CHAPTER I
INTRODUCTION

1.1 Introduction

Antimicrobial resistance is currently one of the major threats facing human being; the rate at which microorganism develop antimicrobial resistance mechanisms outpaces the rate at which new antimicrobials are being developed (Madubuike*etal*., 2017). The wide application of antibiotics in various aspect of life exposed human to different antibiotics and led to development of antibiotic resistant bacteria, and this is thought to be induced by humans due to misuse of antimicrobial agent. There are other factors which could have led to the continuous development of microbial resistant such as patient's poor adherence to treatment regimens, poor hospital hygiene and increased ratios of international travel (Hashim, 2014).

Urinary tract infections (UTIs) are an infection caused by the presence and growth of microorganisms anywhere in the urinary tract. UTIs are very frequent and common pathology that can occur at any age, considering adult hood 48% of women acquired at least one occurrence of UTIs in their life. UTIs and its associated complications are the cause of nearly 150 million deaths per year worldwide (Ahmed, 2015).

Bacterial uropathogens become more resistant to available antibiotics, we need to explore new strategies for managing UTIs, so this leads to increase urgency for new interventions with more availability, lower cost and more effective alternative drugs. A survey of WHO (2000) showed that in Sudan primary health care, there is 62% of patient receive antibiotics and reported this percent as the highest in Africa, so we must alternate antibiotics by more safe substance with high efficiency, such as medicinal plants(Ahmed, 2015).

For a long period of time, a plant has been a valuable source of natural products for maintaining human health, especially in the last decade with more intensive studies for natural therapies. The use of plant compound for the pharmaceutical purpose has gradually increased in the world according to the World Health Organization (WHO, 2005).

The World Health Organization (WHO) has listed more than 21,000 plants which are used for many medicinal purposes around the Word. They observed that about 74% 0f 119 plant-derived pharmaceutical medicines are used in modern medicine (WHO, 2015).

It also estimates that 4 billion people (80 percent of the world population) presently use herbal medicinal plants minerals and organic matter for health care marketed and gaining popularity in developed and developing countries. In the last few years there is an exponential growth in the field of herbal medicine because of their natural origin, availability, efficacy, and safety and less side effects with efficient to cure age-related disorders like memory loss, osteoporosis and immune disorders for which no modern medicine is available. Medicinal plant researchers pursued with several goals like the development of low cost therapeutic compounds and the discovery of prototypic drugs, so the gate is opening for research (Malviya, 2011).

1.2Rationale

The problem of microbial resistance is growing and the outlook for use of antimicrobial drugs in the future is still uncertain, therefore, action must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanism of resistance, and to continue studies to develop new drugs, either synthetic or natural (Spellberg *et al*., 2008).

In Sudan there was high percentage of multi-drug resistant bacteria, so urgent need to develop new drug from our traditional medicine. As*Citrus limon* and *Zingiberofficinale* were used in rural medical care for treatment of many infectious and chronic diseases, thus, to verify the antibacterial activity of those plants against resistant bacteria isolated from urine specimens.It expected to be good treatment for UTIs. So this study was attempted to solve the problem of antimicrobial resistance.

1.3 Objectives

1.3.1 General objective

To determine the antimicrobial activity of *Citrus limon* peels and *Zingiber officinale* roots methanolic and water extract against multi-drug resistant bacteria isolated from patients with urinary tract infections.

1.3.2 Specific objectives

1. To reidentify the pathogenic bacteria isolated from patients with UTIs.
2. To determine the antimicrobial sensitivity pattern of the clinical isolates by disc diffusion method.
3. To determine the multidrug resistant bacteria in UTIs.
4. To evaluate the antimicrobial activity of aqueous and methanolic extract of *Citrus limon* and *Zingiber officinale* against multidrug resistant bacteria isolated from patients with UTIs.
5. To compare between the antimicrobial activity of *Citrus limon* and *Zingiber officinale* extracts on multidrug resistant bacteria isolated from patients with UTIs.
6. To determine the minimum inhibitory concentration (MIC) of aqueous and methanolic extracts of the selected plants using disc-diffusion method.

CHAPTER II
LITERATURE REVIEW

2.1 Medicinal plants

Medical herbalism or medical herbology is the study of herbs and their medical uses. Other terms substituted to herbalism are botanical medicine or phytotherapy, previously defined as the use of plant materials to prevent and treat illness or promote wellness (Sunday *et al*., 2011). A medicinal plant therefore, describe as any plant one or more from its organs contain substance that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Taura *et al*., 2014). Herbal medicines are currently in demand and their popularity is increasing day by day. About 500 plants with medicinal use are mentioned in ancient literature and around 800 plants have been used in indigenous system of medicine (Verma and Singh, 2008). Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Sulieman *et al*., 2015).

The beneficial medicinal effects of plant materials typically result from combination of secondary products present in the plant, this secondary metabolites such as alkaloids, steroids, tannins, phenolic compounds, flavonids, steroids, resins and fatty acid gums which are capable to producing definite physiological action on the body. These compounds were found to be a source of various phytochemical that could be directly used as intermediates for the production of new drugs. So can be used to cure many diseases such as diarrhea, dysentery, cough, cholera, fever, high blood pressure, heart disease, asthma and bronchitis (Saranraj and Sivasakthi, 2014)

2.2 Ginger (*Zingiber officinale*)

Ginger is a member of family *Zingiberaceae*, a small family with more than 45 genera and 800 species. The genus *Zingiber* includes about 85 species of aromatic herbs and it is name derived from a Sanskrit word denoting “horn- shaped” in reference to the protrusions on the rhizome (Singh *et al*., 2014).

2.2.1. Classification

Ginger belongs toKingdom: *plantae,*which in division of*Manoliphyta* under the Class of*Liliopsida* in theOrder*Zingiberales.* TheFamilyisZingiberaceae,Genus*Zingiber* and the Species is *Zingiber officinale* (Gupta and Sharma, 2014).

2.2.2 Distribution

South East Asia is considered as home grown land for ginger production. By tradition, ginger farming is common in number of countries like Japan, China, Indonesia, Nigeria, Brazil, Sri Lanka, the Philippines and Jamaica Islands (Riaz *et al*., 2015).

2.2.3 Botanical description

Zingiber officinale perennial, slender plant that growing from one to three feet in height. The rhizomes are usually branched, fleshy or fibrous, aromatic, white and yellowish to brown, 7-15 cm long and 1-1.5 cm broad and laterally compressed. The branch with thick thumb-like protrusions, thus individual divisions of the rhizomes are known as "hands" (Singh *et al*, 2014).

Leaves are narrowly, up to 20 cm long and 1.5-2 cm wide, flowers are produced in a dense spike, yellow green with purple endings (Sulieman *etal*., 2015)

2.2.4 Chemical and nutritional constituents

Ginger contains up to 3% of a fragrant essential oil whose main constituents are sesquiterpenoids, with gingeberene as the main components. The pungent taste of ginger is due to nonvolatile phenylpropanoid- derived compounds, gingerols and shogaols. In the fresh ginger rhizome, the gengerols were identified as the major active components and gingerol-1 is the most abundant constituent in the gingerol series. In dried ginger powder, shogaols "a dehydrated product of gingerol" is a predominant pungent constituent up to biosynthesis (Malu *etal*., 2009).

Nutrient composition: fresh ginger contains 80.9% moisture, 2.3% protein, 0.9% fats, 1.2% minerals, 2.4% fiber and 12.3% carbohydrates. The minerals present in ginger are iron, calcium and phosphorous. It also contains vitamins such as thiamine, riboflavin, niacin and vitamin C (Sulieman *etal*., 2015).

2.2.5 Medicinal uses

Ginger has a wide range of action on the human body and has been found to be effective in the treatment of heart disease, chronic fatigue, cold, flue, coughs, bronchitis, fever, kidney stones, renal disease and viral infections. Its natural diuretic stimulates the kidney to flush out toxin faster. In addition there is evidence that ginger may increase stomach acid production (Taura *etal.,* 2014). Also in multiple studies ginger has been found effective for treating nausea caused by sea sickness morning sickness and chemotherapy (Sebiomo *etal*., 2011).

Ginger promotes the release of bile from the gall bladder, decrease joint pain from arthritis and cholesterol lowering. Not only but also it has been used for treating

cramps, rheumatism, sprains, muscular aches, pains, vomiting, indigestion, hypertension, dementia and infectious diseases (Islam *etal*., 2014).

The gingerols increase the motility of the gastrointestinal tract and have analgesic, sedative and antibacterial properties (Malu *etal*., 2009). The volatile oil gingerol and other pungent principles not only give ginger its pungent aroma, but the most medically powerful because they inhibit prostaglandin and leukotriene formation, which are products that influence blood flow and inflammation (Adetunde *etal*., 2014). In addition, it has been reported that the main ingredients of ginger like volatile oil, gingerol, shagaol and diarylheptanoids work as antioxidant, anti-inflammatory, anti-lipid, anti-diabetic, analgesic, antipyretic and anti-tumor (Hassan *etal*., 2017).

