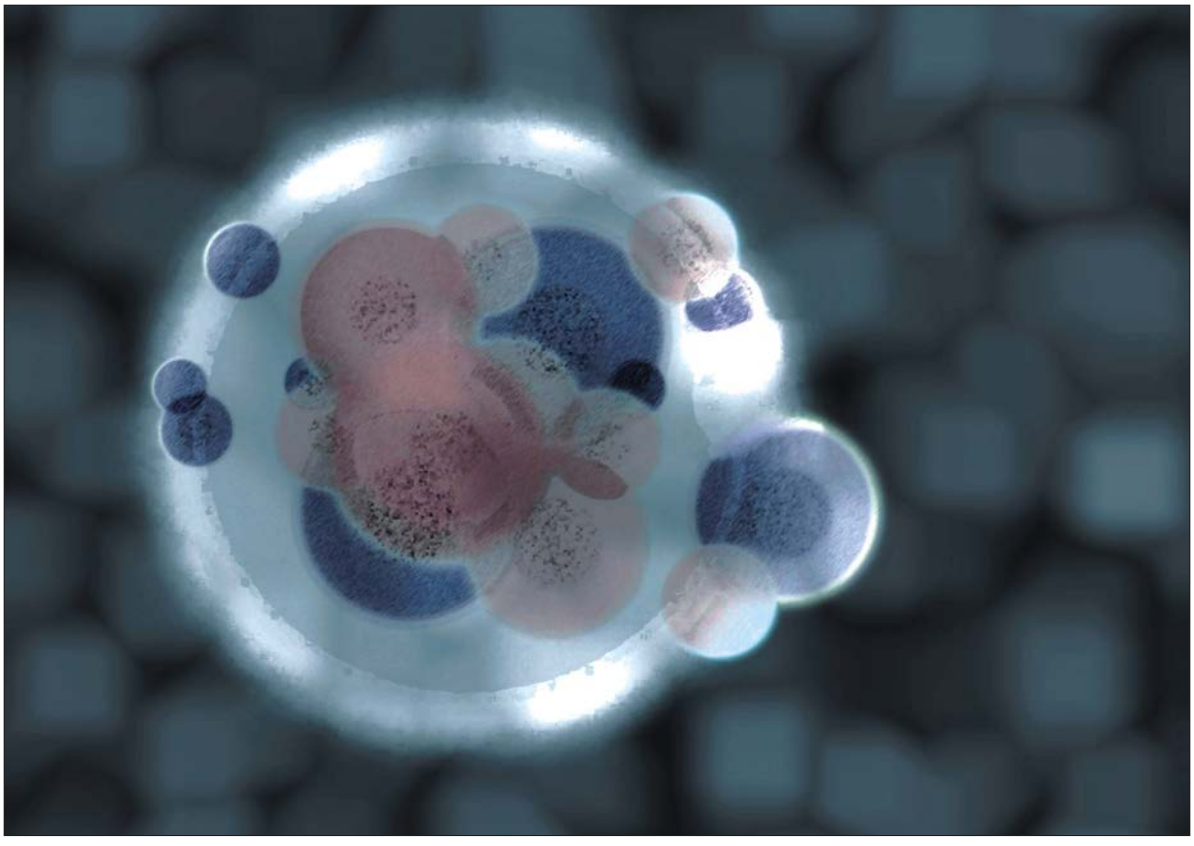

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In vitro Antibacterial Regimes of Crude Aqueous and Acetone Extracts of *Garcinia kola* Seeds

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Abstract: Aqueous and acetone extracts of *Garcinia kola* seeds were screened for activity against 27 bacterial isolates at 30 and 10 mg mL^{-1}, respectively. The aqueous extracts showed activity mainly against Gram positive organisms with MIC values ranging from 5-20 mg mL^{-1}. The acetone extract showed activity against both Gram negative and Gram positive organisms with MIC values ranging from 10-0.156 mg mL^{-1}. The bactericidal activity of the acetone extract was evaluated against *Staphylococcus aureus* ATCC6538 and *Proteus vulgaris* CSIR0030 by time kill assay. The extract showed rapid bactericidal activity achieving a 3.097 log_{10} reduction in counts within 4 h at 0.3125 mg mL^{-1} against *Staph. aureus* ATCC6538 and complete eradication of the organism in 8 h at 2× and 3× MIC levels. A 1.582 log_{10} reduction in counts was observed against *P. vulgaris* CSIR0030 at 5 mg mL^{-1} after 1 h of exposure and a complete eradication was observed in 2 h. We propose that acetone extract of *Garcinia kola* seeds possess strong bactericidal activities and can be chemotherapeutically useful in the treatment of bacterial infections in humans.

