Phytochemical analysis and bioactivity of *Garcinia kola* (Heckel) seeds on selected bacterial pathogens.

By

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DECLARATION

I, the undersigned, declare that this dissertation submitted to the University of Fort Hare for obtaining a Master of Science degree and the work contained herein is original unless cited and has not been submitted at any other University for any degree.

Signature of student.....

Signature of supervisor……

Date January 2012

DEDICATION

I dedicate this work to my mother, Clara Seanego who raised and gave me all the support and strength I needed to further my studies at the University of Fort Hare. I also dedicate this work to my former lecturers at the University of Venda and also to my late brother, Solomon who loved school so much.

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ABSTRACT

Garcinia kola is one of the plants used in folklore remedies for the treatment of microbial infections. Bacterial resistance to commonly used antibiotics has necessitated the search for newer and alternative compounds for the treatment of drug resistant microbial infections. This study focuses on the bioactivity of *G. kola* seeds on *Streptococcus pyogenes* (ATCC 49399), *Staphylococcus aureus* (NCTC 6571), *Plesiomonas Shigelloides* (ATCC 51903) and *Salmonella typhimurium* (ATCC 13311), organisms which can cause illnesses from mild to severe with potentially fatal outcomes. The crude ethyl acetate, ethanol, methanol, acetone and aqueous extracts were screened by agar-well diffusion method and the activities of the extract were further determined by Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays. The inhibition zones ranged from 0 - 24 mm, while MIC and MBC of the extract ranged between 0.04 - 1.25 mg/mL and 0.081 - 2.5 mg/mL respectively. Chloroform/ Ethyl Acetate/ Formic acid (CEF) solvent system separated more active compounds followed by Ethyl Acetate/ Methanol/ Water (EMW) and Benzene/ Ethanol/ Ammonium Hydroxide (BEA). The extracts were fractionated by Thin Layer Chromatography (TLC). Bioautography was used to assess the activity of the possible classes of compounds present in the more active extracts. Column chromatography was used to purify the active compounds from the mixture while Gas Chromatography-Mass Spectrometry (GC-MS) was used to identify the phyto components of the fractions. The MIC of the fractions ranged between 0.0006 - 2.5 mg/mL. CEF 3 (F3), CEF 11 (F11) and CEF 12 (F12) revealed the presence of high levels fatty acids Linoleic acid, 1, 2-Benzenedicarboxylic acid and 2, 3-Dihydro-3, 5-dihydroxy-6-methyl, respectively. The results obtained from this study justify the use of this plant in traditional medicine and provide leads which could be further exploited for the development of new and potent antimicrobials.

CHAPTER ONE

1.1 Introduction

Streptococcus pyogenes is a Gram-positive, non-spore forming, facultative anaerobic bacterium that occurs as long chains of cocci and occasionally in pairs. The metabolism of *S. pyogenes* is fermentative; it is catalase negative. It requires an enriched medium containing blood for growth. It is the cause of many diseases ranging from mild superficial skin infections to life threatening diseases. Infections typically begin in the throat and include pharyngitis (strep throat) and localized skin infection. Severe infections include necrotizing fasciitis and streptococcal toxic shock syndrome; and it is the most common bacterial cause of sore throat (Todar *et al.*, 2005). It evades the immune response and spread by penetrating host tissue layers. *S. pyogenes* releases a number of toxins into its host. The treatment of choice is penicillin for a minimum of 10 days. Macrolides, chloramphenicol and tetracyclines are used if the strain isolated has been shown to be sensitive, but resistance is much more common. An increased macrolide resistance rates in the clinical isolates of *S. pyogenes* have been reported in many parts of the world (Palavecino *et al*., 2001). A decrease in susceptibility of *S. pyogenes* to erythromycin and tetracycline has also been reported (Jasir *et al*., 2000). The application of topical antibiotics such as mupirocin or ointment (Bactroban) and fusidic acid are also used and high resistance rates have been reported with the use of fusidic acid, ranging from 32.5 -50% (Oranje *et al*., 2007).

Staphylococcus aureus is a facultative anaerobic, non-motile, non-spore forming, catalase positive and Gram-positive coccus which appears as grape-like clusters when viewed under the microscope. These potentially pathogenic bacteria are found in nasal or mucous membranes, hair follicles and peritoneum of warm blooded animals. They are able to invade via the broken skin or mucous membranes; hence intact skin is an excellent human defence.

People with weak immune systems and open wounds or who just had surgery are most at risk for developing *S. aureus* infection; it is spread primarily by food handlers with *Staphylococcus* infections on the skin (Tolan, 2000). *S. aureus* can cause food poisoning, toxic shock syndrome and pneumonia. Toxic shock syndrome is characterized by sudden onset of high fever, vomiting, diarrhea and muscle aches followed by hypotension which can lead to death. Penicillins with beta-lactamase-inhibitor such as amoxicillin plus clavulanic acid are used to treat *S. aureus* infections. Patients allergic to penicillin are treated with vancomycin, although for minor infections macrolides such as erythromycin may be adequate. Penicillin resistance in *S. aureus* is due to production of the enzyme beta-lactamase (penicillinase) (Klytmanns *et al*, 1997). The occurrence of vancomycin resistant *S. aureus* has been reported (Kasloom *et al*., 2006). *S. aureus* has been a stumbling block for antimicrobial chemotherapy and introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of pathogens (Aborzi *et al*., 2000).

Plesiomonas shigelloides is a Gram-negative, facultative anaerobic, oxidase positive, flagellated rod belonging to the family *Enterobacteriaceae* (Garrity *et al*., 2004). It is an aquatic microorganism, recognized as a potential human and animal pathogen. *P. shigelloides* is extensively distributed in the environment (Salerno *et al*., 2007). It is commonly found in fresh and brackish water, freshwater fish, shellfish and different types of animals such as cattle, goats, swine, cats, dogs, monkeys, vultures, snakes and toads. Human infections due to this bacterium are mostly waterborne and cause gastroenteritis with fever, chills, abdominal pain, nausea, diarrhea and vomiting (Ueda *et al*., 1999). Extra intestinal complications such as septicemia and death may occur in immunocompromised people. The usual route of transmission is by ingestion of contaminated water or raw shellfish. Most *P. shigelloides* strains are resistant to broad spectrum penicillins such as ampicillin, piperacillin,

carbenicillin, azlocillin and others (Avison *et al*., 2001). Susceptibility of the organism to carbapenems and cephalosporins has been demonstrated (Obi *et al*., 2007).

Salmonella typhimurium is a Gram - negative, non-spore forming, and catalase positive, oxidase negative and facultative anaerobic bacterium. It is a significant cause of morbidity and mortality in humans and animals with multidrug resistance being an emerging problem (Hendrieksen *et al*., 2004). Contaminated food of natural origin, particularly meat products from cattle and pigs, is an important source of *S. typhimurium* in human infections (Evans *et al*., 1996). Although it does not cause clinical disease in pigs, subclinical infection constitutes an important food-safety problem throughout the world (Kranker *et al*., 2003). It causes gastroenteritis in humans and other mammals. It is one of the main serovars of *Salmonella enterica* causing illness like typhoid-like disease in mice. The disease caused by this bacterium in humans is characterized by diarrhea, abdominal cramps, vomiting and nausea and generally last up to seven days. Fluoroquinolones are used for treatment in adults; cephalosporins given by injection are widely used in children, as quinolones are not generally recommended for this group. Other drugs such as chloramphenicol, ampicillin, amoxicillin and trimethoprim-sulfamethoxazole are occasionally used as alternatives. The emergence of multidrug-resistant serotypes, especially *S. typhimurium* definitive phage-type (DT) 104, has become a potential problem of animal husbandry and in human medicine (Butaye *et al*., 2006) and therefore effective antimicrobials are essential for treatment.

Garcinia kola is a medium sized evergreen tree which grows about 15-17m high (Plowden, 1972). It is cultivated and distributed throughout the West and Central Africa and found mostly in moist conditions. It produces a characteristic smooth elliptically shaped seeds, with yellow pulp and brown seed coat. It is also referred to as "bitter *kola"* because of the astringent taste. The seeds are culturally and socially significant in some parts of West Africa and are served for traditional hospitality in private, social and cultural functions. The seeds

have been found to have broad spectrum antibacterial activity (Akinpelu *et al*., 2008). This has been demonstrated with *Bacillus subtilis* (NCIB 3610), *Streptococcus faecalis* (NCIB 775), *Staphylococcus aureus* (NCIB 8588), *Klebsiella pneumoiae* (NCIB 418), *H.pylori* (Akinpelu *at al*., 2008; Njume *et al*., 2011) and other organisms with methanolic extract of the seeds. The phytochemical compounds isolated from *G. kola* seeds includes biflavonoids such as kolaflavone and 2-hydroxybi-flavonoids (Okunji *et al*., 2002), biflavonones (GB -1, GB-2) (Cotterin *et al*., 2000). Other compounds include triterpenes, xanthones and (1-3, 8-11) benzophenones (Okunji *et al*., 2007). Although studies have been done with this plant, the seeds in most cases were obtained from Nigeria and majority of the studies have concentrated on several organisms excluding some of the organisms used in this study. Most studies have been done with aqueous, petroleum, butanol and diethyl ether extracts of the seeds which might limit the antimicrobial potentials of plants. The type of solvent used may have an effect on the nature of compounds extracted and the resulting bioactivity of the extract (Eloff *et al*., 2008). These observations of antibacterial activity of *G. kola* suggest that it could be a potential source of therapeutically useful compounds for the treatment of various infections and therefore further investigation of the plant are required.

1.2 Statement of the problem

The problem of antibacterial resistance, to commonly used antibiotics has led to a search for newer and alternative compounds for the treatment of drug-resistant infections (Sibanda *et al*., 2008). Several findings on chemotherapeutic potentials of plants have shown that they can be sources of antimicrobial compounds of value and a typical example of such plant is *Garcinia kola* (Rabe *et al*., 1997; Njume *et al*., 2011). Presently there are global problems of antibiotic resistance to infections coupled with the emergence of new and re-emerging diseases. There is also a belief that the use of plants for medicinal purposes has been

associated with less side effects (Tolan, 2000). There is therefore a need to search for more efficacious and cost-effective antimicrobial agents of natural origin to complement the existing synthetic antimicrobial drugs that are becoming less potent against pathogenic microorganisms (Ncube *et al*., 2008).

1.3 Hypothesis

G. kola has potent antimicrobial activity against *S. pyogenes, S. aureus, P. shigelloides* and *S. typhimurium.*

1.4 Overall objective

The overall objective was to ascertain the bioactivity of *Garcinia kola* seeds on selected bacterial pathogens.

1.5 Specific objectives

The specific objectives of this study were to:

1. Screen the plant extracts (ethyl acetate, ethanol, methanol, acetone and aqueous) for antimicrobial activity.
2. Determine the Minimum Inhibitory Concentration (MIC).
3. Determine the Minimum Bactericidal Concentration (MBC).
4. Purify and characterize the active compounds by Column chromatography and Gas-Chromatography Mass - Spectrometry (GC-MS).

CHAPTER TWO

Literature Review

2.1 *Streptococcus pyogenes*

2.1.1 Brief history

S. pyogenes was first discovered by Billroth in 1874 in patients with wound infections. In 1883, Fehleisen isolated the chain forming bacteria in pure culture from perierysipelas lesions. The organism was named *S. pyogenes* in 1884 by Rosebach. This name is derived from the word pyogenic which is a classification for the streptococci that are associated with the formation of pus. In 1930's advances in hemolytic and non-hemolytic were made by Lancefield. *S. pyogenes* display an antigen A on its cell wall and because of this it has been classified by Lancefield serotyping as group A. Various studies made by Mueller and Brown in 1903 and 1919 respectively led to knowledge of different patterns of hemolysis as alpha, beta and gamma hemolysis. A later development in this field was the Lancefield classification of beta-hemolytic streptococci by serotyping based on M-protein precipitation reactions. Hippocrates was an ancient physician who studied and recorded observances on the infections and physiology of the body and described the relative symptoms of the flesh-eating bacteria in the early stages. The critical role of M-protein in causing diseases was established by Lancefield. In the early 1900s, Doches, George and Dick identified haemolytic streptococcal infection as the cause of scarlet fever. The epidemiological studies made in the 1900s helped established the link between Group A *Streptococcus* infection and acute rheumatic fever and acute glomerulonephritis (Khan *et al*., 2009).

2.1.2 Morphology

S. pyogenes is a Gram-positive bacterium that grows as long chains or in pairs (Ryan, 2004). Individual cells are round to ovoid cocci, about 0.6 - 1.0 micrometer in diameter. The cell wall of *S. pyogenes* is very complex and chemically diverse. The organism has a rigid cell wall, inner plasma membrane with mesosomal vesicles, cytoplasmic ribosomes and a nucleoid (Joklik *et al*., 2000). It also consists of filamentous pili to adhere mechanically (Manetti *et al*., 2007).

2.1.3 Pathogenesis and clinical manifestation

S. pyogenes has several virulence factors that enable it to attach to host tissues, evade the immune response, and spread by penetrating host tissue layers. As a rule, individual species of *S. pyogenes* are classified based on their hemolytic properties (Patterson, 1996). It is commonly known as beta-hemolytic group A *Streptococcus* or GAS (Todar *et al*., 2002). Hemolysis is the breakdown of red blood cells. Alpha hemolysis is caused by an oxidation of iron in haemoglobin which gives a greenish colour on blood agar. Beta-hemolysis is complete rupture of red blood cells which gives distinct, wide, clear areas around bacterial colonies on blood agar (Facklam, 2002).

The antigenic components of the cell are the virulence factors. The cell surface accounts for many of the bacterium's determinants of virulence. This includes capsular polysaccharides, peptidoglycan, lipoteichoic acids and certain membrane proteins in addition to several structural proteins. These allow bacteria to adhere to surfaces, spread throughout the body, hemolyze blood cells and necrotize tissue. The capsule makes it possible for the organism to become resistant to phagocytosis. M-proteins show clearly virulence factors associated with both colonization and resistance to phagocytosis (Falagas, 2008). M-protein is a macromolecule incorporated in fimbriae present on the cell membrane which protects the

bacterial cell wall. The ability of *S. pyogenes* to cause disease greatly depends upon these proteins. The fibronectin collagen-T-antigen (FCT) is a region that allows the adherence of the *S. pyogenes* to epithelial cells (Baldassarri *et al*., 2006). They have been identified based on the antigenic specificity and are the major cause of antigenic shift and drift among the Group A *Streptococcus pyogenes*. This organism produces a large number of extracellular proteins that cause an increase in its virulence ability which triggers a serious non-specific immunological response in the human host. The extracellular components responsible for diseases include invasins and exotoxins. Another unique property that distinguishes this bacterium from the others is a protein called a fibronectin binding protein that allows it to adhere to respiratory epithelial cells (Hunski *et al*., 1992). The pili found in GAS which are normally found in gram-negative bacteria have important virulence factors that are used for adhesion on target cells. Reports on these pili have shown that they are made up of proteins from the same family, suggesting that they are also found in gram-positive bacteria. They were also investigated further and discovered to have effective antigens that protect against lethal infections (Mora *et al*., 2005). This virulence factor enables the organism to attach and stick to host cell tightly and not let go.

Colonization of the upper respiratory tract and acute pharyngitis may result in spreading to other portions of the upper or lower respiratory tracts which causes infection of the ear, sinuses and lungs (pneumonia). During these aspects of acute diseases, the organism brings into play a variety of secretory proteins that mediate invasion (Clark, 2009). *S. pyogenes* produces a wide range of skin and soft tissue infections and the severity of the illness associated with these infections is extremely variable (Baddour, 1993). The infections typically begin in the throat or skin. In human, it is the cause of many diseases ranging from mild superficial skin infections which include pharyngitis (Strep throat) and localized skin infection such as impetigo, erysipelas and localized cellulitis characterized by fever and

tenderness (Stevens *et al*., 1996; Pierre *et al*., 2003; Smeesters *et al*., 2010). Other infections include acute rheumatic fever, rheumatic heart disease and acute glomerulonephritis (Malin *et al.,* 2006). If strep throat is not treated it can develop into rheumatic fever which is a disease that affects the joints and heart valves. Other toxigenic infections may lead to Streptococcal toxic shock syndrome which can be life threatening (Ryan *et al*., 2004). It can also cause soft tissue infections such as pyoderma myositis, osteomyelitis, pneumonia as well as severe systemic disease and long term non-suppurative complications (Zartash *et al*., 2009).

2.1.4 Laboratory diagnosis

Being responsible for a variety of diseases, various methods are available to diagnose the bacteria. Group A *Streptococcus* (GAS*)* infection is generally diagnosed with a Rapid Strep Test (RST) or by culture. This RST or Rapid Antigen Detection (RADT) is done to determine if a patient has Streptococcal pharyngitis, which is a group A Streptococcal infection of the pharynx and possibly other parts of the body (Robers *et al*., 2002). The bacterial swab is then subjected to either enzyme or acid techniques to extract bacterial antigens (Koo, 2009). Strep throat is commonly diagnosed through a throat culture by taking a throat swab. Once the throat has been swabbed, the cells are smeared onto blood agar that allows the bacteria to grow, with added bacitracin antibiotic disk to show beta-haemolytic colonies and sensitivity (zones of inhibition around the disk). Results are typically available within 24 to 48 hours. Serological identification of the organism involves testing for the presence of group A by Phadebact test (Ulug *et al*., 2009). The gold standard method used to characterize *S. pyogenes* is *emm* typing based on sequence analysis of variable distal portion of the gene that encodes the M-protein which is the virulence factor (Le Hello *et al*., 2010).