2.2.6 Antimicrobial activity

Ginger extracts was demonstrated to have antimicrobial effect especially against the *Staphylococci* species and also exhibits anti-fungal activity against a wide variety of fungi including *Candidaalbicans* (Ficker *etal*., 2003). Other study concluded that the methanolic extract of ginger roots was effective against *Staphylococcusaureus*with 19.0 mm zone of inhibition (Gur *etal*.2006)

According to Malu *etal*. (2009) who studied the antibacterial activity and medicinal properties of ginger extracts showed that ginger roots methanolic extract have antibacterial activities on coliform bacillus, *Staphylococcusepidermidis* and *Streptococcusviridans* while water extract did not have antibacterial activity on these bacterial(Malu *etal*.2009). Furthermore, the inhibition of bacterial growth appeared to be dose dependent since no activity was observed at low concentrations. It was observed that macerated methanolic extract of ginger exhibited maximum inhibitory effect against *pseudomonasaeruginosa* while it showed no effect against *Klebsiellapneumoniae* while the antimicrobial activity against *Escherichiacoli* and *candidaalbicans* were found to be moderate (Joe *etal*., 2009).

According to Sebiomo *etal*. (2011) there was no significant difference in the effects of both water and ethanol extract of ginger on the zone of inhibition of the *Staphylococcusepidermidis* and *Streptococcuspyogens*, while the concentration of the two extracts had significant effect on the zone of inhibition of both organisms *Staphylococcusaureus* and *Streptococcuspyogenes*.

Khalid *etal*. (2011) tested the antibacterial activity of methanolic, water and cold water extracts of ginger roots by using agar discs diffusion technique and result showed that maximum zone of inhibition of methanolic extract of *Zingiberofficinale*

was observed against *Staphylococcus aureus*, while the maximum zone of inhibition of cold water extract against *pseudomonas aeruginosa*, While hot water extract has no activity against *Enterococcusfaecalis* (Khalid *etal*., 2011)

Similarly the result of antimicrobial activity of *Zingiberofficinale* extracts against some selected pathogenic bacteria studied by Akintobi *etal*. (2013) showed that the methanol extracts had a higher inhibitory activity against the test organisms than that of the water extract, also the methanol extract exhibited maximum inhibitory effect against *Pseudomonasaeruginosa* while the water extract did not, both extract showed no effect against *Escherichiacoli*(Akintobi *etal*., 2013).

Redda *etal*. (2014) tested the antibacterial activity of methanolic extract of *Zingiber officinale* using disc diffusion method and concluded that methanolic extract have significant effect on *Salmonellatyphyimurium, Escherichiacoli, Staphylococcusaureus* and *Streptococcusagalactiae* tested bacteria (Redda *etal*., 2014).

In a study in 2017, the antimicrobial activity of methanolic extract of the dried ginger tested against *Salmonella* species, *Staphylococcusepidermidis* and *Staphylococcusaureus* results show that dried ginger is effective against some bacterial growth particularly Gram-positive *Staphylococcus* isolates (Ajayi *etal*., 2017).

2.3Lemon(*Citruslimon*) Lemon is a member of the family Rutaceae, is a family of flowering plant commonly known as citrus family, a large family contains about 160 genera and more than 2000 species (Singh and Gurjaran, 2004).

2.3.1 Classification

Lemon is inKingdom*Plantae*underDivisionof *Angiosperms,* in theClass*Audicots*which inOrder*Sapindales***,**belong toFamily*Rutaceae* in theGenus*Citrus*Speciesof*Citruslimon* (Roose, 2001)**.**

2.3.2 Distribution

The exact origin of the lemon has been disputed but it is believed to have originated in the east Himalayan region. It is native to South Asia, East Asia, Southeast Asia, Melanesia and Australia. From there its cultivation spread into Middle East, Mediterranean and to Europe (Dafna, 2017).

2.3.3Botanical description

These plants are large shrubs or small to moderate sized trees, reaching 5-15m tall, with spiny shoots and alternately arranged evergreen leaves with an entire margin.

The flowers are solitary or in small corymbs, each flower 2-4 cm diameter, with five rarely four) white petals and numerous stamens,the fruit is a hesperidium, a specialized berry, globose to elongated, with a leathery rind or peel called a "pericarp". The outermost layer of the pericarp is an "exocarp" called the flavedo, commonly referred to as the zest. The middle layer of the pericarp is the" mesocarp", which in citrus fruits consists of the white spongy albedo or pith. The innermost layer of the pericarp is the" endocarp". The space inside each segment is a locule filled juice vesicles or "pulp". From the endocarp, string like hairs extend into the locules, which provide nourishment to the fruit as it develops (Blench, 2005**).**

2.3.4 Chemical and nutritional constituents

Lemons are full of vitamins, minerals, phytonutrients and antioxidant.It contain high amount of nutrients such as : water, protein, carbohydrate and fibers, also contain many essential vitamins which include: large amount of vitamin C (31% of recommended daily intake of vitamin C), 3% 0f folate, thiamin, riboflavin, niacin , vitamin B-6, B-12, vitamin A and little amount of vitamin E, the most important minerals in lemon is the potassium, it provide our bodies by 2% of potassium daily need, it also contain calcium, magnesium, phosphorus, iron, small amount of sodium and zinc.Moreover, lemon peel contains many phytochemicals compounds include: alkaloid, flavonoid, saponins, tannin, phytic acid and phenolics, also contains essential oils with high antimicrobial activity known as "terpenes" which include pinene, myrcene and limonene (Mustafa, 2015).

2.3.5Medicinal uses

Lemon is one of the healthiest fruits and has many uses as effective traditional medicineand its juice is an excellent source of the vitamin C, which is great antioxidant that helps our bodies to fight the free radicals, so it may prevent cancer formation. Behind that vitamin C in lemons plays a vital role in collagen formation, which reduces wrinkles and improves over all skin texture. Also vitamin C in lemons helps in fighting cold and flu. In other hand, lemons are natural diuretic stimulates the kidney to flush out toxin faster and reduce water retention and it has high concentration of citrates so it reduce the risk of kidney stones formation. Moreover, lemon improves heart health, it contains hesperidin, which has been known to reduce symptoms of hypertension and also contain pectin and lemonoid compounds, both of which may slow atherosclerosis (Dafna, 2017).

2.4 Urinary tract infections

Urinary tract infections (UTIs) may be defined as presence of bacteria undergoing multiplication in urine within the urinary drainage system (Kumar, 2015).

The urinary system is divided into upper urinary tract which involve two kidneys and two ureters and lower urinary tract which include urethra and urinary bladder (Forbes *etal*., 2007).

Urinary tract infections are among the most prevailing infectious diseases with a considerable financial burden on society. In the United States, UTIs are responsible for >7 million physician visits annually. Approximately 15% of all community-prescribed antibiotics in United States are dispensed for UTI and data from some European countries suggest a similar rate. In the United States, UTIs account for > 100,000 hospital admissions annually, most often for pyelonephritis. At least 40% of all hospital acquired infections are UTIs and the majority of them are catheter associated. Bacteriuria develops in up to 25% of patients using urinary catheters for one week or more with a daily risk for 5-7%. The recent Global Prevalence Infection in Urology (GPIU) studies have demonstrated that 10-12% of patients hospitalized in urological wards has a health care associated infection (HAI). The strains retrieved from these patients are even more resistant (Grabe *etal*., 2015).

Urinary tract infections are categorized into either lower tract infection, located in the bladder and /or urethra (cystitis and urethritis), and upper tract infection, located in the ureters, collecting system and parenchyma (pyelonephritis). It is necessary to understand the differences between the two types to make an accurate diagnosis (Hussein, 2009).

Although several different microorganisms can cause UTIs, including fungi, viruses and bacteria. Bacteria are the major causative organisms and are responsible for more than 95% of UTI cases. *Escherichia coli* are the most prevalent causative organisms of UTIs and are solely responsible for more than 80% 0f these infections. Other bacteria frequently isolated are *Klebsiella* species, other *Enterobacteriaceae*, *Staphylococcus saprophyticus, Staphylococcus aureus* and *Enterococci*. In more complicated UTIs, particularly in recurrent infections, the relative frequency of infection caused by *Proteus, Pseudomonas, Klebsiella* and *Enterobacter* species increases (Forbes *etal*, 2007).

It is very important to recognize and treat UTIs rapidly to minimize the complication of the infection. Selections of appropriate antibiotic depend mainly on the predominant pathogens in the patient's age group, antibacterial sensitivity patterns in the practice area and the clinical status of the patient. The most common antibiotics used in the treatment of UTIs are Nitrofurantoin, Sulphamethoxazole / Trimethoprime, Fluroquinolones (e.g. Ciprofloxacin), Gentamicin, Cephalosporin, Ampicillin and Amoxicillin. Inappropriate use of antimicrobial agent lead to increase resistance to them makes these antibiotics of less value (Hussein, 2009).

Sharma *etal*. (2013) showed that most *Escherichiacoli* isolates are highly resistant to commonly prescribed antiobtics (Ampicillin, Cephalexin, Quinolones and CO-trimoxazole), but are still susceptible to Nitrofurantoin which should be considered as preferred therapeutic agent once the organism is identified (Sharma *etal*., 2013).

Resistance of Gram-negative to Trimethoprim-Sulphamethoxazole is 6.5% in a study in the Department of Emergency Medicine, University of Florida in the United States while appeared to be 55.2% in another study done in Taiwan (Hussein, 2009).

