ASSESSMENT OF THE ANTI-*LISTERIAL* PROPERTIES OF *GARCINIA KOLA* (HECKEL) 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 Doctor of Philosophy Degree 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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SIGNATURE

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DATE

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

MIC	-	Minimum Inhibitory Concentration
MBC	-	Minimum Bactericidal Concentration
HIV	-	Human Immunodeficiency Virus
AIDS	-	Acquired Immune Deficiency Syndrome
NRF	-	National Research Foundation
TLC	-	Thin Layer Chromatography
AEMREG	-	Applied and Environmental Microbiology Research Group
DNA	-	Deoxyribonucleic Acid
et al	-	(*et alii)* and others
PCR	-	Polymerase Chain Reaction
SMX-TMP	-	Sulfamethoxazole/Trimethoprim
GC-MS	-	Gas Chromatography-Mass Spectrometry
EUCAST	-	European Committee for Antimicrobial Susceptibility Testing
SPSS	-	Statistical Package for the Social Sciences
ANOVA	-	Analysis of Variance
CFU	-	Colony Forming Unit
R_f	-	Retention Factor

rRNA	-	Ribosomal Ribonucleic Acid

GENERAL ABSTRACT

A follow-up of traditional medicinal plants uses is an important tool in highlighting their therapeutic potentials, as they have been found to be a source of a wide range of bioactive compounds that can be used as base compounds for new pharmaceutical drugs. This study therefore focuses on assessing the anti-*Listerial* properties of the seeds of *Garcinia kola* (Heckel) plant, which is a traditional medicinal plant of west and central African origin, and was and is still used to traditionally treat several ailments. Four different solvents crude extracts of the seeds were assessed for their anti-*Listerial* activities *in-vitro*, against a panel of 42 *Listeria* bacteria, which included *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria grayi* species. At 10 mg/ml concentration the aqueous extract had activity against 29% of the test isolates while the other three crude extracts namely dichloromethane, n-hexane and the methanol extracts had activity against 45% of the test bacteria. The minimum inhibitory concentration (MIC) ranges of the extracts were 0.079-0.313 mg/ml for the dichloromethane extract; 0.079-0.625 mg/ml for the n-hexane extract; 0.157-0.625 mg/ml for the methanol extract; and 10->10 mg/ml for the aqueous extract. The minimum bactericidal concentration (MBC) ranges of the extracts were 0.625–10 mg/ml for both the n-hexane and the dichloromethane extract; 5-10 mg/ml for the methanol extract; and those for the aqueous extract were above 10 mg/ml against all the susceptible *Listeria* isolates.

The rate of kill analysis was then determined for the three most active crude extracts that is excluding the aqueous extract and it was assessed against four representative *Listeria* species namely *L. monocytogenes* (LAL 8), *L. grayi* (LAL 15), *L. ivanovii* (LEL 30) and *L. ivanovii* (LEL 18). All the three extracts showed a general trend of being concentration and time dependent in their rate of kill profiles such that most bacteria cells were killed at the highest test concentration of 4× MIC value after the maximum exposure time of 2 h. The n-hexane,

dichloromethane and methanol extracts were bactericidal against 4, 3 and 1 isolates out of the four test *Listeria* isolates respectively.

The most bactericidal extract which was the n-hexane extract was analysed for its constituents through the use of thin layer chromatography (TLC) using different solvent combinations namely benzene: ethanol: ammonium hydroxide (B.E.A) in the ratio (36:4:0.4 v/v), ethyl acetate: methanol: water (E.M.W) (40:5.4:4 v/v) and chloroform: ethyl acetate: formic acid (C.E.F) (5:4:1 v/v). The solvent combination of B.E.A (36:4:0.4 v/v) showed better separation of the constituents of the extract in comparison to the other two solvent combinations, with its TLC chromatogram showing the presence of five visible bands after spraying with vanillin spray reagents. TLC coupled with direct bioautography (TLC-direct bioautography) at extract concentration of 100 mg/ml showed two zones of inhibition against *L. grayi* (LAL 15) and *L. ivanovii* (LEL 18) and one zone each against *L. monocytogenes* (LAL 8) and *L. ivanovii* (LEL 30). The R_f values of the zones on the TLC-direct bioautography chromatograms were different among the isolates, which showed the possible multiplicity of the anti-*Listerial* compounds in the extract.

The B.E.A (36:4:0.4 v/v) combination was then further used for the column chromatography process to fractionate the constituents of the n-hexane extract. The column chromatography process resulted in eight fractions which were named in the order in which they were eluted and the solvent used in the elution. The process started with three fractions eluted with 100% benzene solvent and these were named Benz1, Benz2 and Benz3, whilst those eluted by B.E.A (36:4:0.4 v/v) were five and named BEA1, BEA2, BEA3, BEA4 and BEA5.Through MIC determination five out of the eight fractions were found to be active against the four representative *Listeria* isolates used previously for the rate of kill determination and the TLC-direct bioautography assessments whilst three fractions namely Benz1, BEA1 and BEA5 were found to be not active against any of the four test *Listeria* isolates.The Benz2, Benz3, BEA2

and BEA3 fractions were chosen on the basis of their lower MIC values for gas chromatography coupled to mass spectrometry (GC-MS) analysis in-order to identify the constituents of each fraction. GC-MS identified a total of 9, 7, 18 and 27 compounds in the Benz2, Benz3, BEA2 and BEA3 fractions respectively.

The compound 9,19-cyclolanost-24-en-3-ol, (3.beta.) was found to be the most abundant in all the four fractions while the compound 9,19-cyclolanostan-3-ol, 24-methylene-, (3.beta.) was next in abundance in three fractions (Benz2, Benz3 and BEA2) and being the fourth most abundant in the BEA3 fraction and both these compounds are plant sterols. Carboxylic acids and fatty acids derivatives were also found in substantial amounts in the BEA2 and BEA3 fractions respectively. The commercial compound of 9,19-cyclolanost-24-en-3-ol, (3.beta.) was found not to exhibit any anti-*Listerial* activities at a test concentration of 5 mg/ml, such that it is highly likely that the observed anti-*Listerial* activities could have been due to the synergistic activities of the compounds in the different fractions. The interactions of the n-hexane crude extract, Benz2, Benz3 and BEA2 fractions with some antibiotics namely penicillin G, ampicillin and ciprofloxacin showed varying results ranging from synergy to indifference, with the n-hexane crude extract in particular exhibiting 100% synergism with all the test antibiotics.

All the four different crude extracts of *Garcinia kola* seeds used in this study possessed varying anti-*Listerial* potentials, with the non-polar extracts being more effective in comparison to the polar extracts. The bactericidal nature of the n-hexane extract in particular and its synergistic interactions with some conventional antibiotics were a major reflection of the potency of this particular extract. Some of the eluted column chromatography fractions of the n-hexane extract showed appreciable anti-*Listerial* activities and some beneficial interactions with some antibiotics, such that in the light of all these findings *Garcinia kola* seeds have major potential in the treatment of infections caused by *Listeria* bacteria.

CHAPTER ONE

General Introduction

The *Listeria* genus is closely related to the genera *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (Hain *et al*., 2006). It is comprised of eight species which are ubiquitous in nature, namely, *Listeria monocytogenes*, *Listeria grayi*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria innocua*, *Listeria seeligeri* (Stephan *et al*., 2003; Chen *et al*., 2010), *Listeria marthii* sp. nov (Graves *et al*., 2010) and *Listeria rocourtiae* sp. nov (Leclercq *et al*., 2010). *Listeria* species are non-motile Gram positive bacilli, oxidase negative, facultatively anaerobic, catalase positive and non-spore forming bacteria (Bal *et al*., 2008; Salimnia *et al*., 2010), with the human pathogenic species causing the rare but fatal infectious disease listeriosis which has an average case fatality rate of between 20 and 30% (Camejo *et al*., 2011).

L. ivanovii is a known animal pathogen while *L. monocytogenes* is pathogenic for both humans and animals. Pregnant women (including their fetuses), newborns, immune compromised patients (AIDS, cancer, organ transplant and diabetic patients amongst others) and the elderly (60 years and above) are at increased risk of acquiring listeriosis (Bortolussi, 2008; Salimnia *et al*., 2010). Although *L. monocytogenes* is the most common cause of human listeriosis whilst the other species have been regarded as non-pathogenic to humans, they however have been reported cases of human listeriosis being caused by other *Listeria* species other than *L. monocytogenes* such as *L. ivanovii*, *L. grayi* and *L. seeligeri* (Rocourt *et al*., 1986; Guillet *et al*., 2010; Salimnia *et al*., 2010), thereby making them potential human pathogens.

Listeriosis is essentially a foodborne disease as the primary vehicle for *Listeria* infection is food, normally meat and meat products, vegetables, fish, dairy products, minimal processed food and ready-to-eat foods (Adzitey and Huda, 2010; Allerberger and Wagner, 2010). In some rare cases it can be transmitted, directly from infected animals to humans, between humans and also nosocomial transmission through contaminated material or patient-to-patient transmission via healthcare workers (Allerberger and Wagner, 2010). Between 1998 and 2002 *L. monocytogenes* resulted in 38 outbreak-related deaths among 256 cases which were statistically more deaths and a higher case-fatality rate (15%) in comparison to other pathogens causing foodborne illnesses in the United States during that period (Lynch *et al*., 2006). While in Canada in 2008 there was a listeriosis outbreak linked to deli meats that caused 53 confirmed cases, 6 suspected and 20 deaths, recording a very high case fatality rate of 37.7% from the confirmed cases (Warriner and Namvar, 2009).

Commonly reported listeriosis symptoms include fever, watery diarrhoea, nausea, headache, and pain in joints and muscles (Schuppler and Loessner, 2010), while the clinical manifestations of invasive listeriosis are usually severe and include abortion, sepsis, meningoencephalitis, neuro-encephalitis, chorioamnionitis, gastroenteritis and bacteraemia (Khelef *et al*., 2006; Sukhadeo and Trinad, 2009; Adzitey and Huda, 2010).The mechanism by which *L. monocytogenes* causes diarrhoea is highly likely a result of its direct invasion of the epithelial cells of the host intestinal mucosa inducing mucosal inflammation since *L. monocytogenes* also produces enterotoxins and this could then explain the fever and gastroenteritis symptoms (Ooi and Lorber, 2005; Schuppler and Loessner, 2010).

Listeria detection tests rely mainly on an enrichment protocol that promotes the growth of the *Listeria* while suppressing the growth of competitive organisms (Bosilevac *et al*., 2009). *L. monocytogenes* can be readily cultured from clinical specimens such as blood, cerebrospinal fluid, amniotic fluid, placenta, meconium, lochia, gastric washings or ear swabs from

newborns, by directly plating the material onto blood agar plates and incubating overnight at 35°C in an ambient atmosphere, while stool specimens (other than meconium) are selectively enriched for *Listeria* before being plated onto selective agar media (Allerberger and Wagner, 2010). The biochemical tests useful for discriminating between the *Listeria* species are acid production from D-xylose, L-rhamnose, α-methyl-D-mannoside, and D-mannitol (Allerberger, 2003). Molecular-based methods such as the polymerase chain reaction (PCR) for the rapid identification of *Listeria* grown in culture or directly in specimen, can also be utilised and its of particular importance when prior administration of antimicrobial agents compromises culture (Allerberger, 2003; Allerberger and Wagner, 2010).

Treatment of listeriosis usually involves the administration of penicillin G or ampicillin alone or in combination with an aminoglycoside such as gentamicin. However, for patients with penicillin allergies the combinations of sulfamethoxazole/trimethoprim (SMX-TMP) or vancomycin/teicoplanin are used and the doses of the drugs vary depending on factors such as the patient's age and weight (Swaminathan and Gerner-Smidt, 2007; Mardis *et al*., 2012).

Treatment of infections due to opportunistic bacteria such as *Listeria* may become a problem, since most of these bacteria are becoming resistant to most antibiotics, indicating the necessity of continous monitoring of the antimicrobial susceptilibity of these organisms (Arslan and Ozdemir, 2008). In this connection various studies have shown the resistance to antibiotics of both clinical and environmental strains of *Listeria* even against some antibiotics generally used for the treatment of listeriosis (Odjadjare *et al*., 2010; Chen *et al*., 2010; Nwachukwu *et al*., 2010; Lotfollahi *et al*., 2011; Soni *et al*., 2013).

The noted increases in antibiotic resistance among *Listeria* species follows a general worldwide pattern of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistance among many groups of bacteria, of which such an increase in antimicrobial

resistance in *Listeria* species is a major public health concern owing to the high case fatality rates associated with listeriosis (Arslan and Ozdemir, 2008), even despite early and proper antibiotic treatment (Swaminathan and Gerner-Smidt, 2007). In this regard, pharmaceutical companies have been motivated to develop new antimicrobial drugs in recent years to curb the constant emergence of microorganisms resistant to conventional antibiotics (Silva and Fernandes Junior, 2010), such that numerous studies on the pharmacology of traditional medicinal plants have been accomplished, as they constitute a potential source for the production of new medicines (Silva and Fernandes Junior, 2010).

There are different goals of using plants as sources of therapeutic agents. It can be primarily to isolate bioactive compounds for direct use as drugs, or to use the whole plant or part of it as a herbal remedy such as with cranberry and garlic, or to use the bioactive agents as pharmacologic tools such as lysergic acid diethylamide. It can also be essentially to produce bioactive compounds of novel or known structures that can be used as semi-synthesis lead compounds in the production of patentable entities which have higher activities and are of lower toxicity levels (Fabricant and Farnsworth, 2001).With this in mind, this study focuses on assessing the anti-*Listerial* properties of the *Garcinia kola* plant's seeds. *Garcinia* (Guttiferae family) is a large genus of polygamous trees or shrubs which can be found in tropical Asia, Africa and Polynesia and is a rich source of bioactive molecules including xanthones, flavonoids, benzophenones, lactones and phenolic acids (Matsumoto *et al*., 2003; Varalakshmi *et al*., 2010).

Garcinia kola (Heckel) is an angiospermae that can be found in central and west Africa, mostly in moist conditions (Anegbeh *et al*., 2006; Kagbo and Ejebe, 2010). The traditional medicinal and therapeutic uses of the *Garcinia kola* plant are numerous particularly its seeds. The seeds have a bitter taste hence the plant is commonly called bitter kola in Nigeria and as a result of this bitter taste the seeds have been consumed as stimulants (Atawodi *et al*., 1995; Tebekeme

and Prosper, 2007). The seeds are used in the treatment of bronchitis and throat infections (Iwu *et al*., 2009) and also to prevent and relieve colic, cure headaches and chest colds as well as to relieve cough (Iwu, 1993; Iwu *et al*., 1999). They are also used for the treatment of Asthma (Sonibare and Gbile, 2008), and in addition they have also been associated with curing diabetes, palpitations, intestinal pains, jaundice, anaemia, angina ,liver disorders, threatened abortions as well as being an antidote against poison (Adegoke *et al*., 1981; Adeleke *et al*., 2006). They are also used in the treatment of diarrhoea, hepatitis, dysmenorrheal (menstrual cramps) (Dalziel, 1937; Agada and Braide, 2009) amongst other medicinal and social uses. Split stems and twigs of the plant are used as chewing sticks in many parts of Africa, and are commercialised for dental care (Agyili *et al*., 2007; Okoko and Oruambo, 2008).

Studies have shown the plant to possess antimicrobial, antiviral, antiparasitic and anti-inflammatory activities (Iwu *et al*., 1999). While some studies have shown that cytotoxic benzophenone derivatives garcinol, isogarcinol, and xanthochymol from *Garcinia* species display a strong apoptosis-inducing effect against human leukemia cell lines (Matsumoto *et al*., 2003), and that some *Garcinia* species have cytotoxic and anti-HIV activities (Magadula and Suleimani, 2010).

Popular observations on the use and efficacy of medicinal plants has a significant impact on the disclosure of their therapeutic properties (Silva and Fernandes Junior, 2010), and in addition medicinal plants are known to usually have multiple beneficial effects on the body such that their actions often act beyond the symptomatic treatment of the disease (Iwu *et al*., 1999), while also having the potential to enhance the effects of conventional antimicrobials which may probably decrease costs and improve the treatment quality (Silva and Fernandes Junior, 2010). Such that given the varied medicinal uses of *Garcinia kola* seeds mentioned above and their long term use in traditional medicine against a background that listeriosis mainly affects the immune-compromised individuals (meaning patients with underlying illnesses such as diabetic

patients), of which the seeds of *Garcinia kola* have been linked to the treatment of some of these illnesses it becomes a viable option both economically and therapeutically to assess the possible anti-*Listerial* activities of the *Garcinia kola* seeds as well as their interactions with some antibiotics used in the treatment of listeriosis. This study is therefore aimed at assessing the in-*vitro* anti-*Listerial* properties of different extracts of *Garcinia kola* seeds and then further isolate and characterise the active compound(s) thereof. The specific objectives of the study include:

- To prepare and screen crude aqueous, methanol, dichloromethane and n-hexane extracts of *Garcinia kola* seeds for their anti-*Listerial* activities *in-vitro*.
- To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts against the susceptible *Listeria* isolates.
- To determine the rate of kill of the three most active extracts against selected susceptible *Listeria* species.
- To determine the extract-antibiotics interactions of the most bactericidal extract.
- To partition the most bactericidal extract through the use of different chromatography techniques.
- To determine the anti-*Listerial* activities of the partitioned fractions.
- To identify the constituents of the most bioactive fractions of the extract through Gas chromatography linked to mass spectrometry (GC-MS).
- To determine the anti-*Listerial* activities of the major compound found in the active fractions.
- To determine the interactions of some of the active fractions with some conventional antibiotics.

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CHAPTER TWO

Literature Review

2.1. *Listeria* species

Listeria species are Gram-positive, facultatively anaerobic, non-spore-forming, rod-shaped bacteria which are approximately 0.5 µm in width and 1–1.5 µm in length (Liu, 2006), they have a low guanine-cytosine (G+C) content in their DNA ranging between 36% to 42% (Allerberger, 2003). They are also catalase positive, indole and oxidase negative, they can hydrolyse aesculin, but not urea (Liu, 2006). *Listeria* can grow at high salt concentrations (10% NaCl) and at wide pH and temperature ranges of (4.5-9) and (0-45°C) respectively (Grau and Vanderlinde, 1990; Hain *et al*., 2006a). *Listeria* can also even survive at low water activity (aw 0.91) (Lado and Yousef, 2007; Warriner and Namvar, 2009). The optimum temperatures for growth for *Listeria* species is between 30°C and 37°C and they are generally motile at 20-28°C by means of one to five peritrichous flagella (Allerberger, 2003).

Listeria species are ubiquitous in nature and can be found in different environments such as soil, water, effluents and in foods (Liu, 2006). There are six well known species namely *Listeria monocytogenes*, *Listeria grayi*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria innocua* and *Listeria seeligeri* (Stephan *et al*., 2003; Salimnia *et al*., 2010) and also an additional two newly discovered *Listeria* species namely *Listeria marthii* sp. nov (Graves *et al*., 2010) and *Listeria rocourtiae* sp. nov (Leclercq *et al*., 2010).

Key virulence genes in *Listeria* include members of the internalin family and also the six genes (*prfA, plcA, hly, mpl, actA*, and *plcB*) that are clustered in a genomic element, designated the *prfA* virulence cluster or the *Listeria* pathogenicity island (LiPI) (Schmid *et al*., 2005; den Bakker *et al*., 2010). The phylogeny of the *Listeria* genus members suggests two lines of

descent, one containing *L. monocytogenes* and *L. innocua* and a second line of descent containing *L. seeligeri*, *L. ivanovii* and *L. welshimeri*. Both of these lines of descent contain species with and without the virulence gene cluster, which is found in the same genomic location in all the species with this cluster (Graves *et al*., 2010). *L. grayi* on the other hand is a more distinct and distantly related species (Graves *et al*., 2010; den Bakker *et al*., 2010). Out of all the *Listeria* species there are only two known pathogenic species namely *L. ivanovii* which causes disease almost exclusively in ruminants and sheep while *L. monocytogenes* causes disease in both humans and animals (Orndorff *et al*., 2006).

2.2. Identification of *Listeria* species in the Laboratory

The identification of *Listeria* species in the laboratory involves culture based methods as well as molecular techniques. The culture based methods involve an enrichment protocol which is subsequently followed by morphological, biochemical and serological characterisation. A *Listeria* selective enrichment broth is used to promotes growth of *Listeria* while suppressing the growth of competitive organisms in the sample (Bosilevac *et al*., 2009), after-which the homogenates are often inoculated onto *Listeria* selective agar (El-Malek *et al*., 2010). Morphological characterisation tests usually involve the motility tests as well as microscopic analysis such as the Gram stain among others. The motility test detects the motility of the test organism at temperature ranges of 20 to 28°C or even up to 30°C that *Listeria* are motile at. In semi solid motility agar 0.2-0.4% a 1 cm long stab of an 8-24 h old broth culture of *Listeria*, a cloudiness will be observed with the naked eye as the *Listeria* swims through the 'motility medium'. At a distance of approximately 0.5 cm below the surface of the agar a layer of increased growth showing an umbrella shaped motility pattern can be observed, as in that zone

of reduced oxygen tension *Listeria* grows better than under aerobic or strictly anaerobic conditions (Allerberger, 2003; El-Malek *et al*., 2010; Graves *et al*., 2010).

Biochemical characterisation involves a number of tests for *Listeria* identification. A positive catalase test reaction should be expected as well as negative oxidase and indole tests to match the *Listeria* species descriptions (Graves *et al*., 2010). Some of the biochemical tests that can be used for differentiation of species in the *Listeria* genus are as shown in Table 1 below. These tests are based on the presence or absence of acid production (without gas) from D-xylose, L-rhamnose, α-methyl-D-mannoside, and D-mannitol (Allerberger, 2003; Johnson *et al*., 2004).

Table 1: Biochemical differentiation of the six well known species in the genus *Listeria* as well as the two newly discovered *Listeria* species

	L. monocytogenes	*L. seeligeri*	*L. ivanovii*	*L. innocua*	*L. welshimeri*	*L. grayi*	*L. marthii*	*L. rocourtiae*
D-xylose	-	+	+	-	+	-	-	+
L-Rhamnose	+	-	-	v	v	v	-	+
α-Methyl-D-Mannoside	+	-	-	+	+	+	N/A	-
D-Mannitol	-	-	-	-	-	+	-	+

Modified from (Allerberger, 2003): Key: +denotes positive; - denotes negative; V denotes variable; N/A denotes data not available.

The ability of the *Listeria* species to lyse red blood cells (haemolysis) from most mammalian animals is also another species differentiating factor as only three *Listeria* species namely *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* are haemolytic (Graves *et al*., 2010). The haemolysing activity can be demonstrated using horse blood or sheep blood containing agar,

whereby zones of haemolysis can be viewed in the agar plates and the CAMP (Christie, Atkins, Munch-Petersen) test is usually used for this analysis. The CAMP test uses a β-haemolysin-producing *Staphylococcus aureus* and a *Rhodococcus equi* strain streaked in one direction on a sheep blood agar plate. The test *Listeria* isolates will then be streaked at right angles to (but not touching) the *S. aureus* and *R. equi* lines. Haemolysis of *L. monocytogenes* (and to a lesser extent *L. seeligeri*) is enhanced in the vicinity of the *S. aureus* streak, and *L. ivanovii* haemolysis is enhanced in the vicinity of *R. equi* (Allerberger, 2003). Serotyping although not allowing species identification is also a useful technique for confirming the genus diagnosis *Listeria* and for allowing a first-level subtyping for epidemiological purposes, as *Listeria* strains are divided into serotypes on the basis of somatic (O) and flagellar (H) antigens (Allerberger, 2003).

Molecular techniques are more discriminatory and accurate methods for *Listeria* detection, and are even useful in cases when the phenotypic culture based methods test's reaction is atypical in an uncharacteristic strain, for example in cases of non-haemolytic strains of *L. monocytogenes*, in which case for example a molecular technique such as to tests for *L. monocytogenes* specific 16S rRNA with DNA probe kits can be carried out to confirm the identity of the isolate (Johnson *et al*., 2004). There are also different other molecular techniques that are used for *Listeria* species identification and these include ribotyping, pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) (Atil *et al*., 2011), PCR and real time PCR (O'Grady *et al*., 2009). Validation of these techniques is also quite essential for uniform and accurate detection and identification of *Listeria* species in the food industry, learning institutions and the medical sector. However, apart from the above mentioned characteristics there are other additional specific traits that may be applicable to a particular *Listeria* species alone.

2.2.1. L. monocytogenes

L. monocytogenes is a soil organism, which has evolved the ability to invade and mobilise within eukaryotic cells, probably to some lower eukaryotic multi-cellular soil organisms (McLauchlin, 1997), hence the bacterium is not host adapted to man such that it is rather a marginal and opportunistic pathogen (McLauchlin *et al*., 2004). Somewhat unusual for a human pathogen, *L. monocytogenes* is flagellated and motile at temperatures of 30°C and below but is non-motile at the normal human body temperature of 37°C and above (Sleator *et al*., 2009). The flagellar filament of *L. monocytogenes* is composed of one major subunit, the flaA-encoded flagellin, which is produced and assembled at the cell surface when *L. monocytogenes* grows between 4°C and 30°C, whereas its production is markedly reduced at 37°C (Bigot *et al*., 2005).

The virulence of *L. monocytogenes* is due to its capacity to invade and multiply within host cells, including macrophages and hepatocytes, as well as epithelial, endothelial, and neuronal cells (Bigot *et al*., 2005). Each step of the infectious process is dependent on the production of virulence factors, including invasion proteins (InlA and InlB), pore-forming toxin listeriolysin O, two phospholipases (PlcA and PlcB), the Mpl metalloprotease, the secreted protein InlC, the sugar uptake system UhpT and actin polymerization factor ActA, which are controlled by the pleiotropic transcriptional activator PrfA (Dussurget *et al*., 2004; Bigot *et al*., 2005; Cossart *et al*., 2011).

Haemolysis of *L. monocytogenes* resembles that of *Streptococcus agalactiae* and the zone of haemolysis on horse or sheep blood agar is narrow, mostly not extending much beyond the edge of the colonies (Allerberger, 2003). *L. monocytogenes* is composed of 13 serotypes namely 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Allerberger, 2003) with 1/2b, 3b, 4b, 4d and 4e of genetic lineage I being most virulent (Rocourt and Buchrieser, 2007).

2.2.2. L. ivanovii

All *L. ivanovii* strains belong to serovar 5, and reciprocally, all serovar 5 strains are members of *L. ivanovii*. These organisms are members of the only species in the genus *Listeria* which gives a positive CAMP reaction with *Rhodococcus equi* and a negative CAMP reaction with *Staphylococcus aureus* (Boerlin *et al*., 1992). They produce a particularly wide zone or even multiple zones of haemolysis on sheep blood or horse blood agar (Allerberger, 2003).

Two subspecies of *L. ivanovii* have been described which are *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* (Boerlin *et al*., 1992) which can be distinguished by the ability to degrade ribose and N-acetyl-P-D-mannosamine. *L. ivanovii* subsp. *londoniensis* does not produce acid from ribose but produces acid from N-acetyl-P-D-mannosamine after 18 to 24 h of incubation at 37°C (Boerlin *et al*., 1992).

L. ivanovii contains variants of the main *Listeria* virulence gene cluster which includes the virulence genes *hly* (which encodes a haemolysin), *prfA*, *plcA*, *mpl*, *actA* and *plcB* (Graves *et al*., 2010). *L. ivanovii* has an ability to grow in the host cytoplasm and also to accumulate and reorganize host cell actin to effect intracellular and cell-to-cell spread (Karunasagar *et al*., 1993; Chakraborty *et al*., 1995).

2.2.3. L. grayi

Listeria murrayi, which was a separate species in the genus *Listeria*, is now included in the species *L. grayi* (Rocourt *et al*., 1992). *L. grayi* is non-haemolytic and grows in both aerobic and anerobic conditions. It grows as small translucent grey colonies on both blood and

chocolate agar. (Salimnia *et al*., 2010) and lacks listeriolysin. *L. grayi* is not closely related to all the other *Listeria* species such that not much literature is available on it.

2.2.4. L. seeligeri

Although *L. seeligeri* is generally characterized as haemolytic, there are also some atypical non-haemolytic strains, with the haemolytic strains containing a homologue of the main virulence gene cluster that is the *prfA* cluster which carries key virulence genes in both *L. monocytogenes* and *L. ivanovii*, although *L. seeligeri* is considered as being non-pathogenic (Graves *et al*., 2010; Sauders *et al*., 2012). *L. seeligeri* produces even narrower zones of haemolysis, in comparison to *L. monocytogenes* and *L. ivanovii* on horse blood or sheep blood agar plates (Allerberger, 2003).

2.2.5. L. innocua

L. innocua is a close relative of *L. monocytogenes* and has the same environmental niches as *L. monocytogenes*. *L. innocua* shares 2523 orthologous genes with *L. monocytogenes*, representing 88.4% of *L. monocytogenes* protein-coding genes (Glaser *et al*., 2001; Wurtzel *et al*., 2012). It has a circular chromosome made up of 3,011,209 base pairs, with a 37% G+C content. Only 2 973 protein-coding genes were discovered in *L. innocua*, while no function could be predicted for 37% of the genes (Glaser *et al*., 2001), also it has a circular plasmid of 81,905 base pairs whose function is also not yet known (Glaser *et al*., 2001).

L. innocua is considered non-pathogenic and most strains lack the *prfA* virulence gene cluster, although a small proportion of its strains do however carry this cluster (den Bakker *et al*., 2010).

This species is typically non-haemolytic, but some uncharacteristic haemolytic strains have also been documented (Johnson *et al*., 2004).

2.2.6. L. welshimeri

The species *L. welshimeri* appears to have been derived from early evolutionary events and an ancestor more compact than *L. monocytogenes* that led to the emergence of non-pathogenic *Listeria* species (Hain *et al*., 2006b). This particular *Listeria* species is non-haemolytic (Allerberger, 2003), is also considered as non-pathogenic and lacks the *prfA* virulence gene cluster (den Bakker *et al*., 2010). It has a circular chromosome of 2,814,130 base pairs and an average G+C content of 36.4% and 2780 protein-coding genes (Hain *et al*., 2006b). Serovars 6b, 1/2a, 1/2b, 6a, 4c, and 4f (Kluge and Hof, 1986; Jones and Seeliger, 1992; Hain *et al*., 2006b) have also been reported for this species. In this species, the lack of genes required for intracellular replication have been compensated for by the acquisition of genes/gene clusters for uptake systems and metabolic pathways to exploit plant-specific cell wall components (Hain *et al*., 2006b).

2.2.7. L. marthii

This species was first reported and proposed by Graves *et al*. (2010) where they described polyphasic analysis of four *Listeria*-like bacilli that were isolated from the natural environment in the Finger Lakes region of New York and proposed that they should be placed in a new species within the genus *Listeria* as *L. marthii* (Graves *et al*., 2010), since the bacteria exhibited characteristics of the genus *Listeria* as reported by Rocourt and Buchrieser (2007) but did not fit into any of the six known *Listeria* species groups. The bacterium is non-haemolytic, negative

for rhamnose and xylose assimilation whilst by colony morphology it resembles *L. innocua* and other non-haemolytic species of the *Listeria* genus (Graves *et al*., 2010).

By conventional biochemical profiles the species is positive for catalase activity, aesculin hydrolysis, hydrogen sulfide production, and the methyl red test; negative for oxidase activity; tolerant of sodium chloride; positive for assimilation of D-glucose, lactose and maltose; and negative for sucrose assimilation, nitrate reduction, urease activity, indole production and gelatin hydrolysis. It forms an umbrella-type growth appearing 3-5 mm below the surface of a stabbed semisolid motility medium incubated at 20-30°C but is non-motile at 37°C. This *Listeria* species does not grow on MacConkey, *Salmonella-Shigella* citrate or cetrimide agars (Graves *et al*., 2010). PCR-based screens showed that the species lacks the *prfA* virulence gene cluster as well as *inlA* gene (Graves *et al*., 2010). The temperature range for growth is 1-45°C (optimal growth at 30-37°C) (Graves *et al*., 2010) and data by den Bakker *et al*. (2010) included *L. marthii* into the same clade as *L. welshimeri*, *L. innocua* and *L. monocytogenes*.

2.2.8. L. rocourtiae

This *Listeria* species was first identified and proposed by Leclercq *et al*. (2010), from analysis of a *Listeria* like CIP 109804^{T} strain that was isolated in Austria from pre-cut lettuce. The bacteria strain resembled *Listeria* but did not fit into the six known *Listeria* species groups. This *Listeria* species is characterised as being Gram-positive, non-spore forming, non-capsulated and rod-shaped bacterium with optimum growth at 30°C. The bacterium is also facultatively anaerobic, catalase-positive, nitrate reductase-positive, aesculin hydrolysis-positive, oxidase-negative and non-haemolytic. The bacterium is motile at 4-30°C but non-motile at 37°C and shows the umbrella motility form in mannitol-mobility semi solid agar. The

colonies appear blue-green when viewed under a magnifying glass with oblique transmitted light, and bluish grey by normal illumination (Leclercq *et al*., 2010).

The species was characterised as being non-virulent as it does not form plaques in HT-29 cell culture and is also unable to colonize the spleens of Swiss mice in 3 days from initial day of subcutaneous inoculation. Typical *Listeria* genes, such as those known to encode 11 LPxTG anchor containing surface proteins, were detected in the strain as well as many other transcriptional regulators and teichoic acid biosynthesis genes present in other *Listeria* genomes although it has no specific serotype or PCR group described for the genus *Listeria* (Leclercq *et al*., 2010).

2.3. *Listeria* infections

2.3.1. Modes of Transmission

The main source of *Listeria* bacterial infections is through consumption of contaminated food making them mainly food-borne infections (Mead *et al*., 1999; Hain *et al*., 2006a). The fetus on the other hand can acquire the infection from its infected mother through the placenta, or orally through passage in an infected birth canal (Swaminathan and Gerner-Smidt, 2007; Allerberger and Wagner, 2010; Camejo *et al*., 2011).

