

Full Length Research Paper

***In-vitro* anti-bacterial activities of crude n-hexane extracts of *Garcinia kola* (Heckel) seeds against some *Vibrio* bacteria isolated from wastewater effluents**

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

Key words: n-hexane extract, *Garcinia kola*, *Vibrio* species, minimum inhibitory concentrations, rate of kill.

INTRODUCTION

Wound infections, gastroenteritis and primary septicemia are the three well recognized clinical syndromes of *Vibrio* infections (Tantillo et al., 2004; Penduka and Okoh, 2011). These infections are generally acquired either through ingestion of foods and water contaminated with human faecal matter or sewage, raw seafood, or from exposure to skin lesions such as cuts, open wounds and abrasions, to aquatic environments and marine animals (Lee and Younger, 2002). The infections are usually more life-threatening in people with underlying medical conditions or weakened immune systems (Tantillo et al., 2004; Di Pinto et al., 2008) such as people with liver diseases, acquired immune deficiency syndrome (AIDS) and diabetes. In developing countries, the fraction of treated wastewater effluents being discharged into water

bodies such as rivers has increased resulting in high densities of disease causing bacteria such as *Vibrio* species in these water bodies (Igbinosa and Okoh, 2008). Most of these water bodies are used for drinking water, household and recreational purposes such as swimming and fishing by the people living in the surrounding communities and they are therefore at risk of acquiring *Vibrio* infections. Although *Vibrio* species are autochthonous of the aquatic environment, the final effluents discharged into water sources add on to the *Vibrio* population and also become a source of nutrients which favour abundant growth and proliferation of the organism. Several authors have emphasized that there is a broad consensus on the need to monitor the presence of *Vibrios* in the environment and to study their pathogenicity potential in order to properly protect human health (Baffone et al., 2006; Jones and Oliver, 2009; Canigral et al., 2009). Several studies have shown the presence of *Vibrio* species in chlorinated final effluents

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from several wastewater treatment plants (Igbinosa et al., 2009; Dungeni et al., 2010). Antibiotics used in *Vibrio* infections treatments include tetracycline and its derivatives such as doxycycline, fluoroquinolones (for example ciprofloxacin), third-generation cephalosporins (for example, ceftazidime) and aminoglycosides (for example, gentamicin) (Daniels and Shafaie, 2000; Schwartz and Jagar, 2010).

As a consequence of increasing incidences of resistance to these antibiotics, most of them are no longer recommended as first-line therapy and treatment protocols are thus based on local antibiogram data (Daniels and Shafie, 2000). Also, the use of these antibiotics is limited in pregnant women and pediatrics because of their toxicity. Medicinal plants have been used as folklore remedies over the years to treat, manage or control man's ailments as they contain large varieties of chemical substances that possess important therapeutic properties used in the treatment of these ailments (Akinpelu et al., 2008) with the added advantage of being safer to use in terms of their less toxicity deduced from their long term use (Fabricant and Farnsworth, 2001) in comparison with synthetic antibiotics. A typical example of such medicinal plants is *Garcinia kola* (Onasanwo et al., 2011) which is a medium sized forest tree that is well known in its origins of west and central Africa for its vast medicinal properties. *G. kola* seed powder has successfully been used traditionally since time immemorial to treat intestinal pains, diarrhoea, menstrual pains, fevers, jaundice, headaches, diabetes, anaemia, angina, liver disorders and also as an antidote against ingested poison (Adegoke et al., 1981). Several of the specified folklore claims about *G. kola* have been verified (Akinpelu et al., 2008). Studies have been carried out that show the antimicrobial activity of extracts of *G. kola* seeds and other parts of the plant. The seed has shown broad spectrum antibacterial activities against clinical and environmental strains of both gram negative and positive bacteria (Sibanda and Okoh, 2008; Akinpelu et al., 2008; Okigbo and Mmek, 2008). It also has proven adaptogenic properties (Esimone et al., 2007) and analgesic/anti-inflammatory effects in knee osteoarthritis patients (Adegbehingbe et al., 2008). The seeds of the plant have shown appreciable medicinal properties that aroused our interest to test its efficacy against *Vibrio* bacteria. The *Vibrio* species used in this study were shown in a study by Igbinosa et al. (2009) to have survived the treatment processes of a wastewater treatment facility either as free-living organisms or as plankton-associated entities and showed resistance to chlorine disinfection at normal recommended concentrations in water. Hence, these *Vibrio* species posed a potential health risk to the rural communities which depend on the watershed for domestic and recreational purposes. Some of the *Vibrio* isolates were

also shown to be resistant to more than one antibiotic (Okoh and Igbinosa, 2010).