Study done in Sudan by Derese *etal*. (2016) who found that most of clinical isolates were showed resistant against Ampicillin, Amoxicillin, Tetracycline, Sulphamethoxazole /Trimethoprim and Chloramphenicol and the majority of clinical isolates were sensitive to Ciprofloxacin, Erythromycin and Gentamicin(Derese *etal*. 2016).

2.5 General characteristics of isolated bacteria

2.5.1*Escherichiacoli*

Is a Gram- negative, facultative anaerobic rod, usually motile and majority of strains are capsulate. It produces 1-4 mm diameter colonies in blood agar after overnight incubation. The colonies may appear mucoid. Some strains are haemolytic and ferments lactose, producing smooth pink colonies on MacConkey agar (Cheesbrough, 2006).

This organism is associated with a variety of diseases, including gastroenteritis and extraintestinal infections, such as UTIs, meningitis and sepsis. A multitude of strains are capable of causing disease, with some serotypes associated with greater virulence (e.g. *Escherichia coli*0157 is the most common cause of hemorrhagic colitis and hemolytic uremic syndrome). *Escherichia coli* is the most common cause of both community and hospital-acquired UTI and Gram negative- rod sepsis (Murray *etal*., 2009). Study conducted in Sudan showed that *Escherichiacoli* have variety degrees of

resistance to the tested antimicrobial agents, the most common resistance were encountered for Co-trimoxazole, followed by Ciprofloxacin, which are the most commercially used antibiotics by public (Abdelhalim and Ibrahim, 2013).

2.5.2*Klebsiella pneumonia*

It is large, non-motile bacilli that possess a luxurious capsule. *Klebsiella* species exhibit mucoid growth due to the large polysaccharide capsules and they usually give positive tests for lysine decarboxylase, citrate and Voges-Proskauer (VP) reactions (Harvey *etal*., 2013).

Klebsiella may survive drying for months and remain viable for many weeks at room temperature. *Klebsiella* are primarily a cause of infections involving the urinary tract but may also cause soft tissue infections and sever bronchopneumonia. The capsule is a major pathogenic mechanism providing strains with protection against opsonization and the action of serum complement (Greenwood*etal*., 2012).

Mukhtar and Saeed (2011) perform a research on profile of antibiotic sensitivity and resistance of some pathogenic bacteria isolated from clinical specimens in Sudan. The study showed that the resistance rate of *Klebsiella pneumonia* was 20% to Gentamicin, 46.7% to Ciprofloxacin, 20% to Nitrofurantoin, 40% to Nalidixic acid and 20% to Tetracycline (Mukhtar and Saeed, 2011).

2.5.3*Proteus vulgaris*

It's Gram-negative, actively motile, non-capsulate. In blood agar when cultured aerobically, most *proteus* culture has a characteristic fishy odor and show swarming on blood agar. On MacConkey agar produce individual non lactose fermenting colonies after overnight incubation, but swarming is prevented on MacConkey agar because these media contain bile salt (Cheesbrough, 2006).

Proteus species is distinguished from other members of the *Enterobacteriaceae* by their ability to produce the enzyme phenylalanine deaminase. In addition, they produce the enzyme urease, which cleaves urea to form NH3 and CO2 (levienson, 2016).

Members of these genera are agents of urinary tract and other extra intestinal infections. *Proteus* species are relatively common causes of uncomplicated as well as nosocomial UTI. Other extra intestinal infections, such as wound infections, pneumonia and septicemia are associated with compromised patients. *Proteus* organisms produce urease, which catalyzes the hydrolysis of urea to ammonia. The

resulting alkaline environment promotes the precipitation of struvite stones containing insoluble phosphates of magnesium and phosphate (Harvey *etal*., 2013).

A study conducted by Nurain *etal*., (2015) showed that significant resistance to Ampicillin, Ciprofloxacin and Gentamicin (Nurain *etal*., 2015)

2.5.4*Pseudomonasaeruginosa*

Is an obligatory aerobe, motile, Gram- negative rod that is slimmer and paler staining than members of the *Enterobacteriaceae*. It's most striking bacteriologic feature is the production of colorful water-soluble pigments. It is usually recognized by the pigments it produces including pyocyanin a blue-green pigment, and pyoverdin (fluorescein) a yellow-green fluorescent pigment. A minority of strain are non-pigment producing. Confluent growth often has a characteristic metallic sheen and emits an intense fruity odor. Hemolysis is usually produced on Blood agar(Cheesbrough, 2006).

The positive oxidase reaction of *pseudomonasaeruginosa* differentiates it from the *Enterobacteriaceae*. However, once established, infections are particularly virulent and difficult to treat. The main infections caused by this bacterium are burn, wound, urinary tract, skin, ear and respiratory infections, bacteremia and osteomyelitis.

It is also a common cause of otitis externa, conjunctivitis, keratitis or endophthalmitis. Keratitis can progress rapidly and destroy the cornea within24 to 48 hours (Harvey, 2013).

Study conducted in 2017 showed that pseudomonas aeruginosa was resistant to Ampicillin, Amikacin, Chloramphenicol, Nalidixic acid, Norofloxacin and Ciprofloxacin but were sensitive to Gentamicin (Saeed, 2017).

2.5.5*Staphylococcusaureus*

Staphyle means bunch of grapes; *coccus* means grain or berry (grape like cocci) and *aureus* means golden (golden or yellow). *Staphylococci* are Gram-positive cocci arranged in clusters, large β-hemolytic colonies, catalase and coagulase-positive. *Staphylococci* commonly found on the skin of healthy individuals because they are resistant to dry condition and high concentration of salt. The species *Staphylococcusaureus* is the major pathogen and present in the nose of 30% of healthy individuals. It causes pyogenic infection like boils, carbuncles, wound infection, impetigo, blood stream infections, osteomyelitis, UTI, pneumonia and endocarditis *Staphylococcusaureus* also because toxin mediated infections such as

scalded skin syndrome, toxic shock syndrome and food poisoning (Murray *etal*., 2009).

According to a study conducted by Saeed, (2017) in Sudan, *Staphylococcusaureus* showed 77.8% resistance to Cefotaxime and Penicillin, and 66.7% resistance to Ampicillin and Erythromycin, while resistance to Augumentin and Co-trimoxazole were 55.6% (Saeed, 2017).

2.6 Multidrug resistant pathogen (MDR)

During the last few decades, the incidence of microbial infections has increase dramatically. Continuous development of antimicrobial drugs in treating infections has led to emergence of resistance among the various strains of microorganisms. Multidrug resistance is defined as insensitivity or resistance of microorganisms to the administrated of antimicrobial medicine despite earlier sensitivity to it (Singh, 2013 and Popeda *etal*., 2014). According to WHO, these resistant microorganisms are able to attacked by antimicrobial drugs, which lead to ineffective treatment resulting in persistence and spreading of infections. Studies from WHO report has shown very high rates of resistant in bacteria such as *Escherichiacoli* against Cephalosporin and Fluroquinolones, *Enterococci* resist Vancomycin, *Klebsiellapneumonia* against Cephalosporin and *Staphylococcusaureus* against Methicillin (WHO, 2014).

2.7 Classification of MDR pathogen

2.7.1 Primary resistance

It occurs when the organism has never encountered the drug of interest in particular host (Loeffler and Stevens, 2003).

2.7.2 Secondary resistance

Also known as acquired resistance these terms are used to describe the resistant that only arises in an organism after exposure to the drug (Khalilzedeh *etal*., 2006). It may further be classified as follows:

2.7.2.1 Intrinsic resistance

It refers to insensitivity of all microorganisms of a single species to certain common first-line drugs, which are used to treat disease based on the clinical evidence of the patient (Marks and Flood, 2014).

2.7.2.2 Extensive resistance

It is the ability of microorganisms to withstand the inhibitory effects of at least one or two most effective antimicrobial drugs, these seemed to arise in patient after they have undergone treatment with first line drugs (Lee *etal*., 2013).

2.7.3 Clinical resistance

Situation in which the infecting organism is inhibited by the concentration of antimicrobial that is associated with a high likelihood of therapeutic failure (Loeffler and Stevens, 2003).

CHAPTER III
MATERIALS AND METHODS

3.1Study design

This is a descriptive, cross sectional based study.

3.2 Study area and duration

Clinical isolates were collected from Ultra Lab, Fedail Hospital, Almoalim Hospital and Khartoum University Medical Health Services Center, in Khartoum State, Sudan, during the period from February to October 2019.

3.3Sampling technique

Non probability, convenience sampling technique was used.

3.4Sample size

One hundred (n=100) urinary isolates were enrolled in this study.

3.5Data collection

Data were collected from hospital records.

3.6 Ethical considerations

Ethical approval was taken from Scientific Research Committee, College of Medical Laboratory Science, Sudan University of Science and Technology, and hospitals administrations was taken before samples collection.

3.7Laboratory processing

3.7.1 Subculture of isolates

Urinary isolates were subculture on CLED (Cysteine Lactose Electrolyte Deficient) medium under aseptic conditions, using sterile loop, and then incubated aerobically at 37°C for overnight.

3.7.2 Purification and preservation of isolates

Purification was carried out by sub-culturing of well grown colonies on CLED medium. The pure cultures were preserved by incubation in Nutrient agar slope at 37°C for 18-24 hours, then preserved at 4°C. Isolates for long preserved in suspension of 20% (v/v) peptone glycerol broth medium at -20°C.

