IN-VITRO ANTI-*VIBRIO* ACTIVITIES OF CRUDE EXTRACTS OF *GARCINIA KOLA* SEEDS

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DECLARATION

I, the undersigned, declare that this dissertation and the work contained herein being submitted to the University of Fort Hare for the degree of Master of Science in Microbiology in the Faculty of Science and Agriculture, is my original work with the exception of the citations. I also declare that this work has not been submitted to any other university in partial or entirety for the award of any degree

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GENERAL ABSTRACT

The n-Hexane, dichloromethane, methanol and aqueous crude extracts of *Garcinia kola* (Heckel) seeds were screened for their anti-*Vibrio* activities against 50 *Vibrio* bacteria isolated from wastewater final effluents. The 50 isolates consisted of different *Vibrio* species namely *V. fluvialis* (14), *V. vulnificus* (12), *V. parahaemolyticus* (12), *V. metschnikovii* (3) and 9 others unidentified to the specie level. The n-Hexane, dichloromethane and methanol extracts had activities against 16 (32%) of the Vibrio isolates, while the aqueous extracts had activities against 12 (24%) all at a screening concentration of 10 mg/ml. The minimum inhibitory concentrations (MICs) were 0.313-0.625 mg/ml, 0.313-0.625 mg/ml, 0.313-2.5 mg/ml and 10 mg/ml for n-Hexane, dichloromethane, methanol and aqueous extracts respectively. Rate of kill studies were carried out against three different *Vibrio* species namely *V. vulnificus* (AL042), *V. parahaemolyticus* (AL049) and *V. fluvialis* (AL040) using the n-Hexane, dichloromethane and methanol extracts at 1× to 4 × MICs and 2 hour exposure. About 96.3%, 82.2%, and 78.1% (*V. fluvialis* AL040); 92.6%, 87.8% and 68.9% (*V. parahaemolyticus* AL049); and 91.6%, 64.4%, 60% (*V. vulnificus* AL042) of the bacteria were killed by the crude n-Hexane, dichloromethane and methanol extracts respectively after 2 hour exposure time at 4× MIC. The patterns of activity were bacteriostatic, with the n-Hexane extracts being most effective in activity. We conclude that the *Garcinia kola* seeds have promise in the treatment and management of infections caused by *Vibrio* species.

CHAPTER ONE

GENERAL INTRODUCTION

The genus *Vibrio* is a member of the family *Vibrionaceae* and consists of at least 34 recognised species. *Vibrio* species are gram negative straight or curved rod-shaped bacteria. They produce colonies 2-3 mm in diameter on blood agar and colonies on thiosulphate citrate bile salt sucrose (TCBS) (except *V. hollisae*) are either yellow in the case of sucrose fermenters or green in the case of non-sucrose fermenters. They are facultative anaerobes, motile by a single polar flagellum and are oxidase positive (except *V. metschnikovii*) (Health protection agency, 2007; Tantillo, GM *et al*., 2004; Farmer and Hickman-Brenner, 1992). Their growth is stimulated by sodium ions (halophilic) and the concentration required is reflected in the salinity of their natural environment and all of them utilize D-glucose as a sole or main source of carbon and energy and do not form endospores or microcysts (Farmer and Hickman- Brenner, 1992).

There are 12 species of the genus *Vibrio* incriminated in gastrointestinal and extra-intestinal diseases in man and the most important of these is *V. cholerae*. The other species are *V. alginolyticus, V. carchariae, V. cincinnatiensis, V. damsel, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus* (Farmer and Hickman-Brenner, 1992; Health protection agency, 2007).

V. cholerae is non-invasive, affecting the small intestine through secretion of an enterotoxin (Todar, 2005). *V. cholerae* can be serogrouped into 155 groups on the basis of somatic antigens. Epidemic strains usually belong to Ogawa and Hikojima subtypes. Epidemic strains of *V. cholerae* O1 can be further differentiated into E1 Tor and classical biotypes. Strains not belonging to serogroup O1 are generally referred to as *V. cholerae* non O1 (Sack, DA *et al*., 2004).

Vibrio infections are generally acquired either through ingestion of foods and water contaminated with human faeces or sewage, raw fish and seafood, or they are associated with the exposure of skin lesions, such as cuts, open wounds and abrasions, to aquatic environments and marine animals (West, 1989; Toti, L *et al*., 1996; Lee and Younger, 2002; Tantillo, GM *et al*., 2004).

Cholera *Vibrios* have previously been the focus of many research studies because of the severity of cholera but now studies are focusing even on non-cholera causing *Vibrio* species which cause mild to severe human diseases (Tantillo, GM *et al*., 2004).

V. parahaemolyticus, V. mimicus and *V. vulnificus* are food-poisoning bacteria which are normal inhabitants of estuarine and marine environments, and are frequently isolated from seawater and seafood. *V. parahaemolyticus* and *V. vulnificus* are invasive organisms affecting primarily the colon, *V. vulnificus* is an emerging human pathogen and it causes wound infections, gastroenteritis, or a syndrome known as primary septicaemia (Todar, 2005). Primary septicemia is a systemic illness caused by bacteria entering into the bloodstream through the portal vein or the intestinal lymphatic system. Symptoms include fever, hypotension, prostration, chills and occasionally abdominal pain, nausea, vomiting and diarrhoea (Tacket, CO *et al*., 1984).

Although *V. parahaemolyticus* is the most common non cholera *Vibrio* species reported to cause infection, *V. vulnificus* is associated with 94% of reported deaths. Foodborne non-cholera *Vibrio* infections may occur at rate of 0.2-0.3 per population of 100,000. Three thousand cases of *V. parahaemolyticus* infection are estimated to occur annually, resulting in 40 hospitalizations and 7 deaths. Ninety-five cases of *V. vulnificus* infection are estimated to occur annually, resulting in 85 hospitalizations and 35 deaths (Ho, H *et al*., 2009). *V. fluvialis* is commonly isolated from water, animal faeces, human faeces, sewage, and seafood product. *V. fluvialis* is an important cause of cholera-like bloody diarrhoea and causes wound infection with

primary septicemia in immunocompromised individuals and it remains among those infectious diseases posing a potentially serious threat to public health from developed to underdeveloped countries, especially in regions with poor sanitation (Igbinosa and Okoh, 2010).

Because clinical laboratories do not routinely use the selective medium thiosulphate-citrate-bile salts-sucrose (TCBS) for stool culture, many cases of *Vibrio* gastroenteritis are not identified and the surveillance systems underestimate the true incidence of *Vibrio* infections (Angulo and Swerdlow, 1995; Ho, H *et al*., 2009).

Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century. They not only are more severe and require longer and more complex treatments, but they are also significantly more expensive to diagnose and to treat (Alanis, 2005). Epidemiological surveillance of antimicrobial resistance is indispensable for empirical treatment of infections and in preventing the spread of antimicrobial resistant microorganisms (Adeleye, A *et al*., 2008).

It has been observed in epidemiological surveillances that some *Vibrio* species are becoming resistant to antibiotics and that their antibiotic susceptibility is dynamic and varies with the environment (Ottaviani, D *et al*., 2001, Jun, L *et al*., 2003, Adeleye, A *et al*., 2008). *Vibrio* strains isolated from waste water effluents showed the typical multidrug-resistance phenotype of an (sulfamethoxazole-trimethoprim) SXT element in a study by Okoh and Igbinosa (2010). The *Vibrio* species were resistant to sulfamethoxazole (Sul), trimethoprim (Tmp), cotrimoxazole (Cot), chloramphenicol (Chl), streptomycin (Str), ampicillin (Amp), tetracycline (Tet) nalidixic acid (Nal), and gentamicin (Gen). The antibiotic resistance genes detected include dfr18 and dfrA1 for trimethoprim; floR, tetA, strB, sul2 for chloramphenicol, tetracycline, streptomycin and sulfamethoxazole respectively. Some of the genes were only recently described from clinical

isolates, demonstrating genetic exchange between clinical and environmental *Vibrio* species (Okoh and Igbinosa, 2010).

A separate study by Adeleye, A *et al*. (2008) revealed a high prevalence of antibiotic resistance also in *Vibrio* isolates. The resistance patterns detected varied between four to ten drugs respectively with all isolates being resistant to amoxicillin, augmentin, chloramphenicol and citrofurantoin. The isolates included *V. alginolyticus, V. cholera, V. parahaemolyticus, V. mimicus* and *V. harveyi*. Several studies in different parts of the world on *Vibrio* species have also highlighted the presence of multiple drug resistant *Vibrio* species some isolated from sea foods (Coppo, A *et al*., 1995; Ottaviani, D *et al*., 2001; Jun, L *et al*., 2003).

As resistance to old antibiotics spreads, the development of new antimicrobial agents has to be expedited if the problem is to be contained. However, the past record of rapid, widespread and emergence of resistance to newly introduced antimicrobial agents indicate that even new families of antimicrobial agents will have a short life expectancy (Coates, A *et al*., 2002). Traditional medical treatment, supported mainly by the use of medicinal plants, represents the main alternative method which is mainly undocumented scientifically and is still communicated verbally from one generation to the next and many leads for further investigation could be discovered. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies (Wani, MS *et al*., 2007).

Garcinia kola (Heckel) often called bitter kola, is an indigenous medicinal tree belonging to the family *Guttiferae* (Anegbeh, PO *et al*., 2006). The *Garcinia kola* plant is an evergreen, well branched medium-sized tree growing up to 12 metres tall and 1.5 metres wide in 12 years. It has a regular fruiting cycle and it produces fruits yearly. The tree is found in moist forest areas and is distributed throughout West and Central Africa and has been located in Sierra Leone,

Ghana, Nigeria, Cameroon and Congo (Adedeji, OS *et al*., 2006; Anegbeh, PO *et al*., 2006). It produces a characteristic orange-like pod, with edible portion contained in the pod (Iwu, 1993).

Garcinia kola plant parts are used extensively in traditional medicine for the treatment of various diseases. The stem bark is used for the treatment of malignant tumors; the latex (gum) is used internally to treat gonorrhea and is applied externally to fresh wounds whilst the fresh seeds and the dry seed powder are chewed to prevent or to relieve colic pains, cure headache, chest colds and to relieve cough (Iwu, 1993). The sap from *Garcinia kola* is used to treat parasitic skin diseases (Esomonu, UG *et al*., 2005). The seed has long been used as a traditional medicine in sub-Saharan Africa for a variety of indications including hepatitis and other viral infections such as those caused by influenza and Ebola viruses, and as antidote for ingested poison and for oral hygiene (Iwu, 1999).

The identified primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu, MW *et al*., 1999).

In light of the increasing trend of antibiotic resistance in *Vibrio* species and the therapeutic potentials of the *Garcinia kola* plant along with the paucity of information on the use of this plant in the treatment of infections caused by *Vibrio* species, this study is therefore aimed at assessing the *in-vitro* anti-*Vibrio* activities of the *Garcinia kola* seeds. The specific objectives include:

- To prepare crude extracts of *Garcinia kola* seeds using such solvents as n-Hexane, dichloromethane, methanol and water.
- To screen the different crude extracts for anti-*Vibrio* activities.

- To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts against the susceptible Vibrio species.
- To determine the rate of kill of susceptible *Vibrio* species by the crude extracts.
- To compare the anti-*Vibrio* efficacies of the different crude extracts of the *Garcinia kola* seed.

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CHAPTER TWO

LITERATURE REVIEW

2.1. *Vibrio* Species

Vibrios are gram-negative, motile, nonspore-forming, curved or straight rod shaped bacteria that belong to the family Vibrionacea which are autochthonous inhabitants of the marine and estuarine environments. They occur in saline aquatic environments, both free in the water and bound to animate and inanimate surfaces (Farmer and Hickman-Brenner, 1992; Montanari, MP *et al*., 1999; Cavallo and Stabili, 2002). With the exception of non halophilic *Vibrio* species, such as *V. cholerae* and *V. mimicus*, all *Vibrio* species require saline for growth (halophilic) and are oxidase positive except *V. metschnikovii* (Ho, H *et al*., 2009; Health protection agency, 2007).

Vibrio species are acid-sensitive and grow well at neutral and alkaline pH values of up to pH 9, therefore, the pH values of both selective and enrichment media are generally 8-8.8 (Tantillo, GM *et al*., 2004). The main factors influencing the occurrence and distribution of *Vibrio* species in aquatic environments are water temperature, salinity, nutrient availability and the association with marine organisms. It is however difficult to assess the effect of a single parameter on Vibrios ecology as some factors are interactive (Cavallo and Stabili, 2002; Tantillo, GM *et al*., 2004). They vary in their nutritional versatility, but some species will grow on more than 150 different organic compounds as carbon and energy sources (Maugeri, TL *et al*., 2000). Most *Vibrio* species can grow in synthetic media with glucose as a sole source of carbon and energy requiring 2–3% sodium chloride or a seawater base for optimal growth (Maugeri, TL *et al*., 2000; Igbinosa and Okoh, 2008).

Water temperature is considered the most important factor governing the distribution and abundance of pathogenic *Vibrios*. Their density generally remains rather low at temperatures below 20 °C and the highest concentrations occur when water temperature is between 20°C and 30 °C. *Vibrios* of clinical interest are less frequently isolated when the temperature of natural aquatic environments is below 10 °C or exceeds 30 °C (Arias, CR *et al*., 1999; Heath, D *et al*., 2002). The direct relation between *Vibrio* species and water temperature determines seasonal and geographical variations in bacterial distribution as has been observed in the USA, Asia and Europe (Arias, CR *et al*., 1999; Heath, D *et al*., 2002).

Most *Vibrio* species occur when water salinity is from 5% to 30% but ecological observations indicate that they may also occur outside the optimum range in case of elevated nutrient concentrations and high water temperatures (Tantillo, GM *et al*., 2004). *Vibrio* persistence in the aquatic environment is also favoured by their capability to activate a survival state called 'viable but non-culturable' (VBNC), in response to environmental stress factors such as nutrient deficiency, unfavourable temperature and salinity levels (Stabili, L *et al*., 2000; Colwell, 2000; Johnston and Brown, 2002). In the VBNC state, the cells are unable to form colonies on conventional culture media but they maintain metabolic activity however some species are capable of reverting to the vegetative state for their growth and multiplication under suitable environmental conditions which are often season-dependent (Oliver, 1995; Colwell, 2000).

2.1.1. Laboratory Detection of Vibrio Species

Proper identification of *Vibrio* species in the laboratory is of importance as without it treatment decisions are skewed and can be potentially fatal to patients. The identification of *Vibrio* species can be based on the traditional phenotyping techniques or on recent molecular

techniques (Serratore, P *et al*., 1999; Kong, RYC *et al*., 2002). The traditional phenotyping techniques basically employ conventional culture-based methods involving selective pre-enrichment of samples, plating onto selective solid media after which morphological, biochemical and serological characterization are then performed. Detection and identification of *V. cholera* as well as enumeration of *V. parahaemolyticus* and *V. vulnificus* follows standard operating procedures (ISO method 8914, 1990; Food and Drug Administration, 1998) which include inoculation of the test sample into the selective enrichment medium - alkaline peptone water (APW) and incubation at optimum temperatures, followed by streaking onto the selective solid medium thiosulphate citrate bile salt agar (TCBS). Presumptive colonies will thereafter be sub-cultured on trypticase soya agar (TSA) and subjected to microscopic and biochemical analysis, which include Gram staining, motility, oxidase, arginine dihydrolase, lysine decarboxilase, ortho-nitrophenil-galactopyranoside (ONPG), acid–gas from glucose, saccharose, cellobiose, and halophilic characteristics according to Bergey's Manual of Systematic Bacteriology (Farmer and Hickman-Brenner, 1992; Tantillo, GM *et al*., 2004). Other methods that have been used for the successful identification of Vibrio species in food and the aquatic environment are serological methods such as serotyping *Vibrio* species to both somatic 'O' and capsular polysaccharide 'K' antigens. Enzyme-linked immunosorbent assays (ELISA) with monoclonal antibody species-specific for an intracellular antigen have also been used to confirm *V. vulnificus* strains (Tantillo, GM *et al*., 2004).

Differential solid media such as cellobiose polymyxin B colistin (CPC), blood agar (flooded with oxidase reagent after incubation), or mannitol–maltose agar may be used for isolation of many *Vibrio* species (Hagen, CJ *et al*., 1994; Donovan and van Netten, 1995). However, traditional culture-based techniques are slow, laborious and often require several days to be performed and the phenotypic assays are characterized by low sensitivity such that they may fail to detect low levels of the *Vibrio* strains in samples (Kaysner, CA *et al*., 1994; Aono, E

et al., 1997; Farmer and Hickman-Brenner,1992). To the contrary, molecular techniques of identification are rapid, sensitive and highly specific alternative methods for routine microbial screening and monitoring of environmental and food samples. Molecular techniques are mostly useful in the discrimination and comparison of toxigenic and non-toxigenic strains whilst some can be used in studies of the VBNC isolates that are difficult to identify by traditional culture based methods because of morphology, metabolic status and individual interpretation variations (Tantillo, GM *et al*., 2004; Igbinosa and Okoh, 2008).

Molecular methods such as polymerase chain reaction (PCR): both qualitative and quantitative; amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP); fluorescence *in situ* hybridization; microarrays; multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST); and ribotyping are frequently used for *Vibrio* species detection (Arias, CR *et al*., 1995; Igbinosa and Okoh, 2008). The study of the inter- and intra-generic relationships based on 16S rRNA sequences of ten representative *Vibrio* species singled out the presence of variable regions, which could be used as target sites for genus- and species-specific oligonucleotide probes and polymerase chain reaction (PCR) primers for molecular identification (Dorsch, M *et al*., 1992).

There are at least 34 recognised Vibrio species with 12 of the species being pathogenic to humans namely *V. alginolyticus*, *V. carchariae*, *V. cholera*, *V. cincinnatiensis*, *V. damsel*, *V. fluvialis*, *V. furnissii*, *V. hollisae*, *V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus* (Farmer and Hickman- Brenner, 1992; Health protection agency, 2007).

2.2. *Vibrio* Infections

Vibrio infections are largely classified into two distinct groups: *Vibrio* cholera infections and non cholera *Vibrio* infections. Isolates belonging to serogroups O1 and O139 of *V. cholerae* are responsible for *V. cholera* infections. Cholera is a devastating disease that produces severe,

dehydrating diarrhea and death unless rapid therapy is provided (Sack, DA *et al*., 2004). Other *Vibrios* other than *V. cholerae* O1 and O139 cause non cholera *Vibrio* infections.

