THE EFFECT OF PLANT EXTRACTS AND ESSENTIAL OILS ON ANTIMICROBIAL RESISTANT *Escherichia coli* AND *Klebsiella pneumoniae* ISOLATED FROM ENVIRONMENTAL SOURCES

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CERTIFICATION

This is to certify that the research work "**The effect of plant extracts and essential oils on antimicrobial resistant *Escherichia coli* and *Klebsiella pneumoniae***" was carried out by **Okere, Chinyere. E.** (20164025068) in partial fulfillment of the requirements for the award of Master of Science (M.Sc) in Medical Microbiology in the Department of Microbiology, Federal University of Technology, Owerri.

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DEDICATION

This research project is dedicated to God Almighty, who in his infinite mercies has been my source of inspiration.

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ABSTRACT

The continuous increase in resistance to antimicrobials amongst the Enterobacteriaceae constitutes a growing public health threat and so has necessitated the need to continuously search for alternative antimicrobial chemotherapy. This study was aimed at evaluating the effect of plant extracts and essential oils on antimicrobial resistant Enterobacteriaceae isolated from environmental sources. Three hundred isolates of *Klebsiella pneumoniae* and *Escherichia coli* were recovered from three (3) different environmental sources. The susceptibility of the isolates to ten (10) different antimicrobials was examined by the Kirby-Bauer technique. Thirty (30) isolates from each of the different sample types were also tested for extended β-lactamase production by the double disc synergy test and the susceptibility or otherwise of the isolates to essential oils and extracts from *Moringa olifera* seeds and *Ocimum gratissimum (*scent leaf) was analysed using the well in agar diffusion assay. The rates of resistance to the antibiotics were as follows: ampicillin (95.4%), cefotaxime (66.1%), ertapenem (57.4%) ceftazedime (65.1%), imipenem (60.6%), meropenem (53.7%), azetreonam (42.7%), ciproflaxacin (44.9%), lavafloxacin (25.3%) and gentamycin (32%). Twenty (22.2%) Isolates were positive for ESBL production. Antimicrobial analysis of the essential oils against the ESBL producers showed no inhibitory activity while the plant extracts produced zones of inhibition and minimum inhibitory concentrations of between 1.32 and 1.78 mg/ml for the two plant extracts tested. Phytochemical analysis showed the presence of alkaloids, saponins, tanins, flavonoids and glycosides in different quantities. *Moringa olifera* seed and *Ocimum gratissimum* leaf extracts could be used as effective alternatives for the treatment of antimicrobial resistant Enterobacteriaceae.

Keywords: Plant Extracts and Essential oil, Antimicrobial Resistant, *E.coli*, *Klebsiella pneumoniae*, Environment

CHAPTER ONE

1.0 INTRODUCTION

Enterobacteriaceae are rod-shaped, gram-negative bacteria that are normal inhabitants of the intestinal flora and among the most common human pathogens causing infections that range from cystitis to pyelonephrities, wound sepsis, pneumonia, bacteriemia and meningitis (Mitchell & Yehuda, 2008). They are the most common source of both community and hospital acquired infections with *Echerichia coli* being by far the most important pathogens for humans.

Enterobacteriaceae spread easily between humans by hand carriage as well as contaminated food and water and have a propensity to acquire genetic material through horizontal gene transfer, mediated mostly by plasmids and transposons (Partridge, 2011; Stokes, 2011; Toleman & Walsh, 2011).This combination is why emerging muti drug resistance in enterobacteriaceae is of the utmost importance for clinical therapy.

β- lactams are diverse antibiotic molecules that are classified according to their chemical structures. Since the year 2000, the spread of community acquired *E.coli* isolates producing extended-spectrum β- lactamases (ESBLs) capable of hydrolyzing almost all antibiotic β-lactam has been reported world-wide (Pitout & Laupland, 2008). Potential multi-drug resistant Enterobacteriaceae producers are screened first by susceptibility testing using breakpoint values (Nordmann *et al*., 2011).

Environmental sources have been acclaimed as a potential source of antimicrobial resistant bacterial, acting as possible reservoir for the dissemination of these organisms to man via the food chain, person to person contacts (handlers) and environment (waste disposal, organic fertilizers).This environment plays a role as reservoir for antibiotic resistant bacteria stemmed from the uncontrolled use of subethal doses of antibiotics as growth promoters. According to

Casanova & Sobsey (2016) Lagoon waste water has been found to contain bacterial pathogens and antibiotic resisttance gene.

Traditional chemical based therapy such as essential oils and plant extracts may be open pathways for antibiotherapy.

The challenge of the next few years will be the race between the creation of effective molecules and the spread of multi-drug resistant Enterobacteriaceae world-wide.

Essential oils and plant extracts have great potential in the field of bio-medicine to open new pathways for antibiotherapy as they effectively destroy several bacterial, fungal and viral pathogens

1.1 STATEMENT OF THE PROBLEM

The continuous increase in resistance to antimicrobials among the Enterobacteriaceae constitutes a growing public health threat and so has necessitated the need to continuously search for alternative antimicrobial chemotherapy.

1.2 AIM OF THE STUDY

The aim of this work was to evaluate the effect of plant extracts and essential oils on antimicrobial resistant *Escherichia coli* and *Klebsiella pneumoniae* isolated from environmental sources.

1.3 OBJECTIVES

1. To isolate and characterize *Escherichia.coli* and *Klebsiella pneumoniae* from environmental samples viz: River water, fish pond and poultry environments.
2. TO determine antimicrobial resistance profile of the isolates against commonly used antibiotics.
3. To determine Extended Spectrum βeta-Lactamase (ESBLs) producing isolates among resistant *E.coli* and *Klebsiella pneumoniae* isolates.

4. To extract essential oils from scent leaf (*Ocimum gratissimum*) and *Moringa olifera* seeds.
5. To determine efficacy of the extracted essential oils and crude plant extracts against the resistant isolates.
6. To determine the constituents that make up the extracted essential oils and crude plant extracts.

CHAPTER TWO

LITERATURE REVIEW

2.0 ENTEROBACTERIACEAE

Enterobacteria can cause disease by attacking their host in a number of ways. The most important factors for pathogenicity are motility, colonization factors, endotoxin, and enterotoxin production. Those enterobacteria that are motile have several flagella all around their perimeter (peritrichous). This allows them to move swiftly through their host fluid. Enterobacterial colonization factors are filamentous appendages, called fimbriae, which are shorter than flagella and bind tightly to the tissue under attack, thus keeping hold of its host. Endotoxins are the cell wall components, which trigger high fevers in infected individuals. Enterotoxins are bacterial toxins which act in the small intestines and lead to extreme water loss in vomiting and diarrhea (Encyclopedia.com., 2019).

A number of tests exist for rapid identification of enterobacteria. Most will ferment glucose to acid, reduce nitrate to nitrite, and test negative for cytochrome oxidase. These biochemical tests are used to pin-point specific intestinal pathogens. *Escherichia coli*, *Shigella* species, *Salmonella*, and several *Yersinia* strains are some of these intestinal pathogens.

E. coli is indigenous to the gastrointestinal tract and generally benign. However, it is associated with most hospital-acquired infections as well as nursery and travelers diarrhea. *E. coli* pathogenicity is closely related to the presence or absence of fimbriae on individual strains. Although most *E. coli* infections are not treated with antibiotics, severe urinary tract infections usually are.

The *Shigella* genus of the Enterobacteriaceae can produce serious disease when its toxins act in the small intestine. *Shigella* infections can be entirely asymptomatic, or lead to severe dysentery. *Shigella* bacteria cause about 15% of pediatric diarrheal cases in the United States. However, they are a leading cause of infant mortality in developing countries. Only a few

organisms are needed to cause this fecal-orally transmitted infection. Prevention of the disease is achieved by proper sewage disposal and water chlorination, as well as personal hygiene such as handwashing. Antibiotics are only used in the more severe cases (Encyclopedia. com 2019).

Salmonella infections are classified as nontyphoidal or typhoidal. Nontyphoidal infections can cause gastroenteritis, and are usually due to contaminated food or water and can be transmitted by animals or humans. These infections cause one of the largest communicable bacterial diseases in the United States. They are found in contaminated animal products such as beef, pork, poultry, and raw chicken eggs. As a result, any food product that uses raw eggs, such as mayonnaise, homemade ice cream, or Caesar salad, could carry these bacteria. The best prevention when serving these dishes is to adhere strictly to refrigeration guidelines .

Typhoid *Salmonella* infections are also found in contaminated food and water. Typhoid Mary was a cook in New York from 1868 to 1914. She was typhoid carrier who contaminated much of the food she handled and was responsible for hundreds of typhoid cases. (Marineli *et al*., 2013; Strochlic, 2020). Typhoid fever is characterized by septicemia (blood poisoning), accompanied by a very high fever and intestinal lesions. According to Thomas utler *et al* (2001), Typhoid fever is treated with the drugs ampicillin and chloramphenicol, trimethoprim, sulphamethoxazole, ciprofloxacin ofloxacin.

Certain *Yersinia* bacteria cause one of the most notorious and fatal infections known to man. *Yersinia pestis* is the agent of bubonic plague and is highly fatal without treatment. The bubonic plague is carried by a rat flea and is thought to have killed at least 100 million people in the sixth century as well as 25% of the fourteenth century European population. This plague was also known as the "black death," because it caused darkened hemorrhagic skin patches. The last widespread epidemic of *Y. pestis* began in Hong Kong in 1892 and spread to India and eventually San Francisco in 1900. The bacteria can reside in squirrels, prairie dogs,

mice, and other rodents, and are mainly found (in the U.S.) in the Southwest. (Hong Kong Meseum of Medical Science Society 2006). Since 1960, fewer than 400 cases have resulted in only a few deaths, due to rapid antibiotic treatment

Two less severe *Yersinia* strains are *Y. pseudotuberculosis* and *Y. enterocolitica*. *Y. pseudotuberculosis* is transmitted to humans by wild or domestic animals and causes a non-fatal disease which resembles appendicitis. *Y. enterocolotica* can be transmitted from animals or humans via a fecal-oral route and causes severe diarrhea (Encyclopedia.com., 2019).

2.1 ANTIBIOTIC RESISTANCE

Antibiotic-resistant bacteria pose a significant challenge to patients, physicians, and health organizations. In the United States, over 2 million people acquire infections annually from antibiotic resistant bacteria. Over 250,000 of these people require hospitalization, costing the US healthcare system over $21 billion per year (White, 2011; Center for Disease Control (CDC), 2013). In Europe, antibiotic-resistant bacterial infections cause over 20,000 deaths per year and cost over €1.5 billion per year. A 2014 study commissioned by the UK estimates that annual world-wide deaths attributable to antibiotic-resistant infections will rise from a current 700,000 to 10 million by 2050 if action to counter this crisis is not taken immediately (White, 2011).

Due to the increased use of antibiotics in a growing immuno-impaired population (those with advanced age, cancer, organ transplant or diabetes) and rapid dispersion of resistant organisms through travel, drug-resistant organisms have become increasingly common. VenatoRx is primarily targeting Carbapenem Resistant Enterobacteriaceae (CRE) and Carbapenem Resistant Pseudomonas aeruginosa (CRPA). The latter have been termed ‘super-bugs’ due to their resistance to virtually all known antibiotics. Notably, CRE and CRPA have been named two of the most urgent three priority pathogens by WHO and declared Urgent and Serious drug-resistant threats, respectively, by Center for Disease Control (CDC)(2013).

In addition, VenatoRx is engaged in biodefense efforts against less commonly known bacterial organisms such as *Salmonella* spp, *Shigella* spp, *Vibrio* spp, *Burkholderia mallei*, and *Burkholderia pseudomallei*, which could be deliberately engineered for resistance and used as biological weapons.

2.2 ANTIMICROBIAL RESISTANCE IN ENTEROBACTERIACEAE

Antimicrobials are important drugs for the prevention and treatment of bacterial infections in humans and animals (Schwarz and Chaslus-Dancla 2001; DANMAP, 2011). The introduction and use of antimicrobials have contributed remarkably to the sustenance and growth of the livestock industry (Schwarz & Chaslus-Dancla 2001). As a result of the benefits derivable from antimicrobial usage, these drugs have been used without restriction in livestock production. Many livestock producers depend on antimicrobials to cover-up for unhygienic and inadequate management practices that expose animals to potential pathogens and increase their susceptibility to infections (Soulsby, 2007; Silbergeld *et al*., 2008). Also in agriculture and aqua culture, there is substantial use of antibiotics to promote growth and reduce diseases (Prestinaci *et al*., 2015). Oftentimes, antimicrobials are administered without due consideration for the possible deleterious effects they exert on the micro and macro-ecosystem (WHO, 2007).

Over the years, the continuous use of antimicrobials has boomerang into a situation where the continued efficacy of these drugs is under threat due to the occurrence of highly resistant bacterial strains which are refractory to antimicrobial therapy (Barbosa & Levy, 2000). The increasing widespread emergence and dissemination of these multi-drug resistant bacteria is a result of the combined effects of overdependence on antimicrobials, inadequate management practices, climate change, globalization and international trade (Harbarth & Samore, 2005; MacPherson *et al*., 2009). Globally, there is an increase in reports of resistant bacteria of human and animal origins. The socio-economic consequences associated with increased

morbidity and mortality from refractory infections have reached such a magnitude that calls for concerted efforts by all local and international stakeholders in tackling the problem of antimicrobial resistance in bacteria (WHO, 2001). *Escherichia coli* is an important pathogen in humans and animals. Pathogenic *E. coli* is capable of causing devastating intestinal and extra-intestinal diseases in infected hosts (Nataro & Kaper, 1998). *Escherichia coli* is a major cause of morbidity and mortality in poultry and can be transmitted to humans through the consumption of contaminated poultry products (van den Bograad *et al*., 2001; Stordeur *et al*., 2002; Kabir, 2010). The organism is also used as an indicator bacterium for the surveillance of antimicrobial resistance in the ecosystem and also for tracing faecal contamination of food products, hence, the possible presence of other pathogenic bacteria (Momtaz *et al*., 2012; Bergeron *et al*., 2012). Antimicrobial resistance in commensal *E. coli* plays important roles in the maintenance and dissemination of resistant traits in the community (Kijima-Tanaka *et al*., 2003). Drug-resistant *E. coli* may serve as important reservoirs of resistant genes for pathogenic and nonpathogenic recipient bacterial species (Osterloh, 2004; Sunde & Norström 2006).

Surveillance programmes for monitoring antimicrobial resistance in bacteria are important in the development of strategies for the prevention and control of antimicrobial resistance. However, in developing countries, scarcity of data complicates attempts to assess the magnitude of threat to the livestock industry and public health by resistant bacteria. Inadequate documentation of observable trends in antimicrobial resistance hampers risk assessment and development of suitable interventions to mitigate the menace of antimicrobial resistance in developing countries.

2.3 RESISTANT ENTERIC BACTERIA IN ENVIRONMENTAL WATERS

Hog or pig production is a major North Carolina industry, with 8.7 million hogs in the state as of 2016, second only to Iowa (NASSNCFO, 2015). Over time hog production has become more integrated and consolidated, and is now conducted mostly in large concentrated animal feeding operations (CAFOs) that can house thousands of hogs each (Mac Donald & MC Bride, 2009).