3.7.3Bacterial identification

The isolates were identified according to the morphology of colonies, Gram's stain and biochemical tests.

3.7.3.1Gram's stain

Smear was prepared, air dried and fixed by flame, the smear was covered with crystal violet stain for 30-60 seconds then washed by clean tap water and covered with

lugol's iodine for 30-60 seconds then washed by tape water and decolorized with acetone-alcohol for 20-30 seconds then washed with clean water, finally the smear covered with saffranine for 2 minutes, then washed by clean water and let to dry and examined by using oil immersion lens (Cheesbrough, 2006).

3.7.3.2Biochemical tests

Biochemical tests were carried out according to Gram's stain (Cheesbrough, 2000)**.**

3.7.3.2.1Biochemical tests forGram-negative rods

3.7.3.2.1.1Kliger Iron Ager (KIA)

A small part of the tested colony was picked off using a straight loop and inoculated in KIA medium. First stabbing the butt, then streaking the slope in the zigzag pattern, and then incubated at 37°C aerobically overnight. Then the results were interpretedasfollowing:

A yellow butt red –pink slope indicated the fermentation of glucoseonly.

A yellow slope and butt indicated the fermentation of lactose and glucose.

A red-pink slope and butt indicated no fermentation of glucose andlactose.

Blacking along the stab line or throughout the medium indicated H^2S production.

Cracks and bubbles in the medium indicated gas production from glucose fermentation.

3.7.3.2.1.2Indole test

The test colony was inoculated in sterile peptone water using a sterile wire loop and then incubated at 37°C aerobically overnight. Few drops of Kovac's reagent were added to medium and shakengently to test for indole. A positive result was indicated by the production of red color in the surface layer within 10 minutes.

3.7.3.2.1.3Citrate utilization test:

Slopes of Simmon's citrate agar medium were prepped, by using sterile straight wire loop, the slope was streaked and the butt was stabbed with a small part of the test colony. Then the slops of medium were incubated overnight at 35°C. A positive reaction was indicated by the change in medium color into blue color while the negative reaction was indicated by no change in the color.

3.7.3.2.1.4Oxidase test

A piece of filter paper was placed on a clean glass slid and three to four drop of freshly prepared oxidase reagent (tetra methyl para phenylene diamine dihydrocholoride) were added using sterile Pasteur pipette, a wooden stick was used

to pick a colony of the test organism and placed on the filter paper. The positive reaction was indicated by the production of blue-purple color within 10 seconds.

3.7.3.2.1.5Motility test

The tested colony was taken by a sterile straight loop and inoculated by stabbing a semi-solid media, then incubated aerobically at 37°C for overnight. The motility was shown by spreading turbidity from the stab- line or turbidity throughout the medium.

3.7.3.2.2Biochemical tests for Gram-positive cocci

3.7.3.2.2.1Catalase test

Apure of 2-3 ml of hydrogen peroxidase solution was added in a test tube, by sterile wooden stick several colonies of test organism were immersed in hydrogen peroxidase solution. The positive result indicated by immediate budding.

3.7.3.2.2.2Coagulase test

A drop of physiological saline was placed on each end of the slide, a colony of the test organism was emulsified in each of drops to make too thick suspensions, drop of plasma was added to one drop of the suspensions and mixed gently by rotating. The positive result indicated by producing clump within 10 seconds.

3.7.3.2.2.3Deoxyribonuclease (DNAase) test

The test organism was cultured on a medium which contain DNA. After overnight incubation, the colonies were tested for DNAse producing by flooding the plate with a weak hydrochloric acid solution. The acid precipitated unhydrolized DNA. DNAse producing colonies were surrounding by clear areas due to DNA hydrolysis.

3.7.3.2.2.4Mannitol Salt Agar

This medium was used to differentiate *Staphylococcusaureus* from other *Staphyloccoci* species. Apportion of the colony was incubated aerobically at 37°C for 18-24 hours, *Staphylococcusaureus* ferment manitol producing yellow colonies.

3.7.3.2.2.5Bile Esculin test

The tested organism was inoculated on the slope surface of Bile Esculin medium by a sterile wire loop in zigzagging manner, and then incubated overnight at 37°C aerobically. The positive result was indicated by growth and blacking of the agar slant while the negative result was indicated by growth and no blacking of media.

3.7.4Antimicrobial susceptibility tests

The sensitivity testing was doneusing modified Kirby-Bauer disk diffusion method.

Under aseptic conditions, well isolated 2-3 colonies of similar appearance were selected and emulsified by using sterile wire loop in 2ml of a sterile normal saline,

and then the turbidity of suspension was matched to the turbidity of 0.5% McFarland standard in good light for adjustment then a sterile swab was immersed in the suspension, excess was removed by pressing and rotating the swab against the side of the test tube above the level of suspension. Muller- Hinton media surface was inoculated by swabbing evenly and allowed to dry for 2 minutes, andusing sterile forceps the antibiotic discs were placed on the inoculated plate, incubated aerobically at 37°C for overnight. The antibiotics used in this study were: Nalidixic acid (30 µg), Nitrofurantoin (300 µg), Norofloxacin (30 µg), Co-amoxiclav (30 µg), Cefxime (5 µg), Ampicillin (10 µg), Imipenem (10 µg) and Amikacin (30 µg).

The inhibition zone was measured in (mm) and interpreted by aid of interpretative chart and the organism reported as sensitive, intermediate or resistant according manufacturer company(Cheesbrough, 2006).

3.7.5Extraction of medicinal plants

3.7.5.1Collection and preparation of plants sample

The selected plants for this study were *Zingiber officinale* which obtained from Sinnar State, Central Market, Sudan and *Citrus limon* which collected from lemon trees in Sinnar State, Sudan.

The fresh rhizomes of Chinese's *Zingiber officinale* were washed with clean water then peeled and cut into small pieces, and then allowed to dry in shadow for 2-3 days, after that crushed into powder by clean electrical blender. Also the fresh peels of *Citruslimon* were washed with clean water then allowed to dry for2-3 days in shadow, then crushed into powder using clean electricalblender.

3.7.5.2Plant extraction

Extraction was carried out in National Research Center according to method described by (Sukhdev *et al*., 2008).

3.7.5.2.1Preparation of methanolic extracts

One hundred (100) grams of ginger and hundred and fifty (150) grams of lemon powdered plants materials were coarsely powdered using mortar and pestle. Coarsely samples extracted with absolute methanol (400 ml) using Soxhlet tractor apparatus. Extraction carried out for about five hours till the color of solvents at the last siphoning time returned colorless. Solvents were evaporated under reduced pressure using rotary evaporator apparatus. Finally extract allowed to air in Petri dish until complete dryness. The methanolic extract of each plant was weighted and the

yield percentage calculated as follow: weight of extracts obtained / weight of plant sample × 100.

3.7.5.2.2Preparation of water (aqueous) extracts

About 45grams of ginger and 55grams of lemon peels were macerated in distilled water with intermittent stirring at room temperature. Extracts were then filtered and froze, then were dried using freeze drier until powder extract were obtained, finally the yield percentages were calculated as previous mentioned.

3.7.5.2.3 Preparation of plants extract for antimicrobial activity testing

About 0.1 gram of water extracts of both plants were dissolved in 1ml of distilled water in separate tubes then 2 fold serially diluted to obtain final concentration of (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, and 3.125 mg/ml).

About 0.1 gram of methanolic extracts of lemon was dissolved in 1ml of methanol while 0.2 gram of methanolic extract of ginger was dissolved in 1ml of DMSO in separate tubes then 2 fold serially diluted to obtain final concentration of (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125) in both extracts (Elgailany, 2015).

3.7.6Antimicrobial susceptibility of plant extract (disc diffusion method) and determination of Minimum Inhibitory Concentration (MIC)

Under aseptic conditions, bacterial suspensions from overnight isolates were prepared by using sterile normal saline. 2-3 colonies were emulsified from each isolates in a separate tube and the density was compared with turbidity 0f 0.5% McFarland standard under good light illumination. Then sterile cotton swab was dipped into the bacterial suspension to inoculate the entire surface of Muller-Hinton agar plates. Sterile blank discs of 6mm were previously prepared from Whatman No.1 filter paper (Sigma-Aldrich). By using micropipette 20µl from *Zingiberofficinale* and *Citrus limon* methanolic and water extract of different concentration (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125mg/ml) were poured directly into the papers (Pre-experimental measurements showed that the 6 mm disc absorb about 20µl), then put in the plate using sterile forceps. Plates were allowed to stand for 1 hour in refrigerator to allow diffusion of extract before growth of the bacteria, and then incubated aerobically at 37°C after incubation the diameters of inhibition zones were measured in millimeters and recorded. Negative control was the same solution by which plant extracts were prepared. Minimum Inhibitory Concentration (MIC)determined, which is the lowest concentration of plants extracts that show

antibacterial activity. Many Standard microorganisms**:** *Escherichia coli ATCC* 25922, *Staphylococcus aureus ATCC* 26380, *Pseudomonas aeruginosaATCC* 27853 and *ProteusvulgarisATCC*6380. These standard strains were used to determine the potency of lemon and ginger extracts.

3.7.6.1Interpretation of results

The diameter of resultant growth inhibition zone was measured in mm then results were recorded. The inhibition zones with diameter less than 7mm were considered as no antibacterial activity (Mohammed, 2016).

