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In Vitro Antibacterial Activity of Selected Medicinal Plants from Zimbabwe

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ABSTRACT

This study sought to give a scientific basis to plants already used for traditional purposes and also probe new antibacterial constituents from randomly selected plants whose anti-infective properties have not been evaluated. The antibacterial activity of ethanolic extracts from 19 Zimbabwean plants was assessed using the agar diffusion assay, minimum inhibitory concentration and minimum bactericidal using ampicillin as reference. Accumulation of rhodamine 6G in bacteria was used to determine the activity of extracts as drug efflux pump inhibitors (EPIs). Test bacteria were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus* and *Bacillus subtilis*. At least 8 extracts exhibited antibacterial activity against all bacteria. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination of plant extracts ranged from 0.05 to 0.5 mg/ml and 0.06 mg/ml to > 1 mg/ml, respectively. *Mangifera indica*, *Callistemon citrinus* and *Vernonia adoensis* were bactericidal for all bacteria while the other extracts were bacteriostatic, and were thus the most potent. These 3 extracts were effective EPIs in the uptake of R6G with activity greater than the standard inhibitor reserpine. Hence, the compounds in these plants can serve as templates for the development of new antibacterial agents as well as efflux pump inhibitors.

Keywords: bacteria, *Callistemon citrinus*, *Mangifera indica*, plant extracts, traditional medicine, *Vernonia adoensis*
Abbreviations: ATCC, American type culture cell; **CFU**, colony forming units; **DMSO**, dimethyl sulphoxide; **EPI**, efflux pump inhibitor; **MBC**, minimum bactericidal concentration; **MIC**, minimum inhibition concentration; **MRSA**, methicillin-resistant *S. aureus*; **PBS**, phosphate buffered saline; **R6G**, rhodamine 6G; **VRE**, vancomycin-resistant enterococci; **VRSA**, vancomycin-resistant *S. aureus*

INTRODUCTION

Bacteria cause serious infections in humans as well as other animals. *Staphylococcus aureus*, for example, causes superficial skin lesion, localized abscesses and food poisoning (Lotifpour *et al*. 2008). *Pseudomonas aeruginosa* is one of the most commonly isolated nosocomial pathogens accounting for a significant percentage of hospital-acquired infections (Abu-Shanab *et al*. 2004). The spread of multidrug-resistant *P. aeruginosa* is resulting in an increasing trend of nosocomial infections in hospitals and health care centres because there are no effective antimicrobial agents against it (Abu-Shanab *et al*. 2004). Intestinal disorders caused by bacteria such as *Vibrio cholera*, especially diarrhoea, are a major cause of morbidity and mortality in developing countries. The rate of occurrence of intestinal disorders is usually high in infants and children (Aboaba *et al*. 2006). In Zimbabwe, more than 4,300 people died in a cholera epidemic that hit at least 98,000 people in just one year (WHO 2009). Thus, there is a need to find an effective means of controlling these harmful pathogens.

The indiscriminate use of antibiotics has led to the development of multidrug-resistant pathogens. Around 90-95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin (Casal *et al*. 2005) and in most Asian countries, 70-80% of the same strains have become methicillin resistant (Chambers 2001). There are reports on the development of resistance to the last line of antibiotic defence, which has led to a search for reliable methods to control vancomycin-resistant enterococci (VRE), *S. aureus* (VRSA), and methicillin-resistant *S. aureus* (MRSA) (Shanmugam *et al*. 2008). The rapid spread of bacteria expressing multidrug resistance (MDR) has necessitated the discovery of new antibacterials and resistance-modifying agents. Since the initial discovery of bacterial efflux pumps in the 1980s, many have been characterized in community- and hospital-acquired Gram-positive and Gram-negativepathogens, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and mycobacteria (Stavri *et al*. 2007). Efflux pumps are able to extrude structurally diverse compounds, including antibiotics used in a clinical setting, rendering the drug therapeutically ineffective (Amusan *et al*. 2007). Antibiotic resistance can develop rapidly through changes in the expression of efflux pumps. It is, therefore, imperative that new antibiotics, resistance-modifying agents and, more specifically, efflux pump inhibitors (EPIs) are characterized (Stermitz *et al*. 2000). The use of bacterial resistance modifiers such as EPIs could facilitate the re-introduction of therapeutically ineffective antibiotics back into clinical use and might even suppress the emergence of MDR strains (Stavri *et al*. 2007).

Natural product medicines have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms and terrestrial vertebrates and invertebrates (Raja *et al.* 2010). The use of indigenous knowledge of traditional medical practitioners as leads provides a useful route employed in the search for novel drugs (Amusan *et al*. 2007). This is rewarding, and since most indigenous plants used in traditional medicine have not been explored in detail, the potential for discovery of more novel therapeutic compounds through bioprospecting of the flora is tremendous (Amusan *et al*. 2007). The antimicrobial potential of different medicinal plants are being extensively studied all over the world, but only a few studies have been carried out in a systematic manner (Arora *et al*. 2009). Numerous investigations have proved that medicinal plants contain diverse classes of bioactive compounds such as tannins, alkaloids and flavonoids, which ex-

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Table 1 The scientific and vernacular names of some of the plant extracts used in this study as well as the traditional uses of some of the plants.