Antibiotics are one of the most important groups of pharmaceuticals such that antibiotic resistance is one of the major challenges for human and veterinary medicine (Kummerer, 2009). In this study we report on the *in-vitro* anti-*Vibrio* activities of n-hexane extracts of the seeds of *G. kola* in an attempt to identify alternative compounds of relevance in anti-*Vibrio* chemotherapy.

MATERIALS AND METHODS

Plant material

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

Preparation of extracts

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

Test *Vibrio* strains

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

Preparation of the inoculum

The inoculums of the test organisms were prepared using the colony suspension method (EUCAST, 2003). 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 susceptibility of the *Vibrio* isolates to the crude n-hexane

extract 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 50°C in a MacCarthy bottle, mixed gently and then poured into a sterile petri dish and allowed to solidify. A sterile 6 mm diameter cork borer was used to bore wells into the agar medium. The wells were then filled up with approximately 100 µl of the extract solution at a concentration of 10 mg/ml taking care to prevent spillage onto the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of the extract into the medium after which the plates were incubated at 37°C for 24 h and thereafter the plates were observed for zones of inhibition which were then measured. Ciprofloxacin (2 µg/ml) was used as a positive control and distilled water was used as the negative control while 5% dimethyl sulphoxide (DMSO) was also tested to determine its effect on each organism.

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

The MICs were determined only for the test *Vibrio* that had shown susceptibility to the crude extracts using the broth microdilution method as outlined by the EUCAST (2003) in sterile disposable flat-bottomed 96-well microtiter plates. Two-fold serial dilutions using sterile distilled water were carried out from 10 mg/ml stock plant extracts to make 9 test concentrations ranging from 0.039 to 10 mg/ml. A 100 µl volume of double strength Mueller-Hinton broth was introduced into all the 96 wells and 50 µl of the varying concentrations of the extracts were added in decreasing order along with 50 µl of the test organism suspension. Column 1 was used as the sterility wells containing 100 µl of the Mueller-Hinton broth and 100 µl sterile distilled water, column 2 was used as the positive control wells containing 100 µl of the broth, 50 µl of Ciprofloxacin and 50 µl of the test organism whilst column 3 was used as the negative control wells containing 100 µl of the broth, 50 µl sterile distilled water and 50 µl of the test organism whilst columns 4 to 12 were used at test wells containing 100 µl of the broth, 50 µl of the extract concentration and 50 µl of the test *Vibrios*. The plates were then incubated at 37°C for 18 to 24 h. Results were read visually by adding 40 µl of 0.2 mg/ml of p-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 minimum bactericidal concentration (MBC) was determined from the MIC broth microdilution assays by subculturing 10 µl volumes from each well that did not exhibit growth after 24 h of incubation and spot inoculating it onto fresh Mueller-Hinton agar plates (Sudjana et al., 2009). The plates were then incubated for 48 h after which the number of colonies were counted. The MBC was defined as the lowest concentration killing more than or equal to 99.9% of the inoculum compared with initial viable counts (Sudjana et al., 2009).

Rate of kill assay

The time kill assay was done according to the method of Odenholt et al. (2001). Three selected test of *Vibrio* isolates namely: *V. vulnificus* (AL042), *V. parahaemolyticus* (AL049) and *V. fluvialis* (AL040) were used for the rate of kill studies on the basis of

grouping on MIC levels namely: 0.313 and 0.625 mg/ml and medical importance of the species. The turbidity of the 18 h old test *Vibrio* was first standardized to 10^8 cfu/ml. Four different concentrations of the plant extract were made starting from the MIC to $4 \times$ MIC for each test organism. A 0.5 ml volume of known cell density from each organism suspension was added to 4.5 ml of different concentrations of the extracts solutions, held at room temperature and the rate of kill determined over a period of 2 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.

RESULTS

Anti-*Vibrio* activities of the crude extracts

The results of the anti-*Vibrio* activities of the n-hexane extract of *G. kola* seeds are shown in Table 1. The crude extract had activity against 16 (32%) of the test bacteria. The zones of inhibition ranged from 10 to 14 mm with the highest zones being observed from *V. vulnificus* (AL042) and *V. fluvialis* (AL040) at 14 mm and the least being from *V. fluvialis* (AL004), *V. fluvialis* (AL019) and *V. vulnificus* (AL048) at 10 mm. The 5% DMSO and sterile distilled water negative controls had no anti-*Vibrio* activity on all tested *Vibrio* species.