Listeria may also inhabit the gastrointestinal tract of animals and humans hence raw sewage also represents a significant source of the bacteria (Warriner and Namvar, 2009). Contaminated manure can also introduce the pathogen to the feed and the natural environment and due to the psychrotrophic nature of the bacterium it can grow in the environment and become endemic within processing facilities. Therefore, foods can be contaminated during the primary

production phase or during processing which ultimately can introduce the pathogen into food-processing environments (Warriner and Namvar, 2009).

Listeria infections have been associated with a wide range of vegetables, meats, dairy and seafood products, such that it is highly likely that there is a wide range of interactions with different food matrices (McLauchlin *et al*., 2004). The *Listeria* bacterium's persistence in food processing environments could be due to several factors such as the ubiquity of the *Listeria* species which enables them to enter the food chain, the ability of the human pathogenic species *L. monocytogenes* to form biofilms and as well as its ability to grow at low temperatures down to freezing point and the normal refridgerator temperatures of 4-10°C (Swaminathan and Gerner-Smidt, 2007; Bortolussi, 2008; Warriner and Namvar, 2009).

L. monocytogenes can also grow and survive in the amniotic fluid which can result in widespread contamination of newborn infant, maternal sites at delivery and the postnatal environment which may result in additional neonatal cases due to cross-infection (McLauchlin *et al*., 2004). *Listeriosis* is a fatal opportunistic infection/disease of humans and animals caused by the pathogenic *Listeria* bacteria. *Listeriosis* in humans is fatal in up to 30% of the cases or even higher and may involve severe clinical manifestations such as meningoencephalitis, abortion and septicemia (Vazquez-Boland *et al*., 2001a; Schuppler and Loessner, 2010).

2.3.2. Cell Infection and pathogenesis

The pathogenic *Listeria* bacteria *L. monocytogenes* and *L. ivanovii* are typical facultative intracellular parasites. They are able to proliferate within macrophages and a variety of normally non-phagocytic cells, such as epithelial and endothelial cells as well as hepatocytes due to their virulence factors (Vaquez-Boland *et al*., 2001a).

The bacteria utilizes the cell wall adhesion proteins internalin A and internalin B to bind to the host-cell membrane receptors E-cadherin and Met (Mardis *et al*., 2012) and enters the host cell in a form of a phagocytic vacuole and once in these cells, the bacterium develops a characteristic intracellular life cycle which involves early escape from the phagocytic vacuole through production of a phagolysosome (listeriolysin O) by the bacterium which lyses the vacuole and allows it to enter the host cell's cytoplasm where the bacterium starts to multiply (Vaquez-Boland *et al*., 2001a; Mardis *et al*., 2012).

After the *Listeria* bacteria's multiplication, actin-based intracytosolic motility occurs whereby the bacterium's ActA surface proteins initiate actin polymerisation which allows the bacterium to propel to the host cell's membrane and create filopods which are basically an elongated protrusion of the cell membrane that encompasses the bacterial cell and extends to adjacent cells, which then ingest the filopods. This therefore enables the *Listeria* to enter the cytosol of another new cell in the host allowing direct cell to cell spread and reinitiation of the cell cycle in the new cell (Vaquez-Boland *et al*., 2001a; Seveau *et al*., 2007; Mardis *et al*., 2012).

The gastrointestinal tract is the primary site of entry of pathogenic *Listeria* bacteria since contaminated food is the major source of listeriosis infections (Vaquez-Boland *et al*., 2001b). This is then followed by the colonisation of the intestine which leads to intestinal translocation whereby the bacterium crosses the intestinal barrier and is absorbed from the intestinal lumen, traversing the epithelial cell layer, and if the immune system does not control the infection, the pathogen disseminates to the bloodstream and mesenteric lymph nodes (Orndorff *et al*., 2006; Camejo *et al*., 2011). The bacterium may then replicate in the liver and spleen preferentially inside splenic and hepatic macrophages or epithelial cells, resolution of the bacterium at this stage is dependent upon T cell-mediated immunity. In persons with T-cell impaired immunity or the immune-compromised persons in general, there is then hematogenous spread of the *Listeria* to the central nervous system or it transverses through the placental barrier to infect

the fetus in pregnant women (Orndorff *et al*., 2006; Warriner and Namvar, 2009; Camejo *et al*., 2011).

2.3.3. Listeriosis risk groups and clinical manifestastions

Despite there being only two known pathogenic *Listeria* species there have however been some reports as emphasised in chapter one that have shown the potential pathogenicity of some other *Listeria* species: *L. seeligeri* (Rocourt *et al*., 1986), *L. grayi* (Todeschini *et al*., 1998; Rapose *et al*., 2008; Salimnia *et al*., 2010) and *L. ivanovii* (Cummins *et al*., 1994; Guillet *et al*., 2010) have been implicated in human listeriosis cases while *L .innocua* was implicated in an animal listeriosis case (Walker *et al*., 1994), such that these species should also be treated as potential pathogenic species.

Certain conditions have however been identified as risk factors for acquiring listeriosis such as old age, malignancies, diabetes mellitus, alcoholism, liver diseases, renal diseases, autoimmune diseases and other immune-suppressing conditions or treatments (Swaminathan and Gerner-Smidt, 2007; Bortolussi, 2008). Pregnant women, their fetuses and neonates (younger than four weeks) are also at increased risk of listeriosis (Allerberger and Wagner, 2010).

The major defense of the body against listeriosis is cell-mediated immunity such that people with T-cell mediated immunity dysfunction seem to be particularly prone to contracting the disease (Swaminathan and Gerner-Smidt, 2007), such that most maternal infections occur during the third trimester of pregnancy when T-cell immunity is most impaired (Allerberger and Wagner, 2010). Of malignancies, hematological diseases carry the highest risk of listeriosis, while patients with immunological suppression due to HIV infection are considered to be at increased risk for listeriosis as well (Swaminathan and Gerner-Smidt, 2007; Bortolussi,

2008). However, highly virulent strains of *L. monocytogenes* belonging to serotype 4b can be fatal even in healthy individuals (Drevets and Bronze, 2008; Warriner and Namvar, 2009).

The incubation period between exposure that is consumption of contaminated foods and onset of listeriosis symptoms varies between 2-70 days (Bortolussi, 2008). The approximate infective dose of *L. monocytogenes* is estimated to be 10–100 million colony forming units (CFU) in healthy hosts, and only 0.1–10 million CFU in people at high risk of infection (Farber *et al*., 1996; Bortolussi, 2008).

Listeriosis can manifest in either of three forms of infections which are non-invasive gastrointestinal, invasive or focal listeriosis infections. Most cases of listeriosis are non-invasive gastrointestinal and present as flulike symptoms with febrile/feverish gastroenteritis and may often involve watery non-bloody diarrhoea, vomiting, nausea and abdominal pains (Ooi and Lorber, 2005; Allerberger and Wagner, 2010; Mardis *et al*., 2012), usually due to the colonization of the intestine and the subsequent replication of the bacterium in the liver and spleen (Salamina *et al*., 1996; Orndorff *et al*., 2006). These symptoms usually prompt the affected person to seek medical attention, however, cases of diarrhoeal disease with fever are rarely investigated for listeriosis since the media for the isolation of *Listeria* from faeces and the use of blood cultures are not routinely used to investigate such types of infections (McLauchlin *et al*., 2004).

Non-invasive gastrointestinal listeriosis is usually self limiting in immune-competent individuals but in the listeriosis risk groups it can develop into invasive listeriosis. Invasive listeriosis can be fatal and occurs when the bacteria reaches the blood-stream or the central nervous system (CNS). Consequently, this form of listeriosis can lead to clinical manifestastions such as meningitis, rhombencephalitis and septicemia (Vazquez-Boland *et al*., 2001a; Schuppler and Loessner, 2010; Mardis *et al*., 2012). Invasive listeriosis during

pregnancy can lead to abortion, birth of a stillborn fetus, birth of a baby with generalized infection (granulomatosis infantiseptica), and sepsis or meningitis in the neonate (Allerberger and Wagner, 2010).

Symptoms of CNS infection may include intense headache, nuchal rigidity, confusion, loss of balance, convulsions, fever, nausea, vomiting and signs of meningeal irritation (Allerberger and Wagner, 2010; Mardis *et al*., 2012). In addition to fever, Kernig's and Brudzinski's signs may occur in adults with acute meningitis. Although symptoms of acute meningitis are usually less pronounced in children, they commonly include bulging fontanelles, purpuric rash and seizures (Mardis *et al*., 2012), while rhombencephalitis involving the brainstem is an unusual form of listeriosis (Allerberger and Wagner, 2010).

Listeriosis focal infections are rare and are a result of hematogenous spread. The symptoms are diverse and highly site specific (Mardis *et al*., 2012). A wide variety of them have been described including myocarditis and necrotizing fasciitis (Sendi *et al*., 2009), joint infection (Kleemann *et al*., 2009), endocarditis (Kelesidis *et al*., 2010), eye infection (Swaminathan and Gerner-Smidt, 2007) and cutaneous infection (Gilchrist, 2009), with cutaneous infection being a potential hazard to mainly veterinarians and farmers working with infected animals (Schlech III, 2000).

Given the high average case fatality rates of listeriosis, it is of the essence for health care professionals to know its modes of transmission as well as be able to recognize the clinical manifestastions thereof in due time especially in high-risk populations such that proper treatment protocols can be followed earlier (Swaminathan and Gerner Smidt, 2007; Mardis *et al*., 2012).

2.4. Treatment of Listeriosis

Treatment recommendations for listeriosis are made secondary to the location, severity and extent of infection. Immune-competent patients with non-invasive gastrointestinal listeriosis that is self limiting rarely require antibiotic therapy (Ooi and Lorber, 2005; Mardis *et al*., 2012), since the progression from gastroenteritis to more invasive disease is relatively uncommon in immune-competent individuals unlike in the listeriosis risk groups.

Progression from non-invasive gastroenteritis to serious invasive listeriosis forms can to some extend be prevented by administration of oral ampicillin or sulfamethoxazole-trimethoprim (SMX-TMP) (Ooi and Lorber, 2005; Mardis *et al*., 2012). Treatment in invasive listeriosis cases is of vital importance and the first-line antibiotics of choice for the treatment are ampicillin and penicillin G. General dosing of these antibiotics varies based on patient's age and weight (Lorber, 2009; Mardis *et al*., 2012). These penicillins (ampicillin and penicillin G) can also be used synergistically in combinations involving an aminoglycoside mostly gentamicin especially in patients with severe listeriosis conditions, of which gentamicin dosing for synergy should be patient-specific and based on serum peaks and troughs (Swaminathan and Gerner Smidt, 2007; Lorber, 2009).

Patients allergic to penicillin may need to undergo skin testing and subsequent desensitization protocols, while individuals whose allergy is limited to mild-to-moderate maculopapular rash are sometimes prescribed meropenem (Mardis *et al*., 2012). In patients with severe penicillin allergy SMX-TMP may be utilized, with the higher end of the dosing spectrum favoured for more advanced conditions and when feasible, patients taking SMX-TMP intravenously can be converted to oral therapy at the same dosage (Mardis *et al*., 2012). Also, Vancomycin/teicoplanin can be used in patients allergic to penicillins (Swaminathan and Gerner Smidt, 2007).

However, several potential medication-related issues may arise in the treatment of a patient with listeriosis. Antibiotic therapy is often ineffective for several reasons with the most central reason being the ability of *L. monocytogenes* to multiply intracellularly and spread cell-to-cell without leaving the protective environment of the host's cells (Orndorff *et al*., 2006). This property not only limits the choice of antibiotic therapy, but also necessitates a vigorous cell-mediated host immune response (Orndorff *et al*., 2006), and might also explain the differences between *in-vivo* and *in-vitro* results of *Listeria* isolates' antimicrobial susceptibility tests (Allerberger and Wagner, 2010). Addition of gentamicin has not been proven to be clinically advantageous, as synergy has only been demonstrated *in-vitro* (Mitja *et al*., 2009), as in some animal models, gentamicin does not reliably show a synergistic effect (Blanot *et al*., 1999). A gentamicin supplemented protocol should also not be prescribed for pregnant women, because of possible teratogenic effects (Hof, 2003; Jacobson, 2008; Allerberger and Wagner, 2010).These factors therefore impact negatively on listeriosis treatment options.

In-vitro isolates of *L. monocytogenes* are generally susceptible to a wide range of antibiotics such as penicillins, aminoglycosides, trimethoprim, tetracycline, macrolides, and vancomycin. They show reduced susceptiblity or resistance to fosfomycin, sulfomethoxazole, third generation cephalosporins and first generation quinolones but are generally susceptible to fluoroquinolones (Troxler *et al*., 2000; Swaminathan and Gerner-Smidt, 2007; Allerberger and Wagner, 2010). This general trend of susceptibility is however taking a shift due to antibiotic resistance, as most clinical and environmental isolates of *L. monocytogenes* and other *Listeria* species are becoming resistant to most conventional antibiotics (Charpentier and Courvalin, 1999; Arslan and Ozdemir, 2008).

2.5. Antibiotic Resistance with respect to *Listeria* species

Antibiotics are one of the most important weapons in fighting bacterial infections and since their introduction they have significantly improved the health-related quality of human life (Rakholiya and Chanda, 2012). However, over the past years these health benefits have come under threat as many of the conventional antibiotics have reduced efficacy against some of the bacterial infections, primarily due to the constant emergence of antibiotic resistant bacteria in addition to the antibiotics also having adverse side effects on the patients, such that the antibiotic's efficacy lifespan is reduced (Rakholiya and Chanda, 2012).

2.5.1. Bacterial resistance mechanisms against some antibiotics used in listeriosis treatment.

Beta-lactam (β-lactam) antibiotics are a class of antibiotics whose mechanism of action against bacteria is through cell wall inhibition. Bacteria have built resistance to β-lactam antibiotics such as the penicillins through alteration of the target site which is the penicillin binding proteins (PBPs) in the cell wall such that they have less binding affinity to the penicillins or they cannot bind to the penicillin at all. The second mechanism of resistance is through production of β-lactamase enzymes by the bacteria which are able to hydrolyse the β-lactam ring amide bond in the β-lactam antibiotic rendering the antibiotic ineffective (Deshpande *et al*., 2004; Jury *et al*., 2010).

Sulfonamides and trimethoprim are antibiotics that competitively inhibit the bacterial enzymes required for the synthesis of tetrahydrofolic acid (THF), which is necessary for DNA and protein synthesis (Masters *et al*., 2003; Levy and Marshall, 2004). Sulfonamides act as competitive inhibitors of dihydropteroate synthase (DHPS), while trimethoprim inhibits dihydrofolate reductase (DHFR) (Jury *et al*., 2010). Resistance to sulfonamides and

trimethoprim is mostly associated with plasmid-encoded genes, such as the *Sul1*, *Sul2* and *Sul3* genes that have been identified as sulfonamide resistance genes coding for different types of DHPS that are resistant to sulfonamides effects (Grape *et al*., 2003). Resistance to sulfonamides can also be due to mutations within the chromosomally located dihydropteroate synthase gene (*folP)* (Jury *et al*., 2010). Trimethoprim resistance is widespread amongst pathogenic bacteria due to dihydrofolate reductase (*dfr)* resistance genes, most of which are associated with integrons and use elaborate transfer mechanisms to laterally spread and proliferate within the bacterial community (Jury *et al*., 2010).

Gentamicin, kanamycin and streptomycin are classified under the aminoglycosides antibiotics and their mode of action is through protein synthesis inhibition. They bind to bacterial ribosomes thereby preventing the initiation of protein synthesis. The primary mechanism of resistance in aminogylcosides is through chemical alteration of the antibiotic by some bacterial modifying enzymes which then inhibit the antibiotic from binding to the bacterial ribosome which is its target site (Wright, 1999; Levy and Marshall, 2004). Mutations associated with ribosomal genes and efflux systems may also be linked to aminoglycoside resistance (Jury *et al*., 2010).

Vancomycin and teicoplanin are glycopeptide antibiotics and their mode of action is through inhibition of cell wall synthesis (Levy and Marshall, 2004). Vancomycin inhibits cell wall formation of Gram-positive bacteria by binding to its target site within the peptidoglycan assembly preventing cross-linking. Resistance to vancomycin has been associated with seven *van* genes, which code for the promotion of an abnormal target site with lower affinity for the antibiotic (Poole, 2002). The *vanA* genotype also confers a high level of bacterial resistance to teicoplanin (Jury *et al*., 2010).

2.5.2. Studies showing in-vitro antibiotic and multi-antibiotic resistance in Listeria species.

There are numerous reports in literature on the *in-vitro* resistance to antibiotics of both clinical and environmental *Listeria* species isolates (Bertrand *et al*., 2005; Rodas-Suarez *et al*., 2006; Arslan and Ozdemir, 2008; Davis and Jackson, 2009; Odjadjare *et al*., 2010; Lotfollahi *et al*., 2011; Soni *et al*., 2013). The antimicrobial susceptibility to various antimicrobial agents of 47 *Listeria* isolates from homemade white cheese which included all the six well known *Listeria* species showed resistance to penicillin (12.8%), chloramphenicol (8.5%), clarithromycin (6.4%), amikacin (4.3%), tetracycline (2.1%) and cefaclor (2.1%), with the highest resistance percentage being observed against penicillin (Arslan and Ozdemir, 2008).

L. monocytogenes strains isolated from fish and estuarine water showed varying levels of resistance to the different antibiotics with the antibiotic resistance percentages being; gentamicin (5.9%), dicloxacillin (9.7%), tetracycline (13.2%), erythromycin (30.9%), trimethoprim-sulfamethoxazole (37.4%), penicillin (57.4%), ampicillin (60.3%), ceftazidime (67.6%) and pefloxacin (73.5%). Multi-antibiotic resistant strains were also noted in the study with 6% of the *L. monocytogenes* strains showing multi antibiotic resistance to ampicillin, erythromycin, tetracycline, dicloxacillin, and trimethoprim-sulfamethoxazole (Rodas-Suarez *et al*., 2006).

L. monocytogenes and *L. innocua* isolates from human and food-processing sources were found to exhibit tetracycline resistance due to the presence of the *tet (M)* gene (Bertrand *et al*., 2005). Sequence analysis, showed that the *tet (M)* genes in two of the isolates belonged to the sequence homology group (SHG) II, which is a group comprising of chromosomally encoded *tet(M)* genes and was previously found in *Staphylococcus aureus* and in *Lactobacilli*. The *tet (M)* genes found in the other two *L. monocytogenes* strains in the study were associated with a

member of the Tn916–Tn1545 family of conjugative transposons and were closely related to SHG III, which harbours enterococcal *tet (M)* genes associated with Tn916 of which in the study one of the transposon-containing strains was able to transfer the *tet (M)* gene to *Enterococcus faecalis* recipient strain JH2-2 (Bertrand *et al*., 2005). In general, the sequence and conjugation data indicated that the acquisition of the *tet (M)* gene by *Listeria* strains may have been triggered by successive transfers between other Gram-positive organisms (Bertrand *et al*., 2005). The *tet (M)* gene was also found present in tetracycline resistant *Listeria* isolates in the study by Davis and Jackson (2009), whilst other resistance genes such as *aadA, strA–B, sul I–II, penA, vat(A–E), vga(A–B),* and *vgb(A–B)* were negative in the *Listeria* isolates which were however showing phenotypic resistance to the respective antibiotics.

Studies by Odjadjare *et al*. (2010) showed higher resistance to penicillin G, ampicillin, sulphamethoxazole and erythromycin by *L. innocua* and *L. ivanovii* strains isolated from waste water effluents. In a separate study *L. monocytogenes* strains isolated from clinical samples that included blood, urine, placental tissue, fecal and vaginal swabs which were collected from patients with spontaneous abortions, 77.8% of the *L. monocytogenes* isolates was found to be resistant to one or more antibiotics. Resistance to both penicillin G and cephotaxim singly was found in 77.8% of the isolates while resistance to both chloramphenicol and streptomycin singly was found in 11.1% of the isolates (Lotfollahi *et al*., 2011). A study by Soni *et al*. (2013) showed 90% resistance to ampicillin by some isolates of *L. monocytogenes* recovered from water, human clinical and milk samples. Variable resistance by the same isolates to cefoxitin, cotrimoxazole, gentamicin, oflaxacin, rifampin, and tetracycline was also observed and all the isolates showed multi-antibiotic resistance patterns (Soni *et al*., 2013).

From most of the studies mentioned above, resistance against penicillins was evident in both clinical and environmental *Listeria* isolates and some multi-antibiotic resistance strains were also noted such that these results are of major concern as penicillins are the first line antibiotics

of choice against listeriosis. The resistance that pathogenic bacteria have built against conventional antibiotics has drawn much attention to extracts and biologically active compounds from traditional medicinal plants as they represent a potential source of new antibacterial agents, since in many parts of the world traditional medicinal plants are already used for antibacterial, antifungal, and antiviral activities (Essawi and Srour, 2000).

2.6. The use of plants as alternative or supplementary forms of medicines

The success story of chemotherapy is invested in the continuous search for new drugs to combat antibiotic resistance, the investigation of certain indigenous or rather traditional medicinal plants for their antimicrobial properties is an avenue with great potential of yielding successful results (Doughari *et al*., 2008). Since time immemorial, plants have been used to treat various infectious diseases as the idea that they possessed what is currently characterized as antimicrobial principles was generally accepted and up to the present day traditional plant medicines still play a pivotal role in the treatment of numerous diseases or infections (Doughari *et al*., 2008), such that many of these plants have been investigated for the development of novel pharmaceutical drugs or templates thereof (Dogan *et al*., 2010).

2.6.1. Plants as sources of antimicrobial compounds

Plants produce a variety of secondary metabolites which are a significant part of the chemical diversity of the plants that serves to protect them against microbial pathogens (Dixon, 2001; Tegos *et al*., 2002). Many of the plant secondary metabolites are constitutive, existing in healthy plants in their biologically active forms and are classified as phytoanticipins, but others occur as inactive precursors and are activated in response to tissue damage or pathogen attack

such that their levels increase in response to microbial invasion and are classified as phytoalexins (Tegos *et al*., 2002: Ncube *et al*., 2008). In several cases mutant plants that lack the ability to produce a particular phytoalexin have been shown to have higher levels of sensitivity to microbial pathogens (Tegos *et al*., 2002). The phenolics, polyphenols, terpenes (including essential oils, sterols and triterpenes) and alkaloids are amongst some of plants' secondary metabolites that have antimicrobial activities (Ncube *et al*., 2008).

The phenolics and polyphenols include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols and tannins. This group also includes metabolites derived from the condensation of acetate units namely tepernoids (which are terpenes that contain an additional element such as oxygen) and those produced by the modification of aromatic amino acids (phenylpropanoids and coumarins) (Ncube *et al*., 2008).

Essential oils which can also be called volatile oils are obtained from different plant parts (flowers, buds, seeds, twigs, leaves, bark, fruits and roots), they (essential oils) are aromatic and are responsible for the fragrance of most plants (Prabuseenivasan *et al*., 2006). Essential oils possess antimicrobial properties and are mainly constituted of isoprene structure based compounds (Das *et al*., 2010). The proposed mechanisms of action of essential oil components are related to disintegration of the cytoplasmic membrane, destabilization of the proton motive force (PMF), disturbance of active transport and coagulation of the cell content, of which not all of these mechanisms are separate targets as some are affected as a consequence of another mechanism being targeted (Burt, 2004).

Sterols and triterpenes can accumulate as glycoside conjugates in substantial quantities in plants which are commonly referred to as saponins. These saponins can be divided into three groups that include the triterpenoid, steroid and steroidal glycoalkaloids which are classified depending on the structure of their aglycones (Osbourn, 1996; Ncube *et al*., 2008; Gonzalez-

Lamothe *et al*., 2009). Saponins are a form of glycosylated phytoanticipins and they have potent antimicrobial activities and their natural proposed role is to protect plants against invading pathogens (Osbourn, 2003; Gonzalez-Lamothe *et al*., 2009).

Some plants' secondary metabolites have potential as anticancer agents, for example, the flavonoid-rich extracts from the mature roots of *Scutellaria baicalensis* which were shown to possess anti-proliferative effects on an array of cancer lines (Scheck *et al*., 2006), and also a diterpene known as Taxol from the Pacific yew plant which is used for ovarian and breast cancer treatment (Iwu *et al*.,1999; Ncube *et al*., 2008).

2.6.2. Plant compounds as antibiotic potentiators and virulence attenuators

Some plant compounds can be defined as antibiotic potentiators as they may enable some conventional antibiotics to recover some of their potent therapeutic applications which could have been lost due to antibiotic resistance (Gonzalez-Lamothe *et al*., 2009). Examples of antibiotic potentiators include examples of cell wall acting agents and membrane destabilizing agents, such as essential oils of which a major fraction of essential oils from plant extracts is composed of terpenoids and synergy between conventional antibiotics and sesquiterpenoids such as farnesol, nerolidol and others has been demonstrated (Gonzalez-Lamothe *et al*., 2009). Efflux pump inhibitors are also antibiotic potentiators as shown by epigallocatechin-gallate (EGCg) which is a major catechin in green tea extracts which was shown to enhances the activity of tetracycline in Staphylococci isolates by inhibiting tetracycline efflux from the bacterial cells and also reversed tetracycline resistance in *Staphylococcus* isolates which showed resistance due to the expression of Tet(K) efflux pump proteins (Roccaro *et al*., 2004).

Some plant compounds termed virulence attenuators may also assist/enable the host immune system to sufficiently respond to pathogen/microbial invasion. The reasoning behind this theory is that anti-pathogenic molecules that may for example prevent the production of toxins or eliminate the ability of bacteria to adapt to the mammalian environment would give a competitive advantage to the host immune system thereby enabling for the clearance of the pathogen (Gonzalez-Lamothe *et al*., 2009). An example would be the garlic extract which was shown in a study by Bjarnsholt *et al*. (2005) to render *Pseudomonas aeruginosa* biofilms sensitive to the antibiotic tobramycin, and to the phagocytosis effects of polymorphonuclear leukocytes (PMNs). The garlic extract also significantly improved the clearing of the infecting bacteria which was *P. aeruginosa* that was instilled in the left lung of a mouse pulmonary infection model (Bjarnsholt *et al*., 2005).

These antibiotic potentiators and virulence attentuators may also allow for both synergistic and additive interactions between plant extracts and conventional antibiotics as there will be a combined effect of the active compound(s) from the extracts and the conventional antibiotics (Stefanovic *et al*., 2012). Synergism between conventional antibiotics and medicinal plant compounds is a well accepted theory (Silva and Fernandes Junior, 2010). The advantages of using plant antimicrobial being that there are often fewer side effects (as there are comparatively lower incidences of adverse reactions to plant remedies in contrast to conventional antibiotics), better patient tolerance, relatively less expensive (as they are accessible at the local level), acceptance due to long history of use and them also being a renewable resource (Vermani and Garg, 2002). Given all these advantages against a background of increasing antibiotic resistance reports, the search for new antibiotics of plant origin continues unabated (Joshi *et al*., 2011).

2.6.3. Potential of plants as sources of anti-Listerial compounds

A number of studies on different plants' extracts have shown them to possess anti-*Listerial* activities. Studies by Bayoub *et al*. 2010 have shown ethanol extracts of the plants *Artemisia herba alba, Lavandula officinalis L., Matricaria chamomilla, Eugenia caryophylata , Cistus salvifolius, Mentha suaveolens subsp. timija, Thymus serpyllum L., Lippia citriodora, Cinnamomum zeylanicum, Rosa centifolia, Thymus vulgaris L, Rosmarinus officinalis* and *Pelargonium graveolens* to have activities against *L. monocytogenes in-vitro* and the gas chromatography coupled to mass spectrometry (GC-MS) analysis of the extracts showed the presence of various different volatile compounds responsible for the anti-*Listerial* activities observed.

Crude aqueous and ethanolic extracts of the plants *Balanites aegyptiaca* (L) Del. (Balanitaceae), *Hyptis sauveolens* Poit (Lamiaceae), *Leucas aspera* L (Lamiaceae), *Lobelia nicotianaefolia* Roth. ex. Roem. and Schult (Lobeliaceae), *Phyllanthus madraspatana* L (Euphorbiaceae) and the crude ethanolic extract of *Lawsonia inermis* L (Lathyraceae) were found to exhibit anti-*Listerial* activities against *L. monocytogenes in-vitro*. These plants are medicinal plants that are used by traditional medical practitioners in South India (Karmegam *et al*., 2008). The antibacterial activities of the methanol extracts of *Rhizoma Curcumae longae* (Turmeric), *Rhizoma zingiberis* (ginger root) and *Semen lini* (linseed) plants against *L. monocytogenes in-vitro* were observed in a study by Gur *et al*. (2006).

The inhibitory effect of essential oils (1% v/w) of *Thymus daenensis Celak* (Lamiaceae), *Thymbra spicata* L.(Lamiaceae) and *Satureja bachtiarica Bunge* (Lamiaceae) applied to the surface of chicken frankfurters was determined on *L. monocytogenes* and the results showed that the application of the plant's essential oil to frankfurter surfaces could significantly reduce

the *L. monocytogenes* population in comparison to the control after 7 and 14 days of storage at 4°C (Pirbalouti *et al*., 2010). The plant *Rosmarinus officinalis* L. was found to exhibit activity against *L. grayi*, *L. innocua*, *L. monocytogenes* and *L. ivanovii* strains isolated from both environmental and clinical sources (Rozman and Jersek, 2009).

In addition to the above mentioned studies there are also other separate studies carried out by several authors that have shown the inhibitory effects of plant extracts against *Listeria* species (Alzoreky and Nakahara, 2003; Celikel and Kavas, 2008; Mbata and Saikia, 2008; Bocanegra-Garcia *et al*., 2009; Dogan *et al*., 2010), these studies therefore show the potential of plants in anti-*Listerial* therapy.

2.7. *Garcinia kola* as a possible source of anti-*Listerial* compounds

Many plants belonging to the genus *Garcinia* (Guttiferae family) have been reported ethnomedically to exhibit many pharmacological effects and also to be rich sources of polyisoprenylated benzophenones, xanthones, phenolic compounds and steroidal compounds with varying cytotoxic activities (Magadula and Suleimani, 2010; Gao *et al*., 2012). Some of the polyisoprenylated benzophenones isolated from some *Garcinia* species such as isogarcinol, garcinol and xanthochymol which were isolated from the pericarps of *Garcinia purpurea* were shown to exhibit growth inhibition in four human leukemia cell lines due to apoptosis (Matsumoto *et al*., 2003). The xanthones in *Garcinia* species have useful biological activities such as anti-inflammatory, antibacterial, antifungal, anti-oxidant, cytotoxic ,anti-proliferate, antiplasmodial, immunostimulatory and anti-viral (Kosela *et al*., 2000; Kardono *et al*., 2006; Karthiga *et al*., 2012).

The *Garcinia kola* (Heckel) plant is found mostly in central and western Africa (Igbozulike and Aremu, 2009). Its nut also known as the false kola nut is chewed by the natives of Nigeria and Ghana and some other African countries together with the kola nut before a meal to promote digestion and to improve the flavour of the meal (Han *et al*., 2005). The believed beneficial effects of chewing the *Garcinia kola* nut include its mechanical cleansing effect in the mouth and the anti-microbial substances in the seeds (Han *et al*., 2005).

The *Garcinia kola* nut contains an average of four seeds with the seed being popular for its numerous medicinal and cultural uses in Africa and beyond. Apart from the seeds the fruit/nut, leaves, roots, barks, stems and twigs of the plant are also valued for various social, cultural and medicinal purposes (Igbozulike and Aremu, 2009). The stem bark is used for the treatment of malignant tumours, the latex is used internally to treat gonorrhoea and externally on wounds for healing (Iwu, 1993; Adedeji *et al*., 2006). The fruit pulp is used in the treatment of jaundice (Igbozulike and Aremu, 2009), whilst the sap is used for the treatment of parasitic skin disease (Esomonu *et al*., 2005). The fruit extracts of the plant were found to be effective at inhibiting Ebola virus replication (Yamaguchi *et al*., 2000; Magadula and Suleimani, 2010).

Garcinia kola seeds have a bitter astringent taste resembling that of raw coffee seeds which is then followed by a slight sweetness, such that they are also known as bitter kola because of the bitter taste (Niemenak *et al*., 2008). *Garcinia kola* seeds are smooth elliptically shaped, with a yellow pulp and brown seed coat (Eleyinmi *et al*., 2006). The seed is a masticatory used in traditional hospitality, cultural and social ceremonies (Farombi *et al*., 2000) and is also believed to be a snake repellent in addition to possessing aphrodisiac and purgative properties (Igbozulike and Aremu, 2009). The seeds are used in the treatment of bronchitis and throat infections (Mboto *et al*., 2009) and also have shown potential as a substitute for hop in lager beer brewing (Aniche and Uwakwe, 1990).

The whole seed and or its constituents have also been found to exhibit some beneficial therapeutic effects in some controlled experiments. Kolaviron which is a flavonoid extract of *Garcinia kola* seeds comprising of a mixture of *Garcinia* biflavonoid GB1, GB2 and kolaflavanone, has been patented for the treatment of hepatic disorders, inflammation and mouth infections (Farombi *et al*., 2002) and kolaviron's antioxidant and scavenging properties have also been demonstrated (Farombi *et al*., 2002). It was also observed that *Garcinia kola* seeds produce a dose-dependent inhibition of gastric acid secretion and indomethacin-induced ulceration in male albino rats (Ibironke *et al*., 1997).

In-vitro the seeds extracts have also been shown to possess broad spectrum antimicrobial activities (Ezeifeka *et al*., 2004; Nwaokorie *et al*., 2010; Ejele *et al*., 2012). Some synergistic interactions were also shown *in-vitro* between the extracts of *Garcinia kola* seeds and some conventional antibiotics against some Gram positive bacteria (Ofokansi *et al*., 2008; Sibanda and Okoh, 2008). Despite all the researches done, the information on the assessment of the anti-*Listerial* activities of different extracts of *Garcinia kola* seeds is still scanty and given the high mortality rates of listeriosis, the side effects of some of the antibiotics used for listeriosis treatment it becomes imperative to look for alternative remedies, of which *Garcinia kola* seeds are a promising source of antimicrobial compounds given its many medicinal uses.