Hog waste is managed by storing in large lagoon for several months, allowing for anaerobic decomposition and microbial die-off to take place before the waste is land applied on adjacent land (Cole *et al*., 2000). Hog habour and excrete focally several kinds of bacteria that are potential human pathogens (Barton, 2000).

According to Casanova and Sobsey (2016) lagoon waste water has been found to contain bacterial pathogens and antibiotic resistance gene. Studies of North Carolina hog farms have found *Salmonella,* including antibiotic resistant *Salmonella,* in hogs. The farm environment, and waste lagoons (Keelara *et al.,* 2013).Both lagoon leakage and land application of wastes may create opportunities for transport of bacteria to surface and ground water sources (Anderson and Sobsey,2006; Chee-Sanford *et al*., 2001) which may serve as a route of exposure for humans. While enteric bacteria resistant to clinically relevant antibiotics have been identified in hog waste and on farm water sources, it is less clear whether these bacteria are present in environmental surface and ground waters that are not on or adjacent to concentrated animal feeding operations (CAFOs). Identifying the presence and resistance patterns of enteric bacteria in non- CAFO environmental surface and ground waters is a first step to determining the scope of antibiotic resistance and its possible sources in environmental waters not associated with CAFOs or farm settlements.

Casanova and Sobsey (2016), in their study reported that *Salmonella* were detected at low levels in some surf*ace* but not groundwater. *E. coli;* were in surface waters but not ground in non-CAFO environmental samples. *Enterococci* were present in surface water and a small number of ground water sites. For *Salmonella* in surface water, the most frequent type of resistance was to surfamethoxazole. There was no ciprofloxacin resistance. There were a few surface water *E.coli* isolates resistant to chloramphenicol, gentamicin and ampicillin, and streptomycin. *E.coli* and enterococci are present more frequently and at higher levels in surface water than *Salmonella*, but ground water contamination with any of these organisms was rare, and low levels of resistance can be found sporadically in the eastern North Carolina surface and ground waters studies but they could pose a risk of human exposure via ingestion or primary contact recreation.

2.4 POULTRY ENVIRONMENT AS SOURCE OF ANTIMICROBIAL RESISTANT BACTERIA

According to Cookey & Otokunefor (2016), the poultry environment has long been acclaimed as a potential source of antimicrobial resistant bacteria, acting as a possible reservoir for the dissemination of these organisms to man via the food chain (poultry meat), person to person contact (handlers) and environment (poultry waste disposal, organic fertilizers). Initial concerns for the possible role this environment plays as a reservoir of antibiotic resistant bacteria stems from the uncontrolled use of sublethal doses of antibiotics in the poultry industry as "growth promoters". Over the years, this was thought to have caused the high levels of resistance in both commensals and pathogens associated with poultry. Several strict guidelines were therefore put in place limiting the use of medically important antibiotics as growth promoters with the expectation that this would result in a reduction in risk to man. A 2007 European Union report still however noted nalidixic acid or flumequine resistance rates of up to 50% in broiler isolates from EU countries

highlighting the need for continuous surveillance and monitoring of the situation (Fagbamila *et al*., 2010; Omeiza *et al*., 2012). In Nigeria specifically however, with poultry farming mainly characterised by small scale farming (<500 birds), there appears to be a lack of proper veterinary monitoring and a misuse of antibiotics both in prophylaxis and therapy, compounded with a lack of adhesion to "withdrawal" time prior to sale and consumption (Fagbamila *et al*., 2010; Omeiza *et al*., 2012).

Cookey and Otokunefor (2016), reported that as the scourge of antimicrobial drug resistance increases worldwide posing an ever-pressing public health problem, more and more research is geared towards reducing the development of drug resistant pathogens and halting this negative trend. One of such approaches has been to determine possible reservoirs of antibacterial drug resistance and assessing possible effects on man. One such environment with the potential to act as a reservoir of antimicrobial drug resistance due to the application of large amounts of antibiotics as growth promoters, prophylaxis and therapy, is the poultry environment. With the poultry environment noted to generate up to 6.69 kg of poultry litter per day (Ogundiran *et al.*, 2015), results from the study showed that in the absence of adequate treatment, the poultry environment could serve as a source of introduction of large numbers of bacteria (6.7×10^7 to 1.6×10^9 CFU/g) into the environment.

In addition to the high bacterial load observed, the high rates of occurrence of bacteria belonging to known pathogenic genera further highlight the potential public health hazard posed by these poultry environments. *Escherichia coli* is the number one indicator organism for faecal contamination and its presence in poultry litter unsurprising. It however highlights a major risk of the utilisation of poultry litter as organic manure and points at one of the suspected routes of transmission of antibiotic resistant bacteria to man, via contaminated vegetables. Fruits and vegetables have been implicated as the leading foods associated with

interstate foodborne outbreaks in the US and organic manure noted as a possible source of contamination of these items (Atidegla *et al*., 2016).

The poultry environment is expected to harbour a higher level of antibiotic resistant bacteria due to industry practices. Results of this study indicate such a similar trend of the role of the poultry environment as a reservoir of antibacterial resistant isolates with >50% resistance observed to majority (7 of 12) of the antibiotics tested. The rates of resistance noted in this study are similar to the majority of published reports from Nigeria and the rest of the world (Adelowo *et al.,* 2014). That the poultry environment serves not just as a reservoir of antibiotic resistant organisms but also possibly as the perfect environment for the development of such resistance may perhaps be seen in the consistently lower levels of resistance observed in poultry feed isolates opposed to faecal matter and litter isolates. Unlike the case of poultry feed whereby the isolates present do not originate from the chickens but depend on production and storage conditions, this is not so for isolates obtained from faecal matter and poultry litter (Cookey & Otokunefor, 2016).

These isolates are rather, a reflection of poultry industry practices, with the higher levels of resistance in these isolates probably a reflection of the indiscriminate use of antibiotics in the poultry industry. More worrisome however is the high diversity index (0.96 in total) based on the antibiograms of three of the major isolate classes with 31 antibiotic resistance patterns observed from 55 isolates. This points at the possibility that the antibiotic resistant isolates present in these poultry environments, rather than being a result of the spread of a single drug resistant clone, may have resulted from multiple acquisitions of drug resistant genes by susceptible isolates (Cookey & Otokunefor 2016).

2.5 PLANT EXTRACTS IN HEALTH-CARE

Plants have been classified as an essential source of medicinal agents from natural sources. Many of these plants and their extracts have been used for centuries and a huge number of novel drug components have been isolated in traditional medicine. Medicinal plants play a key role in health-care with about 80% of the world's population relying on the use of traditional medicine which is predominantly based on plants (Owolabi *et al*., 2007). According to the World Health Organisation (WHO) medicinal plants would be the best source to obtain a variety of drugs. Plant derived medicines have made large contributions to human health (El-Astal *et al*., 2005). This is due to the significant healing power of the traditional systems (Adebolu & Oladimeji 2005). Medicinal plants are distributed worldwide but they are most abundant in tropical countries (Elvin-Lewis, 2001; Naovi *et al*., 1991). Natural products from plants may offer new agents for antimicrobial use, therefore the use of natural compounds such as organic acids and medicinal plants as antibiotics alternatives become a trend. (Abou-Kassem *et al*., 2021., Abdelnour *et al*., 2020., Alagawany *et al*., 2021., Ashour *et al*., 2020., Reda *et al*., 2020a., Reda *et al*., 2020b., Sheiha *et al*., 2020., Reda *et al*., 2021) A special feature of higher plants is their capacity to produce a large number of organic chemicals of high structural diversity and metabolites (Naovi *et al*., 1991). Plants are rich in a wide variety of secondary metabolites with anti microbial properties such as tannins, terpenoids, alkaloids and flavonoids (Al-momani *et al.,* 2007; Bisignano *et al;* 2000; Bouzada *et al*., 2009 & Campean & Ynalvez, 2014).

The abundance of medicinal plants in nature and the traditional knowledge has increased the understanding of the medicinal plant properties, safety, and efficacy (Nascimento *et al*., 2000). This concern has been expressed because of the resistance of clinically pathogenic microorganisms to the antibiotics that have been produced in the last decades (Cohen, 1992).

Furthermore, since the last decade, studies based on extraction of biologically active compounds from plant species used for medicinal purposes have intensively increased (Nascimento *et al.*, 2000; Rios & Recio, 2005).

2.6 ESSENTIAL OILS (EOs)

An essential oil is a concentrated hydrophobic liquid containing volatile (easily evaporated at normal temperatures) chemical compounds from plants. Essential oils are also known as volatile oils, ethereal oils, aetherolea, or simply as the oil of the plant from which they were extracted, such as oil of clove. An essential oil is "essential" in the sense that it contains the "essence of" the plant's fragrance - the characteristic fragrance of the plant from which it is derived (Oxford English Dictionary, 2014). In addition to the work of (Butnariu & Sarac (2018), Ragno *et al* . (2020) & Abd El-Hack *et al*., (2016), EOs are also seen as naturally occurring extracts of petals, seeds, leaves, stems or roots. The term essential used here does not mean indispensable as with the terms essential amino acid or essential fatty acid which are so called since they are nutritionally required by a given living organism (Reeds, 2000). In contrast to fatty oils, essential oils typically evaporate completely without leaving a stain or residue.

Essential oils are generally extracted by distillation, often by using steam. Other processes include expression, solvent extraction, sfumatura, absolute oil extraction, resin tapping, wax embedding, and cold pressing. They are used in perfumes, cosmetics, soaps and other products, for flavoring food and drink, and for adding scents to incense and household cleaning products (Oxford English Dictionary, 2014).

Essential oils are often used for aromatherapy, a form of alternative medicine in which healing effects are ascribed to aromatic compounds. Aromatherapy may be useful to induce relaxation, but there is not sufficient evidence that essential oils can effectively treat any condition (Lee. & Choi, 2012). Improper use of essential oils may cause harm including

allergic reactions and skin irritation, and children may be particularly susceptible to the toxic effects of their improper use (Posadzki *et al.,* 2012).

In the light of developments made in the scientific field, the medicinal properties of plants have received a great interest because of their low toxicity, pharmacological activities and economic viability (Auddy *et al.,*2003). Such studies have focused on the benefits of plant-extracted phytochemicals and their effect on human health. Additives obtained naturally from plants can be compounds, groups of compounds, or essential oils. In the recent times, there has been an increase in the food industry's interest in natural compounds either for direct addition or for use in synergy with other compounds. It has been reported that direct addition of aromatic plant essential oils and extracts to foodstuffs exert an antioxidant or antimicrobial effect (Costa *et al.*, 2015). Among compounds of natural origin, biological activities have been shown by essential oils from aromatic and medicinal plants and have received particular attention because of their radical-scavenging properties (De Sousa Barros *et al.,* 2015). Several pathologies such as cancer, deterioration of the organoleptic and hygienic quality of food, and neurodegenerative diseases have been attributed to free radicals (Hale *et al.,* 2008). Massive use of antibiotics has resulted in the emergence of resistance against them, which is another problem affecting public health (Lushniak, 2014). *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella* Spp, coagulase-negative *Staphylococcus, Shigella, Enterococcus* sp. and *Escherichia coli* are amongst some of the main bacteria with multidrug resistance and are included in the category of community and hospital acquired pathogens. This has resulted in the strong demand of new antibiotics by consumers against pathogens (Fisher & Phillips, 2008) and an interest has been developed by the scientific community for using herbal medicines with antimicrobial properties. Plants and other natural sources can provide a huge range of complex and structurally diverse compounds.

Plant extracts and essential oils possess antifungal, antibacterial, and antiviral properties and have been screened on a global scale as potential sources of novel antimicrobial compounds, agents promoting food preservation, and alternatives to treat infectious diseases (Safaei-Ghomi & Ahdu, 2010). Essential oils have been reported to possess significant antiseptic, antibacterial, antiviral, antioxidant, anti-parasitic, antifungal, and insecticidal activities (Burt, 2004; Kaloustian *et al.*, 2008). Therefore, essential oils and plant extracts can serve as a powerful tool to reduce bacterial resistance (Stefanakis *et al.*, 2013). Aromatic oily liquids called essential oils (also called volatile oils) are obtained from plant materials (leaves, buds, fruits, flowers, herbs, twigs, bark, wood, roots and seeds).

A steam or hydro-distillation method was first developed in the middle ages by Arabs, and is the most common method for commercially obtaining essential oils. Having a density generally lower than that of water, essential oils are volatile, liquid, limpid, lipid soluble, rarely colored, and soluble in organic solvents. Being natural mixtures of very complex nature, essential oils may consist of about 20–60 components at quite different concentrations. Essential oils are characterized by two or three major components being present at fairly high concentrations (20–70%) in comparison to other components that are present in trace amounts. The amount of the different components of essentials oils varies amongst different plant parts and different plant species as they are chemically derived from terpenes and their oxygenated derivatives i.e., terpenoids that are aromatic and aliphatic acid esters and phenolic compounds.

An important characteristic of essential oils and their components is hydrophobicity, which enables them to partition with the lipids present in the cell membrane of bacteria and mitochondria, rendering them more permeable by disturbing the cell structures. This eventually results in the death of bacterial cell due to leakage of critical molecules and ions from the bacterial cell to a great extent. Some compounds modulate drug resistance by

targeting efflux mechanisms in several species of Gram-negative bacteria (Devi *et al.*, 2010).

An important role of essential oils in nature is protection of plants by acting as antifungal, antibacterial, antiviral, and insecticidal agents and also protection against herbivores by reducing appetite of herbivores for plants with such properties. Health and Human Services Public Health Services have recognized essential oils as safe substances and some essential oils contain compounds that can be used as antibacterial additives (Stefanakis *et al.*, 2013). Efficacy of essential oils (EOs) has been reported in several studies against pathogens and food contaminants, suggesting their applications in the food industry (Burt, 2004).

Essential oils have been used in folk medicine throughout history. The earliest recorded mention of the techniques and methods used to produce essential oils is believed to be that of Ibn al-Baitar (1188–1248), an Al-Andalusian (Muslim Spain) physician, pharmacist and chemist (Houtsma, 1993).

Rather than refer to essential oils themselves, modern works typically discuss specific chemical compounds of which the essential oils are composed. For example: methyl salicylate rather than "oil of wintergreen" (Klaassen *et al*.,1991).

Interest in essential oils has revived in recent decades with the popularity of aromatherapy, a branch of alternative medicine that uses essential oils and other aromatic compounds. Oils are volatilized, diluted in a carrier oil and used in massage, diffused in the air by a nebulizer, heated over a candle flame, or burned as incense.

Medical applications proposed by those who sell medicinal oils range from skin treatments to remedies for cancer and often are based solely on historical accounts of use of essential oils for these purposes. Claims for the efficacy of medical treatments, and treatment of cancers in particular, are now subject to regulation in most countries.

2.7 ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

In recent years there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat antimicrobial resistance. Many essential oils (EOs) posses antimicrobial activity but despite their widespread use for multiple purposes, only a small proportion are commercially used (Millezi *et al*., 2016, Reda *et al*., 2020a, Reda *et al*., 2020b, Ghabraie *et al*., 2016, Ragno *et al*., 2020). Therefore, greater attention has been paid to the screening of antimicrobial activity and its evaluation methods.