Family	Botanical name	Local name	Voucher	Plant part tested	Major traditional use (Reference)
Asteraceae	*Vernonia adoensis* Sch.Bip. ex Walp.	*Musikavakadzi*	C1 E7	Leaves	Boiled and decoction drunk to cure TB (Kisangau *et al*. 2007)
Anacardiaceae	*Mangifera indica* L.	*Mumango*	N 17 E4	Stems and twigs	Used for coughs and diarrhoea
Chrysobalanaceae	*Parinari curatellifolia* Planch. ex Benth.	*Muhacha*	C6 E1, E7	Stems and twigs, leaves	Herpes
Leguminosae	*Xeroderris stuhlmannii* (Taub.) Mendoça & E.P. Sousa	*Murumanyama*	C4 E4	Stems and twigs	Stomach ailments (Iwawela *et al*. 2007), mastitis, backache (Ruffo 1991)
Proteacea	*Faurea saligna*	*Mutsatsati*	C13 E7	Leaves	
Myrtaceae	*Callistemon citrinus* Skeels		UZ2 E7	Leaves	Antibacterial, Haemorrhoid treatment (Oyedeji *et al*. 2009)
Moraceae	*Ficus sycomorus* L.		SCC1 E7	Leaves	
Rubiaceae	*Fadogia stenophylla* Welw. ex Hiern		SCC2 E7	Leaves	
Vitaceae	*Cyphostemma viscosum* (Gilg & R.E.Fr.) Desc. ex Wild & R.B.Drumm.	*Fodya yemusango*	SCC4 E7	Leaves	
Rubiaceae	*Fadogia ancylantha* Schweinf.	*Masamba emusango*	SCC7 E7		
Ranunculaceae	*Clematopsis scabiosifolia* Hutch.		SCC8 E7	Leaves	
	Salons delagoense	*Nhundurwa*		Leaves, fruits	Used to treat scabies in children (Chigora *et al*. 2007)
Myrtaceae	*Syzigium cumini* Linn. Skeels	*Muboo*	C15 E7, E 12	Leaves, fruits	Anti-inflammatory herb, treatment of dysentery (Kumar *et al*. 2008)
Rhamnaceae	*Zyziphus mucronata* Willd.	*Muchecheni*	C7 E7	Stems	Snake bites and stomach ache (Ruffo 1991)
Celastraceae	*Gymnosporia senegalensis* Loes	*Chizhuzhu musosawafa*	N5 E7	Leaves	
Verbenaceae	*Lantana camara* var. *aculeata* (L.) Moldenke	*Mbarambati*	UZ1 E7	Leaves	Ring worm infections (Chigora *et al*. 2007)
Fabaceae	*Brachystegia boehmii* Taub	*Muphuti*	N7 E7	Leaves	
Rubiaceae	*Catunaregum spinosa* Thunb	*Mutsvairachuru*	C 17	Leaves	

hibit various pharmacological properties (Emam 2010). However, in the absence of any scientific proof of their effectiveness, the validity of these remedies remains questionable and their use by local communities remains restricted (Kaur and Arora 2009). Phytochemical and pharmacological investigations of several plants have already led to the isolation of some natural antimicrobials such as berberine and harmane whose mechanism of action is attributed to their ability to intercalate with DNA (Kumar *et al*. 2007). The known success of these traditional therapies has guided the search for new chemotherapeutic alternatives to fight respiratory and other infections caused by drug-resistant bacteria (Bocanegra-García *et al*. 2009).

In the present study, 19 plants of different families were selected to assess their antibacterial potential. Some of the plants are known for their use as traditional medicines to cure common ailments such as diarrhoea, tuberculosis, stomach pains, skin infections and coughs while most of them were randomly selected with no history of use in traditional medicine (Kisangau *et al*. 2007). To give a scientific basis to the use of these plants, this study was carried out to assess the antibacterial potential of selected Zimbabwean plants against some clinically important bacteria using polar ethanol extracts to mimic traditional extraction protocols.

MATERIALS AND METHODS

Plant material

Plants were collected on the basis of indigenous knowledge as well as random selection from three provincial locations in Zimbabwe: Mashonaland Central (Centenary), Harare (University of Zimbabwe) and Mashonaland West (Norton). The plants were authenticated by a taxonomist, Mr. Christopher Chapano of the National Botanic Gardens, Harare, Zimbabwe. Voucher specimens were deposited in the herbarium, Department of Biochemistry, University of Zimbabwe. **Table 1** shows the list of plants used in this study.

Preparation of extracts

The preparation of plant extracts was described by Mukanganyama *et al*. (2011). Briefly, plant samples were ground in a two-speed blender (Cole Parmer Instrument Co., Vernon Hills, USA). Plant A volume of 8 ml of ethanol was added to 2 g of powder and shaken for 5 min on a vortex and left to sit for 10 min. A syringe was prepared by inserting a piece of fine sieve. The plant suspension was then transferred into a syringe and filtered into a small glass vial. The sterile suspension was filtered again using 0.45 µM Millipore sterile filter (Sigma-Aldrich, Taufkirchen, Germany) into a labelled small glass vial. Ethanol was left to evaporate overnight in fume hood with an air stream. A constant dry weight of each extract was obtained and the residues were stored at 4°C.

Materials

All the chemicals, including ethanol, dimethylsufoxide (DMSO), rhodamine 6G, the standard antibiotic ampicillin and the standard drug efflux pump inhibitor reserpine were purchased from Sigma Chemical Co. (Taufkirchen, Germany) and all the solvents used were of analytical grade.

Microorganisms and growth conditions

Five bacterial strains (*Staphylococcus aureus* (ATCC 9144), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 11229), *Bacillus cereus* (ATCC 11778) and *Bacillus subtilis* (ATCC 6633)) were obtained from the Division of Microbiology, Department of Biological Sciences, University of Botswana. All strains were maintained as stock strains in 50% glycerol in Eppendorf® microtubes and kept at -30°C until use. Bacteria were grown in nutrient broth at 37°C for 24 h and adjusted to a concentration of 1×10^6 colony forming units per ml (CFU/ml).