Vibrios cause 3 major syndromes of clinical illness in man which are gastroenteritis, wound infections, and primary septicemia (Tantillo, GM *et al*., 2004). *Vibrio* species can produce multiple extracellular cytotoxins and enzymes that are associated with extensive tissue damage and that may play a major role in the development of sepsis (Ho, H *et al*., 2009). *Vibrio* infections are usually more life-threatening in people with underlying medical conditions or weakened immune systems (Tantillo, GM *et al*., 2004; Di Pinto, A *et al*., 2008) such as people with liver diseases, Acquired immune deficiency syndrome (AIDS) and diabetes.

Many cases of *Vibrio*-associated gastroenteritis are substantially under recognized because *Vibrios* are not readily identified in routine stool cultures as most laboratories do not use the selective media TCBS agar (Angulo and Swerdlow, 1995; Daniels and Shafaie, 2000) although it should be noted that *V. hollisae* does not grow on TCBS agar but can grow on blood agar (Health protection agency, 2007).

Vibrio infections are generally acquired either through ingestion of foods or water contaminated with *Vibrio* containing human faeces or sewage, raw fish and seafood, or they are associated with the exposure of open wounds to aquatic environments and marine animals which are natural habitats of *Vibrio* species (Lee and Younger, 2002). Primary septicemia is acquired through ingestions of food or water contaminated with some pathogenic *Vibrio* species which then invade the bloodstream through the gastro intestinal tract. Gastroenteritis occurs after ingestion of food or water containing the pathogenic *Vibrio* specie whilst wound infections result when skin lacerations or abrasions come in direct contact with water containing the pathogenic *Vibrio* specie. Additionally, wound infections can occur during acute, penetrating marine

injuries. The clinical presentation of *Vibrio* infections symptoms differ with respect to the infecting *Vibrio* specie (Daniels and Shafaie, 2000).

This particular study involved four different halophilic *Vibrio* species namely *V. vulnificus, V. parahaemolyticus, V. fluvialis* and *V. metschnikovii*. These were isolated from final waste water effluents which are discharged into water bodies such as rivers. Their presence increases the *Vibrio* loads in these waterbodies and in addition the effluents provide a source of nutrients for proliferation of the organisms, thus exposing the people in the surrounding communities who rely on these waterbodies for domestic and recreational purposes such as swimming and fishing at major health risks of *Vibrio* infections (Igbinosa, EO *et al*., 2009; Igbinosa and Okoh, 2008).

2.2.1. Vibrio vulnificus Infections

V. vulnificus is a motile, gram-negative, curved rod-shaped bacterium with a single polar flagellum. It is oxidase positive, halophilic and can be distinguished from other members of the *Vibrio* genus in its ability to ferment lactose. It is a naturally occurring, free-living inhabitant of estuarine and marine environments (Strom and Paranjpye, 2000; Bross, MH *et al*., 2007). *V. vulnificus* poses a significant health threat to humans especially those who suffer from immune disorders, liver disease, or hemochromatosis (iron overload). *V. vulnificus* enters human hosts via wound infections or consumption of raw shellfish (primarily oysters), and infections frequently progress to septicemia and death in susceptible individuals such that its associated with 95% of sea food related deaths (Harwood, VJ *et al*., 2004; Todar, 2005). Iron is an important growth factor for *V. vulnificus* such that clinical conditions associated with increased free iron, such as hemochromatosis or hemolytic anemia, represents a major risk factor for disseminated infections (Bisharat, 2002).

V. vulnificus infection is extremely invasive and case-fatality rates are greater than 50 percent for primary septicemia and about 15 percent for wound infections (Bross, MH *et al*., 2007). A number of extracellular cytotoxins and enzymes produced by *V. vulnificus* are associated with its virulence: septicemia is associated with proteases, endotoxic and lipopolysaccharides, whilst the wound infections are associated with proteases, hemolysin, lipase, cytolysin and DNAase and the gastroenteritis is associated with secretion of cytotoxin and hemolysin (Ho, H *et al*., 2009; Linkous and Oliver, 1999; Bisharat, 2002).

Gastroenteritis is a less common presentation whilst wound infection and primary septicemia are the common presentations of *V. vulnificus* infection (Daniels and Shafaie, 2000). Patients with gastroenteritis have a relatively milder syndrome consisting of vomiting, diarrhea and abdominal cramps, they may require hospitalization but it is normally not life threatening (Strom and Paranjpye, 2000).

Symptoms of primary septicemia caused by *V. vulnificus* infection are characterized by an onset of fever and chills often accompanied by nausea, vomiting, diarrhea and abdominal pain as well as pain in the extremities. A sharp drop in blood pressure commonly occurs, with possible outcomes of intractable shock and death. The majority of patients also develop painful skin lesions on the extremities including cellulitis, bullae and ecchymosis (Strom and Paranjpye, 2000; Bross, MH *et al*., 2007).

Wound infections typically begin with swelling, redness, and intense pain around the infected site. Fluid-filled blisters often develop and progress to necrotizing cellulitis. Wounds infections often require early surgical debridement because of the invasiveness of *V. vulnificus* to avoid limb amputation. In some patients the wound infection spreads to the bloodstream leading to secondary septicemia with symptoms identical to *V. vulnificus* primary septicemia and in such cases it is usually fatal (Ulusarac and Carter, 2004; Jones and Oliver, 2009).

2.2.2. Vibrio fluvialis Infections

V. fluvialis is a halophilic, oxidase positive gram-negative bacterium which has a straight to slightly curved rod cell morphology that is motile by means of a polar flagellum. It ferments D-glucose and other carbohydrates with the production of acid and gas (Lee, JV *et al*., 1978). *V. fluvialis* infections remain among those infectious diseases posing a potentially serious threat to public health (Igbinosa and Okoh, 2010). Studies by Bhattacharjee, S *et al*. (2010) in India following cyclone Aila indicated that *V. fluvialis* behaves more aggressively than *V. cholerae* O1 in an epidemic situation with a higher attack rate and a different clinical picture.

Gastroenteritis is the common clinical presentation of *V. fluvialis* infections whilst wound infections and primary septicemia are rare presentations (Daniels and Shafaie, 2000). Production of extracellular cytotoxin and hemolysin are *V. fluvialis* gastroenteritis virulence factors, whilst protease, hemolysin, lipase, cytolysin and DNAase are linked to its virulence in wound infections. Proteases, endotoxic and polysaccharide production are incriminated virulence factors in *V. fluvialis* primary septicemia (Ho, H *et al*., 2009; Chakraborty, R *et al*., 2005).

V. fluvialis infection gastroenteritis is characterized by watery diarrhea with the presence of blood in stool, abdominal pain, vomiting and mild fever in some cases (Lesmana, M *et al*., 2002; Huq, MI *et al*., 1980; Bhattacharjee, S *et al*., 2010; Sanyal, SC *et al*., 1992). In a rare case *V. fluvialis* was found to be the cause of necrotizing fasciitis and septicemia in the absence of gastroenteritis. Debridement of the infected areas proved unsuccessful leading to the amputation of the infected phalanges which was effective in preventing the spread of the infection (Mirfendereski, S *et al*., 2008).

2.2.3. Vibrio metschnikovii Infections

V. metschnikovii is a gram negative, halophilic, motile and slightly curved rod shaped bacterium which is a natural inhabitant of the aquatic environment. It can be differentiated from other *Vibrio* species by its inability to produce cytochrome oxidase (oxidase negative) and to reduce nitrate even though its colonial morphology on TCBS agar is typical of the genus *Vibrio* (Hansen, W *et al*., 1993; Matte, MH *et al*., 2007; Linde, H *et al*., 2004; Health protection agency, 2007). *V. metschnikovii* is often isolated from the environment but rarely isolated from human clinical specimens although the original strain(s) of *V. metschnikovii* was isolated in 1884 from cultures of fecal samples from cholera patients (Farmer III, JJ *et al*., 1988). Jean-Jacques, W *et al.* (1981) described a case of septicemia due to *V. metschnikovii* isolated from the blood of a patient with peritonitis and an inflamed gall bladder. *V. metschnikovii* has also been shown to be associated with other cases of septicemia (Hardardottir, H *et al*., 1994; Hansen, W *et al*., 1993) but in both cases they were from patients above 70 years old with underlying illnesses such as diabetes and in one case it was fatal (Hansen, W *et al*., 1993).

V. metschnikovii has also been associated with cases of wound infections (Hansen, W *et al*., 1993; Linde, H *et al*., 2004), diarrhea (Dalsgaard, A *et al*., 1996; Lesmana, M *et al*., 2002). Miyake *et al*.(1988) described a cytolysin specific for *V. metschnikovii* with hemolytic properties and the findings of Linde, H *et al*.(2004) showed the production of hemolysin and cytotoxin from a *V. metschnikovii* isolated from a wound infection at physiological temperature points pointing to their possible contribution to the pathological process in *V. metschnikovii* infections.

2.2.4. Vibrio parahaemolyticus Infections

V. parahaemolyticus is a gram negative, halophilic, oxidase positive, curved rod shaped bacterium which is a natural inhabitant of estuarine marine water. Its high motility in liquid media is attributed to a polar flagellum. It also possesses lateral flagella, which enable the microorganism to migrate across semi-solid surfaces in a phenomenon called swarming (Yeung and Boor, 2004). *V. parahaemolyticus* is usually responsible for acute gastroenteritis associated with the consumption of contaminated seafood, such as raw or slightly cooked shellfish (Yeung and Boor, 2004; Shimohata and Takahashi, 2010).

Gastroenteritis is the most common presentation of *V. parahaemolyticus* infections which is characterized by watery diarrhea, vomiting, nausea, abdominal cramps and fever. The infection is often self limiting but can cause septicemia that may be life-threatening to persons with underlying medical conditions such as diabetes and compromised immune systems such as those with Acquired immune deficiency syndrome (Lesmana, M *et al*., 2001; Di Pinto, A *et al*., 2008). Less frequently it causes wound infections and a variety of extracellular cytotoxins and enzymes have been implicated as possible virulence determinants for *V. parahaemolyticus* such as the production of cytotoxin and hemolysin in *V. parahaemolyticus* gastroenteritis whilst the protease, hemolysin, lipase, DNAase and cytolysin are possible virulence determinants for its wound infections (Ho, H *et al*., 2009; Yeung and Boor, 2004; Shimohata and Takahashi, 2010).

2.3. Treatment of *Vibrio* Infections

Treatment of *Vibrio* infections may require antibiotics, aggressive wound therapy and supportive care depending on the severity of the infections. Antibiotics that are used for the treatment of moderate to severe *Vibrio* infections include tetracycline and its synthetic derivative doxycycline, fluoroquinolones (for example, ciprofloxacin), third-generation cephalosporins (for example,

ceftazidime), and aminoglycosides (for example, gentamicin) (Daniels and Shafaie, 2000; Schwartz and Jagar, 2010; Qadri, F *et al*., 2003). In cases of mild *Vibrio* infections no antibiotics need to be taken for treatment but in order to prevent rapid dehydration in diarrhea cases, oral rehydration which can either be in the form of oral rehydration salts or oral electrolyte rehydration are recommended for replacement of lost body fluids. In addition to antibiotic treatment; early fasciotomy and debridement of infected wounds is generally recommended to avoid limb amputation (Daniels and Shafaie, 2000; Wisconsin Division of Public Health, 2008).

The use of some of the antibiotics is however limited in pregnant women and pediatrics (Daniels and Shafaie, 2000; Schwartz and Jagar, 2010) because of their toxicity with also the severity of the diseases being more complicated in people with underlying medical conditions and the elderly some of the antibiotics will not be suitable for use.

Antimicrobial therapy has been shown to reduce the duration and severity of symptoms of *Vibrio* infections in severe cases. However, as a consequence of increasing incidences of resistance to these antibiotics, most of them are no longer recommended as first-line therapy and treatment protocols are thus based on local antibiogram data (Daniels and Shafaie, 2000). The indiscriminate and inappropriate use of antibiotics in outpatient clinics, hospitalised patients and in the food industry is the single largest factor leading to antibiotic resistance (Alanis, 2005).

2.4. Antibiotics and Antibiotic Resistance

An antibiotic in a broader sense is defined as a chemotherapeutic agent that inhibits or abolishes the growth of microorganisms such as bacteria, fungi or protozoa. The classical definition of an antibiotic is a compound produced by a microorganism which inhibits the growth of another

microorganism and over the years this definition has been expanded to include synthetic and semi-synthetic products (Kummerer, 2009).

Antibiotics are used extensively in human and veterinary medicine as well as in aquaculture for the purpose of preventing (prophylaxis) or treating microbial infections (Kummerer, 2009) Antibiotics can be grouped by their chemical structure or mechanism of action into different classes such as beta-lactams (ß-lactams), quinolones, tetracyclines, macrolides, sulphonamides, aminoglycosides, glycopeptides, sulphonamides, cyclic lipopeptides, oxazolidononones, metronidazole, streptogramins, ketolides, fluoroquinolones, lincosamides, trimethoprim,polymyxins among others (Alanis, 2005; Tenover, 2006). The mechanism of action of the different major antibiotics classes vary as shown in Table 1 below.

Table 2.1: Major antibiotics classes by mechanism of action

Mechanism of Action	Antibiotic classes
Inhibition of cell wall synthesis	Beta-lactams (penicillins, cephalosporins, carbapenems, monobactams); Glycopeptides; Cyclic lipopeptides (daptomycin)
Inhibition of protein synthesis	Tetracyclines; Aminoglycosides; Oxazolidononones (linezolid); Streptogramins (quinupristin-dalfopristin); Ketolides; Macrolides; Lincosamides
Inhibition of DNA synthesis	Fluoroquinolones
Inhibition of RNA synthesis	Rifampin
Competitive inhibition of folic acid synthesis Inhibition	Sulphonamides; trimethoprim
Membrane Disorganizing agents	Polymyxins (Polymyxin-B,Colistin)
Other mechanisms	Metronidazole

Source: Alanis (2005).

The therapeutic use of an antibiotic, in human or animal populations, creates a selective pressure that favours survival of bacterial strains resistant to the antibiotic. The result is that many bacteria strains to which the antibiotic is used against become resistant and in many cases multi-resistant rendering the antibiotics ineffective as treatment of choice for severe infections caused by the bacteria (Altekruse, SF *et al*., 1997; Tenover, 2006). Diseases and disease agents that were once thought to have been controlled by antibiotics are returning in new forms resistant to antibiotic therapies resulting in simultaneous resistance to several antibiotic classes creating very dangerous multi antibiotic resistant bacterial strains (Levy and Marshall, 2004; Alanis, 2005; Sibanda and Okoh, 2007).

2.4.1. Mechanisms of Antibiotic Resistance in Pathogenic Bacteria

Bacterial resistance to antibiotics has its foundation at the genetic level meaning that changes in the genetic make up of the previously susceptible bacteria takes place either via a mutation or by introduction of new genetic information. The resistance can be natural (intrinsic) or acquired and can be transmitted horizontally or vertically (Alanis, 2005). The natural form of resistance is caused by a spontaneous gene mutation in the absence of selective pressure due to the presence of antibiotics. Once the genetic mutation occurs and causes a change in the bacterial deoxyribonucleic acid (DNA), genetic material can be transferred among bacteria by several mechanisms of genetic transfer such as conjugation, transformation and transduction resulting in acquired resistance (Alanis, 2005; Tenover, 2006).

The expression of the resistance gene and the subsequent production of tangible biological effects results in loss of activity of the antibiotic. The expression of the resistance gene can occur via three general biological mechanisms which are antibiotic destruction or

modification, antibiotic efflux from the cell and alteration of target site/receptor modification (Wright, 2005; Sibanda and Okoh, 2007).

Prevention of interaction of the antibiotic with the target occurs when the intracellular target or receptor of the antibiotic is altered by the bacteria resulting in the lack of binding or reduced affinity of the antibiotic to its binding site and consequently the lack of antibacterial effect (Alanis, 2005; Lambert, 2005). Modifications are usually mediated by constitutive and inducible enzymes. Examples of this mechanism includes modifications in the structural conformation of penicillin-binding proteins (PBPs) observed in certain types of penicillin resistance, ribosomal alterations that can render antibiotics that inhibit protein synthesis such as aminoglycosides, macrolides or tetracyclines inactive and DNA-gyrase modifications resulting in resistance to fluoroquinolones (Sefton, 2002; Levy and Marshall, 2004).

Antibiotic efflux from the bacterial cell takes place when the microorganism is capable of developing an active transport mechanism that pumps the antibiotic molecules that penetrated into the cell to the outside milieu until it reaches a concentration below that necessary for the antibiotic to have antibacterial activity (Alanis, 2005). This means that the efflux transport mechanism must be stronger than the influx mechanism inorder to be effective (Hooper, 2005). Efflux is common in tetracyclines, macrolides and fluoroquinolones among others (Roberts, 1996; Sefton, 2002; Leclercq, 2002; Hooper, 2005). Multi antibiotic resistance efflux pumps are ubiquitous proteins present in both gram positive and gram negative bacteria as either chromosomally encoded or plasmid encoded (Akama, H *et al*., 2005). Although such proteins are present constitutively in bacteria, the continued presence of the substrate induces over-expression (Teran, W *et al*., 2003) whilst the increased transcription is responsible for the acquired resistance (Sibanda and Okoh, 2007).

Destruction or modification of the antibiotic is mainly through enzymatic inactivation and this affects the action of several antibiotics. Antibiotic hydrolysing enzymes and group tranferases production by bacteria are the main factors leading to antibiotic destruction or modification as they chemically degrade or modify the antibiotic rendering it inactive against the bacteria. Group transferases covalently modify antibiotics resulting in structural alterations that impair target binding. Antibiotic modification can be through acyltransfer, phosphorylation, glycosylation, nucleotidylation, ribosylation and thiol transfer (Wright, 2005). Resistance to aminoglycosides in gram negative bacteria is most often mediated by a variety of enzymes that modify the antibiotic molecule by acetylation, adenylation or phosphorylation (Over, U *et al*., 2001). The production of beta-lactamases by bacteria confer resistance by hydrolysis of the amide bond of the four membered beta-lactam ring whose intergrity is central to the biological activity in beta lactam antibiotics (Jacoby and Munoz-Price, 2005; Wilke, MS *et al*., 2005).