2.1.5 Epidemiology

It is estimated that between 5-15 % of normal individuals harbour *S. pyogenes* usually in the respiratory tract without signs of disease. It has been reported that invasive GAS infections carry a mortality rate of 5-15 % with Streptococcal Toxic Shock Syndrome (STSS) and necrotizing fasciitis with fatality rates over 35% and approximately 25% respectively (CDC, 2008). About 9,000-11.5000 cases of GAS disease occur each year in the United States, resulting in 1.000-1.800 deaths, with STSS and necrotizing fasciitis each accounting for 6-7% of these invasive cases. There are several million cases of Strep throat and impetigo every year. Streptococci are also part of the normal commensal flora of the mouth, skin, intestine and upper respiratory tract of humans. Studies have reported that *S. pyogenes* also exists in the vagina (Larsen *et al*., 2001). Initially, it was not considered as a cause of bacterial vaginosis (Donald *et al*., 1991; Stricker *et al*., 2003). A report on incidence of vaginosis showed an increased rate in the past two decades (Efstratiou *et al*., 2001; Sweet *et al*., 2001; Peterson *et al*., 2006).

Invasive GAS infections occur when the bacteria get pass the defences of the person who is infected. This may occur when a person has sores or other breaks in the skin that allow the bacteria to get into tissues. Respiratory droplet spread is the major route of transmission associated with upper respiratory tract infection. Children with untreated acute infections spread organisms by airborne salivary droplets and nasal discharge (Schleiss, 2010). Studies regarding *S. pyogenes* vaginosis suggest that the organism is introduced to the genital area by people that carry the bacterium in their respiratory tract either as normal flora or pharyngeal infection (Stricker *et al*., 2003).

2.1.6 Treatment

Streptococcal infections can be treated with many antibiotics. Diseases caused by *S. pyogenes* typically respond to antibiotics such as penicillin which is the most commonly prescribed medication to treat these diseases for about 10 days (Falagas *et al*., 2008). High doses of penicillin and clindamycin are recommended for treatment of STSS and necrotizing fasciitis. Studies have reported increasing MIC or less susceptibility to penicillin (Amabile-Cuevas *et al*., 2001). Failure of treatment with penicillin is due to other local commensal organisms producing β-lactamase, or failure to achieve adequate tissue levels in the pharynx. The genes encoding beta-lactamases are found to be located on either bacterial plasmids or chromosomes (Aarts et al., 2006). However, individuals with severe illnesses may need supportive and intensive care. Necrotizing fasciitis often involves the surgical removal of all infected tissue, since the bacteria spreads rather quickly. Macrolides, chloramphenicol and tetracyclines are also used if the strain isolated has been shown to be sensitive (Watthaiou *et al.,* 2008). Tetracycline resistance have been reported as a result of new genes uptake (Chopra *et a*l., 2001). Frequent *S. pyogenes* resistance to macrolides antibiotics have been reported in some countries (Richter *et al*., 2008). About 5-50 % of macrolide resistance has been shown to increase worldwide (Shuckcloth *et al*., 2004; Erdem *et al*., 2005; Shibl *et al*., 2005). Macrolides resistance is usually developed by efflux (encoded by *mef A*) or target modification due to ribosomal methylation (encoded by *erm B* or *erm A*) (Leclercq, 2002).

Resistance to fluoroquinolones by this organism has also been reported (Yan *et al*., 2008). They have been reported to be potent inhibitors of bacterial DNA replication and point mutations and reduced intracellular accumulation are the two mechanisms of resistance to fluoroquinolones. The growing number of macrolide resistant strains of *S. pyogenes* is an increasing worldwide problem. This resistance has been shown to differ among countries in

different geographic areas (Palevecino *et al*., 2001; Bergman *et al*., 2004; Rubinstein *et al*., 2005). The ability of the organism to produce biofilm has been reported to be one of the other reasons for antibiotic failure. This was tested by isolating *S. pyogenes* strains and analysing their ability to form biofilms and results indicated that most of the strains (90%) isolated produced biofilm. The data showed that these biofilm forming isolates entered epithelial cells with significantly lower efficiency that those which are not able to produce biofilm (Baldassarri *et al*., 2006). This simply means that if particular strains are unable to enter epithelial cells in order to escape the antibiotic they will instead result in the formation of biofilms as an alternative way of surviving in the host.

2.1.7 Prevention and control

The spread of *S. pyogenes* can be reduced by good hand washing, especially after coughing and sneezing and before preparing food. Earlier studies have reported protective immunity against mucosal *S. pyogenes* challenge by intranasal administration of the M-protein C repeat region expressed on vaccinia virus, which led to the reduction in nasopharyngeal colonization (Rabinowits *et al*., 2009). One strategy to develop a broad strain coverage *S. pyogenes* vaccine is the design of vaccines based on the conserved C repeat region of the M protein (Fischetti *et al*., 1989). Although current measures for treatment and prophylaxis for Streptococcal diseases rely on chemotherapy, there is a consensus that in the longer term, vaccination might constitute the most effective strategy to diminish the global burden caused by this pathogen. The development of an efficient anti-*S. pyogenes* vaccine is widely regarded as the major priority and there is consensus that vaccination might constitute the most effective strategy to slow down global disease caused by this organism (Siegert *et al*., 2006).

2.2 *Staphylococcus aureus*

2.2.1 Brief history

This organism was discovered in Arberdeen, Scotland in 1880 by a surgeon, Sir Alexander Ogston in pus collected from surgical abscesses (Ogston, 1984). Alexender Ogston and Louis Pasteur had reproduced human pyemic infections in mice and rabbits by inoculating pus-derived cultures (Parker *et al*., 1931). Staphylococcal infections were found in 5,000000 patients in American hospitals every year (Ogston, 1984).

2.2.2 Morphology

S. aureus is a gram-positive coccus, which appears as grape-like clusters when observed under the microscope. It has large, round, smooth, slightly raised, golden to yellow colonies often with hemolysis when grown on agar plates (Ryan *et al*., 2004; Jumidge *et al*., 2008). The carotenoid pigment Staphyloxanthin is responsible for *S. aureus* characteristic golden colour. The Staphylococci are perfectly spherical cells about 1 micrometer in diameter. These organisms grow in clusters because the cells divide successively in three perpendicular planes with the sister cells remaining attached to one another following each successive division (Todar, 2008). The cell wall contains peptidoglycan and teichoic acid.

2.2.3 Pathogenesis and clinical manifestation

The portal of entry for *S. aureus* may be a hair follicle, but usually is a break in the skin which may be a minute needle stick or a surgical wound. Methicillin-resistant *S. aureus* colonizes the skin particularly in the rectum and perineal area (Lee *et al*., 2011). Report have also showed that this strain also colonize the pharynx, gut and vagina (Nowrouzian *et al*., 2011; Milstone *et al*., 2010; Nakamura *et al*., 2010). During colonization of this organism to host tissues virulence genes are believed to be triggered (Novick, 2003). This organism may

cause diseases through tissue invasion and toxin production (Barlett *et al*., 2010). *S. aureus* show many potential virulence factors such as surface proteins that promote colonization of the host tissues. Invasins such as leukocidin, kinases and hyaluronidase promote the spreading of bacteria in tissues. Surface factors such as the capsule and protein A inhibits phagocytic engulfment (Todar, 2004). A number of complement inhibitors prevent opsonins from binding the bacterium and therefore avoid destruction (Foster *et al*., 2005; Rooijakkers *et al*., 2005). The variety of molecules that *S. aureus* uses in adhering and invading the host epithelial cells are collectively called Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) (Weidenmaier *et al.,* 2004). These molecules as well as the bacterial products play a role in adhesion and attachment to nasal epithelial cells. Two factors (clumping factor B and wall associated teichoic acid) have proven roles in nasal colonization of humans (Wertheim *et al*., 2008). Nasal carriage is the most important because nose-picking could effectively disseminate the bacterium to other body surfaces (Verbrugh *et al*., 2006).

The organism can cause a variety of pus-forming infections and toxinoses in humans. Strains producing enterotoxin cause foodborne diseases. The patient present acute onset of vomiting and diarrhea after two to six hours of ingestion. It causes superficial skin lesions such as styes, boils, and furuncules; more serious infections such as pneumonia, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. It is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices (Todar, 2008). There have been reports on *S. aureus* causing mastitis in cattles and this result in economic loss (Younis *et al*., 2000).

2.2.4 Laboratory diagnosis

An appropriate specimen is obtained accordingly depending on the type of infection and sent to the laboratory for identification by using biochemical or enzyme-based tests. A gram-positive test is performed first to show typical gram-positive bacteria, cocci in clusters. After, the isolates are cultured on mannitol salt agar, which is a selective medium with 7-9% of Sodium Chloride (NaCl). Sodium Chloride allows *S. aureus* to grow, producing yellow-coloured colonies as a result of mannitol fermentation and a drop in pH of the medium. The ability of the organism to clot plasma is also the other identification of this organism. Phenotypic methods in developing countries are important in diagnosis of infections; coagulase tests are used to confirm the presence of *S. aureus*. These kinds of test are done in the laboratory using a tube coagulase test (clumping factor) and tube slide coagulase methods (Koneman *et al*., 1997). A tube test detects free coagulase whereas slide test detect bond coagulase. The bond coagulase causes organims to aggregate by reacting with fibrinogen. Extracellular staphylocoagulase reacts with prothrombin in order to form staphylothrombin, which is capable of converting fibrinogen to fibrin. The performance of this test vary in the ability to produce results even though is effective; improvement on this method is required in order to give excellent results (Martineau *et al*., 1998; Bello *et al*., 2006).

Studies have reported that approximately 97% of *S. aureus* organisms isolated from humans have both form of coagulase (Tolan *et al*., 2011). The tube test is more preferred due to its reliance on human plasma, other plasmas from rabbits and horses are recommended but if available they can be expensive or be of poor quality. The presence of the enzyme coagulase differentiates between the virulent and less virulent strain of *S. aureus*. Techniques such as real time and quantitative PCR are increasingly employed in clinical laboratories (Francois *et al*., 2008). The tools support infection control strategies to limit spread of the bacteria

(Schrenze *et al*., 2008). The clones of this organism have been found to be relatively stable and differentiate if there is no frequent interstrain recombination, this make it possible to discern different clones as well as clonal lineages by molecular typing (Feil *et al*., 2003).

2.2.5 Epidemiology

It has been estimated that about 30-50 % of the human population are *Staphylococcus* carriers (Bhatia *et al*., 2007). It is a frequent part of the skin flora found in the nose and on the skin. Children carry the organism more persistently than adults (Verkaik *et al*., 1999). More than 80 % of health care-associated *S. aureus* infections are endogenous (Eliff *et al*., 2001; *Wertheim et al*., 2004). It has been found that among healthy adults, carrier rates of 11-32 % were detected in the general population and a prevalence of 25 % was detected in hospital personnel (Wencel *et al*., 1995). Remarkably, 20 % of individuals are persistently colonized in the nose and 30 % are transiently colonized. Colonization of *S. aureus* is also more frequent in patients with HIV and diabetes (Wertheim *et al*., 2005).

Mortality rate caused by Staphylococcal infections varies. Untreated bacteremia showed a mortality rate exceeding 80 % and that of Staphylococcal toxic shock syndrome 3.5% (Thomas, 2011). Mortality in patients suffering from *S. aureus* bacteremia associated with thrombocytopenia has been reported (Gafter-Gvili *et al*., 2011). Infections occur as a result of inoculation of the organism into an open wound; mucosal surfaces that harbour the organism include the nose, throat, vaginal wall and gastrointestinal tract (Wertheim *et al*., 2004). Infection can spread through contact with pus from an infected wound, skin to skin contact with an infected person by producing hyaluronidase that destroys the tissues**.** It can also spread through the air and contact with objects such as towels, sheets, clothing or athletic equipment used by an infected person. Individuals can carry the bacteria from one area of their body to another or pass it to other people through dirty hands and fingernails.

2.2.6 Treatment

The treatment of choice for Staphylococcal infections is penicillin which can be resistant in some countries. First line therapy is most commonly a penicillinase-resistant beta-lactam antibiotic. Combination therapy with gentamicin may be used to treat serious infections such as endocarditis (Campion *et al*., 2009). Methicillin used to be the standard agent remaining for treatment but is no longer used very much due to the emergence and spread of methicillin-resistant *S. aureus* (Nigro *et al*., 2005). This community acquired methicillin-resistant strain is virulent and is frequently occurring (Baba *et al*., 2002). Vancomycin is often a treatment of choice in infections with methicillin-resistant *S. aureus* but the occurrence of vancomycin resistant *S. aureus* has been found (Kalsoom *et al*., 2006). Mupirocin is applied for decolonization and prevention of invasive infections in patients taking long time dialysis treatment (Laupland *et al*., 2003). In other non-surgical patients the application of mupirocin had no effect on the rate of health care-associated infections with this organism (Wertheim *et al*., 2004). Drainage of pus collection, surgical removal of dead tissues and removal of foreign bodies can be applied for treatment (Simms *et al*., 1992). This organism has a record of developing resistance quickly to antibiotics and this has been noticed as one of the paramount microbial threats (Smoliski *et al*., 2003). It has been recognized as having the ability to develop changes in their sensitivity to antimicrobials (Oliveira *et al*., 2006).

2.2.7 Prevention and control

Emphasis on basic hand washing techniques and the use of disposable aprons and gloves are effective in preventing transmission. Natural deterrents such as honey and garlic can help fight or prevent *S. aureus* infections (Buhner, 1999). It can also be prevented by covering any open skin such as abrasions and cuts with clean dry bandages as well as avoiding of sharing personal items such as towels and razors. Decolonization can also be prevented by washing

with disinfecting soap such as chlorhexidine gluconate products (Lonneke *et al*., 2010). Recent studies support the recommendation for careful cleaning of environmental surfaces and disinfection items (Muto, 2003). *S. aureus* has been a stumbling block for antimicrobial chemotherapy and the introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of pathogens (Alborzi *et al*., 2000).

2.3 *Plesiomonas shigelloides*

2.3.1 Brief History

P. shigelliodes was first isolated from the faeces of a patient by Ferguson and Henderson in 1947 and called this bacterium Paracolon C27, a motile organism which possessed sonnei phase I antigen. This microorganism has been renamed several times. In 1954, Bader suggested to include the bacterium within the genus *Pseudomona*s with the species name *shigelloides* (Bader, 1954). It was moved from the *Pseudomona*s genus and included within the genus *Aeromonas* with the species s*higelloides* within the family *vibrionaceae*, because of the flagella morphology and the cytochrome oxidase activity (Ewing *et al*., 1961). In an evaluation of the amplified fragment length polymorphism (AFLP) technique for genotyping *Aeromonas* species, Huys and Sings found out that *P. shigelloides* is phylogenetically related to the genus *Proteus* (Huys and Sings, 1999). The genus *Plesiomonas* has been moved to the family *Enterobacteriaceae*, in the light of these research finding (Garrity *et al*., 2001).

2.3.2 Morphology

P. shigelloides is gram-negative, capsulated, non-spore forming, flagellated rod belonging to the family *Enterobacteriaceae* (Garrity *et al*., 2004). The colonies vary from flat, round, 1-2 mm size with smooth edges on blood agar, irregular edge shape and 1mm size when cultured

on deoxycholate agar. They reveal different colonial appearance depending on the selective and differential agar used.

2.3.3 Pathogenesis and clinical manifestations

Infections caused by *P. shigelloides* are divided in two main groups: gastrointestinal infections and extra-intestinal infections. It is regarded as a pathogen causing mainly gastrointestinal infections. It subsequently enters the human intestinal $CaCO_2$ cells *in vitro* through phagocytic-like process. It was found that live bacteria escape from cytoplasmic vacuoles (Theodorpoules *et al*., 2001) and induce apoptic cell death (Tsugawa *et al*., 2005). Several reports have implicated that the organism as a cause of diarrheal disease in humans and animals (Famer *et al*., 1992, Ndip *et al*., 1995). Patients present a watery or secretory diarrhea but some have a more invasive dysenteric illness. Human infections due to this bacterium are mostly water borne, are strongly associated with drinking water. *P. shigelloides* infections occur in summer months and correlate with environmental contamination of freshwater. Gastroenteritis is usually a mild self-limiting disease with fever, abdominal pain, diarrhea and vomiting. Symptoms occur 20-24 hours after eating contaminated water and food (Ueda *et al*., 1999). Extra intestinal infections have included meningitis or meningocephalitis, bacteremia, wound infections, cellulitis and congenital endophthalmitis.