Several bioassays such as well diffusion, disk-diffusion, and broth or agar dilution are well known and commonly used methods (Balouiri *et al.*, 2016). The lowest concentration of antimicrobial agent that completely inhibits growth of the organism in micro-dilution wells or tubes as detected by the unaided eye is called minimum inhibitory concentration (MIC). The most appropriate bioassays for the determination of MIC values are these dilution methods, as these bioassays offer the possibility of estimating the concentration of the tested antimicrobial agent in the agar (agar dilution) or broth medium (macro dilution or micro-dilution). The most common estimation of bactericidal activity is the determination of minimum bactericidal concentration (MBC) which is defined as the concentration killing 99.9% or more of the initial inocula (Canillac & Mourey, 2001).

An increase in bacterial resistance to antibiotics and the lack of new antibiotics introduced into the market resulted in a need to find alternative strategies so as to cope with infections resulting from drug-resistant bacteria (Bajera *et al.*, 2017). Development of alternatives for antibiotics and the discovery or development of adjuvants are amongst the potential strategies proposed (Bush *et al.*, 2011). In order to increa se or restore antimicrobial efficacy against multi-drug-resistant bacteria, some efforts have been made. Addition of essential oils to antibiotics can induce a reduction in the antimicrobial MIC and the maximum effect has been observed with aminoglycosides, such as amikacin (Sousa *et al.*, 2010). It has been shown in

assays that geraniol demonstrates good activity in modulating drug resistance of various Gram-negative bacterial species (*Enterobacter aerogenes, E. coli, P. aeruginosa*) by targeting efflux pumps and could restore susceptibility to drugs in strains that over-express efflux pumps. This modification of drug resistance by Essential Oils (Eos) is more evident for drugs such as chloramphenicol, β-lactams and fluoroquinolones (Lorenzi *et al.*, 2009). *S. aureus* is a common Gram-positive bacterium that can cause pathogenic conditions including food-borne diseases and infections ranging from minor localized skin disturbances to life-threatening deep tissue and systemic illness. Different components of EOs obtained from *Alpinia pahangensis*, *Origanum vulgare*, *Origanum dictamnus*, *Mentha piperita*, *Lavandula hybrida*, *Zataria multiflora* and *Hofmeisteria schaffneri* have been tested against *S. aureus*, and all were found to possess potential inhibitory activity (Pérez-Vásquez *et al.*, 2011). Furthermore, combination of essential oil with an antimicrobial agent produces a synergistic effect against multidrug-resistant *S. aureus*, and in many cases, a substantial decrease in the MIC has been observed (D'Arrigo *et al.*, 2010).

2.7.1 MECHANISM OF ACTION

The *most* appropriate method for determining the bactericidal effect as well as a strong tool for obtaining information about the dynamic interaction between the anti-microbial agent and the microbial strain is the time-kill test. Also a time-dependent or a concentration-dependent antimicrobial effect is revealed by the time-kill test. Li *et al*.(2014) reported that the kinetic curves (antibacterial) of *Litsea cubeba* oil at 0.0625% (v/v) was able to prolong the lag phase growth of *E. coli* cells to approximate 12 h while the cells were completely killed at 0.125% (v/v) within 2 h, as shown by transmission electron microscopy and that the destruction of the *E. coli* outer and inner membrane could be attributed to the penetration of the *Litsea cubeba* oil with the observation of many holes and gaps on the damaged cells, which led to killing them eventually. Therefore, a broad application of the *Litsea cubeba* oil

in the antimicrobial industry would be possible due to its antimicrobial properties. The time-kill assay of *Foeniculum vulgare* (Fennel) oil against *Shigella dysenteriae* revealed destruction of the membrane integrity (Diao *et al.*, 2014).

Similarly, it was reported that the leaf essential oil of *Forsythia koreana* acted on the cytoplasmic membrane against food-borne and other pathogenic bacteria, resulting in loss of membrane integrity and increased permeability (Yang *et al.*, 2015).

Factors determining the activity of essential oils are composition, functional groups present in active components, and their synergistic interactions (Dorman & Deans 2000). The antimicrobial mechanism of action varies with the type of EO or the strain of the microorganism used. It is well known that in comparison to Gram-negative bacteria, Gram-positive bacteria are more susceptible to Essential oils (Huang *et al.*, 2014., Azhdarzadeh & Hojjati, 2016). This can be attributed to the fact that Gram-negative bacteria have an outer membrane which is rigid, rich in lipopolysaccharide (LPS) and more complex, thereby limiting the diffusion of hydrophobic compounds through it, while this extra complex membrane is absent in Gram-positive bacteria which instead are surrounded by a thick peptidoglycan wall not dense enough to resist small antimicrobial molecules, facilitating the access to the cell membrane (Hyldgaard *et al.*, 2012 ; Zinoviadou *et al*., 2009). Moreover, Gram-positive bacteria may ease the infiltration of hydrophobic compounds of EOs due to the lipophilic ends of lipoteichoic acid present in cell membrane (Cox *et al*., 2000).

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 smembrane. 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.,* 2017 determined the mechanism behind the antibacterial activity of cinnamon Essential oils against *E. coli* and *S. aureus* and reported that the bacterial cell membrane was destroyed after addition of cinnamon Essential oil at the MIC level, whereas addition of cinnamon Essential oil at the MBC levels resulted in the killing of the bacterial cell (Zhang *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 (Lakehal *et al*., 2016).

Antimicrobial activity of Essential oils is therefore attributed to a cascade of reactions involving the entire bacterial cell (Macwan *et al*., 2016).

2.8 COMPONENTS OF ESSENTIAL OILS WITH ANTIMICROBIAL ACTIVITY

The major constituents of EOs can constitute up to 85%, whereas other components are present in trace amounts(Bauer *et al.,* 2001)α-phellandrene (36%) and limonene (31%) in Anethum graveolens leaf oil, d-limonene (over 80%) in citrus peel oils, α-phellandrene (36%) and limonene (31%) in Anethum graveolens leaf oil, carvacrol (30%) and thymol (27%) in Origanum compactum oil, α/β-thujone (57%) and camphor (24%) in Artemisia herba-alba oil, carvone (58%) and d-limonene (37%) in Anethum graveolens seed oil, and menthol (59%) and menthone (19%) in Mentha piperita oil are among the constituents present at relatively higher concentrations in essential oils (Shaaban *et al.,* 2012). Generally, the biological properties of the essential oils are determined by their major components including two groups of distinct bio-synthetical origin (Pichersky *et al.,* 2006). Terpenes and terpenoids comprise the main groups whereas aromatic and aliphatic constituents comprise the other group, all characterized by low molecular weight.

Biochemical modifications of terpenes via enzymes that add oxygen molecules and move or remove methyl groups result in the formation of terpenoids. Terpenoids can be sub-divided into alcohols, phenols, esters, aldehydes, ethers, ketones, and epoxides. Thymol, carvacrol, linalool, linalyl acetate, citronellal, piperitone, menthol, and geraniol are the examples of terpenoids. In one study, α-cedrol was reported as the bioactive constituent of the essential oil from fresh leaves of Thuja orientalis with a minimum inhibitory amount (MIA) of 30.5 μg against A. alternate (Guleria *et al.*,2008).

Monoterpenoid phenols present in the essential oil of Origanum vulgare, thyme, pepperwort and wild bergamot are carvacrol or cymophenol. Diarrheal toxin production by Bacillus cereus and growth of vegetative bacteria were inhibited by carvacrol. The precursor of carvacrol is p-cymene which is a monoterpene with a benzene ring without any functional groups on its side chains. When used alone, p-cymene is not an efficient antimicrobial compound (Aligiannis *et al.,* 2001), but the activity of compounds like carvacrol is potentiated by p-cymene and polymyxin B nona peptide (Mann *et al.*,2000). It has been shown that p-cymene is hydrophobic in nature and causes swelling of the cytoplasmic membrane to a greater extent (Burt *et al.*,2007). Also, p-Cymene had an effect on the synthesis of protein in *E. coli* cells.

It is expected that the antimicrobial action of phenolic compounds such as thymol and carvacrol is attributed to structural and functional damages in the cytoplasmic membrane (Sikkema *et al.*,1995). The primary mode of antibacterial action of thymol is not completely understood, but is believed to involve disruption of outer and inner membrane and interaction with membrane proteins and intracellular targets. Thymol (or 2-isopropyl-5-methylphenol), a natural monoterpene phenol derivative of cymene, is isomeric with carvacrol present in thyme essential oil and is extracted from Thymus vulgaris (common thyme) and various other plants. In a study by Di Pasqua *et al.* (2010), interaction of thymol with membrane proteins

was further supported by exposing Salmonella enterica to sub-lethal concentrations of thymol, and accumulation of outer membrane proteins in misfolded pattern and upregulation of genes involved in synthesis of outer membrane proteins was also observed (Di Pasqua *et al* ., 2010). The citrate metabolic pathway was also impaired by thymol and many enzymes involved directly or indirectly in ATP synthesis. Intracellular action of thymol indicates that it affects important energy-generating processes, which lower the ability of a cell to recover after exposure to thymol. Studies pertaining to investigation of the mode of action of thymol against yeast and fungi point towards the interaction of thymol with the cell envelope and intracellular targets. It has been shown that thymol disrupted vesicles and cell membranes, and impaired biosynthesis of ergosterol in Candida strains, which consequently affected the integrity of cell membrane because membrane fluidity and asymmetry is regulated by ergosterol similarly to cholesterol in animal cells (Ahmad *et al* ., 2011). Rao et al. proposed that specific signaling pathways are activated by thymol in yeast, rather than causing non-specific lesion of membranes. This was based on the observation that cytosolic Ca_2^+ bursts caused by thymol and transcription responses similar to those in Ca_2^+ stress and nutrient starvation are activated. Moreover, an increase in the permeability of P. aeruginosa and S. aureus cells was observed in ethidium bromide (fluorescence nuclear stain), dissipated pH gradients irrespective of glucose availability, and leakage of inorganic ions. These results were in accordance with a study that utilized a mixture of thymol and carvacrol (Lamberte *et al.*,2001).

A major constituent of oregano is carvacrol (a phenolic monoterpenoid). Carvacrol is one of the most extensively studied essential oil constituents together with its closely related isomer thymol. EOs rich in carvacrol have been reported to possess remarkable antimicrobial activity (Mcetin *et al.*,2011). Although the outer membrane is affected by carvacrol, the cytoplasmic membrane is thought to be its site of action, causing passive transport of ions across the

membrane. As an adaptation mechanism to maintain optimal membrane function and structure, it has been proposed that cells exposed to carvacrol change the fatty acid composition of the membrane because of the effect of carvacrol on fluidity (Di Pasqua *et al.,* 2006). It has been demonstrated that carvacrol affects the outer membrane of Gram-negative bacteria (La Storia *et al.*,2011).

According to Friedman *et al*. based on the time they take to produce significant action, essential oils can be divided into the following two types: compounds that act slowly and compounds with fast action. Examples of some antimicrobials considered as fast acting compounds are carvacrol, cinnamaldehyde, and geraniol, since they inactivate organisms like *E. coli* and *Salmonella* in a short time of five minutes. It was reported that a time duration of 30–60 min was required to show efficient antimicrobial activity for the compounds acting slowly (Friedman *et al.*,2004). Carvacrols' mechanism of antifungal activity is similar to thymol, showing H+ homeostasis and disruption of Ca_2^+, up- and down-regulation of gene transcription similar to that found in Ca_2^+ stress and nutrient starvation (Rao *et al.,* 2010), disruption of membrane integrity, and impairment of biosynthesis of ergosterol in *Candida* strains (Silva-Angulo *et al.*,2015). Silva-Angulo *et al*. (2015) , also showed that citral exhibited antilisterial activity against *L. innocua* and *L. monocytogenes* and can be applied in active packaging to control possible recontamination of foods or in combination with other preservation technologies (Klein *et al., (*2013). Similarly, Klein *et al. (*2013) determined the antimicrobial activity of six essential oil components against the potential food spoilage bacteria *Aeromonas hydrophila*, *Escherichia coli*, *Brochothrix thermosphacta*, and *Pseudomonas fragi* for single use and in combination with each other .

2.9 SYNERGISM BETWEEN COMPONENTS / CONSTITUENTS OF ESSENTIAL OILS AND ANTIBIOTICS

Components of essential oils namely thymol and carvacrol were found to show synergism with penicillin against *E. coli* and *S. typhimurium* (Palaniappan & Holley, 2010). Similarly, cinnamaldehyde, that possesses a prop-2-enal side group to the benzene ring, had synergistic activity with fewer of the antibiotics as compared to the phenols carvacrol and thymol(Zhang *et al*.,2011), which could provide some initial indications on the mechanism function of these components of volatile oils. An examination of the synergistic action between eugenol and antibiotics against a number of reference strains of carcinogenic and period onto pathogenic bacteria has also been carried out. It was reported that eugenol exhibited synergism with ampicillin against *S. criceti* and *Streptococcus gordonii* and with gentamicin against *Streptococcus sanguinis* and *Porphyromonas gingivalis* (Moon *et al*., 2011). However, carvacrol was found to be synergistic in combination with both ampicillin and nitrofurantoin against *Klebsiella oxytoca* that was isolated from animal feed whilst thymol was indifferent (Zhang *et al*.,2011).

2.10 ANTIMICROBIAL ACTIVITY OF SOME ESSENTIAL OILS (EOS)

Food safety is an increasingly important public health issue. Antimicrobial drugs are subjected to the microbial resistance and this has become a growing problem in recent years. Therefore, sufficient research to discover potent natural antibiotics is desirable and compulsory. Essential oils are aromatic oily liquids obtained from plant material which have been long known for exhibiting antibacterial, antifungal, antiviral activities and they are regularly used in medicine and in the food industry (Bassole & Juliani, 2012). It is now acceptable by public and green food consumers to use essential oils with low taste concentrations in food to keep it safe and extend its shelf-life (Burt, 2004).

Essential oils or their components have been shown to exhibit insecticidal, antiviral, antitoxigenic, antiphrastic and antimycotic properties. Those characteristic are possibly related to the function of those compounds in plants (Mahmoud & Croteau, 2002). The essential oils or their compounds extracted from various types of plants i.e., mustard, thyme, coriander, ginger, chamomile, nigella, liquorice and marjoram have been used for preserving foods and drinks have an inhibitory effect on the growth of microorganisms. They are also used as spices or aromatic herbs to give special aromas or flavors to foods are also known to have antimicrobial properties (Burt, 2004). Essential oils showed antimicrobial activity against a wide range of bacteria including antibiotic resistant and fungal species. They can affect both Gram positive and Gram negative bacteria in addition to yeasts and filamentous fungi (Soni & Soni, 2014).

Elgazzar *et al.,* (2001) evaluated the inhibition of essential oils from some herbs and mentioned that organo, coriander and basil showed the greatest inhibition effect while anis has no particularly inhibitory effect on bacteria. Donaldson *et al*., (2005) tested the activity of different essential oils and essential oil blends against *Staphylococcus aureus* and *Candida albicans* and found that all essential oils were active against both microorganisms.