MIC and MBC assay

The results of the MIC and MBC assays are presented in Table 2. The extracts showed low MIC values with 10 isolates having an MIC value of 0.313 mg/ml and the remaining 6 isolates having MIC values of 0.625 mg/ml. *V. fluvialis* (AL031) and *V. parahaemolyticus* (AL032) had the lowest MBC value of 5 mg/ml whilst the rest of the isolates had MBC values of 10 mg/ml.

Rate of kill assay

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

Table 1. The Anti- *Vibrio* activities of crude n-hexane extract of *Garcinia kola* seeds.

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

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

increased as the time and the concentration of the extract increased.

DISCUSSION

The n-hexane extract of *G. kola* seeds showed activity against the five *Vibrio* species used in this study, namely: *V. vulnificus*, *V. parahaemolyticus*, *V. fluvialis*, *V. metschnikovii* and *Vibrio* species which are recognized to be pathogenic to humans (Health protection agency, 2007). The minimum inhibitory concentrations of the extract were low and varied between 0.313 and 0.625 mg/ml, while the minimum bactericidal concentrations ranged between 5 to 10 mg/ml. Other plants extracts have been reported to exhibit anti-*Vibrio* activities. For example, Hernandez et al. (2003) assessed the antibacterial effect of hexane extracts of eight plants namely: *Lippia graveolens*, *Lantana achyranthifolia*, *Turnera diffusa*, *Lippia oaxacana*, *Gymnaloena oaxacana*, *Cordia curassavica*, *Lantana camara* and *Acalypha hederacea* and reported MICs ranging between 0.25 to 2

mg/ml against 3 *Vibrio cholerae* isolates. In another study, Srinivasan et al. (2007) assessed the effect of the hexane extract of *Vicoa indica* and reported that, at a test concentration of 12.5 mg/ml, the extract exhibited anti-*Vibrio* activities against *V. parahaemolyticus* and *V. cholerae* with zones of inhibition of 21 and 29 mm respectively. Phytochemical analysis of the extracts of *V. indica* showed the presence of steroids, triterpenes, phenolics groups and aminoacids (Srinivasan et al., 2007). Similarly, *G. kola* seeds have been shown to contain steroids (Adegboye et al., 2008) and a class of terpenoids similar to those found in hops used for brewing (Ogu and Agu, 1995). Bioactivity portrayed by non- polar extracts such as n-hexane is often associated with complex mixtures of triterpenoid and/or steroid compounds (Regasini et al., 2009). The rate of kill studies showed appreciable killing rate by the n-hexane extract. The trend of kill was generally time and concentration dependent with the highest concentration of the extracts (4 × MIC) value achieving the highest percentage of bacteria cells killed after 2 h of exposure time. The highest percentage (96.3%) of bacteria cells killed was

Table 2. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the hexane extract against susceptible *Vibrio* isolates.

Organism	n-hexane extract	
	MIC (mg/ml)	MBC (mg/ml)
<i>V. vulnificus</i> (AL042)	0.625	10
<i>V. fluvialis</i> (AL019)	0.313	10
<i>V. parahaemolyticus</i> (AL049)	0.313	10
<i>V. parahaemolyticus</i> (AL045)	0.313	10
<i>Vibrio</i> . species (AL021)	0.625	10
<i>V. fluvialis</i> (AL022)	0.625	10
<i>V. metschnikovii</i> (AL023)	0.625	10
<i>V. parahaemolyticus</i> (AL030)	0.313	10
<i>Vibrio</i> . species (AL020)	0.313	10
<i>V. fluvialis</i> (AL040)	0.313	10
<i>V. fluvialis</i> (AL031)	0.313	5
<i>V. parahaemolyticus</i> (AL032)	0.313	5
<i>V. parahaemolyticus</i> (AL043)	0.313	10
<i>V. parahaemolyticus</i> (EL009)	0.313	10
<i>V. fluvialis</i> (AL004)	0.625	10
<i>V. vulnificus</i> (AL048)	0.625	10