2.3.4 Laboratory diagnosis

It is identified by common bacteriological analysis, serotyping, genotyping and antibiotic sensitivity testing (Winn *et al*., 2006; Chen *et al*., 2007). The isolation and identification of this bacterium from samples depend on the screening of colonies for oxidase and indole positivity as well as appropriate use of selective and differential media (Krovacek *et al*., 2000). The specimens can be cultured on MacConkey agar and after incubation the suspected colonies of *P. Shigelloides* are tested for cytochrome activity (Obi *et al*., 2007). As compared

to other phenotypic methods, serology has been used more successfully for distinguishing different strains. Serotyping schemes based on Somatic (O) and flagella (H) antigens are two main majors used. Different commercial kits such as TTE-AS and the API 20E can also be used for identification of this bacterium (Krovacek *et al.*, 2000). The use of molecular techniques in genotyping bacteria has increased. Currently, different DNA based methods have been introduced as routine analysis in clinical laboratories. DNA based techniques such as repetitive extragenic palindromic-PCR (REP-PCR), enterobacterial repetitive intergenic consensus (ERIC-PCR) and random amplified polymorphic DNA (RAPD) are used (Gonzalez-Rey *et al*., 2003; Olive and Bean, 1999; Shigematsu *et al*., 2000).

2.3.5 Epidemiology

In an evaluation of *P. shigelloides* in animals, 55 cases were reported which represented 1.21% of the 4.552 samples examined. However, 60% of the strains were isolated during investigations of the deaths or disease of animals (Bardon, 1999). Cats ranked first among animals from which *P. shigelliodes* were isolated. Aquarium fishes and trouts presented a high percentage of *P. shigelloides* cases. *P. shigelloides* is extensively distributed in the environment (Saterno *et al*., 2007). The primary reservoir for this bacterium is aquatic environment such as fresh and estuarine water, animals such as goats, swine, cats, monkeys, snakes, toads and vultures. It can also be isolated from the gut of several animals and human faeces (Ramalivhana *et al*., 2008), numerous animals such as fish, birds and insects. Isolation of this organism has been reported mainly in tropical and subtropical countries (Gonzalez *et al.*, 2003). Studies in Africa have shown that *P. shigelloides* is globally distributed (Obi *et al*., 1995; Svenungsson *et al*., 2000). Isolation of Plesiomonads from a lake situated North of polar circle has been reported (Gonzalez-Rey *et al.*, 2003). It has also been isolated from clinical specimens such as cerebrospinal fluid, wounds and the respiratory tract (Evgeny *et*

al., 2011). The organism may be transmitted through unsanitary water, which has been used as a drinking water, recreational water or water used to rinse food that are consumed without boiling them. It is also transmitted by oral-faecal route (Moussa *et al*., 2010).

2.3.6 Treatment

Most of the *P. shigelloides* strains are resistant to a broad-spectrum of penicillins, including ampicillin, piperacillin, ticarcillin, carbenicillin, azlocillin, ticarcillin and others (Stork and Wiedemann, 2001). Results of various studies show that *P. shigelloides* is naturally resistant to a diversity of Beta-lactams. *P. shigelloides* strains can be resistant to other aminoglycosides. Plesiomonads can be sensitive to cephalosporins (except cefoperazone, ceftazidime and cerfepime), carbapenems, trimethoprim + sulfamethoxazole, nalidixic acid, chloramphenicol and quinolones (Stock and Wiedemann, 2001). Trimethoprim resistance is mediated by *dfr* gene that encodes resistant dihydrofolate reductase (Aarts *et al*., 2006; Schwarz *et al*., 2006). Strains resistant to chloramphenicol were also detected (Wong *et al*., 2000).

2.3.7 Prevention and control

The risks of *P. shigelloides* infections may be reduced by avoiding consumption of raw or undercooked shellfish particularly during warmer months (Shock *et al*., 2009). Control in food by chill storage and moderate salting or acidifying conditions will prevent the growth of the organism. Hands should be washed with clean running water, educating visitors regarding animal contact in public setting and avoiding of eating, drinking and placing things in the mouth after animal contact can help in controlling diseases caused by the organism (CDC, 2011).

2.4 *Salmonella typhimurium*

2.4.1 Brief History

Salmonella organisms were first isolated from infected pigs in 1885 by Theobald Smith. The genus *Salmonella* was named after Daniel Elmer Salmon, an American pathologist who discovered it in 1885.

2.4.2 Morphology

Salmonella typhimurium is a gram-negative, non-spore bacillus, motile with peritrichous flagella (Yan *et al*., 2004). The organism occurs as single cells or in short chains and the fully matured cells average size of 2-3 µm.The outer membrane consists of lipopolysaccharide (LPS) layer which surrounds the thin peptidoglycan layer, which makes it a gram negative organism (Quintela *et al*., 1997).

2.4.3 Pathogenesis and clinical manifestations

S. typhimurium is the most common serovar causing human food-poisoning in some parts of the world (Casadevall *et al*., 2001). Definitive Type (DT) 104, is a particularly virulent form of *Salmonella typhimurium.* It has been reported as world health problem (Helms *et al*., 2005) and its spreading has been associated with intensive use of antibiotics in human medicine and agricultural field (Threlfall, 2002). As pathogens, they have developed complex virulence mechanisms to evade host defence mechanisms (Casadevall *et al*., 2001). When the bacterial cells enter the epithelial cells lining the intestines, they cause host cell ruffling which temporarily damages the microvilli on the surface cells (McCormick *et al*., 2007). Its toxicity is due to an outer membrane consisting of lipolysaccharides (LPS) which protect the bacteria from its environment. Pathogenesis begins when *S. typhimurium* enters the body and if contaminated food is ingested. After ingestion, colonization takes place in the ileum and

colon as *S. typhimurium* invaders, grow and multiply within the lymphoid follicles and endothelium (Hendrieksen *et al*., 2004). Virulence factors allow the microbe to invade the cells, replicate intracellulary and if possible it disseminates the toxin. Toxin injected by *Salmonella typhimurium* works to inhibit the innate immune system by virtue of threonine acyltransferase activity and requires binding to eukaryotic target cell (Mittal *et al*., 2010).

It is an important cause of morbidity and mortality in humans and animals, with *S. enterica* serovar Typhimurium becoming an emerging problem (Abouzeed *et al*., 2000; Hendriksen *et al*., 2004; Smith-Palmer *et al*., 2003). It causes gastroenteritis in humans and other mammalian species (McClelland *et al*., 2001). *Salmonella* infections are often fatal in the elderly, young and people with depressed immune systems. When bacterial cells enter epithelial cells lining the intestines, they cause host cell ruffling which damages the microvilli on the surface of the cell temporarily (McCormick *et al*., 2007). *Salmonella* infections usually resolve in 5-7 days and many times require no treatment unless the patient is severely dehydrated or infection spreads from intestines. In mice, this organism cause symptoms resembling typhoid like fever (Everest *et al*., 2007). Although the organism does not cause the clinical disease in pigs, subclinical infections constitute an important food-safety problem throughout the world (Kranker *et al*., 2003). The disease is characterized by diarrhea, abdominal cramps, vomiting and nausea which generally last up to 7 days. The infection can spread from the intestines to the blood stream as well as other body sites and cause death if the infected person does not receive treatment early (CDC, 2011).

2.4.4 Laboratory diagnosis

Diagnosis is based on culture of the organism from clinical specimens such as blood and faeces to specifically isolate and identify *Salmonella* from patient specimens. These culture plates are observed for the presence of bacterial colonies which are further tested using

biochemical tests. Biochemical tests are confirmation tests that help identify the specific species and strains of *Salmonella* species by observing their ability to ferment sugars, decarboxylate amino acids and utilize citrate (Fraser *et al*., 1999). Since culture takes time to identify the organism, serological tests such as agglutination test can give results in 1-2 hours and these tests are reliable. A small amount of serum is obtained from a patient's blood sample and mixed with *Salmonell*a antigens on a slide. A positive test is indicated by the formation of clumps (Rao *et al*., 1999). Rapid immunological identification and confirmation test based on latex agglutination, enzyme immunoassay (EIA) as well as enzyme-linked immunosorbent assay (ELISA) have been developed for *Salmonella*. Molecular methods are also available, such as DNA hybridization and PCR assays for the identification of *Salmonella typhimurium* in food and environmental samples (Correa *et al*., 2006). Pulsed-field gel electrophoresis, phage typing and plasmid profile analysis are other methods used for characterizing this bacteria (Torpdahl *et al*., 2007; Corbett- Feeney *et al*., 1998).

2.4.5 Epidemiology

Salmonellosis is an important medical problem causing food-borne diseases with an estimated 1.4 million illnesses and 500 deaths occurring annually (Jammy *et al*., 2008). *S. typhimurium* remains a common cause of bloodstream and focal infections in sub-Saharan Africa for adults and children with HIV infection, malaria and malnutrition. *Salmonella enterica* serovar Typhimurium definitive phage type 104 (DT104), is the most well known, multi-resistant strain which disseminated globally (Hancok *et al*., 2000). This bacterium has been reported in 41 states of the USA since April 2009; in this report infected individuals ranged from less than 1 year to 67 years of age and fifty one percent of patients were females. Among these ill people, 30 % were hospitalized but no death cases were reported (CDC, 2010). *S. typhimurium* was isolated in the 1980s in Japan and spreading occurred widely in

food-producing animals and it was among the five serovar found frequently in *Salmonella* foodborne illness in Japan between 2006-2010 (Sameshma *et al*., 2010). Studies have indicated that more than 80% of all salmonenollosis cases occur individually rather than as outbreaks. *S. typhimurium* organisms are common inhabitants of the gastrointestinal tracts of all animals, including cattles (Kunze *et al*., 2008). This can lead to mortality and morbidity as well as a disease burden worldwide (Cobum et *al*., 2007). Animal products are the main vehicles of salmonellosis due to the ability of salmonellae to survive in meat and animal products that are not thoroughly cooked or not properly handled (Hariharan *et al*., 2000). It is readily transmitted through the faeces of people or animals. *S. typhimurium* can survive in the environment and may spread from farm through exchange of livestock, wildlife or runoff from fields (Akoachere *et al*., 2009). There have been reports about commercial chicken layers being infected by *S. typhimurium* DT104, followed by problems in oral and aerosol routes. Most human cases are foodborne and spreading can occur mainly by consumption or handling of meat, consumption of unpasteurized milk and untreated water, however non-foodborne infections can occur during contact with animals, contaminated water or environment (Besser *et al*., 2000; Schiellerup *et al*., 2001).

2.4.6 Treatment

The patient can be treated with ampicillin, gentamicin, amoxicillin, sulfamethoxazole or ciprofloxacin. The length of treatment varies depending on the extent of a patient's illness and can range from 14 days for enteric fever and 6 weeks with bacteremia (Mayo, 2007). Some *Salmonella* bacteria have become more resistant like *S. typhimurium* DT 104 which is multidrug resistant to five antimicrobials: ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines. Although DT 104 is presently the dominant resistant clone of *S. typhimurium*, many other phages such as phage type (D29, 04, 193 and D204 C) of this

serovars have been associated with multidrug resistance (Sanchez *et al*., 2002). Moreover, new strains with resistance to additional antibiotics such as trimethoprim and fluoroquinolones are beginning to emerge. The emergence of *Salmonella* strains to first line therapy and increased minimum inhibitory concentrations of second generation of quinolones is a serious problem which make treatment ineffective (Birosova *et al*., 2009; Ricci *et al*., 2009). This multidrug resistance have posed a major problem in the treatment of complications resulting from these *Salmonella* infections.

2.4.7 Prevention and control

Prevention depends on good food processing practices, proper refrigeration and adequate good hand-washing practises. Food should be thoroughly cooked; hands, surfaces, cutting boards and dishes should be washed with hot soapy water before handling food. Control of drug resistant *Salmonella* strains, is efficiently achieved by reducing the use of antimicrobials. Prevention of the infection can be done if people working in the microbiology laboratory can wash their hands properly before any contact with young children or baby bottles. Food, drinks or personal items such as keys or cell-phones should not be touched while working with microorganisms in the laboratory. Stationary such as notebooks, pens and other items should not be taken out of the microbiology laboratory and protective uniform such as lab-coats should be left in the laboratory after working (CDC, 2011). Control can be achieved by training the students and employees using the laboratory for biosafety practices. Educating people about the symptoms of *Salmonella* infection such as diarrhea, abdominal cramps and other related symptoms to seek medical attention quickly can help eradicate problems associated with salmonellosis.

2.5 Medicinal plants as complementary and alternative therapeutic options

There is a search for phytochemicals from medicinal plants which can be developed for the treatment of infectious diseases especially when there is such an emergence of drug resistant microorganisms (Ncube *et al*., 2008; Anam *et al*., 2010). Medicinal plants are successful natural sources for the treatment of various infectious diseases of human. Scientists are focusing on discovering natural compounds from medicinal plants, with the aim of introducing new drugs which will be more effective than those available in the market (Parekh *et al*., 2006). Recent research has focused on natural plant product as alternatives for existing drugs for curing diseases in developing countries (Aiyegoro *et al*., 2007). They have formed the basis of sophisticated traditional medicine and make an excellent lead for new drug development (Newman *et al*., 2000). They are a good choice as they have less side effects, less expensive and effective against broad spectrum drug resistant microorganisms (Vermani *et al*., 2002; Pareck *et al*., 2005; Motamedi *et al*., 2010).

Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and infectious diseases (Shrisha *et al*., 2011). These chemical constituents have great potential for medicinal use and both traditional healers and pharmaceutical drug companies exploit them (Magwa *et al*., 2008). Medicinal plants have been used as an ancient tradition especially where modern drugs are not affordable or inaccessible (Njume *et al*., 2009). Even today, plants are the most exclusive source of drugs for the majority of the world and people in developing countries especially use traditional medicine for their primary health care (Palombo *et al*., 2001). The world health organization has reported that about 80 % of the world's population is depending on traditional medicine (WHO, 1993). Herbal remedies continue to play a role in the cure of diseases (Tabuti *et al*., 2003).

Commercially proven drugs used in modern medicine were initially used in crude form in traditional folk healing practices. The amount of active components in crude extracts from medicinal plants small or diluted, when fractionated become concentrated and therefore show activity (Adeniyi *et al*., 2004). Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic benefits. Many plants in Africa such as *Garcinia kola* have been investigated for their chemical components and some isolated compounds have been shown to possess interesting biological activity (Ezeifeka *et al*., 2004; Sibanda *et al*., 2008; Njume *et al*., 2011).

2.6 *Garcinia kola*

Garcinia kola is a species of flowering plant belonging to the family *Guttiferae.* It is found mostly in Central and Western Africa; in countries such as Cameroon, Benin, Democratic Republic of Congo, Ivory Coast, Gabon, Ghana, Liberia, Nigeria, Senegal and Sierra Leone (Eyog-Matig *et al*., 2007). This plant has a popular acronym "wonder'' plant among the South-Western Nigerian people because every part of it has been found to be of medical importance (Iwu *et al*., 2002). It consists of fruits, leaves, roots, barks, stems and twigs and an average of four seeds is contained in a fruit. All parts of the plant are used in traditional medicine for various therapeutic purposes. The fruits are redish or yellowish in colour and each fruit contain two to four seeds and pulp that have a sour taste (Nzegbule and Mbakwe, 2001; Adesanya *et al*., 2007). The seeds are smooth, oval shaped with yellow pulp and covered with a brown seed coat. They have been reported to possess antibacterial activity, antidiabetic, antiviral and antihepatotoxicity potentials (Farombi *et al*., 2002; Akoachere *et al*., 2002). They are used to prevent or relieve colic pains, cure head and chest colds (Iwu, 1993). Traditional cough mixture is made by mixing dried and ground seeds with honey

(Odebunmi *et al*., 2009). This plant is also known in commerce as "bitter *kola"* and when the seed is chewed, a bitter astringent and resinous taste comes out of it.

The mechanical cleansing effect and antimicrobial substances of the seed are seen as major beneficial effects of chewing the nut (Han *et al*., 2005). The seeds are chewed raw with both the pulp and can be chopped and steeped in water. They are believed to clean the digestive system, without side effects such as abdominal problems (Odebunmi *et al*., 2009). Traditionally the seeds are chewed to stimulate the flow of saliva (Leakey, 2001); and are also consumed as snack in West and Central Africa. The seed is a highly valued ingredient in African ethnomedicine because of its varied and numerous uses which are social and medicinal, making the plant essential in folk medicine (Igwe *et al*., 2007).

2.7 Solvents for plant extraction

The type of solvents used has an effect on the nature of compounds extracted and the resulting bioactivity of the extract (Dogruoz *et al*., 2008; Eloff *et al*., 2008). This clearly implies that polarity of solvents (non-polar, polar and less polar) play a vital role in the extraction of bioactive compounds, which influence the antimicrobial activity (Bizimeya *et al*., 2002; Parekh *et al.,* 2006; Ndip *et al*., 2007). It is important for the efficiency of extraction to be optimized in order to ensure that many potential active constituents are extracted as possible. The rate of extraction, quantity extracted, handling of extracts, toxicity of solvent are some factors that need to be evaluated in order to ensure the value of a solvent. A variety of solvents is applied in the extraction of antimicrobial compounds (Masoko *et al*., 2007).