The antibacterial activity of essential oils of thyme, marjoram, chamomile was investigated against *Bacillus cereus* and *Bacillus subtilis* by Gurgulova *et al.* (2006) and Abd *et al.* (2009) and they reported that the highest antibacterial activity recorded for the essential oils of thyme and wild marjoram for all strains and the oil of chamomile had low antibacterial activity against the same strains. Also, Witkowska *et al.* (2013) indicate that crude extracts of some herbs and spices possess *in vitro* activity against food spoilage and pathogenic bacteria**,** displaying bactericidal or bacteriostatic activities with consequent damage to bacterial cell membranes of both Gram positive and Gram negative bacteria. The reduction of the microbial population depends on the concentration of the essential oil where high

concentrations of essential oil gave high antibacterial effect and may completely inhibit the growth of microorganisms (Kalemba & Kunicka, 2003). There are many methods that quantify microbiological activity of essential oils including assays such as optical density, agar well diffusion, disk diffusion and hole plate diffusion (Burt, 2004).

The major constituents of EOs can constitute up to 85%, whereas other components are present in trace amounts. α-phellandrene (36%) and limonene (31%) in Anethum graveolens leaf oil, d-limonene (over 80%) in citrus peel oils, α -phellandrene (36%) and limonene (31%) in Anethum graveolens leaf oil, carvacrol (30%) and thymol (27%) in Origanum corn pactum oil, α / β -thujone (57%) and camphor (24%) in Artemisia herba-alba oil, carvone (58%) and d-limonene (37%) in Anethum graveolens seed oil, and menthol (59%) and menthone (19%) in Mentha piperita oil are among the constituents present at relatively higher concentrations in essential oils(Chouhan *et al*., 2017). Generally, the biological properties of the essential oils are determined by their major components including two groups of distinct bio-synthetical origin. Terpenes and terpenoids comprise the main groups whereas aromatic and aliphatic constituents comprise the other group, all characterized by low molecular weight (Chouhan *et al*., 2017).

2.11 ANTIBACTERIAL POTENTIAL OF MORINGA OLEIFERA

Moringa oleifera (family Moringaceae) commonly known as Drumstick tree is indigenous to Northwest India. Most of the parts of the plant possess antimicrobial activity (Saadabi & Abu, 2011). They are well known for their pharmacological actions and are used for the traditional treatment of diabetes mellitus, hepatotoxicity, rheumatism and venomous bites and also for cardiac stimulation. Leaves of *M. oleifera* has been used as antiulcer, diuretic, anti-inflammatory and for wound healing (Udupa *et al.,* 1994). They are also used to treat anxiety, diarrhea, inflammation of the colon, skin infections, scurvy, intestinal parasites and many other conditions (Farooq *et al.,* 2012).

These antimicrobial principles are actually the defensive mechanisms of the plants against different pathogens. It is speculated that the antimicrobial activities of bioactive compounds depend on interactions between their lipid components with the net surface charge of microbial membranes. Furthermore, the drugs might cross the cell membranes, penetrating into the interior of the cell and interacting with intracellular sites critical for antibacterial activity (Trombetta *et al.,* 2005). Petroleum ether extract as well as chloroform extract of both leaves showed antimicrobial activity which certainly indicates that these extracts contain higher concentration of active antimicrobial agents. These may include alkaloids, glycosides, volatile oils or tannins (Hugo & Russell, 1992).

2.12 ANTIMICROBIAL ACTIVITIES OF CLOVE BASIL (OCIMUM GRATISSIMUM)

Ocimum gratissimum Linn (Lamiaceae) is an herbaceous shrub notably found in tropical countries including Nigeria, where it is commonly called Clove basil, Sweet basil, teabush, Scent leaf or fever plant; but it is also popularly known with different local names in Nigeria (Nupe: *Tan-motsungi-wawagi*; Ebira: *Ireru*; Hausa: *Dai doya ta gida;* Yoruba: *Efinrin ajase*; Ibo: *Nchanwu*. Burkill,1985). Many species of the genus *Ocimum* namely: *Ocimum americanum, Ocimum basilicum, Ocimum canum, Ocimum gratissimum, Ocimum sanctum* and *Ocimum suave* have been reputed for various medicinal uses (Adjanahoun, 1991). Several ethnobotanical surveys show that *Ocimum gratissimum* was among the plants reported in Nigerian communities to be used traditionally to treat bacterial infections such as enteric diseases viz: diarrhoea, dysentery and other gastrointestinal infections; upper respiratory tract infections associated with coughing, pneumonia, asthma and bronchitis; urogenital infections including sexually transmitted diseases, skin infections (dermatitis, eczema, scabies), wounds and ulcers, headache, ophthalmic conditions, insect bites, nasal bleeding, stroke, measles, paludism, and bacterial fevers such as typhoid fever and diabetes

and veterinary problems (Elujoba, 2005). It is also used in the treatment of epilepsy, shigellosis, trypanosomiasis, convulsion, pile and anaemia in Nigeria (Idika, 2008), and implicated in oral hygiene and veterinary practice in Nigeria (Abu *et al*., 2009., Matekaire & Bwakur, 2004).

Comprehensive biological activities of *O. gratissimum* have been reviewed and it is associated with antibacterial, antifungal, hypoglycaemic, antipyretic, antinociceptive, antioxidant, anti-inflammatory, anthelmintic, chemo-preventive, anti-carcinogenic, free radical scavenging, radio protective, antidermatophytic activities, and numerous other pharmacological uses (Aprioku & Obianime, 2008). Earlier reports have also shown the smooth muscle contracting and antimutagenic activity as well as its anti-diarrhoeal effects in experimental animals, high antiviral indices against HIV-1 and HIV-2; shigellocidal properties (Iwalokun *et al.*, 2003), anti- trypanosomal effects, immunobiological activity, gastro- protective properties, controlling agent for food spoilage and mycotoxin producing fungi (Ravikumar *et al*., 2007), disintegrant properties of its seed mucilage, and as a relaxant on isolated ileum from guinea pig. Its essential oil has mosquito repellant, insecticidal properties (Kéita *et al.*, 2001). The essential oil of *O. gratissimum* and its main component eugenol were reported to be efficient in inhibiting Haemonchus contortus (Hussien *et al.*, 2011). Currently, basil is mainly used as a culinary herb as well as perfumes and cosmetics. It is therefore important that phytochemical composition be correlated to the antimicrobial activity in order to verify the therapeutic value proclaimed by the traditional healers.

Ocimum gratissimum Linn (Lamiaceae) is reputed for many medicinal and agronomic practices amongst Nigerian peasant farmers. The plant extract are potential sources of antimicrobial and preservative agents.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1.0 DESCRIPTION OF STUDY AREAS

(1) FEDERAL UNIVERSITY OF TECHNOLOGY OWERRI AQUA CULTURE (FISH POND) AND POULTRY FARM

The Federal University of Technology Owerri (FUTO) is a Federal Government University located in Owerri, the capital of Imo State.The University is bounded by communities such as Eziobodo, Umuchima, Ihiagwa and Obinze, in Imo State, Nigeria. FUTO is one of the oldest University of Technology in Nigeria and was established in 1980 by Executive fiat with the composition and appointment of the first provisional Council by Nigeria's First Executive President, Shehu Shagari. The fish ponds and poultry farms are located inside the University campus. These are owned by the school and managed by the school authorities.

(2) OTAMIRI RIVER

The Otamiri River is one of the main rivers in Imo State, Nigeria. The river runs south from Egbu past Owerri and through Nekede, Ihiagwa, Eziobodo, Olokwu Umuisi, Mgbirichi and Umuagwo to Ozuzu in Etche, in Rivers State, from where it flows to the Atlantic Ocean.The length of the river from its source to its confluence at Emeabiam with the Uramiriukwa River is 30 kilometres (19 miles). The Otamiri watershed covers about 10,000 square kilometres (3,900 sq mi) with annual rainfall of 2,250 to 2,500 millimetres (89 to 98 in). The watershed is mostly covered by depleted rain forest vegetation, with mean temperatures of 27 °C (81 °F) throughout the year. Conversion of the tropical rainforest to grassland with slash and burn practices is degrading soil quality. The rivers joined by Nworie River at Nekede in Owerri, a river about 9.2 kilometres (5.7 miles) long.

Otamiri River is subject to intensive human and industrial activities, and is used as a source of drinking water by the poor when the public water system fails. It is usually polluted by

organic and inorganic wastes, being subjected to intensive human and industrial activities such as dumping of solid and liquid wastes resulting in the discharge of a wide range of pollutants into the water body. The river also serves for various domestic as well as economic activities by the lower segment of the inhabitants of Owerri.

3.1.1 COLLECTION OF SAMPLES

(A) RIVER WATER SAMPLES

The method of Mgbemena *et al.*, (2012) was adopted for river water sample collection. Samples were collected on weekly basis. Three sampling points were chosen along the longitudinal stretch of the Otamiri River viz: upstream, midstream and downstream. A total of fifteen (15) samples were collected on each sampling day, five (5) samples from each point. The sampling was done during the month of July, 2018, which fell within the rainy season in Nigeria. Sample collection was done aseptically using clean sterile plastic containers. These sample bottles were immersed below the water surface at an elbow depth, filled to overflowing, and the cap affixed securely to eliminate contamination. The water samples were transported immediately to the laboratory in ice block box and analyzed within one hour of collection.

(B) CHICKEN CLOCAL SWAB SAMPLES

A total of forty (40) samples from chicken cloacal swab were collected from Federal University of Technology Poultry farm. The method described by Ejikeukwu *et al.* (2017) was adopted for sample collection. Samples were aseptically collected by inserting a sterile swab sticks in the cloacal region of the poultry birds at a depth of 3cm and rotated at angle 360^0C. The swab sticks were thereafter returned to their respective containers and labeled.

The chickens were given a mark on the body with marker pen in order to avoid resampling from the same chicken.

(C) AQUA CULTURE (FISH POND) WATER SAMPLES

The method of Njoku *et al*. 2015 was adopted for samples collection. A total of thirty two (32) water samples were collected from Federal University of Technology Owerri (FUTO) fish pond. Water samples were aseptically collected from the ponds once a week using sterile 200mls screw capped bottles. Composite samples were obtained by collecting at different sampling points in each pond compartments at a depth of 30cm below the water surface using the sampling bottle tied with a sterile rope. Duplicate samples were collected from each pond. The water samples were placed in ice block box and transported to the laboratory within one hour for analysis.

3.1.2 PREPARATION OF CULTURE MEDIA

The culture media used for analysis of the samples were prepared according to the manufacturers descriptions and they include Nutrient agar, MacConkey agar, MacConkey broth, Muller hinton agar, Nutrient broth, Trypyon broth, MR-VP medium and Simmons citrate agar. Moisture formed on solidified agar plates was dried off by placing the agar plates in hot-air-oven at 50^0C. The plates were checked at interval until the moisture dries off (Cheesbrough, 2003). The culture media plates and nutrient agar slants in bijou bottles thus prepared were subjected to sterility test prior to use by incubating the plates in an incubator at 37^oC for 18hours and the agar slants at room temperature .Absence of visible growth indicates that the culture media and agar slants thus prepared were sterile (Cheesbrough, 2003).

3.1.3 BACTERIOLOGICAL ANALYSIS OF SAMPLES

(1) ISOLATION OF *Klebsiella* sp AND *Escherichia coli* FROM FISH POND WATER SAMPLES

The method of Douglas and Isor (2016) was adopted. Briefly 1ml of pond water sample was inoculated into 9mls of MacConkey broth in McCartney bottles. The bottles were incubated at 37^0C for 18hours. A loopful of the culture was streaked onto freshly prepared MacConkey agar plates. The plates were left on the laboratory bench for twenty (20) minutes, inverted and incubated at 37^0C for 24hours in an incubator and examined thereafter. Colonies of *Klebsiella* sp appear pink and mucoid while colonies of *Escherichia coli* appear pink and non mucoid.

(2) ISOLATION OF *Klebsiella* sp AND *Escherichia coli* FROM RIVER WATER SAMPLES

The method of Njoku *et al.,* (2015) was adopted. Tenfold serial dilution of the sample was performed using 9% w/v normal saline as diluent. From the appropriate dilution, 0.1ml of the diluted samples were inoculated onto freshly prepared MacConkey agar plates and the inoculum spread with sterile L- shaped glass rod. The plates were left on the laboratory bench for twenty (20) minutes, inverted and incubated at 37^0C for 24 hours and examined thereafter. Colonies of Klebsiella sp appear pink and mucoid while colonies of *Escherichia coli* appear pink and non mucoid.

(3) ISOLATION OF *Klebsiella* sp AND *Escherichia* coli FROM CHICKEN CLOACAL SWAB

The method of Ejikeukwu *et al.,* (2017) was adopted. Each of the swab sticks were broken into McCartney bottles containing 5mls of freshly prepared MacConkey broth and the bottles were covered with its cap, and incubated at 37°C for about 18 hours. Bacterial growth was indicated by the presence of turbidity in the bottles. Bottles showing turbidity (as indication

of bacterial growth) were aseptically subcultured by streaking a loopful onto freshly prepared MacConkey agar plates. The plates were left on the laboratory bench for twenty (20) minutes, inverted and incubated at 37°C for 24 hours and examined thereafter. Colonies of *Klebsiella* sp appear pink and mucoid while colonies of *Escherichia coli* appear pink and non mucoid.

3.1.4 PURIFICATION AND PRESERVATION OF ISOLATES

Colonies which develop on MacConkey agar plates were sub cultured onto sterile nutrient agar plates, incubated at 37^0C for 24 hours in order to obtain pure culture. Once confirmed pure, a loopful of the isolates was inoculated into sterile screw capped bottles (bijou) containing nutrient agar in slanted positions and incubated in the dark at room temperature (Sule *et al.*, 2011).

3.1.5 GRAM STAIN AND BIOCHEMICAL TESTS FOR CONFIRMATION OF BACTERIAL ISOLATES

Gram staining and the following biochemical tests were performed on 18 hours old pure cultures of bacterial isolates using the method of Ayandele *et al.* (2015) and they included:

Indole test, Methyl red test, Vogues proskauer test, Citrate test.

(1) GRAM STAINING

Gram staining is used to differentiate bacteria as gram positive or gram negative based on their gram reaction. Briefly a thin smear of 18 hours old pure culture of the isolate was prepared using a drop of sterile water on a clean grease free glass slide. The smear was allowed to air dry, then heat fixed by passing the slides over a Bunsen burner flame 3-4 times. The heat-fixed smear was then covered with crystal violet stain for 30 seconds. The stain was quickly washed off with clean water. The water was tipped off and the smear was covered with Lugols iodine for 30 seconds. The iodine was washed with clean water. The smear was decolourized rapidly for 15 seconds with 95% ethanol. The smear was quickly washed with clean water and then covered with dilute safranine for 30seconds.The stain was

washed off with clean water and the stained slide placed in a draining rack and allowed to air dry at room temperature. A drop of immersion oil was added to the stained slides and the preparation examined microscopically using oil immersion objective lens (x100), observing the cellular morphology of bacteria, such as colour of the cell shape of the cell and arrangement of the cell.

(2) INDOLE TEST

An aliquot of 5mls tryptone broth prepared according to manufacturer's guide was added into a test tube. The tube was inoculated with a loopful of 18 hours old culture of the test isolate and incubated for 48hours at 37^0C. 0.5ml. Kovac's reagent, was added into the culture in a test tube after incubation. The formation of a red ring at the surface interface indicates a positive indole test.