1. Determination of antibacterial activity by agar diffusion method

The disc diffusion method for antimicrobial susceptibility testing was carried out according to the standard method by Zaidan *et al*. (2005) to assess the presence of antibacterial activities of the plant extracts. Plant extracts were screened for antibacterial activity against five species of bacteria; *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*. Nutrient agar mixed with bacteria at a concentration of 1×10^6 cfu/ml was poured in Petri dishes and allowed to cool. The plant extracts equivalent to 1000 µg, dissolved in ethanol, were applied to sterile paper discs (6 mm diameter, Cartridge Susceptibility Discs, Mast Diagnostics, Mast Group Ltd., Merseyside, UK). The solvent was allowed to evaporate from filters deposited on 96-well plates at room temperature. The discs were then deposited on the surface of the innoculated agar plates. Plates were then incubated for 24 h at 37°C (Jeio Tech Incubated Shaker, SI-300, Gasan-Dong, Geumcheon-Gu, Seoul, Korea). Ampicillin was used as the positive control at concentrations of 500, 50, 5 and 1 µg/ml and de-ionized water was used as the negative control for the antibiotic. Zones of inhibition were measured in mm after 24 h of growth. The experiment was performed in quadruplicate.

2. Determination of minimum inhibitory concentration (MICs) and minimal bactericidal concentrations (MBC)

The microplate method of Eloff (1998) was used to determine the MIC values for plant extracts with antibacterial activity. Residues of plant extracts were dissolved to 25 mg/ml using the extracting solvent ethanol. All extracts are tested at 1000 µg/ml (Al-Fatimi *et al*. 2007) and serially diluted two-fold to 1.95 µg/ml in a 96-multiwell polystyrene flat-bottomed microplate(Sigma-Aldrich, St. Louis, MO, USA) after which 100 µl (1×10^6 CFU/ml) of bacteria are added to each well. The antibiotic ampicillin was added as reference antibiotic in each assay. Extract-free solution was used as the negative control. Pre-incubation absorbance values were read from an ELISA reader (Biokinetic Reader EL 350, Bio-Tek™ Instruments, Winooski, VT, USA). The microplates were then incubated overnight at 37°C and absorbance values were read after 24 h. MIC values are recorded as the lowest concentration of the extract that completely inhibited bacterial growth. The experiment was performed in duplicate. Bacterial cells from the MIC test plate were sub-cultured on solid nutrient agar by making streaks on the surface of the agar. The plates were incubated overnight at 37°C and the MBCs were determined after 24 h. Plates that did not show growth were considered to be the MBC for the extract or drug used. The experiment was carried out in duplicate.

Rhodamine 6G uptake

Initial screening results showed that extracts from *Mangifera indica*, *Callistemon citrinus* and *Vernonia adoensis* were potent antibacterials. These extract activities were tested for effectiveness as MDR inhibitors in Rhodamine 6G accumulation experiments using the method of Maesaki *et al.* (1999) with some modifications. Bacteria were cultured overnight at 37°C at 110 rpm with shaking. After 24 h cells were centrifuged using a Rotofix 32 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) at 4000 rpm for 5 min and washed twice with phosphate buffered saline (PBS) (pH 7.2). Cells were centrifuged again and re-suspended at 40 mg/ml in PBS containing 10 mM sodium azide (NaN_3). R6G was added to a final concentration of 10 µm and cells placed in an incubator for 1 h. Cells were then divided into two aliquots, tube A and tube B. Cells were centrifuged for 5 min at 4000 rpm. Cells in tube A were re-suspended in PBS containing 1 M glucose while the cells in the tube B were re-suspended in PBS alone. Plant extract was then added to the cells containing glucose to a final concentration of 100 µM. A standard inhibitor reserpine was also used at a final concentration of 100 µM. Both tubes were then placed in an incubator with agitation for 30 min at 37°C. Cells were centrifuged and the supernatant was discarded. The remaining pellet was re-suspended in 0.1 M glycine HCl, pH 3 and placed in a shaking incubator overnight. After 24 h, cells were centrifuged for 10 min at 4000 rpm and the supernatant collected for measuring absorbance at 527 nm using a Shimadzu UV/VIS UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

Statistical analysis

A comparison of the antibacterial activity of the samples with standard antibiotics was evaluated by applying a two tailed-unpaired *t*-test. All values are expressed as the mean ± standard deviation and $P < 0.05$ values were considered to indicate statistically significant differences. Numerical data were analysed using the Student's *t*-test using Graphpad™ version 4 for Windows (Graphpad™ Software Inc., San Diego, California, USA).

RESULTS AND DISCUSSION

Thirteen extracts from the 19 plants selected exhibited antibacterial activity against the test microorganisms (**Table 2**). Extracts were not strain specific and showed antibacterial activity for all five bacterial species. Exceptions were observed for the extract of *Asteraceae* that showed no activity at all for *E. coli* and *S. aureus* and hardly any activity for *P. aeruginosa* and *B. cereus* (7 and 8 mm, respectively) but resulted in a zone of inhibition of 25 mm for *B. subtilis*, *Mangifera indica*, *Callistemon citrinus* and *Vernonia adoensis* were shown to be the most potent extracts for all the test microorganisms in all the assays carried out in this study (**Tables 2, 3**). Results from the R6G uptake experiment showed that *Mangifera indica*, *Callistemon citrinus* and *Vernonia adoensis* extracts were a potential source of compounds that had efflux pump inhibitor activity (**Fig. 1**).