Key words: *Garcinia kola*, acetone extract, bactericidal potency, killing rate

INTRODUCTION

The problem of bacterial resistance to commonly used antibiotics has necessitated the search for newer and alternative compounds for the treatment of drug resistant infections and the high cost of conventional drugs particularly in resource poor communities of the developing world has led to the increased use of plants as an alternative for the treatment of infectious diseases. Medicinal plants have for generations been used for the treatment of ailments including infectious diseases. Several findings on the chemotherapeutic potentials of some plants have shown that they can be sources of antimicrobial compounds of value (Rios and Recio, 2005).

Garcinia kola (Heckel), of the family of Guttiferae is a tropical tree of evergreen forests found in moist semi deciduous forest zones and savannah (Agyili *et al*., 2006). It is cultivated and distributed throughout West and Central Africa where it is valued for its medicinal properties. The medicinal properties of the plant have been a subject of numerous investigations. The seeds commonly known as "bitter kola" are used by communities for the treatment of bronchitis and throat infections (Iwu *et al*., 1999) and also to prevent and relieve colic, cure head or chest colds and relieve cough (Farombi, 2000). The stems and twigs of the plant are used as chewing sticks in maintenance of oral hygiene (Ndukwe *et al*., 2005).

Aqueous, ethanolic and petroleum ether extracts of the seeds have been observed to possess antibacterial properties (Ezeifeka *et al*., 2004) and Kolaviron, (a fraction of the defatted ethanol extract of the seed, containing Garcinia biflavonoid GB1, GB2 and kolaflavanone) has been reported to possess numerous therapeutic potentials (Farombi *et al*., 2002; Uko *et al*., 2001) along with such components as mixtures of phenolic compounds, biflavonoids, xanthones, benzophenones and related triterpenes (Han *et al*., 2005).

While numerous work have been done on the antimicrobial potentials of this plant, the overwhelming majority of the studies have concentrated on oral and respiratory tract pathogens (Akoachere *et al*., 2002; Ndukwe *et al*., 2005), thus underestimating the antimicrobial potentials the plant. Also, reports so far available on the antibacterial potentials of the seed show that activity has been demonstrated for the aqueous, ethanolic and petroleum ether extracts of the seed (Ezeifeka *et al*., 2004). There are no documented reports in literature on the antimicrobial potentials of the acetone extract of the seeds, despite the fact that acetone has

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been shown to be an efficient extractant of bioactive components (Eloff, 1998). In addition, while previous researchers have used MICs and MBCs as prediction tools for antimicrobial action of the plant extracts, there are limitations to the use of such data since it does not consider time-related antimicrobial effects (Kiem and Schentag, 2006), such as killing rate. The bactericidal potencies of the extracts of the plant in terms of the kinetics of bacterial death of the extracts of the seeds have not been reported. In this study, we report the antibacterial potentials of the aqueous and acetone extracts of the seeds of *Garcinia kola* against a widened panel of bacterial pathogens especially those not normally related to respiratory tract infections and using the killing rate of the extract as a predicting tool of their bactericidal efficiency.

MATERIALS AND METHODS

Plant material: This study was conducted at the University of Fort Hare's Department of Biochemistry and Microbiology between the period April to July 2007. The plant material was prepared following the description of Farombi (2000). Peeled seeds of *Garcinia kola* were cut into pieces and dried in an oven at 40°C for 48 h. The material was then ground into a powder using a mechanical blender.