A study reported that methanol extract of the rhizome and leaves had better average activity against all tested organisms, while hexane extract had no activity (Masoko *et al*., 2008). Petroleum ether and methanolic extract of the leaves of *Cassia occidentalis* was effective

against *E.coli* at a concentration of 400 mg/mL with 5 - 11 mm inhibition zone, respectively (Vedpriya *et al*., 2010). Water is a universal solvent used and is usually used in preparation of folkloric medicine (Das *et al*., 2010). Natural products such as pigments and bioactive components are soluble in water which point out high yield of extract; while some solvents are only selective for certain bioactive compounds. This has been associated to a better solubility of active agents; xanthones, benzophenones and flavonoids especially biflavonoid type GB1 (Nwaokorie *et al.,* 2010). In most studies water extract demonstrated weak activity (Doughari *et al*., 2007; Igbinosa *et al*., 2009; Sanjay *et al*., 2010). A study reported that acetone is a good solvent for extraction; and that it is usually preferred because of its ability to extract polar and non-polar components (Samie *et al*., 2005; Masoko and Eloff, 2006; Adefuye *et al*., 2011**)**..

It was also reported that n-hexane extract of *G. kola* seeds showed best activity against five different *Vibrio* species (Okoh *et al.*, 2011). Ethyl acetate extract showed a broad spectrum activity against gram - positive and gram - negative organisms (Adefuye *et al*., 2011). The study of Rajasekaran *et al*. (2008) exhibited ethanol and chloroform leaf extracts of *Aegel marmelos,* showing maximum inhibition against both gram-positive and gram-negative bacteria. This study was carried out with ethyl acetate, ethanol, methanol, acetone and distilled water with a view to find out the potentiality of *G.kola* seeds. It has been found that organic solvent extracts revealed the promising antibacterial activity (Parker *et al*., 2008; Njume *et al.,* 2011).

2.8 Phytochemical constituents

Plants derived preparations and isolated phytochemicals may be potentially useful in the treatment of infectious diseases (Ncube *et al*., 2008). A number of compounds with *in vitro* antibacterial activity have been isolated from the extracts of this seeds. Phytochemical

analysis of the extracts of *G. kola* seeds have shown that they contain reasonable amounts of phenolic compounds including biflavonoids (GB-1, GB-2), xanthones and benzophenones (Okoko *et al*., 2009; Okunji *et al*., 2007). The wide spectrum of these organic compounds confer some anti-microbial activities against gram - negative and gram - positive bacteria. The therapeutic activity has been attributed to flavonoids, which are the dominant compounds and some new biflavonoids still continue to be identified along with such mixtures of phenolic compounds, related triterpenes and tannins (Han *et al*., 2005; Adeboye *et al*., 2006). The biological activities of flavonoids include action against allergies, inflammation, free radicals and hepatoxins (Tarishma *et al*., 2002).

Some plant secondary metabolites such as alkaloids, phenols, tannins, glycosides, terpenoids, saponins, and steroids have been implicated in their ability to inhibit the formation of pro-inflammatory signalling molecules (Polya *et al*., 2000). The phenolic compounds, when present at high concentration, act as a protoplasmic poison penetrating and disrupting bacterial cell wall in addition to precipitating cell proteins (Rubino *et al.*, 1991). Saturated fatty acids (myristic, pentadecanoic, palmitic, margaric, stearic, arachdic, behemic); monosaturated fatty acids [(myristoleic, palmitoleic (trans), palmitoleic (cis), oleic (cis), vaccenic (cis)]; polysaturated fatty acids (linoleic, eiscosadienoic, polyenes) and essential amino acids (arginine, aspartic acid, glutamic acid, serine, histidine, proline, glycine and alanine were reported to be found in *G. kola* seed and hulls (Afolabi *et al*., 2006). The seeds have also been found to contain 10% carbohydrate, 10 % crude fat, 5 % protein and sodium (Agyili *et al*., 2006). Two chromanols: garcionic and garcinal together with tocotrienol have been reported (Terishma *et al*, 2002). The seeds have also been reported to contain phlobaphenes, anthrocyans red pigment, betaine, protein and starch (Alolaiye *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Bacterial strains

Standard strains of the following organisms were used in this study. They included: *Streptococcus pyogenes* (ATCC 49399), *Staphylococcus aureus* (NCTC 6571), *Plesiomonas shigelloides* (ATCC 51903) and *Salmonella typhimurium* (ATCC 13311). These organisms are human pathogens and are leading causes of hospital and acquired infections. They were selected based on their prevalence and increasing trend of resistance to antibiotics (Tolan, 2008).

3.2 Resuscitation of bacterial strains

Bacterial isolates of test organisms were obtained from stock cultures maintained at the Department of Biochemistry and Microbiology, University of Fort Hare. Resuscitation of the cultures was done by inoculating *S. pyogenes* and *S. aureus* on Mueller Hinton and *P. Shigelloides* and *S. typhimurium* on *Salmonella - Shigella* agar and plates incubated at 37°C for 24 hours. They were frequently subcultured on Mueller Hinton or Nutrient agar slants and stored in the refrigerator.

3.3 Preparation of plant extract

Garcinia kola seeds were purchased from a local market in Cameroon. They were blended into powder and kept in an air-tight container for further use. The extracts of the seeds were prepared in accordance with the method of Basri and Fan (2005). One hundred grams of the powdered seeds were steeped in 500 mL of ethyl acetate, ethanol, methanol, acetone and distilled water for 24 hours with 140 rev/min shaking. The resulting extracts were filtered

using Whatman No 1 Filter paper. The extracts were further concentrated to dryness under reduced pressure at 37 °C using a rotary evaporator (Strike 202 Steroglass, Italy) to remove the solvents.

3.4 Antimicrobial Susceptibility testing

The sensitivity testing of *G. kola* seeds extract was determined using the agar well diffusion method as previously described (Irobi *et al*., 2000) with minor modifications. The bacterial isolates of *S. aureus, S. pyogenes, P. shigelloides and S. typhimurium* were grown in Mueller Hinton agar. The medium was poured into plates and allowed to solidify. The plates were swabbed with cotton wool impregnated with the organisms prepared at 0.5 McFarland standard. Wells were bored into the agar medium using sterile 6mm cork borer and labelled. Five holes were bored in one plate. The first three wells were filled with solution of the extract at concentrations of 200, 100 and 50mg/mL. The other two wells were filled with a positive control (ciprofloxacin) and negative control [dimethylsulfoxide (10 % DMSO)]. The plates were then allowed to stand for 20 minutes to allow proper diffusion of the solution into the medium before incubation. They were then incubated at 37 °C for 24 hours. Antimicrobial activity was evaluated by measuring the zones of inhibition against the test organisms. The experiment was replicated two times.

3.5 Determination of Minimum Inhibitory Concentration (MIC_{90})

The microdilution method was employed to determine the MIC of the plant extract that gave best activity using 96 well microtitre plates as previously described (Njume *et a*l., 2011). A twofold serial dilution was carried out. Two to three colonies of *S. aureus, S. pyogenes, P. shigelloides* and *S. typhimurium* were grown in BHI broth. Approximately 20µL of each bacterial suspension was added to 180 µL of wells containing extract (methanol) which

showed best antimicrobial activity. Control wells were prepared by adding 100 µL of ciprofloxacin at a concentration of 1.25 mg/mL. The plates were incubated overnight at 37 °C and read with an automatic enzyme-linked Immunosorbent assay microplate reader (Model 680, Bio-Rad, Japan). The MIC_{90} was taken as lowest concentration of the test extract resulting in inhibition of 90% of bacterial growth.

3.6 Determination of Maximum Bactericidal Concentration (MBC)

The MBC was determined using the method of Vila et *al*. (2010) with little modifications. Approximately, 2µL of the sample from MIC assay was spread onto freshly prepared agar plates and incubated at 37 °C for 24 hours in order to determine the MBC. *S. pyogenes*, *S. aureus*, *P. shigelloides* and *S. Typhimurium* were subcultured on Mueller Hinton agar. This also included the positive control; the experiment was done separately for all organisms. The plates were monitored for the presence of bacterial growth after 24 hours of incubation. The MBC were taken as the lowest concentration that did not allow bacterial growth on the surface of the agar plates.

3.7 Phytochemical and Antimicrobial analysis

3.7.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography was used to analyse the chemical constituents of the *G. kola* seeds extracts using aluminium-backed TLC plates (Merck, silica gel 60 F254) according to the method of Kotse and Eloff (2002). The plates were spotted with 50 and 100mg/mL of methanol*ic* extract of *G. kola* seeds. The TLC plates were developed with three eluent systems i.e.: ethyl acetate/methanol/water (40:5.4:5): [EMW], chloroform/ethyl acetate/formic acid (10:8:2): [CEF], benzene/ethanol/ammonium hydroxide (18:2:0.2): [BEA]. Development of the chromatograms was done in a closed TLC chamber containing

solvent mixture and shaken. The chamber was kept covered in order for evaporation not to change the composition of the developing solvent mixture. A line was drawn with a pencil were migration of the extract had ended on the TLC plate. The plates were sprayed with vanillin solution (0.2 g vanillin + 28 mL of methanol +1mL of Sulphuric acid) and allowed to dry for 5 minutes; the chromatograms were heated at 100°C and allowed for optimal colour development. The spots or bands were visualized in broad daylight and also under ultraviolet light at 302 and 365 nm. The following formula was used to measure the retention factor (R_f) which is distance the compound travels to the distance the solvent travels.

$$R_f = \frac{\text{distance moved by the compound}}{\text{distance moved by solvent}}$$

3.7.2 Antimicrobial activity assay by bioautography

The method developed by Masoko and Eloff (2005) was used to determine active compounds. The plates developed in the three different mobile systems used: CEF, BEA and EMW were dried for up to a week at room temperature under a stream of air to remove the remaining solvent. The plates were sprayed with concentrated bacterial cultures and incubated in a humidified container at 37°C overnight. The following day the plates were sprayed with 0.2 mg/mL solution of *p*-iodonitrotetrazolium violet (Sigma®) (INT). Purple/pink colour indicated an area were the organism grew and clear zones indicated the absence of the organism due to the presence of compound(s) that inhibited the growth of tested microorganisms. R_f of the zones on the plates were compared with that on the reference plates to find the R_f of the active compound.

3.8 Column Chromatography

Column Chromatography was used as a purification technique. The mixture of compounds to be purified was dissolved in small amount of the appropriate solvents as earlier described by

Kotse and Eloff (2002). A 40cm long x 2.5cm diameter glass column was packed to a height of 31cm with a slurry of silica gel 60; particle size 0.063 - 0.2mm/ 70 – 230 mesh (Merck, Germany). The column was quilibrated with 100% chloroform for 30 minutes. Five grams of the methanolic extract of the seeds were mixed with 10g of silica gel powder and ground to very fine particles. A dry loading method was applied. The mixture was dissolved in chloroform and put under extreme air temperature until the solvent evaporated leaving only dry powder. The mixture was then loaded onto a silica gel column equilibrated first with chloroform. The combination which gave good activity, CEF (10:8:2) was then used to elute the column; fractions (200 mL) were collected in bottles and coded C for fractions collected in chloroform and CEF for Chloroform/Ethyl Acetate/Formic acid. They were concentrated on a rotary evaporator (Strike 202 Steroglass, Italy) to remove excess solvents at a reduced pressure. Fractions were dried and weighed to determine the mass extract and stored in air tight containers for further bioassay.

3.9 Determination of Minimum Inhibitory Concentration (MIC_{90}) of fractions.

The MIC_{90} of the fractions was determined by micro-broth dilution method performed in 96-well plate as previously described (Njume *et a*l., 2011) and active fractions were further analyzed on TLC to deternine the purity. Test fractions were prepared at a concentration of 2.5 mg/mL. Two to three colonies of *S. aureus, S. pyogenes, P. shigelloides* and *S. typhimurium* were grown in BHI broth. Approximately 20µL of each bacterial suspension was added to 100 µL of fraction that contain culture medium. Two-fold dilutions of each fraction was made in the test wells in BHI broth. Control wells were prepared with culture medium only and bacterial suspension plus broth. Ciprofloxacin was used as a positive control at 1.25 mg/mL. An automatic ELISA microplate reader (SynergyMx,Biotek^{R} USA)

adjusted to 620 nm was used to measure the absorbance of the plates before and after incubation at 37^{o}C. The absorbencies were compared to detect an increase or deacrease in bacterial growth. The lowest concentration of the fraction resulting in inhibition of 90% bacterial growth was recorded as the $MIC_{90.}$

3.10 Gas-Chromatography/Mass-Spectrometry (GC-MS)

The chemical constituents of fractions were analysed by GC/MS using a Hewlett-Packard HP 5973 mass spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column (30 m ×0.25 mm i.d, 0.25 µm film thickness) and MS detector. Helium was used as a carrier gas. The oven temperature was set at 70◦C for 2 minutes to 325◦C at a temperature of 4◦C per minute. The final temperature was 240 ◦C. The samples were injected into GC-MS inlet port using a syringe. The ion source was at a temperature of 250◦C and electron ionization at 70 Ev. The compounds were identified based on the match with their mass spectra and retention indices with those of the Wiley 275 library (Wiley, New York, NY) in the computer library and literature.

3.11 Statistical analysis

Analysis was performed using SPSS version 18.0 (Illinois, USA, 2009). The one way ANOVA test was used to determine if there was any statistically significant difference in the diameter of zones of inhibition of the plant extracts and ciprofloxacin; the MIC of the most active extract (methanol), fractions and positive control (ciprofloxacin). P values < 0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 Susceptibility testing

The zones of inhibition (clear zones on agar) for all four organisms were measured in mm. All the extracts with the exception of water extract were active against the test organisms with inhibition zone diameters ranging from 0 - 24mm (Table 1). The methanol extract demonstrated a bigger zone diameter of 24mm for *S. pyogenes* ($P < 0.05$). Positive control zones ranged between 23 - 31mm. Extract considered very active in this assay was further evaluated to determine the MIC.

Table 1: Antibacterial activity of the seed extracts of *G. kola* and ciprofloxacin.

		Zones of inhibition (mm) at different concentration (mg/mL)			
Organism	Extract	50mg/mL	100mg/mL	200mg/mL	Ciprofloxacin
S.pyogenes	Ethyl Acetate	21	23	20	25
	Acetone	19	22	23	24
	Ethanol	19	19	22	24
	Methanol	20	24	21	23
	Water	0	0	0	25
S.aureus	Ethyl Acetate	17	17	14	26
	Acetone	19	19	21	24
	Ethanol	18	23	20	27
	Methanol	21	22	19	23
	Water	0	0	0	24
P.shigelloides	Ethyl Acetate	0	0	0	30
	Acetone	0	15	12	30
	Ethanol	10	19	11	31
	Methanol	18	21	19	30
	Water	0	0	0	31
S.typhimurium	Ethyl Acetate	0	0	0	30
	Acetone	16	14	0	30
	Ethanol	0	18	14	29
	Methanol	17	18	15	30
	Water	0	0	0	31

4.2 MIC and MBC determination

The MIC and MBC were evaluated on the plant extract (methanol) that exhibited best activity against the test organisms. The MIC of the extract and ciprofloxacin ranged from 0.04 - 1.25 mg/mL and 0.0012 - 0.0195 mg/mL, respectively while the MBC of the extract and ciprofloxacin ranged from 0.081 - 2.5 mg/mL and 0.0781 - 0.3125 mg/mL, respectively. *S. pyogenes* and *S. aureus* had the lowest MIC of 0.04 mg/mL (Table 2).

Table 2: MIC_{90} and MBC of methanol extracts of *G. kola* and ciprofloxacin on test organisms.

Organism	MIC value of Extract (mg/mL)	MIC value of ciprofloxacin (mg/mL)	MBC value of extract (mg/mL)	MBC value of ciprofloxacin (mg/mL)
S.pyogenes	0.04	0.0012	0.081	0.0781
S.aureus	0.04	0.0024	0.25	0.1563
P.shigelloides	1.25	0.0049	2.5	0.3125
S.typhimurium	0.63	0.0195	1.25	0.1563

4.3 Thin Layer Chromatography analysis of methanol extract of *G. kola*.

The three solvents combination used were CEF (10:8:2), EMW (40:5.4:5) and BEA (18:2:0.2) at 50 and 100 mg/mL of methanol extract. CEF separated more compounds, followed by EMW and BEA. Bands which were not seen on TLC plates in daylight were visible when viewed under ultraviolet light at 302 and 365nm (Figure 1 a - b). Most bands were visible in daylight with CEF plates while for BEA and EMW the bands were visible when viewed under 365 nm UV. In BEA, compound 3 and 6 showed the highest R_f value of 0.12 and 0.32; compound 1 showed lowest R_f value of 0.08 and 0.01 at 50 mg/mL and 100 mg/mL, respectively. In CEF, compound 10 and 12 showed the highest R_f value of 0.87 and 0.88 whereas compound 1 showed lowest R_f value of 0.09 and 0.08 at 50 mg/mL and 100 mg/mL, respectively. In EMW, compound 9 and 10 showed the highest R_f value of 0.75 and 0.88 while compound 1 showed the lowest R_f value of 0.03 and 0.09 at 50 mg/mL and 100 mg/mL, respectively. A good separation was observed at a concentration of 100 mg/mL (figure 1 a - c).

Table 3: R_f values for the different systems at different concentrations.