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CHAPTER THREE

In-Vitro Anti-*Listerial* Properties of Crude Methanol Extracts of *Garcinia kola* (Heckel) Seeds

(Published in The Scientific World Journal)

Abstract

Crude methanol extracts of *Garcinia kola* (Heckel) seeds were screened for their anti-*Listerial* activities against 42 *Listeria* bacteria isolated from wastewater effluents. The extract had activity against 45% of the test bacteria and achieved minimum inhibitory concentrations (MICs) ranging between 0.157 and 0.625 mg/ml. The rate of kill of the extract was determined against four representative *Listeria* species in the study, and the results showed that the highest percentage of bacteria cells were killed after the maximum exposure time of 2 h at the highest concentration of 4× MIC value, with the maximum number of bacteria cells killed being for *L. ivanovii* (LEL30) 100%, *L. monocytogenes* (LAL 8) 94.7%, *L. ivanovii* (LEL 18) 60.3%, and *L. grayi* (LAL 15) 56.1%. The nature of inhibition of the crude methanol extracts of *Garcinia kola* seeds can therefore be either bactericidal or bacteriostatic depending on the target *Listeria* isolate as it can also differ among same specie *Listeria* as evidenced by *L. ivanovii* strains LEL 30 and LEL 18.

Key words: *Listeria* species, *Garcinia kola* seeds, Methanol extract, Rate of kill, MIC

Introduction

Medicinal plant remedies are an integral part of the history and culture of people in developing countries where they are widely used to cover basic health care needs and their use is also becoming part of the integrative healthcare system of developed nations as complementary and alternative systems of medicines (Arif *et al*., 2009). Plant remedies are favoured as a cheaper and readily available alternative form of treatment and Borris (1996) estimated that between 250 000 and 500 000 plant species exist on earth thereby representing a biologically and chemically diverse resource.

Plants are also a source of many useful phytochemicals of great diversity, which have inhibitory effects on different types of microorganisms *in-vitro* (Arif *et al*., 2009). Examples of these phytochemicals include terpenoids, essential oils, alkaloids, lectins, polypeptides, polyacetylenes and phenolics. Phenolics can be subdivided into phenolic acids, flavonoids, quinones, tannins, coumarins and simple phenols (Cowan, 1999). These antimicrobial compounds from plants also have potential to inhibit bacteria through different mechanisms other than those presently used by conventional antibiotics and this may have clinical value in the treatment of antibiotic resistant microbial strains (Eloff, 1998).

In this connection *Listeria* species is one such bacteria specie with increasing reports of its resistance to conventional antibiotics (Abuin *et al*., 1994; Arslan and Ozdemir, 2008; Chen *et al*., 2010; Nwachukwu *et al*., 2010). *Listeria* species are Gram positive bacteria that are widespread in nature and they have been recovered from raw vegetables, raw milk, fish, poultry, and meats, such that infection most likely begins following ingestion of the organism in contaminated food, and clinical manifestations of the invasive listeriosis are usually severe and may include abortion, sepsis, and meningoencephalitis (Snapir *et al*., 2006).

Listeria crosses the mucosal barrier of the intestine and, once in the bloodstream, may disseminate hematogenously to any site although the liver is thought to be the first target organ, where active multiplication occurs until cell-mediated immune response gains control of the infection (Vazquez-Boland *et al*., 2001, Snapir *et al*., 2006). In healthy individuals the continual exposure to *Listerial* antigens may result in maintenance of anti-*Listeria* memory T cells, however, in immune-compromised individuals this exposure may result in prolonged bacteremia and progress to overt listeriosis such that approximately 70% of nonperinatal *Listerial* infections occur in individuals with malignancies, AIDS, organ transplants, or in those receiving corticosteroid therapy (Snapir *et al*., 2006). The case-fatality rates of *Listeria* infection vary from country to country, but invariably the highest mortality is among newborns (25%–50%) due to infection acquired from their mothers, whilst mortality among those over 60 years of age is also high ranging between 10%–20% (Bortolussi, 2008)

Given the high mortality rates of *Listeria* infection against a background of antibiotic resistant strains it becomes imperative to explore for alternative forms of treatment, and having acknowledged the medical importance of plant remedies and their potential in curbing antibiotic resistance, this study therefore focuses on the anti-*Listerial* activities of the crude methanol extract of *Garcinia kola* seeds. *Garcinia kola* is a traditional medicinal plant which has been used since time immemorial for its medicinal purposes mainly in its indigenous origins of central and west Africa (Iwu, 1993). Almost every part of the plant has been found to be of medical importance; the nut is used for nervous alertness, induction of insomnia and also as a masticatory; the root of the plant is used as bitter chew-sticks; the stem bark is used as a purgative; the latex is externally applied to fresh wounds to prevent sepsis, thereby assisting in wound healing (Uko *et al*., 2001). Various studies have focused on the antimicrobial and therapeutic potentials of the *Garcinia kola* plant (Adeleke *et al*., 2006; Tebekeme and Prosper, 2007; Ogbulie *et al*., 2007; Adegbehingbe *et al*., 2008; Penduka and

Okoh, 2011), but despite all that, there is still paucity of information on the anti-*Listerial* activities of the crude methanol extract of the seeds.

Materials and Methods

Plant Material

The ground seed powder of *Garcinia kola* was 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 extracts were prepared following the descriptions of Basri and Fan (2005) and Dogruoz *et al*. (2008). A 100 gram of the seed powder was steeped in 500 ml of methanol solvent for a 48 h period with shaking in an orbital shaker (Stuart Scientific Orbital Shaker, UK). The resultant extract was centrifuged at 3000 rpm for 5 min at 4°C (Beckman Model TJ-6RS Centrifuge, Great Britain), the supernatant was then filtered through Whatman No.1 filter paper while the residue was then used in the second extraction process involving 300 ml of the solvent. The combined extracts were concentrated using a rotary evaporator (Steroglass S.R.L, Italy) at 65°C, after which they were dried 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 extract when making test concentrations.

Test *Listeria* strains

The 42 test *Listeria* isolates 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 previously isolated from wastewater effluents in the Eastern Cape Province of South Africa and belonged to three *Listeria* species groups which are *L. ivanovii*, *L. grayi* and *L. monocytogenes* (Odjadjare *et al*., 2010).

Preparation of the Inoculum

The EUCAST (2003) colony suspension method was used to prepare the inoculum of the test organisms. In brief, colonies picked from 24 h old cultures grown on nutrient agar plates were suspended in saline solution (0.85% NaCl) to give an optical density of approximately 0.1 at 600 nm, after which the suspension was then diluted a hundred-fold before use.

Antibacterial susceptibility test

The agar well diffusion method according to Irobi *et al*. (1996) with some modifications was used to determine the sensitivity of the test *Listeria* to the extract. The prepared bacterial suspension (100 µl) was inoculated into sterile molten Mueller-Hinton agar medium at 50ºC in a MacCarthney 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 after which the wells were filled up with approximately 100 µl of 10 mg/ml extract solution. The plates were then allowed to stand on the laboratory bench for 1 h to allow proper diffusion of the extract into the medium before incubation at 37ºC for 24 h, and thereafter the zones of

inhibition were observed and measured. Ciprofloxacin (2 µg/ml) was used as a positive control, and sterile distilled water was used as the negative control whilst 5% 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 of the extract against the susceptible *Listeria* isolates were determined using the broth microdilution assay method of EUCAST (2003) and carried out in sterile disposable flat-bottomed 96-well microtiter plates. Two-fold serial dilutions using sterile distilled water were carried out from 10 mg/ml of the stock plant extract to make 9 test concentrations ranging from 0.039 to 10 mg/ml. Double strength Mueller-Hinton broth was used for the test. Column 1 was used as the sterility wells containing 100 µl of sterile distilled water in addition to the 100 µl of Mueller-Hinton broth, 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, 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 100 µl of the broth, 50 µl of the test extract concentration and 50 µl of the test organism. The plates were then incubated at 37°C for 18-24 h. 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 *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 h of incubation (EUCAST, 2003).

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

Rate of kill assay

The time kill assay was done according to the method of Odenholt *et al*. (2001) following the descriptions of Akinpelu *et al*. (2008). The selected test *Listeria* isolates namely *L. ivanovii* (LEL18), *L. grayi* (LAL15), *L. monocytogenes* (LAL8) and *L. ivanovii* (LEL 30) were used for the rate of kill studies as representatives of the *Listeria* species used in the study. The turbidity of an 18 h old test *Listeria* culture was standardized to 10^8 cfu/ml by matching with a 0.5 McFarland standard. Four different concentrations of the plant extract were made starting from the MIC value to 4× MIC value obtained against each test organism. A 0.5 ml volume of the standardised organism suspension was added to 4.5 ml of the different extract's concentrations, held at room temperature and the rate of kill determined over a period of 2 h. A 0.5 ml volume of each suspension was withdrawn at 15 min 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 *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 h. The control plates contained the test organism without the plant extracts. The emergent colonies from the tests were counted and compared with the counts of the controls.

Statistical Analysis

The SPSS 19.0 version for windows program (SPSS, Inc.) at a 95% confidence level was used to determine the means and standard deviations of the zones of inhibitions, with the one-way analysis of variance (ANOVA) of the same program being used to determine the means and standard deviations of the rate of kill results.

Results

Antibacterial susceptibility tests

Table 1 shows the zones of inhibitions observed against the susceptible *Listeria* isolates. The methanol extract was active against 19 of the 42 test *Listeria* used in the study giving a percentage activity of 45%. The highest zone of inhibition was against *L. ivanovii* (LDB 7) with a zone of inhibition of 19 mm whilst the lowest zone was 10 mm observed against 7 isolates namely *L. ivanovii* (LDB 11), *L. ivanovii* (LEL 9), *L. ivanovii* (LAL 9), *L. grayi* (LAL 12), *L. grayi* (LAL 15), *L. ivanovii* (LDB 3) and *L. ivanovii* (LAL 11). The positive control ciprofloxacin was active against all the 42 isolates whilst the negative control (sterile distilled water) and 5% DMSO were both not active against any of the test isolates.

Minimum inhibitory concentration and Minimum bactericidal concentration.

The results of the MICs and MBCs of the extract against the susceptible *Listeria* isolates are shown in Table 2. The MICs ranged from 0.157 – 0.625 mg/ml, with MIC values of 0.157 mg/ml and 0.625 mg/ml being recorded against 5 isolates each whilst the MIC value of 0.313 mg/ml was observed against 9 isolates only. The MBCs ranged between 5 and 10 mg/ml with the extract's lowest MBC value of 5 mg/ml being recorded against 3 isolates, namely *L.*

ivanovii (LEL 30), *L. ivanovii* (LDB 12) and *L. ivanovii* (LDB 10), whilst an MBC value of 10 mg/ml was observed against the rest of the isolates. The overall mean MIC and MBC values of the extract were 0.354 mg/ml and 9.21 mg/ml respectively against the 19 test *Listeria* isolates.

Rate of kill assay

The rate of kill results are shown in Figures 1 to 4 for the four isolates tested with the standard deviations also being included in the curves. The extract was bactericidal against *L. ivanovii* (LEL 30) as it killed all (100%) of the initial bacterial population at 75 min exposure time at 4× MIC value as shown in Figure 1. The extract was however bacteriostatic after the maximum exposure time of 2 h at the highest concentration of 4× MIC value against the other three isolates managing to kill 94.7% bacteria cells against *L. monocytogenes* (LAL 8) (Figure 2), 60.3% bacteria cells against *L. ivanovii* (LEL 18) (Figure 3) and 56.1% bacteria cells against *L. grayi* (LAL 15) (Figure 4).

Table 1: Zones of inhibition including the standard deviations of ciprofloxacin and the crude methanol extract of *Garcinia kola* seeds against *Listeria* isolates.

Test organisms	Zones of inhibition (mm)		Test organisms	Zones of inhibition (mm)	
	Meth	**Cipro**		**Meth**	**Cipro**
L. grayi (LAL 13)	0	20±3.06	*L. ivanovii* (LEL 18)	12±0.577	20±3.22
L. ivanovii (LEL 17)	11±1.16	19±1.53	*L. ivanovii* (LEL 29)	0	8±0.577
L. ivanovii (LEL 30)	15±0.577	30±0.577	*L. ivanovii* (LEL 15)	0	13±2.08
L. ivanovii (LDB 11)	10±1.16	20±1	*L. ivanovii* (LDB 9)	13±1.16	25±2.08
L. ivanovii (LEL9)	10±2.52	16±2.08	*L. ivanovii* (LDB 10)	15±1.16	25±0.577
L. ivanovii (LEL 1)	17±0.577	17±0.577	*L. ivanovii* (LEL 2)	0	28±1.53
L. ivanovii (LEL 5)	0	11±0.577	*L. ivanovii* (LEL 6)	0	11±1.73
L. ivanovii (LEL 3)	0	35±3.06	*L. ivanovii* (LEL 4)	0	14±1
L. ivanovii (LEL 19)	0	25±4.04	*L. ivanovii* (LEL 10)	0	20±2.08
L. ivanovii (LAL 9)	10±0.577	25±1.73	*L. ivanovii* (LAL 11)	10±1.73	17±2.65
L. grayi (LAL 12)	10±1.53	17±1.16	*L. ivanovii* (LAL 10)	11±2.08	15±2.08
L. grayi (LAL 15)	10±2	18±2.08	*L. ivanovii* (LAL 14)	0	30±2.52
L. ivanovii (LDB 1)	0	15±2.08	*L. ivanovii* (LDB 2)	0	14±0
L. ivanovii (LAL 6)	0	19±1.16	*L. ivanovii* (LAL5)	0	20±1.53
L. ivanovii (LAL 7)	0	20±1.53	*L. monocytogenes* (LAL8)	18±1.53	12±1
L. ivanovii (LDB 7)	19±0.577	27±0.577	*L. ivanovii* (LDB 12)	14±1.16	25±1.53
L. ivanovii (LDB 3)	10±0.577	15±1	*L. ivanovii* (LDB 8)	0	20±1.73
L. ivanovii (LEL 7)	0	9±1	*L. ivanovii* (LEL 8)	0	30±1.53
L. ivanovii (LEL 14)	0	35±2	*L. ivanovii* (LEL 16)	11±0.577	15±1.53
L. grayi (LAL 3)	0	13±3.06	*L. ivanovii* (LAL 4)	0	20±2
L. ivanovii (LAL 2)	11±1.16	16±1	*L. ivanovii* (LAL 1)	0	20±2

Key: Meth denotes methanol extract; Cipro denotes ciprofloxacin; number ± number denotes zone of inhibition in mm ± standard deviation in mm.

Table 2: Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs) of the crude methanol extracts of *Garcinia kola* (Heckel) seeds against susceptible *Listeria* isolates.

Organism	**Methanol Extracts**	
	MIC (mg/ml)	**MBC (mg/ml)**
L. ivanovii (LEL9)	0.313	10
L. ivanovii (LEL 18)	0.313	10
L. ivanovii (LAL 10)	0.625	10
L. ivanovii (LEL 30)	0.625	5
L. ivanovii (LEL 16)	0.313	10
L. monocytogenes (LAL 8)	0.157	10
L. ivanovii (LDB 12)	0.625	5
L. ivanovii (LDB 10)	0.313	5
L. ivanovii (LEL 1)	0.625	10
L. ivanovii (LAL 11)	0.157	10
L. ivanovii (LDB 3)	0.313	10
L. grayi (LAL 15)	0.157	10
L. grayi (LAL 12)	0.313	10
L. ivanovii (LDB 11)	0.313	10
L. ivanovii (LAL 2)	0.157	10
L. ivanovii (LEL 17)	0.313	10
L. ivanovii (LDB 7)	0.313	10
L. ivanovii (LDB 9)	0.625	10
L. ivanovii (LAL 9)	0.157	10

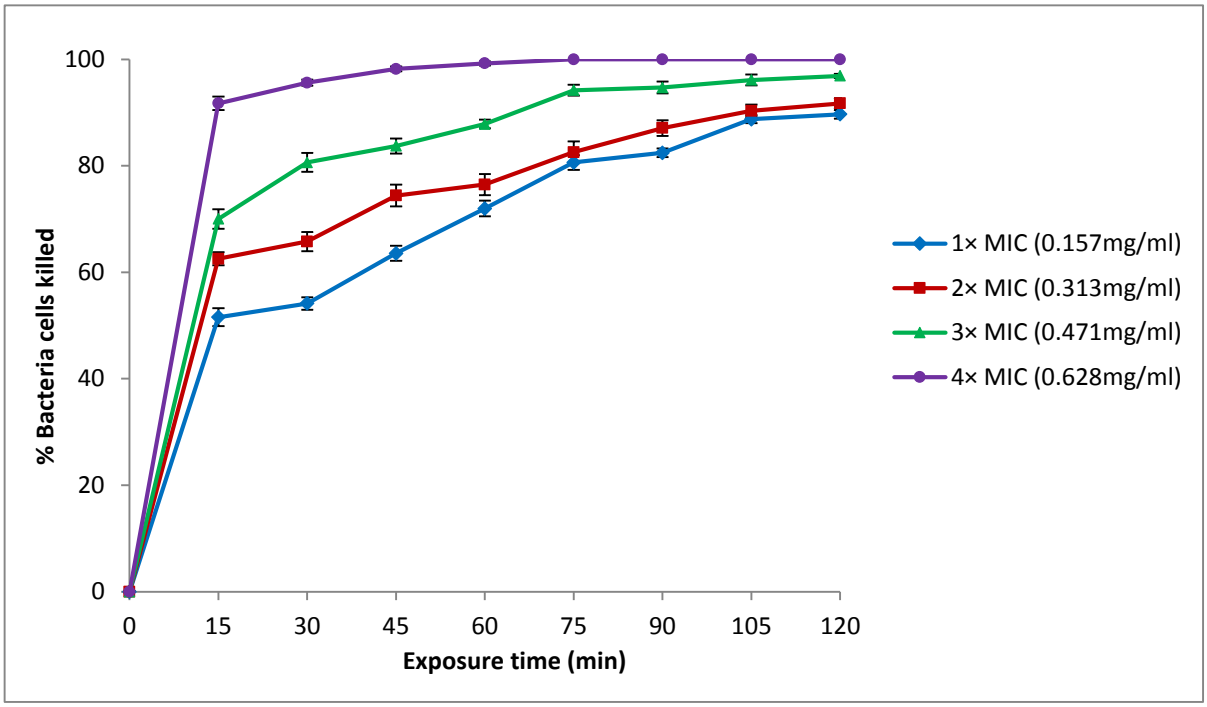

Figure 1. Rate of kill profile for *L. ivanovii* (LEL 30) by crude methanol extracts of *Garcinia kola* seeds.

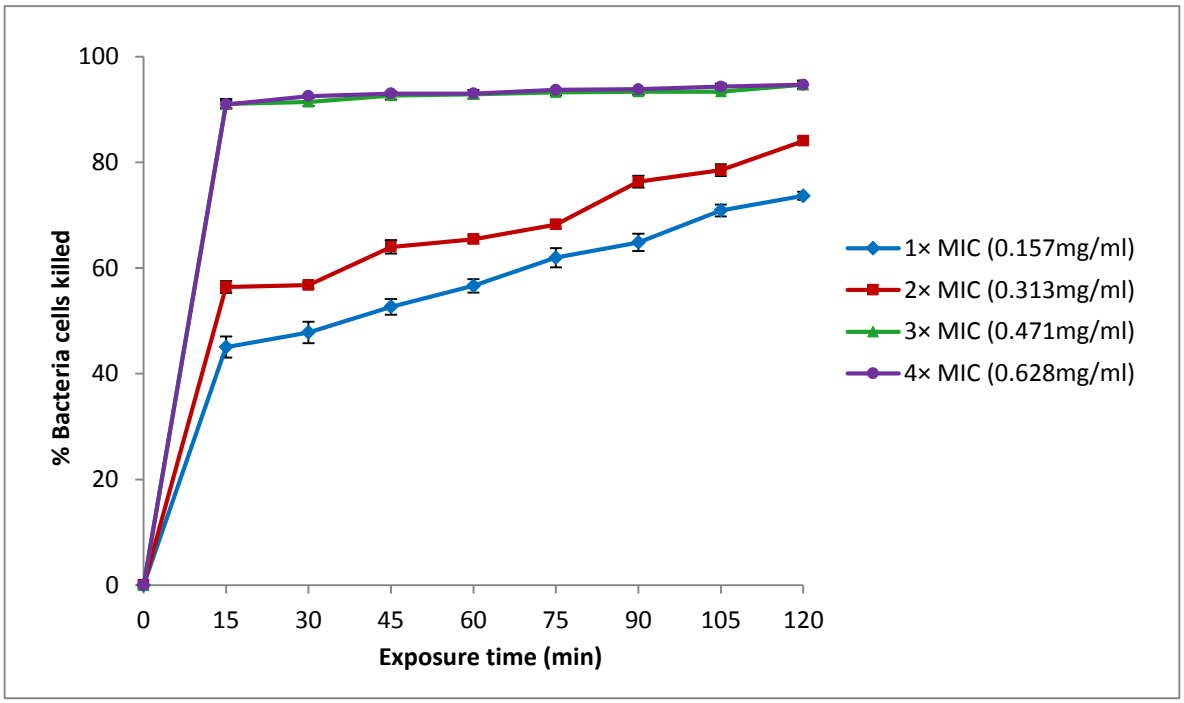

Figure 2. Rate of kill profile for *L. monocytogenes* (LAL 8) by crude methanol extracts of *Garcinia kola* seeds.

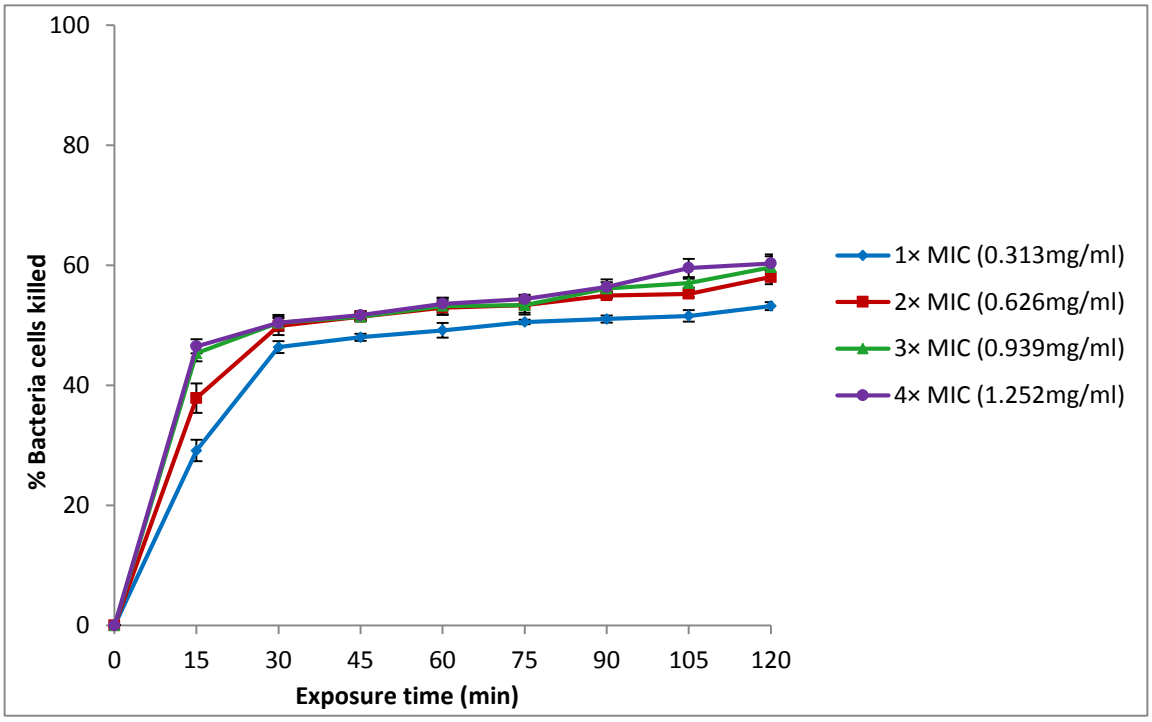

Figure 3. Rate of kill profile for *L. ivanovii* (LEL 18) by crude methanol extracts of *Garcinia kola* seeds

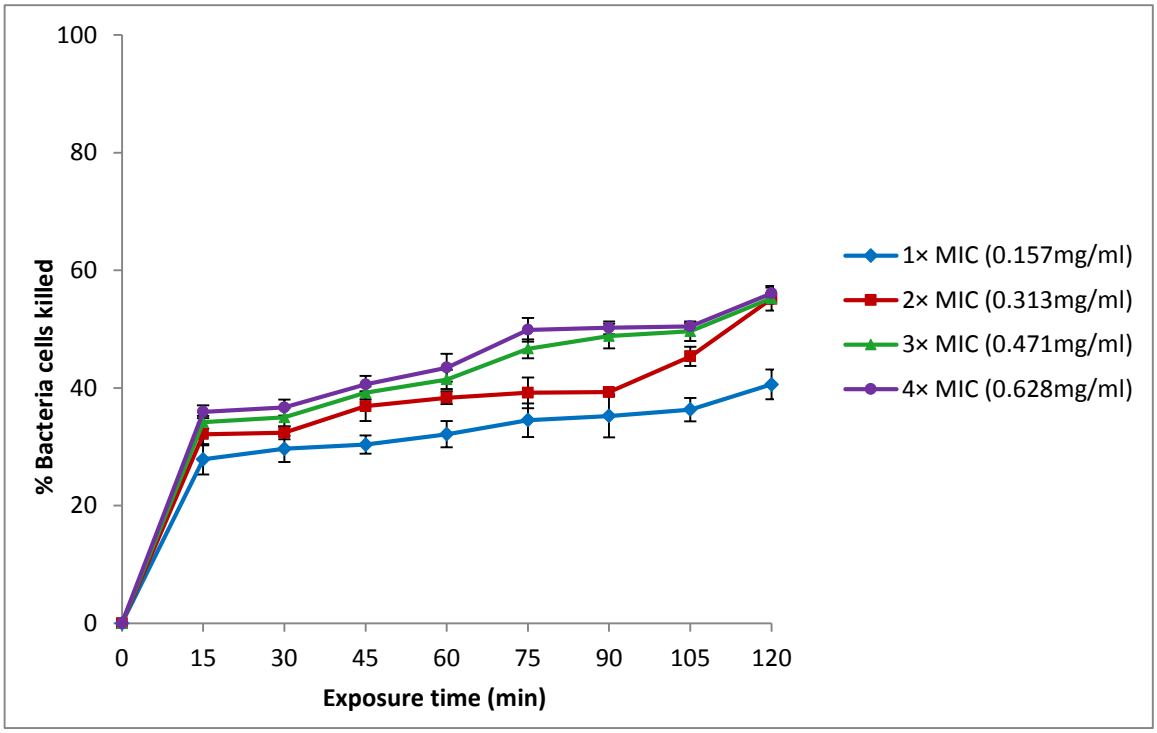

Figure 4. Rate of kill profile for *L. grayi* (LAL 15) by crude methanol extracts of *Garcinia kola* seeds.

Discussion

This study revealed the anti-*Listerial* activities of the methanol extract of *Garcinia kola* seeds. The extract was active against each *Listeria* specie used in the study and had a 45% activity. Similary, the methanol extract of *Garcinia kola* seeds in other studies has been found to be also active against other Gram positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumonia* (Adeleke *et al*., 2006) and *Staphylococcus sciuri* (Sibanda *et al*., 2010).

The extract's MIC values against the test *Listeria* bacteria ranged from 0.157- 0.625 mg/ml. A separate study involving the methanol extract of *Garcinia kola* seeds against Gram positive bacteria also showed MIC values within the same range as those observed in this study; the findings of Sibanda *et al*. (2010) showed that the extract had an MIC value of 0.312 mg/ml against all four *Staphylococcus* strains tested. However, findings involving Gram negative bacteria revealed higher MIC ranges, the study by Penduka *et al*. (2011) involving *Vibrio* isolates revealed higher MIC ranges with values ranging from 0.313 mg/ml to 2.5 mg/ml whilst that by Nwaokorie *et al*. (2010) involving *Fusobacterium nucleatum* species showed MIC values ranging from 1.25 mg/ml to 12.5 mg/ml, and the higher MIC values against Gram negative bacteria could be due to the presence of the outer membrane present in Gram negative bacteria which acts as a barrier against antibiotics that work inside the cell a factor attributing to resistance.

The highest number of bacteria cells killed was achieved at the highest concentration of 4 × MIC value and at the maximum exposure time of 2 h for all the four organisms. The extract was bactericidal against *L. ivanovii* (LEL 30) only but was however bacteriostatic against the other three *Listeria* isolates since by definition a ≥99.9% or ≥3$\log_{10}$ killing rate is characteristic of a cidal agent whilst a lower killing rate is characteristic of a bacteriostatic agent (Pankey and Sabath, 2004). The results show that the methanol extract can be either bactericidal or bacteriostatic depending on the *Listeria* isolate tested and the rate of kill can vary even within

same species isolates as evidenced by *L. ivanovii* (LEL 30) and *L. ivanovii* (LEL 18) in this study.

Garcinia kola seeds methanol extract's rate of kill studies by Penduka *et al*. (2011) involving *Vibrio* isolates similarly showed the highest number of bacteria cells killed being achieved at the highest concentration of 4× MIC after maximum exposure time of 2 h; however, only a bacteriostatic nature of inhibition was noted in the study, whilst in a study by Nwaokorie *et al*. (2010) involving *Fusobacterium nucleatum* clinical isolates and a biofilm produced by the association of *Fusobacterium nucleatum* isolates, *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* and *Prevotella intermedia* at 100 mg/ml concentration of the extract showed a bactericidal activity by killing the entire bacterial population after 1 h exposure time. A point to note however in the study by Nwaokorie *et al*. (2010) is the concentration of the extract used in the study which was 4× MIC value against the biofilm and 8× MIC or 80× MIC value against the *Fusobacterium nucleatum* clinical isolates and this achieved a bactericidal effect, this can therefore mean that an increase in the concentration of the seed's methanol extract to MIC levels above the 4× MIC value can result in bactericidal activity of the extract at minimum exposure time.

The methanol solvent is known to extract a wide range of phytochemicals such as anthocyanins, terpenoids, saponins, tannins, xanthoxyllines, totarol, quassinoids, lactones, flavones, phenones and polyphenols (Cowan, 1999). Some of these phytochemicals such as flavonoids, tannins, cardiac glycoside, saponins, steroids and reducing sugars have also been found to be present in *Garcinia kola* seeds (Adegboye *et al*., 2008). Flavones are a class of flavonoids of which flavonoids are well known for possessing a wide range of therapeutic properties such as antioxidant, antipyretic (fever-reducing), analgesic and spasm inhibiting properties (Krishnaiah *et al*., 2009), in addition to also possessing antibacterial, antiviral, antiallergic and anti-inflammatory activities (Cook and Samman, 1996). Saponins have been reported to have anti-

fungal properties (Sodipo *et al*., 1991) and tannins are known to possess antiviral, antibacterial and anti-tumor activities (Kunle and Egharevba, 2009), such that either of these phytochemicals could have been responsible for the observed antibacterial activities of the extract in this study.

Conclusion

The results found in this study are therefore good preliminary findings that are good foundations for the further isolation and characterisation of the anti-*Listerial* compounds in *Garcinia kola* seeds as the purified active compound could be more potent in comparison to the crude extract and these are subjects of ongoing research.

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CHAPTER FOUR

Antibacterial Potentials of the Crude Dichloromethane Extract of *Garcinia kola* (Heckel) Seeds against some *Listeria* Species Isolated from Wastewater Effluents

(Published in Journal of Pure and Applied Microbiology)

Abstract

The anti-*Listerial* activities of the dichloromethane extract of *Garcinia kola* seeds were assessed against a panel of 42 *Listeria* bacteria. The extract was active against 19 of the isolates with the minimum inhibitory concentrations (MICs) ranging between 0.079 mg/ml and 0.313 mg/ml whilst the minimum bactericidal concentrations (MBCs) ranged between 0.625 mg/ml and 10 mg/ml. The extract's rate of kill against four representative *Listeria* strains showed a concentration and time dependent profile, being more lethal to the bacteria at the highest concentration (4× MIC) at the maximum exposure time of 2 h. The extract was bacteriostatic against *Listeria grayi* (LAL 15) giving a less than $3\log_{10}$ decrease in the viable cell counts after 2 h exposure time at all four MIC values. However, the extract was bactericidal against *Listeria ivanovii* (LEL 18) and *Listeria monocytogenes* (LAL 8) after 105 min and 120 min respectively at 4× MIC value. The extract was also bactericidal against *Listeria ivanovii* (LEL 30) achieving complete elimination of all the viable cells at 3× MIC and 4× MIC values after 90 min and 45 min exposure times respectively. These results therefore showed the possible presence of therapeutic compounds in *Garcinia kola* seeds that have potential in listeriosis treatment.

Key words: *Listeria* species; Rate of kill; *Garcinia kola* seeds; MIC; Dichloromethane.

Introduction

Antibiotics are defined as either naturally occurring, semi-synthetic or chemically synthesised antimicrobial compounds used mainly in the treatment and prevention of diseases in both humans and animals and also as growth promoters in animal intensive industries (Jury *et al*., 2010). The therapeutic use of an antibiotic, in either human or animal population, 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 to it, rendering the antibiotic ineffective as treatment of choice against that respective bacterial strain (Altekruse *et al*., 1997; Tenover, 2006). Some of the mechanisms of resistance include: alteration of permeability barriers across bacterial outer membranes, prevention of antibiotic uptake through inhibiting its corresponding transport carrier, modification of the antibiotic' s target binding sites to prevent recognition of the antibiotic, and the ability by the bacteria to chemically and/or enzymatically degrade the antibiotic (Jury *et al*., 2010).

