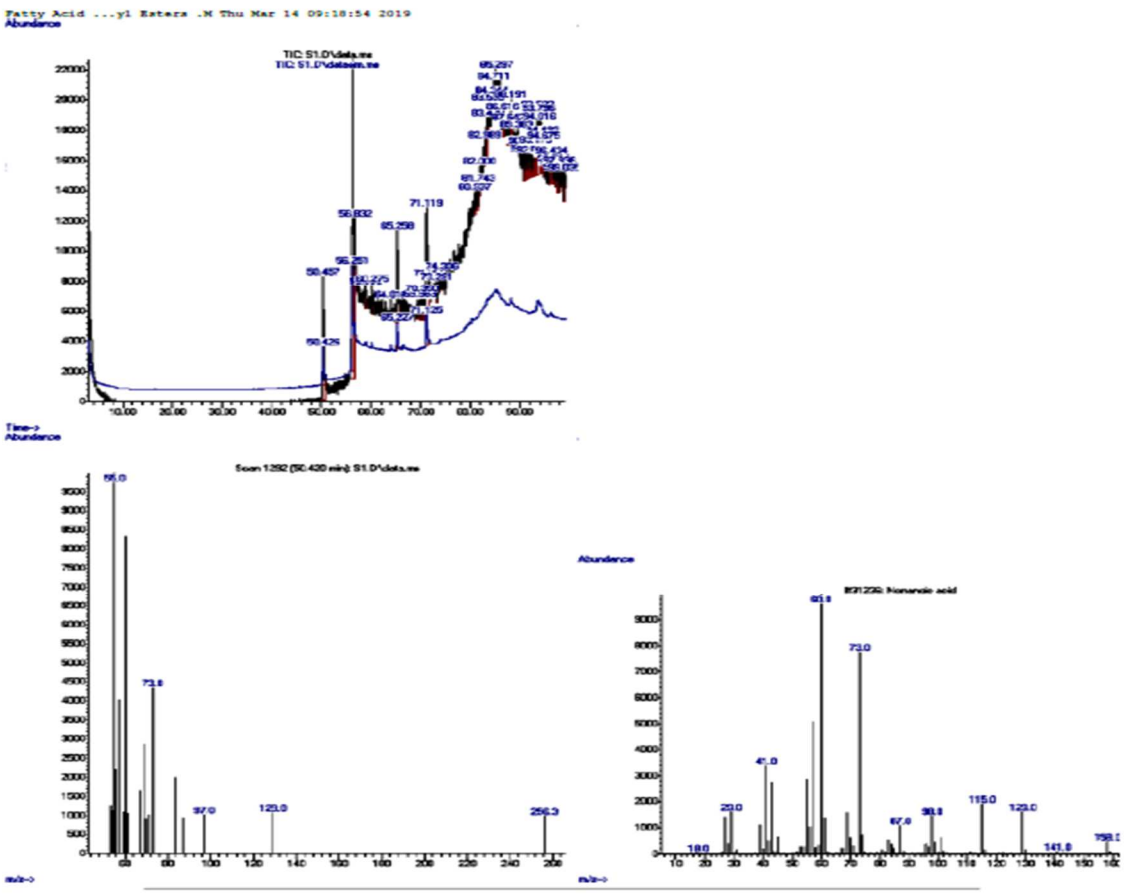

Figure 9: Chromatogram of *Citrus aurantium* using GC-MS