(3) CITRATE UTILIZATION

Simmons citrate agar was prepared based on manufacturer's instruction and sterilized. Ten (10) mls of it was added into a sterile test tube using ten (10) mls sterile syringe and the tubes allowed to solidify in a slanted position. A loopful of an 18 hours old culture of the test organism was streaked onto the surface of the slanted agar in a test tube and then incubated for 1-5 days at 37^0C. A change in the colour of agar medium from green to royal blue following growth of the organism on the slant indicated a positive test, while no colour change in the medium indicates a negative citrate test.

(4) METHYL RED TEST

Two (2) ml of sterile glucose phosphate peptone water (MR-VP medium) in a sterile test tube was inoculated with a loopful of an 18 hours old cuture of the bacterial isolate. The culture was incubated at 37^0C for 48hours. Three to five (3-5) drops of methyl red indicator was added, mixed and read immediately. A bright red coloured ring at the surface layer indicates a positive test while yellow colour indicates a negative test.

(5) VOGUES-PROSKAUER TEST

Two (2) mls of sterile glucose phosphate peptone water (MR- VP medium) in a test tube was inoculated with 18 hours old culture of the test isolate. The culture was incubated at 37^0C for 48 hours. Then, 1ml of 40% potassium hydroxide was added followed by 3ml of 5% alcoholic alpha-naphthol. The test tube was mixed very well and observed for colour change. A pink-red colour within 2-5 minutes shows a positive test.

3.1.6 PREPARATION OF 0.5 McFARLAND SOLUTION

The 0.5 Mcfarland standard solution was prepared by adding 1ml of concentrated sulphuric acid to 99ml of distilled water to make 1% v/v solution of sulphuric acid. Similarly 0.5g of dehydrated barium chloride ($Bacl_2.H_20$) was dissolved in 50ml of distilled water to make 1% w/v solution of barium chloride. The 0.5ml barium chloride was added to 99.5ml, thus 0.5 Mcfarland standard solution produced (Uzoigwe and Agwa, 2011).

3.1.7 BACTERIA INOCULUM STANDARDIZATION USING 0.5 McFARLAND STANDARD SOLUTION

The method adopted by Uzoigwe and Agwa. (2011) was used. Briefly the turbidity of an 18 hours old pure culture of the bacteria isolates in test tubes containing 5mls of nutrient broth was matched with the turbidity of 0.5 Mcfarland solution in a test tube in a good light.

The turbidities were examined against a printed card or a sheet of paper. The turbidities of the bacterial suspension were adjusted to that of 0.5 Mcfarland solution by adding a sterile distilled water to the suspension. A standardized inoculum corresponds to $1.5x10^8$cfu/ml.

3.1.8 ANTIBACTERIAL SUSCEPTIBLITY TEST ON BACTERIAL ISOLATES

The method described by CLSI. (2011) was adopted for antibiotics susceptibility test on the bacterial isolates. Using a standardized bacterial inoculum in a test tube, a sterile swab stick was dipped into the culture test tube so as to absorb the inoculum. Excess fluid inoculum on the swab was removed by gently rotating the swab against the walls of the test tube. The

swab stick was gently removed from the tube and used to inoculate freshly prepared sterile Muller Hinton agar plates by streaking the entire surface of the agar plate. The agar plates were allowed to absorb the inoculum while on the laboratory inoculating bench for thirty (30) minutes before antibiotics discs were seeded onto the inoculated plates using sterile forceps. Five (5) well spaced different antibiotics were seeded per agar plate and in all ten different antibiotics were seeded on duplicate agar plate. The ten (10) different antibiotics from oxoid United Kingdom used were: imipenem, (IPM, 30 mcg), meropenem, (MEM, 10 mcg), ertapenem, (ETP, 10 mcg), ampicillin, (AMP, 10 mcg), gentamycin, (CN, 10 mcg), ceftazidime, (CAZ, 30 mcg), cefotaxime, (CTX, 30 mcg), aztreonam, (ATM, 30 mcg), ciprofloxacin, (CIP, 5 mcg) and levofloxacin, (LEV, 5 mcg). The plates were inverted and incubated at 37^0C for 18 hours. Diameter of zones of inhibition which develop after incubation were carefully examined and measured in millimeter using a meter rule or ruler. The zones of inhibition were judged as being sensitive or resistant comparing their diameter to that in Clinical Laboratory and Standard Institute (CLSI, 2011) for antibiotics susceptibility testing standard.

3.1.9 COLLECTION OF PLANT SAMPLES, PROCESSING, EXTRACTION AND TREATMENT

Plants used were Moringa Olifera and scent leaf (Ocimum gratissimum also known as clove basil) material. Scent leaf and Moringa olifera seeds were collected from villages in Owerri North Local Government of Imo State, Nigeria. The scent leaf and Moringa seeds were dried at room temperature at $28^oC \pm 2^o$ for one month. The dried leaves and seeds were ground with sterilized grinding machine. Each of the powdered samples was weighed for two hundred grams each (200g). Crude athanol extraction produced (CEEs) was carried out by soaking the powdered leaves and powdered seeds in ethanol for 4 days with constant shaking.

This mixture was filtered using whatman number 1 fitter paper, centrifuged at 1500xg for 20 minutes, capped and stored in refrigerator as CEEs. The extracts were divided into two portions: The first part was for phytochemical analysis and the second part for antimicrobial test. Ethanol used for extraction was removed using a water bath at 40^{o}C.

3.1.10 STERILITY TEST OF THE EXTRACTS

The ethanol extracts of the plants were tested for sterility using the method of Dalitha (2008). 1ml of each of the extract was added into test tube containing 5ml of sterile nutrient broth. It was then incubated at 37^{o}C for 24 hours. The set up or test cultures were clear after incubation indicating the absence of contaminant which would have caused a turbid appearance in the tubes.

3.1.11 DETERMINATION OF PHYTOCHEMICAL CONSTITUENT OF THE PLANT EXTRACTS.

The extracts were subjected to various standard phytochemical analysis to identify the chemical constituents such as tannins, Alkaloids, Flavonoids Saponnins, Steroids, Tannins, Alcoloids, Tepernoids, and Glycosides as described by Amadi *et al.*, (2004). The phytochemicals constituents determined include:

1 Test for Tannins:

A 0.5g of powdered sample of each plant extract was boiled in 20ml distilled water in a test tube for 5 minutes and then filtered. One milliter of $FeCl_3$ was added to 2ml of the filtered samples and observed for brownish green or blue black colouration, which showed the presence of tannins.

2. Test for Saponins:

A 2g of powdered samples of each plant extract was boiled together with 20ml of distilled water in a bath and filtered. A 10ml of the filtered samples was mixed with 5ml of distilled water in a test tube and shaken vigorously to obtain a stable persistent froth. The frothing was

them mixed with 3 drops of olive oil and observed for the formation of emulsion, which indicated the presence saponins.

3. Test for Flavonoids:

A few drops of 1% NH_3 solution was added to the ethanol extract of each sample in a test tube. A yellow colouration was observed showing the presence of flavonoid compounds.

4. Test for Glycosides:

A 1ml concentrated H_2So_4 was put in a test tube. Five milliter (5ml) of ethanol extract from each plant extract sample was mixed with 2ml of glacial CH_3COOH containing 1 drop of $Fecl_3$. The above mixture was carefully added to 1ml of concentrated H_2SO_4 so that the concentrated H_2SO_4 was underneath the mixture. The presence of glycoside in the sample was indicated by the appearance of a brown ring indicating the presence of glycoside constituents

5 Alkaloids

A 200mg plant material boiled in 20ml of 1% H_2SO_4 and filtered. Five drops of conc. H_4H0H was added to the filterate, after which 20ml chloroform was added. The separated chloroform layer was extracted with 20ml dilute H_2SO_4. To the extract, 5 drops of Mayer's/Wagner's/Dragendorff's reagent, a cream/brownish-red/orange-red precipitate indicated the presence of alkaloids.

6 Test for Glucosides.

The powdered extract was evaporated. About 0.5g of the extract was placed in a clean test tube of water. A moist sodium picrate paper was suspended in the neck of the test tube using cork to trap it. The closed tube was placed in a clean test tube of water. A moist sodium picrate paper was suspended in the neck of the test tube using cork to trap it. The closed tube was placed in a warm water bath at 45^oC for 1 hour. The colour change was noted.

7 Test For Steriods:

Two milliters of the extracts were evaporated to dryness in a separated test tube and the residues dissolved in acetic anhydride followed by addition of Chloroform. Concentrated sulphuric acid was added by means of a pipette via the side of the test tubes. Formation of brown ring at the interface of the two liquids and violet colour in the supernatant layer denoted the presence of steroids.

8. Test for Terpenoids:

About 0.5g of plant extract in test tube was taken with 2ml of chloroform; 5ml of concentrated sulphuric acid was carefully added to form a layer and observed for presence of reddish brown color interface to show positive results for the presence of terpenoid.

3.1.12 METHODS FOR EXTRACTION OF PLANT SAMPLES

(1) Extraction of Plant Essential Oil

The techniques adopted by Adepoju *et al.*, (2014) was followed strictly. A 250ml soxhlet extractor apparatus and petroleum ether as solvent were used.The quantity of the oil yield was determined gravimetrically as the ration of the weight of the extracted oil to the weight of the plant powder sampled used. The obtained oil was kept in a refrigerator until it was used.

(2) Crude Plant Extraction

Crude ethanol extraction procedure was carried out by soaking the powdered leaves and powdered seeds in ethanol for 4 days with constant shaking, the mixture was filtered using Whatman number 1 filter paper, centrifuged at 150xg for 20 minutes.The extracts were capped and stored in refrigerators as crude ethanol extraction. Extracts were divided into two potions. The first part was for phytochemical analysis and second part was for antimicrobial test. Ethanol used for extraction was removed using a water bath at 40^0C

3.1.13 DETERMINATION OF ESBL PRODUCING ISOLATE AMONG RESISTANCE ISOLATES

All the strains which showed resistance to ceftazidime and cefotaxime were selected for checking for ESBL production. The ESBL test was conducted by the modified Double Disk Synergy Test (Mchanty, *et al.*, 2010) by using a disk of amoxicillin – clavulanate (20/10μg) along with four cephalosporins, cefotaxime, ceftazidime, ceftriazone, cofpopdoxime, (3GC) and cofepime (4GC). A lawn culture of the organisms were made on muller-Hinton agar plates as was recommended by Clinical and Laboratory Standards Institute (CLSI, 2009). A disc which contained amoxicillin-clavulanate (30μg) was placed in the centre of the plate.

The discs of the other antibiotics were placed 15mm and 20mm apart respectively, centre to centre to that of the amoxicillin-clavulanate disc (Paterson, 2006). Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for ESBL production.

3.1.14 ANTIMICROBIAL SUCESPTIBILITY TEST OF THE PLANTS ESSENTIAL OILS AGAINST EXTENDED SPECTRUM βeta-LACTAMASE (ESBLs) PRODUCING POSITIVE ISOLATES

Disc Preparation for Plant Essential Oils

Filter paper disc were punched from whatman No. 1 fitter paper and sterilized in separate bijou bottles (100 discs per bottle). The different concentration solutions used in the assay were 10μ1 (1ml of essential oil) 7.5μ1 (0.75m1 of essential oil and 0.25ml of DMSO), 5.0μ1 (0.5ml of essential oil and 0.5ml of DMSO) and 2.5μ1 (0.25ml of essential oil and 0.75ml of DMSO) with each disc capable of adsorbing 0.01ml of the solution (Serban *et al*., 2011).

Standardization of inoculum

Using inoculation loop, enough material from an overnight culture of the test organism was transferred into a test tube containing normal saline until the turbidity of the 0.5 McFarland standard is gotten as described by the National committee for clinical laboratory standard (NCCLS, 2008).

3.1.15 WELL DIFFUSION ASSAY OF ESSENTIAL OILS

Standardized inocula of the isolates were swabbed on the surface of prepared and solidified Muller Hinton agar in duplicates, 0.1ml of the active cultures was spread over the plate using a sterile glass spreader in order to get a uniform microbial growth for all the plates (Donaldson *et al.,* 2005). Wells were dug using a 6mm diameter cork borer on the agar plates, and the wells were filled with 10, 20 and 30μl of the essential oils extracts. All the plates were sealed by parafilm under sterile laboratory conditions to avoid evaporation of the essential oils. (Eos). The plates were then left for 30 minutes at room temperature to allow for the diffusion of the oil and were incubated at 37^{o}C for 24 hours.

3.1.16 ANTIMICROBIAL ACTIVITY OF THE CRUDE PLANTS EXTRACTS AGAINST THE ESBL PRODUCING POSITIVE ISOLATES

Activity of the two plant extracts against ESBL Isolates was screened by using the agar- well diffusion method (Perez *et al.,* 1990) An inoculum suspension was swabbed uniformly to solidified 20ml Mueller Hinton Agar (MHA) for bacteria and the inoculum was allowed to dry for 5 minutes. Holes of 6mm in diameter were made in the seeded agar using sterile cork borer. Aliquots of 100mg/ml, 50 mg/ml, 25 mg/ml and 12.5mg/ml from each plant extract was added into each well on the seeded medium and allowed to stand on the bench for one hour for proper diffusion and thereafter incubated at 37^{0}C for 24 hours. After incubation, the diameters of the growth inhibition zones were measured in millimeters. (mm). Three replicates were carried out for each extract against each of the test organisms that produced Extended Spectrum βeta-Lactamases (ESBL).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 ANTIMICROBIAL RESISTANT PROFILE OF *E.coli* AND *Klesbiella pneumoniae* ISOLATES FROM RIVER WATER SAMPLES.

The results of antimicrobial resistant profile of *E.coli and Klebsiella pneumonia* isolates are shown in tables below.The susceptibility of the isolates of ten (10) different antibiotics was examined by disc diffusion test. Out of the hundred (100) isolates of *E.coli* and *Klebsiella pneumoniae* from River Water assessed for antimicrobial susceptibility, the highest resistant rates of (99%), was observed for ampicillin (AMP) in all the isolates from the two samples. This was followed by cefotaxime (CTX), imipenem (IPM), aztreonam (ATM), meropenem (MEM), ertapenem (ETP) and cefazidime with resistant rates of (83%), (77%), (75%), (74%), (70%) and (53%) respectively. The resistant rates were moderate for ciprofloxacin (CIP) and gentamycin (CN) at (40%) and (33%) while resistance rate was low for lavofloxacin (19%), hence the antimicrobial resistant profile of *E.coli* and *Klebsiella pneumoniae* isolates from River Water are as shown on Table 4.1.1

4..1.2 ANTIMICROBIAL RESISTANT PROFILE OF *E.coli* AND *Klebsiella pnumoniae* ISOLATED FROM AQUA CULTURE WATER SAMPLES.