2.5. Antibiotic Resistance Among *Vibrio* Species

Antibiotics used against *Vibrio* infections are also prone to indiscriminate and inappropriate use like all other antibiotics posing a potential threat to human health due to the presence of individual and multiple antibiotic resistance strains among both human and non-human pathogenic *Vibrio* species. In a study by Okoh and Igbinosa (2010) antibiotic resistance genes were detected in environmental isolates of *Vibrio* species and the genes included dfr18 and dfrA1 for trimethoprim; floR, tetA, strB, sul2 for chloramphenicol, tetracycline, streptomycin and sulfamethoxazole respectively of which some of the antibiotic resistance genes were only recently described from clinical isolates, demonstrating genetic exchange between clinical and environmental *Vibrio* species.

Exchange of resistance genes among *Vibrio* species was also shown in studies by Garg, P *et al*. (2000) where dissipation of some of the resistant patterns commonly found among clinical strains of *V. cholerae* non-O1, non-O139 or O1 serogroups to the O139 serogroup and vice versa was observed during succeeding years from 1992 to 1997 (Garg, P *et al*., 2000). According to Alanis (2005), the development of antibiotic resistance tends to be related to the degree of simplicity of the DNA present in the microorganism becoming resistant and to the ease with which it can acquire DNA from other microorganisms, such that studies such as those by Okoh and Igbinosa (2010) and by Garg, P *et al*. (2000) show the presence of genetic exchange of resistance genes among *Vibrio* species showing the development of antibiotic resistance. Individual and multiple antibiotic resistance among clinical and environmental *Vibrio* species has been shown through several antibiotic susceptibilty research studies (Garg, P *et al*., 2000; Jun, L *et al*., 2003; Manjusha, S *et al*., 2005; Adeleye, A *et al*., 2008; Okoh and Igbinosa, 2010).

In a study including 840 clinical isolates of *V. cholerae* isolated in a period of six years from 1992 to 1997 by Garg, P *et al*. (2000) it was found that among *V. cholerae* serogoup O1 and O139, ampicillin resistance increased from 35 and 70% respectively to 100% for both serogroups from the year 1992 to 1997. Resistance to furazolidone and streptomycin were also constantly high among *V. cholerae* O1 strains with gradual increase in resistance to other antibiotics such as ciprofloxacin, cotrimoxazole, neomycin and nalidixic acid. *V. cholerae* non-O1, non-O139 strains exhibited high levels of resistance to virtually every class of antibiotics tested in that study which included tetracyclines, aminoglycosides and quinolones among others. Studies by Jun, L *et al*. (2003) detected multiple antibiotic resistances to ampicillin, cefuroxime, amikacin, kanamycin and trimethoprim among fifty-one pathogenic *Vibrio* species from sea fishes in Hong Kong. In a separate study by Manjusha, S *et al*. (2005) out of a total of 119 *Vibrio* strains 83.19% were found to exhibit resistance to one or more of the antibiotics used and 54% showed multiple antibiotic resistance. The highest incidence of antibiotic resistance was evident against

amoxycillin, ampicillin, carbencillin and cefuroxime followed by rifampicin and streptomycin and lowest against chloramphenicol, tetracycline, chlortetracycline, furazolidone, nalidixic acid, gentamycin, sulphafurazole, trimethoprim, neomycin and amikacin (Manjusha, S *et al*., 2005).

Susceptibility patterns to ten antibiotics namely amoxylin, ofloxacin, tetracycline, gentamycin, nitrofurantoin, augmentin, chloramphenicol, cotrimozazole, ceftriazone and ciprofloxacin were investigated in 44 potentially pathogenic halophilic *Vibrio* species which included *V. cholerae*, *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus* and *V. mimicus* isolated from sea foods in Lagos. All the isolates (100%) were resistant to amoxicillin, augmentin, chloramphenicol and nitrofurantoin. Multiple resistance patterns to gentamycin, nitrofurantoin, tetracycline, augmentin, chloramphenicol, amoxycilin, ofloxacin, cotrimozazole, ceftriazone, and ciprofloxacin were observed whilst resistance to all ten antibiotics occured in 18% of the isolates (Adeleye, A *et al*., 2008).

Most recent antibiotic susceptibility tests showed *V. metschnikovii*, *V. parahaemolyticus*, *V. fluvialis*, *V. vulnificus* isolates to portray varying degrees of individual and multiple antibiotic resistances to 21 antibiotics. All the isolates in the study were resistant to ampicillin and sulfamethoxazole, and sensitive to imipenem, meropenem and norfloxacin. All the different species were found to contain one to six of the antibiotic resistance genes of the (sulfamethoxazole-trimethoprim) SXT-like element (Okoh and Igbinosa, 2010). These studies are among numerous other studies that show antibiotic resistance among *Vibrio* species. When infections become resistant to first choice or first line antibiotics, treatment has to be switched to second or third line drugs, which are nearly always expensive (Sibanda and Okoh, 2007). In many poor countries, the high cost of such replacement drugs is prohibitive with the result that some diseases can no longer be treated in areas where resistance to first line antibiotics is widespread (WHO, 2002).

The number of new antibiotics licensed for human use in different parts of the world has become lower than in the previous research and development. The pharmaceutical industry, large academic institutions or the governments are not investing the necessary resources to produce the next generation of newer safe and effective antimicrobial drugs. The potential negative consequences of all these events are relevant because they put society at risk as they may lead to the spread of potentially serious multi antibiotic resistance bacterial infections (Alanis, 2005).

Plants used traditionally as medicines constitute a potentially useful resource for new and safe antibiotics for the treatment of bacterial infections and other diseases (Moshi, MJ *et al*., 2009). The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains (Eloff, 1998).

2.6. Potential of Plants as Sources of New Antibiotics

Plants continue to be a rich source of therapeutic agents, their remarkable contribution to the drug industry was possible because of the large number of phytochemical and biological studies all over the world (Kianbakht and Jahaniani, 2003). Continual research on bioactive substances from plants could possibly lead to the discovery of new compounds, resulting in the formulation of new and more potent antibiotics, to overcome the problem of resistance to currently available antibiotics (Sharma, A *et al*., 2009).

Plants have an intrinsic defense mechanism against predation by pathogenic microorganisms, insects and herbivores. The secondary metabolites synthesized by plants have been found to be a major part of plants' defense mechanism and have also been shown to possess antimicrobial activities *in-vitro.* These metabolites include phenols, phenolics acids, quinones,

terpenoids, essential oils, alkaloids, lectins, polypeptides, flavonoids, flavonols and tannins among other plant compounds (Das, K *et al*., 2010; Cowan, 1999).

Studies on crude extracts of some plants have shown the potential of plants to also possess some antibiotic resistance modifying compounds whilst further studies also led to the isolation of some plant compounds which have been proven to have antibiotic resistance modifying properties. The aqueous extracts of *Camellia sinensis* have been shown to reverse methicillin and penicillin resistance in methicillin resistance *Staphylococcus aureus* and in beta-lactamases producing *Staphylococcus aureus* respectively (Stapleton, PD *et al*., 2004). Synergistic activities against ciprofloxacin resistance *Staphylococcus aureus* were shown in separate combinations of ciprofloxacin with the ethanolic extracts of the plants *Isatis tinctoria* and *Scutellaria baicalensis* (Yang, ZC *et al*., 2005). Studies by Ahmad and Aqil (2006) revealed synergistic interactions among the crude extracts of the plants, *Acorus calamus, Hemidesmus indicus*, *Holarrhena antidysenterica* and *Plumbago zeylanica* with tetracycline and ciprofloxacin against an extended spectrum of beta-lactamase producing multi antibiotic resistant enteric bacteria. The plant compounds ferruginol and 5-Epipisiferol from *Chamaecyparis lawsoniana* (Smith, ECJ *et al*., 2007) , Carsonic acid carnosol from *Rosmarinus officinalis* (Oluwatuyi, M *et al*., 2004) and ethyl gallate from *Caesalpinia spinosa* (Shibata, H *et al*., 2005) have been shown to possess antibiotic resistance modifying properties.

It is universally believed that plants provide an unlimited source of novel and complex chemical structures that most likely would never be the subject or starting point in new drug development synthetic programs for example vinblastine, vincristine, taxol, d-tubocurarine and digoxin, in addition plants are believed to offer a renewable source of starting material in many but not all cases (Fabricant and Farnsworth, 2001).

Most useful drugs derived from plants have been discovered by follow up of ethnomedical / traditional uses (Fabricant and Farnsworth, 2001). In this connection relying on information suggesting that specific plants may yield useful drugs based on long-term use by humans (ethnomedicine) it can be rationalized that any isolated active compounds from the plants are likely to be safer than active compounds from plants with no history of human use and it is also economical in that lesser resources are used in the preliminary search for possible plants with antimicrobial activities (Fabricant and Farnsworth, 2001). The use of traditional medicinal plants still plays a vital role to cover the basic health needs in the developing countries and the use of herbal remedies from these plants has also increased in the developed countries (Srinivasan, K *et al*., 2007).

Traditional medicinal plants are readily available at a local level offering affordable therapeutic alternatives in comparison to conventional antibiotics and medicinal compounds from plants usually have multiple beneficial effects on the body and their actions often act beyond the symptomatic treatment of the disease (Iwu, MW *et al*., 1999). It is therefore essential for systematic evaluation of plants used in traditional medicine for various ailments in search for new antibiotic compounds (Panda, SK *et al*., 2009).

2.7. *Garcinia kola* as a Potential Source of Antibacterial Compounds

Garcinia kola is a traditional medicinal plant that is cultivated and distributed throughout west and central Africa's rain forests and grows as a medium size tree up to 12 m high (Iwu, MW *et al*., 1999). It is extensively used in west and central Africa as a herbal medicine and as a food source since time immemorial (Nzegbule and Mbakwe, 2001). The plant is commonly called "bitter kola" or "male kola" because of its bitter taste, or for its claimed aphrodisiac activity

respectively and it is popular among the people of Nigeria for nervous alertness and induction of insomnia (Uko, OJ *et al*., 2001).

The roots of the plant are used as bitter chew-sticks, the nut is chewed extensively as a masticatory, whilst the stem bark is used as a purgative, the latex is externally applied to fresh wounds to prevent sepsis, thereby assisting in wound healing and the fruit pulp is used in the treatment of jaundice (Uko, OJ *et al*., 2001; Igbozulike and Aremu, 2009). The plant is used for the treatment of liver disorders and has been shown to possess anti-inflammatory, antimicrobial, antioxidant, antiviral, antidiabetic and anti-hepatotoxic activities (Iwu, 1993; Okwu, 2005). The seeds are used in the treatment of bronchitis, throat infections, colics, headaches, chest colds, coughs, diarrhoea, hepatitis, asthma and dysmenorrheal/menstrual cramps (Iwu, 1993; Dalziel, 1937; Okojie, AK *et al*., 2009). The seed has also shown broad spectrum antibacterial activities (Ezeifeka, GO *et al*., 2004; Sibanda and Okoh, 2008; Okigbo and Mmeka, 2008).

Esimone, CO *et al*. (2007) showed the adaptogenic potentials of *Garcinia kola* seeds through *in-vivo* studies in rats and the results proved the seeds' ability to increase non-specific resistance to biologic, physical and chemical stressors. The seeds protected the rats from bacteria-induced mortality and morbidity and also significantly reduced infection-induced leucocytosis. Despite the numerous studies on the antibacterial activities and therapeutic potentials of *Garcinia kola* seeds, there is scanty information on the anti-*Vibrio* activities of the seeds of the plant. Most traditional medicinal plants have been shown to possess anti-*Vibrio* activities such as *Dissotis brazzae*, *Isoglossa lacteal*, *Whitfieldia elongate*, *Strombosia scheffleri* and *Canarium schweinfurthii* (Moshi, MJ *et al*., 2009), *Vicoa indica* (Srinivasan, K *et al*., 2007), *Lippia graveolens, Lantana achyranthifolia, Turnera difusa, Lippia oaxacana, Gymnaloena oaxacana, Cordia curassavica, Lantana camara* and *Acalypha hederacea* (Hernandez, T *et al*., 2003).

Phytochemical analysis of *Garcinia kola* seeds showed the presence of steroids, cardiac glycosides, flavonoids, tannins, saponins and reducing sugars (Adegboye, MF *et al*., 2008). *Garcinia kola* seeds flavonoids have been shown to possess anti-inflammatory (Braide, 1993), anti-hepatotoxic (Braide, 1991) and antimicrobial activities (Madubunyi, 1995). Saponins are known to possess antibacterial activities (Gonzalez-Lamothe, R *et al*., 2009; Cowan, 1999) whilst tannins play an important role in wound healing (Okwu and Josiah, 2006) and also possess some antimicrobial activities (Das, K *et al*., 2010). Complex mixtures of steroid compounds from plants are known to exhibit some bioactivity (Regasini, LO *et al*., 2009).

Studies by Eleyinmi, AF *et al*. (2006) showed the presence of unsaturated and saturated fatty acids mainly linoleic, linolenic and oleic acids in *Garcinia kola* seeds. These fatty acids in plants have been shown to exhibit antimicrobial activities (Walters, D *et al*., 2004; Zheng, CJ *et al*., 2005; Kilic, T *et al*., 2005; Won, S *et al*., 2007; Skalicka-wozniak, K *et al*., 2010). *Garcinia kola* seeds also possess essential oils (Aniche and Uwakwe, 1990) and in this regards, separate studies by Saeed and Tariq (2008) and Snoussi, M *et al*. (2008) showed anti-*Vibrio* activities of some essential oils derived from plants, thus suggesting the need for further assessment of the potentials of the seeds of the plant as a source of anti-*Vibrio* compounds. The adaptogenic potentials of the seeds can also be useful in alleviating the toxic side effects caused by continuous intake of some conventional antibiotics as its seeds were shown to alleviate the hepatic degenerative changes associated with ciprofloxacin in rats (Esimone, CO *et al*., 2007).

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CHAPTER THREE

In-vitro Anti-*Vibrio* Activities of Crude Aqueous and Methanolic Extracts of *Garcinia kola* (Heckel) Seeds.

Abstract

The methanolic and aqueous extracts of *Garcinia kola* seeds were screened for their anti-*Vibrio* activities against 50 *Vibrio* isolates obtained from wastewater final effluents in the Eastern Cape Province, South Africa. The crude extracts at 10 mg/ml exhibited appreciable inhibitory activities against most of the test *Vibrio* isolates with zones of inhibition ranging from 10-19 mm for methanol extract and 8-15 mm for the aqueous extracts. The minimum inhibitory concentrations (MIC) of the methanol extract varied from 0.313 to 2.5 mg/ml while that for the aqueous extract was 10 mg/ml for all the susceptible *Vibrio* isolates. Rate of kill assay of the methanolic extracts against three selected Vibrio species showed bacteriostatic activities against all of them achieving (after 2h exposure time) 58% and 60% (*Vibrio vulnificus* AL042); 68% and 69% (*Vibrio parahaemolyticus* AL049); and 70% and 78% (*Vibrio fluvialis* AL040) killing of the test bacteria at 3× and 4 ×MICs values respectively. We conclude that *Garcinia kola* seeds hold promise as a potential source of therapeutic compounds of relevance in *Vibrio* infections management.

Key words: *Vibrio* species, *Garcinia kola*, Methanol extract, Aqueous extract, MIC, Rate of kill.

Introduction

Vibrio species are ubiquitous in aquatic environment. They appear at particularly high densities in and/or on marine organisms including corals, fish, molluscs, sea grass, sponges, shrimps and zooplankton (Thompson, J *et al*., 1997). Among the major diseases caused by *Vibrio* species is cholera which occurs when *V. cholerae* colonizes the small intestine and releases an enterotoxin (Gopal, S *et al*., 2005). Also, *V. parahaemolyticus, V. alginolyticus* and *V. vulnificus* are known to cause seafood borne infections such as septicemia and wound infections, and *V. vulnificus* has been reported to be responsible for 95% of sea foods related deaths (Todar, 2005).

Extra intestinal *Vibrio* infections often result in serious disability or death (Whitman and Griffin, 1993). Infections by *V. vulnificus*, *V. parahaemolyticus*, and possibly *V. cholerae* non-01 are more likely to cause primary septicemia in persons with pre-existing liver disease such as (chronic active hepatitis, cirrhosis, or iron-storage diseases) or compromised immune systems (for example chronic renal insufficiency, cancer, or diabetes) (Angulo and Swerdlow,1995).

Vibrio species are not an exception when it comes to antibiotic resistance strains. Several studies (Ottaviani, D *et al*., 2001; Jun, L *et al*., 2003; Adeleye, A *et al*., 2008; Okoh and Igbinosa, 2010) have reported the emergence of some antibiotic resistant strains. The development of antibiotic resistance outpaces the development of new drugs such that it has become a worldwide problem that has deleterious long- term effects (Planta, 2007). In developing countries, factors such as inadequate access to effective drugs, unregulated dispensing and manufacture of antibiotics and truncated antibiotic therapy because of cost are contributing to the development of multi-drug resistant organisms (Planta, 2007).

Traditional medicines represented mainly by plants become an alternative as it is relatively safer and is affordable when compared to synthetic antibiotics. Hence the need to increase the body of knowledge on the antimicrobial activities of some traditional medicinal

plants such as *Garcinia kola* towards curbing the effects of antibiotic resistance in such virulent pathogens as *Vibrio* species becomes imperative.

Garcinia kola is a plant of west and central African origin (Iwu, 1993). It is commonly referred to as bitter kola for its bitter taste and has the popular acronym "wonder plant" amongst the south western Nigerian people because every part of it has been found to be of medicinal importance (Dalziel, 1937). In Nigeria, the seed is chewed for the relief of cough, colds, colic, hoarseness of voice, and throat infections. The plant is also used for the treatment of liver disorders, jaundice, high fever and as a purgative and chewing stick (Iwu, 1993). The seed has proven antimicrobial activities (Nwaokorie, F *et al*., 2010; Sibanda and Okoh, 2008; Akinpelu, DA *et al*., 2008; Ezeifeka, GO *et al*., 2004) and it has been employed in the treatment of various ailments.