Unused and or unmetabolised antibiotic substances such as those from hospital effluents are sometimes disposed off into the sewage system (Kummerer, 2003). The biological treatment process in a conventional wastewater treatment plant may result in a selective increase of the antibiotic resistant bacteria population and the increased occurrence of multi-antibiotic resistant bacteria (Zhang *et al*., 2009). Wastewater treatment plants may facilitate the spread of antibiotics, antibiotic resistance genes and antibiotic resistance bacteria in the aquatic environment as they link different aquatic environments including municipal sewage and surface waters (Schluter *et al*., 2007). The occurrence and spread of antibiotic resistant bacteria species is a major threat to public health as it is limiting treatment options thereby causing an increase in morbidity and mortality (Zhang *et al*., 2009). Most medicinal plants and their purified constituents have been proven to possess beneficial therapeutic potentials

(Venkatachallam *et al*., 2010), such that they can be a useful and effective alternative in mitigating the spread of antibiotic resistance.

Garcinia kola is one such traditional medicinal plant that is an evergreen plant found in the equatorial forest of Sub-Saharan Africa where it grows wild and can also be domesticated due to its numerous medicinal values (Oze *et al*., 2010). The plant is also known as "bitter kola" because of its seed's bitter taste or "male kola" because of its claimed aphrodisiac activity (Uko *et al*., 2001). *Garcinia kola* seeds form a major part of the herbal preparation used for the treatment of various respiratory tract diseases including asthma (Okojie *et al*., 2009). Studies by Olaleye and Farombi (2006) showed that treatments with kolaviron extracted from the powdered seeds of *Garcinia kola* significantly inhibited gastric lesions produced by indomethacin and acidified ethanol in rats.

There are some studies that have proven the antibacterial activities of *Garcinia kola* seeds extracts *in-vitro* (Ofokansi *et al*., 2008; Okigbo and Mmeka, 2008; Sibanda *et al*., 2010; Penduka *et al*., 2011), however, information on the anti-*Listerial* activities of the seeds is very rare. *Listeria* species are Gram positive, facultatively anaerobic, psychrotrophic and catalase positive rod shaped bacterium (Warriner and Namvar, 2009). The genus *Listeria* is composed of eight species namely *Listeria grayi, Listeria innocua, Listeria ivanovii, Listeria welshimeri, Listeria seeligeri, Listeria marthii, Listeria rocourtiae* and *Listeria monocytogenes* (Camejo *et al*., 2011), however, only *L. monocytogenes* and *L. ivanovii* are considered pathogenic (Rocourt and Buchrieser, 2007; Warriner and Namvar, 2009; Camejo *et al*., 2011). Human listeriosis is a food borne disease normally caused by *L. monocytogenes* (Singh *et al*., 2012), which because of its ubiquitous nature, commonly contaminates raw produce and through cross-contamination infects other food items such that humans are routinely exposed to the organism (Swaminathan and Gerner-Smidt, 2007), but the defined high risk groups to listeriosis are the pregnant, neonates, aged and immune-compromised persons (Zunabovic *et al*., 2011).

Most reported cases of listeriosis present as life-threatening illness in one of three clinical syndromes: maternofetal listeriosis or neonatal listeriosis, blood stream infection, and meningoencephalitis (Swaminathan and Gerner-Smidt, 2007). Despite efficient antibiotic therapy, listeriosis is fatal in up to 30% of the cases making it a major public health threat (Yucel *et al*., 2005). A number of authors have reported the resistance of *Listeria* species to antibiotics (Rahimi *et al*., 2010; Odjadjare *et al*., 2010; Acciari *et al*., 2011; Adetunji and Isola, 2011). The need to provide alternative listeriosis treatment options become a necessity and in this paper, the anti-*Listerial* activities of the dichloromethane extract of *Garcinia kola* seeds is reported.

Materials and Methods

Plant Material

The ground seed powder of *Garcinia kola* was 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 method of Basri and Fan (2005) and Dogruoz *et al*. (2008) was used to prepare the dichloromethane solvent extracts. A 100 gram measurement of the seed powder was steeped in 500 ml of the solvent for 48 h with shaking on an orbital shaker (Stuart Scientific Orbital Shaker, UK). The resultant extract was centrifuged at 3000 rpm for 5 min at 4°C (Beckman Model TJ-6RS Centrifuge, Great Britain) and the supernatant filtered through Whatman No.1 filter paper while the residue was used in the second extraction process involving 300 ml of the

solvent. The combined extracts were concentrated using a rotary evaporator (Steroglass S.R.L, Italy) at 50°C, after which they were dried to a constant weight under a stream of air in a fume cupboard at room temperature. Dimethyl sulphoxide (DMSO) at a concentration of 5% (v/v) was used to aid the reconstitution of the dried extract when making different test concentrations.

Test *Listeria* strains

The 42 test *Listeria* isolates 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 previously isolated from wastewater effluents in the Eastern Cape Province of South Africa and belonged to three *Listeria* species groups which are *L. ivanovii*, *L. grayi* and *L. monocytogenes* (Odjadjare *et al*., 2010).

Preparation of the Inoculum

Colonies were picked from 24 h old cultures grown on nutrient agar and suspended in saline solution (0.85% NaCl) to give an optical density of approximately 0.1 at 600 nm for each organism. The suspension was then diluted a hundred-fold before use (EUCAST, 2003).

Antibacterial susceptibility test

The susceptibility of the *Listeria* bacteria to the extract was determined using the agar well diffusion method described by Irobi *et al*. (1996) with some modifications. A 100 µl volume

of the prepared bacterial suspension was inoculated into sterile molten Mueller-Hinton agar medium at 50ºC in a MacCarthney bottle, mixed and poured into a sterile petri dish. A sterile 6 mm diameter cork borer was used to bore wells into the solidified agar medium after which approximately 100 µl of 10 mg/ml extract solution was put in the wells. The plates were then left to stand on the laboratory bench for 1 h to allow proper diffusion of the extract into the medium before incubation at 37ºC for 24 h, and thereafter the zones of inhibition were observed and measured. Ciprofloxacin (2 µg/ml) was used as a positive control, and sterile distilled water was used as the negative control while 5% DMSO was also tested to determine its effect on each organism.

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

The MICs of the susceptible *Listeria* bacteria were determined using the broth microdilution assay method of EUCAST (2003) and carried out in sterile disposable flat-bottomed 96-well microtiter plates. Two-fold serial dilutions using sterile distilled water were carried out from 10 mg/ml of the stock plant extract to make 9 test concentrations ranging from 0.039 to 10 mg/ml. The assay procedures follow after the report of Penduka *et al*. (2011). Double strength Mueller-Hinton broth (100 µl) was introduced into all the 96 wells. Column 1 was used as the sterility wells containing 100 µl of sterile distilled water in addition to the 100 µl of Mueller-Hinton broth, 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 as test wells containing 100 µl of the broth, 50 µl of the test extract concentration and 50 µl of the test organism. The plates were then incubated

at 37°C for 18-24 h. 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 *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 h of incubation (EUCAST, 2003).

The method of Sudjana *et al*. (2009) was used to determine the MBC from the MIC broth microdilution assays through subculturing 10 µl volumes from each well that did not exhibit growth after 24 h of incubation and spot inoculating it onto Mueller-Hinton agar plates. The plates were incubated for 48 h after which the numbers of viable colonies were counted. The MBC was defined as the lowest concentration killing ≥99.9% of the inoculum compared with initial viable counts (Sudjana *et al*., 2009).

Rate of kill assay

The time kill assay was done according to the method of Odenholt *et al*. (2001) as described by Akinpelu *et al*. (2008). The selected test *Listeria* isolates namely *L. ivanovii* (LEL18), *L. grayi* (LAL15), *L. monocytogenes* (LAL8) and *L. ivanovii* (LEL 30) were used for the rate of kill studies as representatives of the *Listeria* species used in the study. The turbidity of an 18 h old test *Listeria* culture was standardized to 10^8 cfu/ml by matching with a 0.5 McFarland standard. 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 each organism suspension was added to 4.5 ml of the extract's different concentrations, held at room temperature and the rate of kill determined over a period of 2 h. After 15 min intervals a 0.5 ml volume of each suspension was withdrawn 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 *et al*., 2008). The suspension was then serially diluted and 0.5 ml was plated out for viable counts and incubated at 37°C for 48 h. The control plates contained the test organism without the plant extract. The emergent colonies were counted and compared with the counts of the culture control.

Statistical Analysis

The SPSS 19.0 version for windows program was used to determine the means and standard deviations of the zones of inhibitions results, with the one way analysis of variance (ANOVA) of the same program being used to determine the means and standard deviations of the rate of kill results.

Results

Antibacterial susceptibility test

The results of the antibacterial susceptibility test are as shown in Table 1. The zones of inhibition ranged from 8-16 mm and 19 out of the 42 isolates were susceptible to the extract. The highest zone of inhibition was observed against *L. ivanovii* (LDB 7), whilst the lowest zones of inhibitions were observed against *L. ivanovii* (LEL 17) and *L. ivanovii* (LDB 9). The 5% DMSO and the sterile distilled water negative controls had no antibacterial activity on all the tested *Listeria* isolates.

MIC and MBC

The results of the MIC and MBC of the extract are as shown in Table 2. The MICs ranged between 0.079 mg/ml and 0.313 mg/ml, of which the extract had MIC values of 0.079 mg/ml against 13 *Listeria* isolates, of 0.157 mg/ml against 4 isolates and of 0.313 mg/ml against 2 isolates. The MBC values ranged from 0.625mg/ml to 10 mg/ml with the lowest MBC value of 0.625 mg/ml being recorded against *L. ivanovii* (LEL 30) isolate only. The extract had an MBC value of 5 mg/ml against four isolates, whilst against the remaining 14 isolates it had an MBC value of 10 mg/ml.

Rate of kill

The highest number of viable cells killed was noted at the maximum exposure time of 2 h at all the test concentrations with the highest concentration of 4× MIC value being most lethal for all the four test isolates. The extract was bactericidal against *L. ivanovii* (LEL 18) (Figure 1) and *L. monocytogenes* (LAL 8) (Figure 2) at 105 min and 120 min at 4 × MIC values only. The extract was also bactericidal against *L. ivanovii* (LEL 30) (Figure 3) at 15 min at 4× MIC value and after 60 min at 3× MIC value and also went on to achieve a complete elimination of all viable cells of the organism after 45 min at 4× MIC value and after 90 min at 3× MIC value of the extract. However, for *L. grayi* (LAL 15) (Figure 4) the extract was bacteriostatic at all four test concentrations even after 2 h exposure time achieving a maximum of 2.35$\log_{10}$ decrease in viable cell count after 2 h at 4×MIC value concentration.

Table 1: The anti-*Listerial* activities of ciprofloxacin and the crude dichloromethane extract of *Garcinia kola* seeds

Organism	**Zones of inhibition (mm)**		**Organism**	**Zones of inhibition (mm)**	
	DCM	**Cipro**		**DCM**	**Cipro**
L. grayi (LAL 13)	0	20±3.06	*L. ivanovii* (LEL 18)	11±0.577	20±3.22
L. ivanovii (LEL 17)	8±0	19±1.53	*L. ivanovii* (LEL 29)	0	8±0.577
L. ivanovii (LEL 30)	10±1.53	30±0.577	*L. ivanovii* (LEL 15)	0	13±2.08
L. ivanovii (LDB 11)	9±0.577	20±1	*L. ivanovii* (LDB 9)	8±0.577	25±2.08
L. ivanovii (LEL9)	10±1.16	16±2.08	*L. ivanovii* (LDB 10)	13±1.16	25±0.577
L. ivanovii (LEL 1)	13±2.65	17±0.577	*L. ivanovii* (LEL 2)	0	28±1.53
L. ivanovii (LEL 5)	0	11±0.577	*L. ivanovii* (LEL 6)	0	11±1.73
L. ivanovii (LEL 3)	0	35±3.06	*L. ivanovii* (LEL 4)	0	14±1
L. ivanovii (LEL 19)	0	25±4.04	*L. ivanovii* (LEL 10)	0	20±2.08
L. ivanovii (LAL 9)	11±0.577	25±1.73	*L. ivanovii* (LAL 11)	10±0.577	17±2.65
L. grayi (LAL 12)	9±1.155	17±1.16	*L. ivanovii* (LAL 10)	11±0	15±2.08
L. grayi (LAL 15)	11±1.73	18±2.08	*L. ivanovii* (LAL 14)	0	30±2.52
L. ivanovii (LDB 1)	0	15±2.08	*L. ivanovii* (LDB 2)	0	14±0
L. ivanovii (LAL 6)	0	19±1.16	*L. ivanovii* (LAL5)	0	20±1.53
L. ivanovii (LAL 7)	0	20±1.53	*L. monocytogenes* (LAL 8)	13±0.577	12±1
L. ivanovii (LDB 7)	16±0.577	27±0.577	*L. ivanovii* (LDB 12)	14±1	25±1.53
L. ivanovii (LDB 3)	11±1	15±1	*L. ivanovii* (LDB 8)	0	20±1.73
L. ivanovii (LEL 7)	0	9±1	*L. ivanovii* (LEL 8)	0	30±1.53
L. ivanovii (LEL 14)	0	35±2	*L. ivanovii* (LEL 16)	12±1	15±1.53
L. grayi (LAL 3)	0	13±3.06	*L. ivanovii* (LAL 4)	0	20±2
L. ivanovii (LAL 2)	13±2.08	16±1	*L. ivanovii* (LAL 1)	0	20±2

Keynotes: DCM denotes dichloromethane, Cipro denotes ciprofloxacin, number±number denotes mean zone of inhibition±standard deviation.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of dichloromethane extract of *Garcinia kola* seeds against *Listeria* species

Organism	**Dichloromethane extract**	
	MIC (mg/ml)	**MBC (mg/ml)**
L. ivanovii (LEL9)	0.157	10
L. ivanovii (LEL 18)	0.079	10
L. ivanovii (LAL 10)	0.079	10
L. ivanovii (LEL 30)	0.157	0.625
L. ivanovii (LEL 16)	0.157	10
L. monocytogenes (LAL 8)	0.079	5
L. ivanovii (LDB 12)	0.157	10
L. ivanovii (LDB 10)	0.079	10
L. ivanovii (LEL 1)	0.079	10
L. ivanovii (LAL 11)	0.079	10
L. ivanovii (LDB 3)	0.079	5
L. grayi (LAL 15)	0.079	10
L. grayi (LAL 12)	0.313	10
L. ivanovii (LDB 11)	0.079	10
L. ivanovii (LAL 2)	0.313	10
L. ivanovii (LEL 17)	0.079	10
L. ivanovii (LDB 7)	0.079	5
L. ivanovii (LDB 9)	0.079	5
L. ivanovii (LAL 9)	0.079	10

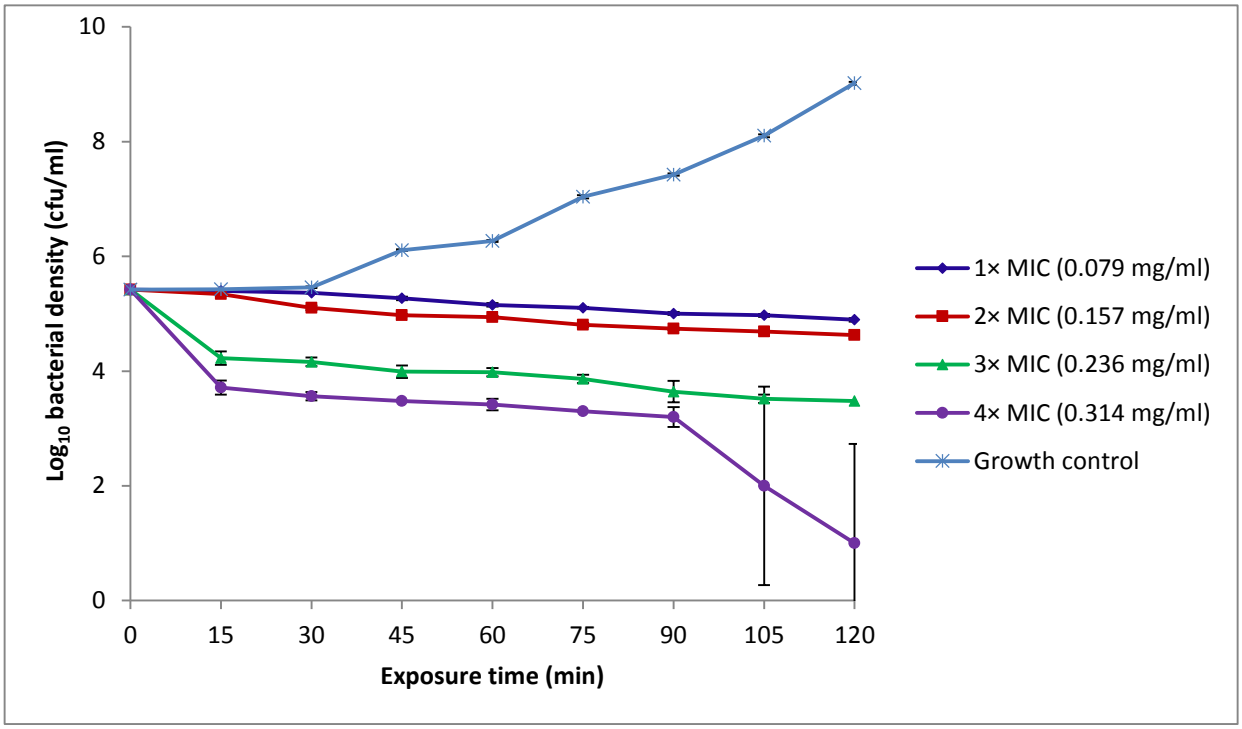

Figure 1. Rate of kill for the dichloromethane extract of *Garcinia kola* seeds against *L. ivanovii* (LEL 18).

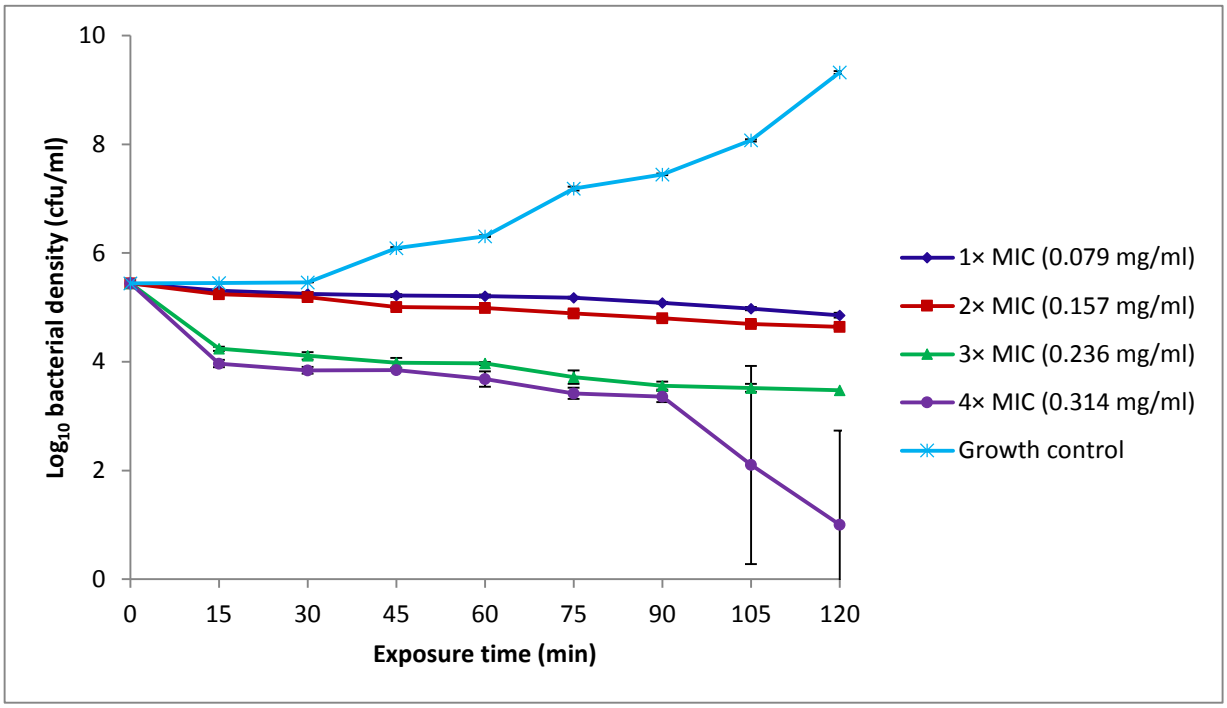

Figure 2. Rate of kill for the dichloromethane extract of *Garcinia kola* seeds against *L. monocytogenes* (LAL 8).

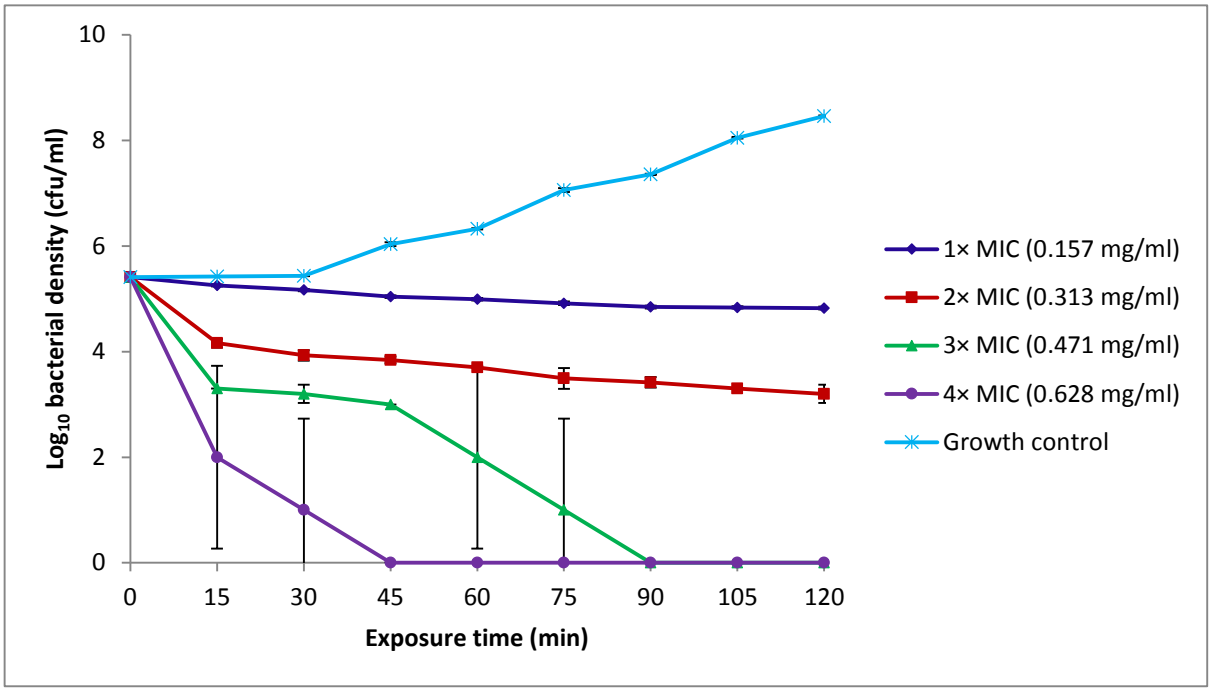

Figure 3. Rate of kill for the dichloromethane extract of *Garcinia kola* seeds against *L. ivanovii* (LEL 30).

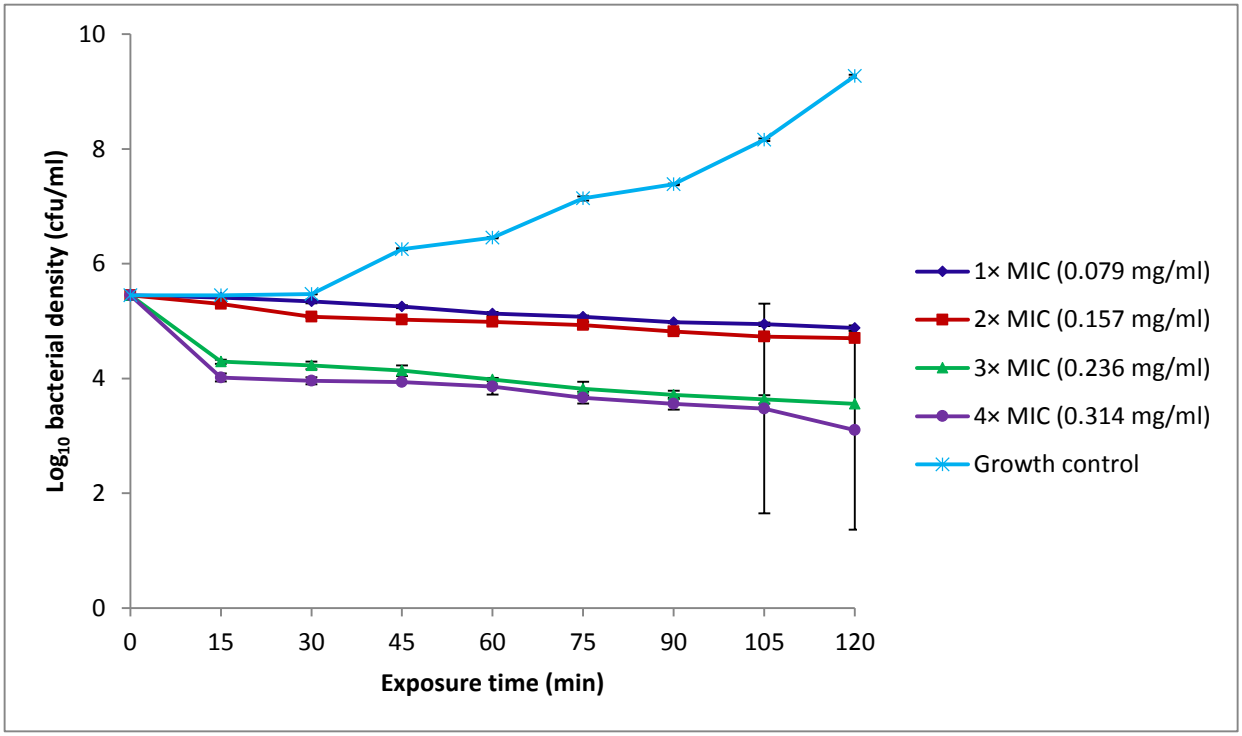

Figure 4. Rate of kill for the dichloromethane extract of *Garcinia kola* seeds against *L. grayi* (LAL 15).

Discussion

The dichloromethane extract of *Garcinia kola* seeds was active against 45% of the test bacteria including the pathogenic species *L. ivanovii* and *L. monocytogenes*. The MIC values ranged from 0.079 mg/ml to 0.313 mg/ml whilst the MBC values ranged between 0.625 mg/ml and 10 mg/ml. In a similar study (Penduka and Okoh, 2011) involving the dichloromethane extract of *Garcinia kola* seeds against *Vibrio* species which are Gram negative bacteria, higher MIC values were reported, with the MICs and MBCs ranging from 0.313 mg/ml-0.625 mg/ml and 5 mg/ml-10 mg/ml respectively. Similary results from studies by Sibanda and Okoh (2008) also showed the Gram positive bacteria tested in that particular study to be more susceptible than the Gram negative bacteria to the aqueous and acetone extracts of *Garcinia kola* seeds. These findings suggest that the active compounds in *Garcinia kola* seeds have broad spectrum activity and they are more antagonistic towards Gram positive bacteria than Gram negative ones,

Rate of kill curves are used to determine the kinetics of bacterial killing *in- vitro* and can be used in distinguishing whether bacterial killing is concentration and/or time dependent (Pankey and Sabath, 2004). In this particular study, the rate of kill studies showed a concentration and time dependent characteristic for all the four test *Listeria* isolates as shown in Figures 1 to 4, since an increase in the concentration of the extract from MIC value to 4× MIC value resulted in more bacteria cells being killed in shorter exposure times and also for each MIC value the highest bacterial cells were killed at the maximum exposure time of 2 h. For an antibacterial agent to be termed bactericidal, it should be able to kill bacteria by achieving a ≥99.9% or $\geq 3\log_{10}$ reduction in viable bacterial density, whilst a bacteriostatic agent does not reach the above required killing activity points (Pankey and Sabath, 2004). The extract proved to be bactericidal against three of the tested *Listeria* isolates namely *L. ivanovii* (LEL 18), *L. monocytogenes* (LAL 8) at 4× MIC value only and *L. ivanovii* (LEL 30) at 4× MIC and at 3×

MIC values only and this was within the 2 h exposure time whilst it was bacteriostatic against *L. grayi* (LAL 15) at all test concentrations even at the maximum exposure time of 2 h used in the study. The results suggest that the extract can be either bactericidal or bacteriostatic against *Listeria* species, which is not an unexpected result in any antibacterial agent (Pankey and Sabath, 2004), although in this instance the extract appears to be more of a bactericidal nature than of a bacteriostatic one since it was bactericidal against three of the four tested isolates.

Phytochemical analysis of the crude methanolic extract of *Garcinia kola* seeds showed the presence of flavonoids, tannins, cardiac glycoside, steroids, saponins and reducing sugars which are known to play vital roles in the bioactivity of medicinal plants (Akinpelu *et al*., 2008). Dichloromethane solvent as shown by some studies on plants can also extract some of these bioactive compounds from plant material such as saponins and tannins (Doughari *et al*., 2008) and steroids (Nazifi *et al*., 2008). Besides these phytochemicals, dichloromethane solvent is also known to extract essential oils from plant material (Gamiz-Gracia and Luque deCastro, 2000), of which most plant species are known to exhibit antimicrobial activity due to their essential oils content. Anti-*Listerial* activities of different plants' essential oils have also been reported by several authors (Smith-Palmer *et al*., 2001; Singh *et al*., 2003; Sokovic *et al*., 2007; Celikel and Kavas, 2008). The mode of action of essential oils and their components is based on their lipophilic nature, which enables them to partition the lipids of the bacterial cell membrane. This disrupts the membrane's intergrity causing a loss of chemiosmotic control which leads to bacterial cell death (Sikkema *et al*., 1995; Cox *et al*., 2000; Prabuseenivasan *et al*., 2006). Studies by Aniche and Uwakwe (1990) have shown the presence of essential oils in *Garcinia kola* seeds which also may have attributed to the observed antibacterial activities in this study.

Conclusion

The dichloromethane extract of *Garcinia kola* seeds has been shown in this study to exhibit anti-*Listerial* activities which could be bacteriostatic or bactericidal in nature. Isolation and characterization of the active compounds in the extract remain the vital follow up steps and these are subjects of ongoing research.

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CHAPTER FIVE

In-Vitro Anti-*Listerial* Activities of Crude N- Hexane and Aqueous Extracts of *Garcinia kola* (Heckel) Seeds.

(Published in International Journal of Molecular Sciences)

Abstract

The anti-*Listerial* activities of the crude n-hexane and aqueous extracts of *Garcinia kola* seeds were assessed against a panel of 42 *Listeria* isolates previously isolated from wastewater effluents in the Eastern Cape Province of South Africa and belonging to *Listeria monocytogenes*, *Listeria grayi* and *Listeria ivanovii* species. The n-hexane extract was active against 45% of the test bacteria with zones of inhibition ranging from 8-17 mm, while the aqueous extract was active against 29% of the test *Listeria* with zones of inhibition ranging from 8-11 mm. The minimum inhibitory concentrations (MIC) were within the ranges of 0.079-0.625 mg/ml for the n-hexane extract and 10 to >10 mg/ml for the aqueous extract. The rate of kill experiment carried out for the n-hexane extract only, revealed complete elimination of the initial bacterial population for *L. grayi* (LAL 15) at 3× and 4× MIC values after 90 and 60 min; *L. monocytogenes* (LAL 8) at 3× and 4× MIC values after 60 and 15 min; *L. ivanovii* (LEL 18) at 3× and 4× MIC values after 120 and 15 min; *L. ivanovii* (LEL 30) at 2×, 3× and 4× MIC values after 105, 90 and 15min exposure times respectively. The rate of kill activities were time and concentration dependant and the extract proved to be bactericidal as it achieved a more than $3\log_{10}$ decrease in viable cell counts after 2 h exposure time for all the four test organisms at 3× and 4× MIC values. The results therefore showed the potential presence of anti-*Listerial* compounds in *Garcinia kola* seeds that can be exploited in effective anti-*Listerial* chemotherapy.

Key words: *Garcinia kola* seeds; *Listeria* species; rate of kill; bactericidal

Introduction

The genus *Listeria* consists of Gram-positive, non-sporeforming rod shaped bacteria which are facultatively anaerobic, catalase positive, oxidase negative and ubiquitous in nature (Salimnia *et al*., 2010). There are eight characterized *Listeria* species namely *Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria innocua, Listeria grayi, Listeria welshimeri* (Hain *et al*., 2006; Salimnia *et al*., 2010), *Listeria marthii* and *Listeria rocourtiae* (Camejo *et al*., 2011). Among these *Listeria* species only two species *L. ivanovii* and *L. monocytogenes* are regarded as pathogenic; *L. monocytogenes* is pathogenic to humans and animals whilst *L. ivanovii* is pathogenic to animals mainly sheep and cattle causing the bacteremia listeriosis (Vazquez-Boland *et al*., 2001; Hain *et al*., 2006; Camejo *et al*., 2011). Some studies have however implicated *L. seeligeri* (Rocourt *et al*., 1986), *L. grayi* (Todeschini *et al*., 1998; Rapose *et al*., 2008; Salimnia *et al*., 2010) and *L. ivanovii* in human listeriosis (Cummins *et al*., 1994; Guillet *et al*., 2010) and *L .innocua* in animal listeriosis (Walker *et al*., 1994) thereby also showing the potential pathogenicity of some of the *Listeria* species apart from *L. monocytogenes* and *L. ivanovii*.

Listeriosis is a severe food-borne disease characterized by bacteremia, meningitis and encephalitis, the individuals usually at high risk are those with impaired cell-mediated immunity, including neonates, pregnant women, elderly persons, and the immune-compromised patients (Schuppler and Loessner, 2010). *L. monocytogenes* is an invasive, intracellular pathogen that can transverse the placenta in pregnant women and infect the fetus, although some intrauterine infection may be the result of ascending spread of the bacteria from vaginal colonization resulting in abortion, birth of a stillborn fetus or a baby with generalized infection (granulomatosis infantiseptica), and sepsis or meningitis in the neonate such that it is of particular high risk for pregnant women (Schuchat *et al*., 1991; Davis and Jackson, 2009; Allerberger and Wagner, 2010).