3.8 Data analysis

Statistical Package for Social Science (SPSS) version 16 for windows was used for data analysis. Frequencies were presented in tables and one sample and independent t-tests were used at *p-value* $\geq$0.05 statistically significant.

CAHAPTER IV

RESULTS

In this study 100 urinary isolates were collected as follow: 21 were Gram-positive cocci (21%) and 79 (79%) were Gram- negative rods.

The frequency of isolated species as follow: *Escherichia coli* 37/100 (37%), *Proteus vulgaris* 21/100 (21%), *Enterococcus faecalis* 18/100 (18%), *Klebsiella pneumoniae* 13/100 (13%), *Pseudomonas aeruginosa* 8/100 (8%) and *Staphylococcusaureus* was only 3/100 isolates (3%) as shown in table 4.1.

Table 4.1: Frequency of different bacterial species among urinary isolates

Bacterial species	Frequency	Percentage
Escherichia coli	37	37%
Proteus vulgaris	21	21%
Enterococcus faecalis	18	18%
Klebsiella pneumoniae	13	13%
Pseudomonas aeruginosa	8	8%
Staphylococcus aureus	3	3%
Total	100	100%

Among selected antimicrobial agents,Nitrofurantoin showed the highest sensitivity (7%) while Co-Amoxiclav revealed the lowest one (31%) as shown in table 4.2.

Table 4.2: Frequency and percentage of overall antimicrobial sensitivity testing

Antimicrobial agents	Sensitive N=100	ResistantN=100
Nitrofurantoin	78 (78%)	22 (22%)
Cefxime	65 (65%)	35 (35%)
Norofloxacin	57 (57%)	43 (43%)
Nalidixic acid	50 (50%)	50 (50%)
Co-Amoxiclav	31 (31%)	69 (69%)

From 37 isolated *Escherichia coli*,18 isolates were multidrug resistant (15 resistant for three antimicrobial agents and 3 resistant for 4 antimicrobial agents) as shown in table 4.3. The highest resistance was for Amoxiclav (62.2%) followed by Norofloxacin (51.4%) then Nalidixic acid (48.6%).

Out of 21 isolated *Proteus vulgaris*,12 isolates were multidrug resistant (5 isolates were resistant for 3 antimicrobial agents and 7 isolates were resistant for 4 antimicrobial agents) as shown in table 4.3. The highest resistance was for Co-Amoxiclav (81%) followed by Cefxime (57.1%) then Norofloxacin (52.4%).

The total isolated *Klebsiella pneumoniae* were 13, from which 8 isolates were multi drug resistant (4 isolates were resistant for 4 antimicrobial agents, and 4 isolates were resistant to five antimicrobial agents) as shown in table 4.3.The highest resistance was for Co-Amoxiclav (100%) followed by Cefxime (84.6%) then both Norofloxacin and Nalidixic acid (53.8%).

Table 4.3: Antimicrobial sensitivity testing of *Escherichia coli, Proteus vulgaris* and *Klebsiellapneumoniae*against selected antimicrobial agents

Isolate	Antimicrobial agents														
	NA			NOR			NIT			CF			AMC		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
E. coli	13 35.1 %	6 16.2 %	18 48.6 %	15 40.6 %	3 8.1%	19 51.4%	23 62.2%	9 24.5 %	5 13.5 %	5 13.5 %	28 75.7%	4 10.8%	4 10.8 %	10 27.0 %	23 62.2%
Pr.vulgaris	0%	0%	9 42.9 %	0%	0%	11 52.4%	0%	0%	5 23.8 %	0%	0%	12 57.1%	0%	0%	17 81%
K. pneumoniae	6 46.2 %	0%	7 53.8 %	6 46.2 %	0%	7 53.8%	8 61.5%	0%	5 38.5 %	2 15.4 %	0%	11 84.6%	0%	0%	13 100%

NA: Nalidixic acid NOR: Norofloxacin NIT: Nitrofurantoin

AMC: Co- AmoxiclavCF: Cefxime

Pseudomonas aeruginosa showed high resistant, in which all isolates were multidrug resistant, out of 8 isolates 5 isolates were resistant for 4 antimicrobials, 2 resistant for 5 antimicrobial agents and one isolate resist to all used antimicrobial agents.

Table 4.4: Antimicrobial sensitivity testing of *Pseudomonas aeruginosa* against selected antimicrobial agents

Isolate	Antimicrobial agents													
	NA		NOR		NIT		CF		AMC		IMP		AK	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R
Ps. Aeruginosa	2 25%	6 75%	5 62.5%	3 37.5%	2 25%	6 75%	0 0%	8 100%	0 0%	8 100%	7 87.5%	1 12.5%	2 25%	6 75 %

Keys:

NA: Nalidixic acid AMC: Co- AmoxiclavNOR: Norofloxacin CF: CefximeNIT: Nitrofurantoin IMP: Impenem

AK: Amikacin

From total three isolated*Staphylococcus aureus*there was no multidrug resistant only one isolate was resistant for 2 antimicrobial agents, the other two isolates were sensitive for all used antimicrobial agents.

Table 4.5: Antimicrobial sensitivity testing of *Staphylococcus aureus* against selected antimicrobial agents

Isolate	NOR		NIT		CF		AMC	
	S	R	S	R	S	R	S	R
S. aureus	2 66.7%	1 33.3%	2 66.7%	1 33.3%	3 100%	0 0%	2 100%	0 0%

Keys:

AMC: Co- Amoxiclav NIT: Nitrofurantoin

NOR: Norofloxacin CF: Cefxime

Out of 18 isolated *Enterococcus faecalis*, there was no multidrug resistant, 7 isolates were resistant for one antimicrobial agent and 4 were resistant for two antimicrobial agents.There were 7 isolates sensitive for all used antimicrobial agents.

Table 4.6: Antimicrobial sensitivity testing of *Enterococcus faecalis* against selected antimicrobial agents

Isolates	NOR		NIT		CF		AMC	
	S	R	S	R	S	R	S	R
E. faecalis	16 88.9%	2 11.1%	18 100%	0 0%	11 61.1%	7 38.9%	18 100%	0 0%

Keys: AMC: Co- AmoxiclavNOR: Norofloxacin CF: CefximeNIT: Nitrofurantoin

Only the isolated bacteria which was multidrug resistanttested for their sensitivity to *Zingiber officinale* and *Citrus limon* extracts. They were distributed as follow: *Escherichia coli* 18/37 (48.6%), *Proteus vulgaris* were12/21 (57.1%), *Klebsiella pneumoniae* 8/13(61.5%) and *Pseudomonas aeruginosa*8/8(100%).

Table 4.7: Distribution of multidrug resistant bacteria among isolates

Isolates	**No. of multi drug resistant bacteria (%)**
Escherichia coli (N=37)	18 (48.6%)
Proteus vulgaris (N=21)	12 (57.1%)
Klebsiella pneumonia(N=13)	8(61.5%)
*Pseudomonas aeruginosa (*N=*8)*	8 (100%)
*Staphylococcus aureus (*N=*3)*	0 (0%)
*Enterococcus faecalis (*N=*18)*	0 (0%)
Total (N=100)	47 (47%)

The following table is about the MICs of some bacterial standard strains,for both *Zingiber officinaleextracts* and *Limon peels extracts*.The MICs obtained by serial dilution to only one isolate for each bacterium,after that the last concentration which give reading reported as MIC.

Table 4.8: Antimicrobial activity (zone inhibition in mm) and MIC of *Zingiber officinale* and *Citruslimon*extracts on the Standard strains

Bacteria	***Zingiber officinale* methanolic extract**		***Zingiberofficinale* water extract**		***Citruslimon*methanolic extract**		***Citruslimon* water extract**	
	MIC mg/ml	Zone diameter(mm)	MIC mg/ml	Zone diameter(mm)	MIC mg/ml	Zone diameter(mm)	MIC mg/ml	Zone Diameter (mm)
E.coli ATCC 2592	100	17	50	12	12.5	14	3.12	10
Ps. aeruginosa ATCC 27853	3.12	10	3.12	12	3.12	14	3.12	15
Pr. vulgaris ATCC 6380	-	0	-	0	6.25	12	3.12	9

4.1.*Zingiber officinale* extract

Zingiber officinale with different concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/mland 3.125 mg/ml) showed antimicrobial activity against most multi drug resistant isolated bacteria.

Both methanolic and water extracts of *Zingiber officinale* was effective against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, but not showed reaction against *Proteus vulgaris*.