Compounds	Solvent systems					
	BEA		CEF		EMW	
	50	100mg/mL	50	100mg/mL	50	100mg/mL
1	0.08	0.01	0.09	0.08	0.03	0.09
2	0.1	0.13	0.14	0.11	0.08	0.16
3	0.12	0.16	0.39	0.19	0.11	0.19
4	-	0.22	0.5	0.39	0.16	0.31
5	-	0.28	0.52	0.53	0.31	0.49
6	-	0.32	0.53	0.55	0.49	0.56
7	-	-	0.63	0.63	0.56	0.64
8	-	-	0.72	0.64	0.72	0.67
9	-	-	0.83	0.72	0.75	0.76
10	-	-	0.87	0.83	-	0.88
11	-	-	-	0.85	-	-
12	-	-	-	0.88	-	-

-, No R_f values determined

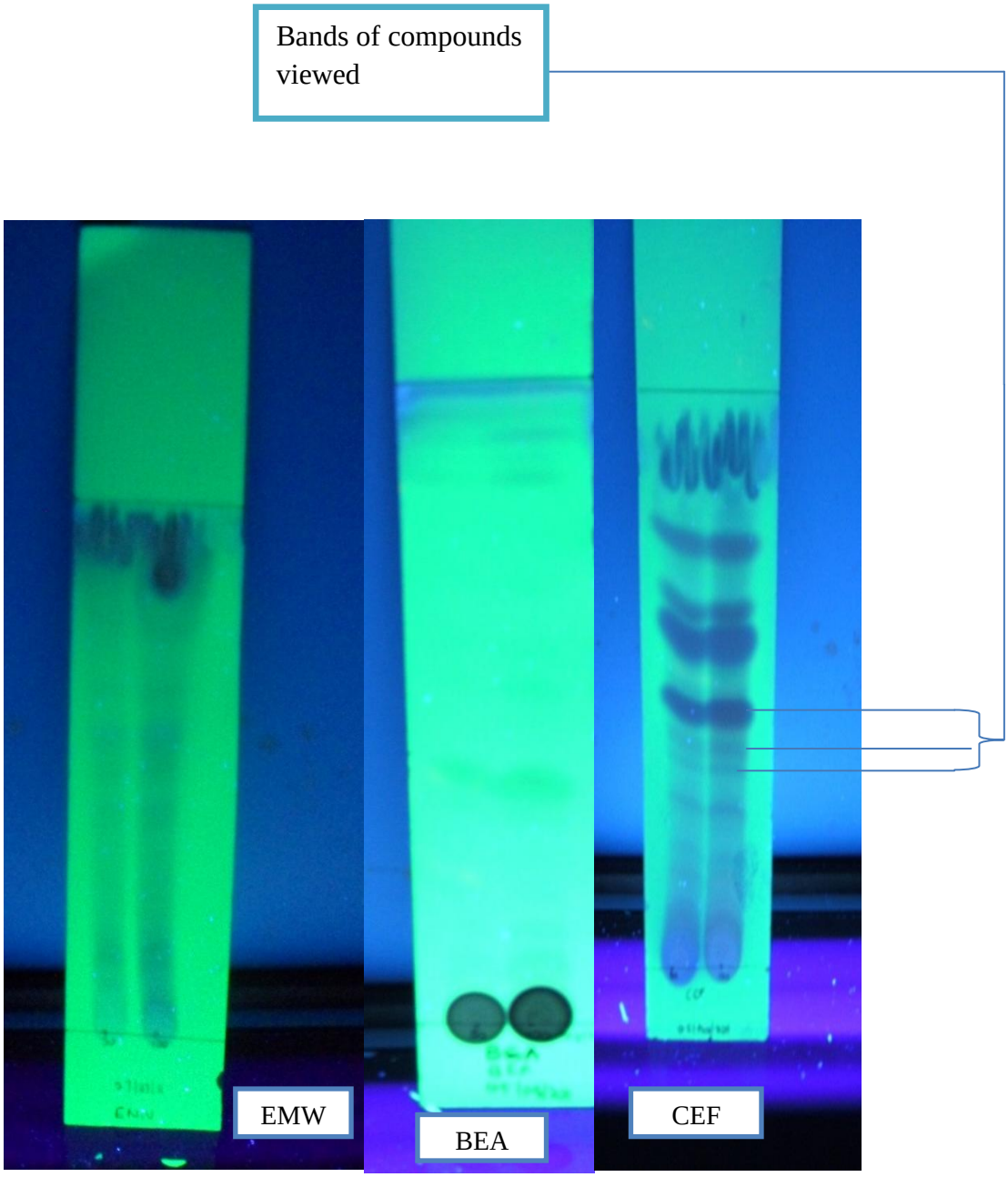

Figure 1a: TLC plates showing separation of compounds for (EMW, BEA, CEF), viewed under 302 nm (UV).

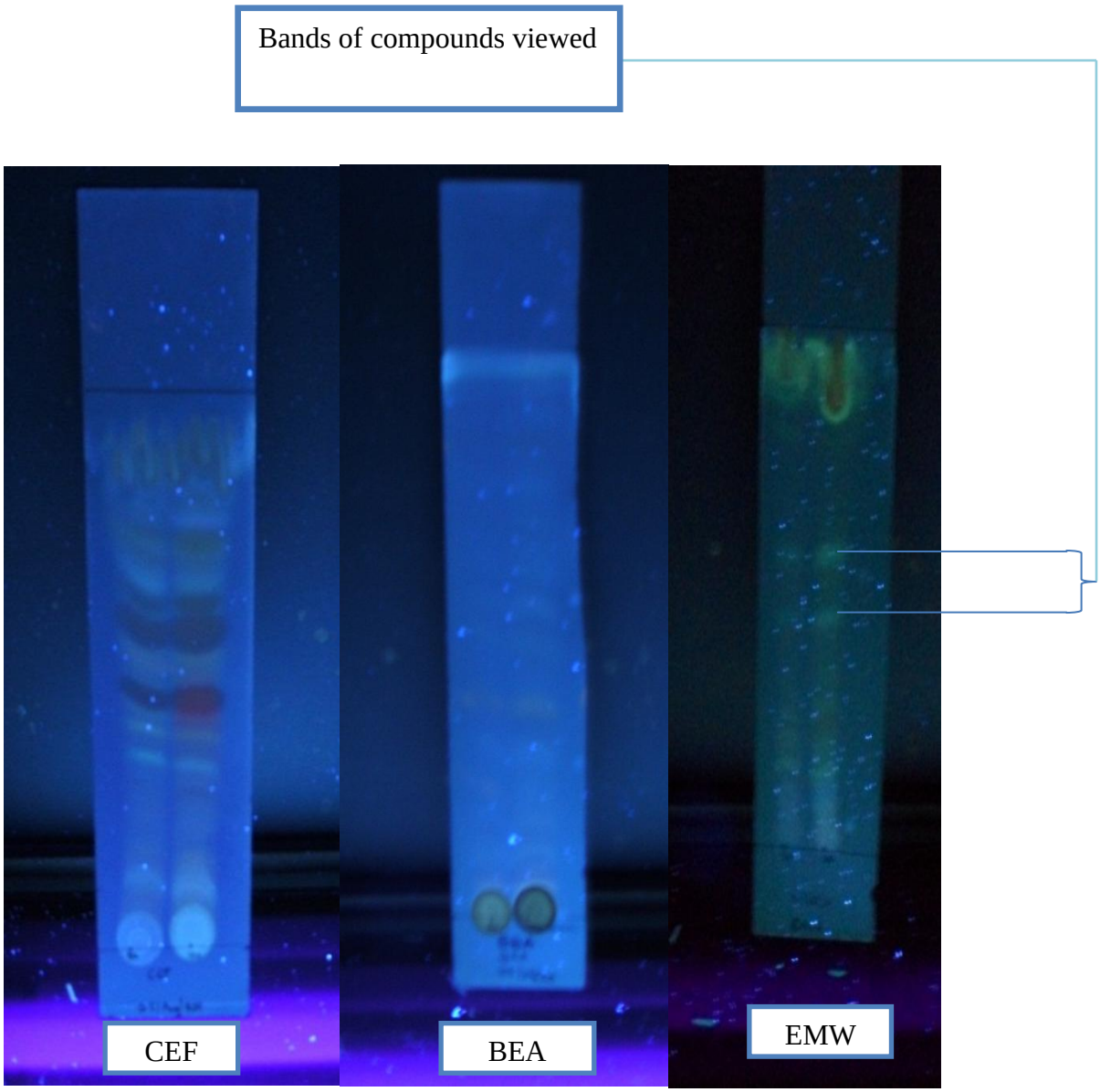

Figure 1 b: TLC plates showing separation of compounds for (CEF, BEA, EMW), viewed under 365 nm (UV).

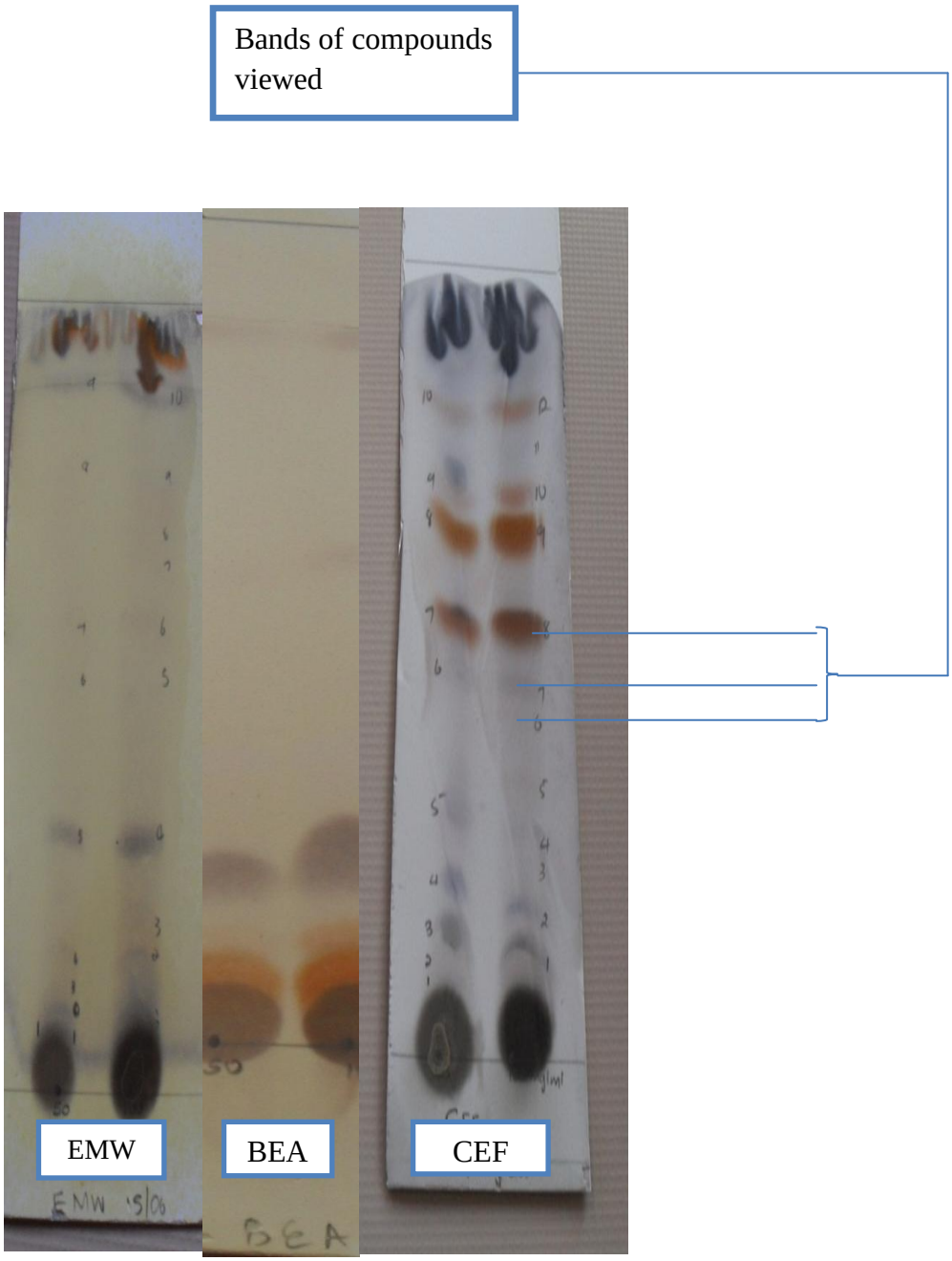

Figure 1c : TLC plates showing separation of compounds for (EMW, BEA, CEF), viewed after staining with vanillin.

4.4 Antimicrobial activity assay by bioautography

The areas of inhibition coloured white/ light yellow were compared with the R_f on the related spot on the reference plate. In CEF, active compounds against *S. pyogenes* were found at varying R_f values (0.53, 0.64) , *S. aureus* (0.82) , *P. shigelloides* (0.63, 0.72, 0.85) and *S. typhimurium* (0.53, 0.63, 0.55, 0.64) at 50 and 100 mg/mL, respectively. Compounds containing inhibitory potential in EMW for *S. pyogenes* were located in R_f (0.30, 0.31), *S. aureus* (0.56, 0.63), *P. shigelloides* (0.75, 0.76, 0.88) and *S. typhimurium* (0.52). In BEA, inhibitory compounds were found at the origin for both *S. pyogenes* and *S. typhimurium* only. Most compounds having inhibitory effect were found in CEF chromatograms, followed by EMW and lastly BEA (Table 4). A very high inhibition was observed in chromatograms developed in CEF against *S. typhimurium* (0.63) and BEA against *S. pyogenes* at the origin (figure 2 a - b).

Table 4 : Inhibition of bacterial species by methanol extract using bioautography.

Organisms	Methanolic extract				Solvent system
	a		b		
	(50 mg/mL	100 mg/mL)	(50 mg/mL	100 mg/mL)	
S.pyogenes	0.53	0.53	+++	+++	CEF
	0.64	0.64	+++	+++	CEF
	0.30	0.31	+	+	EMW
	origin	origin	++++	++++	BEA
S.aureus	-	0.82	-	+	CEF
	0.56	0.63	+++	+++	EMW
	-	-	-	-	BEA
P.Shigelloides	0.63	0.63	++	++	CEF
	0.72	0.72	++	+++	CEF
	-	0.85	-	++	EMW
	0.75	0.76	++	++	EMW
	-	0.88	-	++	EMW
	-	-	-	-	BEA
S.typhimurium	0.53	0.55	++	++	CEF
	0.63	0.64	++++	+++	CEF
	0.52	0.52	++	+++	EMW
	origin	origin	++	++	BEA

a, Component R_f ; b, Degree of inhibition; R_f, Ratio of the distance travelled by compound to the distance travelled by solvent up plate; - , Component not active; +, slight inhibition ; ++, moderate inhibition; +++, high inhibition; ++++, very high inhibition; origin, spot on the TLC plate where the extract was initially applied.

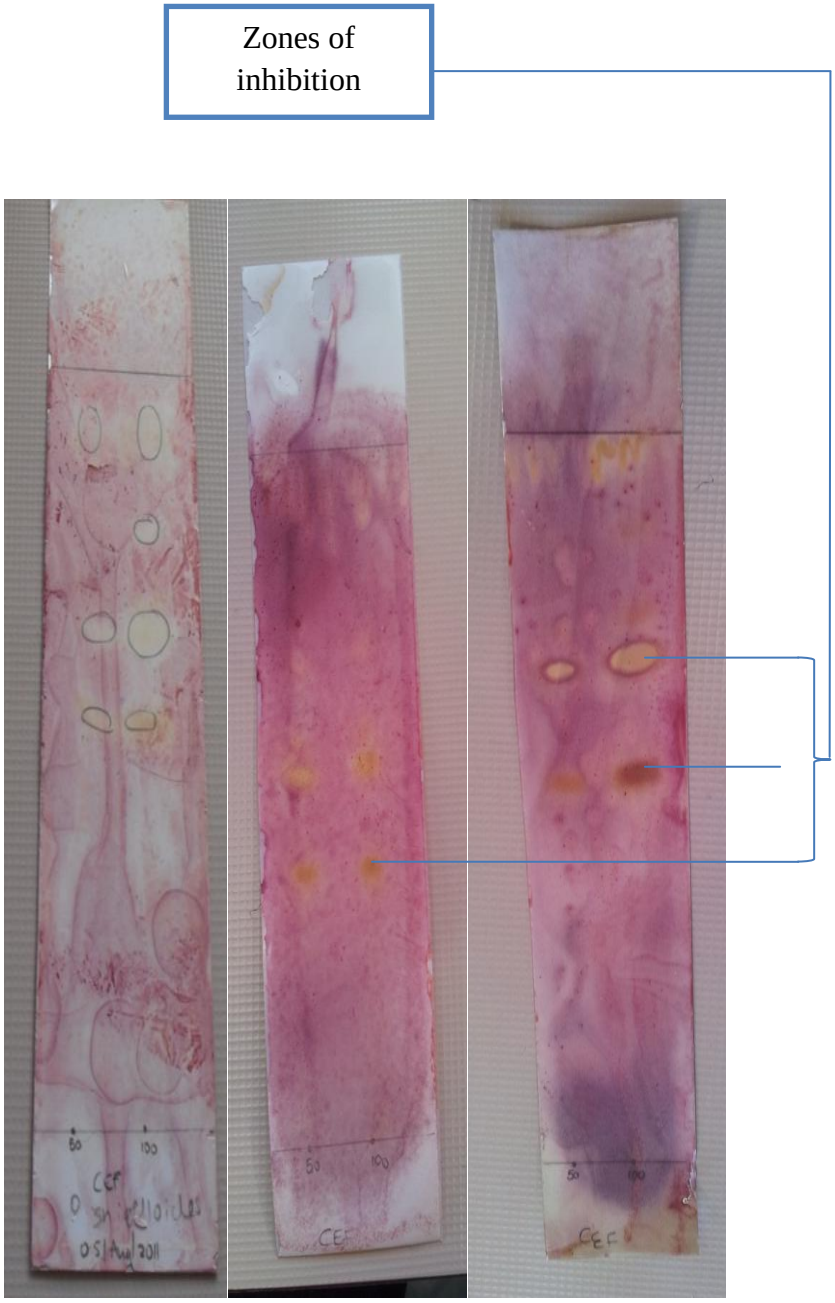

Figure 2a: Bioautography of chromatograms developed in CEF solvent system showing clear zones of inhibition for *P. shigelloides*, *S. pyogenes* and *S. typhimurium*.