Out of the hundred isolates of *E.coli* and *Klebsiella Pneumoniae* assessed for antimicrobial resistance, high resistants rate were observed for ampicillin (95%) and ceftazidime (86%). The resistant rates were moderate for ertapenem (65%), imipenem (56%), cefotaxime (44%), ciprofloxacin (43%), meropenem (41%) and lavofloxacin (33%). While resistants rate were low for gentamycin (18%) and aztreonam (8%). (Table 4.1.2)

Table 4.1.1: Antimicrobial RESISTANT PROFILE OF *E.coli and Klebsiella pneumoniae* Isolated from River Water samples

ANTICIOTICS	**No(%) of Resistant Isolates**		
	Ecoli	***Klebsiella pneumoniae***	**Total Resistant**
	(n=50	***(n=50)***	**(n=100)**
Ampicillin	50 (100)	49 (98)	99 (99)
Cefotaxime	39 (78)	44 (88)	83 (83)
Imipenem	43 (86)	34 (68)	77 (77)
Aztreonam	36 (72)	39 (78)	75 (75)
Meropenem	36 (72)	38 (76)	74 (74)
Ertapenem	37 (74)	33 (66)	70 (70)
Ceftazidime	20 (40)	33 (66)	53 (53)
Ciprofloxacine	22 (44)	18 (36)	40 (40)
Genetamycin	16 (32)	18 (36)	34 (34)
Lavofloxacin	8 (16)	11 (22)	19 (19)

Table 4.1.2: ANTIMICROBIAL RESISTANT PROFILE OF *E.coli AND Klebsiella pneumoniae* ISOLATED FROM AQUA CULTURE WATER SAMPLES.

ANTICIOTICS	**No(%) of Resistant Isolates**		
	Ecoli (n=50	***Klebsiella pneumoniae (n=50)***	**Total Resistant (n=100)**
Ampicillin	45(90)	50(100)	95(95)
Ceftazidime	43(86)	43(86)	86(86)
Ertapenem	27(54)	38(76)	65(65)
Imipenem	31(62)	25(50)	56(56)
Cefotaxime	23(46)	21(42)	44(44)
Ciprofloxacin	17(34)	26(52)	43(43)
Meropenem	4(8)	37(74)	41(41)
Lavofloxacin	17(34)	16(32)	33(33)
Gentamycin	8(16)	10(20)	18(18)
Aztreonam	4(8)	4(8)	8(8)

4.1.3 ANTIMICROBIAL RESISTANT PROFILE OF *E.coli* AND *Klebsiella pneumoniae* ISOLATED FROM CHICKEN CLOACAL SWAB SAMPLES

Out of the 100 isolates of *E.coli* and *Klebsiella pneumoniae* assessed for antimicrobial resistance, high resistant rates were observed for ampicillin (92%), and cefotaxime 71%. The resistant rates were moderate for ceftazidime (56%), ciprofloxacin (52%), imipenem (49%), meropenem (45%), gentamycin (44%) and ertapenem (37%). While resistant rate was low for lavofloxacin (24%). Hence, the antimicrobial Resistant Profile of *E.coli* and *Klebsiella pneumoniae* Isolated from Chicken Cloacal Swab is shown on Table 4.1.3.

Table 4.1.3 Antimicrobial RESISTANT PROFILE OF *E.coli AND Klebsiella pneumoniae* Isolated from Chicken Cloacal Swab Samples.

ANTICIOTICS	**No(%) of Resistant Isolates**		
	Ecoli (n=50	***Klebsiella pneumoniae (n=50)***	**Total Resistant (n=100)**
Ampicillin	43(86)	49(98)	92(92)
Cefotaxime	24(48)	47(94)	71(71)
Ceftazidime	20(40)	36(72)	56(56)
Ciprofloxacin	18(36)	34(68)	52(52)
Imipenem	11(22)	38(76)	49(49)
Meropenem	6(12)	40(80)	46(46)
Aztreonam	5(10)	40(80)	45(45)
Gentamycin	9(18)	35(70)	44(44)
Ertapenem	11(22)	26(52)	37(37)
Lavofloxacin	6(12)	18(36)	24(24)

4.1.4 COMPARATIVE ANALYSIS OF RESISTANT RATES (%) FROM THE THREE DIFFERENT SAMPLES ANALYSED.

The total rates of resistance to the antibiotics were as follows: ampicillcin (95.4%), cefotaxime (66.1%), ceftazedime (65.1%), imipenem (60.6%), ertapenem (57.4%), meropenem (53.7%), azetreonam (42.7%), ciproflaxacin (44.9%), lavafloxaxin (25.3%) and gentamycin (32%). The total resistant profile of *E.coli* and *Klebsiella pneumoniae* is as shown on Table 4.1.4.

TABLE 4.1.4 :COMPARATIVE RESISTANT RATES (%) OF THE ISOLATES FROM THE THREE DIFFERENT ENVIRONMENTAL SAMPLES.

Antibiotics	River water (n=100)	Aquaculture (n = 100)	Chickhen Cloacal Swab (n = 100)	Total (n = 300)
Ampicillin	99(33)	95 (31.7)	92(30.7)	286(95.4)
Cefotaximo	83(27.7)	44(14.7)	71(23.7)	198(66.1)
Ceftazedime	53 (17.7)	86(28.7)	56(18.7)	195(65.1)
Imiperiem	77(25.7)	56(18.6)	49(16.3)	182(60.6)
Ertapenem	70(23.5)	65(21.6)	37(12.3)	172(57.4)
Meropenem	74(24.7)	41(13.7)	46(15.3)	161(53.7)
Azetreonam	75(25)	8(2.7)	45(15)	128(42.7)
Ciprofloxacin	40(13.3)	43(14.3)	52(17.3)	135(44.9)
Lavafloxacin	19(6.3)	33(11)	24(8)	76(25.3)
Gentamycin	34(11.3)	18(6)	44(14.7)	96(32)

4.1.5 ANTIBIOTIC RESISTANT PATTERNS OF *E.coli* FROM RIVER WATER SAMPLES.

A high level of variability of resistance was seen among the isolates *E.coli* from River water, where a total of twenty-six (26) different patterns were noticed, the most predominant pattern being ; CTX.ETP IPM MEM AMP ATM. This is as shown on Table 4.1.5.

4.1.6 ANTIBIOTIC RESISTANT PATTERNS OF *E.coli FROM* AQUA CULTURE WATER SAMPLES

A high level of variability of resistance was seen among *E.coli* in Aqua Culture, where a total of thirty six (36) different patterns were noticed , the most predominant patterns being CAZ ATM AMP IPM; CAZ CTX ETP ATM AMP; CAZ CTX ETP AMP IPM. This is as shown in Table 4.1.6.

TABLE 4.1.5: ANTIBIOTIC RESISTANT PATTERNS OF *E.coli* FROM RIVER WATER SAMPLES

S/N	PATTERN	NUMBER OF ISOLATES (n=50)
1	CTX ETP IPM MEM AMP ATM	8
2	CTX CAZ ETP 1PM MEM CN CIP AMP ATM	7
3	CTX CAZ ETP IPM MEM AMP ATM	4
4	CTX ETP IPM MEM LEV CIP AMP	2
5	CTX CAZ ETP IPM MEM LEV CN CIP AMP ATM	2
6	CTX ETP IPM MEM CN CIP AMP	2
7	CTX ETP IPM AMP ATM	2
8	CTX IPM MEM AMP ATM	2
9	ETP IPM AMP	2
10	IPM APM ATM	2
11	CTX ETP IPM MEM CN AMP ATM	2
12	CTX ETP IPM MEM AMP	1
13	CTX CAZ ETP IPM CN CIP AMP ATM	1
14	ETP IPM MEM AMP	1
15	CTX CAZ ETP MEM CIP AMP ATM	1
16	CTX ETP IPM MEM LEV CN CIP AMP ATM	1
17	CTX CAZ ETP IPM MEM LEV CIP AMP	1
18	CTX CAZ AMP ATM	1
19	CAZ MEM AMP	1
20	CAZ ETP IMP AMP ATM	1
21	CTX IPM AMP ATM	1
22	ETP IPM MEM AMP ATM	1
23	CTX ETP IPM MEM CIP AMP ATM	1
24	IPM LEV AMP	1
25	CTX AMP	1
26	IPM CIP AMP	1

KEY: CN (GENTAMYCIN), IMP (IMIPENEM), AMP (AMPICILLIN), LEV (LEVOFLOXACIN), CAZ (CEFTAZIDIME), ATM (AZTREONAM), CTX (CEFOTAXIME), CIP (CIPROFLOXACIN), MEM (MEROPENEM), ETP (ERTAPENEM).

TABLE 4.1.6: ANTIBIOTIC RESISTANT PATTERNS OF *E.coli* FROM AQUA CULTURE WATER SAMPLES

S/N	PATTERN	NUMBER OF ISOLATES (n=50)
1	CAZ ATM AMP IPM	3
2	CAZ CTX ETP ATM AMP	3
3	CAZ CTX ETP AMP IPM	3
4	CAZ CTX ATM AMP IPM CN CIP	2
5	CAZ CTX ATM AMP IPM	2
6	CAZ CTX AMP	2
7	ETP AMP IPM	2
8	CAZ LEV ATM AMP IPM	2
9	CAZ LEV AMP	2
10	CAZ ATM AMP	2
11	CAZ LEV ATM AMP IPM CN	2
12	ETP LEV ATM AMP	1
13	CAZ CTX ETP ATM AMP IPM CIP	1
14	CAZ CTX ETP LEV ATM AMP IPM CN	1
15	CAZ CTX ETP ATM AMP IPM	1
16	CAZ CTX ETP LEV ATM AMP IPM	1
17	CAZ ETP AMP	1
18	CAZ CTX ETP IPM	1
19	CAZ CTX ETP	1
20	CAZ ETP AMP IPM	1
21	CAZ CTX ETP LEV ATM AMP IPM CIP	1
22	CAZ ETP LEV ATM AMP IPM CIP	1
23	CAZ CTX ETP ATM AMP IPM CIP MEM	1
24	CAZ CTX ETP ATM AMP IPM CIP MEM	1
25	CAZ ATM AMP IPM CN	1
26	CAZ LEV ATM AMP CN CIP	1
27	CAZ LEV AMP CN CIP	1
28	CAZ LEV ATM AMP	1
29	CAZ CTX ETP ATM AMP CN	1
30	AMP	1
31	CAZ ETP LEV ATM AMP IPM	1
32	CAZ ATM AMP IPM CIP	1
33	CAZ ETP LEV ATM AMP IPM MEM	1
34	CAZ ETP LEV ATM AMP IPM MEM	1
35	ETP LEV AMP IPM MEM	1
36	ETP	1

KEY: CN (GENTAMYCIN), IMP (IMIPENEM), AMP (AMPICILLIN), LEV (LEVOFLOXACIN), CAZ (CEFTAZIDIME), ATM (AZTREONAM), CTX (CEFOTAXIME), CIP (CIPROFLOXACIN), MEM (MEROPENEM), ETP (ERTAPENEM).

4.1.7: ANTIBIOTIC RESISTANT PATTERNS OF *E.coli* FROM CHICKEN CLOACAL SWAB SAMPLES.

The result of Antibiotic resistant pattern of chicken cloacal swab is as shown on Table 4.7. A high level of variability of resistant pattern was seen among *E.coli* in chicken cloacal swab. Where a total of twenty nine (29) different pattern were noticed. Among the *E.coli* in chicken cloacal swab, the highest number of resistant pattern occurred among the following antibiotics AMP. (Table 4.1.7) .

TABLE 4.1.7: ANTIBIOTIC RESISTANT PATTERNS OF *E.coli* FROM CHICKEN CLOACAL SWAB SAMPLES.

S/N	PATTERN	NUMBER OF ISOLATES (n=50)
1	AMP	13
2	IPM AMP	3
3	CTX CAZ IPM CN CIP AMP	3
4	CTX CAZ ETP IPM CIP AMP	2
5	CTX CAZ AMP	2
6	IPM CIP AMP	2
7	CTX CAZ IPM CIP ATM	2
8	CTX CAZ ETP LEV CN CIP AMP	2
9	CAZ MEM CIP AMP	1
10	ETP MEM AMP	1
11	CTX AMP	1
12	CTX CN CIP AMP	1
13	IMP CN AMP	1
14	CTX LEV CIP AMP	1
15	CTX CAZ IPM MEM LEV AMP	1
16	CTX CAZ ETP IPM MEM AMP	1
17	ETP IPM AMP	1
18	CTX CAZ ETP IPM AMP	1
19	CN AMP	1
20	CTX ETP MEM AMP ATM	1
21	CTX CN AMP	1
22	CTX CAZ ETP LEV AMP	1
23	CTX CAZ ETP IPM CIP AMP ATM	1
24	CTX CAZ IPM LEV CIP AMP	1
25	CTX CAZ IPM	1
26	ETX IPM LEV CIP AMP	1
27	EAZ ETP CIP AMP	1
28	CIP AMP	1
29	ETP AMP	1

KEY: CN (GENTAMYCIN), IMP (IMIPENEM), AMP (AMPICILLIN), LEV (LEVOFLOXACIN), CAZ (CEFTAZIDIME), ATM (AZTREONAM), CTX (CEFOTAXIME), CIP (CIPROFLOXACIN), MEM (MEROPENEM), ETP (ERTAPENEM).

4.1.8: ANTIBIOTIC RESISTANT PATTERNS OF *Klebsiella pneumoniae ISOLATED FROM* RIVER WATER SAMPLES.

A high level of variability of resistant pattern was seen among the *Klebsiella pneumoniae* on River water. Where a total of thirty six (36) different pattern were noticed. Among the *Klesbiella pneumoniae*, the highest number of resistant pattern occurred among the following antibiotics; CTX CAZ ETP IPM MEM LEV CN CIP APM ATM. The resistant pattern can be seen as shown on Table 4.1.8

4.1.9: ANTIBIOTIC RESISTANT PATTERNS OF *Klebsiella pneumoniae* ISOLATED FROM AQUA CULTURE WATER SAMPLES.

The result of Antibiotic resistant pattern of *Klebsiella pneumoniae* on Aqua culture is as shown on Table 4. 1.9 . A total of forty three (43) resistant patterns was noticed among the isolates with the most predominant being CAZ ETP AMP IPM CIP MEM.

TABLE 4.1.8: ANTIBIOTIC RESISTANT PATTERNS OF *Klebsiella Pneumoniae* FROM RIVER WATER SAMPLES

S/N	PATTERN	NUMBER OF ISOLATES (n=50)
1	CTX CAZ ETP IPM MEM LEV CN CIP APM ATM	6
2	CTX CAZ ETP IPM MEM CN CIP APM ATM	3
3	CTX CAZ ETP IPM MEM CN APM ATM	3
4	CTX CAZ ETP IPM MEM LEV APM ATM	3
5	CTX ETP IPM MEM APM ATM	2
6	CTX CAZ ETP IPM MEM CIP AMP ATM	2
7	CTX ETP IPM AMA ATM	2
8	CTX CAZ ETP MEM APM ATM	1
9	CTX ETP IPM MEM APM	1
10	CTX CAZ IPM MEM CN CIP AMP ATM	1
11	CTX CAZ IPM MEM APM ATM	1
12	CTX CAZ ETP MEM CIP APM ATM	1
13	CTX CAZ MEM APM ATM	1
14	CTX CAZ IPM APM	1
15	CTX CAZ ETP IPM MEM CN APM ATM	1
16	CTX ETP IPM MEM CIP ATM	1
17	CTX CAZ MEM CIP AMP ATM	1
18	CTX MEM CIP AMP	1
19	MEM	1
20	CTX MEM AMP	1
21	CAZ MEM AMP	1
22	CTX CAZ ETP LEV AMP ATM	1
23	CTX IPM AMP	1
24	CTX CAZ IPM MEM CN AMP	1
25	CTX CAZ ETP AMP	1
26	CTX MEM CN CIP AMP	1
27	CTX MEM LEV AMP ATM	1
28	CTX CAZ ETP IPM CN AMP ATM	1
29	IPM CN AMP ATM	1
30	ETP IPM MEM AMP ATM	1
31	CTX CAZ ETP AMP ATM	1
32	CTX AMP ATM	1
33	ETP AMP ATM	1
34	CAZ IPM AMP ATM	1
35	CTX CAZ ETP IPM MEM AMP	1
36	CTX ETP MEM AMP ATM	1

KEY: CN (GENTAMYCIN), IMP (IMIPENEM), AMP (AMPICILLIN), LEV (LEVOFLOXACIN), CAZ (CEFTAZIDIME), ATM (AZTREONAM), CTX (CEFOTAXIME), CIP (CIPRPFLOXACIN), MEM (MEROPENEM, ETP (ERTAPENEM).