Table 4.9: Distribution of multidrug resistant bacteria sensitive to methanolic extract of *Zingiber officinale*

No. of sensitive isolates	Concentrations of *Zingiber officinale* methanolic extract					
	100 mg/ml	**50 mg/ml**	**25mg/ml**	**12.5 mg/ml**	**6.25mg/ml**	**3.125mg/ml**
E.coli N=18	18 (100%)	2 (11.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pr. Vulgaris N=12	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
K.pneumoniae N=8	8 (100%)	8 (100%)	4 (50%)	0 (0%)	0 (0%)	0 (0%)
Ps.aeruginosa N=8	8 (100%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)

Table 4.10: Sensitivity of *Zingiber officinale* water extract against multidrug resistant bacteria

No. of sensitive isolates	Concentrations of *Zingiber officinale*water extract					
	100mg/ml	**50 mg/ml**	**25 mg/ml**	**12.5mg/ml**	**6.25 mg/ml**	**3.125 mg/ml**
E.coli N=18	18 (100%)	14 (77.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pr. Vulgaris N=12	0 (0%)	0 (0%)	0 (0%)	0 0%)	0 (0%)	0 (0%)
K.pneumoniae N=8	8 (100%)	8 (100%)	8 (100%)	4 (50%)	0 (0%)	0 (0%)
Ps.aeruginosa N=8	8 (100%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)	0 (0%)

The methanolic extract at the highest concentration of 100mg/ml were exhibited the largestinhibition zone against *Klebsiella pneumoniae* (22±1.408mm), followed *by Pseudomonas aeruginosa* (19 ±.518mm) then *Escherichia coli* (16±1.o555mm).

Table 4.11: Bacterial activity of *Zingiber officinale* methanolic extract

Bacteria	100 mg/ml		50 mg/ml		25 mg/ml		12.5mg/ml		6.25 mg/ml		3.125 mg/ml	
	M ± STD	*P-value*	M ± STD	*P-value*	M ± STD	*P-value*	M ± STD	*P-value*	M ± STD	*P-Value*	M ± STD	*P-Value*
E.coli	16 ± 1.o555	.000	9± 3.474	.003	6 ± 1.258	.468	5± 0.00	1.34	0 ± 0.00	1.56	0 ± 0.00	1.56
Pr. Vulgaris	6 ± 0.00	0.504	4± 0.00	0.901	0 ± 0.00	-	0 ± 0.00	-	0 ± 0.00	-	0 ± 0.00	-
K.pneumoniae	22 ± 1.408	.004	17± 3.137	.002	7± 5.902	.336	0 ± 0.00	-	0 0.00	-	0 ± 0.00	-
Ps.aeruginosa	19 ± .518	.000	17± 1.282	.000	16 ± 1.753	.000	15± 1.959	0.00	13± 2.357	.000	12± 2.357	.001

Keys:

STD: Standard Deviation. **M:** Means of inhibitory zonesinmm**.**

While the water extract showed thelargest zones at the highest concentration of 100mg/ml. The largest inhibition zone was 22mm against both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* followed by *Escherichia coli* (15±2.149mm).

Table 4.12: Bacterial activity of *Zingiber officinale* water extract

Bacteria	100mg/ml		50mg/ml		25mg/ml		12.5mg/ml		6.25mg/ml		3.125mg/ml	
	M ± STD	*P-value*	M± STD	*P-Value*	M ± STD	*P-Value*	M ± STD	*P-Value*	M ± STD	*P-value*	M ± STD	*P-Value*
E.coli	15 ± 2.149	.000	10 ± 3.222	.023	0 ± 0.00	-	0 ± 0.00	-	0 ± 0.00	-	0 ± 0.00	-
Pr. Vulgaris	0 ± 0.00	-	0 ± 0.00	-	0 ± 0.00	-	0 ± 0.00	-	0 ± 0.00	-	0 ± 0.00	-
K.pneumoniae	22 ± 2.449	.000	20 ± 1.506	.000	13 .535	.000	5 ± 5.120	.366	0 ± 0.00	-	0 ± 0.00	-
Ps.aeruginosa	22 ± .756	.000	21± 1.061	.000	19 1.165	.000	17 ± 1.165	.000	16 ± 1.126	.000	13 ± 1.847	.000

STD: Stander Deviation.**M:** Means of inhibitory zones in mm.

The MIC values of *Zingiber officinale*methanolic extract were as follows: 3.125mg/ml for *Pseudomonas aeruginosa*, 25mg/ml *for Klebsiella pneumoniae*and 50 mg/ml for *Escherichia coli*.

Its MIC values of *Zingiber officinale* water extract were as follows: 3.125mg/ml for *Pseudomonasaeruginosa*, while *Klebsiella pneumoniae* was 12.5mg/ml and 50 mg/ml for *Escherichia coli*.

Table 4.13: Minimum inhibitory concentrations (MIC) for methanolic and water extracts of *Zingiber officinale*

Isolates	MIC for Methanolic extract	MIC for Water extract
Escherichia coli	50 mg/ml	50 mg/ml
Proteus vulgaris	-	-
Klebsiella pneumoniae	25 mg/ml	50 mg/ml
Pseudomonasaeruginosa	3.125 mg/ml	3.125 mg/ml

4.2 *Citrus Limon* extracts

It showed strong antimicrobial activity against all tested bacteria, and showed activity in all different concentration 100mg/ml, 50mg/ml, 25mg/ml 2.5, 6.25% mg/ml and 3.125mg/ml.

Both methanolic and water extracts of *Citruslimon* was effective against *Escherichiacoli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Proteusvulgaris*. The methanolic extract at the highest concentration of 100mg/ml were exhibited strong inhibition zone against *Pseudomonasaeruginosa*(26±.916 mm in diameter), followed *by Proteusvulgaris*(25±.879mm)and*Klebsiellapneumoniae* (22±.991) mm followed by *Escherichia coli* (21 ±1.381mm in diameter). The MIC value was 3.125 mg/ml for all bacteria.

While the water extract showed increase in the volume of zones at the highest concentration of 100 mg/ml in whichthe largest inhibition zone was 27±0.354 mm against*Pseudomonas aeruginosa*, then *Proteusvulgaris*(26±0.343 mm), and *Klebsiella pneumoniae*(25 ±1.126 mm) and the least zone(19±1.798mm) for *Escherichia coli*. The MIC values were 3.125mg/ml for all tested bacteria.

Table 4.14: Minimum inhibitory concentrations (MIC) for methanolic and water extracts of *Citrus limon*

Bacteria	Methanolic MIC	Water MIC
Escherichia coli	3.125 mg/ml	3.125 mg/ml
Proteus vulgaris	3.125 mg/ml	3.125 mg/ml
Klebsiella pneumonia	3.125 mg/ml	3.125 mg/ml
Pseudomonasaeruginosa	3.125 mg/ml	3.125 mg/ml

Table 4.15: Sensitivity of *Citrus limon* methanolic extract against multidrug resistant bacteria

Bacteria	Concentration					
	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.12mg/ml
E.coli N=18	18 (100%)	18 (100%)	18 (100%)	18 (100%)	18 (100%)	18 (100%)
Pr.vulgaris N=12	12 100%	12 100%	12 (100%)	12 (100%)	12 (100%)	3 (25%)
K.pneumoniae N=8	8 100%	8 100%	8 (100%)	8 (100%)	8 100%)	8 (100%)
P.aeruginosa N=8	8 100%	8 100%	8 (100%)	8 (100%)	8 (100%)	8/ (100%)

Table 4.16: Bacterial activity of *Citrus limon* methanolic extract

Bacteria	100mg/ml		50mg/ml		25mg/ml		12.5mg/ml		6.25mg/ml		3.125mg/ml	
	M ± STD	***P-value***	**M ± STD**	***P-value***	**M ± STD**	***P-Value***	**M ± STD**	***P-value***	**M ± STD**	***P-value***	**M ± STD**	***P-Value***
E.coli	21 ± 1.381	.000	19 ± 2.146	.000	17 ± 3.209	.000	14 ± 3.989	.000	12 ± 3.316	.000	9 ± 5.659	.187
Pr.vulgaris	25 ± .879	.000	22 ± .999	.000	18 ± 1.865	0.00	16 ± 2.198	.000	12 ± 2.665	.000	7 ± 4.983	.809
K.pneumoniae	22 ± .991	.000	19 ± .991	.000	17 ± 1.847	.000	16 ± 2.066	.000	14 ± .991	.000	12 ± .991	.000
P.aeruginosa	26 ± .916	.000	23 ± .916	.000	10 ± 1.642	.000	17 ± 1.165	.000	14 ± .463	0.00	13 ± 1.642	.000

Table 4.17: Sensitivity of *Citrus limon* water extract against multidrug resistant bacteria

Bacteria	Concentration					
	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	3.12mg/ml
E.coli N=18	18 (100%)	18 (100%)	18 (100%)	18 (100%)	18 (100%)	18 (100%)
Pr.vulgaris N=12	12 100%	12 100%	12 (100%)	12 (100%)	12 (100%)	8 (66.7%)
K.pneumoniae N=8	8 100%	8 100%	8 (100%)	8 (100%)	8 100%)	8 (100%)
P.aeruginosa N=8	8 100%	8 100%	8 (100%)	8 (100%)	8 (100%)	8/ (100%)

Table 4.18: Bacterial activity of *Citrus limon* water extract

Bacteria	100 mg/ml		50 mg/ml		25 mg/ml		12.5 mg/ml		6.25 mg/ml		3.125 mg/ml	
	M± STD	***P-value***	**M ± STD**	***P-Value***	**M ± STD**	***P-value***	**M ± STD**	***P-value***	**M ± STD**	***P-Value***	**M ± STD**	***P-Value***
E.coli	19 ± 1.798	0.00	15 ± 2.413	.001	12 ± 2.176	.000	11 ± 2.713	.000	10 ± 2.516	.000	8 ± 4.802	.387
Pr.vulgaris	26 ± 0.343	0.00	23 ± 1.052	.000	19 ± 1.788	.000	16 ± 1.792	.000	15 ± 1.792	.000	12 ± 2.456	.017
K.pneumoniae	25 ± 1.126	0.00	23 ± 1.069	.000	19 ± .835	.000	15 ± .835	.000	12 ± .916	.000	11 1.669	.000
P.aeruginosa	27± 0.354	0.00	22 ± 1.069	.000	20 ± 1.4040	.000	17 ± 1.061	.000	16 ± 1.061	.000	14 .744	.000

Keys:

STD: Stander Deviation. **M:** Means of inhibitory zones in mm**.**

Both Methanolic and water extracts are effective against selected organisms except*Zingiber officinale* water extract were ineffective against *Proteus vulgaris*. The Citrus *limon* extracts are more effective than those of *Zingiber officinale* for all selected bacterial isolates.