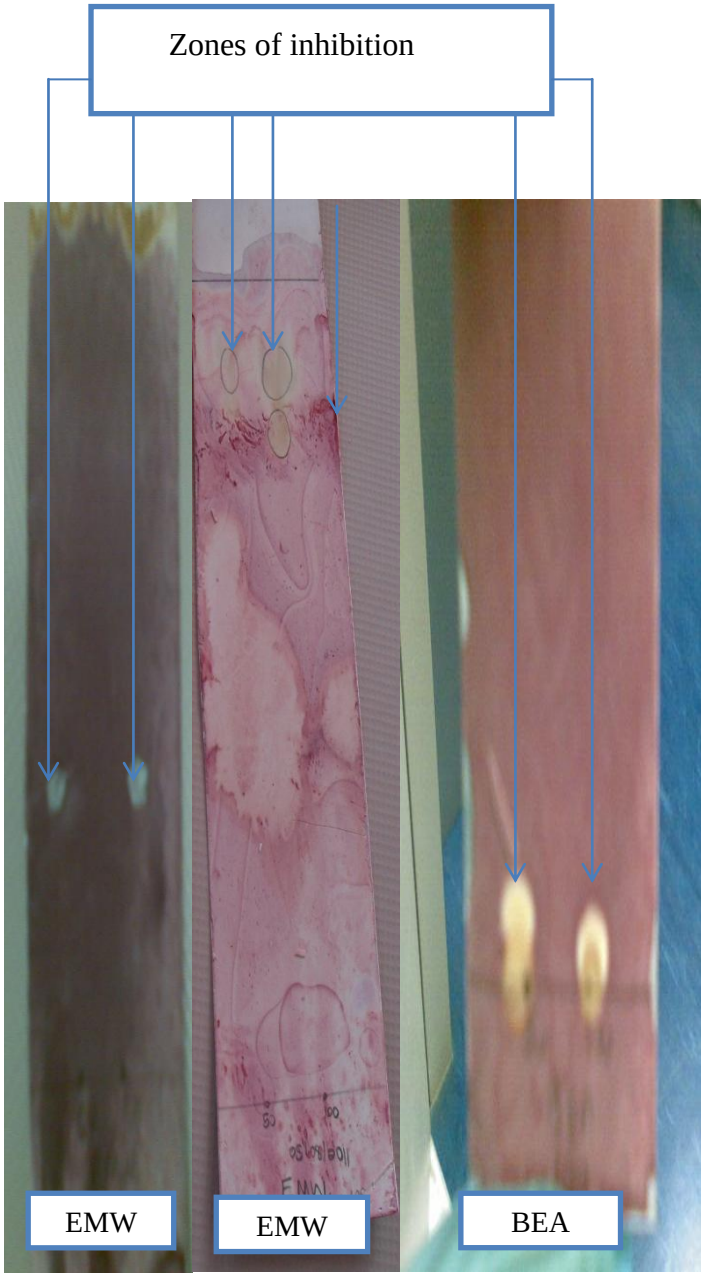

Figure 2b: Bioautography of chromatograms developed in EMW and BEA solvent systems showing clear zones of inhibition for *S. aureus*, *P. Shigelloides* and *S. pyogenes*.

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4.5 Column Chromatography analysis and MIC_{90} determination of fractions.

Approximately four fractions of 200mL of chloroform were collected, followed by eighteen fractions of Chloroform/Ethyl Acetate/Formic acid (CEF). Fraction CEF 4-6 and CEF 8-10 were combined due to the presence of similar compounds showing same mobility or (R_fs) on TLC profile. This yielded a total of sixteen major fractions including those collected from chloroform. CEF 3 and CEF 11 showed one band with R*f* values 0.153 and 0.13, respectively. A maximum of three bands were observed on fractions analysed by TLC. Minimum Inhibitory concentrations of fractions were tested against all the organims. Lowest MIC values of 0.0012 mg/mL against *S. pyogenes* (compared favourably to that of ciprofloxacin), 0.0006, 0.0195, 0.012 mg/mL for *S. aureus*, *P. shigelloides* and *S. typhimurium*, respectively (Table 5). CEF (1, 2, 3, 8 - 10, 11, 12) demonstrated bacterial inhibition against all the organisms tested. The MIC of the fractions ranged between 0.0006 - 2.5 mg/mL and that of ciprofloxacin between 0.0012 - 0.781 mg/mL.

Table 5: R_f values of fractionated compounds and MICs in mg/mL against test organisms.

Fraction	R*f* value of fractionated compounds	MIC_{90} against test organisms			
		S.pyogenes	***S.aureus***	***P.shigelloides***	***S.typhimurium***
C1	0.008, 0.159, 0.31	ND	ND	ND	ND
C2	0.09, 0.12, 0.36	ND	ND	ND	ND
C3	0.13, 0.17, 0.29	0.0195	0.00781	ND	ND
C4	0.156, 0.286, 0.294	0.00871	0.0195	ND	ND
CEF 1	0.2, 0.366, 0.42	0.049	0.0098	0.625	0.3125
CEF 2	0.34, 0.4, 0.52	0.625	0.024	0.625	1.25
CEF 3	0.153	0.1563	0.0781	0.0781	0.1563
CEF 4-6	0.13, 0.226, 0.3	ND	0.024	2.5	2.5
CEF 7	0.2, 0.33	0.625	0.0049	ND	0.1563
CEF 8-10	0.16, 0.306, 0.36	0.0195	0.024	2.5	2.5
CEF 11	0.13	0.049	0.0006	2.5	0.012
CEF 12	0.15, 0.18	0.3125	0.635	0.0781	1.25
CEF 13	0.13, 0.18, 0.28	0.0049	0.024	1.25	ND
CEF 14-16	0.15, 0.2	0.3125	1.25	1.625	ND
CEF 17	0.11, 0.38	0.0024	0.049	0.325	ND
CEF 18	0.12, 0.27	0.0012	0.1563	0.0195	ND
Ciprofloxacin		0.0012	0.0049	0.012	0.0781

C = Chloroform, CEF = (Chloroform: Ethyl Acetate: Formic acid), ND = Not Determined (value not within susceptible range)

4.6 GC-MS Analysis of fractions

Three fractions CEF 3 (F3), CEF 11 (F11) and CEF 12 (F12) were analysed by GC-MS because they were more active and pure. Figure 3 (a, b, c) show chromatograms, which are plots of the total mass eluting from GC and detected by MS as a function of time. Each peak represents a discrete chemical compound. GC-MS chromatogram of CEF 3 (F3) showed 10 peaks indicating the presence of 10 compounds (Figure 3a), with the highest peak of RT 34.24 and lowest peak RT 29.89. The three major compounds present in CEF3 (F3) were Linoleic acid constituting a proportion percentage of 26.60 %, followed by Hexadecanoic acid (25.07 %) and 9-Octadecenoic acid (24.81 %); minor compounds were Hexadecanoic acid, methyl ester (0.73 %) and 9-Octadecanoic methyl ester (0.91%) (Table 6). Most compounds prevalent in CEF 3 (F3) were fatty acids. A different GC-MS chromatogram was shown in CEF 11 (F11) which had a single peak (RF 41.20) and 100 % area percentage of a single compound, 1, 2- Benzenedicarboxylic acid, a carboxylic fatty acid ester (Figure 3b). CEF 12 (F12) demonstrated 14 peaks, indicating the presence of 14 compounds (Figure 3c) with the highest and lowest peak (RT 40.34 and RT 37.76), respectively. The three major compounds present were 2.3 - Dihydro - 3, 5 - dihydroxy - 6 - methyl (24.16 %), 1- Butanol (15.72 %) and 9 - Octadecenamide (13.82 %) and minor compounds were Hexadecanamide, Amide (1.59 %) and cyclohexadecane (1.84 %).

Figure 3a: GC-MS chromatogram of CEF 3 (F3)

```
File        : D:\DATA\CHRIS\F3D.D
Operator    : Bola
Acquired    : 19 Oct 2011  15:04      using AcqMethod BOLAZULU
Instrument :   GC/MS Ins
Sample Name: F3
Misc Info   : 19-10-2011
Vial Number: 1
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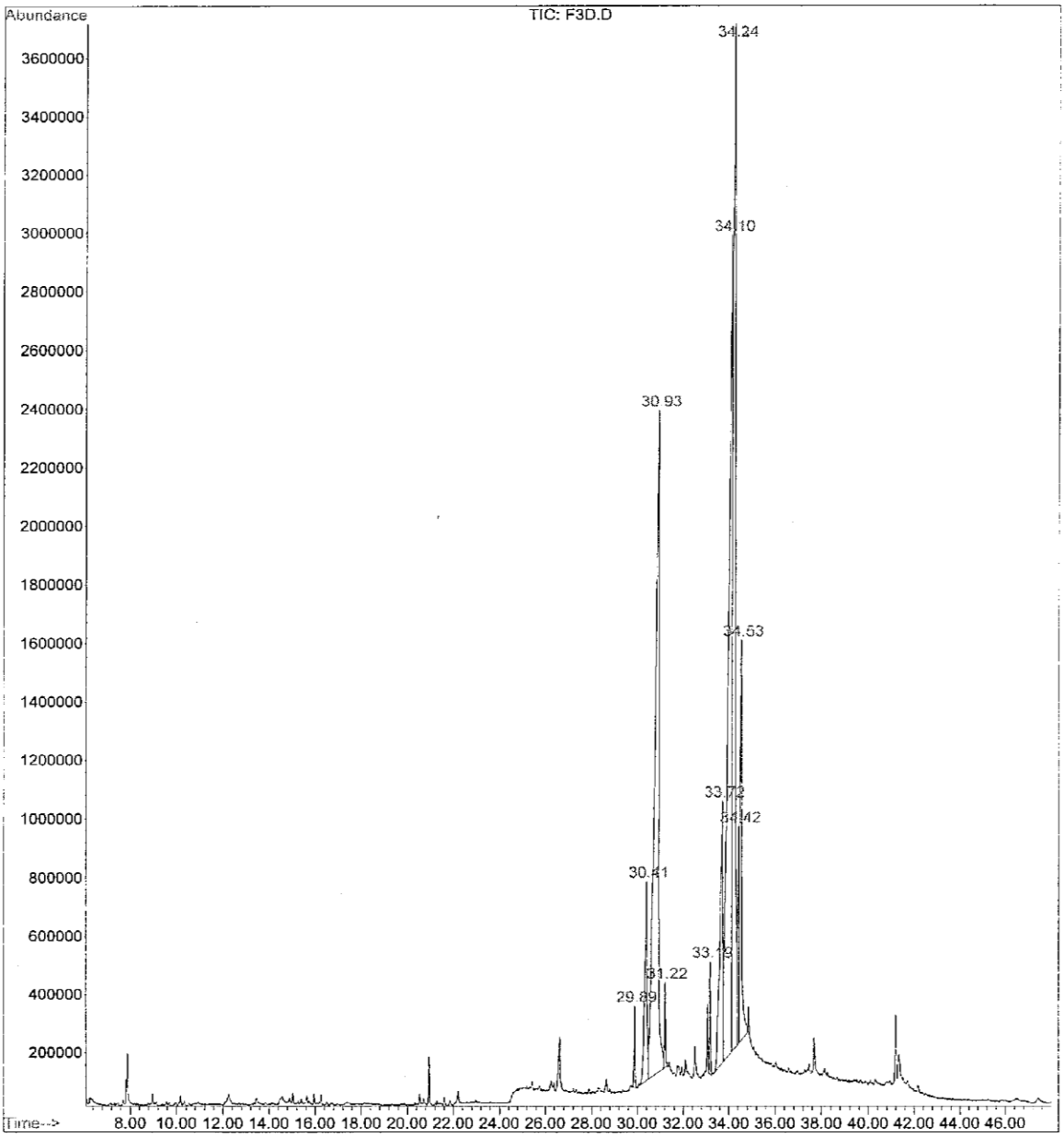

Figure 3b: GC/MS chromatogram of CEF 11 (F11)

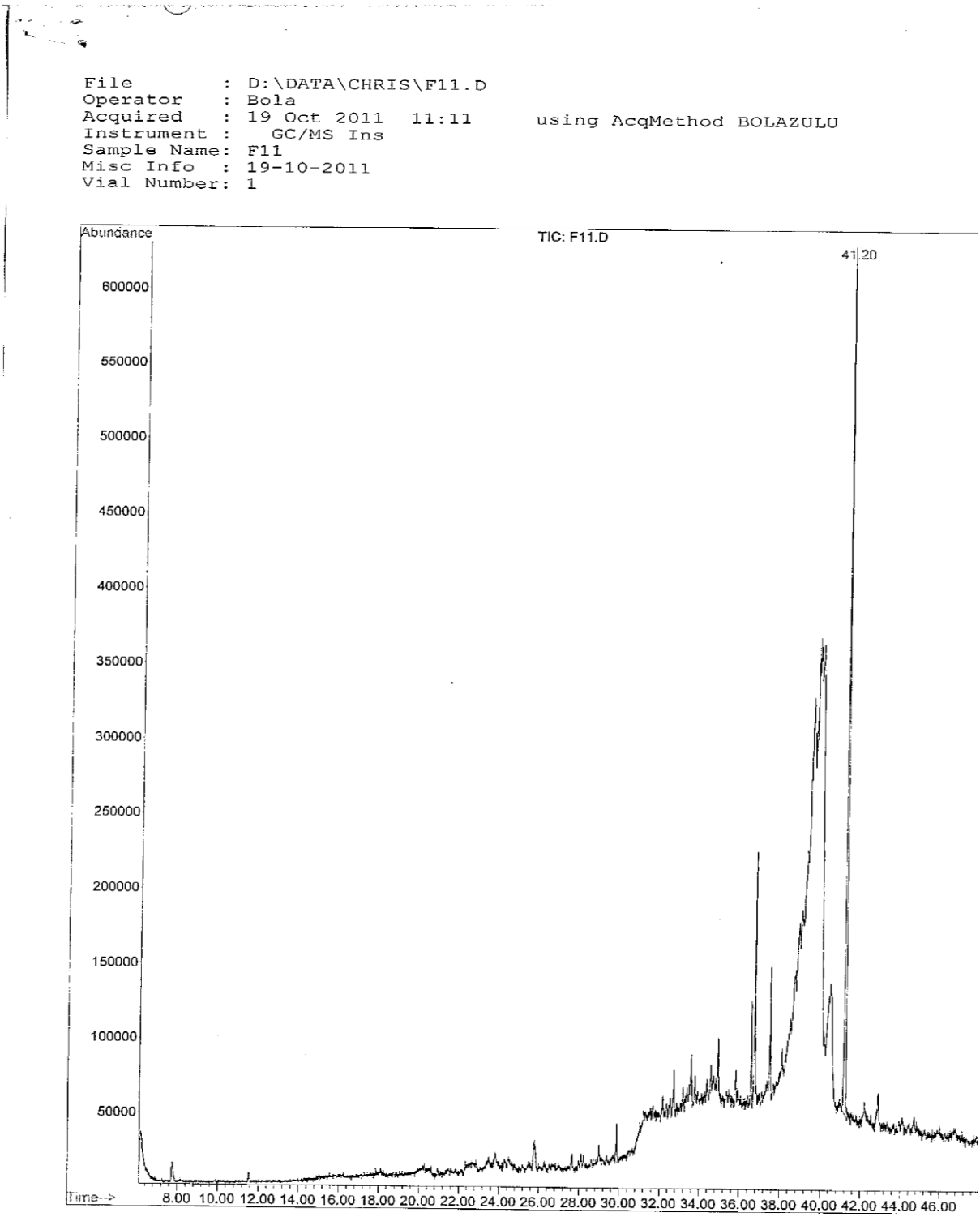

Figure 3c: GC-MS chromatogram of CEF 12 (F12)