Preparation of extracts: The acetone and aqueous extracts of the plant were prepared in accordance to the description of Basri and Fan (2005). One hundred grams of seed powder was extracted in 2 steps with 500 and 300 mL of the respective solvent for 48 h. Aqueous extracts were freeze dried at -50°C under vacuum and acetone extracts were concentrated under reduced pressure using a rotary evaporator at 50°C. The concentrated acetone extracts were then allowed to dry at room temperature to a constant weight. When not immediately used, the extracts were stored in air tight bottles at 4°C.

Bacterial isolates used in the study: Bacterial strains used in this study consisted of reference strains obtained from the South African Bureau of Standards (SABS), namely, *E. coli* ATCC8739, *E. coli* ATCC25922, *Staphylococcus aureus* ATCC6538, *Streptococcus faecalis* ATCC29212, *Bacillus cereus* ATCC10702, *Bacilus pumilus* ATCC14884, *Pseudomonas aeruginosa* ATCC7700, *Enterobacter cloacae* ATCC13047, *Klebsiella pneumoniae* ATCC10031, *Klebsiella pneumoniae* ATCC4352, *Proteus vulgaris* ATCC6830, *Proteus vulgaris* CSIR0030, *Serratia marcescens* ATCC9986, *Acinetobacter calcoaceticus* Aci1, *Acinetobacter calcoaceticus* Aci2. Also included in this study were environmental bacterial strains of *Klebsiella pneumonia*, *Bacillus subtilis*, *Shigella flexineri*, *Salmonella* sp., *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterococcus faecalis*, *E. coli*, *Staphylococcus aureus*, *Micrococcus luteus* and *Micrococcus kristinae*.

Assay for antibacterial activity: Antibacterial activities of the crude extracts were carried out using the agar dilution method (Afolayan and Meyer, 1997) with modification. Stock solutions of the extract were prepared by reconstituting the dried extract in the extracting solvents. This was used to prepare dilutions of the extract in molten Mueller Hinton agar maintained in a water bath at 50°C to achieve concentrations of 30 mg mL^{-1} for the aqueous extract and 10 mg mL^{-1} for the acetone extract and also achieving a final of acetone concentration of 5% in the acetone extract media. An inoculum of each test organism was prepared as described by Nostro *et al.* (2000) and used to seed the agar plates by streaking in duplicates. The inoculated plates were incubated under aerobic conditions at 37°C for 24 h. Positive controls for the aqueous extract consisted of extract free plates of Mueller Hinton agar inoculated with the test organism and negative control consisted of uninoculated plates. Control plates for the acetone extract consisted of Mueller Hinton agar plates with 5% acetone (which represented the final acetone concentration in the test plates). The absence of growth on the test plate compared with the positive control was used to indicate the inhibitory activity of the extracts (Afolayan and Meyer, 1997).

Determination of the Minimum Inhibitory Concentration (MIC): The minimum inhibitory concentration of the extracts was determined using the agar dilution method following the standard protocol of the European Committee for Antibacterial Susceptibility Testing (EUCAST, 2000). Acetone extracts were diluted in such a way that the highest concentration of the solvent in agar was 5% (at this concentration, the solvent was found to have no inhibitory effect on the test organisms). Control plates contained extract free Mueller Hinton agar for the aqueous extracts. Control plates for the acetone extract contained Muller Hinton agar with 5% acetone. Plates were inoculated with overnight broth cultures of the test organisms diluted 1:100 with fresh sterile nutrient broth and incubated for 18 h at 37°C. The MIC was defined as the lowest concentration of the extract that was able to inhibit the visible growth of the test organism (EUCAST, 2000).

Determination of the rate of kill of the crude extracts: The rate of kill determination was done by monitoring of bacterial cell death over time as in accordance with the description of Okoli and Iroegbu (2005). The assay was done for the acetone extract on *Staphylococcus aureus*

ATCC6538 and *Proteus vulgaris* CSIR0030 as representative Gram positive and Gram negative organisms, respectively. The inocula were prepared using the colony suspension method following the guidelines described in the EUCAST Discussion Document (2003). The resultant suspension was diluted 1:100 with fresh sterile broth and used to inoculate 50 mL volumes of Mueller Hinton broth incorporated with extract at multiples of the MIC to a final cell density of approximately 5×10^5 cfu mL^{-1}. The flasks were incubated with shaking at 37°C. Samples (100 μL) were withdrawn at intervals and diluted appropriately and known volumes of diluted samples were plated out in triplicate on Mueller Hinton agar. Plates were incubated at 37°C for 24 h after which the numbers of survivors were enumerated. Controls consisted of extract free Mueller Hinton broth inoculated with the test organism.