Antibacterial activity of plant extracts

The ethanolic extracts of all the potent plants resulted in variable zone of inhibition (7-25 mm) for all bacteria tested (**Table 2**). *Callistemon citrinus* and *Vernonia adoensis* extracts showed potent antibacterial activity compared with the extracts of *Parinari curatellifolia* and *Mangifera indica*, with the zone of inhibition diameter ranging from 12-25 mm and 15-25 mm, respectively. In the disc diffusion assay, the extract from *Mangifera indica* was not very potent in terms of inhibiting bacterial growth but showed good antibacterial activity with the zone of inhibition diameters ranging from 9-15 mm. The extract from the leaves and stems of *Parinari curatellifolia* also showed antibacterial activity with diameters of zone of inhibition ranging from 9-20 mm. Diameters of zone of inhibition exhibited by *Callistemon citrinus* and *Vernonia adoensis* extracts were comparable to those exhibited by the reference antibiotic ampicillin which had diameters of zone of inhibition ranging from 25-45 mm. The different bacterial cells were susceptible to the standard antibiotic ampicillin and resulted in variable inhibitory zones of 21-45 mm (**Table 2**). Extracts of *Callistemon citrinus* and *Vernonia adoensis* were as active as the standard antibiotic but all the other extracts were less potent than ampicillin. All bacterial strains were sensitive to ampicillin.

Minimum inhibitory concentration and minimum bactericidal concentration

The five most potent extracts showing considerably good antibacterial activity for each test organism were selected to determine MIC. Values for MICs were dependent on the bacterial species. Generally, the MIC values were low (0.06-0.5 mg/ml) showing that the extracts are potent (**Table 3**). However, ampicillin was a more potent antibacterial than all the extracts with MIC values ranging from 0.002-0.008 mg/ml. *Mangifera indica*, *Callistemon citrinus* and *Vernonia adoensis* were the most potent extracts against all the test bacteria. *M. indica* was the most potent showing the same trend of inhibition against all bacteria (0.04-0.047 mg/ml) followed by *C. citrinus* with MIC values ranging from 0.02-0.13 mg/ml. *V. adoensis* had MIC values ranging from 0.09-0.19 mg/ml. Extracts of *Parinari curatellifolia* and *Vernonia adoensis* also showed good inhibitory activity

Table 2 Zones of inhibition (mm) induced by ethanol extracts from selected Zimbabwean plants against bacteria.

Plant species	Bacterial species				
	Pseudomonas aeruginosa	***Escherichia coli***	***Staphylococcus aureus***	***Bacillus subtilis***	***Bacillus cereus***
Syzigium cumini	9 ± 0.6	NA	11 ± 0	NA	9 ± 0.6
Callistemon citrinus	12 ± 0****	25 ± 0*****	24 ±0 ****	25 ± 0*****	12 ± 0.5****
Ficus sycomorus	8 ± 0	NA	NA	NA	7 ± 0.6
Vernonia adoensis	20 ± 0 *****	15 ± 0****	25 ± 0*****	20 ± 0***	16 ± 0.6*****
Parinari curatellifolia/stems	12 ± 0***	12 ± 0***	15 ± 0**	11 ± 0	10 ± 0.5***
Parinari curatellifolia/leaves	11 ± 0**	10 ± 0**	20 ± 0***	11 ± 0	9 ± 1*
Gymnsporia senegalensis	6 ± 0	NA	NA	NA	
Mutsvairachuru	6 ± 0	7 ± 0	NA	10 ± 0	
Lantana camara	7 ± 0	NA	NA	NA	9 ± 0**
Brachystegia boehmii	8 ± 0	8 ± 0	10 ± 0	NA	8 ± 0
Mangifera indica	9 ± 0*	10 ± 0*	10 ± 0	15 ± 0*	
Faurea spp	9 ± 0	10 ± 0	11 ± 0*	18 ± 0**	7 ± 0.6
Asteraceae family/flowers	7 ± 0	NA	NA	25 ± 0****	8 ± 0.6
Ampicillin (500 mg) A	25 ± 0	45 ± 0	30 ± 0	45 ± 0	21 ± 0

*****: most potent, *: least potent
Values are expressed as mean ± standard deviation. (n = 4).

Table 3 A summary table for the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays.

Microorganism	G+/G-	Plant species	MIC[a] µg/ml	MBC[b] µg/ml	MIC amp	MBC amp
Escherichia coli	G-	*Callistemon citrinus*	125	250	8	16
		Vernonia adoensis	188	500		
		Parinari curatellifolia (stems)	188	500		
		P. curatellifolia	300	500		
		Mangifera indica	47	250		
Pseudomonas aeruginosa	G-	*Vernonia adoensis*	188	1000	16	125
		Callistemon citrinus	63	250		
		P. curatellifolia (stems)	ND	> 1000		
		P. curatellifolia	188	500		
		M. indica	46	250		
Staphylococcus aureus	G+	*Vernonia adoensis*	94	1000	< 2	8
		Callistemon citrinus	47	250		
		P. curatellifolia	375	500		
		P. curatellifolia (stems)	250	500		
		Faurea sp	ND	>1000		
Bacillus subtilis	G+	*Callistemon citrinus*	24	63	4	8
		Asteraceae family (flowers)	292	1000		
		Vernonia adoensis	188	1000		
		Faurea sp	500	> 1000		
		M. indica	40	125		
Bacillus cereus	G+	*Vernonia adoensis*	188	1000	4	8
		Callistemon citrinus	63	125		
		P. curatellifolia (stems)	179	500		
		Lantana camara	ND	> 1000		
		P. curatellifolia	375	> 1000		

a: minimum inhibitory concentration
b: minimum bactericidal concentration
ND: no activity detected

Table 4 Percentage increase of R6G accumulation in bacterial cells. Results show the percentage accumulation of R6G inside the cell after exposure to glucose, plant extracts and the standard inhibitor reserpine. The value of drug accumulation in the presence of glucose alone was taken as the control at 0 %. Values are expressed as mean ± standard deviation. (n = 4).