The phytochemical analysis of methanol and sterile distilled water in the ratio 3:2 extract of *Garcinia kola* seeds powder revealed the presence of flavonoids, tannins, cardiac glycoside, steroids, saponins and reducing sugars. These phytochemical compounds are known to play important roles in bioactivity of medicinal plants (Akinpelu, DA *et al*., 2008). Although studies have been carried out that show the antimicrobial activities of crude extracts of *Garcinia kola* seeds, to the best of our knowledge there is paucity of information of the anti-*Vibrio* potentials of the aqueous and methanolic extracts of the seeds of this plant especially against environmental strains of the bacteria such as those isolated from wastewater environments. In the light of the increasing trend of multiple antibiotic resistance in *Vibrio* species isolated in the South African aquatic milieu (Okoh and Igbinosa, 2010) and the pathogenicity of *Vibrio* species to humans (Health protection agency, 2007) the exploration for new anti-*Vibrio* compounds especially of plants origin becomes necessary. In this paper therefore we report on the anti-*Vibrio* potentials of crude aqueous and methanol extracts of *Garcinia kola* seeds.

Materials and Methods

Plant Material

Ground powder of the *Garcinia kola* seeds were obtained from the plant material collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, University of Fort Hare Alice. South Africa.

Preparation of extracts

The solvent extracts of the plant were prepared in accordance with the description of Basri and Fan (2005). Briefly, 100 grams of the seed powder was steeped in 500 ml of the respective solvent (methanol or water) for 48 h with shaking. The resultant extract was centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was then filtered through Whatman No.1 filter paper while the residue was then used in the second extraction with 300 ml of the respective solvents. After the second extraction process, the aqueous extract was freeze-dried at -50 °C under vacuum, whereas methanol extracts were concentrated under reduced pressure using a rotary evaporator at 65 °C. The concentrated extracts were then allowed to dry to a constant weight under a stream of air in a fume cupboard at room temperature. Dimethyl sulphoxide (DMSO) at a concentration equal to 5% of the total volume which was made up with sterile distilled water was used to aid the reconstitution of the dried methanol extract when making different test concentrations whilst the water extracts were reconstituted in sterile distilled water.

Test *Vibrio* strains

The test *Vibrio* isolates (50 in all) used in this study were obtained from the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice, South Africa. The bacteria were isolated from wastewater effluents (Igbinosa, EO *et al*., 2009; Okoh and Igbinosa, 2010) and belonged to five species groups viz. *Vibrio*. sp.(unidentified to the species level), *V. parahaemolyticus, V. fluvialis*, V. *vulnificus*, *V. metschnikovii*.

Preparation of the Inoculum

The inoculums of the test organisms were prepared using the colony suspension method (EUCAST, 2003). Colonies picked from 24 hour old cultures grown on nutrient agar plates were used to make suspensions of the test organisms in saline solution (0.85% NaCl) to give an optical density of approximately 0.1 at 600 nm. The suspension was then diluted a hundred-fold before use.

Antibacterial susceptibility test

The sensitivity of each crude extract of the plant was determined using the agar well diffusion method as described by Irobi, ON *et al*. (1996), with modifications. The prepared bacterial suspension (100 µl) was inoculated into sterile molten Mueller- Hinton agar medium at 50 ºC in a MacCartney bottle, mixed gently and then poured into a sterile petri dish and allowed to solidify. A sterile 6 mm diameter cork borer was used to bore wells into the agar medium. The wells were then filled up with approximately 100 µl of the extract solution at a concentration of 10 mg/ml taking care to prevent spillage onto the surface of the agar medium. The plates were allowed to

stand on the laboratory bench for 1 hour to allow proper diffusion of the extract into the medium after which the plates were incubated at 37 ºC for 24 hours, and thereafter the plates were observed for zones of inhibition and measured. Ciprofloxacin (2 µg/ml) was used as a positive control, and distilled water was used as the negative control while 5% Dimethyl sulphoxide (DMSO) was also tested to determine its effect on each organism.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The MICs were determined only for the test *Vibrio* that had shown susceptibility to the crude extracts using the broth microdilution method as outlined by the EUCAST (2003) in sterile disposable flat-bottomed 96-well microtiter plates. Two-fold serial dilutions using sterile distilled water were carried out from 10 mg/ml stock plant extracts to make 9 test concentrations ranging from 0.039 to 10 mg/ml for each solvent extract. A 100 µl volume of double strength Mueller-Hinton broth was introduced into all the 96 wells and 50 µl of the varying concentrations of the extracts were added in decreasing order along with 50 µl of the test organism suspension. Column 1 was used as the sterility wells containing 100 µl of the Mueller-Hinton broth and 100 µl sterile distilled water, column 2 was used as the positive control wells containing 100 µl of the broth, 50 µl of Ciprofloxacin and 50 µl of the test organism whilst column 3 was used as the negative control wells containing 100 µl of the broth, 50µl sterile distilled water and 50 µl of the test organism whilst columns 4 to 12 were used as test wells containing 100µl of the broth, 50µl of the extract concentration and 50 µl of the test *Vibrios*. The plates were then incubated at 37 °C for 18-24 hr. Results were read visually by adding 40 µl of 0.2 mg/ml of ρ-iodonitrotetrazolium violet (INT) dissolved in sterile distilled water into each well (Eloff, 1998). A pinkish coloration is indicative of microbial growth because of their ability to convert INT to red formazan

(Iwalewa, EO *et al*., 2009).The MIC was recorded as the lowest concentration of the extract that prevented the appearance of visible growth of the organism after 24 hour of incubation (EUCAST, 2003).

The minimum bactericidal concentration (MBC) was determined from the MIC broth microdilution assays by subculturing 10 µl volumes from each well that did not exhibit growth after 24 hours of incubation and spot inoculating it onto fresh Mueller-Hinton agar plates (Sudjana, AN *et al*., 2009). The plates were incubated for 48 hours after which the numbers of colonies were counted. The MBC was defined as the lowest concentration killing more than or equal to 99.9% of the inoculum compared with initial viable counts (Sudjana, AN *et al*., 2009).

Rate of kill assay

The time kill assay was done according to the method of Odenholt, I *et al*. (2001). Three selected test *Vibrio* isolates namely *V. vulnificus* (AL042), *V. parahaemolyticus* (AL049) and *V. fluvialis* (AL040) were used for the rate of kill studies on the basis of grouping on MIC levels viz 0.625, 1.25 and 2.5 mg/ml and medical importance of the species. The assay was done for the methanol extract only which proved to be more active when compared to the aqueous extract. The turbidity of the 18 hour old test *Vibrio* was first standardized to 10^8 cfu/ml. Four different concentrations of the plant extract were made starting from the MIC to 4 × MIC value for each test organism. A 0.5 ml volume of known cell density from each organism suspension was added to 4.5 ml of different concentrations of the extracts solutions, held at room temperature and the rate of kill determined over a period of 2 hours. Exactly 0.5 ml volume of each suspension was withdrawn at 15 minutes intervals and transferred to 4.5 ml of nutrient broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compound carryovers on the test organisms (Akinpelu, D *et al*., 2008). The suspension was then serially diluted and 0.5 ml was

plated out for viable counts using the pour plate method. The plates were thereafter incubated at 37 °C for 48 hours. The control plates contained the test organism without the plant extracts. The emergent colonies were counted and compared with the counts of the culture control.

Results

Anti-*Vibrio* activities of the crude extracts

The results of the anti-*Vibrio* activities of the methanol and aqueous crude extracts of *Garcinia kola* seeds are shown in Table 3.1.The methanol extract showed activity against 16 (34%) of the test bacteria whilst the aqueous extract had activity against 12 (24%) out of the 50 *Vibrio* isolates. The zones of inhibition ranged from 8-20 mm for methanol extracts and 8-14 mm for the aqueous extracts. *V. fluvialis* (AL040) had the highest zones of inhibition for both extracts. All the isolates that were susceptible to the aqueous extract were all susceptible to the methanol extract as well. It appears that the methanol extract has more potent bacterial activity compared to the aqueous extract. The 5% DMSO and sterile distilled water negative controls had no anti-*Vibrio* activity on all tested *Vibrio* species.

MIC and MBC assays

The MIC and MBC results are presented in Table 3.2. The methanol extract had MIC and MBC values ranging from 0.313-2.5 mg/ml and 10- >10 mg/ml respectively, whilst for the aqueous extracts the MIC and MBC values were higher ranging from 10- >10 mg/ml and above 10 mg/ml respectively. *V. fluvialis* (AL031) and *V. parahaemolyticus* (AL032) had the lowest MIC values of 0.313 mg/ml for methanol and the highest MIC value was from *V. vulnificus* (AL042) (2.5

mg/ml). For the aqueous extract *V. vulnificus* (AL042) and *V. fluvialis* (AL022) had the highest MIC values of >10 mg/ml whilst all the other *Vibrios* had MIC values of 10 mg/ml. The MBC values for the methanol extract were above 10 mg/ml for 5 *Vibrio* isolates whilst the rest of the isolates had values of 10 mg/ml, for aqueous extracts all the isolates had MBC values of above 10 mg/ml.

Rate of kill assay

The rate of kill assay was carried out for the methanol extract only based on its higher activity compared to the aqueous extract. Three different *Vibrio* species namely *V. vulnificus* (AL042), *V. parahaemolyticus* (AL049) and *V. fluvialis* (AL040) were selected for this analysis and the results are as shown in Figures 3.1, 3.2 and 3.3 respectively. The percentage of bacteria cells killed at 1×, 2×, 3× and 4 × MIC values respectively for each *Vibrio* specie after 2 hour exposure time, were 48.8, 53.6, 58 and 60% for *V. vulnificus* (AL042) (Figure 3.1); 63.7, 64.1, 68.2 and 68.9% for *V. parahaemolyticus* (AL049) (Figure 3.2); and 52.0, 62.5, 70.3 and 78.1 % for *V. fluvialis* (AL040) (Figure 3.3). The number of bacteria cells killed for each *Vibrio* specie increased as the time and the concentration of the extract increased.

Table 3.1: Anti- *Vibrio* activities of crude methanol and aqueous extracts of *Garcinia kola* seeds.

ORGANISM	EXTRACTS		ORGANISM	EXTRACTS	
	METHANOL	AQUEOUS		METHANOL	AQUEOUS
Vibrio species (EL 031)	- (0)	- (0)	*Vibrio* species (AL 020)	+ (15)	+ (8)
V. parahaemolyticus (AL 043)	+ (15)	+ (8)	*V. vulnificus* (AL 001)	- (0)	- (0)
V. fluvialis (AL 025)	- (0)	- (0)	*V. fluvialis* (AL002)	- (0)	- (0)
Vibrio species (AL021)	+ (13)	- (0)	*Vibrio* species (AL035)	- (0)	- (0)
V. vulnificus (AL042)	+ (13)	+ (10)	*V. vulnificus* (AL048)	+ (8)	- (0)
V. metschnikovii (AL012)	- (0)	- (0)	*V. vulnificus* (AL018)	- (0)	- (0)
V. vulnificus (AL041)	- (0)	- (0)	*V. fluvialis* (AL036)	- (0)	- (0)
Vibrio species (AL 050)	- (0)	- (0)	*V. fluvialis* (AL013)	- (0)	- (0)
V. fluvialis (AL 022)	+ (12)	+ (9)	*V. parahaemolyticus* (AL017)	- (0)	- (0)
V. vulnificus (AL 024)	- (0)	- (0)	*V. vulnificus* (AL038)	- (0)	- (0)
V. fluvialis (AL014)	- (0)	- (0)	*V. parahaemolyticus* (AL049)	+ (12)	+ (9)
V. parahaemolyticus (AL009)	- (0)	- (0)	*V. vulnificus* (AL011)	- (0)	- (0)
V. fluvialis (AL037)	- (0)	- (0)	*V. fluvialis* (AL033)	- (0)	- (0)
V. vulnificus (AL039)	- (0)	- (0)	*V. fluvialis* (AL004)	+ (11)	- (0)
V. parahaemolyticus (DM 015)	- (0)	- (0)	*V. parahaemolyticus* (AL003)	- (0)	- (0)
Vibrio species (AL005)	- (0)	- (0)	*V. fluvialis* (AL006)	- (0)	- (0)
V. fluvialis (AL031)	+ (15)	+ (8)	*V. fluvialis* (AL027)	- (0)	- (0)
V. fluvialis (AL040)	+ (20)	+ (14)	*Vibrio* species (EL 027)	- (0)	- (0)
V. parahaemolyticus (AL008)	- (0)	- (0)	*V. vulnificus* (AL015)	- (0)	- (0)
V. parahaemolyticus (AL030)	+ (11)	+ (8)	*V. parahaemolyticus* (AL032)	+ (13)	+ (8)
V. parahaemolyticus (EL009)	+ (14)	- (0)	*V. vulnificus* (AL044)	- (0)	- (0)
V. vulnificus (AL029)	- (0)	- (0)	*V. parahaemolyticus* (AL045)	+ (12)	+ (9)
V. metschnikovii (AL023)	+ (12)	+ (8)	*Vibrio* species (AL047)	- (0)	- (0)
V. fluvialis (AL019)	+ (11)	+ (8)	*V. metschnikovii* (AL 016)	- (0)	- (0)
V. parahaemolyticus (AL028)	- (0)	- (0)	*Vibrio* species (EL 047)	- (0)	- (0)

Key: (+) denotes susceptible to the extract, (-) denotes not susceptible, (number) denotes diameter of zone of inhibition in mm.

Table 3.2: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the methanol and aqueous extracts against the susceptible *Vibrio* isolates.

ORGANISM	**EXTRACTS**			
	METHANOL		**AQUEOUS**	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
V. vulnificus (AL042)	2.5	>10	>10	>10
V. fluvialis (AL019)	1.25	>10	10	>10
V. parahaemolyticus (AL049)	1.25	>10	10	>10
V. parahaemolyticus (AL045)	1.25	>10	10	>10
Vibrio. species (AL021)	0.625	10	_	_
V. fluvialis (AL022)	0.625	10	>10	>10
V. metschnikovii (AL023)	0.625	10	10	>10
V. parahaemolyticus (AL030)	0.625	10	10	>10
Vibrio. species (AL020)	0.625	10	10	>10
V. fluvialis (AL040)	0.625	10	10	>10
V. fluvialis (AL031)	0.313	10	10	>10
V. parahaemolyticus (AL032)	0.313	10	10	>10
V. parahaemolyticus (AL043)	0.625	10	10	>10
V. parahaemolyticus (EL009)	0.625	10	_	_
V. fluvialis (AL004)	0.625	10	_	_
V. vulnificus (AL048)	1.25	>10	_	_

Key: MIC denotes minimum inhibitory concentration, MBC denotes minimum bactericidal concentration, - denotes not determined.

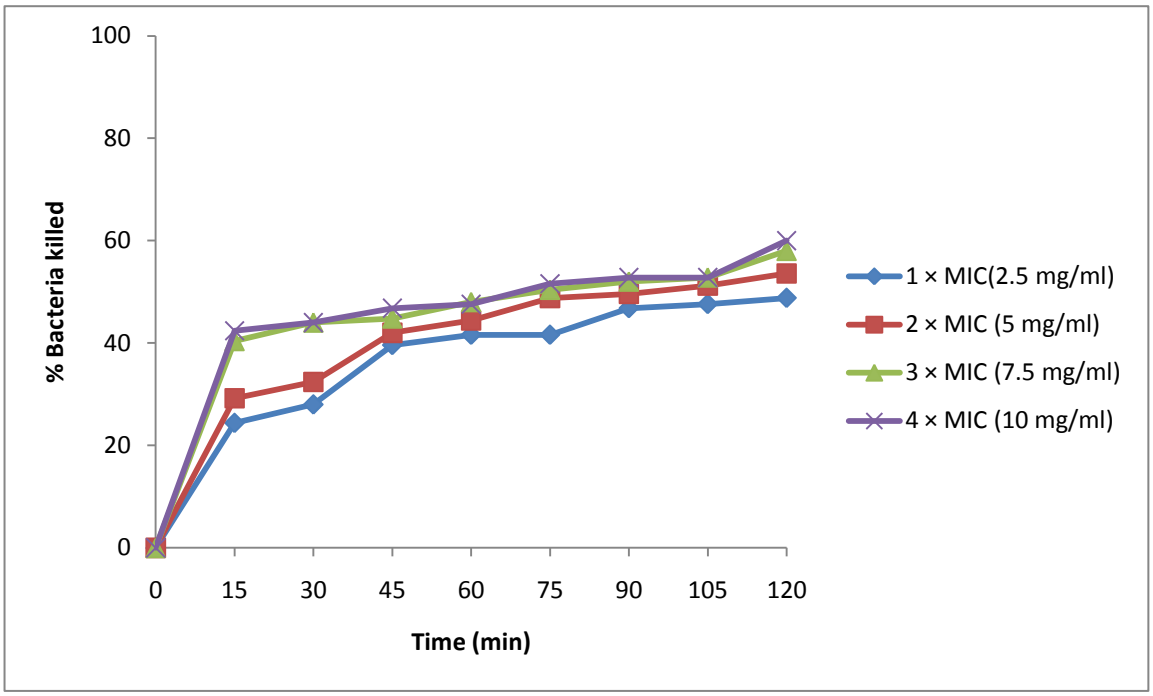

Figure 3.1: Rate of kill of *V. vulnificus* (AL042) by crude methanol extract of *Garcinia kola* seeds.