RESULTS

Of the twenty seven bacterial isolates tested 17 were susceptible to the aqueous extract at a concentration of 30 mg mL^{-1}, while 24 isolates were susceptible to the acetone extract at a concentration of 10 mg mL^{-1} (Table 1). *Enterobacter cloacae* ATCC13047, *Klebsiella pneumoniae* ATCC10031 and an environmental strain of *Klebsiella pneumoniae* were not susceptible to either the aqueous or acetone extract of the seeds. The Minimum Inhibitory Concentrations (MIC) of the aqueous extract ranged between 5 and 20 mg mL^{-1}, while that of the acetone extracts were generally lower and ranged between 0.156 and 10 mg mL^{-1} (Table 2). Differences were also observed in the susceptibilities of the gram positive (MIC values, 0.156-0.625 mg mL^{-1}) and gram negative test organisms (2.5-10 mg mL^{-1}) to the acetone extract.

The killing rate studies revealed that the acetone extract exhibited bactericidal activities against *Staph. aureus* (ATCC6538) and *P. vulgaris* (CSIR0030) resulting in the eradication of approximately 10^5 cfu mL^{-1} in 2 to 8 h. Both the 2× MIC and 3× MIC of the acetone

Table 1: Antimicrobial activity of aqueous and acetone extracts of *Garcinia kola* seeds against bacterial isolates

	Crude extracts	
Test organisms	Aqueous (30 mg mL^{-1})	Acetone (10 mg mL^{-1})
E. coli ATCC8739	-	+
E. coli ATCC25922	-	+
Staphylococcus aureus ATCC6538	+	+
Streptococcus faecalis ATCC29212	+	+
Bacillus cereus ATCC10702	+	+
Bacillus pumilus ATCC14884	+	+
Pseudomonas aeruginosa ATCC7700	+	+
Enterobacter cloacae ATCC13047	-	-
Klebsiella pneumoniae ATCC10031	-	-
Klebsiella pneumoniae ATCC4352	-	+
Proteus vulgaris ATCC6830	+	+
Proteus vulgaris CSIR0030	+	+
Serratia marcesceus ATCC9986	-	+
Acinetobacter calcoaceticus Aci1	+	+
Acinetobacter calcoaceticus Aci2	+	+
Klebsiella pneumoniae	-	-
Bacillus subtilis	+	+
Shigella flexineri	-	+
Salmonella sp.	-	+
Staphylococcus epidermidis	+	+
Pseudomonas aeruginosa	+	+
Proteus vulgaris	+	+
Enterococcus faecalis	+	+
E. coli	-	+
Staphylococcus aureus	+	+
Micrococcus luteus	+	+
Micrococcus kristinae	+	+

+: Susceptible to the extract, -: Not susceptible

Table 2: The Minimum Inhibitory Concentrations (MIC) of the crude extracts of *Garcinia kola*

	MIC (mg mL^{-1})	
Test organisms	Aqueous extract	Acetone extract
Staph. aureus ATCC6538	10	0.1560
Str. faecalis ATCC29212	10	0.3125
B. cereus ATCC10702	10	0.1560
B. pumilus ATCC14884	5	0.1560
Ps. aeruginosa ATCC7700	10	10.0000
Pr. vulgaris ATCC6830	20	5.0000
Pr. vulgaris CSIR0030	20	2.5000
B. subtilis	10	0.1560
Staph. epidermidis	10	0.3125
Pr. vulgaris	20	5.0000
Ent. faecalis	20	0.3125
Staph. aureus	10	0.3125
Micro. luteus	10	0.6250
Micro. kristinae	10	0.6250
E. coli ATCC8739	-	10.0000
E. coli ATCC25922	-	10.0000
Aci. calcoaceticus Aci1	-	5.0000
Aci. calcoaceticus Aci2	-	5.0000