Microorganism	Sample causing highest R6G influx (%)	Second highest (%)	Sample causing least influx (%)	% influx by Reserpine
Staphylococcus aureus (Gram+)	*V. adoensis* (116 ± 2)	*M. indica* (87 ± 1)	*C. citrinus* (54 ± 3)	90 ± 1
Pseudomonas aeruginosa (Gram-)	*C. citrinus* (100 ± 1)	*M. indica* (73 ± 1)	*V. adoensis* (64 ± 1)	58 ± 1
Bacillus cereus (Gram+)	*V. adoensis* (49 ± 3)	*M. indica* (21 ± 1)	*C. citrinus* (14 ± 2)	18 ± 2
Bacillus subtilis (Gram+)	*V. adoensis* (40 ± 1)	*C. citrinus* (38 ± 1)	*M. indica* (9 ± 0.5)	44 ± 1
Escherichia coli (Gram-)	*M. indica* (83 ± 1)	*V. adoensis* (29 ± 1)	*C. citrinus* (11 ± 1)	7 ± 0.5

(0.19-0.5 mg/ml).

Results from the MBC assays supported the data obtained from the disc diffusion assay and the MIC determination assay. These results further confirmed that *Mangifera indica*, *Callistemon citrinus* and *Vernonia adoensis* were the most potent extracts. These three extracts exhibited a bactericidal nature as observed from their MBC values that ranged from 0.06-1 mg/ml (**Table 3**). However, plant extracts seemed to be species specific as they did not show the same trend of bactericidal activity. Other extracts were bacteriostatic with MBC values ranging from 0.5-1 mg/ml. From the MIC and MBC assays, Gram-negative species seemed to be more resistant to plant extracts than Gram-positive species as indicated by their high MIC and MBC

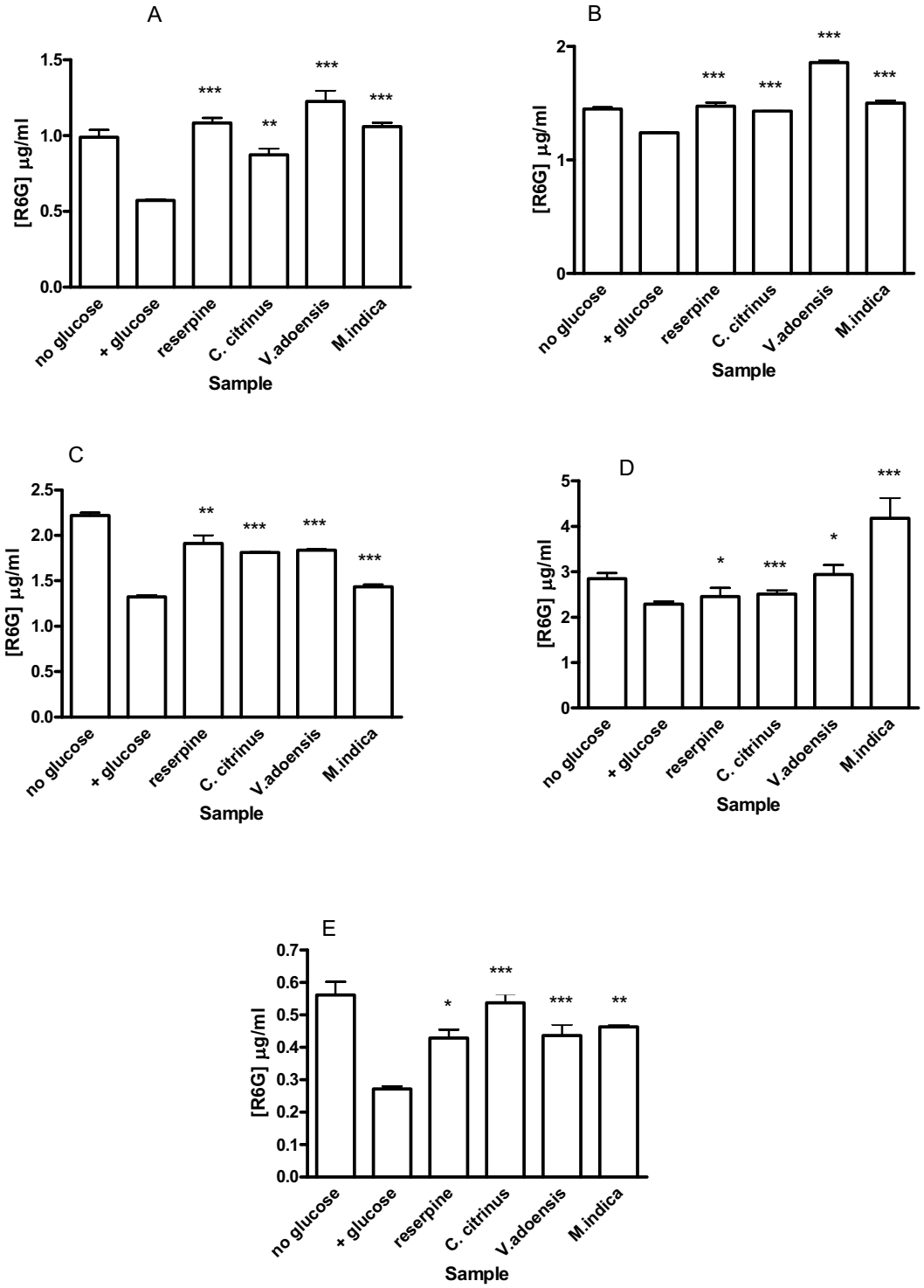

Fig. 1 The accumulation of R6G in bacterial cells over time. The graphs are a plot of R6G concentration inside the cell after 30 min of incubation against the sample used (plant extract, ampicillin or reserpine). Glucose was used to provide energy for the efflux pumps in the form of ATP. Key: A = *Staphylococcus aureus*; B = *Bacillus cereus*; C = *Bacillus subtilis*; D = *Escherichia coli*; E = *Pseudomonas aeruginosa.* Error bars denote the standard deviations from mean (n = 4). The asterisks (*) indicate statistical significant differences with the control (+ glucose) ($P < 0.05$).