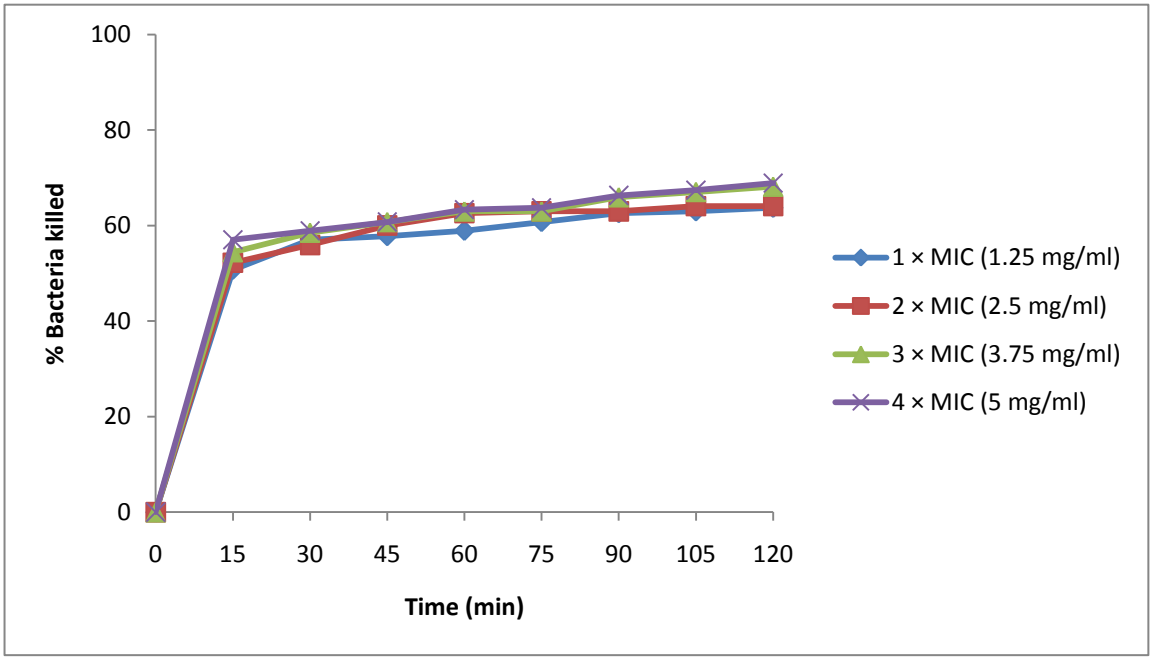

Figure 3.2: Rate of kill of *V. parahaemolyticus* (AL049) by crude methanol extract of *Garcinia kola* seeds.

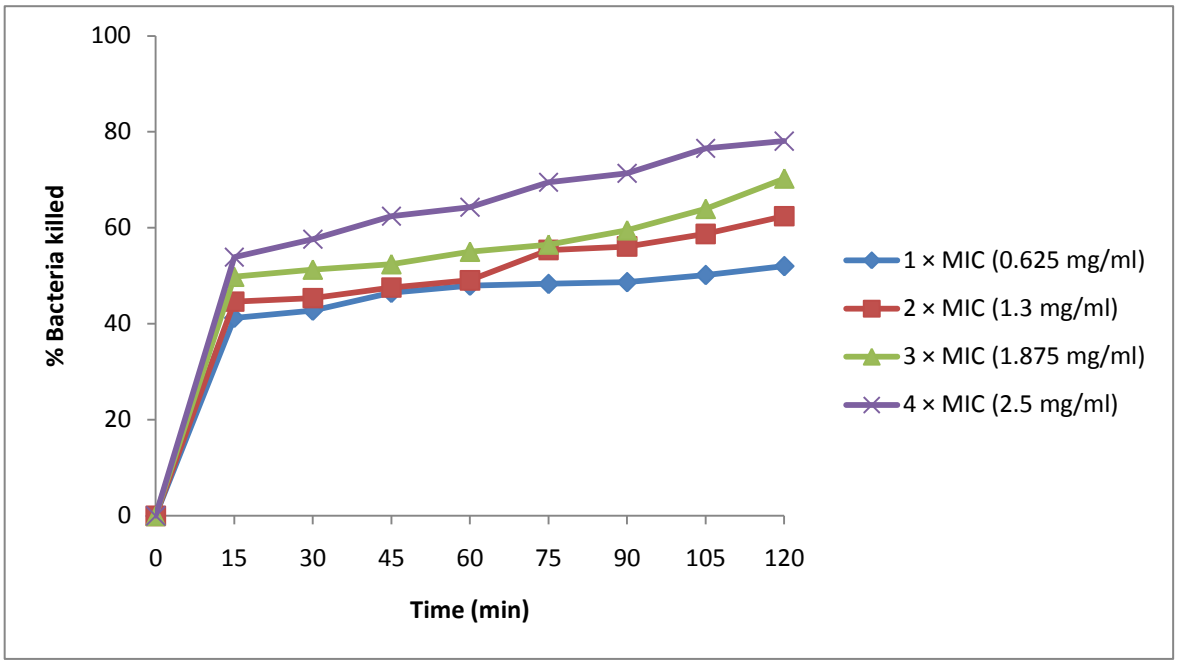

Figure 3.3: Rate of kill of *V. fluvialis* (AL040) by crude methanol extract of *Garcinia kola* seeds.

Discussion

This study has revealed that both methanolic and aqueous extracts of the *Garcinia kola* seeds have antagonistic activities against *V. vulnificus*, *V. fluvialis*, *V. parahaemolyticus* and *V. metschnikovii* and some unidentified *Vibrio* species. The antagonistic activity exhibited by the aqueous extract validate the traditional use of the plant for the treatment of diarrhoea (Dalziel, 1937), high fever (Iwu, 1993), stomach aches (Ajebesone and Aina, 2004) and abdominal colicky pain (Adaramoye, OA *et al*., 2005) as some *Vibrio* species such as *V. parahaemolyticus* and *V. vulnificus* are food poisoning bacteria that can cause such symptoms (Ho, H *et al*., 2009). Also, our finding corroborates previous reports on the antibacterial activities of *Garcinia kola* seeds (Sibanda and Okoh, 2008; Nwaokorie, F *et al*., 2010; Ezeifeka, GO *et al*., 2004).

Nevertheless, Ogbulie, JN *et al*. (2007) reported contrary findings to ours. In that study, they showed that both hot and cold aqueous and ethanolic extracts of *Garcinia kola* seeds had no activity against a *Vibrio* specie used in the study, although they had variable activities against other gram negative bacteria such as *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* with the ethanolic extracts having more activity compared to the aqueous extracts. The limitations of the study by Ogbulie, JN *et al*. (2007) in comparison to ours is that only one *Vibrio* isolate was tested such that it cannot conclusively be used to determine the anti-*Vibrio* potential of *Garcinia kola* seeds.

Although both the aqueous and methanol extract of the *Garcinia kola* seeds had anti-*Vibrio* activities, the methanol extract was more active. The difference in activity between the two solvent extracts has been attributed to a better solubility of the active agents; xanthones, benzophenones, and flavonoids especially biflavonoid type GB1 (Xu and Lee, 2001, Han, QB *et al*., 2005) in organic solvents than in water (Taiwo, O *et al*., 1999; Obi and Onuoha, 2000; Ogueke, CC *et al*., 2006: Ogbulie, JN *et al*., 2007; Nwaokorie, F *et al*., 2010). These phytochemical compounds have been known to play different roles in antimicrobial potentials of medicinal plants. For example, previous reports have demonstrated the anti-diarrhoeal activity of tannins (Dharmananda, 2003), flavonoids (Galvez, J *et al*., 1993), saponins and reducing sugars (Otshudi, AL *et al*., 2000) containing plant extracts. The phytochemicals outlined above are also present in *Garcinia kola* seeds and might be responsible for the anti-*Vibrio* activities found in this study more so as most *Vibrio* species are implicated in diarrhoea

The rate of kill of the selected test *Vibrio* species by the methanol extract proved to be generally concentration and time dependent with the rate of kill increasing with increasing concentration of the extract and times of exposures (Figures 3.1, 3. 2 and 3.3). Also, the pattern

of activity suggest that the extract is bacteriostatic against all the three *Vibrio* species tested at 1×, 2×, 3× and 4 × MIC in line with the interpretation key of Pankey and Sabath (2004) which recommended a 99.9% reduction in cell counts to be considered bactericidal. The percentages of cells killed during the rate of kill experiment varied from 48.8 to 78.1%, thus suggesting *Garcinia kola* to be a potential source of active compounds of relevance in anti-*Vibrio* chemotherapy.

Conclusion

This study has shown that both aqueous and methanol extracts of the seeds of *Garcinia kola* have antagonistic activities against *Vibrio* species, although the methanol extract appears to be more active. The low MIC values observed for the methanol extract are good starting points for further research that can lead to the isolation, purification and characterization of active compounds for new anti-*Vibrio* drug development purposes which is a subject of on-going investigation in our group.

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CHAPTER FOUR

The *In-vitro* Anti-bacterial Activities of Crude Dichloromethane Extracts of *Garcinia kola* (Heckel) Seeds Against Potentially Pathogenic *Vibrio* Species

Abstract

Dichloromethane extracts of the seeds of *Garcinia kola* were screened for anti-*Vibrio* activities against a panel of 50 *Vibrio* isolates comprised of five different species namely *V. parahaemolyticus, V. vulnificus, V. fluvialis* and *V. metschnikovii* and *Vibrio* sp. (not identified to the specie level). The extract had activity against 16 of the *Vibrio* isolates at a screening concentration of 10 mg/ml, with zones of inhibition ranging from 9-15 mm. The minimum inhibitory concentration (MIC) varied between 0.313 mg/ml and 0.625 mg/ml, while the minimum bactericidal concentration (MBC) ranged between 5 and 10 mg/ml. The rate of kill analysis revealed about 87.8%, 82.2% and 64.4% of *V. parahaemolyticus* (AL049), *V. fluvialis* (AL040) and *V. vulnificus* (AL042) respectively were killed during 2 hours of exposure to 4 × MIC of the extract. We conclude that the dichloromethane extract of *Garcinia kola* seeds could be an important source of compounds of value in the treatment of infections caused by *Vibrio* species.

Keywords: Dichloromethane extract, *Garcinia kola* seeds, *Vibrio* species, MIC, Rate of kill.

Introduction

The use of traditional medicine supported mainly by medicinal plants is on the increase in many developing countries due to limited availability, accessibility and high costs of pharmaceutical drugs, whilst in comparison traditional health care is familiar, often available at the local level and is affordable, thereby playing an important role in national healthcare globally (Patwardhan, 2005). Plants have been used as remedies for a wide range of diseases since time immemorial, and although this knowledge has been passed down through oral history there still remains a need for the identified plants to be scientifically screened for new drug development purposes.

Arihan and Ozkan (2007) reported that plant antimicrobials derived from traditional indigenous knowledge benefit pharmaceutical companies in terms of less cost of screening as often the plants would be active compared to screening of tens of thousands molecules with perhaps only one emerging as a new drug in a very expensive process that could take on the average 15 years and a conservative estimate of about $800 million for a single drug to be developed.

Plants contain bioactive compounds which normally serve as plant defence mechanisms against microorganisms, insects and herbivores. These compounds also evolve to increase plants resistance as some of the plant predators become resistant to the plant's defence mechanism compounds (Cowan, 1999; Gamboa-Angulo, MM *et al*., 2008). Also, numerous plants and their compounds such as phenolics (quinones, flavonoids, tannins and coumarins), terpenoids, essential oils, alkaloids, lectins, polypeptides and poly acetylenes have been shown to possess antimicrobial activity (Cowan, 1999).

Garcinia kola is one such medicinal plant and it is used in folklore remedies in sub-Saharan Africa for the treatment of various ailments such as liver disorders, hepatitis, diarrhoea

and bronchitis (Iwu, 1993; Iwu, 1999). The plant has also been found to be useful in the treatment of stomach ache and gastritis (Ajebesone and Aina, 2004). The seed is masticatory and is also used to prevent and relieve colic, chest colds, and cough and can be used as well to treat headaches (Iwu, 1993). The wide variety of ailments treated by this plant shows its vast potential as an effective antimicrobial agent. Numerous studies have shown the antimicrobial activities and therapeutic potentials of this plant (Adefule-Ositelu, AO *et al*., 2010; Ezeifeka, GO *et al*., 2004; Sibanda and Okoh, 2008; Tebekeme and Prosper, 2007). However, there is paucity of information on the potential of the seeds of this plant for treatment of infections caused by *Vibrio* species.

Vibrio species are such gram negative rod shaped bacteria that have shown grown resistance to conventional antibiotics (Okoh and Igbinosa, 2010; Adeleye, A *et al*., 2008; Jun, L *et al*., 2003). The taxonomy of *Vibrio* genus of the family *Vibrionaceae* contains at least 34 recognised species (Health protection agency, 2007) and is continuously being updated due to the addition of new species. Studies are now also focusing on other emerging *Vibrio* species other than cholera causing *Vibrios* only as they have been implicated in causing mild to severe *Vibriosis* in humans (Tantillo, GM *et al*., 2004). *Vibriosis* is a disease caused by an infection with bacteria of the *Vibrio* genus, most commonly *Vibrio parahaemolyticus* or *Vibrio vulnificus*. The infections are variable in severity and are characterized by diarrhoea, vomiting, primary septicemia (illness associated with bacteria in the bloodstream), or wound infections (Wisconsin Division of Public health, 2008) and are common in both developed and developing countries, and currently, there are 12 *Vibrio* species that are pathogenic to humans namely *V. cholera*, *V. cincinnatiensis*, *V. damsel*, *V. fluvialis*, *V. furnissii*, *V. hollisae*, *V. metschnikovii*, *V. mimicus*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. carchariae* (Farmer and Hickman-

Brenner, 1992; Health protection agency, 2007). In this paper, we report on the anti-*Vibrio* activities of dichloromethane extracts of the seeds of *Garcinia kola* (Heckel).

Materials and Methods

Plant Material

Ground powder of the *Garcinia kola* seeds were obtained from the plant material collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, University of Fort Hare Alice. South Africa.

Preparation of extracts

The dichloromethane solvent extract of the plant was prepared in accordance with the description of Basri and Fan (2005). Briefly, 100 grams of the seed powder was steeped in 500 ml of dichloromethane for 48 h with shaking. The resultant extract was centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was then filtered through Whatman No.1 filter paper while the residue was then used in the second extraction with 300 ml of dichloromethane. After the second extraction process, the extracts were concentrated under reduced pressure using a rotary evaporator at 50 °C. The concentrated extracts were then allowed to dry to a constant weight under a stream of air in a fume cupboard at room temperature. Dimethyl sulphoxide (DMSO) at a concentration equal to 5% of the total volume which was made up with sterile distilled water was used to aid the reconstitution of the dried extracts when making different test concentrations.

Test *Vibrio* strains

The test *Vibrio* isolates (50 in all) used in this study were obtained from the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice, South Africa. The bacteria were isolated from wastewater effluents (Igbinosa, EO *et al*., 2009; Okoh and Igbinosa, 2010) and belonged to five species viz. *V. parahaemolyticus*, *V. fluvialis*, V. *vulnificus*, *V. metschnikovii* and some *Vibrio* sp. (unidentified to the specie level).

Preparation of the Inoculum

The inoculums of the test organisms were prepared using the colony suspension method (EUCAST, 2003). Colonies picked from 24 hour old cultures grown on nutrient agar plates were used to make suspensions of the test organisms in saline solution (0.85% NaCl) to give an optical density of approximately 0.1 at 600 nm. The suspension was then diluted a hundred-fold before use.

Antibacterial susceptibility test

The sensitivity of the crude extract of the plant was determined using the agar well diffusion method as described by Irobi, ON *et al*. (1996), with modifications. The prepared bacterial suspension (100 µl) was inoculated into sterile molten Mueller- Hinton agar medium at 50 ºC in a MacCartney bottle, mixed gently and then poured into a sterile petri dish and allowed to solidify. A sterile 6 mm diameter cork borer was used to bore wells into the agar medium. The

wells were then filled up with approximately 100 µl of the extract solution at a concentration of 10 mg/ml taking care to prevent spillage onto the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 hour to allow proper diffusion of the extract into the medium after which the plates were incubated at 37 ºC for 24 hours, and thereafter the plates were observed for zones of inhibition and measured. Ciprofloxacin (2 µg/ml) was used as a positive control, and distilled water was used as the negative control while 5% Dimethyl sulphoxide (DMSO) was also tested to determine its effect on each organism.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The MICs were determined only for the test *Vibrio* that had shown susceptibility to the crude extracts using the broth microdilution method as outlined by the EUCAST (2003) in sterile disposable flat-bottomed 96-well microtiter plates. Two-fold serial dilutions using sterile distilled water were carried out from the 10 mg/ml stock plant extract to make 9 test concentrations ranging from 0.039 to 10 mg/ml for each solvent extract. A 100 µl volume of double strength Mueller-Hinton broth was introduced into all the 96 wells and 50 µl of the varying concentrations of the extract were added in decreasing order along with 50 µl of the test organism suspension. Column 1 was used as the sterility wells containing 100 µl of the Mueller-Hinton broth and 100 µl sterile distilled water, column 2 was used as the positive control wells containing 100 µl of the broth, 50 µl of Ciprofloxacin and 50 µl of the test organism whilst column 3 was used as the negative control wells containing 100 µl of the broth , 50µl sterile distilled water and 50 µl of the test organism whilst columns 4 to 12 were used as test wells

containing 100µl of the broth, 50µl of the extract concentration and 50 µl of the test *Vibrios*. The plates were then incubated at 37 °C for 18-24 hr. Results were read visually by adding 40 µl of 0.2 mg/ml of ρ-iodonitrotetrazolium violet (INT) dissolved in sterile distilled water into each well (Eloff, 1998). A pinkish coloration is indicative of microbial growth because of their ability to convert INT to red formazan (Iwalewa, EO et al., 2009).The MIC was recorded as the lowest concentration of the extract that prevented the appearance of visible growth of the organism after 24 hour of incubation (EUCAST, 2003).

The minimum bactericidal concentration (MBC) was determined from the MIC broth microdilution assays by subculturing 10 µl volumes from each well that did not exhibit growth after 24 hours of incubation and spot inoculating it onto fresh Mueller-Hinton agar plates (Sudjana, AN *et al*., 2009).The plates were incubated for 48 hours after which the number of colonies were counted. The MBC was defined as the lowest concentration killing more than or equal to 99.9% of the inoculum compared with initial viable counts (Sudjana, AN *et al*., 2009).

Rate of kill assay

The time kill assay was done according to the method of Odenholt, I *et al*. (2001). Three selected test *Vibrio* isolates namely *V. vulnificus* (AL042), *V. parahaemolyticus* (AL049) and *V. fluvialis* (AL040) were used for the rate of kill studies on the basis of grouping on MIC levels viz 0.313 and 0.625 mg/ml and medical importance of the species. The turbidity of the 18 hour old test *Vibrio* was first standardized to 10^8 cfu/ml. Four different concentrations of the plant extract were made starting from the MIC to 4 × MIC value for each test organism. A 0.5 ml volume of known cell density from each organism suspension was added to 4.5 ml of different

concentrations of the extracts' solutions, held at room temperature and the rate of kill determined over a period of 2 hours. Exactly 0.5 ml volume of each suspension was withdrawn at 15 minutes intervals and transferred to 4.5 ml of nutrient broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compound carryovers on the test organisms (Akinpelu, D *et al*., 2008). The suspension was then serially diluted and 0.5 ml was plated out for viable counts using the pour plate method. The plates were thereafter incubated at 37 °C for 48 hours. The control plates contained the test organism without the plant extracts. The emergent colonies were counted and compared with the counts of the culture control.