-: Represents not done

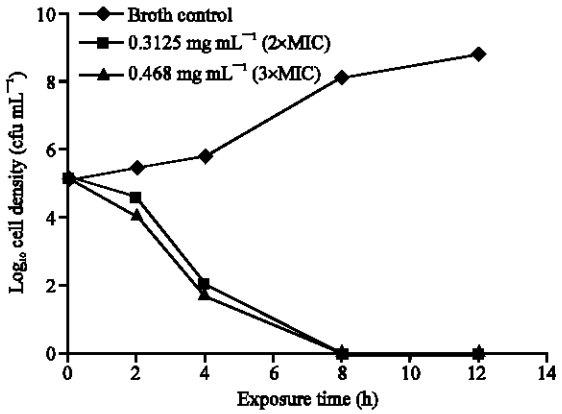

Fig. 1: The killing rate regimes of acetone extract of *Garcinia kola* on *Staphylococcus aureus* ATCC6538

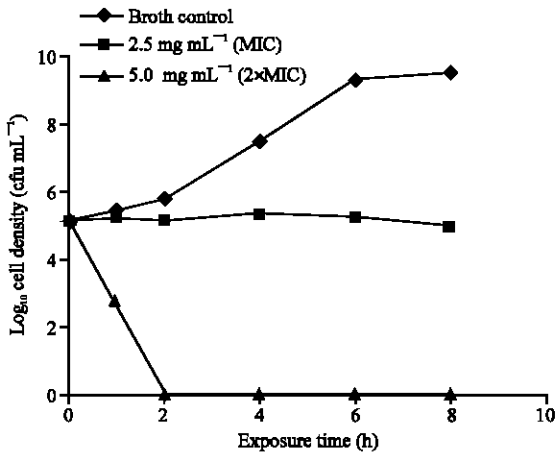

Fig. 2: The killing rate regimes of acetone extract of *Garcinia kola* on *P. vulgaris* CSIR0030

extracts killed 100% of *Staph. aureus* (ATCC6538) in 8 h (Fig. 1) while total eradication of *P. vulgaris* (CSIR0030) was achieved in 2 h by the 2×MIC of the extract (Fig. 2).

DISCUSSION

Garcinia kola is a plant that has shown tremendous potential as a source of novel chemotherapeutic agents. The plant is extensively utilized in traditional medicinal practices in West and Central Africa. It is therefore one of the prime medicinal plants of Africa that can provide relief to the millions of the poor people of the continent if its potentials are adequately explored.

The antibacterial activity of the aqueous extract of the seed was observed against all gram positive organisms tested with limited activity against gram negatives. Results from this study confirm that the aqueous extracts of the seeds of *Garcinia kola* seeds possess antibacterial properties thus validating the traditional use of the seeds in treatment of oral and respiratory tract infections. The limited activity of the aqueous extract against gram negatives is in agreement with the findings of Ezeifeka *et al.* (2004) who reported that crude aqueous extracts of *Garcinia kola* seeds had activity against *Pseudomonas aeruginosa* by disc diffusion method; although it is worth noting that the concentration of the extract used in that study was not reported. It is likely that the aqueous extracts can exhibit inhibitory activity against gram negative organisms at higher concentrations than was used in this study. We considered 30 mg mL^{-1} comparable to the levels often reported in literature for the screening of crude extracts in the absence of a standard. Ndukwe *et al.* (2005) observed that aqueous extracts of *Garcinia kola* stems and twigs had no antimicrobial activity against reference strains of *Escherichia coli* and *Pseudomonas aeruginosa* but had activity against *Staphylococcus aureus* and *Bacillus cereus*, although the same extracts exhibited activity against clinical isolates of gram negative organisms from cases of oral infections. The differences in the susceptibilities of gram positive and gram negative bacteria to plant extracts have been observed by several researchers (Nostro *et al.*, 2000; Suffredini *et al.*, 2006; Parekh and Chanda, 2006). Gram negative bacteria are inherently more resistant to antimicrobials than Gram positive organisms and this has been ascribed to the combined exclusion of the antimicrobial compounds by the double membrane barrier and transmembrane efflux present in this group of organisms (Zgurskaya and Nikaido, 2000).