values (**Table 4**). Other studies carried out have also shown Gram-negative strains to be less sensitive to antibiotics than Gram-positive bacteria (Stavri *et al*. 2007; Doughari and Manzara 2008). Gram-negative bacteria and mycobacteria both possess thick outer membranes that are highly hydrophobic, providing these organisms with a permeability barrier especially towards hydrophilic compounds such as macrolide antibiotics such as erythromycin. This in part explains the observed less sensitivity to antimicrobials by Gram-negative bacteria than by Gram-positive organisms (Stavri *et al*. 2007). Some of the extracts used in this study have shown antibacterial activity in other studies as well. *Vernonia species* possesses antibacterial properties against *K. pneumoniae* and *M. tuberculosis* (Kisangau *et al*. 2007; Maregesi *et al*. 2008) and were used in the cure of tuberculosis. A crude extract of *Vernonia adoensis* was found to have high activity against *E. coli*, a gram-negative bacterial species of clinical importance (Kisangau *et al*. 2007). The leaves of *M. indica* have been reported to possess antibacterial activity against *E. coli* and other bacteria in the family enterobacteriaceae (Doughari and Manzara 2008). Another different part of the mango plant, the kernel seed was also observed to possess high antibacterial activity against foodborne pathogenic bacteria (Kabuki *et al*. 2000). In another study, *Mangifera indica* was found to contain alkaloids and glycosides, giving it great pharmacological importance

(Nwinuka *et al*. 2008). Preliminary phytochemical analysis of *M. indica* also showed the presence of tannins, saponins and phenols in addition to the glycosides and the alkaloids (Doughari and Manzara 2008). The presence of these phytoconstituents in leaf extracts is thought to be responsible for antibacterial activity. Alkaloids and their derivatives have activities against *Staphylococcus aureus* and methicillin-resistant *S. aureus.* The mechanism of action of highly aromatic planar quaternary alkaloids is attributed to their ability to intercalate with DNA (Kumar *et al*. 2007). *Mangifera indica* also has traditional uses for curing coughs, diarrhoea and dysentery (Kisangau *et al*. 2007). These findings on the antibacterial activity of the mango plant, makes it a potential candidate to focus on in terms of discovering new lead compounds for drug development. *Callistemon* species have also been used in traditional medicine (Seyyed *et al*. 2010), ornamental horticulture and essential oil production (Oyedeji *et al*. 2009). The ethanolic and methanolic extract of *Callistemon citrinus* exhibited a varied range of antimicrobial activity against both gram positive as well as Gram-negative bacteria (Seyyed *et al*. 2010). In a number of studies carried out in different countries, essential oils extracted from *Callistemon* species, including *C. citrinus* were found to contain 1,8-cineole, α-pinene, β-pinene, myrcene, limonene, linalool and menthyl acetate (Oyedeji *et al*. 2009). These oils were also found to have antibacterial activity against both Gram-positive and Gram-negative bacteria. C-methyl flavonoids, triterpenoids and phloroglucinol derivatives were also some of the compounds identified in *Callistemon* species (Oyedeji *et al*. 2009).

Effects of extracts on Rhodamine 6G uptake

Bacterial efflux pumps offer potential targets to combat problematic infectious diseases such as those caused by MRSA, *E. coli* and *P. aeruginosa* (Stavri *et al*. 2007). In Gram-positive organisms, the pumps studied in greatest detail include the NorA, Tet (K) and Msr (A) transporters while in Gram-negative bacteria, studies have focused on the tripartite AcrAB-TolC and MexAB-OprM efflux pumps of *E. coli* and *P. aeruginosa*, respectively (Stavri *et al*. 2007).

Our results showed that the accumulation of R6G was both strain- and extract-specific with a different trend of R6G accumulation from one bacterium to another (**Table 4**). A study to determine the consequences of inhibiting the efflux pumps of *P. aeruginosa* was undertaken by Lomovskaya *et al.* (1999). Inhibition of efflux pumps significantly decreased MICs for both antibiotic-susceptible and -resistant bacteria, reversed acquired resistance, and resulted in a decreased frequency of emergence of *P. aeruginosa* mutants highly resistant to fluoroquinolones (Lomovskaya *et al*. 1999).

For *S. aureus*, the *V. adoensis* extract inhibited efflux pumps to a greater extent, thus, resulting in the highest accumulation of R6G (116% increase from the control) within the cell compared to *M. indica* (87%) and *C. citrinus* (54%) (**Table 4**). For *P. aeruginosa*, *C. citrinus* extract inhibited drug efflux pumps the most, accumulating R6G within the cell by 100% more than the control. *M. indica* extract was the second most potent drug efflux pump inhibitor increasing R6G uptake by 73% with *V. adoensis* extract increasing uptake up to 64%. *B. cereus* extract showed the same trend of inhibition of efflux pumps as that of *S. aureus* with percentage increases of 49, 21 and 14%, respectively (**Table 4**). However, inhibition of pumps was to a greater extent in *S. aureus* than in *B. cereus*. For *B. subtilis*, accumulation of R6G decreased from *V. adoensis* extract to *M. indica* extract with percentage increase values of 40, 38 and 19%, respectively. *M. indica* increased R6G accumulation 83 times more than the control while *V. adoensis* and *C. citrinus* increased accumulation by 29 and 11%, respectively (**Table 4**). These results show that the plant extracts used in this study were able to increase the accumulation of R6G within the bacterial cells even in the presence of glucose, which is needed to provide energy for the efflux pumps to initiate the efflux process.