```
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Acquired     : 19 Oct 2011   12:32      using AcqMethod BOLAZULU
Instrument :    GC/MS Ins
Sample Name: F12
Misc Info    : 19-10-2011
Vial Number: 1
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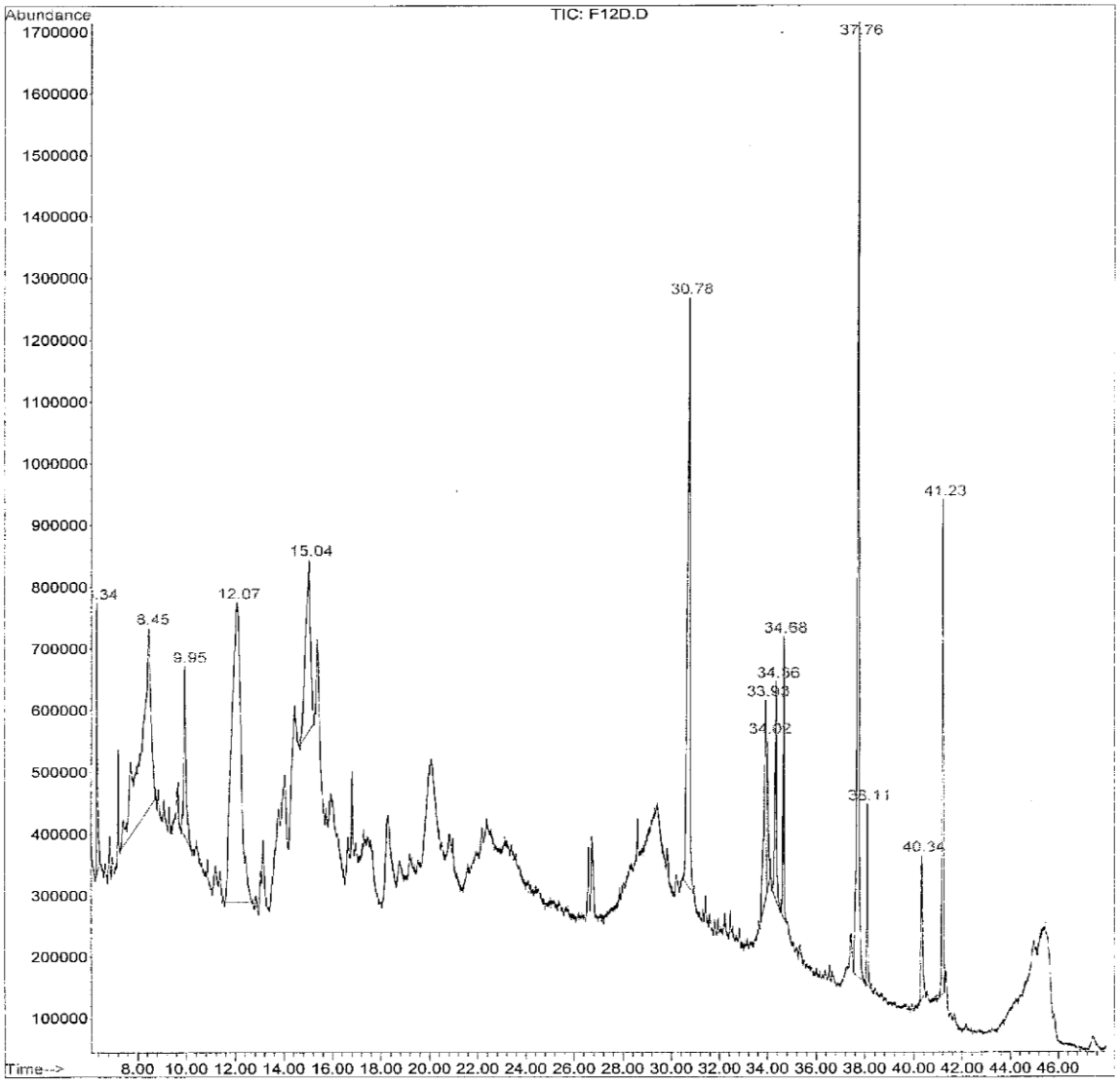

Table 6: Chemical compounds identified by GC-MS

Fraction	Peak	RT (mins)	Area %	Compounds identified
CEF 3 (F3)	1	29.89	0.73	Hexadecanoic acid, methyl ester
	2	30.42	4.41	Hexadecanoic acid, Palmitic acid
	3	31.93	25.07	Hexadecanoic acid, Palmitic acid
	4	31.22	0.83	Hexadenoic acid , ethyl ester
	5	33.19	0.91	9-Octadecenoic acid, methyl ester
	6	33.71	6.63	Heptadecene –(8)-Carbonic acid
	7	34.10	26.60	Linoleic acid
	8	34.24	24.81	9-Octadecadienoic acid
	9	34.42	2.62	14-Pentadenoic acid
	10	34.53	7.49	Octadenoic acid, Stearic acid
CEF 11 (F11)	1	41.20	100	1,2-Benzenedicarboxylic acid, bis
CEF 12 (F12)	1	6.63	2.31	Formide, N, N-Diethyl
	2	8.45	15.72	1-Butanol
	3	9.95	3.03	3-Isothiazolecarboxamide
	4	12.07	24.16	2,3-Dihydro-3,5-dihydeoxy-6-methyl
	5	15.04	7.06	2,5-Di (hydroxymethyl)-furan
	6	30.78	12.91	Hexadecanoic acid, Palmitic acid
	7	33.94	4.08	9-Octadecanoic acid
	8	34.02	1.84	Cyclohexadecane
	9	34.36	3.11	Octadecanoic acid, Stearic acid
	10	34.68	2.55	n-tetradecanoic acid amide
	11	37.75	13.82	9-Octadecenamide
	12	38.11	1.59	Hexadecanamide, Amide
	13	40.34	2.07	3,4,8-Trimethyl-2-nonenal
	14	41.23	5.76	Di-(2-ethylhexyl) phthalate

CHAPTER FIVE

Discussion and Conclusion

5.1 Discussion

Antimicrobial resistance is a major problem which the world is facing and is resulting in increased death rate. The multiple antimicrobial resistant bacteria cause severe problems that result in the complication of treatment of bacterial infections and this has been recognized by the World Health Organization (WHO, 2005). Antibiotics are used as chemotherapeutic agents and they were believed to lead in the complete eradication of infectious diseases (Rosina *et al*., 2009). Despite the progress made in introducing new antibiotics, emergence of drug resistant strains cause failure of infectious disease treatment (Matthias *et al*., 2000; Gibbons *et al.*, 2005). It is believed that consumption of antibiotics in livestock, agriculture and poultry is one of the factors that has caused an increase in the development of drug resistance. Some farmers use antibiotics to make animals grow big and this application in animal and food production promotes the development of antibiotic resistance in humans; also the indiscriminate use of antibiotics by humans accounts for this. Studies have found that antibiotic resistance occur as a result of an intrinsic mechanism that prevents bacteria from destruction (McDonnell *et al*., 2001; Stacey *et al*., 2001). These bacteria usually do not have the structural cellular mechanisms that are needed in order for the antibiotic to act upon (Courvalin *et al.*, 2006). New effective strategy is therefore necessary for the management of resistance bacteria: one of which includes medicinal plants.

It is estimated that more than two thirds of the world's plant species have medicinal value (WHO, 2011); and about 80% of people rely on herbs for their medicines (Arunkumar *et al*., 2009). Medicinal plants are believed to be an important source of new chemical substances

with potential therapeutic benefits (Eisner *et al*., 1990). They contain many biologically active compounds with medicinal properties and largely employed in developing countries (Ifeom *et al*, 2002). They produce a variety of natural products or secondary metabolites which are toxic in nature and are used as defence mechanism against microbial pathogens, insects, herbivores and other predators (Schafer *et al*., 2009; Bakkali *et al*., 2008). In addition to the production of intrinsic antimicrobial compounds, it has been hypothesized that plants also produce multi-drug resistance inhibitors which enhance the activity of antimicrobial compounds (Stermits *et al*., 2000). These active constituents react with other bacterial pathogens and inhibit their growth (Sermakkani *et al.,* 2011). Compounds isolated from natural origin such as medicinal plants are believed to have less side effects, tolerated by patients and can be afforded by most people since they are sold at a cheaper, reasonable price (Vermani and Garg *et al*., 2002).

G. kola plant has been investigated largely due to its diverse bioactivities. Ethyl acetate, ethanol, methanol, acetone and distilled water were the five solvents used in extraction of the *G. kola* seeds in this study. A concentration of 50, 100 and 200 mg/ mL of the dried extract was reconstituted for antimicrobial activity by dissolving extracts in 10 % DMSO (Njume *et al*., 2011). Methanolic extract of the seeds demonstrated good activity against all the organisms with the highest zone diameter of inhibition of 24 mm against *S. pyogenes.* The results for susceptibility testing of the extracts which are indicated in Table 1 confirms the results of previous studies, which reported that methanol is an efficient solvent (Eloff, 1998; Ezeifeka *et al*., 2004, Jayalakshmi *et al*., 2011; Vhagasia *et al*., 2011). The water extract demonstrated poor activity in all the organisms since no clear zones of inhibition were seen on the agar plate; which is in line with previous findings (Eloff, 1988; Manetti *et al*., 2007; Nwaokorie *et al*., 2010). This is an indication that water was not a good solvent; this could probably be that compounds responsible for bioactivity were not soluble in distilled water.

The activity of the positive control was higher than that of plant extracts ($P < 0.05$), although the ethanol extract compared favourably with that of ciprofloxacin (positive control) against *S. pyogenes*. Although methanol extract showed activity against both Gram - positive and Gram - negative, the Gram - positives were more susceptible with greater inhibition zones; these findings agree with previously published reports (Ezeifeka *et al.,* 2004; Jigma and Sumitra, 2006; Sibanda *et al*., 2008).

The activity of the methanol extract against both gram-negative and gram-positive organisms was of great interest because a more profound activity of several extracts is usually expected against gram-positive bacteria. This has been explained by the difference in cell wall composition, with gram-negative being complex (Bhattarai *et al*., 2008; Vhagasiya *et al*., 2011). The antimicrobial activity of *G. kola* seeds extracts showed a broad spectrum activity and this is in harmony with previous findings (Akinpelu *et al*., 2006; Adeboye *et al*, 2008; Njume *et al*., 2011).

The MIC_{90} of the methanol extract was evaluated against all organims as it demonstrated the best antimicrobial activity in the general screening. The MIC values for the extract and positive control (ciprofloxacin) ranged between 0.04 - 1.25 mg/mL and 0.0012 - 0.0195 mg/mL, respectively (Table 2). The MBC of the extract and ciprofloxacin ranged from 0.081 - 2.5 mg/mL and 0.0781- 0.3125 respectively. The MBC values were higher than the MIC values. This suggests that the extract was bacteriostatic at lower concentration and bactericidal at higher concentration. *S. pyogenes* and *S. aureus* were the most sensitive bacteria tested, with the lowest MIC of 0.04 mg/mL. This results show that some medicinal plants can be potential sources of new antibacterial agents.

Plants contain oils or fats and these are found mainly in their seeds. These lipids are usually stored in the form of triglycerides. Essential oils produced by plants are not only used in agriculture or in food industries as food preservatives or additives, but also used pharmaceutically for their therapeutic activities in the treatment of various diseases (Vila *et al*., 2010). The complexity of their chemical constituents is responsible for various biological activities. Linoleic acid, palmitic, and stearic acids are usually found in oil obtained from seeds. Palmitic acid is common saturated fatty acids reported to be found in plants and animals; and one of the major components of oil found in plants and their seeds. Linoleic acid is an organic compound that belongs to a group of essential fatty acids found in many oils produced by plants such as vegetables (Rogers *et al*., 2006). It cannot be produced in human bodies and therefore is acquired through diet. It is a carboxylic acid with 18-carbon chains and most seed oils are sources of this acid. It is regarded as one of the healthiest cooking oil and suitable for most cooking purposes. Studies have proved that it lowers the risk of cardiovascular disease (William *et al*., 2000; Penny *et al*., 2002). Stearic acid is the saturated fatty acid occurring in oils, vegetables and animals. It has an IUPAC name of Octadecanoic acid and is a carboxyl long hydrocarbon chain with carboxyl group at one end and methyl group at the other end.

Success in isolating compounds from the plant material was largely dependent on the type of solvent combination used in the extraction process. CEF separated more compounds (10) at 50mg/mL and 12 at 100 mg/mL, followed by EMW which separated 9 compounds at 50 mg/mL and 10 at 100mg/mL. BEA separated fewer compounds, with 3 at 50 mg/mL and 6 at 100 mg/mL. An interesting observation was noted at 100 mg/mL; most compounds were efficiently separated at this concentration. The total number of compounds extracted using different solvents is shown in Table 3. This implies that solvent with intermediary polarity

separated more active compounds. This findings correlates the findings of Masoko (2007), where the greatest separation was obtained using CEF.

Bioautography was done in order to determine the position of compound(s) showing antibacterial activity. The plates for bioautography were run in duplicates, one set was used as the reference chromatogram and the other for antibacterial activity assay. The areas of inhibition were compared with the R_f of the related spots on the reference TLC plate. Bioautography revealed that the methanol plant extract had components with inhibitory effects against most of the organisms including the gram-negative organisms (Table 4). A very high inhibition (indicated by a clear zone) was observed in the chromatogram developed in CEF at R_f (0.63) against *S. typhimurium* a gram-negative and BEA at the origin against *S. pyogenes*. In some cases no inhibition of microbial growth was observed on some parts of the plate; these findings agree with previously published results of Masoko and Eloff (2005). The absence of activity was interpreted to be the evaporation of active compounds or presence of very little amount of active compounds during the removal of eluents (Masoko and Eloff, 2008). It might also be due to the traces of some solvents left in the chromatograms. Another explanation for the observed non-activity could be due to very weak activity of the extracts against the selected microorganisms. The active spots appeared as white/yellow spots on a purple/pink background.

The solvent system that exhibited the best separation of compounds (CEF) was chosen for column chromatography. CEF separated 12 compounds at 100 mg/mL concentration more than EMW and CEF solvent combinations (Table 3). Initially 22 fractions of 200 mL (Njume *et al*., 2011) were collected in separated bottles and coded C and CEF for fractions collected from chloroform and solvent system Chloroform/ Ethyl Acetate/ Formic acid, respectively. The first few fractions collected in CEF exhibited a light brown colour but TLC profile indicated different compounds and therefore were not combined. TLC analysis of fractions

was therefore compulsory even for fractions which indicated the same colour. Most fractions collected in this study were colourless. TLC fractions of 4-6, 8-10 and 14-16 indicated similar compounds, and were combined to yield 16 compounds (Table 5).

Eluted compounds fractions were assayed for MIC_{90} using 96-well microdilution method. MIC ranged between 0.0006 - 2.5 mg/mL and that of ciprofloxacin ranged between 0.0012 - 0.0781mg/mL (Table 6). The lowest MIC (0.0012 mg/mL) of CEF 18 (F18) against *S. pyogenes* compared favourably to that of ciprofloxacin ($P < 0.05$). This result may indicate that CEF 18 (F18) has the same inhibitory potential with ciprofloxacin (positive control). Most high MIC values of 2.5 mg/mL were observed against gram-negative bacteria (*S. typhimurium* and *P. shigelloides*); this may be related to the thicker cell wall composition of gram - negatives. The results indicate that most fractions demonstrated a good inhibition against the organisms. All fractions collected from CEF demonstrated a good inhibition against *S. aureus*. Poor activity of some fractions may be due to insufficient amount of active ingredients.

Three fractions CEF 3 (F3), CEF 11 (F11), and CEF 12 (F12) were analysed by GC - MS to determine the type(s) of compounds present. These fractions were selected based on their purity [less compounds shown by TLC profile ($\leq$ 2 bands)] and their activity on the organisms. CEF 3 (F3) and CEF 11 (F11) showed one band on the TLC plate indicating a better purity of the compounds while CEF 12 showed two bands. Although CEF 7, CEF 14-16, CEF 17 and CEF 18 indicated two compounds on the TLC plate, they did not show inhibitory activity against all the four tested organisms therefore were not analyzed by GC-MS. CEF 17 was not effective against *P. shigelloides* and CEF (14 -16, 17, 18) were found ineffective against *S. typhimurium.* CEF 3 (F3) showed an R*f* value of 0.153, CEF 11 (F11) 0.13 and CEF 12 (F12) (0.15 and 0.18). The chemical compounds identified by GC-MS are presented in Table 6. CEF 3 (F3) showed high level of Linoleic acid (26.60 %), followed by

Hexadecanoic acid (25.07 %) and 9-Octadecenoic acid (24.81 %), and only 10 peaks were identified which indicate the presence of 10 compounds. CEF 11 (F11) had 1, 2-Benzenedicarboxylic acid [Di-(2-ethylhexyl) phthalate] (100 %) and only one peak was identified from this fraction. CEF12 (F12) showed major compounds of 2, 3-Dihydro-3, 5-dihydroxy-6-methyl (24.16 %), followed by 1-Butanol (15.72 %) and 9-Octadecenamide (13.82 %) and 14 peaks were identified on the GC-MS chromatogram. Linoleic acid is an unsaturated fatty acid and a carboxylic acid that belong to an essential fatty acid. Hexadecanoic acid is also known as palmitic acid, a common saturated fatty acid usually found in plants and animals. Octadecanoic acid (stearic acid) is a saturated fatty acid, one of the common long-chain fatty acid. 1, 2-Benzenedicarboxylic acid (phthalic acid) and 1-butanol (primary alcohol) and 9-Octadecenamide (Amide) were also dominant chemical compounds found. Among other chemical compounds detected by GC-MS were 3, 4, 8-Trimethyl - 2 - nonenal (2.07 %), Hexadecanamide (1.59 %), n-Tetradecanoic acid amide (2.55 %), though small quantities of these compounds were observed (Table 6). The compound 3, 4, 8 - Trimethyl - 2 - nonenal could probably be new since no report exist on it in literature.