In contrast with the aqueous extract, the acetone extract showed activity against both Gram positive and Gram negative organisms at 10 mg mL^{-1} (Table 1). Results from this study confirm that acetone is a preferred solvent for the extraction of bioactive compounds in *Garcinia kola*. Acetone has been observed to be a relatively more efficient extractant of bioactive compounds. Eloff (1998), in a comparison of ethanol, acetone methanol, methylenedichloride, methanol/ chloroform/water and water) observed acetone to be the best in terms of the quantity and diversity of compounds extracted and water the least. It is anticipated that the acetone extract of *Garcinia kola* seeds contains a higher quantity and concentration of active compounds than the aqueous extract since water is only able to extract hydrophilic compounds.

The MIC values for the aqueous extracts which ranged from 5 to 20 mg mL^{-1} were higher than the values for the acetone extract which ranged from 0.156 to 10 mg mL^{-1}. Gram positive bacteria showed more susceptibility to the acetone extract than gram negatives. It is likely that the extraction with acetone could have resulted in an increased diversity of compounds that interact with each other in a synergistic way to account for an increased activity of the active principles (Tegos *et al.*, 2002). The interaction might include the increased permeation of the cell membrane of gram negative bacteria which often presents an intrinsic resistance barrier.

Because the acetone extract showed more activity than the aqueous extract, its bactericidal efficacy was investigated against *Staphylococcus aureus* (ATCC6538) and *Proteus vulgaris* (CSIR0030) by assay of bacterial death time. The extract showed good bactericidal activity (Fig. 1) at 0.3125 mg mL^{-1} (2×MIC) and at 0.468 mg mL^{-1} (3×MIC) against *Staphylococcus aureus*. Reductions of 3.097 $\log_{10}$ and 3.370 $\log_{10}$ cfu mL^{-1}, respectively were

achieved at 0.3125 and 0.468 mg mL^{-1} against *Staphylococcus aureus* ATCC 6538 after 4 h of exposure. At 8 h, no survivors could be recovered in both the 2 and 3×MIC reaction cultures. The rate of killing of *Staphylococcus aureus* (ATCC6538) by the extract appears to be both concentration and time dependent. Preliminary investigation revealed that the extract was rapidly bactericidal at 0.625 mg mL^{-1} (4×MIC) achieving a complete elimination of the organism after 30 min of exposure (data not shown), while at 1×MIC seemed to have a bacteristatic effect on the test organism with no major changes on the bacterial load with time (data not shown). The extract exhibited a strong bactericidal efficacy against *Proteus vulgaris* (CSIR0030) at 5 mg mL^{-1} (Fig. 2). A 1.258log_{10} reduction in counts of the test organisms was achieved after 1 h of exposure with a complete eradication after 2 h.

A 3Log_{10} or ≥99.9% reduction in viable bacterial density in an 18-24 h period is the generally accepted definition of bactericidal activity in antibiotics (Pankey and Sabath, 2004). Although the MIC values of the crude extract were higher than often observed for antibiotics (Anadiotis *et al*., 2002; Osburne *et al*., 2006), the bactericidal potencies against the two test organisms showed a similar pattern to that often exhibited by antibiotics.

The bactericidal potentials of the extracts of Garcinia kola from this study represent a significant finding on the therapeutic potentials of this plant. Since the acetone extracts showed such strong bactericidal activity against the test organisms used in this study, it is expected that if the compounds responsible for this activity could be isolated and crystalised, therapeutically useful drugs could be obtained.

CONCLUSION

This study has shown that the aqueous and acetone extracts of the seeds of Garcinia kola possess antibacterial activity with acetone extract exhibiting activity against both gram negative and gram positive organisms. This confirms that acetone can be a good solvent for the extraction of biologically active components of the plant and also suggests that the acetone fraction could compare favorably well with standard antibiotics with regards to time of action and this is the subject of an accompanying paper.

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