Listeriosis is regarded as a food-borne disease because most of the listeriosis cases are mainly caused by consumption of contaminated food (Mead *et al*., 1999; Hain *et al*., 2006; Allerberger and Wagner, 2010), foods such as ready- to- eat meats products and milk products such as cheese (Swaminathan and Gerner-Smidt, 2007; Allerberger and Wagner, 2010). In addition to its ubiquitous nature, the *Listeria* species presents a particular concern with respect to food handling because of the ability to grow at temperatures of 0–45°C making the species able to grow at refrigerator temperatures commonly used to control pathogens in foods. It can also multiply at high salt concentration (10% Sodium chloride) and at pH values ranging from 4.5-9 (Grau and Vanderlinde, 1990; Hain *et al*., 2006).

Standard antibiotic therapy for the effective treatment of listeriosis consists of the intravenous administration of penicillin G or ampicillin often in combination with an aminoglycoside. The drug of choice in patients with known allergy to penicillins is vancomycin/teicoplanin or trimethoprim/sulfamethoxazole (Swaminathan and Gerner-Smidt, 2007). Listeriosis has an average case-fatality rate of 20-30% despite adequate antibiotic treatment (Swaminathan and Gerner-Smidt, 2007) and case fatality rates as high as 40% have been reported during outbreaks (Schuchat *et al*., 1991; Altekruse *et al*., 1997). Epidemiological surveillances have shown the prevalence of antibiotic resistant strains of *Listeria* species to different antibiotics including those used for the treatment of listeriosis (Bertrand *et al*., 2005; Rodas-Suarez *et al*., 2006; Davis and Jackson, 2009; Odjadjare *et al*., 2010).

The challenge is therefore to develop effective strategies that may be able to help curb antibiotic resistance in such virulent bacteria species such as *Listeria*. Traditional medicinal plants such as *Garcinia kola* which is a plant of central and west African origin have been shown to be potential sources of anti-bacterial compounds that can be effective against antibiotic resistant bacteria species. *Garcinia kola* 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 produces a

characteristic orange-like pod, with edible portion contained in the pod yearly and it belongs to the family *Guttiferae* (Iwu, 1993; Adedeji *et al*., 2006; Anegbeh *et al*., 2006). *Garcinia kola* seed also known as "bitter kola" because of its bitter taste has been and is still used traditionally to treat various medicinal ailments such as diarrhoea, hepatitis, asthma, dysmenorrhea, diabetes, anaemia, angina, liver disorders and also as an antidote against ingested poison (Dalziel,1937; Adegoke *et al*., 1981).

Adedeji *et al*. (2006) studies showed that the inclusion of *Garcinia kola* seed powder into the diet of pullet chickens lowered their mortality rate and also caused significant proliferation of the chickens' white blood cells specifically the lymphocytes. Lymphocytes play an important role in cellular immunity as they form antibodies that attack antigens in the body, this further supports the traditional medicinal value of *Garcinia kola* seeds. There have also been studies by various authors that have also proven the antimicrobial activities of the seeds of this plant (Akoachere *et al*., 2002; Sibanda *et al*., 2010; Nwaokorie *et al*., 2010; Njume *et al*., 2011; Penduka and Okoh, 2011). In studies by Han *et al*. (2005) an antibacterial biflavonoid 3″,4′,4‴,5,5″,7,7″-heptahydroxy-3,8″-biflavanone (GB1) was isolated from the roots of *Garcinia kola* and the GB1 showed antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE).

The vast medicinal properties and therapeutic potentials of *Garcinia kola* seeds and the high listeriosis mortality rates in particular against a background of prevalent antibiotic resistant *Listeria* species, prompted this study to evaluate the anti-*Listerial* properties of the *Garcinia kola* seed. Despite the numerous studies that have been done on this plant, to the best of our knowledge there is no information in literature on the *in-vitro* anti-*Listerial* activities of n-hexane and aqueous extracts of the *Garcinia kola* seed.

Materials and Methods

Plant material

Garcinia kola seeds ground powder was 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 method of Basri and Fan (2005) and Dogruoz *et al*. (2008) was used to prepare the solvents extracts. The seed powder (100 grams) was steeped in 500 ml of the respective solvent (n-hexane or water) for 48 h with shaking in an orbital shaker (Stuart Scientific Orbital Shaker, UK). The resultant extract was centrifuged at 3000 rpm for 5 min at 4°C (Beckman Model TJ-6RS Centrifuge, Great Britain), the supernatant was then filtered through Whatman No.1 filter paper while the residue was then used in the second extraction process with 300 ml of the respective solvents. After which the combined aqueous extract was freeze dried at -50 °C under vacuum, whereas the n- hexane extracts were concentrated under reduced pressure using a rotary evaporator (Steroglass S.R.L, Italy) at 50°C. The concentrated n-hexane 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 n-hexane extract when making different test concentrations whilst the water extracts were reconstituted in sterile distilled water.

Test *Listeria* strains

The test *Listeria* isolates (42 in total) 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 previously isolated from wastewater effluents in the Eastern Cape Province of South Africa and belonged to three specie groups which are *L. ivanovii, L. grayi* and *L. monocytogenes* (Odjadjare *et al*., 2010).

Preparation of the Inoculum

The colony suspension method according to EUCAST (2003) was used to prepare the inoculums of the test organisms. Briefly, colonies picked from 24 h 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 *et al*. (1996), with modifications. The prepared bacterial suspension (100 µl) was inoculated into sterile molten Mueller- Hinton agar medium at 50ºC in a MacCarthney 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 after which the wells were filled up with approximately 100 µl of 10 mg/ml extract solution taking care to prevent spillage onto the surface of the agar medium. The plates were then allowed to

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

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

The broth microdilution assay method of EUCAST (2003) was used to determine the MICs against the susceptible *Listeria* isolates 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 nine test concentrations ranging from 0.039 to 10 mg/ml for each solvent extract. Double strength Mueller-Hinton broth (100 µl) was introduced into all the 96 wells. Column 1 was used as the sterility wells containing 100 µl of sterile distilled water in addition to the 100 µl of Mueller-Hinton broth, 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 100 µl of the broth, 50µl of the extract concentration and 50 µl of the test organism. The plates were then incubated at 37°C for 18-24 h. 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 *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 h of incubation (EUCAST, 2003).

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

Rate of kill assay

The time kill assay was done according to the method of Odenholt *et al*. (2001) as described by Akinpelu *et al*. (2008). The selected test *Listeria* isolates namely *L. ivanovii* (LEL18), *L. grayi* (LAL15), *L. monocytogenes* (LAL8) and *L. ivanovii* (LEL 30) were used for the rate of kill studies as representatives of each *Listeria* species used in the study. The turbidity of the 18 h old test *Listeria* culture was first standardized to 10^8 cfu/ml by matching with a 0.5 McFarland standard. 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 h. Exactly 0.5 ml volume of each suspension was withdrawn at 15 min 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 *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 h. 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.

Statistical Analysis

SPSS 19.0 version for Windows program (SPSS, Inc.) at a 95% confidence level was used to determine the one way ANOVA, means and standard deviations.

Results

Anti-*Listerial* activities of the crude extracts

The results of the anti-*Listerial* activities of the crude extracts are shown in Table 1. The n-hexane extract had activity against 19 isolates whilst the aqueous extract had activity against 12 isolates in total with all the isolates that were susceptible to the aqueous extract also being susceptible to the n-hexane extract. The zones of inhibition ranged from 8-17 mm and 8-11 mm for the n-hexane and aqueous extracts respectively at a concentration of 10 mg/ml. The highest zone of inhibition for the n-hexane extract which was 17 mm was obtained against *L. ivanovii* (LEL30) and *L. ivanovii* (LDB 7) whilst for the aqueous extract it was 11 mm obtained against *L. ivanovii* (LEL 1). The positive control (ciprofloxacin) and negative controls (distilled water and 5% DMSO) were used for quality control purposes, with the positive control showing activity against all the isolates with zones of inhibition ranging from 9-35 mm whilst, the negative controls had no activity against all the test isolates.

MIC and MBC Determination

Table 2 shows the MIC and MBC results for both the extracts against the susceptible *Listeria* isolates. The n-hexane extract had MIC values ranges of 0.079–0.625 mg/ml with a mean value of 0.218 mg/ml, whilst the MBC values ranges were 0.625–10 mg/ml with a mean value of 8.717 mg/ml. The aqueous extract had MIC values ranging from 10 to >10 mg/ml and MBC values of above 10 mg/ml for all the isolates. The n-hexane extract interms of its lower MIC and MBC values proved to be more active in comparison to the aqueous extract.

Rate of Kill assay

The results for the rate of kill assay for the n-hexane extract against the four representative *Listeria* species are shown in Figures 1, 2, 3 and 4 with standard deviations included in the curves for *L. grayi* (LAL 15), *L. monocytogenes* (LAL 8), *L. ivanovii* (LEL 30) and *L. ivanovii* (LEL 18) respectively. The rate of kill proved to be both time and concentration dependent for all the four organisms. A complete bactericidal effect for *L. grayi* (LAL 15) was achieved at both 3× MIC and 4× MIC values after 90 and 60 min exposure times respectively. *L. monocytogenes*' (LAL 8) entire bacterial population was killed at both 3× MIC and 4× MIC values after 60 and 15 min exposure times respectively. *L. ivanovii*'s (LEL 30) entire bacterial population was eliminated at 2× MIC, 3× MIC and 4× MIC values after 105, 90 and 15 min exposure times respectively and a complete bactericidal effect for *L. ivanovii* (LEL 18) was achieved at 3× MIC and 4× MIC values after 120 and 15 min exposure times respectively. The n-hexane extract proved to be bactericidal against all the *Listeria* species giving a more than $3\log_{10}$ decrease in viable cell counts within 2 h exposure time.

Table 1: The anti-*Listerial* activities of ciprofloxacin and the crude n-hexane and aqueous extracts of *Garcinia kola* seeds.

ORGANISM	**N-H**	**A**	**C**	**ORGANISM**	**N-H**	**A**	**C**
L. grayi (LAL 13)	0	0	20±3.06	*L. ivanovii* (LEL 18)	9±1	8±0	20±3.22
L. ivanovii (LEL 17)	8±0.577	0	19±1.53	*Listeria species* (LEL 29)	0	0	8±0.577
Listeria species (LEL 30)	17±0.577	0	30±0.577	*L. ivanovii* (LEL 15)	0	0	13±2.08
L. ivanovii (LDB 11)	9±0.577	0	20±1	*L. ivanovii* (LDB 9)	9±1	0	25±2.08
L. ivanovii (LEL9)	9±0.577	0	16±2.08	*L. ivanovii* (LDB 10)	13±0	8±0.577	25±0.577
L. ivanovii (LEL 1)	16±1.16	11±1	17±0.577	*L. ivanovii* (LEL 2)	0	0	28±1.53
L. ivanovii (LEL 5)	0	0	11±0.577	*L. ivanovii* (LEL 6)	0	0	11±1.73
L. ivanovii (LEL 3)	0	0	35±3.06	*L. ivanovii* (LEL 4)	0	0	14±1
L. ivanovii (LEL 19)	0	0	25±4.04	*L. ivanovii* (LEL 10)	0	0	20±2.08
L. ivanovii (LAL 9)	11±0.577	0	25±1.73	*L. ivanovii* (LAL 11)	10±0.577	8±0	17±2.65
L. grayi (LAL 12)	8±0	0	17±1.16	*L. ivanovii* (LAL 10)	10±5.77	8±0.577	15±2.08
L. grayi (LAL 15)	10±2.08	8±0	18±2.08	*L. ivanovii* (LAL 14)	0	0	30±2.52
L. ivanovii (LDB 1)	0	0	15±2.08	*L. ivanovii* (LDB 2)	0	0	14±0
L. ivanovii (LAL 6)	0	0	19±1.16	*L. ivanovii* (LAL5)	0	0	20±1.53
L. ivanovii (LAL 7)	0	0	20±1.53	*L. monocytogenes* (LAL 8)	13±5.77	10±1.16	12±1
L. ivanovii (LDB 7)	17±0.577	10±0.577	27±0.577	*L. ivanovii* (LDB 12)	16±1.53	10±0.577	25±1.53
L. ivanovii (LDB 3)	11±0	8±0.577	15±1	*L. ivanovii* (LDB 8)	0	0	20±1.73
L. ivanovii (LEL 7)	0	0	9±1	*L. ivanovii* (LEL 8)	0	0	30±1.53
L. ivanovii (LEL 14)	0	0	35±2	*L. ivanovii* (LEL 16)	12±1	8±0.577	15±1.53
L. grayi (LAL 3)	0	0	13±3.06	*L. ivanovii* (LAL 4)	0	0	20±2
L. ivanovii (LAL 2)	13±2.08	8±0.577	16±1	*L. ivanovii* (LAL 1)	0	0	20±2

Key : (number ± number) denotes mean of three replicates zone of inhibition diameter in mm ± standard deviation; N-H: denotes n-Hexane extract; A: denotes aqueous extract; C: denotes ciprofloxacin.

Table 2: The Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the crude n-hexane and aqueous extracts of *Garcinia kola* seeds against susceptible *Listeria* bacteria.

Organism	**Extracts**			
	n-Hexane		**Aqueous**	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
L. ivanovii (LEL9)	0.079	10	_	_
L. ivanovii (LEL 18)	0.079	10	10	>10
L. ivanovii (LAL 10)	0.157	10	10	>10
Listeria species (LEL 30)	0.157	0.625	_	_
L. ivanovii (LEL 16)	0.157	10	10	>10
L. monocytogenes (LAL 8)	0.079	5	10	>10
L. ivanovii (LDB 12)	0.157	5	>10	>10
L. ivanovii (LDB 10)	0.079	10	10	>10
L. ivanovii (LEL 1)	0.157	10	>10	>10
L. ivanovii (LAL 11)	0.625	10	10	>10
L. ivanovii (LDB 3)	0.079	10	10	>10
L. grayi (LAL 15)	0.079	10	10	>10
L. grayi (LAL 12)	0.625	10	_	_
L. ivanovii (LDB 11)	0.079	10	_	_
L. ivanovii (LAL 2)	0.157	10	10	>10
L. ivanovii (LEL 17)	0.625	10	_	_
L. ivanovii (LDB 7)	0.079	10	10	>10
L. ivanovii (LDB 9)	0.625	5	_	_
L. ivanovii (LAL 9)	0.079	10	_	_

Key: - denotes not determined as the organism was not susceptible to the extract.

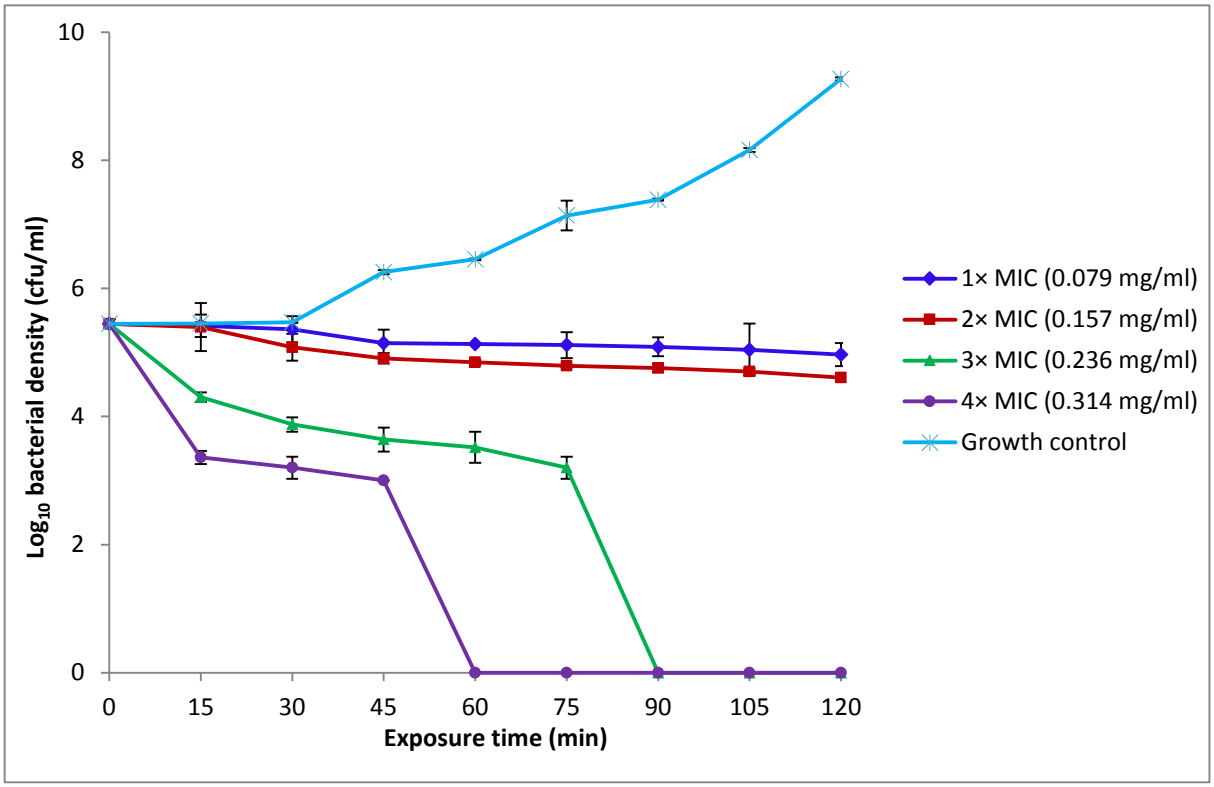

Figure 1. Profile of rate of kill of *L. grayi* (LAL 15) by crude n-hexane extracts of *Garcinia kola* seeds.

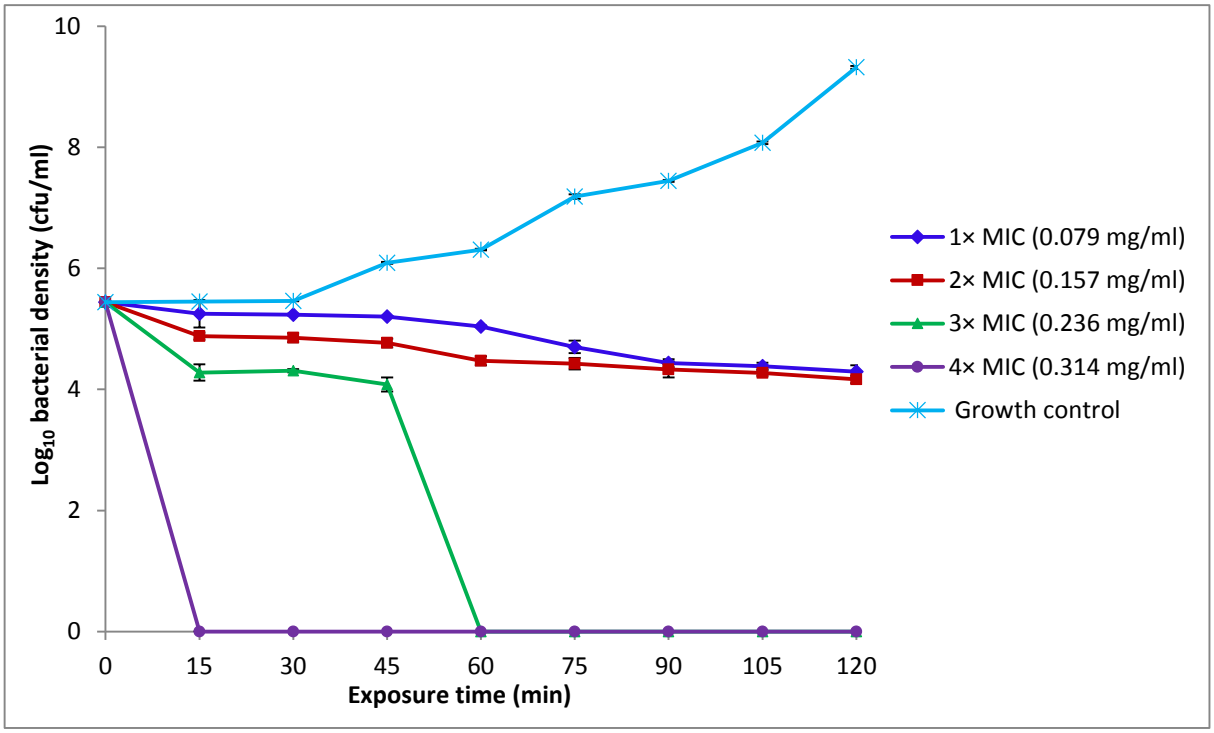

Figure 2. Profile of rate of kill of *L. monocytogenes* (LAL 8) by crude n-hexane extracts of *Garcinia kola* seeds.

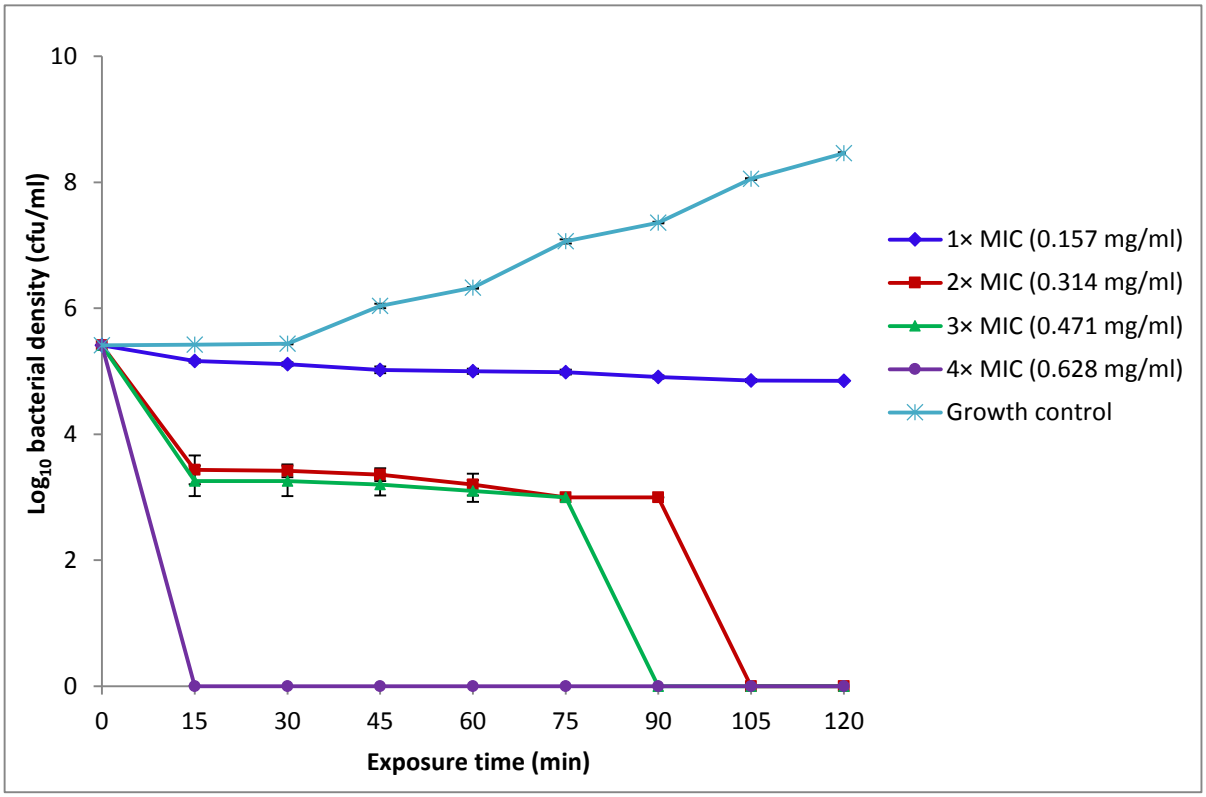

Figure 3. Profile of rate of kill of *L. ivanovii* (LEL 30) by crude n-hexane extracts of *Garcinia kola* seeds.

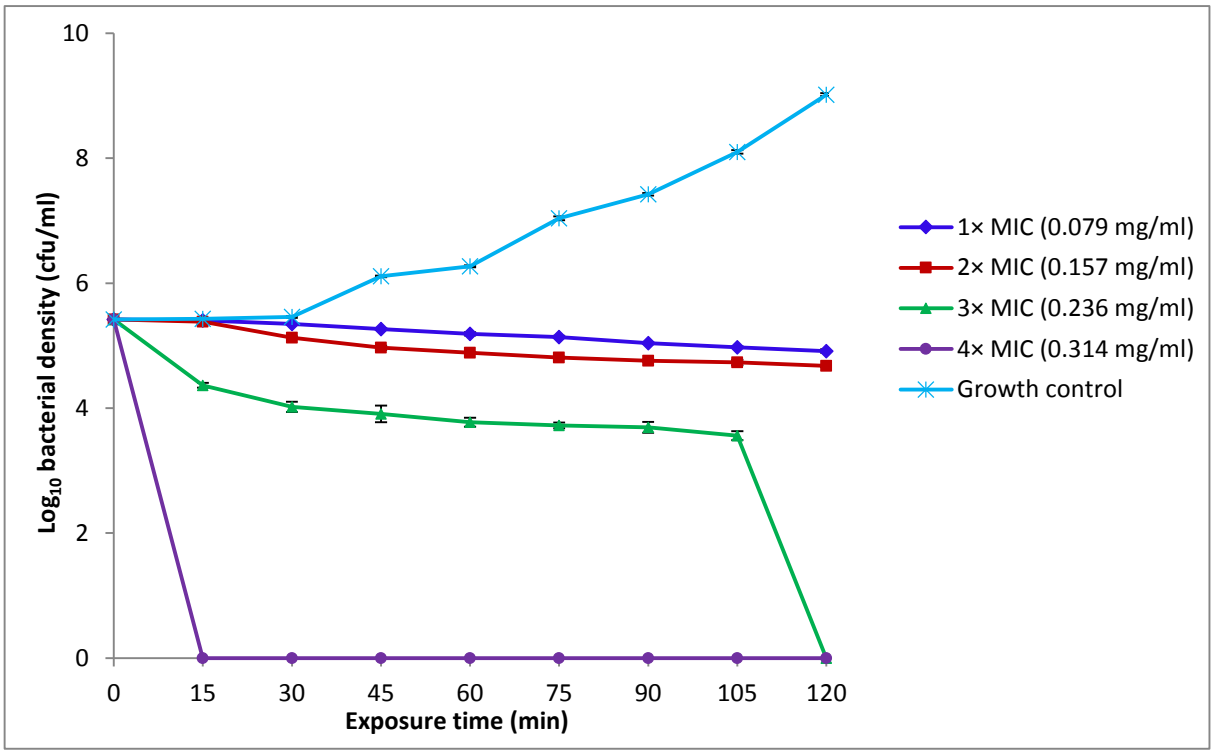

Figure 4. Profile of rate of kill of *L. ivanovii* (LEL 18) by crude n-hexane extracts of *Garcinia kola* seeds.

Discussion

The crude n-hexane and aqueous extracts of *Garcinia kola* seeds showed appreciable anti-*Listerial* activities from the susceptibility tests results with the n-hexane extract achieving a 45% activity which was higher in comparison to a 29% activity of the aqueous extract. The MIC and MBC ranges of the n-hexane extract were ranging from 0.079-0.625 mg/ml and 0.625-10 mg/ml respectively whilst the aqueous extract had higher values with MIC and MBC ranges of 10->10 mg/ml and above 10 mg/ml respectively. These results corroborates other reports (Ezeifeka *et al*., 2004; Ogbulie *et al*., 2007; Sibanda and Okoh, 2008; Sibanda *et al*., 2010; Penduka *et al*., 2011) that showed that the organic solvents extracts of *Garcinia kola* seeds were more antibacterial in comparison to the aqueous extracts, mainly because of the better solubility of the antibacterial agents in *Garcinia kola* such as xanthones, benzophenones, and flavonoids (Xu and Lee, 2001; Han *et al*., 2005; Okunji *et al*., 2007) in organic solvents than in water (Taiwo *et al*., 1999; Obi and Onuoha, 2000; Ogueke *et al*., 2006; Ogbulie *et al*., 2007; Nwaokorie *et al*., 2010).

Some studies on the antibacterial activities of the crude aqueous extracts of *Garcinia kola* seeds have shown MIC values within the ranges of 5-20 mg/ml (Sibanda *et al*., 2010; Sibanda and Okoh 2008; Nwaokorie *et al*., 2010). Similarly in our findings, the crude aqueous extract had MIC values of 10 mg/ml against 10 isolates whilst having MIC values of above 10 mg/ml against 2 isolates. Variations in the methodologies used in the studies becomes the greatest obstacle in comparing results to give concrete evidence of the seeds' crude aqueous extracts MIC ranges but they however support the use of the seed's aqueous extracts in traditional medicine to treat various medical conditions that can originate from bacterial infections, conditions such as such as diarrhoea, high fever and throat infections (Iwu, 1993).

Members of the genus *Garcinia* from the family Guitefferae are considered as a rich and valuable source of bioactive compounds (Monache *et al*., 1984; Almeida *et al*., 2008; Pereira *et al*., 2010; Zhou *et al*., 2010). In a study by Pereira *et al*. (2010), three prenylated benzophenones namely 7-epi-clusianone, garciniaphenone and guttiferone-a were obtained from silica gel chromatography of the hexane extract of powdered *Garcinia brasiliensis* Mart. fruits and these were found to exhibit significant activity on *Leishmania (L.) amazonensis* and having minimum toxicity for mammalian cells (Pereira *et al*., 2010). In a separate study involving non-polar solvents petroleum ether and ethyl acetate a polyisoprenyl benzophenone (kolanone) was found in the petroleum ether fraction whilst a hydroxybiflavanonols was found in the ethyl acetate fraction of *Garcinia kola* seeds and GB1 (a hydroxybiflavanonol) was the main component exhibiting activity against bacteria, *Candida albicans* and *Aspergillus flavus* (Madubunyi, 1995). The activity of the n-hexane extract in this study can therefore be attributed to a number of compounds possibly those mentioned above that can be found in *Garcinia kola* seeds especially those extracted through the use of non-polar solvents such as n-hexane.

Rate of kill assays 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 *et al*., 2009). The rate of kill activity of the n-hexane extract proved to be bactericidal at 2× (for *L. ivanovii* (LEL 30) only), 3× and 4× MIC values after 2 h exposure time for all the test organisms, since a reduction of the viable bacterial density of ≥99.9% or $\geq 3\log_{10}$ in cfu/ml is used as a standard of measurement for bactericidal efficacy (Pankey and Sabath, 2004; CLSI, 2005). The acetone extracts (Sibanda and Okoh, 2008), methanol extracts (Nwaokorie *et al*., 2010), butanol and diethyl-ether fractions of the methanol extract (Akinpelu *et al*., 2008) of *Garcinia kola* seeds were also found to exhibit bactericidal activities against both Gram positive and Gram negative bacteria, with the findings of Akinpelu

et al. (2008) and Sibanda and Okoh (2008) findings showing a concentration and time dependent killing activity similar to this study. This study shows the nature of inhibition of the n-hexane extract of *Garcinia kola* seeds to be bactericidal at 3× and 4× MIC values against *Listeria* species as well as being concentration and time dependant.

Conclusion

This study revealed the anti-*Listerial* activities of both the crude n-hexane and aqueous extracts of *Garcinia kola* seeds with the n-hexane extracts being more active and bactericidal. Further studies to determine the extracts' interactions with standard antibiotics and to also isolate and characterize the active principles in the n-hexane extract are subjects of on-going investigation. This study therefore shows that *Garcinia kola* seeds hold promise as a potential source of therapeutic compounds that can be exploited in effective anti-*Listerial* therapy.

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CHAPTER SIX

***In-Vitro* Anti-*Listerial* Activities of Combinations of the Crude N-Hexane Extract of *Garcinia kola* Seeds and some Conventional Antibiotics**

(Submitted for review in the Pakistan Journal of Pharmaceutical Sciences)

Abstract

The chequerboard method was used to evaluate the effect of combinations of the crude n-hexane extract of *Garcinia kola* seeds and some conventional antibiotics on their *in-vitro* anti-*Listerial* activities. The n-hexane extract had minimum inhibitory concentrations (MICs) ranging from 0.079 to 0.157 mg/ml whilst the antibiotics used namely penicillin G, ampicillin and ciprofloxacin had MIC's ranging from 0.020 to 0.625 µg/ml. The interactions between the extract and the antibiotics were synergistic with all the antibiotics. The solvent combination of benzene: ethanol: ammonium hydroxide (B.E.A) in the ratio (36:4:0.4 v/v) separated the constituents in the extract through the use of thin layer chromatography (TLC) and showed the presence of five bands after spraying with vanillin spray reagents at extract concentrations of 100, 50 and 25 mg/ml. TLC coupled with direct bioautography (TLC-direct bioautography) at extract concentration of 100 mg/ml showed two zones of inhibition against *Listeria grayi* (LAL 15) and *Listeria ivanovii* (LEL 18) and one zone each against *Listeria monocytogenes* (LAL 8) and *Listeria ivanovii* (LEL 30). The R_f values of the zones on the TLC-direct bioautography chromatograms were different among the four isolates, which showed the multiplicity of the anti-*Listerial* compounds in the extract. The n-hexane extract of *Garcinia kola* seeds can be a useful source of compounds that can be used in synergy with conventional antibiotics to treat *Listeria* infections.

Keywords: *Garcinia kola* seeds; n-hexane extract; *Listeria* species; antibiotics; interactions.

Introduction

Although most cases of listeriosis are self-limiting in immune-competent individuals, there is still a need to administer appropriate and effective antibiotic therapy especially in high-risk patients such as the immune-compromised, elderly and the pregnant to curb its progression to invasive listeriosis infections such as central nervous system infections and bacteraemia which can result in fatality (Mardis *et al*., 2012). The first-line antibiotics of choice for the treatment of severe listeriosis are ampicillin and penicillin G. In individuals with penicillin allergies meropenem is used for mild allergies such as those resulting in maculopapular rash while sulfamethoxazole-trimethoprim (SMX-TMP) is used in cases of a severe penicillin allergy such as Stevens-Johnson syndrome [SJS] or toxic epidermal necrolysis (Mardis *et al*., 2012). Vancomycin/teicoplanin can also be used in patients with penicillin allergy, whilst cephalosporins should not be used for treatment of listeriosis and clinical experience on the effectiveness of fluoroquinolones is limited (Swaminathan and Gerner-Smidt, 2007).