Most compounds revealed by GC-MS in this study were fatty acids and they have been reported to have antibacterial and antifungal activity (McGraw *et al*., 2002; Seidel and Tailor, 2004; Russell, 1991). The presence of these chemical compounds correlate the findings of several authors (McGraw *et al*., 2002; Siedel and Taylor, 2004; Eleyinmi *et al*., 2006; Gopalakrishan *et al*., 2011) who using GC-MS analysis revealed the presence of hexadecanoic, benzenedicarboxylic, polyunsaturated fatty acid (Linoleic), octadecanoic, methyl ester Tetradecanoic (revealed by GC-MS in CEF 12), saturated fatty acids (stearic and palmitic). There are few reports on the identification of compounds in *Garcinia kola* seeds by GC-MS analysis. The observed wide range of antimicrobial properties of methanolic

extract and fractions can be explained by the presence of 1, 2-Benzenedicarboxylic acid (100 %), Linoleic acid (26.60 %), Hexadecanoic acid, palmitic (25.07 %), 2, 3-Dihydro-3, 5-dihydroxy-6-methyl (24.16 %), and 9-Octadecadienoic acid (24.81 %). These chemical compounds which are volatiles are the major constituents and occupied large area percentages.

5.2 Conclusion

From the results of this study, the following conclusions can be drawn.

1. All the extracts of *G. kola* seeds with the exception of the water extract showed varying degrees of antimicrobial activity on the microorganisms tested, with methanol extract demonstrating the best activity (bigger zones) against all the organims at all concentrations.
2. The methanol extract inhibited the growth of all organisms, with MIC ranging between 0.0012 - 0.0195 mg/mL and that of fractions from 0.0006 - 2.5 mg/mL against all tested organisms.
3. The MBC of the methanol extract ranged from 0.081 - 2.5 mg/mL.
4. The major chemical compounds revealed by GC-MS analysis were 1, 2-Benzenedicarboxylic acid (100 %), Linoleic acid (26.60 %), Hexadecanoic acid, palmitic (25.07 %), 2, 3-Dihydro-3, 5-dihydroxy-6-methyl (24.16 %) and 9-Octadecadienoic acid (24.81 %) which are believed to be responsible for antimicrobial activity.

5.3 Recommendations

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

1. Further analysis should be done to identify the non-volatile compounds by High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR).
2. The antioxidant activity of *Garcinia kola* seeds since the plant has been reported to have diverse bioactivity.
3. It is also important to evaluate the toxicity of the plant crude extracts in order to determine its safety parameters.

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APPENDIX: STATISTICAL ANALYSIS

Table 1: Multiple comparisons of zone diameters of seeds extracts of *G.kola* and ciprofloxacin.

1, ethyl acetate extract; 2, acetone; 3, ethanol; 4, methanol; 5, water; 6,6 ciprofloxacin

Zone diameter

Tukey HSD

(I) Exts	(J) Exts	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-2.833	2.444	.854	-10.01	4.34
	3	-6.750	2.444	.077	-13.92	.42
	4	-10.250*	2.444	.001	-17.42	-3.08
	5	9.333*	2.444	.004	2.16	16.51
	6	-18.917*	2.444	.000	-26.09	-11.74
2	1	2.833	2.444	.854	-4.34	10.01
	3	-3.917	2.444	.600	-11.09	3.26
	4	-7.417*	2.444	.039	-14.59	-.24
	5	12.167*	2.444	.000	4.99	19.34
	6	-16.083*	2.444	.000	-23.26	-8.91
3	1	6.750	2.444	.077	-.42	13.92
	2	3.917	2.444	.600	-3.26	11.09
	4	-3.500	2.444	.707	-10.67	3.67
	5	16.083*	2.444	.000	8.91	23.26
	6	-12.167*	2.444	.000	-19.34	-4.99
4	1	10.250*	2.444	.001	3.08	17.42
	2	7.417*	2.444	.039	.24	14.59
	3	3.500	2.444	.707	-3.67	10.67
	5	19.583*	2.444	.000	12.41	26.76
	6	-8.667*	2.444	.009	-15.84	-1.49
5	1	-9.333*	2.444	.004	-16.51	-2.16
	2	-12.167*	2.444	.000	-19.34	-4.99
	3	-16.083*	2.444	.000	-23.26	-8.91
	4	-19.583*	2.444	.000	-26.76	-12.41
	6	-28.250*	2.444	.000	-35.42	-21.08

6	1	18.917*	2.444	.000	11.74	26.09
	2	16.083*	2.444	.000	8.91	23.26
	3	12.167*	2.444	.000	4.99	19.34
	4	8.667*	2.444	.009	1.49	15.84
	5	28.250*	2.444	.000	21.08	35.42

*. The mean difference is significant at the 0.05 level.

Table 2: Multiple Comparisons of MIC values of methanol and ciprofloxacin

4, methanol extract; 6, ciprofloxacin

MIC

Tukey HSD

		Mean			95% Confidence Interval	
(I) Exts	(J) Exts	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
0	4	-.4900000	.2359868	.150	-1.148876	.168876
	6	-.0070000	.2359868	1.000	-.665876	.651876
4	0	.4900000	.2359868	.150	-.168876	1.148876
	6	.4830000	.2359868	.157	-.175876	1.141876
6	0	.0070000	.2359868	1.000	-.651876	.665876
	4	-.4830000	.2359868	.157	-1.141876	.175876

*. The mean difference is significant at the 0.05 level.

Table 3: Multiple Comparisons of MBC values of methanol and ciprofloxacin

4, methanol extract; 6, ciprofloxacin

MBC

Tukey HSD

(I) Exts	(J) Exts	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	4	-1.0202500	.4562476	.118	-2.294096	.253596
	6	-.1758000	.4562476	.922	-1.449646	1.098046
4	0	1.0202500	.4562476	.118	-.253596	2.294096
	6	.8444500	.4562476	.208	-.429396	2.118296
6	0	.1758000	.4562476	.922	-1.098046	1.449646
	4	-.8444500	.4562476	.208	-2.118296	.429396

*. The mean difference is significant at the 0.05 level.

Table 4: Mulitiple comparisons of MIC values of fractions of *G.kola* seeds and ciprofloxacin.

1 C1, 2 C2, 3 C3, 4C4, 5 CEF1, 6 CEF 2, 7 CEF 3, 8 CEF 4-6, 9 CEF 7, 10 CEF 8-10, 11 CEF 11, 12 CEF 12, 13 CEF 13, 14 CEF 14-16, 15 CEF 17, 16 CEF 18, 17 Ciprofolxacin.

Turkey HSD

					95% Confidence Interval	
(I) fracn	(J) fracn	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
1	2	.00000000	.69992933	1.000	-2.5464677	2.5464677
	3	1.24317250	.69992933	.928	-1.3032952	3.7896402
	4	1.24733500	.69992933	.926	-1.2991327	3.7938027
	5	2.25092500	.69992933	.140	-.2955427	4.7973927
	6	2.10337500	.69992933	.220	-.4430927	4.6498427
	7	2.38280000	.69992933	.090	-.1636677	4.9292677
	8	.61900000	.69992933	1.000	-1.9274677	3.1654677
	9	1.67845000	.69992933	.586	-.8680177	4.2249177
	10	1.23912500	.69992933	.929	-1.3073427	3.7855927
	11	1.23175000	.69992933	.933	-1.3147177	3.7782177
	12	1.98472500	.69992933	.303	-.5617427	4.5311927
	13	1.54425000	.69992933	.716	-1.0022177	4.0907177
	14	1.07812500	.69992933	.978	-1.4683427	3.6245927
	15	1.77550000	.69992933	.490	-.7709677	4.3219677
	16	1.83075000	.69992933	.437	-.7157177	4.3772177
	17	2.47865000	.69992933	.064	-.0678177	5.0251177
2	1	.00000000	.69992933	1.000	-2.5464677	2.5464677
	3	1.24317250	.69992933	.928	-1.3032952	3.7896402
	4	1.24733500	.69992933	.926	-1.2991327	3.7938027
	5	2.25092500	.69992933	.140	-.2955427	4.7973927
	6	2.10337500	.69992933	.220	-.4430927	4.6498427
	7	2.38280000	.69992933	.090	-.1636677	4.9292677
	8	.61900000	.69992933	1.000	-1.9274677	3.1654677
	9	1.67845000	.69992933	.586	-.8680177	4.2249177
	10	1.23912500	.69992933	.929	-1.3073427	3.7855927
	11	1.23175000	.69992933	.933	-1.3147177	3.7782177
	12	1.98472500	.69992933	.303	-.5617427	4.5311927
	13	1.54425000	.69992933	.716	-1.0022177	4.0907177
	14	1.07812500	.69992933	.978	-1.4683427	3.6245927

	15	1.77550000	.69992933	.490	-.7709677	4.3219677
	16	1.83075000	.69992933	.437	-.7157177	4.3772177
	17	2.47865000	.69992933	.064	-.0678177	5.0251177
3	1	-1.24317250	.69992933	.928	-3.7896402	1.3032952
	2	-1.24317250	.69992933	.928	-3.7896402	1.3032952
	4	.00416250	.69992933	1.000	-2.5423052	2.5506302
	5	1.00775250	.69992933	.988	-1.5387152	3.5542202
	6	.86020250	.69992933	.998	-1.6862652	3.4066702
	7	1.13962750	.69992933	.964	-1.4068402	3.6860952
	8	-.62417250	.69992933	1.000	-3.1706402	1.9222952
	9	.43527750	.69992933	1.000	-2.1111902	2.9817452
	10	-.00404750	.69992933	1.000	-2.5505152	2.5424202
	11	-.01142250	.69992933	1.000	-2.5578902	2.5350452
	12	.74155250	.69992933	1.000	-1.8049152	3.2880202
	13	.30107750	.69992933	1.000	-2.2453902	2.8475452
	14	-.16504750	.69992933	1.000	-2.7115152	2.3814202
	15	.53232750	.69992933	1.000	-2.0141402	3.0787952
	16	.58757750	.69992933	1.000	-1.9588902	3.1340452
	17	1.23547750	.69992933	.931	-1.3109902	3.7819452
4	1	-1.24733500	.69992933	.926	-3.7938027	1.2991327
	2	-1.24733500	.69992933	.926	-3.7938027	1.2991327
	3	-.00416250	.69992933	1.000	-2.5506302	2.5423052
	5	1.00359000	.69992933	.989	-1.5428777	3.5500577
	6	.85604000	.69992933	.998	-1.6904277	3.4025077
	7	1.13546500	.69992933	.965	-1.4110027	3.6819327
	8	-.62833500	.69992933	1.000	-3.1748027	1.9181327
	9	.43111500	.69992933	1.000	-2.1153527	2.9775827
	10	-.00821000	.69992933	1.000	-2.5546777	2.5382577
	11	-.01558500	.69992933	1.000	-2.5620527	2.5308827
	12	.73739000	.69992933	1.000	-1.8090777	3.2838577
	13	.29691500	.69992933	1.000	-2.2495527	2.8433827
	14	-.16921000	.69992933	1.000	-2.7156777	2.3772577
	15	.52816500	.69992933	1.000	-2.0183027	3.0746327
	16	.58341500	.69992933	1.000	-1.9630527	3.1298827
	17	1.23131500	.69992933	.933	-1.3151527	3.7777827
5	1	-2.25092500	.69992933	.140	-4.7973927	.2955427
	2	-2.25092500	.69992933	.140	-4.7973927	.2955427
	3	-1.00775250	.69992933	.988	-3.5542202	1.5387152
	4	-1.00359000	.69992933	.989	-3.5500577	1.5428777
	6	-.14755000	.69992933	1.000	-2.6940177	2.3989177
	7	.13187500	.69992933	1.000	-2.4145927	2.6783427
	8	-1.63192500	.69992933	.632	-4.1783927	.9145427

	9	-.57247500	.69992933	1.000	-3.1189427	1.9739927
	10	-1.01180000	.69992933	.988	-3.5582677	1.5346677
	11	-1.01917500	.69992933	.987	-3.5656427	1.5272927
	12	-.26620000	.69992933	1.000	-2.8126677	2.2802677
	13	-.70667500	.69992933	1.000	-3.2531427	1.8397927
	14	-1.17280000	.69992933	.954	-3.7192677	1.3736677
	15	-.47542500	.69992933	1.000	-3.0218927	2.0710427
	16	-.42017500	.69992933	1.000	-2.9666427	2.1262927
	17	.22772500	.69992933	1.000	-2.3187427	2.7741927
6	1	-2.10337500	.69992933	.220	-4.6498427	.4430927
	2	-2.10337500	.69992933	.220	-4.6498427	.4430927
	3	-.86020250	.69992933	.998	-3.4066702	1.6862652
	4	-.85604000	.69992933	.998	-3.4025077	1.6904277
	5	.14755000	.69992933	1.000	-2.3989177	2.6940177
	7	.27942500	.69992933	1.000	-2.2670427	2.8258927
	8	-1.48437500	.69992933	.769	-4.0308427	1.0620927
	9	-.42492500	.69992933	1.000	-2.9713927	2.1215427
	10	-.86425000	.69992933	.998	-3.4107177	1.6822177
	11	-.87162500	.69992933	.997	-3.4180927	1.6748427
	12	-.11865000	.69992933	1.000	-2.6651177	2.4278177
	13	-.55912500	.69992933	1.000	-3.1055927	1.9873427
	14	-1.02525000	.69992933	.986	-3.5717177	1.5212177
	15	-.32787500	.69992933	1.000	-2.8743427	2.2185927
	16	-.27262500	.69992933	1.000	-2.8190927	2.2738427
	17	.37527500	.69992933	1.000	-2.1711927	2.9217427
7	1	-2.38280000	.69992933	.090	-4.9292677	.1636677
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	3	-1.13962750	.69992933	.964	-3.6860952	1.4068402
	4	-1.13546500	.69992933	.965	-3.6819327	1.4110027
	5	-.13187500	.69992933	1.000	-2.6783427	2.4145927
	6	-.27942500	.69992933	1.000	-2.8258927	2.2670427
	8	-1.76380000	.69992933	.501	-4.3102677	.7826677
	9	-.70435000	.69992933	1.000	-3.2508177	1.8421177
	10	-1.14367500	.69992933	.963	-3.6901427	1.4027927
	11	-1.15105000	.69992933	.961	-3.6975177	1.3954177
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	14	-1.30467500	.69992933	.897	-3.8511427	1.2417927
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	16	-.55205000	.69992933	1.000	-3.0985177	1.9944177
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	2	-.61900000	.69992933	1.000	-3.1654677	1.9274677
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	4	.62833500	.69992933	1.000	-1.9181327	3.1748027
	5	1.63192500	.69992933	.632	-.9145427	4.1783927
	6	1.48437500	.69992933	.769	-1.0620927	4.0308427
	7	1.76380000	.69992933	.501	-.7826677	4.3102677
	9	1.05945000	.69992933	.981	-1.4870177	3.6059177
	10	.62012500	.69992933	1.000	-1.9263427	3.1665927
	11	.61275000	.69992933	1.000	-1.9337177	3.1592177
	12	1.36572500	.69992933	.860	-1.1807427	3.9121927
	13	.92525000	.69992933	.995	-1.6212177	3.4717177
	14	.45912500	.69992933	1.000	-2.0873427	3.0055927
	15	1.15650000	.69992933	.959	-1.3899677	3.7029677
	16	1.21175000	.69992933	.941	-1.3347177	3.7582177
	17	1.85965000	.69992933	.410	-.6868177	4.4061177
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	3	-.43527750	.69992933	1.000	-2.9817452	2.1111902
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	5	.57247500	.69992933	1.000	-1.9739927	3.1189427
	6	.42492500	.69992933	1.000	-2.1215427	2.9713927
	7	.70435000	.69992933	1.000	-1.8421177	3.2508177
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	3	.00404750	.69992933	1.000	-2.5424202	2.5505152
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	6	.86425000	.69992933	.998	-1.6822177	3.4107177
	7	1.14367500	.69992933	.963	-1.4027927	3.6901427
	8	-.62012500	.69992933	1.000	-3.1665927	1.9263427
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	11	-.00737500	.69992933	1.000	-2.5538427	2.5390927
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	13	.30512500	.69992933	1.000	-2.2413427	2.8515927
	14	-.16100000	.69992933	1.000	-2.7074677	2.3854677
	15	.53637500	.69992933	1.000	-2.0100927	3.0828427
	16	.59162500	.69992933	1.000	-1.9548427	3.1380927
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	4	.01558500	.69992933	1.000	-2.5308827	2.5620527
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	6	.87162500	.69992933	.997	-1.6748427	3.4180927
	7	1.15105000	.69992933	.961	-1.3954177	3.6975177
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	4	-.29691500	.69992933	1.000	-2.8433827	2.2495527
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	6	.55912500	.69992933	1.000	-1.9873427	3.1055927
	7	.83855000	.69992933	.998	-1.7079177	3.3850177
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	9	.13420000	.69992933	1.000	-2.4122677	2.6806677
	10	-.30512500	.69992933	1.000	-2.8515927	2.2413427
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	17	1.40052500	.69992933	.836	-1.1459427	3.9469927
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	5	-.22772500	.69992933	1.000	-2.7741927	2.3187427
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