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

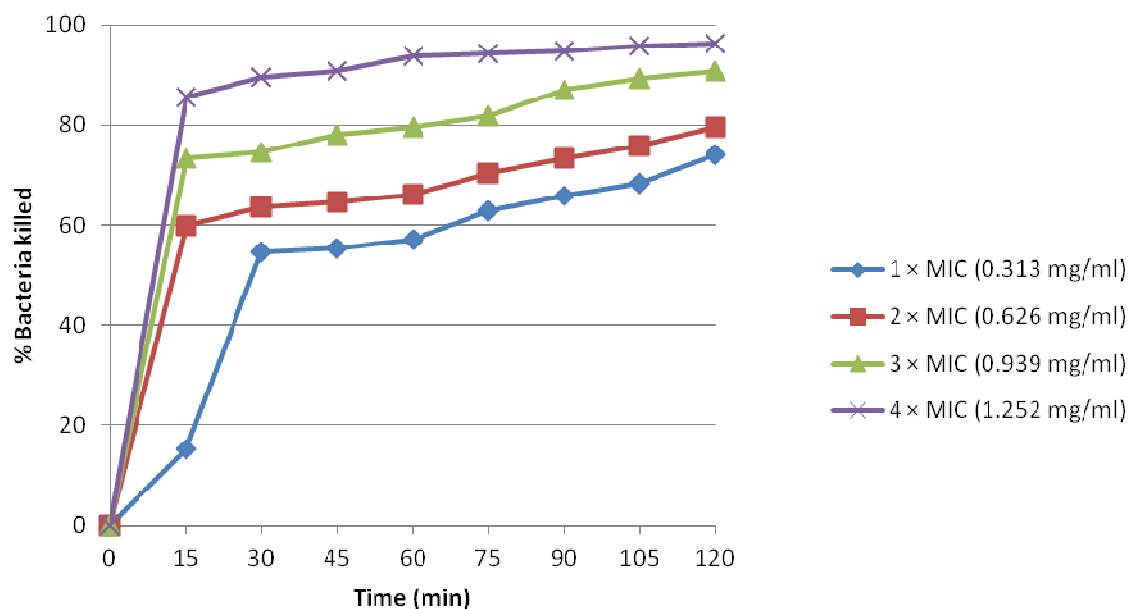


Figure 1. Rate of kill of *V. fluvialis* (AL040) by crude n-hexane extract of *G. kola* seeds.

observed from *V. fluvialis* (AL040). A greater or equal to 99.9% killing activity in 24 h is generally used as a standard of measurement of bactericidal efficacy (CLSI,

2005). The extract did not however achieve the aforementioned 99.9% mark for bacterial efficacy but showed relative cidal properties which are significant.

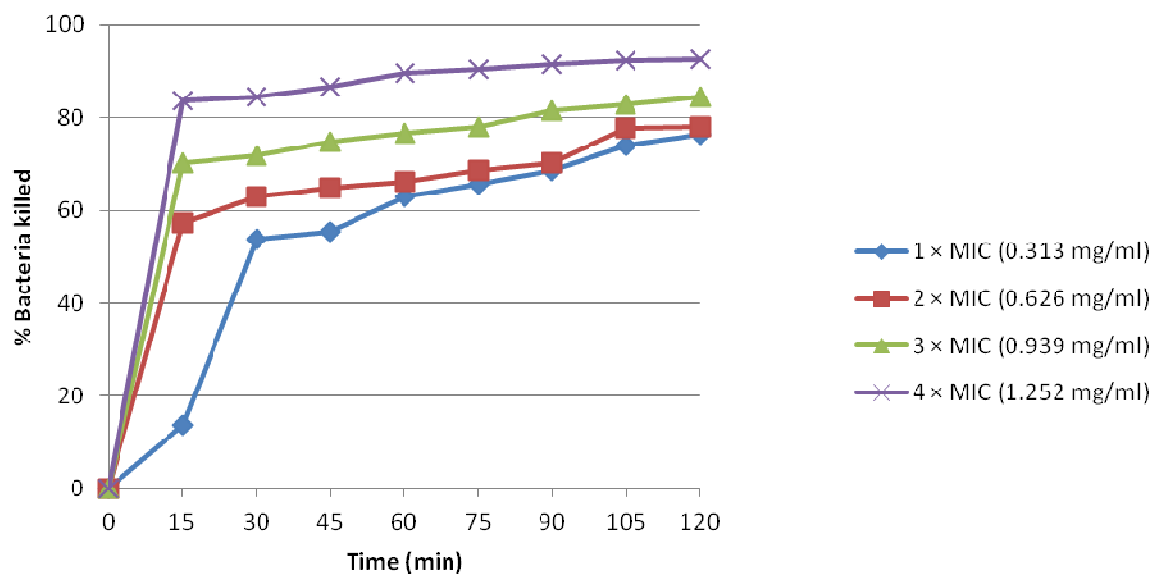


Figure 2. Rate of kill of *V. parahaemolyticus* (AL049) by crude n-hexane extract of *G. kola* seeds.