Table 4.19: Comparison between water and methanolic extract of *Zingiber Officinale* and *Citruslimon* at 100% concentration

Methanolic Extract of *ZingiberOfficinale*			Water Extracts*Zingiber Officinale*		Methanolic Extracts*Citruslimon*		Water Extracts *Citruslimon*	
Bacteria	Mean± STD	*P.value*	Mean± STD	*P.value*	Mean± STD	*P.value*	Mean± STD	*P.value*
E.coli	16.78± 1.555	0.000	15.83± 2.149	0.003	20.56± 1.381	0.000	17.94± 1.798	0.003
Pr.vulgaris	0.00	-	0.00	-	21.88± 0.991	0.427	25.13± 1.126	0.021
K.pneumoniae	2.50±1 .00	0.891	0.00±0 .00	-	23.83± 1.193	0.000	24.42± 1.929	0.000
P.aeruginosa	19.63± 0.518	0.000	22.50± 0.756	0.000	26.38± 0.916	0.000	27.00± 0.000	0.000

CHAPTER V
DISCUSSION, CONCLUSION AMD RECOMMENDATIONS

5.1 Discussion

Antimicrobial drugs provide the main basis for treatment of various microbial infections, however, the high genetic variability of some microorganisms enable them to rapidly develop antimicrobial resistance. Thus, there has been a continuing search for New PotentAntimicrobials (Alfadol and Eltalib 2017). Medicinal plants are cheap and renewable sources of pharmacologically active substances and are known to produce certain chemicals that are naturally toxic to bacteria (Taura *etal*., 2014).

In the present study;the percentage of resistance of isolates against selected antimicrobials was varied, in whichthe highest resistance was for Co- Amoxiclav was 69%, followed by Nalidixic acid (50%), then Norofloxacin (43%), Cefxime (35%), and Nitrofurantoin (22%). This agrees with Ahmed *etal*. (2000) in Sudan and with Alfadol and Eltalib (2017)in Sudan whomfound that the most common urinary isolates were highly resistant when they were tested against Co-Amoxiclav.

Only the isolated bacteria which was multidrug resistant, they were tested for their sensitivity to *Zingiber officinale* and *Citrus limon.* They were distributed as follow: *Escherichia coli* 18/37 isolates (48.6%), *Proteus vulgaris* were12/21 isolates (57.1%), *Klebsiella pneumoniae* 8/13(61.5%), *Pseudomonas aeruginosa* 8/8(100%) and only one *Staphylococcus aureus* 1/3(33.3%).

The antibacterial activity of aqueous and methanolic extracts of *Zingiber officinale* roots showed the highest inhibitory zone at the highest concentration (100 mg/dl). The water extracts of *Zingiber officinale* was effective against all *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae,* andone *Staphylococcus aureus*, while showed no reaction against *Proteus vulgaris,* which show an agreement with Khalid *et al.*(2011) in Sudan, who found that the cold water extracts showed different zones of inhibition against *Pseudomonas aeruginosa*, *Escherichia coli,and Staphylococcus aureus*. While it wasdisagreed with that obtained by Malu *et al*., (2009)in Nigeria and Kakil(2013) in Sudan, who found water extract of *Zingiberofficinale*didn't show antibacterial activity against urinary tract isolates. However, negative result do not indicate the absence of bioactive compounds, nor that the plant is inactive, since active components may be present in insufficient amount in the crude extracts to show activity with the dose level employed (Kakil, 2015).

Like water extract of *Zingiber officinale* the methanolic extract was also effective against all *Escherichia coli*, *Pseudomonas aeruginosa,Klebsiellapneumoniae*, and the one *Staphylococcus aureus*. This result matchedwith Ahmed *etal*. (2012)in Sudanwho reported ginger methanolic extracts showed inhibiter activity against *Staphylococcus aureus,Escherichia coli* and *Klebsiellapneumoniae*. While this mismatched with Pilerood *etal*. (2014) in India who found activity only against *Staphylococcus aureus.* The variation of susceptibility of the tested microorganism to the same extract could be attributed to their intrinsic properties that are related to the permeability of their cell surface to the same extract (Mohammed, 2016).

The antibacterial activity of *Citruslimon* aqueous and methanolic extracts also showed the highest inhibitory zone at the highest concentration (100 mg/dl).

The methanolic extract of *Citruslimon* showed strong activity against all tested isolates which includes: *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*and *Proteusvulgaris*. This result harmonized with Liya and Siddique(2018)in Bangladesh who found that; methanolic extracts of *Citrus limon* showed positive result against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Alsoit wasagreed with Sharma and Rathore (2018)in Indiawho found out strong positive result against *Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. This findings wasconflict with Alhoi *et al*(2018) in Indonesiawhom reportedthat; the methanolic extract of *Citrus limon* has antibacterial activity only against *Escherichiacoli.*

Like the methanolic extract the water extract of *Citruslimon*, showed strongpositive result against all tested bacteria includes: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Staphylococcus aureus* with variation in the zones size. There is no previous studies in water extracts of *Citrus limon* for comparison.

The methanolic and water extracts of each plant have the same affect against the isolated bacteria with the variation in the inhibition zones this variation may due to the different extraction solvents as concluded by (Kakil, 2013).

5.2. Conclusion

This study concluded that; the highest rate of urinary antimicrobials resistance was against Co-Amoxiclav followed by Nalidixic acid then Norofloxacin.

Both methanolic and water extracts of *Zingiber officinale* was effective against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, while showed no reaction against *Proteus vulgaris*. While *Citrus limon* methanolic and water extracts showed good antimicrobial activity against all tested bacteria included *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Proteus vulgaris*.

5.3. Recommendations

Testing the antibacterial activity of both plants using other methods rather than methods used here such as Petroleum ether extracts.

Determine the minimum bactericidal concentration (MBC) for both plants and toxicity for the active ingredients of each plant including in this study.

Analyze the bioactive components of the extracts using gas chromatography and high performance liquid chromatography.

Such studies should be assessed to be affordable for commercial usage.

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Appendices

Appendix(I)

Colored atlas

Colored Plate(1): Methanolic Extract of *Lemon Peels*.

Colored Plate (2): Methanolic Extract of *Zingiber officinale*

Colored Plate (3): Water Extract of *Citrus limon.*

Colored Plate (4): Water extract of *Zingiberofficinale*.

Colored Plate (5): Working Solution of Methanolic Extract of *Citrus limon*.

Colored plate(6): Working Solution of Methanolic Extract of *Zingiber Officinale*

Colored Plate (7): Antimicrobial sensitivity test of *Escherichia coli*..

Colored Plate (8): Result of methanolic Extract of Lemon peels on *Pseudomonas aeruginosa*.

Colored Plate (9): Result of Water Extract of *Citrus limon* on *Staphylococcus aureus.*

Colored Plate (10): Result of methanolic extract of *Citrus Limon* on *proteus vulgaris*.

Colored Plate (11): Result of methanolic extract of *Citrus Limon* on *Klebsiella pneumoniae*

Colored Plate(12): Result of biochemical Tests of Gram negative bacteria.

Appendix (II)

Table 1: Weight and yield percentage of methanolic and water extracts

Sample No	**Sample**	**Weight of sample in gm.**	**Weight of extract in gm.**	**Yield %**
1	Methanolic Extract of *Zingiber officinale.*	100gm	8.5gm	8.5%
2	Methanolic Extract of *Citrus lemon.*	150gm	17.6gm	11.7%
3	Water Extract of *Zingiber officinale.*	45gm	2.5gm	5.6%
4	Water Extract of *Citrus limon*	55gm	3.2 gm	5.8%

Table 2: phytochemical screening of ginger and lemon

Sample	Saponin	Cumarin	Alkaloids	Flavonoids	Tanins	Steroids	Triterpens	Anthraquinone
Zingiber officinale	–	–	–	++	–	–	–	–
Lemon peels	++	–	–	++	++	+	–	–

Key:

(-): Negative **(+):** Trace

(++): Moderate **(+++):** High

Appendix (III)

Preparation of the reagents and culture media

I-1:Nutrient agar:

Contents:

Typical formula in g/L

Peptone……..……5.0g

Meat extract……..3.0g

Agar…...……... ...……..15.0g

Preparation:

A 23g of powder were suspended in 1L of distilled water and heated to boiling, Dispensed into containers and sterilized in the autoclave at 121ºC for 15 minutes. Allowed to cool and stored at 2-8ºC.

MacConkey agar:

This medium was best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents:

Peptone, lactose, bile salts, sodium chloride, neutral red, and agar.