Results

Anti-*Vibrio* activities of the crude dichloromethane extracts

The results of the anti-*Vibrio* assay of the dichloromethane extract of *Garcinia kola* seeds are shown in Table 4.1. The zones of inhibition ranged from 9 mm to 15 mm with the largest zone of inhibition observed against *V. vulnificus* (AL042) and the lowest against *V. fluvialis* (AL004).

MIC and MBC assays

The MIC and MBC results of the susceptible *Vibrio* isolates are shown in Table 4.2. A total of 5 *Vibrio* isolates had MIC value of 0.313 mg/ml whilst the remaining 11 isolates had an MIC value of 0.625 mg/ml. Two isolates *Vibrio* specie (AL020) and *V. parahaemolyticus* (AL032) had MBC values of 5 mg/ml whilst the rest had MBC values of 10 mg/ml.

Table 4.1: The Anti- *Vibrio* activities of the crude dichloromethane (DCM) extract of *Garcinia kola* seeds.

ORGANISM	**DCM EXTRACT**	**ORGANISM**	**DCM EXTRACT**
Vibrio sp. (EL 031)	- (0)	*Vibrio* sp. (AL 020)	+ (11)
V. parahaemolyticus (AL 043)	+ (10)	*V. vulnificus* (AL 001)	- (0)
V. fluvialis (AL 025)	- (0)	*V. fluvialis* (AL002)	- (0)
Vibrio sp. (AL021)	+ (10)	*Vibrio* sp. (AL035)	- (0)
V. vulnificus (AL042)	+ (15)	*V. vulnificus* (AL048)	+ (10)
V. metschnikovii (AL012)	- (0)	*V. vulnificus* (AL018)	- (0)
V. vulnificus (AL041)	- (0)	*V. fluvialis* (AL036)	- (0)
Vibrio sp. (AL 050)	- (0)	*V. fluvialis* (AL013)	- (0)
V. fluvialis (AL 022)	+ (11)	*V. parahaemolyticus* (AL017)	- (0)
V. vulnificus (AL 024)	- (0)	*V. vulnificus* (AL038)	- (0)
V. fluvialis (AL014)	- (0)	*V. parahaemolyticus* (AL049)	+ (11)
V. parahaemolyticus (AL009)	- (0)	*V. vulnificus* (AL011)	- (0)
V. fluvialis (AL037)	- (0)	*V. fluvialis* (AL033)	- (0)
V. vulnificus (AL039)	- (0)	*V. fluvialis* (AL004)	+ (9)
V. parahaemolyticus (DM 015)	- (0)	*V. parahaemolyticus* (AL003)	- (0)
Vibrio sp. (AL005)	- (0)	*V. fluvialis* (AL006)	- (0)
V. fluvialis (AL031)	+ (13)	*V. fluvialis* (AL027)	- (0)
V. fluvialis (AL040)	+ (14)	*Vibrio* sp. (EL 027)	- (0)
V. parahaemolyticus (AL008)	- (0)	*V. vulnificus* (AL015)	- (0)
V. parahaemolyticus (AL030)	+ (11)	*V. parahaemolyticus* (AL032)	+ (11)
V. parahaemolyticus (EL009)	+ (11)	*V. vulnificus* (AL044)	- (0)
V. vulnificus (AL029)	- (0)	*V. parahaemolyticus* (AL045)	+ (11)
V. metschnikovii (AL023)	+ (11)	*Vibrio* sp. (AL047)	- (0)
V. fluvialis (AL019)	+ (13)	*V. metschnikovii* (AL 016)	- (0)
V. parahaemolyticus (AL028)	- (0)	*Vibrio* sp. (EL 047)	- (0)

Key: (+) denotes susceptible to the extract, (-) denotes not susceptible, (number) denotes diameter of zone of inhibition in mm.

Table 4.2: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the Dichloromethane (DCM) extract against susceptible *Vibrio* isolates.

ORGANISM	**DCM EXTRACT**	
	MIC (mg/ml)	**MBC (mg/ml)**
V. vulnificus (AL042)	0.625	10
V. fluvialis (AL019)	0.313	10
V. parahaemolyticus (AL049)	0.313	10
V. parahaemolyticus (AL045)	0.313	10
Vibrio sp. (AL021)	0.625	10
V. fluvialis (AL022)	0.625	10
V. metschnikovii (AL023)	0.625	10
V. parahaemolyticus (AL030)	0.625	10
Vibrio sp. (AL020)	0.313	5
V. fluvialis (AL040)	0.625	10
V. fluvialis (AL031)	0.625	10
V. parahaemolyticus (AL032)	0.313	5
V. parahaemolyticus (AL043)	0.625	10
V. parahaemolyticus (EL009)	0.625	10
V. fluvialis (AL004)	0.625	10
V. vulnificus (AL048)	0.625	10

Key: MIC denotes minimum inhibitory concentration, MBC denotes minimum bactericidal concentration.

Rate of kill assay

The rate of kill results of the dichloromethane extracts against three representative *Vibrio* species are as shown in Figures 4.1, 4.2 and 4.3 for *V. fluvialis* (AL040), *V. vulnificus* (AL042) and *V. parahaemolyticus* (AL049) respectively. The percentage bacteria cells killed after 2 hour

exposure time at 1×, 2×, 3× and 4× MICs respectively were 79.9%, 79.9%, 81.8% and 82.2% for *V. fluvialis* (AL040); 44.8%, 56.4 %, 60.8% and 64.4% for *V. vulnificus* (AL042); and 82.2%, 83%, 86.3% and 87.8% for *V. parahaemolyticus* (AL049).

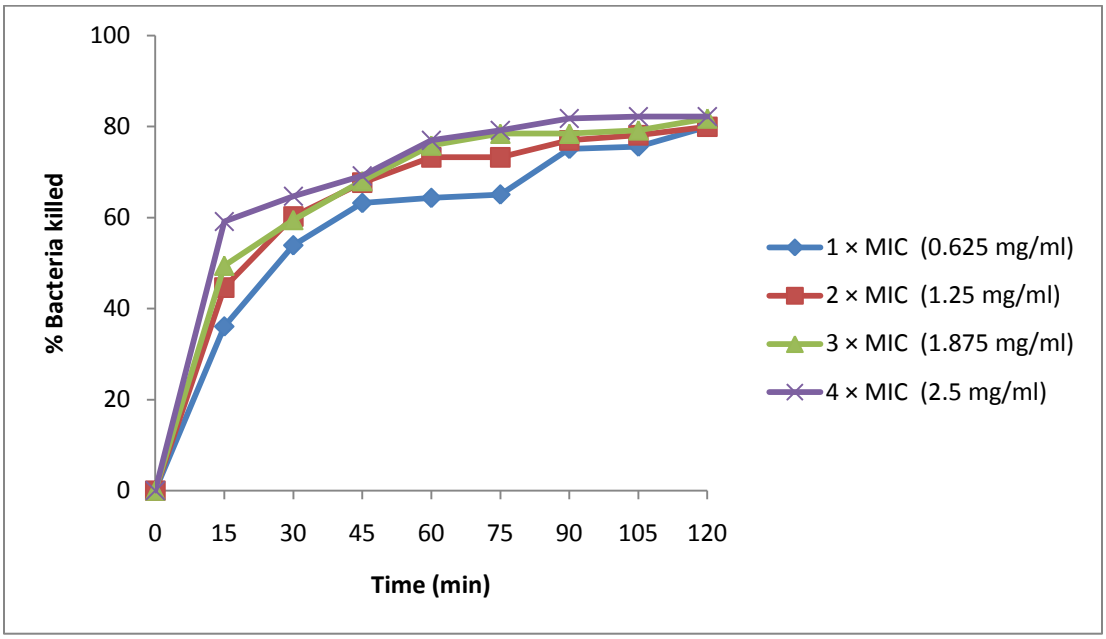

Figure 4.1: Rate of kill for *V. fluvialis* (AL040) by crude dichloromethane extract of *Garcinia kola* seeds.

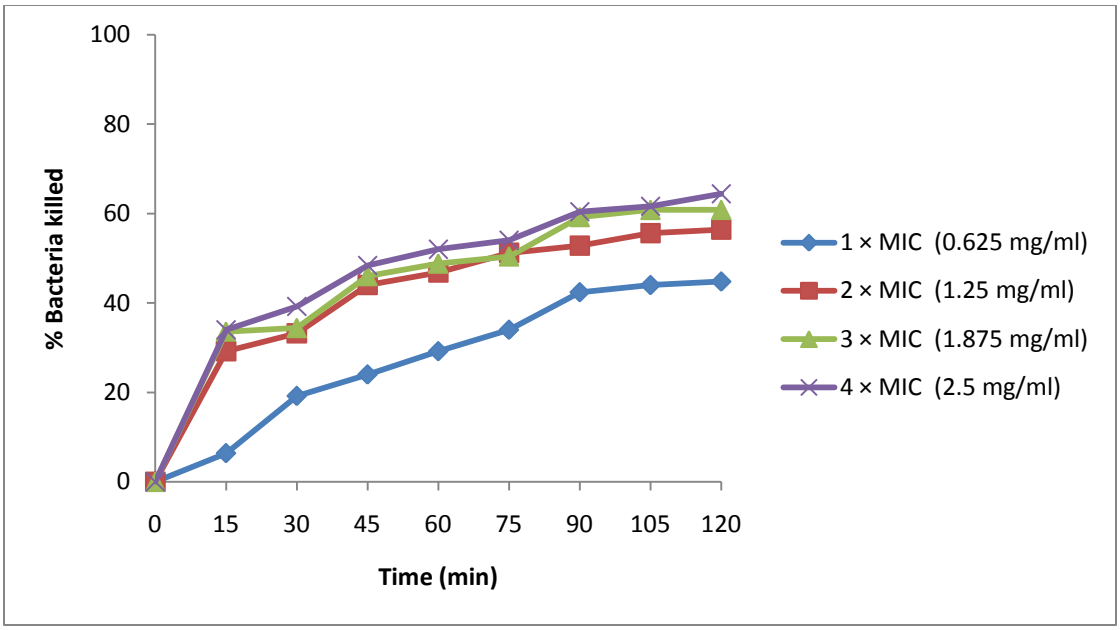

Figure 4.2: Rate of kill of *V. vulnificus* (AL042) by crude dichloromethane extract of *Garcinia kola* seeds

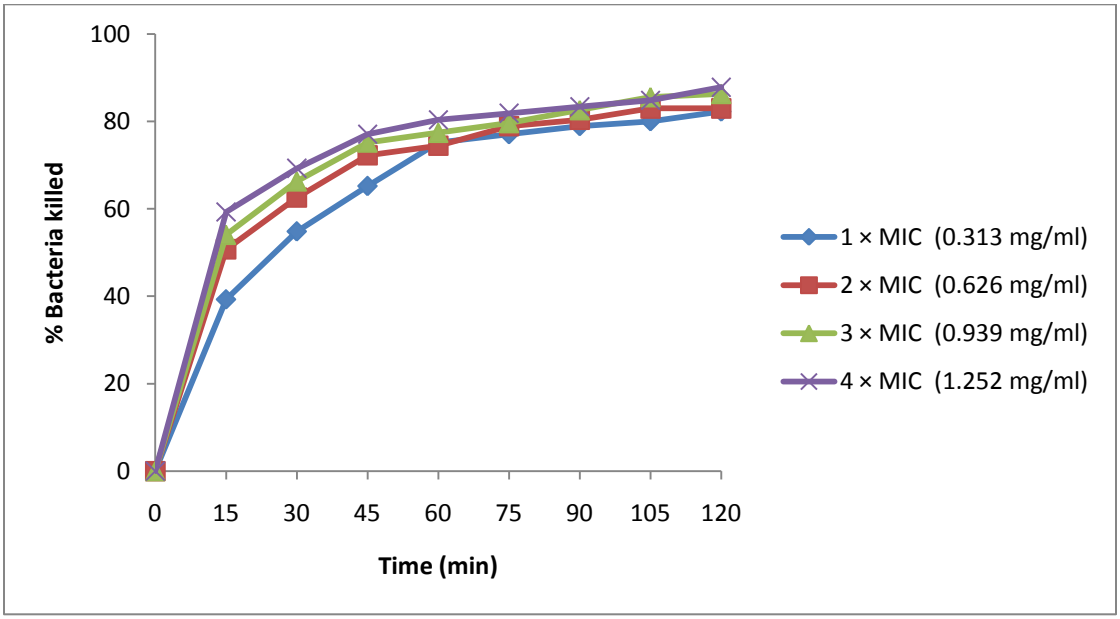

Figure 4.3: Rate of kill of *V. parahaemolyticus* (AL049) by crude dichloromethane extract of *Garcinia kola* seeds.

Discussion

Appreciable anti-*Vibrio* activities were exhibited by the dichloromethane extract of *Garcinia kola* seeds giving zones of inhibition ranging between 9 and 15 mm. The extract was antagonistic to *V. parahaemolyticus, V. fluvialis, V. vulnificus, V. metschnikovii* and some other *Vibrio* sp., with MICs varying from 0.313-0.625 mg/ml and MBCs in the range of 5-10 mg/ml. In a similar report, dichloromethane extracts of the plants *Dissotis brazzae*, *Isoglossa lacteal*, *Whitfieldia elongate*, *Strombosia scheffleri* and *Canarium schweinfurthii* had MICs ranging between 0.16-7.8 mg/ml against a clinical isolate of *Vibrio cholera* (Moshi, MJ *et al*., 2009), although this study was carried our using only one *Vibrio* specie as against ours that utilized several, and as such gave a more dependable picture of the anti-*Vibrio* potential of plant materials.

In another study, dichloromethane extracts of the sea weeds (*Asparagopsis armata*, *Ceramium rubrum*, *Drachiella minuta*, *Falkenbergia rufolanosa*, *Gracilaria cornea* and *Halopitys incurvus*) was antagonistic against *Vibrio anguillarum* MICs ranging from < 100 µg/ml to > 400 µg/ml (Bansemir, A *et al*., 2006). It would appear that dichloromethane is a reliable solvent for extraction of anti-*Vibrio* compounds from plant materials. However, a contrary report (Berahou, A *et al*., 2007) suggest that this characteristic is dependent on the constitution of the plant material as dichloromethane fraction of the methanolic extract of *Quercus ilex* bark had no antagonistic effect on *V. cholerae.*

The time kill experiments suggest that the rate of kill of the *Vibrio* species were both time and concentration dependent such that the highest percentage number of bacteria cells killed were achieved at the highest MIC after 2 hours of exposure in all three test *Vibrio* species, being 82.2% for *V. fluvialis* (AL040), 64.4% for *V. vulnificus* (AL042) and 87.8% for *V.*

parahaemolyticus (AL049). Hence, the dichloromethane extract are by definition bacteriostatic as more than the 99.9% killing rate characteristic for a cidal agent is not met (Pankey and Sabath, 2004). In the treatment of an infection, bacteriostasis is often effective because the killing and elimination of the pathogen are further mediated through host immune defences (Kummerer, 2009).

Dichloromethane is known to extract essential oils from plants extract. Essential oils are mixtures of mono and sesquiterpenic hydrocarbons and their oxygenated compounds such as ethers, alcohols, ketones, aldehydes, lactones, oxides and carboxylic acids. Aniche and Uwakwe (1990) showed the presence of essential oils in *Garcinia kola* seeds. A number of essential oils/volatile oils components have been identified as effective antibacterials, for example carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde and cinnamic acid, (Burt, 2004). Studies on plants essential oils have shown their anti-*Vibrio* properties, in a study by Snoussi, M *et al*. (2008) the essential oils of the plants *Mentha longifolia*, *Mentha pulegium*, *Eugenia caryophyllata*, *Thymus vulgaris* and *Rosmarinus officinalis* were found to exhibit anti-*Vibrio* activities against *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis* and *V. vulnificus*. In a separate study clove essential oil was found to also exhibit anti-*Vibrio* activities against 5 *Vibrio* isolates (Saeed and Tariq, 2008).

Terpenes which are constituents of essential oils occur as diterpenes, triterpens ,tetraterpenes, hemiterpenes and sesquiterpenes and when they contain additional oxygen they are termed terpenoids, and all are known to possess antimicrobial activities. In a study by Onayade, OA *et al*. (1998), *Garcinia kola* seeds were found to contain monoterpenes and degradation products thereof (sesquiterpenes and diterpenes) and their hydrocarbons. The major components of the mixture of the volatiles were 6-methylhept-5-en-2-one and (E, E)-farnesol, 5-

ethenyldihydro-5-methylfuran-2-one (lavender lactone) and linalol. The compound 6-Methylhept-5-en-2-one found in *Garcinia kola* seeds is a constituent of many essential oils and contributes considerably to the formation of total flavours of certain foodstuffs has many biological activities including insecticidal activity (Onayade, OA *et al*., 1998), and these may have contributed to the anti-*Vibrio* activities dichloromethane extract of *Garcinia kola* seeds in this study.

Conclusion

The anti-*Vibrio* activities exhibited by the dichloromethane extract of the seeds of *Garcinia kola* in this study show the plant's potential as a source of compounds that could be of relevance in the treatment and management of infections caused by *Vibrio* species. Elucidation and characterisation of the active compounds through a bioassay directed fractionation of the dichloromethane extract are necessary follow ups to this study.

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CHAPTER FIVE

The *In-vitro* Anti-bacterial Activities of Crude n-Hexane Extracts of *Garcinia kola* (Heckel) Seeds Against Some *Vibrio* Bacteria Isolated From Waste Water Effluents

Abstract

Crude n-Hexane extracts of the seed of *Garcinia kola* (*G. kola*) were screened for their *in-vitro* anti-*Vibrio* activities against 50 *Vibrio* bacteria isolated from wastewater final effluents in the Eastern Cape Province of South Africa. The extract at a screening concentration of 10 g/ml resulted in zones of inhibition ranging from 10-14 mm against the susceptible isolate and the minimum inhibitory concentrations (MIC) varied between 0.313 mg/ml and 0.625 mg/ml. The rate of kill assay was done against 3 representative isolates namely *V. vulnificus* (AL042), *V. parahaemolyticus* (AL049) and V. fluvialis (AL040). The results showed appreciable biocidal activity after 2 hours exposure time at 4 × MIC with *V. vulnificus* (AL042) having 91.6% bacteria cells killed; *V. parahaemolyticus* (AL049) had 92.6% cells killed; and *V. fluvialis* (AL040) had 96.3% killed. We conclude that *Garcinia kola* seeds are a potential source of compounds that could be useful in the treatment of infections caused by *Vibrio* bacteria.