It is expected that Gram-positive bacteria are more sensitive to the extracts because of the single layer of their cell wall while the double membrane of Gram-negative bacteria should make them less sensitive (Kaur and Arora 2009). In this study, *S. aureus* was the most sensitive test bacterium to all the plant extracts used (54-116% R6G accumulation) and for *E. coli* for which two extracts increased accumulation of R6G inside the cell by 29 and 11%. The Gram-negative *P. aeruginosa* produced unexpected drug accumulation results: it was more sensitive to the extracts even though many studies have shown that the presence of a double membrane in Gram-negative bacteria makes them less sensitive to extracts or drugs (Stavri *et al*. 2007). Further work needs to be carried out with the strain especially regarding its medical importance and reported cases of drug resistance. All the extracts that were screened for drug efflux pump inhibitor properties against *P. aeruginosa* were effective in inhibiting the efflux system that was responsible for extruding R6G from the cell. The percentage R6G accumulation in *P. aeruginosa* ranged from 64-100%, thus making it the second most sensitive strain to the plant extracts after *S. aureus*. Phytochemicals from these extracts could be developed to serve as efflux pump inhibitors in the administration of antibiotics curing infections by resistant *P. aeruginosa* to restore their uses. Work has already been done to characterize some efflux pumps in *P. aeruginosa*. Westbrock-Wadman *et al.* (1999) characterized *amrAB* genes that encode a *P. aeruginosa* transporter belonging to the resistance, nodulation, and cell division (RND) family of efflux systems which also appeared to be the same as *mexXY* genes discovered from *P. aeruginosa* earlier (Westbrock-Wadman *et al*. 1999). The other two Gram-positives (*B. cereus* and *B. subtilis*) in this study also produced unexpected result with percentage increase in the amount of R6G in the cell ranging from 9-49%. These results were unexpected because Gram-positive bacteria have been shown to be more susceptible to antibiotics (Kaur and Arora 2009) but in this study *B. cereus* and *B. subtilis* were less susceptible to the plant extracts (**Table 4**).

It is of importance to identify agents that can block the efflux of drugs from within bacteria, so as to extend the life of existing antibacterial drugs. The concept of using a compound that inhibits resistance together with a conventional antibiotic is well proven and co-amoxiclav is an important example (Stermitz *et al*. 2000). A number of MDR pump inhibitors against the human pathogen *S. aureus* have been described and they include berberine isolated from *Berberis fremontii*, which is an alkaloid with only weak antibacterial activity (MIC = 256 mg/L) against a wild-type strain of *S. aureus* (Stermitz *et al*. 2000). However, the isolation of the flavonolignan 5'-methoxyhydnocarpin-D (5'-MHC-D) and a synergistic study between these two compounds led to a 16-fold increase in the antibacterial activity of berberine (MIC = 16 mg/L). 5'-MHC-D also had a synergistic effect with several other substrates of the efflux pump NorA, including norfloxacin (Stavri *et al*. 2007). Although the antibacterial activities of *V. adoensis*, *M. indica* and *C. citrinus* observed in this study confirm the findings of other previous similar studies, it is worth noting that these extracts also showed activity as efflux pump inhibitors, an interesting finding which has not been reported in previous studies investigating the antibacterial activity of these three extracts.

Inhibition of efflux pumps by plant extracts and reserpine

Reserpine is an antihypertensive plant alkaloid that was first isolated from the roots of *Rauwolfia vomitoria* and was found to reverse NorA-conferred MDR (Stavri *et al*. 2007). Thus, reserpine is used as a standard inhibitor of efflux pumps. All the test bacteria in this study responded to the effects of reserpine by increasing R6G influx by 7-90% (**Table 4**). In most cases the plant extracts used in this assay

were more effective in blocking drug efflux pumps than reserpine. A possible reason for this would be the presence of abundant chemical compounds in the extracts with efflux pump inhibitor activity. Hence, these extracts have a promising future for the development of effective EPIs which would augment the antibacterial activities of standard antibiotics.

CONCLUSION

The extracts from *Mangifera indica*, *Callistemon citrinus* and *Vernonia adoensis* were found to have the most antibacterial activity. These extracts also showed activity as efflux pump inhibitors. Lead compounds from such plant extracts need to be isolated so that they can serve as templates for the production of new antibiotics as well as efflux pump inhibitors.

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REFERENCES

Aboaba OO, Smith SI, Olude FO (2006) Antibacterial effect of edible plant extract on *Escherichia coli* 0157:H7. *Pakistan Journal of Nutrition* **5**, 325-327

Abu-Shanab B, Adwan G, Abu-Safiya D, Jarrar N, Adwan K (2004) Antibacterial activities of some plant extracts utilized in popular medicine in Palestine. *Turkish Journal Biology* **28**, 99-102

Al-Fatimi M, Wurster M, Schwoder G, Lindequist U (2007) Antioxidant antimicrobial and cytotoxic activities of selected medicinal plants from Yemen. *Journal of Ethnopharmacology* **111**, 657-666

Amusan OOG, Sukati NA, Dlamini PS, Sibandze FG (2007) Some Swazi phytomedicines and their constituents. *African Journal of Biotechnology* **6**, 267-272

Arora DS, Kaur GJ, Kaur H (2009) Antibacterial activity of tea and coffee: Their extracts and preparations. *International Journal of Food Properties* **12**, 286-294

Bocanegra-García V, Camacho-Corona MR, Ramírez-Cabrera M, Rivera G, Garza-Casal M, Vaquero M, Rinder H, Tortoli E, Grosset J, Rusch-Gerdes S, Gutierrez J, Jarlier V (2005) Synergism between natural products and antibiotics against infectious diseases. *Microbial Drug Resistance* **11**, 62-67

Chambers HF (2001) The changing epidemiology of *Staphylococcus aureus*. *Emerging Infectious Diseases* **7**, 178-182