TABLE 4.1.9: ANTIBIOTIC RESISTANT PATTERNS OF *Klebsiella pneumonia* FROM AQUA CULTURE WATER SAMPLES

S/N	PATTERN	NUMBER OF ISOLATES (n=50)
1	CAZ ETP AMP IPM CIP MEM	6
2	CAZ ETP AMP CIP MEM	2
3	CAZ ETP LEV ATM AMP CIP MEM	2
4	CAZ CTX ETP LEV AMP IPM CIP	1
5	CAZ CTX ETP AMP MEM	1
6	CAZ CTX ETP LEV MEM	1
7	CAZ CTX ETP AMP CIP MEM	1
8	CAZ CTX ETP LEV AMP IPM CIP MEM	1
9	CAZ CTX ETP AMP CN CIP MEM	1
10	CAZ CTX ETP AMP IPM MEM	1
11	CAZ ETP AMP IPM CN MEM	1
12	CAZ ETP AMP IPM MEM	1
13	CAZ CTX ETP LEV AMP CN CIP MEM	1
14	CAZ CTX ETP AMP IPM CN MEM	1
15	CAZ ETP ATM AMP MEM	1
16	CAZ ETP LEV ATM AMP CN CIP MEM	1
17	CAZ ETP LEV ATM AMP IPM CIP MEM	1
18	CAZ ETP LEV AMP MEM	1
19	CAZ CTX ETP LEV ATM AMP IPM CIP MEM	1
20	CAZ CTX ETP ATM AMP IPM MEM	1
21	ETP AMP IPM CIP MEM	1
22	CAZ CTX ATM AMP IPM MEM	1
23	CAZ AMP MEM	1
24	CAZ ATM AMP IPM CIP	1
25	CAZ CTX ETP ATM AMP IMP CIP	1
26	CAZ CTX ETP ATM AMP CN	1
27	CAZ CTX ETP ATM AMP CIP	1
28	CAZ CTX ETP ATM AMP CN CIP MEM	1
29	CAZ CTX AMP MEM	1
30	CAZ LEV ATM AMP IPM CIP MEM	1
31	CAZ ATM AMP IPM MEM	1
32	CAZ ETP AMP IPM MEM	1
33	ETP AMP IPM MEM	1
34	CAZ LEV ATM AMP IPM	1
35	CAZ CTX ETP AMP IPM	1
36	ETP AMP	1
37	CAZ AMP IPM CN CIP	1
38	AMP	1
39	ETP LEV AMP IPM CN CIP MEM	1
40	CAZ ETP LEV AMP IPM CIP MEM	1
41	LEV AMP	1
42	CAZ CTX AMO CIP	1
43	CAZ ETP LEV AMP IPM CN CIP MEM	1

KEY: CN (GENTAMYCIN), IMP (IMIPENEM), AMP (AMPICILLIN), LEV (LEVOFLOXACIN), CAZ (CEFTAZIDIME), ATM (AZTREONAM), CTX (CEFOTAXIME), CIP (CIPRPFLOXACIN), MEM (MEROPENEM, ETP (ERTAPENEM).

4.1.10: ANTIBIOTIC RESISTANT PATTERNS OF Klebsiella pnumoniae FROM CHICKEN CLOACAL SWAB SAMPLES

The result of Antibiotic resistant pattern of klebsiella pneumoniae on chicken cloacal swab is as shown on Table 4. 1. 10. A total of thirty seven (37) resistant pattern was noticed among the isolates with this pattern, CTX CAZ ETP IPM MEM CIP AMP ATM, being the most predominant.

TABLE 4.1.10: ANTIBIOTIC RESISTANT PATTERNS OF *Klebsiella pneumonia FROM* CHICKEN CLOACAL SWAB SAMPLES

S/N	PATTERN	NUMBER OF ISOLATES (n=50)
1	CTX CAZ ETP IPM MEM CIP AMP ATM	7
2	CTX CAZ IPM MEM LEV CIP AMP ATM	3
3	CTX CAZ ETP IPM MEM LEV CN CIP AMP ATM	3
4	CTX IPM CN AMP	2
5	CTX ETP IPM MEM CN CIP AMP ATM	2
6	CTX CAZ IPM MEM CN CIP AMP ATM	2
7	CTX CAZ IPM MEM CIP AMP ATM	1
8	CTX CAZ CN AMP ATM	1
9	CTX ETP MEM LEV CN CIP AMP	1
10	CTX IPM MEM LEV CN CIP AMP ATM	1
11	CTX ETP IPM MEM CN AMP	1
12	CTX CAZ ETP IPM MEM CN AMP	1
13	CTX CAZ IPM MEM LEV CN AMP ATM	1
14	CTX MEM LEV CN AMP ATM	1
15	CTX ETP CN AMP	1
16	CTX ETP MEM CN AMP	1
17	CTX CAZ IPM MEM LEV CN CIP AMP ATM	1
18	CTX CAZ IPM CN AMP ATM	1
19	CAZ ETP MEM CN CIP AMP	1
20	CTX MEM CIP AMP ATM	1
21	CTX MEM LEV CN CIP AMP	1
22	CTX CAZ ETP MEM CN AMP ATM	1
23	CTX CAZ MEM CN CIP AMP	1
24	CTX CAZ IPM CIP AMP ATM	1
25	CTX CAZ ETP IPM MEM LEV CIP AMP ATM	1
26	CAZ IPM CN CIP AMP ATM	1
27	CTX CAZ IPM LEV CN AMP ATM	1
28	CTX CAZ ETP IPM CN CIP AMP ATM	1
29	CTX CAZ ETP IPM MEM LEV CIP ATM	1
30	CTX CAZ ETP IPM MEM CN CIP AMP ATM	1
31	CTX CAZ ETP IPM MEM LEV CN CIP AMP ATM	1
32	IPM MEM AMP ATM	1
33	CTX CAZ ETP IPM MEM AMP ATM	1
34	CTX CAZ IPM LEV CN CIP AMP ATM	1
35	CTX MEM CN CIP AMP ATM	1
36	CTX CAZ IPM MEM CN AMP ATM	1
37	CTX CAZ ETP IPM MEM CN AMP ATM	1

KEY: CN (GENTAMYCIN), IMP (IMIPENEM), AMP (AMPICILLIN), LEV (LEVOFLOXACIN), CAZ (CEFTAZIDIME), ATM (AZTREONAM), CTX (CEFOTAXIME), CIP (CIPRPFLOXACIN), MEM (MEROPENEM, ETP (ERTAPENEM).

4.1.11 FREQENCY (%) OF EXTENDED SPECTRUM BETA LACTAMASE (ESBL) PRODUCERS FROM THE DIFFERENT SAMPLE TYPES

Of the three hundred (300) isolates which were studied. 20 (22.2%) isolates were found to be ESBL producers by the Modified Double Disk Synergy Test. (MDDST). All these Isolates showed a clear extension of the edge of inhibition which was produced by the antibiotic towards the amoxicillin clavulanate disk. (7.7%) of the extended spectrum βeta-lactamase (ESBL) producers were from chicken cloacal swab samples, (4.4%) were from river water samples, (10%) were from Aqua culture water samples. The Table can be seen as shown on Table 4.1.11.

Table 4.1.11: FREQUENCY (%) OF ESBL PRODUCERS FROM THE DIFFERENT SAMPLE TYPES

Sampling Sources	***E.coli***	***Klebsiella Pneumoniae***	**Total**
Chicken cloacal Swab(n=30)	5(16.6)	2(6.6)	7(7.7)
River water (n=30)	3(10)	1(3.3)	4(4.4)
Aqua culture (n=30)	6(20)	3(0.6)	9(10)
TOTAL (90)	**14(46.6)**	**6(19.9)**	**20(22.28)**

4.1.12 EFFECT OF PLANT ESSENTIAL OILS ON EXTENDED SPECTRUM BETA-LACTAMASE (ESBL) PRODUCING ISOLATES

After performing the susceptibility test with well in agar diffusion assay using extract oil from *Moringa Oleifera* and scent leaf *(Ocimum gratissimum*) on extended spectrum βeta-lactamase (ESBL) Isolates, it was noticed that there was no zone of inhibition on the isolates.

4.1.13 ANTIMICROBIAL SUSCEPTIBILITY TEST OF THE PLANT EXTRACTS

The Extended Spectrum βeta-Lactamase (ESBL) producing *E. coli* and *Klebsiella pneumoniae* were tested with extract of scent leaf, (*Ocimum gratissimum*) and *Moringa oleifera* to the MIC of the extracts. It was observed that, there was inhibition of the Isolates by the extracts at different concentations in both aqueous and ethanol extracts and the minimum inhibitory concentrations of between 1.32 mg/ml and 1.78 mg/ml for the two plant extracts (Table 4.1.13)

4.1.14 PHYTOCHEMICAL CONSTITUENTS OF THE CRUDE AQUEOUS AND ETHANOL EXTRACTS OF THE PLANTS.

After performing the phytochemical analysis of both the aqueous and ethanol extracts, it was noticed that some phytochemicals are found more in one extractant while the other lacked such phytochemicals in them. This study, indicates the presence of some phytochemicals, such as Alkaloids, Tannins, Saponina, Flavonoids Glycosides, Terpernoid, in the plant samples.This is as shown on Tables 4.1.13 and 4.1.14.

TABLE 4.1.13: QUALITATIVE PHYTOCHEMICAL SCREENING OF CRUDE ETHANOLIC AND HOT WATER EXTRACTIONS OF SCENT LEAF *(Ocimum gratissimum)*

	OE	Ow
Alkaloids	+	-
Saponins	+	+
Tannins	+	+
Flavonoids	+	+
Steriods	-	-
Glycosides	+	+

OW = Water extract of *O. gratissimum*

Oe = Ethanol extract of *O. gratissimum*

TABLE 4.1.14: QUANTITATIVE PHYTOCHEMICAL SCREENING OF CRUDE ETHANOLIC AND HOT WATER EXTRACTIONS OF MORINGA SEED

Extract type	Tannins	Alkaloids	Flavonoids	Terpernoid	Glycosides	Saponnins
Ethanol extract	++	+++	++	+	+	++
Hot water extract	+	++	+	++	++	++

Key

+ = slightly present

++ = Moderately present

+++ = Abundantly present

4.2 DISCUSSION

Enterobacteriaceae such as *E. coli* and *Klebsiella pneumonia* are among the most important causative agents of hospital acquired and community onset infections of human beings (Paterson, 2006).

In this study, the susceptibility data of environmental enterobacteriaceae isolates demonstrated remarkable resistance to commonly used antibiotics. The susceptibility of the isolates to ten (10) different antibiotics was examined by disk-diffusion test.

The high resistant ot these antibiotics is probably due to wide use of the antibiotics drugs as growth promoter,prophylaxis and therapy in poultry and fishfarms and discharge of waste from households,drug manufacturing units, hospitals and poultry industry in the body of waters.These antibiotics may have lead to evolution of bacteria that are resistant to antibiotics which then grow in numbers and spread in the environment.Bacteria resistant to antibiotics like beta –lactam, multi-drug /efflux are highly abundant in the river water.

The presence of Extended Spectrum βeta-lactamase (ESBL) on plasmids also makes it possible for them to be easily transferred from one organism to another.Some studies have shown that Floroquinoles and Aminoglycosides have greater antimicrobial activity against ESBL organisms than non beta –lactams drugs (Jean *et al*.,2002).Some other studies have also shown that the use of beta-lactamase inhibitors such as amoxicillin – clavulanic acid and piperracillin – tazo bactam in the treatment of ESBL isolates is possible, others states that invitro susceptibility testing does not predict a killing activity of beta-lactamase inhibitor (Burgess, 2001).

The constant use of third generation cephalosporins in the treatment of infections in Nigeria is probably the reason for the current spread of Extended Spectrum βeta-lactamase (ESBL) organisms in our environment (Peterson *et al*., 2000).

Information as regards to ESBL is very uncommon in our environment and as a result, most clinicians probably don't know when to test for ESBL or any preventive measures to adopt which will help to control its spread. This level of ignorance could culminate to devastating consequences. It is very important to note that some apparently efficacious drugs against ESBL producers are not readily available in Nigeria and where available, maybe beyond the reach of the common man. The need to avert the further spread of the enzyme is thus emphasized.

The prevalence of Extended Spectrum βeta-lactamase (ESBLs) among environmental isolates vary greatly worldwide and it is rapidly changing over time. In the west, the Extended Spectrum βeta-lactamase (ESBL) production in Enterobacteriaceae varies from 5 to 52 percent and in other Asian countries, it varies from 10 to 46.5 percent (Clinical & Laboratory Standards Institute (2009) ; Dhillon. & Clark. 2012).

The extended spectrum βeta-lactamase (ESBLs) production coexists with the resistance to several other antibiotics. The extended spectrum βeta-lactamase (ESBLs) are encoded by plasmids which also carry resistant genes for other antibiotics. Jacoby & Sutton. (1991).

A co-resistance to the quinolones and aminoglyycosides is common (Paterson *et al.*, 2000).

We found such an associated resistance with ten (10) different antibiotics used in this study. The high resistance to the non β-lactam antibiotics of the ESBL producing strains possess a threat of treatment failure by these drugs.

Although β-lactam/β-lactamase inhibitor combinations have been suggested as the treatment option for ESBL producers, these drugs must be given in high doses (Gold and Mcellering 1996).

The susceptibility test of the ESBL with essential oil did not show inhibitory activity at all. Oils of *Moringa Oleifera* and scent leaf (*Ocimum gratisimum)* presented no inhibitory activity against ESBL producers. Pathogens causing nosocomial infections have been

reported to be resistant to most antimicrobial agents (Hsueh *et al.,* 2002). The susceptibility of resistant isolates to crude plant extracts show significant levels of inhibition in this study. Previous studies on *Ocimum gratissimum* and *vernonia amygdalina* extracts on antibacterial activity of cold water and ethanol extracts show slight inhibition (Alo *et al.,* 2012).

Antimicrobial analysis of the essential oils against the Extended Spectrum βeta-lactamase (ESBL) producers showed no inhibitory activity while the plant extracts produced zones of inhibition.