Key words: n-Hexane extract, *Garcinia kola*, *Vibrio* species, MIC, Rate of kill.

Introduction

Wound infections, gastroenteritis and primary septicemia are the three well recognized clinical syndromes of *Vibrio* infections (Tantillo, GM *et al*., 2004). These infections are generally acquired either through ingestion of foods and water contaminated with human faecal matter or sewage, raw seafood, or from exposure to skin lesions, such as cuts, open wounds and abrasions, to aquatic environments and marine animals (Lee and Younger, 2002). The infections are usually more life-threatening in people with underlying medical conditions or weakened immune systems (Tantillo, GM *et al*., 2004; Di Pinto, A *et al*., 2008) such as people with liver diseases, Acquired immune deficiency syndrome (AIDS) and diabetes.

In developing countries, the fraction of treated wastewater effluents being discharged into water bodies such as rivers have increased resulting in high densities of disease causing bacteria such as *Vibrio* species in these water bodies (Igbinosa and Okoh, 2008). Most of these water bodies are used for drinking water, household and recreational purposes such as swimming and fishing by the people living in the surrounding communities and they are therefore at risk of acquiring *Vibrio* infections.

Although *Vibrio* species are autochthonous of the aquatic environment, the final effluents discharged into water sources add on to the *Vibrio* population and also become a source of nutrients which favour abundant growth and proliferation of the organism. Several authors have emphasized that there is a broad consensus on the need to monitor the presence of *Vibrios* in the environment and to study their pathogenicity potential in order to properly protect human health (Baffone, W *et al*., 2006; Jones and Oliver, 2009; Canigral, I *et al*., 2009). Several studies have

shown the presence of *Vibrio* species in chlorinated final effluents from several wastewater treatment plants (Igbinosa, EO *et al*., 2009; Dungeni, M *et al*., 2010).

Antibiotics used in *Vibrio* infections treatments include tetracycline and its derivatives such as doxycycline, fluoroquinolones (e.g. ciprofloxacin), third-generation cephalosporins (e.g. ceftazidime), and aminoglycosides (e.g. gentamicin) (Daniels and Shafaie, 2000; Schwartz and Jagar, 2010). As a consequence of increasing incidences of resistance to these antibiotics, most of them are no longer recommended as first-line therapy and treatment protocols are thus based on local antibiogram data (Daniels and Shafaie, 2000). Also, the use of these antibiotics is limited in pregnant women and pediatrics because of their toxicity.

Medicinal plants have been used as folklore remedies over the years to treat, manage or control man's ailments as they contain large varieties of chemical substances that possess important therapeutic properties used in the treatment of these ailments (Akinpelu, DA *et al*., 2008), with the added advantage of been safer to use in terms of their less toxicity long term use (Fabricant and Farnsworth, 2001) in comparison with synthetic antibiotics. A typical example of such medicinal plants is *Garcinia kola*.

Garcinia kola is a medium sized forest tree that is well known in west and central Africa for its vast medicinal properties. The seed powder has been used traditionally since time immemorial to treat intestinal pains, diarrhoea, menstrual pains, fevers, jaundice, headaches, diabetes, anaemia, angina, liver disorders and also as an antidote against ingested poison (Adegoke, EO *et al*., 1981).

Studies have been carried out that show the antimicrobial activity of extracts of *Garcinia kola* seeds and other parts of the plant. The seed has shown broad spectrum antibacterial

activities against clinical and environmental strains of both gram negative and gram positive bacteria (Sibanda and Okoh, 2008; Akinpelu, DA *et al*., 2008; Okigbo and Mmeka 2008). It also has proven adaptogenic properties (Esimone, CO *et al*., 2007) and analgesic/anti-inflammatory effects in knee osteoarthritis patients (Adegbehingbe, OO *et al*., 2008).

The seeds of the plant have shown appreciable medicinal properties that aroused our interest to test its efficacy against *Vibrio* bacteria. The *Vibrio* species used in this study were shown in a study by Igbinosa, EO *et al*. (2009) to have survived the treatment processes of a wastewater treatment facility either as free-living organisms or as plankton-associated entities and showed resistant to chlorine disinfection at normal recommended concentrations in water. Hence, these *Vibrio* species posed a potential health risk to the rural communities which depend on the watershed for domestic and recreational purposes. Some of the *Vibrio* isolates were also shown to be resistant to more than one antibiotic (Okoh and Igbinosa, 2010). Antibiotics are one of the most important groups of pharmaceuticals such that antibiotic resistance is one of the major challenges for human and veterinary medicine (Kummerer, 2009). In this study, we report on the *in-vitro* anti-*Vibrio* activities of n-Hexane extracts of the seeds of *Garcinia kola* in an attempt to identify alternative compounds of relevance in anti-*Vibrio* chemotherapy.

Materials and Methods

Plant Material

Ground powder of the *Garcinia kola* seeds were obtained from the plant material collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, University of Fort Hare Alice. South Africa.

Preparation of extracts

The solvent extracts of the plant were prepared in accordance with the description of Basri and Fan (2005). Briefly, 100 grams of the seed powder was steeped in 500 ml of n-Hexane for 48 h with shaking. The resultant extract was centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was then filtered through Whatman No.1 filter paper while the residue was then used in the second extraction with 300 ml of n-Hexane. After the second extraction process, the extracts were concentrated under reduced pressure using a rotary evaporator at 50 °C. The concentrated extracts were then allowed to dry to a constant weight under a stream of air in a laminar flow at room temperature. Dimethyl sulphoxide (DMSO) at a concentration of 5% of the total volume which was made up with sterile distilled water was used to aid the reconstitution of the dried extracts when making different test concentrations.

Test *Vibrio* strains

The test *Vibrio* isolates (50 in all) used in this study were obtained from the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice, South Africa. The bacteria were isolated from wastewater effluents (Igbinosa, EO *et al*., 2009; Okoh and Igbinosa, 2010) and belonged to five species groups viz. *V. parahaemolyticus*, *V. fluvialis*, V. *vulnificus* and *V. metschnikovii* and some *Vibrio* sp. (unidentified to the specie level).

Preparation of the Inoculum

The inoculums of the test organisms were prepared using the colony suspension method (EUCAST, 2003). Colonies picked from 24 hour old cultures grown on nutrient agar plates were used to make suspensions of the test organisms in saline solution (0.85% NaCl) to give an optical density of approximately 0.1 at 600 nm. The suspension was then diluted a hundred-fold before use.

Antibacterial susceptibility test

The susceptibility of the *Vibrio* isolates to the crude n-Hexane extract was determined using the agar well diffusion method as described by Irobi, ON *et al*. (1996), with modifications. The prepared bacterial suspension (100 µl) was inoculated into sterile molten Mueller-Hinton agar medium at 50 ºC in a MacCartney bottle, mixed gently and then poured into a sterile petri dish and allowed to solidify. A sterile 6 mm diameter cork borer was used to bore wells into the agar medium. The wells were then filled up with approximately 100 µl of the extract solution at a concentration of 10 mg/ml taking care to prevent spillage onto the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 hour to allow proper diffusion of the extract into the medium after which the plates were incubated at 37 ºC for 24 hours, and thereafter the plates were observed for zones of inhibition and measured. Ciprofloxacin (2 µg/ml) was used as a positive control, and distilled water was used as the negative control while 5% Dimethyl sulphoxide (DMSO) was also tested to determine its effect on each organism.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

The MICs were determined only for the test *Vibrio* that had shown susceptibility to the crude extracts using the broth microdilution method as outlined by the EUCAST (2003) in sterile disposable flat-bottomed 96-well microtiter plates. Two-fold serial dilutions using sterile distilled water were carried out from 10 mg/ml stock plant extracts to make 9 test concentrations ranging from 0.039 to 10 mg/ml for each solvent extract. A 100 µl volume of double strength Mueller-Hinton broth was introduced into all the 96 wells and 50 µl of the varying concentrations of the extracts were added in decreasing order along with 50 µl of the test organism suspension. Column 1 was used as the sterility wells containing 100 µl of the Mueller-Hinton broth and 100 µl sterile distilled water, column 2 was used as the positive control wells containing 100 µl of the broth, 50 µl of Ciprofloxacin and 50 µl of the test organism whilst column 3 was used as the negative control wells containing 100 µl of the broth , 50 µl sterile distilled water and 50 µl of the test organism whilst columns 4 to 12 were used at test wells containing 100 µl of the broth, 50 µl of the extract concentration and 50 µl of the test *Vibrios*. The plates were then incubated at 37 °C for 18-24 hr. Results were read visually by adding 40 µl of 0.2 mg/ml of ρ-iodonitrotetrazolium violet (INT) dissolved in sterile distilled water into each well (Eloff, 1998). A pinkish coloration is indicative of microbial growth because of their ability to convert INT to red formazan (Iwalewa, EO *et al*., 2009).The MIC was recorded as the lowest concentration of the extract that prevented the appearance of visible growth of the organism after 24 hours of incubation (EUCAST, 2003).

The minimum bactericidal concentration (MBC) was determined from the MIC broth microdilution assays by subculturing 10 µl volumes from each well that did not exhibit growth

after 24 hours of incubation and spot inoculating it onto fresh Mueller-Hinton agar plates (Sudjana, AN *et al*., 2009). The plates were incubated for 48 hours after which the number of colonies was counted. The MBC was defined as the lowest concentration killing more than or equal to 99.9% of the inoculum compared with initial viable counts (Sudjana, AN *et al*., 2009).

Rate of kill assay

The time kill assay was done according to the method of Odenholt, I *et al*. (2001). Three selected test *Vibrio* isolates namely *V. vulnificus* (AL042), *V. parahaemolyticus* (AL049) and *V. fluvialis* (AL040) were used for the rate of kill studies on the basis of grouping on MIC levels viz 0.313 and 0.625 mg/ml and medical importance of the species. The turbidity of the 18 hour old test *Vibrio* was first standardized to 10^8 cfu/ml. Four different concentrations of the plant extract were made starting from the MIC to 4 × MIC for each test organism. A 0.5 ml volume of known cell density from each organism suspension was added to 4.5 ml of different concentrations of the extracts solutions, held at room temperature and the rate of kill determined over a period of 2 hours. Exactly 0.5 ml volume of each suspension was withdrawn at 15 minutes intervals and transferred to 4.5 ml of nutrient broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compound carryovers on the test organisms (Akinpelu, DA., *et al* 2008). The suspension was then serially diluted and 0.5 ml was plated out for viable counts using the pour plate method. The plates were thereafter incubated at 37 °C for 48 hours. The control plates contained the test organism without the plant extracts. The emergent colonies were counted and compared with the counts of the culture control.

Results

Anti-*Vibrio* activities of the crude extracts

The results of the anti-*Vibrio* activities of the n-Hexane extract of *Garcinia kola* seeds are shown in Table 5.1. The crude extract had activity against 16 (32%) of the test bacteria. The zones of inhibition ranged from 10–14 mm with the highest zones being observed from *V. vulnificus* (AL042) and *V. fluvialis* (AL040) at 14 mm and the least being from *V. fluvialis* (AL004), *V .fluvialis* (AL019) and *V. vulnificus* (AL048) at 10 mm. The 5% DMSO and sterile distilled water negative controls had no anti-*Vibrio* activity on all tested *Vibrio* species.

Table 5.1: The Anti- *Vibrio* activities of crude n-Hexane extract of *Garcinia kola* seeds.

ORGANISM	**n-HEXANE EXTRACT (10 mg/ml)**	**ORGANISM**	**n-HEXANE EXTRACT (10 mg/ml)**
Vibrio species (EL 031)	- (0)	*Vibrio* species (AL 020)	+ (11)
V. parahaemolyticus (AL 043)	+ (11)	*V. vulnificus* (AL 001)	- (0)
V. fluvialis (AL 025)	- (0)	*V. fluvialis* (AL002)	- (0)
Vibrio species (AL021)	+ (11)	*Vibrio* species (AL035)	- (0)
V. vulnificus (AL042)	+ (14)	*V. vulnificus* (AL048)	+ (10)
V. metschnikovii (AL012)	- (0)	*V. vulnificus* (AL018)	- (0)
V. vulnificus (AL041)	- (0)	*V. fluvialis* (AL036)	- (0)
Vibrio species (AL 050)	- (0)	*V. fluvialis* (AL013)	- (0)
V. fluvialis (AL 022)	+ (12)	*V. parahaemolyticus* (AL017)	- (0)
V. vulnificus (AL 024)	- (0)	*V. vulnificus* (AL038)	- (0)
V. fluvialis (AL014)	- (0)	*V. parahaemolyticus* (AL049)	+ (12)
V. parahaemolyticus (AL009)	- (0)	*V. vulnificus* (AL011)	- (0)
V. fluvialis (AL037)	- (0)	*V. fluvialis* (AL033)	- (0)
V. vulnificus (AL039)	- (0)	*V. fluvialis* (AL004)	+ (10)
V. parahaemolyticus (DM 015)	- (0)	*V. parahaemolyticus* (AL003)	- (0)
Vibrio species (AL005)	- (0)	*V. fluvialis* (AL006)	- (0)
V. fluvialis (AL031)	+ (11)	*V. fluvialis* (AL027)	- (0)
V. fluvialis (AL040)	+ (14)	*Vibrio* species (EL 027)	- (0)
V. parahaemolyticus (AL008)	- (0)	*V. vulnificus* (AL015)	- (0)
V. parahaemolyticus (AL030)	+ (10)	*V. parahaemolyticus* (AL032)	+ (11)
V. parahaemolyticus (EL009)	+ (11)	*V. vulnificus* (AL044)	- (0)
V. vulnificus (AL029)	- (0)	*V. parahaemolyticus* (AL045)	+ (11)
V. metschnikovii (AL023)	+ (12)	*Vibrio* species (AL047)	- (0)
V. fluvialis (AL019)	+ (10)	*V. metschnikovii* (AL 016)	- (0)
V. parahaemolyticus (AL028)	- (0)	*Vibrio* species (EL 047)	- (0)

Key: (+) denotes susceptible to the extract, (-) denotes not susceptible, (number) denotes diameter of zone of inhibition in mm.

MIC and MBC assays

The results of the MIC and MBC assays are as presented in Table 5.2. The extracts showed low MIC values, with 10 isolates having an MIC value of 0.313 mg/ml and the remaining 6 isolates had MIC values of 0.625 mg/ml. *V. fluvialis* (AL031) and *V. parahaemolyticus* (AL032) had the lowest MBC value of 5 mg/ml whilst the rest of the isolates had MBC values of 10 mg/ml.

Table 5.2: Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the hexane extract against susceptible *Vibrio* isolates.

ORGANISM	**n-HEXANE EXTRACT**	
	MIC (mg/ml)	**MBC (mg/ml)**
V. vulnificus (AL042)	0.625	10
V. fluvialis (AL019)	0.313	10
V. parahaemolyticus (AL049)	0.313	10
V. parahaemolyticus (AL045)	0.313	10
Vibrio. species (AL021)	0.625	10
V. fluvialis (AL022)	0.625	10
V. metschnikovii (AL023)	0.625	10
V. parahaemolyticus (AL030)	0.313	10
Vibrio. species (AL020)	0.313	10
V. fluvialis (AL040)	0.313	10
V. fluvialis (AL031)	0.313	5
V. parahaemolyticus (AL032)	0.313	5
V. parahaemolyticus (AL043)	0.313	10
V. parahaemolyticus (EL009)	0.313	10
V. fluvialis (AL004)	0.625	10
V. vulnificus (AL048)	0.625	10

Key: MIC denotes minimum inhibitory concentration, MBC denotes minimum bactericidal concentration.

Rate of kill assay

Figures 1, 2 and 3 show the rate of kill of *V. fluvialis* (ALO40), *V. parahaemolyticus* (AL049) and *V. vulnificus* (AL042) by the crude extract. The percentage of bacteria cells killed at 1×, 2×, 3× and 4 × MIC respectively for each *Vibrio* specie after 2 hour exposure time, were 74, 79.6, 90.7 and 96.3 % for *V. fluvialis* (AL040) (Figure 5.1); 76.3, 78.2, 84.4 and 92.6 % for *V. parahaemolyticus* (AL049) (Figure 5.2); and 52.8, 61.2, 71.2 and 91.6 % for *V. vulnificus* (AL042) (Figure 5.3). The number of bacteria cells killed for each *Vibrio* specie increased as the time and the concentration of the extract increased.

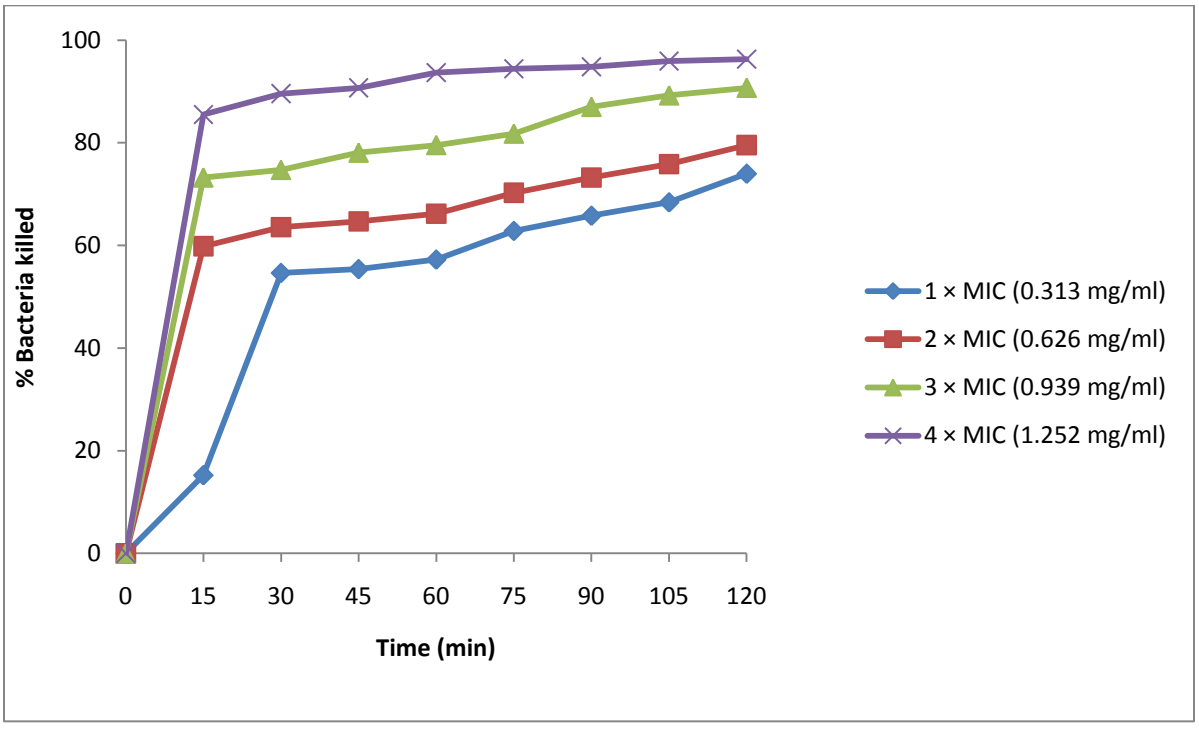

Figure 5.1: Rate of kill of *V. fluvialis* (AL040) by crude n-Hexane extract of *Garcinia kola* seeds.