Preparation:

Prepared as instructed by the manufacturer. Sterilized by autoclaving at 121ºC for 15 minutes. Then the medium was cooled to 50-55ºC, mixed well and dispensed aseptically in sterile petri dishes. the medium was dated and gived a batch number. Steriled plates were stored at 2-8ºC in plastic bags to prevent loss of moisture.

CLED agar:

Contents:

Component (per liter of purified water)

Gelatin peptone…… ...4.0 g

Beef Extract……...…….. 3.0 g

Casein peptone …….. 4.0g

Lactose…….....10.0 g

L- Cystine ……..... 128.0 mg

Bromthymol blue ……..... 20.0 mg

Agar…… 15.0 g.

Blood agar:

Contents:

To make about 35 blood agar plates

Agar……500ml

Sterile defibrinated blood…… ...…25ml

Preparation:

The agar medium was prepared as instructed by the manufacturer. Sterilized by autoclaving at 121ºC for 15 minutes. Transfered to a 50ºC water bath. Then the agar was cooled to 50ºC, the steriled blood was added aseptically and mixed gently and we took into account avoiding forming air bubbles. A 15 ml amounts were dispended aseptically in a steriled petri dishes. The medium was dated and gived a batch number. The plates were stored at 2-8ºC in plastic bags to prevent loss of moisture.

Mannitol salt agar:

The medium was best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents:

Typical formula in g/L:

Pancreatic digest of casein…….....5g

Peptic digest of animal tissue……..……...…5g

Beef extract……..……..…1g

Sodium chloride…….....75g

D-Mannitol…….……..10g

Phenol red……..…….……..…..0.025g

Agar…….……..……..15g

Preparation:

The medium was prepared as instructed by the manufacturer. Sterilized by autoclaving at 121ºC for 15 minutes. Then the plate was cooled to 50-55ºC, mixed well, and dispensed aseptically in sterile petri dishes. The medium was dated and gived a batch number. The plates were stored at 2-8ºC in plastic bags to prevent loss of moisture.

DNA-ase agar:

The medium was best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents:

Tryptose, deoxyribonucleic acid, sodium chloride, and agar.

Preparation:

Prepared and sterilized as instructed by the manufacturer. Then the plate has cooled to 50-55ºC, mixed well, and dispensed aseptically in sterile petri dishes. The medium was dated and gived a batch number. The plates were stored at 2-8ºC in plastic bags to prevent loss of moisture.

Bile Aesculin agar:

The medium was best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents:

Typical formula peptone ……14g

Bile salts…….……...15g

Ferric citrate ……..……..……..……0.5g

Aesculin …..…..……..……..1g

Agar…….……..14g

Preparation:

Prepared and sterilized as instructed by the manufacturer. Then the plate was cooled to 50-55ºC, mixed well, and dispensed aseptically in test tubes (slopes). The medium was dated and gived a batch number. The plates were stored at 2-8ºC in plastic bags to prevent loss of moisture.

Kligler iron agar:

The medium was best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents

Peptone, Lab-Lemco powder, yeast extract, sodium chloride, lactose, glucose, ferrous sulfate, sodium thiosulphate, phenol red, and agar.

Preparation:

Prepared as instructed by the manufacturer. Then cooled to 50-55ºC, mixed well and dispensed in 6ml amounts in large size tubes. Sterilized by autoclaving (with caps loosened) at 121ºC for 15 minutes. The medium was allowed to solidify in a sloped position to give a butt 25-30mm deep and a slope 20-23mm long (the butt should be longer than the slope). The medium was dated and gived a batch number. The medium was stored at 2-8ºC.

Urea medium:

The medium was best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents:

Typical formula in g/L:

Gelatin peptone…….……1g

Dextrose……...……1g

Sodium chloride……...……5g

Monopotassium phosphate……..2g

Phenol red…….……0.012g

Agar…….……..15g

Sterile urea solution, 40%.....50ml

Preparation:

The medium was prepared as instructed by the manufacturer. Sterilized by autoclaving at 121ºC for 15 minutes. Then the medium was cooled to 50-55ºC, aseptically the sterile urea solution was added, and mixed well. Dispensed aseptically in 3ml amounts in steriled tubes and allowed dry in slant position. Labeled and stored at 2-8ºC.

Semi solid nutrient agar:

Preparation:

To make about 20 bottles

About 0.75g nutrient agar and 1.3g nutrient broth were mixed in 100ml distilled water, and heaedt to 100ºC to dissolve the ingredients. Dispensed the medium in 5-7ml amounts in screw-cap bottles. Sterilized by autoclaving at 121ºC for 15 minutes. Then cooled, the bottle caps were tightened. The medium was dated and gived a batch number. Stored at 2-8ºC.

Mueller Hinton agar:

The medium was best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents:

Typical formula g/L

Casein acid hydrolysate…….……..…17.5g

Beef heart infusion……...……2g

Starch, soluble……..1.5g

Agar……..…….17g

Preparation:

About 38g of the powder were suspended in 1L of distilled water (or as the manufacturer's instructions), mixed well and heated to 100ºC to dissolve completely. Sterilized by autoclaving at 121ºC for 15 minutes. Then the medium was cooled to 50-55ºC, mixed well and dispensed aseptically in sterile petri dishes. The medium was dated and gived a batch number. The plates were stored at 2-8ºC in plastic bags to prevent loss of moisture

Peptone water:

Contents:

Peptone……..10g

Sodium chloride…….5g

Distilled water……1L

Preparation:

The peptone and sodium chloride were dissolved in the water and mixed well. Sterilized by autoclaving at 121ºC for 15 minutes. Stored at 2-8ºC.

Crystal violet Gram stain:

Contents:

To make 1L:

Crystal violet……..20g

Ammonium oxalate……9g

Ethanol or methanol, absolute…….95ml

Distilled water……1L

Preparation:

The crystal violet was weighted on a piece of clean pre-weighted paper. Transfered to a brown bottle premarked to hold 1L. The absolute ethanol or methanol was added and mixed until the dye is completely dissolved. Weight The ammonium oxalate was weighted and dissolved in about 200 ml of distilled water, and it was added to the stain. Made up to the 1L mark with distilled water, and mixed well. The bottle was labeled and stored it at room temperature. The stain will be stable for several months.

Lugol's iodine solution:

Contents:

To make 1L:

Potassium iodine……..…….20g

Iodine…….……..10g

Distilled water……...……1L

Preparation:

The potassium iodine was weighted, and transfered to a brown bottle premarked to hold 1L. About a quarter of the volume of water was added, and mixed until the potassium iodine completely dissolved. The iodine was weighted, and added to the potassium iodine solution. Mixed until it dissolved. Made up to the 1L mark with distilled water, and mixed well. The bottle was labeled, and marked as toxic. It was stored in a dark place at room temperature.

Acetone-alcohol decolorizer:

Contents:

To make 1L:

Acetone…….……500ml

Ethanol or methanol, absolute……...……475ml

Distilled water…….25ml

Preparation:

The distilled water was mixed with the absolute ethanol or methanol and the solution was transfered to a screw-caped bottle of 1L capacity. The acetone was measured, and added immediately to the alcohol solution. Mixed well and then the bottle was labeled, and marked as highly flammable. Stored in a safe place at room temperature. The reagent was stable indefinitely.

Safranin stain:

Contents:

Certified safranin-O……..2.5g

95% Ethyl alcohol…….……..100ml

Distilled water…….90ml

Preparation:

Certified safranin-O was added to ethyl alcohol and mixed until it completely dissolved. About 10ml of the solution was added and made to the distilled water. The bottle was labeled and stored at room temperature.

3% hydrogen peroxide solution:

Contents:

Hydrogen peroxide……3g

Distilled water…….100ml

Preparation:

The hydrogen peroxide was added to the water in glass bottle. Mixed well, The solution was labeled and stored at room temperature.

Hydrochloric acid, 1mol/L:

Contents:

To make 100ml

Concentrated hydrochloric acid…….……8.6ml

Distilled water……..……100ml

Preparation:

A 100ml volumetric flask was half fulled with distilled water. The 8.6ml concentrated hydrochloric acid was added. Made up to the 100ml mark with distilled water, and mixed well. Transfered to a screw-caped container.

Kovac's reagent:

Contents:

To prepare 20ml:

4-dimethylaminobenaldehyde…….……..1g

Isoamylalcohol (3-methyl-1-butanol)…….15ml

Concentrated hydrochloric acid……..5ml

Preparation:

The dimethylaminobenaldehyde was weighted, dissolved in the isoamylalcohol. Concentrated hydrochloric acid was added and mixed well. Transfered to clean brown bottle and stored at 1-8ºC.

0.5 McFarland standard:

Contents:

Concentrated sulphuric acid……..……1ml

Dihydrate barium chloride……..…..0.5g

Distilled water……..150ml

Preparation:

A 1% v/v solution of sulphuric acid was prepared by adding the concentrated sulphuric acid to 99ml of water, mixed well. A 1% w/v solution of barium chloride

was prepared by dissolving the dehydrate barium chloride ($BaCl_2.2H_2O$) in 50ml of distilled water. A 0.6ml of barium chloride solution was added to 99.4ml of sulphuric acid solution, and mixed. A small volume of the turbid solution was transfered to a capped tube or screw-caped bottle of the same type as used for preparing the test and control inoculum.