Chigora P, Masocha R, Mutenheri F (2007) The role of indigenous medicinal knowledge (IMK) in the treatment of ailments in rural Zimbabwe: The case of Mutirikwi communal lands. *Journal of Sustainable Development in Africa* **9**, 23-46

Doughari JH, Manzara S (2008*) In vitro* antibacterial activity of crude leaf extracts of *Mangifera indica* Linn. *African Journal of Microbiology Research* **2**, 67-72

Eloff JN (1998) A sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extracts for bacteria. *Planta Medica* **64**, 711-713

Emam SS (2010) Comparative study of the alkaloidal and tannin contents of some *Reseda* species. *Journal of Applied Sciences Research* **6**, 888-896

Iwawela OE, McGaw LJ, Naidoo V, Eloff JN (2007) Inflammation: The foundation of disease and disorders: A review of phytomedicines of Southern Africa origin used to treat pain and inflammatory conditions. *African Journal of Biotechnology* **6**, 2868-2885

Kabuki T, Nakajima H, Arai M, Ueda S, Kuwabara Y, Dosako S (2000) Characterization of novel antimicrobial compounds from mango (*Mangifera indica* L.) kernel seeds. *Food Chemistry* **71**, 61-66

Kaur GJ, Arora DS (2009) Antibacterial and phytochemical screening of *Anethum graveolens*, *Foeniculum vulgare* and *Trachysprmum ammi. BMC Complementary and Alternative Medicine* **9**, 30

Kisangau DP, Hosea KM, Joseph CC, Lyaruu HVM (2007) *In vitro* antimicrobial assay of plants used in traditional medicine in Bukoba rural district, Tanzania. *African Journal of Traditional, Complimentary and Alternative Medicines* **4**, 510-523

Kumar A, Ilavarasan R, Jayachandran T, Deecaraman M, Mohan Kumar R, Aravindan P, Padmanabhan N, Krishan MRV (2008) Anti-inflammatory activity of *Syzygium cumini* seed. *African Journal of Biotechnology* **7**, 941-943

Kumar GS, Jayaveera KN, Kumar ACK, Sanjay UP, Swamy BMV, Kumar DVK (2007) Antimicrobial effects of Indian medicinal plants against acne-inducing bacteria. *Tropical Journal of Pharmaceutical Research* **6**, 717-723

Lomovskaya O, Lee A, Hoshino K (1999) Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **43**, 1340-1346

Lotifpour F, Nazemiyeh N, Fathi-Azad F, Garacei N, Arami S, Talat S, Sadegpour F, Hasanpour R (2008) Evaluation of antibacterial activities of some medicinal plants from North-West Iran. *Iranian Journal of Basic Medical Sciences* **11**, 80-85

Maesaki S, Marichal P, Bossche HV, Sanglad D, Kohno S (1999) Rhodamine 6 G efflux for the detection of CDR1-overexpressing azole-resistant *Candida albicans* strains. *Journal of Antimicrobial Chemotherapy* **44**, 27-31

Maregesi SM, Pieters L, Ngassapa OD, Apers S, Nwinuka NM, Monanu MO, Nwiloh BI (2008) Effects of aqueous extract of *Mangifera indica* L. (mango) stem bark on haematological parameters of normal albino rats. *Pakistan Journal of Nutrition* **7**, 663-666

Mukanganyama S, Ntumy AN, Maher F, Muzila M, Andrae-Marobela K (2011) Screening for anti-infective properties of selected medicinal plants from Botswana. *African Journal of Plant Science and Biotechnology* **5 (Special Issue 1)**, 1-7

Nwinuka NM, Monanu MO, Nwiloh BI (2008) Effects of aqueous extract of *Mangifera indica* L. (mango) stem bark on haematological parameters of normal albino rats. *Pakistan Journal of Nutrition* **7**, 663-666

Oyedeji OO, Lawal OA, Shode FO, Oyedeji AO (2009) Chemical composition and antibacterial activity of the essential oils of *Callistemon citrinus* and *Callistemon Viminalis* from South Africa. *Molecules* **14**, 1990-1998

Raja A, Gajalakshmi P, Mohamed Mahroop Raja M (2010) Drugs from the natural bio sources for human disease. *International Journal of Pharmacology* **6**, 360-363

Ruffo CK (1991) A survey of medicinal plants in Tabora region, Tanzania in Traditional Medicinal Plants, Dar Es Salaam University Press-Ministry of Health, Tanzania, 391 pp

Seyyed MS, Masumeh N, Ismaieel D, Hossein M (2010) Antibacterial Activity of Hydroalcoholic Extract of *Callistemon citrinus* and *Albizia lebbeck*. *American Journal of Applied Sciences* **7**, 13-16

Shanmugam H, Kruthiventi AK, Doble M (2008) Synergism between natural products and antibiotics against infectious diseases. *International Journal of Phytotherapy and Phytopharmacology* **15**, 639-652

Stavri M, Piddock LVJ, Gibbons S (2007) Bacterial efflux pump inhibitors from natural sources. *Journal of Antimicrobial Chemotherapy* **59**, 1247-1260

Stermitz FR, Lorenz P, Tawara JN (2000) Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. *Proceedings of the National Academy of Sciences USA* **97**, 1433-1437

Westbrock-Wadman S, Sherman DR, Hickey MJ, Coulter SN, Zhu YQ (1999) Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Antimicrobial Agents and Chemotherapy* **43**, 2975-2983

World Health Organisation (WHO 2009) Cholera in Zimbabwe - update 4. Available online: http://www.who.int/csr/don/2009_06_09/en/index.html

Zaidan MRS, Noor Rain A, Badrul AR, Adlin A, Norazah A, Zakiah I (2005) *In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method. *Tropical Biomedicine* **22**, 165-170