Treatment of listeriosis can be monotherapy of the penicillin antibiotic of choice alone or be a combinatorial therapy whereby ampicillin or penicillin G is administered together with an aminoglycoside mainly gentamicin (Mitja *et al*., 2009). Action of combined antimicrobial agents can be beneficial (synergism or additive interaction) or deleterious (antagonistic or toxic outcome) (Adwan and Mhanna, 2008). The action is considered to be synergistic if their joint effect is stronger than the sum of effects of the individual agents; additive if their joint effect is equal to the sum of effects of the individual agents; indifferent if their joint effect is equal to the effect of either individual agent; antagonistic if their joint effect is weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent (Stefanovic *et al*., 2012).

There are some raised concerns regarding the use of some of the listeriosis treatment antibiotic combinations. In some monitored clinical listeriosis cases the combination including penicillins and an aminoglycoside primarily gentamicin were found in most instances not to yield synergistic effects and had an unfavourable outcome on the patients' health (Mitja *et al*., 2009; Amaya-Villar *et al*., 2010). This was particularly evident in patients with listeriosis characterised by meningitis and this was attributed to gentamicin's associated nephrotoxicity and inability to cross the blood-brain barrier (Mitja *et al*., 2009; Amaya-Villar *et al*., 2010). On the other hand the combination of SMX and TMP in which the sulfonamide SMX and the folic acid analogue TMP inhibit the enzymatic pathway of bacterial folate synthesis which ultimately inhibits DNA synthesis (Tenover, 2006), has a potential risk of causing potentially serious adverse effects in pregnant women, due to its disturbances of folic acid metabolism and also its use in late pregnancy may cause kernicterus in the child (Mardis *et al*., 2012).

Given the side effects of some of the antimicrobial combinations used in the treatment of listeriosis, it becomes essential to search for newer compounds that could be used in synergy with the current antibiotics with less adverse side effects and also so as to increase the armoury of anti-*Listerial* agents. A combined antimicrobial activity between known antibiotics and bioactive plant extracts is a novel concept (Adwan and Mhanna, 2008). This combination with natural plant compounds can help in mitigating the adverse side effects caused by some of the synthetic antibiotics as the doses of these antibiotics would have been reduced. Also, in reducing the dosage of the antibiotics this can in-turn lower the treatment costs, making it affordable to the common man in developing countries where the use of medicinal plants is a more affordable treatment option in comparison to conventional antibiotics. This communication therefore focuses on assessing the interactions of the antibiotics penicillin G, ampicillin and ciprofloxacin with the n-hexane extract of the seeds of *Garcinia kola* (a

traditional medicinal plant) which was found to possess anti-*Listerial* activities *in-vitro* (Penduka and Okoh, 2011).

Materials and Methods

Bacterial Strains

Four *Listeria* isolates that were isolated from wastewater effluents in the Eastern Cape Province of South Africa (Odjadjare *et al*., 2010) and were found to be susceptible to the n-hexane extract of *Garcinia kola* seeds (Penduka and Okoh, 2011) were used in the study and these included *Listeria grayi* (LAL 15), *Listeria ivanovii* (LEL 18), *Listeria monocytogenes* (LAL 8) and *Listeria ivanovii* (LEL 30).

Plant material and preparation of the n-hexane extract

The *Garcinia kola* seeds were sourced from Nigeria and the ground seed powder was kept in the plant material collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, University of Fort Hare, Alice, South Africa. The solvent extraction method used followed the description of Basri and Fan (2005) and Dogruoz *et al*. (2008). In brief the method involved steeping 100 grams of the seed powder into 500 ml of the n-hexane solvent for 48 h with shaking in an orbital shaker (Stuart Scientific Orbital Shaker, UK), after which the supernatant was centrifuged at 3000 rpm for 5 min at 4°C in a centrifuge (Beckman Model TJ-6RS Centrifuge, Great Britain), followed by filtration through Whatman No. 1 filter paper. The residue from the extraction was steeped in 300 ml of the solvent and subjected to another 48 h of extraction with shaking after which it underwent the same filtration process as mentioned above. The extracts were combined and concentrated using a rotary evaporator

(Steroglass S.R.L, Italy) at 50°C and then dried to a constant weight in a fume cupboard. Prior to testing, the extracts were dissolved in 5% Dimethyl Sulphoxide (DMSO) with the final volume being made up by sterile distilled water to make the test concentrations.

MIC determination

The broth microdilution method was used to determine the minimum inhibitory concentrations (MIC's) of the test antibiotics and the crude n-hexane extract. Double strength Mueller-Hinton broth was used in performing the serial dilutions in 96 well microtitre plates to make the different test concentrations, such that the initial starting concentration of the n-hexane extract was 10 mg/ml whilst that for penicillin and ampicillin was 10 µg/ml and that for ciprofloxacin was 5 µg/ml. A 20 µl volume of the test bacteria suspension in sterile saline prepared using the colony suspension method of EUCAST (2003) was added to each test well. An automatic ELISA microplate reader (SynergyMx, BiotekR USA) at 630 nm was used to measure the absorbance of the plates before and after 18-24 h incubation at 37°C to determine the MIC's of the antimicrobial agents. The growth control wells contained the broth and the organism only; sterility control wells contained the broth only; whilst absorbance control wells contained the broth, antibacterial agent at the different test concentrations and sterile saline (EUCAST, 2003).

Antibiotics – Extract Interactions

The interactions studies were performed in 96 well microtitre plates using the chequerboard methodology as described by Miranda-Novels *et al*. (2006) with some modifications. Each well contained 100 µl of individual antimicrobial combinations. The colony suspension method

according to EUCAST, (2003) was used to prepare the test *Listeria* inoculums. MICs were read after 18-24 h incubation at 37°C. Growth, sterility and absorbance control wells were also included. An automatic ELISA microplate reader (SynergyMx, BiotekR USA) at 630 nm was used to measure the absorbance of the plates before and after incubation to determine the subsequent MIC's of the antimicrobial agents from which the Fractional inhibitory concentration (FIC) indices were calculated as: FIC index of extract (FIC_E)=MIC of extract in combination/MIC of extract alone, and the FIC index of antibiotic (FIC_A)=MIC of antibiotic in combination/MIC of antibiotic alone, whilst the FIC index (ΣFIC)=FIC_E+FIC_A. The interactions were interpreted as: synergism when the ΣFIC index ≤ 0.5; additivity when ΣFIC index is >0.5 and ≤1; indifference when ΣFIC index is >1 and ≤4 whilst antagonism was defined as when the ΣFIC index >4 (Satish *et al*., 2005; Stefanovic *et al*., 2012).

Thin layer chromatography (TLC) of the extract

The crude n-hexane extract was subjected to TLC to determine the phytochemical constituents of the extract after the method of Eloff *et al*. (2005) with some modifications. About 20 µl of the extract on aluminium backed TLC plates was fractionated in a closed TLC tank using the solvent combination of benzene: ethanol: ammonium hydroxide (B.E.A) (36:4:0.4 v/v) which from preliminary studies involving two other solvent combinations namely ethyl acetate: methanol: water (E.M.W) (40:5.4:4 v/v) and chloroform: ethyl acetate: formic acid (C.E.F) (5:4:1 v/v) proved to have the best separation of the constituents in the extract. After drying, the plates were visualised under daylight and also under ultraviolet light (302 nm or 365 nm) before being sprayed with vanillin spray reagents (0.1 g vanillin, 28 ml methanol,1 ml sulphuric acid) (Stahl, 1969), after which they were heated at 105°C for optimal colour development for

4-5 min. The retention factor (R_f) values (ratio of distance moved by the band to distance moved by solvent) of the visible extract's bands were marked.

Qualitative anti-*Listerial* activity assay by bioautography

Bioautographic analyses of the anti-*Listerial* activities of the extract was done in accordance with the descriptions of Begue and Kline (1972) with minor modifications. TLC plates loaded with approximately 20 µl of the extract and developed in B.E.A (36:4:0.4 v/v) solvent combination were left to dry for four days in a fume cupboard to ensure complete evaporation of the solvents. After which they were sprayed with actively growing 18-24 h *Listeria* in Mueller-Hinton broth and incubated overnight at 100% humidity and subsequently sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT). Observation of clear zones on the chromatogram after a further 1-2 h incubation period indicated inhibition of growth.

Statistical analysis

The SPSS 19.0 version for windows program was used to determine the means and standard deviations.

Results

MICs of the different anti-bacterial agents

Table 1 shows the MICs of the different antibacterial agents tested which includes penicillin G, ampicillin, ciprofloxacin and the n-hexane extract of *Garcinia kola* seeds. The MICs of the

three antibiotics ranged from 0.020 to 0.625 µg/ml with ciprofloxacin having the highest MIC values among the three antibiotics. The MIC values of the n-hexane extract ranged from 0.079 to 0.157 mg/ml which were higher in comparison to all the tested antibiotics.

Table1: MIC results of the different anti-bacterial agents

Organism	**Penicillin G (µg/ml)**	**Ampicillin (µg/ml)**	**Ciprofloxacin (µg/ml)**	**n-Hexane extract (mg/ml)**
Listeria monocytogenes (LAL 8)	0.079	0.079	0.313	0.079
Listeria ivanovii (LEL 18)	0.020	0.079	0.313	0.079
Listeria grayi (LAL 15)	0.020	0.079	0.625	0.079
Listeria ivanovii (LEL 30)	0.156	0.039	0.313	0.157

Antibiotics-n-hexane extract interactions

The results of the antibiotics and n-hexane extracts interactions are as shown in Table 2. The n-hexane extract proved to have synergistic interactions with all the three antibiotics used in the study.

Table 2: Results of the antibiotics and n-hexane extract interactions

Organism	Antimicrobial combination	FIC_A	FIC_E	ΣFIC	Interaction
L. monocytogenes (LAL 8)	Penicillin G	0.124	0.062	0.186	Synergy
	Ciprofloxacin	0.016	0.062	0.078	Synergy
	Ampicillin	0.124	0.062	0.186	Synergy
L. ivanovii (LEL 18)	Penicillin G	0.125	0.016	0.141	Synergy
	Ciprofloxacin	0.016	0.062	0.078	Synergy
	Ampicillin	0.124	0.062	0.186	Synergy
L. grayi (LAL 15)	Penicillin G	0.125	0.016	0.141	Synergy
	Ciprofloxacin	0.008	0.062	0.07	Synergy
	Ampicillin	0.062	0.031	0.093	Synergy
L. ivanovii (LEL 30)	Penicillin G	0.125	0.062	0.187	Synergy
	Ciprofloxacin	0.031	0.062	0.093	Synergy
	Ampicillin	0.25	0.031	0.281	Synergy

Thin layer chromatography

The thin layer chromatography results showed one visible band each deep pink in colour when viewed with the naked eye at 25 and 50 mg/ml concentration of the extract, while two bands were visible at 100 mg/ml with one being of a deep pink colour with an R_f value of 0.119±0.017, and another light pink in colour with an R_f value of 0.497±0.031 (appears faint in the image) as shown in Figure 1(a). At 100 mg/ml concentration of the extract, two visible bands could be seen under UV at 365nm, whilst only one band was visible at both 50 and 25 mg/ml extract concentration as shown in Figure 1(b). Five bands were visible at all the three concentrations after spraying the TLC chromatogram with vanillin spray reagents as seen in Figure 1(c). The R_f values of all the visible bands are as shown in Table 3


(a)

(b)

(c)

Figure 1: Thin layer chromatography chromatograms of the n-hexane extract view; (a) with the naked eye; (b) at 365nm wavelength and (c) after spraying with vanillin spray reagents.

Table 3: Retention factor (R_f) values of bands of the crude n-hexane extract of *Garcinia kola* seeds after spraying with vanillin spray reagents.

Band Number From Bottom of TLC Plate	**Mean R_f values for visible bands after vanillin spray reagents at different extract concentrations**		
	100 mg/ml	**50 mg/ml**	**25 mg/ml**
1	0.119±0.017*	0.079±0.005*	0.066±0.004*
2	0.333±0.012	0.277±0.003	0.252±0.016
3	0.497±0.031*	0.469±0.009	0.500±0.036
4	0.761±0.002	0.767±0.002	0.786±0.011
5	0.896±0.012	0.890±0.005	0.893±0.010

Key note: Values with asterisk indicate bands visible at 365nm and also before and after vanillin spray.

Bioautography of the n-hexane extract

One clear zone was seen on the bioautogram of the extract against *L. ivanovii* (LEL 30) with a mean R_f value of 0.178±0.009 and *L. monocytogenes* (LAL 8) with a mean R_f value of

0.182±0.009, as seen in Figure 2a and Figure 2b respectively. Two zones of inhibition were observed on the bioautograms of the extract against *L. grayi* (LAL 15) with mean R_f values of 0.835±0.018 and 0.082±0.018 and against *L. ivanovii* (LEL 18) with mean R_f values of 0.893±0.007 and 0.076±0.009 as shown in Figure 2c and Figure 2d respectively.

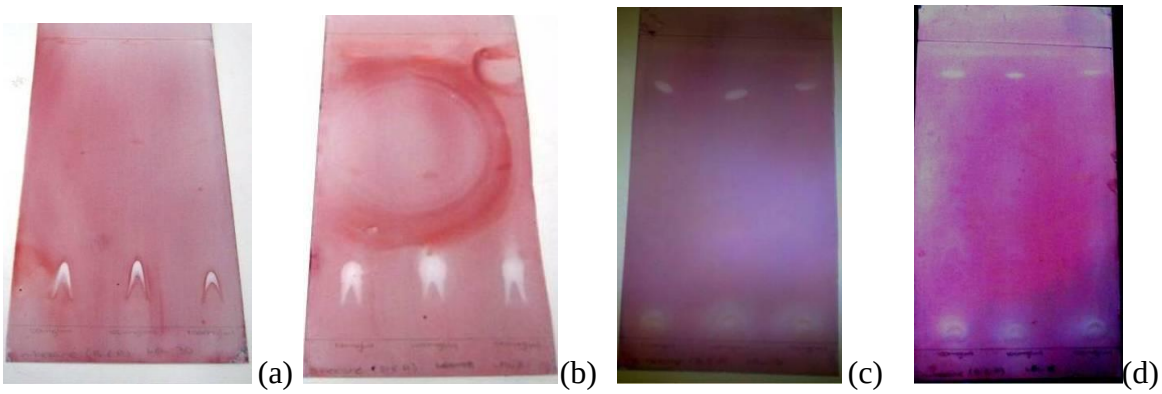
(a) (b) (c) (d)

Figure 2. Bioautograms showing results in triplicates of the fractionated crude n-hexane extract showing antibacterial activity (clear white zones) against (a) *L. ivanovii* (LEL 30), (b) *L. monocytogenes* (LAL 8), (c) *L. grayi* (LAL 15) and (d) *L. ivanovii* (LEL 18).

Discussion

The chequerboard technique is one of the best known laboratory methods used to determine fractional inhibitory concentration (FIC) indices using a two dimensional array of serial concentrations of test compounds. The indices are in turn used to interpret antimicrobial combinations as synergistic, additive, indifferent or antagonistic (Eliopoulos and Moellering Jr, 1991; Odds, 2003; Sweeney and Zurenko, 2003). Combinatorial therapy in the clinic often begins with *in-vitro* interactions that will have shown synergy in inhibiting the growth of the microorganism of interest (Odds, 2003).

In this experiment synergistic interactions were observed between the n-hexane extract of *Garcinia kola* seeds and the β-lactam penicillins namely penicillin G and ampicillin as well as with a fluoroquinolone antibiotic ciprofloxacin. Fluoroquinolone antibiotics exert their antibacterial effects by disrupting DNA synthesis and causing lethal double strand DNA breaks during DNA replication (Tenover, 2006). β-lactam antibiotics on the other hand exert their antibacterial effect by inhibiting cell wall synthesis. The inhibitory action of penicillins is due to their high affinity to the penicillin-binding protein 3 (PBP 3), which is the essential transpeptidase participating in the construction of peptidoglycan, the main constituent of the bacterial cell wall (Hof, 2003).

A number of mechanisms could have attributed to the observed synergistic effect between the extract and the antibiotics. The extract and the antibiotics could have both attacked the cell wall that is the penicillins and the extract, as postulated by Zhao *et al*. (2001) that epigallocatechin gallate (EGCg) a bactericidal plant compound from tea catechins synergizes the activity of β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA) owing to its interference with the integrity of the cell wall through direct binding to the peptidoglycan. It could also be possible that both ciprofloxacin and the extract constituents all disrupted DNA synthesis.

An antimicrobial agent such as a cell wall inhibitor for example β-lactam antibiotics may enhance the entry of a protein synthesis inhibitor such as aminoglycoside antibiotics into bacteria and thus produce synergistic effects or in another case the cell membrane can be affected such that entry of the second antimicrobial agent is facilitated (Toroglu, 2007). In this case, the penicillins could have enhanced the entry of the antibacterial compounds in the n-hexane extract into the bacterial cell wall and this could have resulted in the observed synergistic outcome. The antibacterial compounds in the n-hexane extract could also have caused loss of the cell membrane's intergrity thereby facilitating the entry of ciprofloxacin. This could be due to the presence of essential oils in *Garcinia kola* seeds (Aniche and Uwakwe,

1990) which are postulated to exhibit anti-bacterial activity by disrupting the permeability barrier of microbial membrane structures (Cox *et al*., 2000).

Garcinia kola seeds have different other bioactive compounds such as kolanone (Madubunyi, 1995), fatty acids (Eleyinmi *et al*., 2006) and terpenoids (Ogu and Agu, 1995) which are extractable by non-polar solvents such as n-hexane. It is a possible hypothesis that these compounds together may have prevented the possible inactivation of the antibiotics by microbial enzymes, resulting in the observed synergy (Brooks *et al*., 1995; Toroglu, 2007). Separation of the bioactive constituents in the n-hexane extract could help pinpoint the exact mechanism of action that may have led to the observed synergistic interactions and then again the synergy could have been brought about by the combination of the bioactive constituents in the extract as a whole.

The observed better separation of the constituents of the extract by the B.E.A solvent combination which is mainly non-polar in nature in comparison to the E.M.W combination which is polar and C.E.F combination which is mainly of medium polarity showed that the nature of compounds present in the n-hexane extract were mainly non-polar and this was in-line with the fact that the solvent n-hexane extracts mainly non-polar compounds which are mainly oils from plant material particulary seeds (Ferreira-Dias *et al*., 2003).

The TLC plate showed the presence of five bands after spraying with vanillin spray reagents which were more visible at the highest concentration used of 100 mg/ml of the extract in comparison to the lower concentrations of 50 and 25 mg/ml. There is a possibility that some of the compounds were present in low concentrations or that they do not react with the vanillin spray reagents (Eloff *et al*., 2005) such that they were not visible on the chromatogram or that some volatile compounds could have evaporated during the drying process. TLC-direct bioautography assay showed the presence of one active band/zone against *L. ivanovii* (LEL 30)

and *L. monocytogenes* (LAL 8) and two zones/bands of activity against *L. grayi* (LAL 15) and *L. ivanovii* (LEL 18). An active band can represent a number of compounds which have almost the same R_f values and are all active or are active in synergy against the *Listeria* bacteria. The different R_f values observed among the active fraction bands in bioautography show the potential presence of multiple bioactive constituents in the extract.

Bioactive compounds multiplicity in plant extracts can be a good indication as combinations of antibiotics with plant extracts could become a basis for development of new approaches in antibiotic resistance modifying agents. This is because the use of plant extracts promises a lower risk of increasing bacterial resistance as they contain mixtures of different bioactive compounds with different modes of action, which make microbial adaptability very difficult in comparison to single-constituent antibiotics (Stefanovic *et al*., 2012).

Conclusion

The n-hexane extract of *Garcinia kola* seeds showed synergistic interactions with the selected antibiotics. It appears as a potential alternative for use in combinatorial listeriosis therapy with the selected antibiotics *in-vitro*, although *in-vivo* studies are also necessary to test the combinations and the possible use of the extract as a standardised extract. Preliminary separation and bioactivity testing using TLC-direct bioautography showed the potential presence of multiple anti-*Listerial* compounds in the extract such that further separation and identification of these constituents is a subject of on-going research.

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CHAPTER SEVEN

Gas chromatography-Mass Spectrometry Characterisation of the Anti-*Listeria* Components of *Garcinia kola* Seeds.

(Published in Applied Biochemistry and Microbiology)

Abstract

Column chromatography was used to separate the bioactive constituents of the crude n-hexane extract of *Garcinia kola* seeds. The column fractions were eluted using the solvent combination of benzene: ethanol: ammonium hydroxide (B.E.A) in the ratio combination of 36:4:0.4 v/v respectively. The fractions were tested for their anti-*Listerial* activities by either determining their MIC_{50}, MIC_{90} or MIC against four representative *Listeria* species. The fractions were labelled BEA1 to BEA5 and three out of the five fractions eluted by the B.E.A solvent combination were active against the test *Listeria* species with MIC's ranging from MIC 0.157mg/ml to MIC_{50} 0.625mg/ml. The two most active fractions BEA2 and BEA3 with MIC ranges of 0.157-0.313 mg/ml and 0.313 - MIC_{90} 5 mg/ml respectively were subjected to gas chromatography coupled to mass spectrometry (GC-MS) to identify the composition of the fractions. Fraction BEA2 was composed of 18 compounds mostly sterols and the BEA3 fraction was composed of 27 compounds with the most abundant compounds being fatty acids derivatives. The BEA2 fraction's interactions with antibiotics proved to be 100% synergistic with ciprofloxacin and ampicillin whilst it exhibited 50% additivity and 50% synergism with penicillin G. However, all the interactions of the BEA2 fraction with conventional antibiotics were synergistic against the human listeriosis causative bacteria *L. monocytogenes*.

Keywords: *Garcinia kola* seeds, *Listeria* species, GC-MS, Synergism, MIC

Introduction

Isolation and identification of compounds responsible for antimicrobial activities in crude plants extracts is an important factor and it requires the use of different multi-step chromatographic techniques. Chromatography is a physical method of separating compounds in a mixture. The separation is achieved by the distribution of the compounds between a stationary and a mobile phase (Scott, 2003). Compounds that have more affinity to the stationary phase are retained longer in the system than those that have more affinity to the mobile phase. As a consequence, solutes are eluted from the system in a definite direction according to their elution rates from the stationary phase (Scott, 2003).

Chromatography is a versatile technique that can separate gases and volatile substances by gas chromatography (GC), non-volatile compounds and compounds of extremely high molecular weight (including biopolymers) by liquid chromatography (LC) (Scott, 2003). In GC the mobile phase is a gas whilst in LC the mobile phase is a liquid. The stationary phases of either types of chromatography can either be solid or a liquid depending on the type of chromatography being performed (Scott, 2003).

There are many different variations of LC which are mainly dependent on the stationary phase chemistry. The simplest forms of LC are paper chromatography and thin layer chromatography (TLC). Paper chromatography is based upon the separation of a sample's components on paper with a solvent whilst TLC is based on the separation of the plant's components on a thin two dimensional sheet of stationary phase (usually silica) coated onto a slide/solid material which is placed in closed tank with the solvent system (Allwood and Goodacre, 2010).

TLC is an effective and inexpensive procedure that gives the researcher an idea of how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f value of a compound is compared with the R_f value of a known compound

(Sasidharan *et al*., 2011). Bioautography is a useful technique to determine antimicrobial compounds within a plant extract. TLC combined with bioautographic methods combine chromatographic separation and *in-situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture (Sasidharan *et al*., 2011).

Column chromatography-based LC, is a much more powerful technique than the paper chromatography and TLC methods, and has a greater sample capacity (Allwood and Goodacre, 2010). Column chromatography may employ either isocratic elution which uses a mobile phase of a constant single composition or employ gradient elution whereby the mobile phase composition is altered during the chromatographic separation process. The flow of the mobile phase can either be due to gravity or vacuum where columns are not designed to withstand high pressures (Allwood and Goodacre, 2010). The mobile phase used in plants extracts separation is usually the one that would have shown good separation of the extract's components during TLC and also showed compounds with antimicrobial activity during bioautography. The usual stationary phases used in column chromatography are adsorbents such as silica, alumina, calcium carbonate, calcium phosphate, magnesia, sugar, carbon, magnesium silicate, magnesium carbonate and starch.

High performance liquid chromatography (HPLC) is distinguished from ordinary liquid chromatography because the pressure of HPLC is relatively high, while ordinary liquid chromatography typically relies on the force of gravity to provide pressure. HPLC is usually coupled to mass spectrometry (MS). MS provides abundant information for structural elucidation of the compounds, whilst HPLC is efficient in separating chemical compounds in a mixture. Therefore, the combination of HPLC and MS (HPLC-MS) facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable (Ye *et al*., 2007).

Gas chromatography is a well established analytical technique commonly used for the characterization and identification of volatile organic compounds (Tistaert *et al*., 2011), as well as a large range of semi-volatile organic compounds through chemical derivatisation which can be achieved through the addition of volatile trimethylsilyl (TMS) groups, through the use of compounds such as N-methyl-N-triflouoroacetamide (MSTFA) which causes the formation of volatile trimethylsilyl esters which can be easily characterized and identified by gas chromatography (Roessner *et al*., 2000). Gas chromatography coupled to mass spectrometry (GC-MS) allows for separation, identification and quantification of volatile and semi-volatile organic compounds with good resolution in a plant extract, such that it has emerged as the best technique for characterization of low-polarity compounds, including triterpenes, sterols, glycerols, waxes, and derivatized fatty acids (Regasini *et al*., 2009).

Prior purification of the plant extract through column chromatography allows for more specific identification of antimicrobial compounds by GC-MS or HPLC-MS. Thorough biological evaluation of plant extracts is vital to ensure their efficacy and safety. These factors are of importance if plant extracts are to be accepted as valid medicinal agents (Ncube *et al*., 2008). This paper focuses on the GC-MS characterisation of *Garcinia kola* seeds' n-hexane extract column chromatography fractions eluted by a solvent combination of benzene: ethanol: ammonium hydroxide (B.E.A) at the ratio of 36:4:0.4 v/v. The n-hexane extract as reported in the previous work (Penduka and Okoh, 2011) exhibited bactericidal properties against *Listeria* isolates, this study therefore aims at identifying the compounds responsible for the anti-*Listerial* activities through the use of combined chromatographic techniques.

Materials and Methods

Plant Material

The seeds were sourced from the south western part of Nigeria and the ground seed powder was kept in the plant materials collection of the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, University of Fort Hare, Alice, South Africa.

Bacterial Strains

Four *Listeria* isolates previously isolated from wastewater effluents (Odjadjare *et al*., 2010) were used in the study and included *Listeria grayi* (LAL 15), *Listeria ivanovii* (LEL 18), *Listeria monocytogenes* (LAL 8) and *Listeria ivanovii* (LEL 30). These were also previously found to be susceptible to the n-hexane extract of *Garcinia kola* seeds (Penduka and Okoh, 2011).

Preparation of the n-hexane extract

The extract preparation followed the method by Basri and Fan (2005) and Dogruoz *et al*. (2008). In brief, the method involved steeping 100 grams of the seed powder into 500 ml of n-hexane solvent for 48 h with shaking in an orbital shaker (Stuart Scientific Orbital Shaker, UK), after which the supernatant was centrifuged in a centrifuge (Beckman Model TJ-6RS Centrifuge, Great Britain) at 3000 rpm for 5 min at 4°C, followed by filtration through Whatman No. 1 filter paper. The residue from the extraction was steeped in 300 ml of the solvent and subjected to another 48 h period of extraction with shaking after which it underwent the same filtration process as mentioned above. The extracts were combined and concentrated

using a rotary evaporator (Steroglass S.R.L, Italy) at 50°C and then dried to a constant weight in a fume cupboard.

Column Chromatography

Seven grams of the n-hexane extract was mixed with 14 grams of silica gel 60 (Merck, Gremany; particle size 0.063 to 0.2mm/ 70 to 230 mesh) and benzene to form a homogenous thick paste and was left to dry in the fume cupboard overnight. A suspension of silica gel and benzene was poured into a glass column (40-cm long×2.5-cm diameter) up to a height of 30cm being careful to prevent formation of gaps and bubbles and equilibrated with 100% benzene. The dried silica gel and extract combination was then loaded onto the column and eluted with 100% benzene first and then with the solvent combination of BEA (36:4:0.4 v/v). The eluted fractions were collected on the basis of solvent polarity and or colour separation and then dried in a rotary evaporator (Steroglass S.R.L, Italy) at 50°C, after which they were dried to a constant weight in a fume cupboard following the descriptions of Selowa *et al*. (2010) with some modifications. The phytochemical composition of the fractions was determined by TLC according to the methodology of Eloff *et al*. (2005) and fractions with similar profiles were pooled together.

MIC, MIC_{90} or MIC_{50} determination of the Fractions eluted by BEA (36:4:0.4) solvent combination.

The broth microdilution and the inoculum standardising methods of EUCAST (2003) were used to determine the MICs of the five fractions named BEA1 to BEA5 eluted by the B.E.A solvent combination. Briefly the fractions were dissolved in 5% Dimethyl sulphoxide (DMSO)

with the final volume being made up of sterile distilled water. Two fold serial dilutions of the fractions in Mueller-Hinton broth were carried out in 96 well microtitre plates, with an initial starting concentration of 5 mg/ml. Sterility, growth and positive, control wells were also included together with absorbance control wells which had the test fraction at different concentrations, broth and saline. Ciprofloxacin, penicillin G and ampicillin were used as the positive controls whilst 5% DMSO was also tested to verify if it had any effect on the growth of the organism. An automatic ELISA microplate reader (SynergyMx, BiotekR USA) at 620 nm was used to measure the absorbance of the plates before and after incubation to determine either MIC_{50}, MIC_{90} or MIC of the test fraction. MIC_{50} which is the minimum concentration of the test fraction that inhibited 50% growth of the bacteria, MIC_{90} which is the minimum concentration of the test fraction that inhibited 90% bacterial growth and MIC which is the lowest concentration of the test fraction that inhibited growth of the bacteria.

Gas chromatography-Mass spectrometry (GC-MS) analysis

The test samples were dissolved in 1 ml dichloromethane and then derivatized with pyridine and MSTFA (*N*-Methyl-*N*-trimethylsilyltrifluoroacetamide) followed by incubation at 80°C for 30 min before injection onto a GC column. The analysis was carried out using an Agilent 6890N GC with CTC CombiPAL Autosampler and Agilent 5975B MS with an Rtx®-5MS (30 m, 0.25 mm ID, 0.5 µm film thickness) Restek 12723-127 column. The injector temperature was at 280°C, injection volume was 1 µl using the split injection mode at a split ratio of 15:1 using Helium as a carrier gas at a flow rate of 1ml/min. The MS transfer was at 280°C, mode EI+; Electron energy 70 eV, acquistion mode was scan at a scanning mass range of 40 to 550 m/z and solvent delay of 8 min. The initial oven temperature was at 150°C held for 1 min to a final temperature of 325°C held for 20 min. The compounds were identified by comparison of

their retention indices and mass spectra with those in the National Institute of Standards and Technology (NIST) library.

Antibiotics – BEA2 Interactions

The interactions were determined using the chequerboard method according to the descriptions of Miranda-Novales *et al*. (2006) with some modifications. The interactions were performed in 96 well microtitre plates. Each well contained 100 µL of individual antimicrobial combinations. The inoculum suspension method of EUCAST, (2003) was used to standardise the test *Listeria.* MICs were read after 18-24 h incubation at 37°C. Control wells were also included in each plate. An automatic ELISA microplate reader (SynergyMx, BiotekR USA) at 620 nm was used to measure the absorbance of the plates before and after incubation to determine the subsequent MIC's of the antimicrobial agents from which the Fractional Inhibitory Concentration (FIC) indices were calculated as: FIC index of BEA2 (FIC_B)=MIC of BEA2 in combination/MIC of BEA2 alone, and the FIC index of antibiotic (FIC_A)=MIC of antibiotic in combination/MIC of antibiotic alone, whilst the FIC index (ΣFIC)=FIC_B+FIC_A. The interactions were interpreted as: synergism when the ΣFIC index ≤ 0.5; additivity when ΣFIC index value is >0.5 and ≤1; Indifference when the ΣFIC index is >1 and ≤4 while antagonism was defined as when the ΣFIC index >4 (Satish *et al*., 2005; Stefanovic *et al*., 2012).

Statistical Analysis

The Microsoft office excel 2007 version for windows program was used to determine the means.

Results

Column chromatography and TLC profiles

Eight fractions with volumes ranging from 75 to 200 ml were collected, five of the fractions eluted by the B.E.A solvent combination were named BEA1 to BEA5 are reported here whilst the results of the other three fractions eluted by the benzene solvent only are reported in chapter eight. Their TLC profiles were different from each other and the visible bands after spraying the chromatogram with vanillin spray reagents for each fraction were five for BEA1, four for BEA2, two for BEA3, six for BEA4 and four for BEA5.

MIC determination of the eluted column chromatography fractions.

The different MICs of the column fractions are as shown in Table 1 below. Three out of the 5 B.E.A fractions were found to be active against the different *Listeria* species. The MICs of the active fractions ranged from as low as 0.157 mg/ml to as high as MIC_{90} 5 mg/ml. The 5% DMSO had no antibacterial activities against any of the test *Listeria* isolates, whilst the antibiotics ciprofloxacin, penicillin G and ampicillin had activity against the test *Listeria* isolates.

Table 1: Antibiotics (µg/ml) and B.E.A column chromatography fractions (mg/ml) MIC_{50}, MIC_{90} or MIC against *Listeria* species.