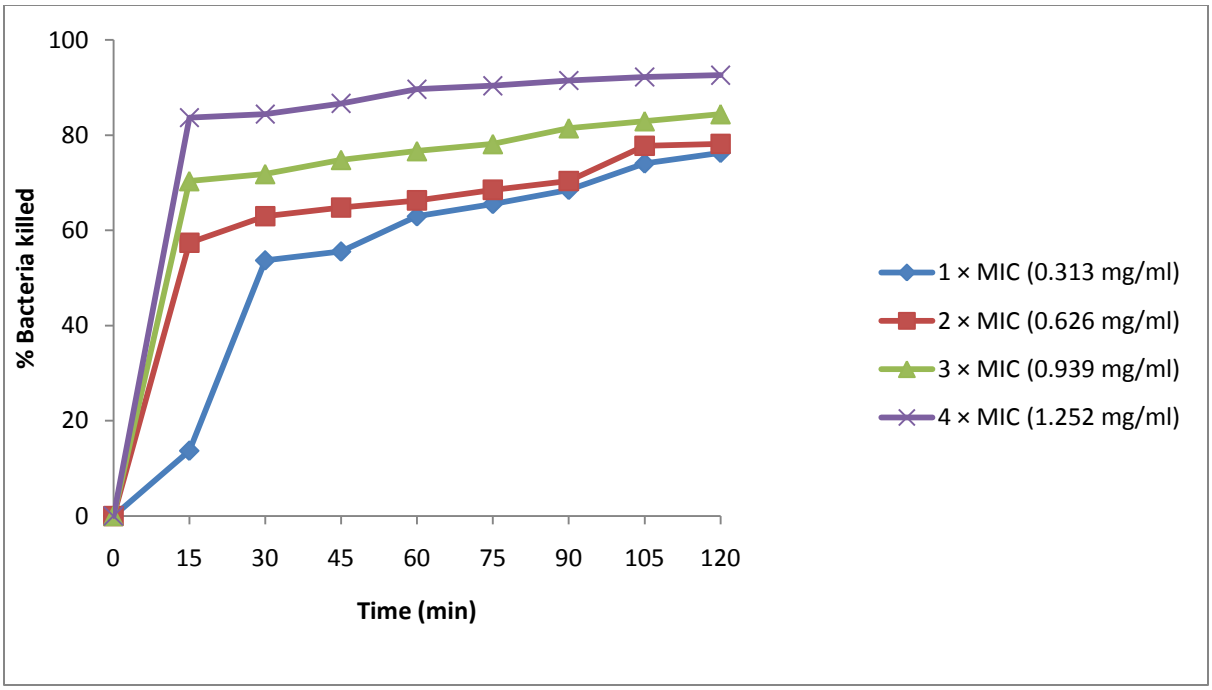

Figure 5.2: Rate of kill of *V. parahaemolyticus* (AL049) by crude n-Hexane extract of *Garcinia kola* seeds.

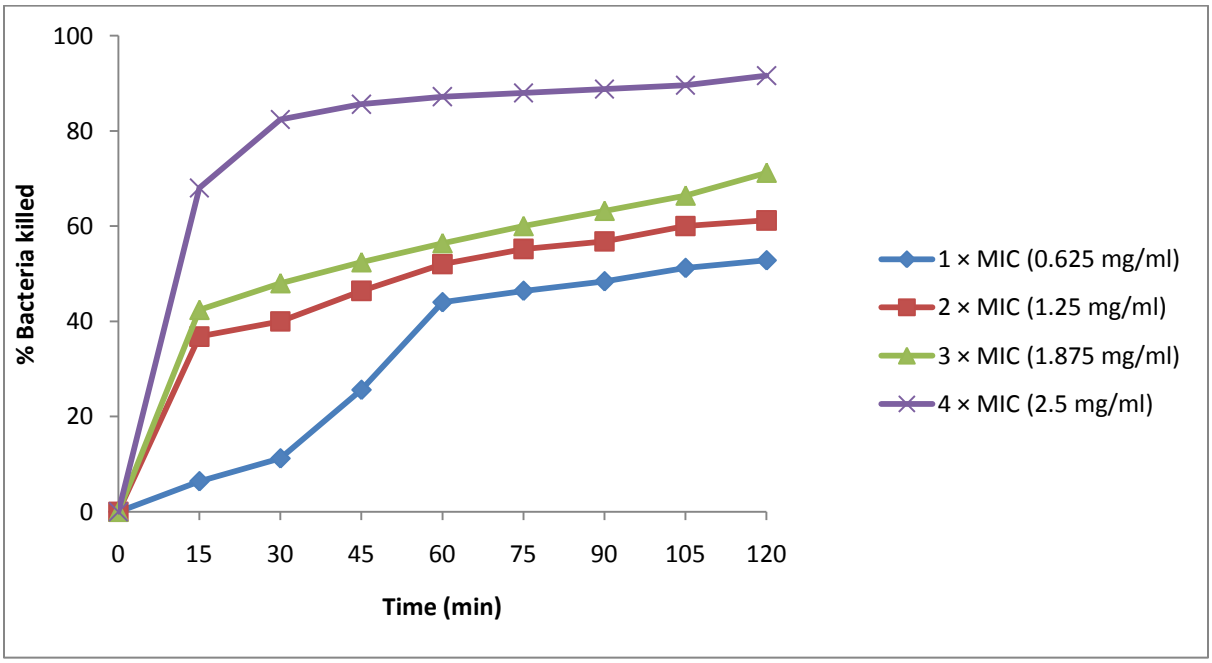

Figure 5.3: Rate of kill of *V. vulnificus* (AL042) by crude n-Hexane extract of *Garcinia kola* seeds.

Discussion

The n-Hexane extract of *Garcinia kola* seeds showed activity against the five *Vibrio* species used in this study, namely, *V. vulnificus*, *V. parahaemolyticus*, *V. fluvialis*, *V. metschnikovii* and *Vibrio* species which are recognized to be pathogenic to humans (Health protection agency, 2007). The minimum inhibitory concentrations of the extract were low and varied between 0.313 mg/ml and 0.625 mg/ml, while the minimum bactericidal concentrations ranged between 5–10 mg/ml. Other plants extracts have been reported to exhibit anti-*Vibrio* activities. For example, Hernandez, T *et al*. (2003) assessed the antibacterial effect of n-Hexane extracts of eight plants namely *Lippia graveolens, Lantana achyranthifolia, Turnera difusa, Lippia oaxacana, Gymnaloena oaxacana, Cordia curassavica, Lantana camara* and *Acalypha hederacea* and reported MICs ranging between 0.25–2 mg/ml against 3 *Vibrio cholerae* isolates. In another study, Srinivasan, K *et al*. (2007) assessed the effect of n-Hexane extract of *Vicoa indica* and reported that at a test concentration of 12.5 mg/ml the extract exhibited anti-*Vibrio* activities against *V. parahaemolyticus* and *V. cholerae* with zones of inhibition of 21 mm and 29 mm respectively. Phytochemical analysis of the extracts of *Vicoa indica* showed the presence of steroids, triterpenes, phenolics groups and aminoacids (Srinivasan, K *et al*., 2007). Similarly, *Garcinia kola* seeds have been shown to contain steroids (Adegboye, MF *et al*., 2008) and a class of terpernoids similar to those found in hops used for brewing (Ogu and Agu, 1995). Bioactivity portrayed by non- polar extracts such as n-Hexane is often associated with complex mixtures of triterpenoid and/or steroid compounds (Regasini, LO *et al*., 2009).

The rate of kill studies showed appreciable killing rate by the n-Hexane extract. The trend of kill was generally time and concentration dependent with the highest concentration of the extracts (4 × MIC) value achieving the highest percentage of bacteria cells killed after 2 hours of

exposure time. The highest percentage (96.3%) of bacteria cells killed was observed from *V. fluvialis* (AL040). A greater or equal to 99.9% killing activity in 24 h is generally used as a standard of measurement of bactericidal efficacy (CLSI, 2005). The extract did not however achieve the above 99% mark for bacterial efficacy but showed relative cidal properties which are significant.

The anti-*Vibrio* activities portrayed by the extract in this study can also be attributed to a number of other factors that are related to their chemical constituents. Non-polar fractions and extracts of *Garcinia kola* have been reported to demonstrate a significant antimicrobial activity attributed to a benzophenone and kolanone (Onayade, OA *et al*., 1998). In a study by Eleyinmi, AF *et al*. (2006) *Garcinia kola* seeds were found to contain saturated and unsaturated fatty acids namely myristic, pentadecanoic, palmitic, margaric, stearic, palmitoleic, oleic, vaccenic, linoleic and α- linolenic acids. The dominant fatty acids in the seeds were oleic, linoleic and palmitic acids. Linoleic, linolenic and oleic acids of different plant species have been shown to possess antimicrobial activities (Kilic, T *et al*., 2005; Won, S *et al*., 2007; Skalicka-wozniak, K *et al*., 2010; Zheng, CJ *et al*., 2005; Walters, D *et al*., 2004). Also, Agoramoorthy, G *et al*. (2007) found the fatty acid methyl esters of the blind-your eye mangrove (*Excoecaria agallocha*) plant to possess antibacterial activity against gram negative bacteria. Findings by Zheng, CJ *et al.* (2005) showed that linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI) of *Escherichia coli* a gram negative rod shaped bacteria. FabI is an essential component of bacterial fatty acid synthesis. Additional unsaturated fatty acids including palmitoleic acid, oleic acid, and linolenic acid also exhibited the inhibition of FabI and all of these fatty acids have been found in different quantities in *Garcinia kola* seeds (Eleyinmi, AF *et al*., 2006).

Conclusion

This study has shown that *Garcinia kola* seeds contain compounds that are antagonistic to *Vibrio* bacteria and could therefore be of relevance in the treatment and management of infections caused by *Vibrio* species. A follow up that includes a bioassay directed fractionation of the n-Hexane extract so as to isolate and identify the active compounds is necessary and is a subject of on-going investigation in our group.

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CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

The burden of antibiotic resistance among bacteria has been progressively increasing since the discovery of antibiotics. Antibiotic resistance genes that were generally at low levels during the initial stages of antibiotics introduction have increased largely due to the selective pressure of antibiotic use and the resulting exposure of bacteria to the antibiotics in humans, food, animals and the environment (Hawkey, 2008). In developing countries, factors such as inadequate access to effective antibiotics; unregulated dispensing and manufacture of antibiotics; and truncated antibiotic therapy due to cost are contributing to the development of multi-drug resistant organisms (Planta, 2007). Plants are studied as potential disease controlling agents in humans as they are relatively safer, affordable and are easily accessible at a local level, such that they can offer an alternative treatment option to the conventional antibiotics.

To protect themselves plants accumulate an armoury of antimicrobial compounds as they are continuously in contact with different microorganisms which include viruses, bacteria and fungi. Most of antimicrobial compounds are secondary metabolites that represent constitutive chemical barriers to microbial attack (phytoanticipins) and others are inducible antimicrobials (phytoalexins) (Gonzalez- Lamothe, R *et al*., 2009) whilst some of the antimicrobial secondary metabolites also, give plants their odours and flavours (terpenoids); others (quinones and tannins) are responsible for plant pigmentation (Cowan, 1999). Fabricant and Farnsworth (2001) reported that most of the useful drugs that have been derived from plants have been discovered by follow-up of ethnomedical uses.

In this study, the anti-*Vibrio* activities of *Garcinia kola* seeds were investigated. The plant has been used in folklore medicine in West and Central African countries to treat many ailments earning it the name "wonder plant" amongst the south western Nigerian people because every part of it has been found to be of medicinal importance (Dalziel, 1937). The aqueous, methanol, dichloromethane and n-Hexane solvent extracts were tested for their activity against 50 *Vibrio* isolates. The aqueous extract had activity against 12 (24%) of the isolates whilst all the other extracts had activity against 16 (32%) of the isolates. The aqueous, methanol, dichloromethane and n-Hexane extracts had MIC values ranging 10->10 mg/ml, 0.313-2.5 mg/ml, 0.313-0.625 mg/ml and 0.313-0.625 mg/ml respectively. The limited activity of the aqueous extract in comparison to the other organic solvents is in agreement with the findings of previous reports on the antibacterial activities of *Garcinia kola* seeds (Sibanda and Okoh, 2008; Nwaokorie, F *et al*., 2010; Ezeifeka, GO *et al*., 2004). The difference in activity between the aqueous and the organic solvents extracts has been attributed to a better solubility of *Garcinia kola* seeds active agents - xanthones, benzophenones, and flavonoids especially biflavonoid type GB1 (Xu and Lee, 2001, Han, QB *et al*., 2005) in organic solvents than in water (Taiwo, O *et al*., 1999; Obi and Onuoha, 2000; Ogueke, CC *et al*., 2006: Ogbulie, JN *et al*., 2007; Nwaokorie, F *et al*., 2010).

All four extracts tested had activity against the 5 *Vibrio* species represented in the study and all the identified species are known to be pathogenic to humans (Health protection agency, 2007) thus suggesting that *Garcinia kola* seeds have potentials in anti-*Vibrio* chemotherapy. Interestingly, in this study, the methanol, dichloromethane and n-Hexane extracts had activity against the same isolates inclusive of those that were susceptible to the aqueous extracts showing that the active compounds present might be the same in all the three solvents extracts but in

different concentrations as highlighted by the MIC results that showed that more isolates had lower inhibitory concentrations against the n-Hexane extract compared to the other solvent extracts. Bioactivity portrayed by non-polar extracts such as n-Hexane is often associated with complex mixtures of triterpenoid and/or steroid compounds (Regasini, LO *et al*., 2009). These compounds can also be extracted by solvents such as methanol (Cowan, 1999) but in lesser concentrations in comparison to a more non-polar solvent such as n-Hexane.

Rate of kill curves are important in that they show the bactericidal activity or the duration of a bacteriostatic effect of a fixed concentration of the antimicrobial agent thereby providing a clear analysis of the relationship between the extent of microbial population mortality and the antimicrobial agent concentration (Burt, 2004; Oliveira JLTM *et al*., 2009). Studies of the rate of kill assays of the n-Hexane, dichloromethane and methanol extracts against *V. fluvialis* (AL040), *V. parahaemolyticus* (AL049) and *V. vulnificus* (AL042) showed a trend of being concentration and time dependent as the highest number of bacteria cells were killed at the highest concentration of 4× MIC and after the maximum exposure time of 2 hours. A greater or 99.9% killing activity in 24 h is generally used as a standard of measurement of bactericidal efficacy (Pankey and Sabath, 2004; CLSI, 2005). All the extracts did not however achieve the 99.9% killing rate required for a bactericidal compound, but rather had killing rates of between 60% and 96.3% which represents bacteriostatic property. In the treatment of an infection, bacteriostasis is often effective because the killing and elimination of the pathogen are further mediated through host immune defences (Kummerer, 2009).

Results from the rate of kill studies showed that the n-Hexane extract resulted in the killing of more bacterial cells followed by dichloromethane and methanol extracts in that order, and in support of the MIC results which also showed the same trend in the efficacy comparison

of the different solvent extracts. These results therefore indicate that the active compounds against *Vibrio* species are mainly non- polar substances. According to Onayade, OA *et al*. (1998) non-polar fractions and extracts of *Garcinia kola* have been reported to demonstrate a significant antimicrobial activity attributed to a benzophenone and kolanone. Also, methanol is known to extract anthocyanins, terpenoids, saponins, tannins, xanthoxyllins, lactones, flavones and polyphenols (Cowan, 1999), dichloromethane extracts mainly volatile/essential oils, whilst n-Hexane extracts mainly fixed oils which are triglycerides of higher fatty acids. Findings from authors have shown the presence of most of these bioactive compounds in *Garcinia kola* seeds such as fatty acids (Eleyinmi, AF *et al*., 2006), essential oils (Aniche and Uwakwe, 1990), flavonoids, tannins, cardiac glycoside, steroids, saponins and reducing sugars (Akinpelu, DA *et al*., 2008).

The choice of solvent for further research purposes would have to depend on what is intended with the extract. If extraction is to screen plants for antimicrobial components, the effect of the extractant on subsequent separation procedures is not important, but the extractant should not inhibit the bioassay procedure. If the plant material is extracted to isolate chemical components without using bioassay, toxicity of the solvent is not important because the solvent can be removed before subsequent isolation procedures (Eloff, 1998).

This research showed the *in-vitro* antagonistic activities of the different solvents crude extracts of *Garcinia kola* seeds against *Vibrio* species and revealed that *Garcinia kola* seeds could be useful in the treatment and management of infections cause by *Vibrio* species. In light of these significant findings this research therefore concludes with the following recommendations:

- There is a need to isolate and identify the active compound(s) responsible for the anti-*Vibrio* activities.
- There is a need to study the activities of the extracts in combination with conventional antibiotics in order to elucidate the nature of interactions such as synergistic, antagonistic or indifference actions.
- An assessment of the *in-vivo* activity of the active compounds pursuant to drug development is necessary and these are subject of future investigations in our group.

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