Antimicrobial susceptibility test indicated that the extracts, inhibited the isolates at different concentrations used. More especially all the extracts inhibited the growth of both *E.coli* and *Klebsiella pneumoniae* more at 100mg/ml concentration especially in *Ocimum gratissium*. Also, all the extracts was unable to inhibit the growth of the test isolates at 12.5mg/ml concentration. Some previous studies however, have shown that further purification of extracts could enhance their antimicrobial properties (Okoli & Iroegbu, 2014).

Phytochemical analysis showed the presence of alkaloids, saponins, tannins, flavonoids and glycosides in different quantities. *Moringa oleifera* seed and *Ocimum gratissimum* leaf extracts could be used as effective candidates for the treatment of antimicrobial resistant Enterobacteriaceae.According to Whitney *et al*., (2002),these phytochemicals are important for their pharmacological effects.In particular,the Flavonoids and Alkaloids were detected in the extracts of the *Ocimum gratissimum* grown in Nigeria.The antimicrobial activity of Flavonoids maybe due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall,thereby,distrupting their membrane intergrity (Tsuchiya *et al*.,1996).It is noteworthy that phytochemicals are the most important antioxidants.Antioxidants are molecules that can delay or prevent an oxidation reaction catalysed by free radicals.

Saponins which are gycocides with soapy characteristics could support the herbal medicinal uses of *Ocimum gratissimum* and Moringa olifera as antioxidant and its edible leaves being used to prepare medicine.The antioxidant effect is mainly due to the presence of phenolic components such as Flavonoids and phenolic acids.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

Resistance to multidrug in Enterobacteriaceae may result from combined effects of β-lactamases and decreased outer membrane permeability.The frequency of ESBL production can easily be under estimated in the clinical isolates of *E.coli* and *Klebsiella pneumoniae* with the use of the current CLSI recommendation methods,since these organisms often produce multiple β –lactamases.

The plant extracts possess antimicrobial activity against bacteria more than the essential oil.The present study reveals more information on environmental research studies of essential oils and plant extracts.However,more efforts are required to conduct clinical trials in the future.Most of these antimicrobial studies using essential oils have failed to provide defined information on their chemical nature as well as their mechanisms of action.This poses ambiguity on the reproducibility and accuracy of their discoveries.Therefore, further research should focus on exploring the molecular mechanisms of essential oils and plant extracts and their individual compounds.

Biopharmaceutical industries are in need of ecofriendly alternative drug molecules to treat diseases associated with microbial pathogens and body metabolism.Thus,plant extracts and essential oils might be a prospective antimicrobial agents and may play an important role in the discovery of new drugs for the treatment of a wide range of pathogenic microorganisms in the near future.

5.2 RECOMMENDATIONS

The present study revealed more information on diverse chemical structures and mechanisms of action of phytochemicals which are attractive therapeutic tools for discovering bioactive product in the next few years. However, continued researches should be carried out for better

understanding of exact mechanisms and also pharmacodynamic and pharmacokinectic properties of the molecules.

The present study also revealed that the antimicrobial susceptibility test indicated that the extracts inhibited the isolates at different concentrations used. More especially all the extracts inhibited the growth of *E. coli* and *Klebsiella pneumonia* more at 100mg/ml concentration especially in *Ocimum gratissium*. Therefore, further purification of extracts could enhance their antimicrobial properties, and can be used in the treatment of infectous diseases caused by resistant microbes.

Finally, researchers can averts the antimicrobial resistance crisis by improving education and knowledge of antimicrobial resistance while providing support for research on novel antimicrobials and implementing strategies to combat misuse and reduce use of antibiotics worldwide.

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APPENDIX

APPENDIX 1

MEDIA PREPARATION

SOLID MEDIA

(a) NUTRIENT AGAR

FORMULA AND PREPARATION

Yeast extract	-	3.0g
Peptone	-	5.0g
Agar No 1	-	15.0g
Distilled water	-	1000.0cm^3

Dissolve 28g in 1000.0cm^3 of distilled water. Boil gently to dissolve completely. Adjust PH to 7.0. Sterilize for 15 minutes at 121^{0}C. Allow to cool 40-45^{0}C before dispensing into bottles and plates.

(b) Mueller Hinton Agar

Ph	7.3$\pm$0.1
Beef infusion solid	2.0
Acid Hydrolysed casein	17.5
Starch	1.5

Disperse 38g in one liter of deiionized water. Soak for 10 minutes, swirl to mix and sterilize by autoclaving for 15 minutes at 121^{0}C. Cool to 47^{0}C and mix before pouring into petri dishes and then dry the agar surface.

(c) MacConkey Agar

Composition a preparation

Ingredient	Amount
Peptone (Pancreatic digest of gelatin)	17gm

Proteose peptone (meat and casein)	3g
Lactose monohydrate	10g
Bile salts	1.5gm
Sodium chloride	5gm
Neutral red	0.03gm
Crystal violet	0.001g
Agar	13.5gm
Distlled water	Add to make 1 liter

Final PH 7.1 +/_ 0.2 at 25^0C

- Suspend 49.53 grams of dehydrated medium in 1000ml purified/distilled water
- Head to boding to dissolve the medium completely
- Sterilize by autoclaving at 15 ibs pressure (121^0C) for 15mins
- Cool to 45.50^0C
- Mix well before pouring into sterile petril plates

d. EMB Agar

Agar 15.0

Methylene blue 0.065

Eosin Y: 0.4

Typical formular: gm/litre

Final PH (at 25^0C)

- Suspend the agar in 1000ml distilled water. Make sure you mix it well in order to achieve a uniform suspension.
- Bring to boil so that medium will be dissolved completely.
- Sterile the medium through autoclaving at 121oC for about 15 minutes. Make sure you do not overheat the medium.

- Allow the medium to cool a shake to oxide the ethylene blue and suspend the precipitate.
- If you are going to inoculate the agar on the same day, then you don't necessarily need to autoclave.

LIQUD MEDIA

a. Nutrient Broth

Formula and Preparation

Yeast extract - 3.0g

Peptone - 5.0g

Distilled water - 1000.0cm^3

Dissolve 13g in 1000.0cm^3 distilled water. Adjust ph to 7.0, dispense into bottles, sterilize for 15 minutes at 121^{0}C.

b. PEPTONE WATER BROTH

FORMULA AND PREPARATION

Peptone - 10.0g

NaCl - 5.0g

Distilled water - 1000.0cm^3

Adjust ph to 7.0 – 7.4. Dispense into bottles and sterilize at 121^{0}C.

PREPARATION OF REAGENTS

(a) METHYL-RED INDICATOR

FORMULA AND PREPARATION

Methyl red - 0.10g

Ethanol (95%) - 30.0ml

Dissolve methyl red in 95% ethanol. Add 200mls distilled water to make up for 500ml.

(b) KOVAC's (INDOLE) REAGENT

FORMULA AND PREPARATION

p-Dimethylaminobenzaldehyde	-	5.0g
Concentrated hydrochloric acid	-	25.0g
Tertamyl-alcohol	-	75.0ml

Dissolve (i) in (ii) and (iii) and mix thoroughly by shaking.

(1) PREPARATION OF TURBIDITY STANDARD (equivalent to McFarland 0.5)

1. Prepare a 1%v/v solution of sulphuric acid by adding 1ml of concentrated sulphuric acid to 99ml of water. Mix well.
   Caution: Concentrated sulphuric acid is hygroscopic and highly corrosive, therefore do not mouth pipette, and never add water to acid.
2. Prepare a 1% w/v solution of barium chloride by dissolving 0.5g of dehydrate barium chloride ($BaCl_2.2H_2O$) in 50ml of distilled water.
3. Add 0.6ml of the barium chloride solution and 99.4ml of the sulphuric acod solution, and mix.

   Transfer a small volume of the turbid solution to a capped tube or screw-cap bottle of the same type as used for preparing the test and control inocula.

   When stored in a well-sealed container in the dark at room temperature (20-280C), the standard can be kept for up to 6 months.

MATERIALS USED

The following materials were used viz:

Nutrient agar, 2. MacConkey agar, 3. Muller hinton agar, 4. MacConkey broth, 5. Nutrient broth, 6. Simmons citrate agar, 7. Tryptone broth, 8. MR- VP medium, 9. 0.5 McFarland standard solution, 10. Gram staining reagents (crystal violet, grams iodine, 95% alcohol, safranine), 11. Swab sticks, bijou bottles, 12. Test tubes, 13. Forceps, 14. Sterile 200mls and 20mls sampling bottles, 15. Hand gloves and nose masks, 16. Microscope, 17. Glass slides, 18. Immersion oil, 19. Antibiotics disc, oxoid product England, e.t.c.

APPENDIX 2

DESCRIPTION OF MICROORGANISM

1. DESCRIPTION OF MORPHOLOGICAL FEATURES OF MICROORGANISMS

In microbiological studies, it is common practice to study the morphological features of organisms. Several descriptions have been used in the study of colonial morphology, and they include;

i. Size of colony: The size of the colony was determined by using a ruler calibrated in millimeter placed at the bottom of the petri-dish. In most cases, they appear like pin point size.

ii. Shape/form of colony: The shape of the organism on agar medium could be circular, irregular, filamentous etc.

iii. Colour/pigmentation of colony: Different organism exhibits varied colour on different bacteriological and mycological media. This is sometimes used in diagnosis. Colours commonly encountered include; Green with a dark centre for the *Aeromonas hydrophila* and brownish colour for the *Aeromonas salmonicida.*

iv. Margin/edge of colony could be entire, lobate, filamentous or serrated in appearance.

v. Elevation of colony: This is the position of the colony relative to the surface of the agar. Commonly encountered are flat, raised, low convex, high convex and umbonate.

2. BIOCHEMICAL TESTS

Species that cannot be distinguished by their cultural and microscopic characteristics may exhibit distinct difference in their biochemical reactions. A description of some of the biochemical reactions is provided by Cheesbrough (2000). These tests were used in differentiating isolated organisms which were preserved on Nutrient agar

slants. The slants were brought to room remperature and sub-cultured unto fresh nutrient agar to obtain fresh cultures that were then used for biochemical tests.

IMVic TEST

This test consists of four different tests; they are Indole production, Methyl red test, Voges Proskaeur tests and Citrate utilization test. This test is specically designed to determine the physiological properties of microorganisms. They are especially useful in the differentiation of Gram negative intestinal bacilli, particularly Escherichiacoli and the Enterobacter-Klebsiella group.

APPENDIX 3

PHYTOCHEMICAL ANALYSIS

REAGENTS AND MATERIALS USED FOR PHYTOCHEMICAL ANALYSIS

- Olive oil
- Filter paper
- Coinc H_2SO_4
- 1% HCl
- 10% KOH
- 10% NaOH
- 5% $FeCl_3$
- 25% H_2SO_4
- Copper (II) sulphate
- Potassium sodium tartarate
- Anhydrous NaOH
- Benzene
- Ammonia
- Mayer's reagent (K_2HgI_4)
- Hydrated NaOH
- Fehling's solution

PHYTOCHEMICAL ANALYSIS

This involves the analysis of chemical substances that are obtained from plants and fruits which are biologically active but not nnutritive. It involves the analysis of plant chemical which are naturally occurring components in fruits, vegetables and legumes (Liu, 2004).

(a) FLAVONOID TEST

Flavonoids are polyphenolic compounds found in fruits, vegetables and certain beverages that have diverse beneficial biochemical and antioxidant effect (Buhler, 2000).

To test for the presence of flavonoid, a few drops of 10% NH_3 solution was added to the ethanol extracts of each sample in a test tube. A yellow colouration was observed showing the presence of flavonoid compounds.

(b) SAPONIN TEST

Saponins are glycosides with a distinctive foaming characteristic. They are found in many plants, but get their name from the soapwort plant (Saponaria), the root of which was used historically as a soap. They consist of a polycyclic aglycone that is either a choline steroid or tritepenoid attached via C_3 and an either bond to a sugar side chain (George et al., 2002). To test for saponins, a 2g of powdered samples of each plant extract was boiled together with 20ml of distilled water in a bath and filtered. A 10ml of the filtered samples was mixed with 5ml of distilled water in a test tube and shaken vigorously to obtain a stable persistent froth. The frothing was them mixed with 3 drops of olive oil and observed for the formation of emulsion, which indicated the presence saponins.

c. STEROID TEST

Steroids are known to be very important for their cardiotonic activities; they possess insecticidal and antimicrobial properties. They are also used in nutrition, herbal medicine and cosmetics. They are routinely used in medicine because of their profound biological activities (Denwick, 2002). Two milliters of the extracts were evaporated to dryness in a separated test tubes and the residues dissolved in acetic anhydride followed by addition of Chloroform. Concentrated sulphuric acid was added by means of a pipette via the side of the test tubes.

Formation of brown ring at the interface of the two liquids and violet colour in the supernatant layer denoted the presence of steroids.

d. ALKALOID TEST

These are group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. This group also includes some related compounds with neutral and even weak acidic properties. They are produced by large variety of organisms, including bacteria, fungi, plant and animals and are part of the group of natural products (also called secondary metabolites. They are toxic to other organisms and also have pharmacological effects and are thus used as medications (Denwick, 2002). To test for alkaloids, a 200mg plant material boiled in 20ml of 1% H_2SO_4 in 50% and filtered. Five drops of conc. H_4H0H was added to the filterate, after which 20ml chloroform was added. The separated chloroform layer was extracted with 20ml dilute H_2SO_4. To the extract, 5 drops of Mayer's/Wagner's/Dragendorff's reagents, a cream/brownish-red/orange-red precipitate indicated the presence of alkaloids.

e. TEST FOR GLUCOSIDES.

Glucosides are common in plants, but rare in animals. Glucose is produced when a glucoside is hydrolysed by purely chemical means, or decomposed by fermentation or enzymes. A glucoside is a glycoside that is derived from glucose. To test for glucoside, the powered extract was evaporated to dryness. About 0.5g of the extract was placed in a clean test tube of water. A moist sodium picrate paper was suspended in the neck of the test tube using cork to trap it. The closed tube was placed in a clean test tube of water. A moist sodium picrate paper was suspended in the neck of the test tube using cork to trap it. The closed tube was placed in a warm water bath 45^{o}c for 1 hour. The colour change was noted.

f. TEST FOR TERPENOIDS:

The terpenoids, sometimes called isoprenoids, are a large and diverse class of naturally occurring organic chemicals derived from terpenes. Most are multicyclic structures with oxygen-containing functional groups. About 60% of known natural products are terpenoids. To test for terpenoids a 0.5g of plant extract in spate test tube was taken with 2ml of chloroform; 5ml of concentrated sulphuric acid was carefully addede to form a layer and observed for presence of reddish brown color interface to show positive results for the presence of terpenoid.

g. TEST FOR TANNINS:

Tannins are a class of astringent, polyphenolic biomolecules that bind to and precipitate proteins and various other organic compounds including amino acids and alkaloids. To test for tannins a 0.5g of powdered sample of each plant extracts was boiled in 20ml distilled water in a test tube for 5 minutes and then filtered. One milliter of $Fecl_3$ was added to 2ml of the filtered samples and observed for brownish green or blue black colouration, which showed the presence of tannins.