Test antibacterial agent	***L. ivanovii* (LEL 30)**	***L. grayi* (LAL 15)**	***L. monocytogenes* (LAL 8)**	***L. ivanovii* (LEL 18)**
BEA 1	Not active	Not active	Not active	Not active
BEA 2	0.313	0.157	0.313	0.157
BEA3	0.313	0.625	MIC_{90}5	0.625
BEA4	0.313	1.25	MIC_{50}0.625	MIC_{50}0.625
BEA5	Not active	Not active	Not active	Not active
Ciprofloxacin	0.313	0.625	0.313	0.313
Penicillin G	0.156	0.020	0.079	0.020
Ampicillin	0.039	0.079	0.079	0.079

GC-MS Analysis Results

Tables 2 and 3 show the identified compounds together with their retention times and area percentages in the respective fractions. Figures 1 and 2 show the obtained GC-MS chromatograms for the BEA2 and BEA3 fractions respectively. The identified compounds as trimethylsilyl (TMS) derivatives are listed in the tables in descending order of their area percentages from the most abundant to the least abundant compound. The compound 9,19-cyclolanost-24-en-3-ol,(3.beta)- was the most abundant in both fractions. A total of 22 and 31 compounds were identified in the BEA2 and BEA3 fractions respectively in-terms of their different retention times. However, some compounds such as 9,19-cyclolanost-24-en-3-ol, (3.beta.)-, cyclotrisiloxane, hexamethyl-,1-benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester were identified more than once though at different

retention times bringing down the total to 18 identified compounds in fraction BEA2. The compounds 9-octadecenamide, (Z)- and 1,2-bis(trimethylsilyl)benzene were identified more than once at different retention times in the BEA3 fraction bringing the total of identified compounds down to 27.

Table 2: GC-MS results for TMS derivatives of identified compounds in the BEA2 column chromatography fraction of the n-hexane extract of *Garcinia kola* seeds.

Retention Time	**Compound Number**	**Compound Identity**	**Area %**
23.697	1	9,19-Cyclolanost-24-en-3-ol,(3.beta)-	37.707
24.276	2	9,19-Cyclolanostan-3-ol,24-methylene-,(3.beta.)-	19.464
21.354	3	2-Piperidinecarboxylic acid, 1-(trimethylsilyl)- ,trimethylsilyl ester	9.148
22.314	4	Acetamide,N-[4-(trimethylsilyl)phenyl]-	4.355
23.098	5	Lanosterol	3.836
23.405	6	12-Oleanen-3-yl acetate, (3.alpha.)-	3.327
22.910	1	9,19-Cyclolanost-24-en-3-ol,(3.beta)-	3.158
22.858	7	Ethanone, 2-(2-benzothiazolylthio)-1-(3,5-dimethylpyrazolyl)-	2.284
21.537	8	Tetrasiloxane, decamethyl-	2.120
21.783	9	1,2-Bis(trimethylsilyl)benzene	2.000
22.124	10	4-Methyl-2-trimethylsilyloxy-acetophenone	1.917
21.924	11	N-Methyl-1-adamantaneacetamide	1.599
20.344	12	Acetic acid, 4,4,6a,8a,11,12,14b-heptamethyl-13-oxo-1,2,3,4,4a,5,6,6a,7,8,8a,9,10,11,12,12a,13,14,14a,14b-eicosahydropicen-3-yl este	1.332
20.638	13	Cyclotrisiloxane, hexamethyl-	1.177
21.879	14	1H-Indole, 5-methyl-2-phenyl-	1.144
25.575	15	Silicic acid, diethyl bis(trimethylsilyl) ester	1.106
23.498	16	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	1.088
20.477	16	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	0.993
23.018	16	1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	0.957
21.093	17	Silane, 1,4-phenylenebis[trimethyl-	0.667
25.493	13	Cyclotrisiloxane, hexamethyl-	0.436
20.255	18	Methyltris(trimethylsiloxy)silane	0.184
		TOTAL	**99.9998**

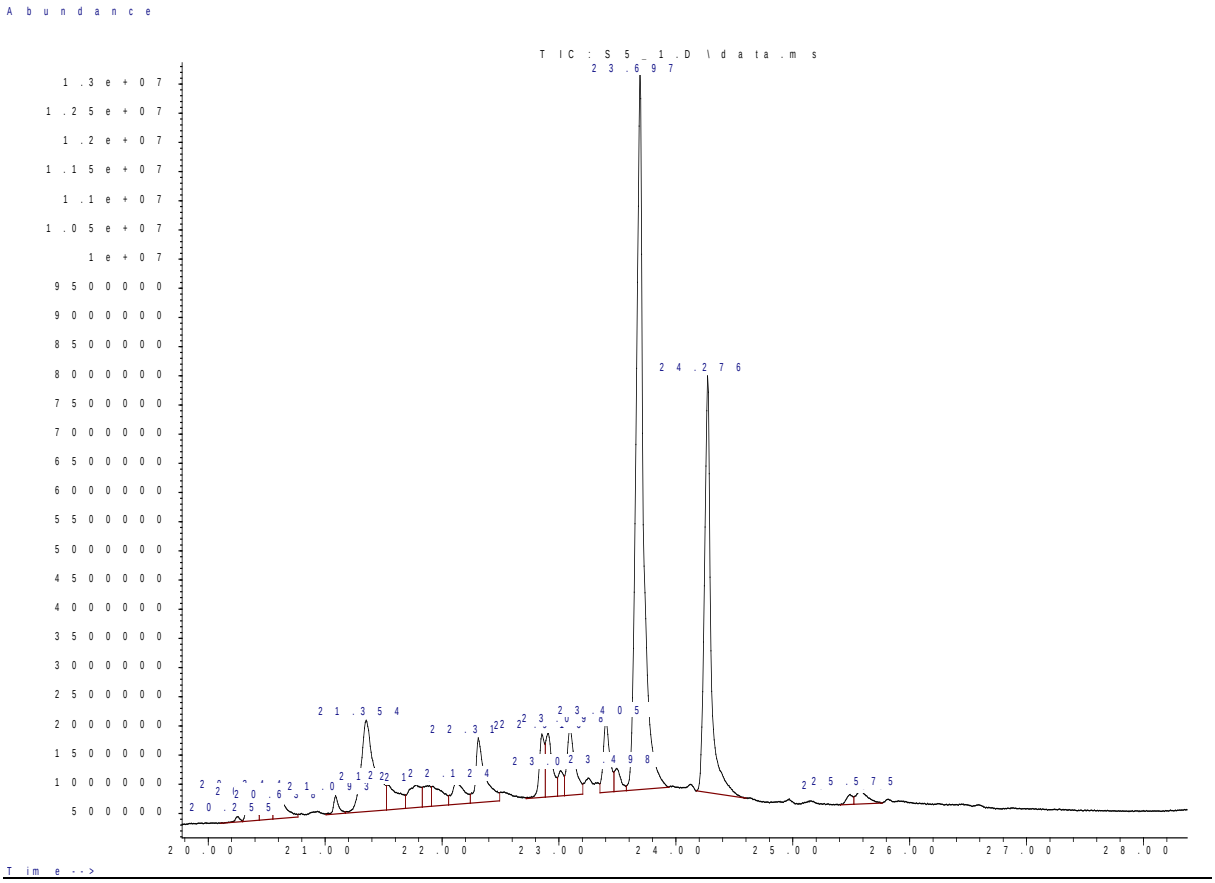

Figure 1. GC-MS chromatogram showing the compounds in the BEA2 column chromatography obtained fraction of the n-hexane extract of *Garcinia kola* seeds.

Table 3: GC-MS results of the TMS derivatised compounds in the column chromatography fraction BEA3.

Retention time	**Compound Number**	**Compound Identity**	**Area %**
23.675	1	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	14.376
13.519	2	9-Octadecenoic acid,ethyl ester	13.615
11.644	3	Hexadecanoic acid,ethyl ester	8.403
24.264	4	9,19-Cyclolanostan-3-ol,24-methylene-, (3.beta)-	7.861
13.457	5	Linoleic acid ethyl ester	5.857
15.379	6	9-Octadecenamide,(Z)-	5.557
13.764	7	Octadecanoic acid,ethyl ester	5.262
20.443	8	1,19-Eicosadiene	4.255
19.508	9	9-Tricosene, (Z)	3.546
19.067	10	13-Docosen-1-ol, (Z)	3.362
24.035	11	Ethane,1-(4,4,4-trifluoro-1,3-dithiobutyl)-2-(3,3,3-trifluoro-1,2-dithiopropyl)-	3.305
26.654	12	Cyclotrisiloxane, hexamethyl-	3.017
23.069	13	N,N-Dimethyl-4-nitroso-3-(trimethylsily)aniline	2.417
23.095	14	Benzo[h]quinoline,2,4-dimethyl-	2.362
20.875	15	1,2-Bis(trimethylsilyl)benzene	2.243
22.329	16	Trimethyl(4-tert.-butylphenoxy)silane	1.661
15.333	17	Isopropyl linoleate	1.443
21.885	15	1,2-Bis(trimethylsilyl)benzene	1.311
21.798	18	Trimethyl[4-(1-methyl-1-methoxyethyl)phenoxy]silane	1.266
22.916	16	Trimethyl(4-tert.-butylphenoxy)silane	1.179
20.668	19	1-Dimethyl(phenyl)silyloxypentane	1.171
13.666	20	Hexadecanamide	1.071
20.364	21	2-Ethylacridine	0.969
22.870	15	1,2-Bis(trimethylsilyl)benzene	0.958
21.332	22	5-Methyl-2-trimethylsilyloxy-acetophenone	0.850
21.104	23	Methyltris(trimethylsiloxy)silane	0.780
15.596	6	9-Octadecenamide, (Z)-	0.719
12.743	24	Heptadecanoic acid, ethyl ester	0.371
19.465	25	Silicic acid, diethyl bis(trimethylsilyl) ester	0.362
21.480	26	Acetamide, N-[4-(trimethylsilyl)phenyl]-	0.242
18.043	27	Hexadecane, 1-chloro-	0.207
		TOTAL	**100**

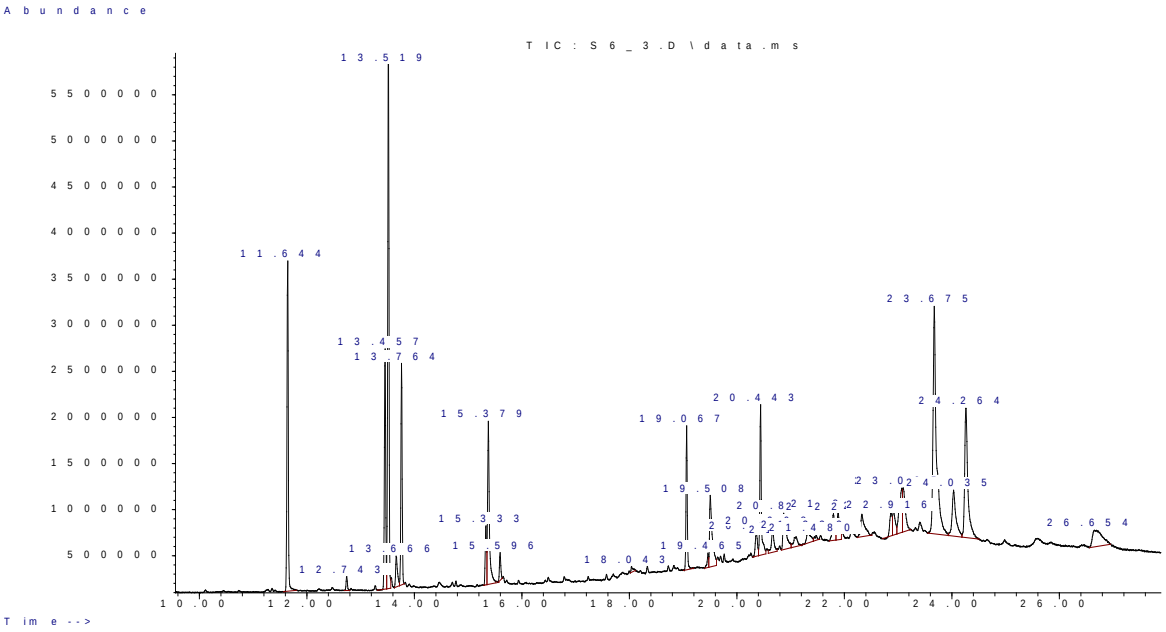

Figure 2. GC-MS chromatogram of the BEA3 fraction of the n-hexane extract of *Garcinia kola* seeds eluted through column chromatography.

Antibiotics-BEA2 fraction interactions

Table 4 shows the results of the interactions of the BEA2 column chromatography fraction and the test antibiotics. The interactions were 100% synergistic with ampicillin and ciprofloxacin but were 50% synergistic and 50% additive with penicillin G.

Table 4: Interactions of BEA2 fraction and antibiotics against some *Listeria* isolates.

Organism	Antimicrobial combination	FIC_A	FIC_B	ΣFIC	Interaction
L. monocytogenes (LAL 8)	Penicillin G	0.248	0.031	0.279	Synergy
	Ciprofloxacin	0.031	0.031	0.062	Synergy
	Ampicillin	0.248	0.031	0.279	Synergy
L. grayi (LAL 15)	Penicillin G	0.5	0.031	0.531	Additive
	Ciprofloxacin	0.016	0.062	0.078	Synergy
	Ampicillin	0.248	0.062	0.311	Synergy
L. ivanovii (LEL 18)	Penicillin G	0.5	0.031	0.531	Additive
	Ciprofloxacin	0.031	0.062	0.093	Synergy
	Ampicillin	0.248	0.062	0.311	Synergy
L. ivanovii (LEL 30)	Penicillin G	0.031	0.008	0.039	Synergy
	Ciprofloxacin	0.016	0.016	0.032	Synergy
	Ampicillin	0.25	0.016	0.266	Synergy

Discussion

The major compounds found in the BEA2 fraction were the plant sterols 9,19-Cyclolanost-24-en-3-ol,(3.beta)- and 9,19-Cyclolanostan-3-ol,24-methylene-, (3.beta)- also known as cycloartenol and 24-methylenecycloartanol respectively which made up 60.3% of the BEA2 fraction, these compounds were also present in the BEA3 fraction although in the fraction carboxylic acids mainly fatty acids TMS ethyl esters were the most abundant in comparison to the cycloartenol and the 24-methylenecycloartanol combined. These results are also in-line with some other studies that found the presence of cycloartenol and 24-methylenecycloartanol in some unsaponifable plant oils such as rape seed oil, olive oil and linseed oil (Schroder and Vetter, 2012). Plant sterols have antibacterial potentials as Singh *et al*. (2012) studies showed

the antibacterial activities of some medicinal plants' sterols, whilst Geyid *et al*. (2005) studies showed the antibacterial activities of medicinal plants' extracts that have sterols and triterpenes in abundance.

Separate studies on *Garcinia kola* seeds have shown the presence of fatty acids in the seeds (Eleyinmi *et al*., 2006; Seanego and Ndip, 2012), and this comes in agreement with the findings in this study as well which showed the presence of derivatised fatty acids as ethyl esters and amides TMS derivatives namely; hexadecanoic acid, ethyl ester (palmitic acid ethyl ester), hexadecanamide (palmitic acid amide), 9-Octadecenamide, (Z)- (oleic acid amide), linoleic acid, ethyl ester and octadecanoic acid, ethyl ester (stearic acid ethyl ester) and these fatty acids may account for the observed anti-*Listerial* activities of the BEA3 fraction. Zheng *et al*. (2005) postulated that the antibacterial activities of long-chain unsaturated fatty acids such as linoleic and oleic acids is due to their inhibition of the bacterial fatty acid biosynthesis process as they are selective inhibitors of FabI which is a bacterial enoyl-acyl carrier protein reductase which is an essential component of bacterial fatty acid synthesis.

Although ethyl ester and amide derivatives of the fatty acids are the major constituents in the BEA3 fraction the presence of cycloartenol and 24-methlylenecycloartanol cannot be ignored as both constitute 22.2% of the fraction. Since fatty acids are known to possess antibacterial activities as well as some plant sterols, it is a possible hypothesis that they could have also worked in synergism.

Acetic acid could have attributed to the observed anti-*Listerial* activities as its ester TMS derivative was identified in the BEA2 fraction. Acetic acid has since been used in medicine for the disinfection of wounds and as an antiseptic agent in the treatment and prophylaxis of the plague, and has been found *in-vitro* to possess broad spectrum bactericidal activities at low

concentrations (Ryssel *et al*., 2009). Its presence in the BEA2 column chromatography fraction may also explain the traditional use of *Garcinia kola* in wound treatment (Uko *et al*., 2001).

Studies by Won *et al*. (2007) showed the presence of an oleic acid-based unsaturated fatty acid in the extract of *Prunus salicina* which proved to be an uncompetitive inhibitor of glucosyltransferase (GTase). GTase is produced mainly by *Streptococcus mutans* and *Streptococcus sobrinus*, which are major causative agents of dental caries. Using GTase inhibitors is considered to be a useful means of preventing glucan formation without disturbing the balance of helpful oral bacteria (Won *et al*., 2007). The findings in this study show the presence of oleic acid amide derivative in the BEA3 fraction, the presence of this fatty acid in *Garcinia kola* seeds can therefore support the traditional chewing of *Garcinia kola* seeds which is believed to be beneficial in aiding the mechanical and microbial cleansing of the mouth (Han *et al*., 2005).

Another compound to take note of in the BEA3 fraction is 9-Tricosene, (Z) which is a hydrocarbon sex pheromone (Hanley *et al*., 2004). This finding is interesting as it can support the traditional belief of the aphrodisiac properties of *Garcinia kola* seeds also known as male kola (Uko *et al*., 2001), and a study by Iwuji and Herbert (2012) indicated that *Garcinia kola* seed meal improves semen characteristics and sexual drive (libido) in matured rabbit bucks.

The antibiotic extracts interactions of the BEA2 fraction showed synergistic interactions with ciprofloxacin and ampicillin against all four *Listeria* isolates. However, a point to note is that all the antibiotics and BEA2 interactions involving the human pathogenic *L. monocytogenes* (LAL 8) were all synergistic. This showed that the compounds in the BEA2 column fraction of *Garcinia kola* seeds have potential for use in synergy together with penicillin G and ampicillin which are the first line antibiotics of choice in human listeriosis treatment. It could have also been interesting to determine the interactions of the BEA3 fraction with conventional

antibiotics but this could not be so as the fraction was obtained in minute quantities that were not sufficient enough to test for interactions. *Garcinia kola* seeds' use as standardised extracts in anti-*Listerial* chemotherapy to lower the MICs of the conventional antibiotics and to also subsequently reduce the dosages of the antibiotics is therefore a viable option that needs to be pursued further with *in-vivo* studies.

Conclusion

The column chromatography fractionated fractions of the n-hexane extract of *Garcinia kola* seeds were found to possess anti-*Listerial* activities *in-vitro*. GC-MS analysis of the fractions revealed the most abundant compounds to be sterols and carboxylic acids ethyl ester TMS derivatives (mostly fatty acids ethyl esters). The BEA2 fraction was found to exhibit synergistic activities *in-vitro* against the human listeriosis causative bacterium *L. monocytogenes*. Further studies to test the most abundant compounds in a highly pure form for their anti-*Listerial* activities are a subject of ongoing studies.

Acknowledgements

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CHAPTER EIGHT

Identification of the Anti-*Listerial* Constituents in Partially Purified Column Chromatography Fractions of *Garcinia kola* Seeds and their Interactions with Standard Antibiotics

(Published in Evidence-Based Complementary and Alternative Medicine)

Abstract

Partially purified fractions of the n-hexane extract of *Garcinia kola* seeds were obtained through column chromatography and their constituents were identified through the use of gas chromatography coupled to mass spectrometry (GC-MS). Three fractions were obtained by elution with benzene as the mobile phase and silica gel as the stationery phase and these were named Benz1, Benz2 and Benz3 in the order of their elution. The anti-*Listerial* activities of these fractions were assessed through MIC determination and only Benz2 and Benz3 were found to be active with MIC's ranging from 0.625 to 2.5 mg/ml. The results of the GC-MS analysis showed Benz2 to have 9 compounds whilst Benz3 had 7 compounds, with the major compounds in both fractions being 9,19-Cyclolanost-24-en-3-ol, (3.beta.) and 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.). The Benz2 fraction was found to have mainly indifferent interactions with ampicillin and penicillin G whilst mainly additive interactions were observed with ciprofloxacin. The Benz3 fraction's interactions were found to be 50% synergistic with penicillin G, 25% synergistic with ciprofloxacin and ampicillin. A commercially available 9,19-Cyclolanost-24-en-3-ol, (3.beta.) was found not to exhibit any anti-*Listerial* activities at a maximum test concentrations of 5 mg/ml, suggesting that the compound could be acting in synergy with the other compounds in the eluted fractions of *Garcinia kola* seeds.

Keywords: *Garcinia kola* seeds; 9,19-Cyclolanost-24-en-3-ol, (3.beta.), *Listeria* species

Introduction

Plants produce a vast diversity of secondary metabolites most of which are phytochemicals that have potential use in the pharmaceutical industry for new drug development purposes. Phytochemicals are naturally occurring bioactive plant compounds that act as a natural defence system for the host plants and also provide colour, aroma and flavour (Caribbean Food and Nutrition Institute, 2005). Some phytochemicals have been shown to possess antimicrobial properties and these include terpenoids, essential oils, alkaloids, lectins, polypeptides, polyacetylenes, and phenolics, of which phenolics can be further divided into phenolic acids, flavonoids, quinones, tannins, coumarins, and simple phenols (Cowan, 1999).

The terpenes are one of the largest and most diverse groups of plant secondary metabolites. They include complex compounds that are formed by the cyclization of 2,3-oxidosqualene (Ncube *et al*., 2008). They include terpenoids and sterols and as well as essential oils which carry the fragrance of the plant. Terpenes possess antimicrobial properties and their mechanism of action is mainly through disruption of the bacterial membrane (Cowan, 1999, Ncube *et al*., 2008).

Flavones, flavonoids and flavonols are phenolic structures with one carbonyl group and are synthesized by plants in response to microbial infection (Dixon *et al*., 1983; Das *et al*., 2010) and often have broad spectrum antibacterial activities *in vitro* (Bennet and Wallsgrove, 1994; Das *et al*., 2010). Flavonoids and flavones modes of action usually involve formation of complexes with cell walls, binding to adhesins and inactivation of bacterial enzymes (Cowan, 1999). Flavonoids also enhance the effects of vitamin C and function as antioxidants and in addition are also known to be biologically active against liver toxins, tumours, viruses, allergies and inflammation (Caribbean Food and Nutrition Institute, 2005).

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency and they are found in almost every plant part (Haslam, 1996; Cowan, 1999). They exert their antimicrobial action in different ways which can include amongst others, binding to proteins, binding to adhesins, bacterial enzyme inhibition, substrate deprivation, formation of complexes with bacterial cell wall, membrane disruption and metal ion complexation (Cowan, 1999). Alkaloids on the other hand intercalate into cell wall and or DNA (Cowan, 1999) causing leakage of bacterial cell contents or disruption of DNA synthesis respectively which eventually lead to bacterial death.

Continual research on bioactive substances from plants can be a possible lead to the discovery and formulation of new potent antibacterial compounds that could help alleviate the problem of antibiotic resistance (Sharma *et al*., 2009). *Garcinia* is a large genus of polygamous trees or shrubs, that can be found in tropical Asia, Africa and Polynesia and is a rich source of bioactive molecules including xanthones, flavonoids, benzophenones, lactones and phenolic acids amongst others (Varalakshmi *et al*., 2010).

Garcinia kola is a traditional medicinal plant that is cultivated and distributed throughout west and central Africa. Its medicinal uses include being an anti-parasitic and antimicrobial agent as well as being a purgative. The seeds are traditionally used to prevent and relieve colic, cure head or chest colds and relieve cough (Iwu *et al*., 1999). Several studies have shown the antibacterial potentials of different extracts of *Garcinia kola* seeds *in-vitro* (Ogbulie *et al*., 2007; Sibanda *et al*., 2010; Penduka *et al*., 2011), whilst other studies have shown some theraupetic effects of the seeds in human clinical trials and in some animal models (Adedeji *et al*., 2006; Adegbehingbe *et al*., 2008). A number of phytochemicals that can account for the antibacterial activities of *Garcinia kola* seeds have been identified in them and these include

tannins, saponins, alkaloids and cardiac glycosides. Biflavonoids such as kolaflavonone and 2 hydroxyflavonoids are the most abundant phytochemicals in the seeds (Adesuyi *et al*., 2012).

Although some authors reported the presence of 9,19-cyclolanost-24-en-3-ol, (3.beta.) which is also known as cycloartenol and 9,19-cyclolanostan-3-ol, 24-methylene-, (3.beta.) also known as 24-methylenecycloartanol in *Garcinia kola* seeds extract (Aplin *et al*., 1967; Madubunyi, 1995). To the best of my knowledge this is among one of the first articles that reports the presence of these sterols amongst other compounds such as lanosterol and β-amyrin in the column chromatography eluted fractions of the n-hexane extract of *Garcinia kola* seeds that exhibited anti-*Listerial* activities *in-vitro*.

Materials and Methods

Listeria Isolates and Plant material

The *Listeria* isolates used in this study were previously isolated from wastewater effluents (Odjadjare *et al*., 2010), and were kept in the bacterial culture collection of the Applied and Environmental Microbiology Research Group (AEMREG) University of Fort Hare Alice, South Africa. The *Garcinia kola* seed powder was also collected from the plant material collection of the above mentioned research group.

Preparation of the Crude N-hexane Extract

The extracts were prepared using the method of Basri and Fan (2005) and Dogruoz *et al*. (2008) as outlined by Penduka and Okoh (2011). Briefly the method involved the steeping of the seed

powder in n-hexane solvent for 48 h with shaking in an orbital shaker (Stuart Scientific Orbital Shaker, UK), followed by centrifugation (Beckman Model TJ-6RS Centrifuge, Great Britain), filtration using a Whatman No.1 filter paper and subsequent evaporation of the solvent using a rotary evaporator (Steroglass S.R.L, Italy) at 50°C. The extract was thereafter dried to a constant weight in a fume cupboard.

Column Chromatography

The method of Selowa *et al*. (2010), with some modifications was used to elute the fractions during column chromatography. A suspension of silica gel and benzene was poured into a glass column (40 cm long × 2.5 cm diameter) up to a height of 30 cm being careful to prevent formation of gaps and bubbles and equilibrated with 100% benzene. An overnight dried mixture of seven grams of the n-hexane extract and 14 grams of the silica gel 60 (Merck, Gremany; particle size 0.063 to 0.2mm/ 70 to 230 mesh) in benzene solvent was loaded onto the column and eluted with 100% benzene first and then with the solvent combination of Benzene: ethanol: ammonium hydroxide (B.E.A) (36:4:0.4 v/v) collected on the basis of solvent polarity and or colour separation. The fractions were dried in a rotary evaporator after which they were dried to a constant weight in a fume cupboard. Thin layer chromatography (TLC) was carried out according to the descriptions of Eloff *et al*. (2005) using the B.E.A solvent combination at a ratio 36:4:0.4 v/v such that fractions with similar profiles could be pooled together.

MIC determination

The starting test concentrations of the fractions were prepared by dissolving them in acetone at 20% of the final volume which was then made up of sterile distilled water. The broth microdilution assay performed in 96 well microtiter plates was used to determine the MICs of the eluted fractions following the method of EUCAST (2003) with some modifications. The EUCAST (2003) colony suspension method was used to standardise the inoculum. A starting concentration of 5 mg/ml was used which was serially diluted two fold in the microtitre plates to make 12 different test concentrations in double strength Mueller-Hinton broth and incubated for 18-24 h at 37°C after addition of the test *Listeria*. An automatic ELISA microplate reader (SynergyMx, BiotekR USA) at 620 nm was used to measure the absorbance of the plates before and after incubation to determine the MIC. Ciprofloxacin, ampicillin and penicillin G were used as the positive controls. Sterility, growth and absorbance control wells were also included.

Gas Chromatography coupled to Mass Spectrometry (GC-MS)

An Agilent 6890N GC with CTC CombiPAL Autosampler and Agilent 5975B MS was used for the GC-MS analyses of the fractions using an Rtx®-5MS (30 m, 0.25 mm ID, 0.5 µm film thickness) Restek 12723-127 column. The test samples were dissolved in 1 ml dichloromethane and then derivatized with pyridine and MSTFA (*N*-Methyl-*N*-trimethylsilyltrifluoroacetamide) followed by a 30 min incubation period at 80°C before injection onto the GC column The instrument settings were ; injector temperature 280°C, injection volume 1 µl, split injection mode, split ratio of 15:1, carrier gas Helium, flow rate 1ml/min, MS transfer 280°C, mode EI+;Electron energy 70 eV, acquistion mode scan, scanning mass range 40 to 550 m/z and a solvent delay of 8 min. The initial oven temperature was at 150°C which was held for 1 min to

a final temperature of 325°C which was then held for 20 min. The compounds were identified by comparison of their retention indices and mass spectra with those in the National Institute of Standards and Technology (NIST) library.

Determination of the anti-*Listerial* activities of 9,19-Cyclolanost-24-en-3-ol, (3.beta.)

The microtiter broth microdilution assay according to EUCAST (2003) was used to determine the anti-*Listerial* activities of the purchased standard 9,19-Cyclolanost-24-en-3-ol, (3.beta.) (ChromaDex). The descriptions of the method are as those explained above for the determination of the MICs of the eluted column chromatography fractions.

Interactions between antibiotics and the eluted column chromatography fractions

The interactions of the column fractions and the selected test antibiotics were interpreted by using the fractional inhibitory concentration (FIC) indices which were determined using the chequerboard method according to the descriptions of Miranda-Novales *et al*. (2006) with some modifications. The tests were perfomed in 96 well microtitre plates and each well contained 100 µl of the test antimicrobial combination. Growth, sterility and absorbance controls were also included in each plate. The inoculum suspension method of EUCAST .(2003) was used to prepare the test *Listeria* inoculums whilst the MICs were read after 18- 24 h incubation at 37°C. An automatic ELISA microplate reader (SynergyMx, BiotekR USA) at 620 nm was used to measure the absorbance of the plates before and after incubation to determine the MIC. The FIC index of a column fraction (FIC_C) = MIC of column fraction in combination/MIC of column fraction alone, and the FIC index of the antibiotic (FIC_A) = MIC of antibiotic in combination/MIC of antibiotic alone and the $\Sigma FIC=FIC_C+FIC_A$. The interactions were

interpreted as: synergism when the ΣFIC index ≤ 0.5, additive when ΣFIC index is between >0.5 and ≤ 1, indifference when the ΣFIC index is >1 and ≤4 whilst antagonism was defined as when an ΣFIC index is >4 (Satish *et al*., 2005; Stefanovic *et al*., 2012).

Statistical Analysis

The Microsoft office excel 2007 version for windows program was used to determine the means and standard deviations.

Results

MIC Determination

The MICs of the different eluted column chromatography fractions are as seen in Table 1 below. Three fractions were eluted by the benzene solvent named Benz1, Benz2 and Benz3, two out of the three fractions eluted by the benzene solvent exhibited anti-*Listerial* activities. Benz2 fraction had MIC values ranging from 0.625- 1.25 mg/ml, Benz3 MIC values were ranging from 0.625-2.5 mg/ml whilst Benz1 fraction did not exhibit any anti-*Listerial* activities. The results of the column fractions eluted by the B.E.A solvent combinations are reported in chapter seven.

Table 1: Minimum inhibitory concentrations (MICs) of the eluted column chromatography fractions in mg/ml and the antibiotics in µg/ml.

Antibacterial agent	***L. monocytogenes* (LAL 8)**	***L. ivanovii* (LEL 30)**	***L. ivanovii* (LEL 18)**	***L. grayi* (LAL 15)**
Benz1	Not active	Not active	Not active	Not active
Benz 2	1.25	0.625	0.625	0.625
Benz 3	2.5	0.625	1.25	1.25
Ciprofloxacin	0.313	0.313	0.313	0.625
Penicillin G	0.079	0.156	0.020	0.020
Ampicillin	0.079	0.039	0.079	0.079

Thin Layer Chromatography

The thin layer chromatography showed one band on each of the three fractions. The band of Benz1 had an R_f value of 0.957±0 and it was also visible at 365nm UV wavelength. Benz2 band had an R_f value of 0.75±0.009 and Benz3 had one band with an R_f value of 0.82±0.004. Bands for both Benz2 and Benz3 were not visible under UV at both 365nm and 302nm.

GC-MS

The GC-MS analysis was carried out for the two active fractions namely Benz2 and Benz3 and the GC-MS chromatograms are as shown in Figure 1 for Benz2 and in Figure 2 for the Benz3 fraction while Table 3 and Table 4 show the compounds found in the Benz2 and Benz3 fractions respectively. Combining all the identical compounds at different retention times Benz2 had a total of 9 compounds as 9,19-cyclolanost-24-en-ol, (3.beta.)- was identified five times at different retention times and 4-methyl-2-trimethylsilyloxy-acetophenone was

identified twice. Benz3 had a total of 7 compounds as 2,4,6-cycloheptatrien-1-one, 3,5-bis-trimethylsilyl- was reported twice at different retention times. The compounds 9,19-cyclolanost-24-en-3-ol, (3.beta.) and 9,19-cyclolanostan-3-ol, 24-methylene-, (3.beta.) - were the most abundant in both fractions. 9,19-cyclolanost-24-en-ol, (3.beta.)- had a total area percentage of 53.6% in Benz2 and of 46.0% in Benz3 after combining area of the compound at different retention times. The compound 9,19-cyclolanost-3-ol, 24-methylene-, (3.beta.)- had a total area percentage of 23.4% in Benz2 and 24.7% in Benz3. The compound 4-methyl-2-trimethylsilyloxy-acetophenone was also common in both fractions.