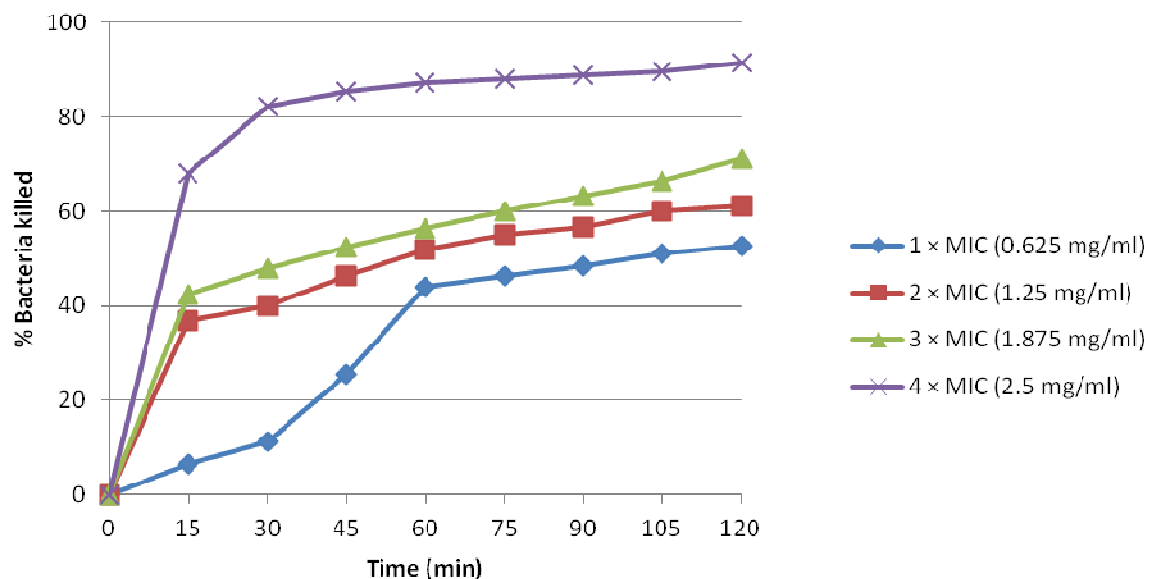


Figure 3. Rate of kill of *V. vulnificus* (AL042) by crude n-hexane extract of *G. kola* seeds.

The anti-*Vibrio* activities portrayed by the extract in this study can also be attributed to a number of other factors that are related to their chemical constituents. Non-polar fractions and extracts of *G. kola* have been reported to demonstrate a significant antimicrobial activity attributed to a benzophenone and kolanone (Onayade et al., 1998). In a study by Eleyinmi et al. (2006), *G. kola* seeds were

found to contain saturated and unsaturated fatty acids namely: myristic, pentadecanoic, palmitic, margaric, stearic, palmitoleic, oleic, vaccenic, linoleic and α -linolenic acids. The dominant fatty acids in the seeds were oleic, linoleic and palmitic acids.

Linoleic, linolenic and oleic acids of different plant species have been shown to possess antimicrobial

activities (Kilic et al., 2005; Won et al., 2007; Skalicka-wozniak et al., 2010; Zheng et al., 2005; Walters et al., 2004). Also, Agoramoorthy et al. (2007) found the fatty acid methyl esters of the blind-your eye mangrove (*Excoecaria agallocha*) plant to possess antibacterial activity against Gram negative bacteria. Findings by Zheng et al. (2005) showed that linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI) of *Escherichia coli*, a Gram negative rod shaped bacteria. FabI is an essential component of bacterial fatty acid synthesis. Additional unsaturated fatty acids including palmitoleic, oleic and linolenic acids also exhibited the inhibition of FabI and all of these fatty acids have been found in different quantities in *G. kola* seeds (Eleyinmi et al., 2006).

Conclusion

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

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