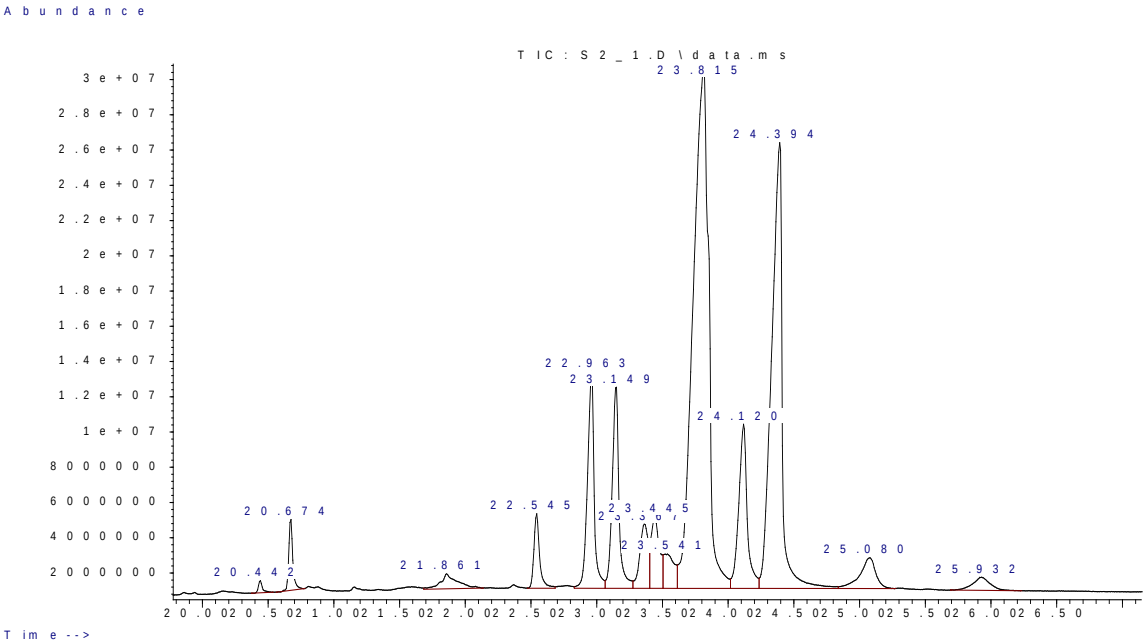

Figure1. GC-MS chromatogram of the compounds in Benz2 column chromatography fraction of the n-hexane extract of *Garcinia kola* seeds.

Table 2: Identification of trimethylsilyl (TMS) derivatised compounds in the Benz2 fraction of *Garcinia kola* seeds.

Retention Time	**Number**	**Compound Identity**	**Area %**
23.815	1	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	39.943
24.394	2	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	23.423
22.963	1	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	7.095
24.120	3	2(1H)-Phenanthrenone, 3,4,4a,4b,5,6,7,8,10,10a-decahydro-1,1,4a,7,7-pentamethyl-,[4aR-(4a.alpha.,4b.beta.,10a.beta.)]-	6.895
23.149	4	Lanosterol	6.758
23.445	5	.beta.-Amyrin	2.949
23.367	1	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	2.653
25.080	6	N-Methyl-1-adamantaneacetamide	2.408
22.545	1	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	2.072
23.541	1	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	1.828
20.674	7	1-(2,4-Dihydroxybenzoyl)-3-ethyl-5-trifluoromethyl-5-hydroxy-2-pyrazoline	1.384
21.861	8	4-Methyl-2-trimethylsilyloxy-acetophenone	1.233
25.932	8	4-Methyl-2-trimethylsilyloxy-acetophenone	1.121
20.442	9	Z-8-Pentadecen-1-ol acetate	0.238
		TOTAL	**99.99**

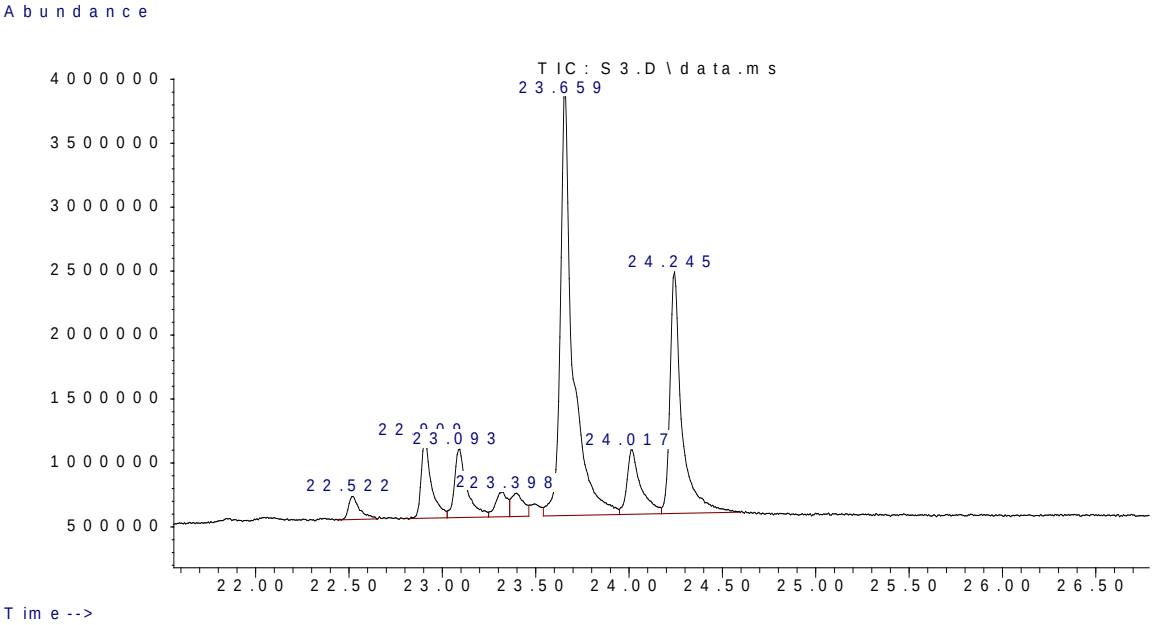

Figure 2. GC-MS chromatogram of the compounds in the Benz3 column chromatography fraction of the n-hexane extract of *Garcinia kola* seeds.

Table 3: Identification of trimethylsilyl (TMS) derivatised compounds in Benz3 fraction in the n-hexane extract of *Garcinia kola* seeds.

Retention Time	**Number**	**Compound Identity**	**Area %**
23.659	1	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	46.041
24.245	2	9,19-Cyclolanostan-3-ol,24-methylene-, (3.beta.)-	24.695
24.017	3	Trimethyl[4-(1,1,3,3,-tetramethylbutyl)phenoxy]silane	7.711
23.093	4	Tetrasiloxane, decamethyl-	7.396
22.909	5	5-Methyl-2-trimethylsilyloxy-acetophenone	6.904
23.398	6	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	2.530
23.324	7	4-Methyl-2-trimethylsilyloxy-acetophenone	2.494
22.522	6	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	2.230
		TOTAL	**100**

Anti-*Listerial* activities of 9,19-cyclolanost-24-en-3-ol, (3.beta.)/ cycloartenol

The commercially obtained compound 9,19-Cyclolanost-24-en-3-ol, (3.beta.) (ChromaDex) which was the most abundant compound in the fractions did not exhibit anti-*Listerial* activities against any of the test *Listeria* isolates at a maximum test concentration of 5 mg/ml.

Interactions of Benz2 fraction with some test antibiotics *in-vitro.*

The interactions of the Benz2 fractions with the test antibiotics are as shown in Table 4 below. The interactions of the fraction and ciprofloxacin were 75% additive and 25% synergistic. The fraction's interaction with penicillin G was 25% additive and 75% indifference, whilst all the interactions of the fraction and ampicillin were all indifferent.

Table 4: Interactions of the Benz2 column chromatography fraction and some antibiotics.

Organism	**Antimicrobial combination**	**FIC index of (antibiotic)**	**FIC index of Benz2**	**ΣFIC index**	**Interaction**
L. grayi (LAL 15)	Benz2/Pen	1	0.063	1.06	Indifference
	Benz2/Cipro	0.125	0.5	0.625	Additive
	Benz/Ampi	1	0.250	1.25	Indifference
L.monocytogenes (LAL 8)	Benz2/Pen	1	0.250	1.25	Indifference
	Benz2/Cipro	0.5	0.5	1	Additive
	Benz2/Ampi	1	0.250	1.25	Indifference
L. ivanovii (LEL 18)	Benz2/Pen	1	0.063	1.06	Indifference
	Benz2/Cipro	0.250	0.5	0.750	Additive
	Benz/Ampi	1	0.250	1.25	Indifference
L. ivanovii (LEL 30)	Benz2/Pen	0.5	0.250	0.750	Additive
	Benz2/Cipro	0.125	0.250	0.370	Synergy
	Benz2/Ampi	1	0.250	1.25	Indifference

Key: Pen denotes penicillin G, Ampi denotes ampicillin and Cipro denotes ciprofloxacin.

Interactions of the Benz3 fraction and the test antibiotics

Table 5 below shows the results obtained for the interactions of the Benz3 fraction and the test antibiotics. Penicillin G had 50% synergistic and 50% additive interactions with the fraction Benz3. Ciprofloxacin had 25% synergistic and 75% additive interactions with the fraction, while the interactions of the fraction with ampicillin were 25% synergistic, 25% additive and 50% indifferent.

Table 5: Interactions of the Benz3 column fraction of *Garcinia kola* seeds and some antibiotics.

Organism	**Antimicrobial combination**	**FIC index of antibiotic**	**FIC index of Benz3**	**ΣFIC Index**	**Interaction**
L. grayi (LAL 15)	Benz3/Pen	0.5	0.031	0.531	Additive
	Benz3/Cipro	0.125	0.5	0.625	Additive
	Benz3/Ampi	1	0.250	1.24	Indifference
L.monocytogenes (LAL 8)	Benz3/Pen	0.5	0.063	0.563	Additive
	Benz3/Cipro	0.25	0.25	0.5	Synergy
	Benz3/Ampi	0.497	0.063	0.560	Additive
L. ivanovii (LEL 18)	Benz3/Pen	0.25	0.016	0.266	Synergy
	Benz3/Cipro	0.125	0.5	0.625	Additive
	Benz3/Ampi	0.248	0.063	0.311	Synergy
L. ivanovii (LEL 30)	Benz3/Pen	0.25	0.25	0.5	Synergy
	Benz3/Cipro	0.125	0.501	0.626	Additive
	Benz3/Ampi	1	0.251	1.25	Indifference

Key: Pen denotes penicillin G, Ampi denotes ampicillin and Cipro denotes ciprofloxacin.

Discussion

Fractions Benz2 and Benz3 though they showed one band on the TLC plate showed multiple compounds during GC-MS analysis such results are similar to those observed by Floriani *et al*. (1998) when they identified three plant phytosterols in a fraction that had given only one band on TLC analysis. This observation in the Benz2 fraction could be attributed to the fact that the sterols identified in it namely 9,19-cyclolanost-24-en-3-ol, (3.beta.)-/cycloartenol, 9,19-cyclolanostan-3-ol,24-methylene-, (3.beta.)-/ 24-methylenecycloartanol and lanosterol as well as the triterpene β-amyrin share a common precursor 2,3-oxidosqualene (Abe *et al*., 1993; Lee *et al*., 2004) and together they make up a major part of the fraction which is 86.7%. In the Benz3 fraction cycloartenol and 24-methylenecycloartanol make up 70.7% of the fraction, which is also a greater percentage of the fraction and could have attributed to the observed single TLC band. In mammals, insects and higher plants, sterols are also converted to steroidal hormones (Ohyama *et al*., 2009) this could support the findings by Adegboye *et al*. (2008) who found the presence of steroids in *Garcinia kola* seeds.

In all fractions cycloartenol was the most abundant compound, although our findings using a commercial standard of the compound showed that it did not exhibit anti-*Listerial* activities alone. This is not surprising as the beneficial medicinal effects of plant materials is typically not attributed to a single compound but to a combination of the compounds in it (Ncube *et al*., 2008). This could then suggest that the observed anti-*Listerial* activities from the fractions could have been a result of the fraction's compounds acting in synergy. Another possible explanation could be that the test concentration of 5 mg/ml could have been too low to effect an anti-*Listerial* activity, such that maybe a higher concentration would have been needed, however, activity of pure plant compounds are routinely classified as being antimicrobial on the basis of susceptibility tests that produce MICs in the range of 100 to 1,000 µg/ml (0.1 mg/ml to 1 mg/ml) against the target organism (Tegos *et al*., 2002).

Separate studies involving non-polar solvents extracts of *Garcinia kola* seeds are also in line with our findings. Aplin *et al*. (1967) studies revealed the presence of cycloartenol and 24-methylenecycloartanol in *Garcinia kola* seeds light petroleum extracts, whilst Madubunyi (1995) also found the presence of cycloartenol and 24-methylenecycloartanol and other compounds namely prenylcouranalactone and kolanone in the petroleum ether extract of the seeds. Studies by Madubunyi (1995) did not attain any antibacterial activity from the cycloartenol and the 24 methylenecycloartanol, although the crude extract had activity against *Staphylococcus aureus* and *Bacillus megaterium* isolates, the kolanone is the only isolated compound that had activity against the *Staphylococcus aureus* and *Bacillus subtilis* but not on *Bacillus megaterium* which the crude extract had activity on, although in our findings there was no kolanone present in the column chromatography fractions.

Some studies involving other different plants showed the presence of either of the two major compounds which we found in the fractions. The studies of Floriani *et al*. (1998) and Rosa *et al*. (2007) on the different parts of the Brazillian plant *Epidendrum mosenii* identified 24-methylenecycloartanol as the main active compound in the dichloromethane extract (a non-polar extract) of the plant and they linked the compound to the antinociceptive/analgesic effects observed using the writhing test in mice. Ragasa *et al*. (2004) showed the presence of cycloartenol in the freeze dried dichloromethane extract of unripe fruit of *Artocarpus heterophyllus* and it had no activity against the test bacteria namely *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis* in that work but had activity against the test fungi namely *Candida albicans, Aspergillus niger* and *Trichophyton mentagrophytes*.

Bioactivity portrayed by non- polar extracts such as n-hexane is often associated with complex mixtures of triterpenoid and/or steroid compounds (Regasini *et al*., 2009). Anti-*Listerial* activities exhibited by the ethanolic extracts of *Eremophila alternifolia* and *Eremophila*

duttonii, in food homogenates and milk were attributed to the presence of terpenes and or sterols in the plants (Owen and Palombo, 2007). This could support the anti-*Listerial* activities of the Benz2 fraction which had the sterols cycloartenol, 24-methylenecycloartanol and lanosterol as well as *β*-amyrin which is a typical pentacyclic triterpene. Cycloartenol is a precursor of all plant sterols whilst lanosterol is the precursor of all mammals and yeasts sterols (Ohyama *et al*., 2009). Some evidence indicates that the biosynthetic pathway of sterols via lanosterol also exists in plant cells (Ohyama *et al*., 2009), this could therefore explain the presence of lanosterol in the Benz2 fraction.

Both fractions had 25% synergistic and 75% additive interactions with ciprofloxacin a floroquinolone antibiotic that inhibits DNA synthesis (Tenover, 2006). Synergistic and additive interactions are a result of a combined effect of active compounds from extracts and antibiotics (Stefanovic *et al*., 2012). The mechanism of action of most lipophilic compounds against bacteria is postulated to be mainly due to their interference effects on the structural and functional properties of the bacterial membrane resulting in the membrane losing its integrity and becoming more permeable (Sikkema *et al*., 1995), such that the observed synergistic and additive interactions could be due to the lipophilic nature of the sterols in fractions causing loss of membrane integrity thereby allowing increased and easier entry of ciprofloxacin into the bacterial cell.

Penicillin G had 50% synergistic and 50% additive interactions with Benz3 fraction whilst it had 25% additive and 75% indifference interactions with Benz2 fraction. However, ampicillin had 100% indifferent interactions with Benz2 whilst with Benz3 it had 25% synergistic, 25% additive and 50% indifferent interactions. It is possible that the presence of the other compounds in Benz2 fraction which are absent in the Benz3 fraction such as lanosterol and β-amyrin caused more indifference interactions to be observed between the Benz2 fraction and the penicillins in comparison to the Benz3 interactions with the penicillins.

The differences observed between how penicillin G and ampicillin interact with the same fraction could be due to the differences in their molecular structures. The penicillins consist of a thiazolidine ring connected to a β-lactam ring attached to a side chain. The side chain determines many of the pharmacologic characteristics of given penicillin. The presence of an amino group on the benzyl side chain of ampicillin distinguishes ampicillin from penicillin G (Glover *et al*., 1996).

Conclusion

The sterols were the major compounds found in both the Benz2 and Benz 3 fractions that exhibited anti-*Listerial* activities *in-vitro*. The observed anti-*Listerial* activities are highly likely to have been a result of the synergistic interactions of the compounds in the fractions. The interactions of the fractions and the chosen antibiotics penicillin G and ampicillin which are the first choice antibiotics of treatment of human listeriosis and ciprofloxacin which is a fluoroquinolone antibiotic showed varying interactions from synergy to indifference. The fractions did not show any antagonistic interactions with the antibiotics which is a good indication of its potential in combination therapy although *in-vivo* tests are also necessary.

Acknowledgements

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CHAPTER NINE

General Discussion and Conclusion

Medicinal plants are of great economic value, as they provide the richest drugs resource of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and as chemical entities for synthetic drugs (Hammer *et al*., 1999; Joshi *et al*., 2011). Preliminary studies of the bioactivities of traditional medicinal plants is the starting point to discovering the different compounds available in plants that can be useful in the medical fraternity in curbing different illnesses.

In that connection *Garcinia kola* (Heckel) is one of the most highly valued plants in African ethnomedicine because of its varied numerous uses which are both social and medicinal (Kagbo and Ejebe, 2010). In this study the seeds of this plant were assessed for their antibacterial activities *in-vitro* against *Listeria* bacteria. The initial starting point in every plant bioactivity assessment is the extraction process of the test substance, of which four different solvents with different polarities were used, namely, n-hexane, dichloromethane, methanol and water (mentioned in the order of increasing polarity). The ground seed powder of *Garcinia kola* was used for the extraction process so as to increase the surface area for extraction thereby also increasing the extraction efficiency (Das *et al*., 2010).

The aqueous (water) extract was found to exhibit the least anti-*Listerial* activity in comparison to the other three organic solvents used. It had activity against 29% of the *Listeria* isolates while all the other solvents extracts were active against 45% of the isolates. This is not an unlikely result as plant extracts from organic solvents have been found to give more consistent and potent antimicrobial activity in comparison to water, though water is a universal solvent

used by traditional healers to extract plant products with antimicrobial activities (Parekh *et al*., 2005; Das *et al*., 2010).

The minimum inhibitory concentration (MIC) is cited by researchers mostly as a measure of the antibacterial performance of a given antibacterial agent (Burt, 2004), of which in this study it is defined according to EUCAST (2003) as the lowest concentration of the test substance that inhibited visible growth of the test organism. In that regard the MIC ranges of the different extracts were 0.079-0.625 mg/ml for n-hexane extract, 10->10 mg/ml for aqueous extract, 0.157-0.625 mg/ml for methanol extract and 0.079-0.313 mg/ml for dichloromethane extract. At this point, the dichloromethane extract had the lowest MIC ranges in comparison to all the other extracts followed by the n-hexane extract and the methanol extract whilst the aqueous extract had the highest MIC ranges against the *Listeria* isolates, suggesting that the anti-*Listerial* activities in *Garcinia kola* seeds were mainly due to non-polar compounds, as the most active extracts were from the non-polar solvents (dichloromethane and n-hexane).

Newly discovered antibacterial agents are tested *in-vitro* not only for the ability to inhibit the bacteria, but also to determine lethality of the agents (Pankey and Sabath, 2004). The antibacterial agents can then be termed bacteriostatic if it inhibits the growth of the bacteria and bactericidal if it kills the bacteria. The rapidity of a bactericidal effect or the duration of a bacteriostatic effect can be determined by time kill/rate of kill analysis (survival curve plot) whereby the number of viable cells remaining in broth after the addition of antibacterial agent is plotted against time (Burt, 2004).

The rate of kill results showed the n-hexane extract to have the most rapid killing activity within a given period in comparison to the dichloromethane extract and the methanol extract as it gave bactericidal activities against all the four representative test *Listeria* isolates from an extract concentrations as low as 2× and 3× MIC values and at exposure periods as low as 15 min

depending on the extract's concentration. The dichloromethane extract was however bactericidal against *L. grayi* (LAL 15) but was bactericidal against the other three isolates from concentrations as low as 3× MIC value and at exposure periods as low as 45 min depending on the extract's concentration. The methanol extract was bactericidal only against *L. ivanovii* (LEL 30) isolate whilst being bacteriostatic against all the other three *Listeria* isolates.

The clinically effective antibiotics penicillin G and ampicillin are found to be only bacteriostatically effective against *L. monocytogenes*, thus emphasizing the importance of the body's own cellular defence mechanisms (Hof, 2003; Allerberger and Wagner, 2010), although this is then complicated by the aspect that listeriosis affects mainly the immune-compromised hosts such that the body's own defence mechanism is not able to help the antibiotic to overcome the invading bacteria (Hof, 2003). In this background the study then targeted the most bactericidal extract for the possible identification of the anti-*Listerial* constituents in *Garcinia kola* seeds with a bactericidal effect on *Listeria* species, of which the n-hexane extract exhibited bactericidal activities at lower extract concentrations against all the *Listeria* species in the study, in comparison to all the other extracts.

The preliminary separation of the n-hexane extract showed the presence of five bands on the thin layer chromatography (TLC) chromatogram after spraying with vanillin spray reagents and a presence of active bands in the TLC-direct bioautography analysis showing the good separation of the chosen solvent combination of Benzene: ethanol: ammonium hydroxide (B.E.A) at a ratio of 36:4:0.4 v/v and also the multiplicity of the anti-*Listerial* compounds in the extract as shown by the different R_f values obtained amongst the four representative *Listeria* isolates. TLC- direct bioautography is a good guiding method for the further fractionation of plant extracts as it is an *in-situ* method that detects plant compounds with relevant biological activities by combining the separation of the plant extract's constituents and the localisation of

the active constituents on the TLC plate (Sharififar *et al*., 2009; Martson, 2011), such that the same solvent combination used for TLC-direct bioautography was then adopted as the mobile phase for the next stage which was column chromatography.

The column chromatography process resulted in eight different fractions being eluted from the n-hexane extract, of which only five of the fractions exhibited activity against the four representative *Listeria* isolates through MIC determination. Four of the active fractions chosen on the basis of their low MIC values were then further analysed through gas chromatography coupled to mass spectrometry (GC-MS) in-order to identify the constituents of each fraction. These were namely Benz2, Benz3, BEA2 and BEA3 fractions with MIC ranges of 0.625-1.25 mg/ml, 0.625-2.5 mg/ml, 0.157-0.313 mg/ml and 0.313 - MIC_{90} 5 mg/ml respectively. In GC-MS, compounds are separated by GC and then transferred inline to the mass spectrometer (MS) for further separation and detection. This combines two strongly complementary technologies of which GC can separate metabolites that have almost identical mass spectra (such as isomers), while MS provides fragmentation patterns that differentiate between co-eluting but chemically diverse metabolites (Kopka *et al*., 2004).

The GC-MS analysis afforded 99.99 – 100% identification of the peaks representing the different constituents in the fractions which is an important factor as it depicts the cumulative power of GC-MS in identifying novel metabolites despite its high sensitivity and resolution (Kopka *et al*., 2004). A total of 18 and 27 compounds were identified through GC-MS in the BEA2 and BEA3 fractions respectively, whilst Benz2 had 9 and Benz3 had 7 identified compounds. The compound 9,19-cyclolanost-24-en-3-ol, (3.beta.) was found to be the most abundant in all four fractions while the compound 9,19-cyclolanostan-3-ol, 24-methylene-, (3.beta.) was next in abundance in the three fractions (Benz2, Benz3 and BEA2) and being the fourth most abundant in the BEA3 fraction. The presence of these compounds in *Garcinia kola* seeds extracts is also supported by the findings of Aplin *et al*. (1967) and Madubunyi (1995).

Interestingly, the fractions with the least number of compounds - Benz2 and Benz3 had 77.0% and 70.7% of their composition being made up of the sterols 9,19-cyclolanost-24-en-3-ol, (3.beta.) and 9,19-cyclolanostan-3-ol,24-methylene-, (3.beta.)- such that it is highly likely that the anti-*Listerial* activities observed could be due to the lipophilic nature of these sterols which can cause membrane disruption by partitioning lipids in the bacterial cell membrane causing increased fluidity or disordering of the membrane structure (Sikkema *et al*., 1995; Cox *et al*., 2001) which can lead to bacterial cell death. These sterols could also have been working in synergy, a reason which could account for the lack of anti-*Listerial* activity of the commercial 9,19-cyclolanost-24-en-3-ol, (3.beta.) compound singly, as sometimes quantitative analysis of the bioactivities of one or several compounds in a medicinal plant does not represent its quality because its efficacy results from multiple components acting at different target sites (Liang *et al*., 2009).

However, the BEA2 fraction had more activity in terms of its lower MIC values in comparison to the Benz2 and Benz3 fractions a result that can be attributed to the possibility of synergy between the sterols and the carboxylic acids contents of which the latter were absent in the Benz2 and Benz3 fractions. The GC-MS analyses showed that the BEA2 fraction constituted of 60.3% of the above mentioned sterols and 14.6% of some carboxylic acids derivatives such as 2-piperidinecarboxylic acid, 1-(trimethylsilyl)-, trimethylsilyl ester, acetic acid, 4,4,6a,8a,11,12,14b-heptamethyl-13-oxo1,2,3,4,4a,5,6,6a,7,8,8a,9,10, 11,12, 12a, 13,14,14a,14b-eicosahydropicen-3-yl este, silicic acid, diethyl bis(trimethylsilyl) ester and 1-benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester of which some carboxylic acids such as acetic acid are known to possess antibacterial activities (Ryssel *et al*., 2009).

The observed anti-*Listerial* activities could however be biased towards a higher amount of the sterols in comparison to the carboxylic acids derivatives, as the BEA3 fraction which had

22.2% of the above mentioned plant sterols but more abundant carboxylic acids mainly fatty acid derivatives which accounted for 42.9% of the fraction had higher MIC ranges in comparison to the other fractions which had higher sterol content ratio (BEA2 fraction) and those which had sterols but without carboxylic acids derivatives (Benz2 and Benz3 fractions). However, the mechanism of action of carboxylic acids and fatty acids is generally not known but some authors have presumed that some carboxylic acids cause physical alteration of the bacterial cell wall (Ryssel *et al*., 2009), while some demonstrated that the antibacterial action of unsaturated fatty acids is through the inhibition of bacterial fatty acid synthesis (Zheng *et al*., 2005), of which fatty acid synthesis in bacteria is essential for the production of a number of lipid-containing components, including the cell membranes (Heath *et al*., 2001; Zheng *et al*., 2005).

In the light of these results, its highly likely that the anti-*Listerial* activities observed in the different fractions could have been due to the complex mixture of the sterols and or the carboxylic acids and fatty acids (depending on the column chromatography fraction), of which complex mixtures of such compounds is usually postulated to be one of the factors behind the antibacterial activities exhibited by most non-polar plant extracts (Regasini *et al*., 2009). Some other minor compounds in the fractions could have been by-products or artifacts of the derivatisation process during GC-MS analysis which involved the use of pyridine and MSTFA (*N*-Methyl-*N*-trimethylsilyltrifluoroacetamide) (Little, 1999; Azizan *et al*., 2012). This therefore could account for compounds such as 4-Methyl-2-trimethylsilyloxy-acetophenone, N-methyl-1-adamantaneacetamide, methyltris(trimethylsiloxy)silane, 1-(2,4-dihydroxybenzoyl) -3-ethyl-5-trifluoromethyl-5-hydroxy-2-pyrazoline as well as 5-methyl-2-trimethylsilyloxy-acetophenone.

The interactions of the three fractions Benz2, Benz3 and BEA2 with the antibiotics penicillin G, ampicillin and ciprofloxacin gave different results ranging from synergy to indifference but

no antagonism was observed. The BEA2 fraction had the most synergistic interactions with the antibiotics in comparison to the other two fractions. The interactions of the BEA2 fractions were 100% synergistic with ciprofloxacin and ampicillin while 50% synergistic and 50% additive with penicillin G. These results were almost similar to those of the crude extract which had 100% synergistic interactions with all the test antibiotics. The synergy and additive results observed with the penicillins could be due to the combined action of the penicillins together with the action of the sterols and or the carboxylic acids on the bacterial cell membrane. While the synergy and additive interactions of ciprofloxacin with the fractions could be attributed to the membrane disrupting properties of both the sterols and or the carboxylic acids which could have assisted the entry of the ciprofloxacin to disrupt DNA synthesis in the bacteria cell. In a separate study by Sibanda and Okoh (2008) the crude acetone extracts of *Garcinia kola* seeds were also found to exhibit some synergistic interactions with the antibiotics penicillin G and ciprofloxacin against some Gram positive bacteria.

New findings of anti-*Listerial* agents showing synergistic interactions are significant as the current combinations involving gentamicin have nephrotoxicity risks (Mitja *et al*., 2009; Amaya-Villar *et al*., 2010), whilst those involving sulfamethoxazole-trimethoprim (SMX-TMP) have potential risks of causing kernicterus to the unborn child and also causing disturbances of folic acid metabolism in pregnant women (Mardis *et al*., 2012). Combined antibiotic therapy has been shown to delay emergence of bacterial resistance (Adwan and Mhanna, 2008) and these synergistic findings could be helpful especially in the light of the observed antibiotic resistance of *Listeria* bacteria to antibiotics (Odjadjare *et al*., 2010; Acciari *et al*. 2011; Adetunji and Isola 2011; Soni *et al*., 2013) as combinations of antimicrobial agents that demonstrate an *in-vitro* synergism are more likely to result in successful therapeutic outcome (Sibanda and Okoh, 2008).

The crude n-hexane extract had better activity in comparison to all the eluted column chromatography fractions in terms of its lower MIC values and also its synergistic interactions with the test antibiotics, with only the BEA2 fraction exhibiting almost similar activities. This could imply that the composition of the fraction is about similar to that of the crude extract, this could be helpful in the formulation of standardised extracts of *Garcinia kola* seeds in anti-*Listerial* therapy. Standardised extracts have an unmatched chemical diversity (Cosa *et al*., 2006; Sasidharan *et al*., 2011), which is helpful in delaying antibiotic resistance because of different modes of actions by the different compounds in the extract targeting different targets sites on the bacteria.

It would however, be economical to choose the crude extract over the eluted column chromatography fractions, as it appears the purification process of the extract leads to loss of activity, as noted from the commercial product of 9,19-cyclolanost-24-en-3-ol, (3.beta.) and the observed lesser activities of the fractionated column chromatography fractions. This loss of activity could be due to the removal of some beneficial compounds or them being left in lesser concentrations that result in poorer or no activity against the *Listeria* bacteria. This could be attributed also to the fact that the bioactivity of plant materials typically results from complex combinations of the metabolites/compounds in the plant in this case the sterols and the carboxylic acids amongst other compounds (Ncube *et al*., 2008). As traditional medicinal plants have been used in folk medicine for centuries there are relatively lower incidences of adverse reactions to plant standardised extracts in comparison to modern conventional pharmaceuticals. This coupled with their reduced cost becomes encouraging for both the consuming public and national health care institutions to consider plant medicines as alternatives to synthetic drugs (Nair *et al*., 2005; Joshi *et al*., 2011).

This study therefore has shown that the crude n-hexane extract of *Garcinia kola* seeds possess compounds with a bactericidal property against *Listeria* bacteria including the human

pathogenic species *L. monocytogenes* as well as having synergistic interactions with the antibiotics of choice against listeriosis namely penicillin G and ampicillin. The GC-MS results further show that its eluted column chromatography fractions with anti-*Listerial* activities consists of complex mixtures of mainly sterols and or carboxylic acids including fatty acids which could be the bioactive compounds. Given a background of such significant findings the pursuing of *Garcinia kola* n-hexane extract in listeriosis treatment becomes a very viable option which promises successful therapeutic outcomes. In this connection, this study therefore concludes with the following recommendations for future attention:.

- Isolation and identification of the anti-*Listerial* compounds in the dichloromethane extract of *Garcinia kola* seeds.
- Assessment for the possible anti-*Listerial* activities of 9,19-cyclolanostan-3-ol,24-methylene-, (3.beta.) singly and also in combination with the major compound (9,19-cyclolanost-24-en-3-ol, (3.beta.) as well as with the antibiotics penicillin G and ampicillin.
- Determination of the *in-vivo* activities of the crude n-hexane extract and the other eluted active column chromatography fractions and as well as their subsequent interactions with the antibiotics penicillin G and ampicillin *in-vivo*.
- Determination of toxicity properties of the n-hexane extract as well as the active eluted column chromatography fractions.

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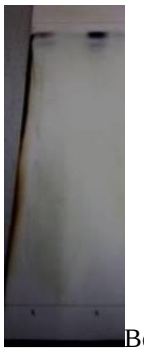
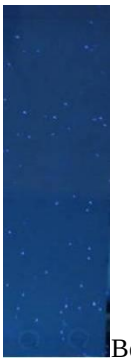
APPENDICES

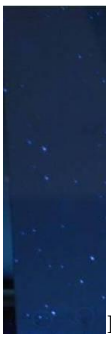
APPENDIX 1


Benz1 at 365nm wavelength


Benz1 (After vanillin spray reagents spraying)


Benz2 at 365nm wavelength

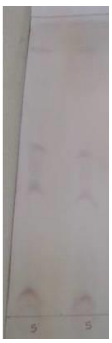

Benz2 (After vanillin spray reagents spraying)


Benz3 at 365nm wavelength


Benz3 (After vanillin spray reagents spraying)


BEA1 at 365nm wavelength


BEA1 (After vanillin spray reagents spraying)

 BEA2 at 365nm wavelength  BEA2 (After vanillin spray reagents spraying)

 BEA3 at 365nm wavelength  BEA3 (After vanillin spray reagents spraying)

 BEA4 at 365nm wavelength  BEA4 (After vanillin spray reagents spraying)

 BEA5 at 365nm wavelength  BEA5 (After vanillin spray reagents spraying)

Figure A1. Images of the thin layer chromatography performed in duplicates for all the eluted column chromatography fractions viewed at 365nm wavelength and after spraying with vanillin spray reagents.

APPENDIX 2

Manuscripts

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APPENDIX 3

Figure A3. Image of the peeled *Garcinia kola* (Heckel) seeds.