African Journal of Pharmacy and Pharmacology Vol. 6(23), pp. 1653-1667, 22 June, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.107
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Multi-drug resistance, verotoxin production and efficacy of crude stem bark extracts of *Curtisia dentata* among *Escherichia coli* (non-O157) and *Acinetobacter* species isolates obtained from water and wastewater samples

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Accepted 16 May, 2012

Drug resistant diarrhea and nosocomial infections caused by verotoxic *Escherichia coli* and some *Acinetobacter* species has posed serious therapeutic challenges, especially in developing countries. The aim of this work is to investigate multi-drug resistance, verotoxin-production and susceptibility of *E. coli* and *Acinetobacter* spp. isolated from some water samples to crude stem bark extracts of *Curtisia dentata.* Culture of 62 water samples on Brilliance *E. coli* coliform selective medium (BECSM, Oxoid), Eosin Methylene Blue (EMB) agar, or Baumann's enrichment medium (BEM) and Leeds Acinetobacter Medium (LAM) yielded 69 isolates of *E. coli* and 41 isolates of *Acinetobacter* spp. with 26 (53.06%) of the *E. coli* and 6 (14.63%) of the *Acinetobacter haemolyticus* isolates producing verotoxins, and no *Acinetobacter lwoffii* isolate produced the toxins. Multi-drug resistance index (MDRI) values of isolates ranged between 7 to 33.00% for both isolates with 12 (17.39%) of the *E. coli* and 10 (24.39%) of the *Acinetobacter* spp. resistant to 3 or more classes of the antibiotics. *C. dentata* stem bark extracts demonstrated low minimum inhibitory concentration (MIC) values of 150 to 300 µg/ml for *E. coli* and 150 to 2000 µg/ml for *Acinetobacter* spp. The plant also contained saponins, tannins, glycosides, anthraquinones, flavonoids, steroids, and phenols. The presence of verotoxic multidrug resistant *E. coli* and *Acinetobacter* spp. in the environments investigated calls for further surveillance of more water bodies and other environments. Proactive control measures need to be in place to curtail possible contamination of food and drinking water sources. Purification of *C. dentata* phytoconstituents, toxicological as well as *in vivo* studies for their antimicrobial potentials against pathogenic bacteria, should be carried out with a view of utilizing the plant in developing novel antibiotic substances.

Key words: *Acinetobacter* spp., Baumann's enrichment medium, *Curtisia dentata*, *Escherichia coli*, multi-drug resistance, plant extracts, verotoxins.

INTRODUCTION

Contamination of food and water with faecal bacteria is and remains a common persistent problem impacting public health and local and national economies. Water related diseases are the major cause of morbidity and mortality worldwide. Among these, diarrhea is estimated to be responsible for 2.0 million deaths per annum, particularly, in developing countries (Sausa, 2006). Among the causative agents of this gastrointestinal disease are bacteria (diarrhogenic *Escherichia coli, Shigella, Salmonella*, and *Campylobacter*), viruses

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(norovirus, Hepatitis A), and protozoa (*Cryptosporidium, Giardia*) (Ishii and Sadowsky, 2008). Although, the verotoxin producing *E. coli* O157:H7 (VTEC) has been the mainly implicated and widely reported strain as the causative agent of bloody diarrhea, emergence of non O157:H7 VTEC serotypes including O111:H, O26:H11, O103:H2, and O145 have been reported (Duffy and Garvey, 2000; Verweyen, 2006). These strains have also been linked to outbreaks of food poisoning (Duffy and Garvey, 2000).

Members of the genus *Acinetobacter* (Gram-negative coccobacilli) have also emerged as significant notorious antibiotic resistant nosocomial infectious agents in hospital settings. The bacteria are ubiquitous, free-living, and fairly stable in the environment (Smith et al., 2007). Clinically important species include *Acinetobacter baumannii, Acinetobacter johnsonnii, Acinetobacter haemolyticus, Acinetobacter junii*, and *Acinetobacter* genomospecies 3, and 13. Apart from multidrug resistant (MDR) nosocomial infections, these bacteria are implicated in endocarditis, bacteremia, sepsis in neonatal intensive care units, and paediatric oncology units, as well as community acquired infections, such as meningitis, peritonitis, and endophthalmitis (Crawford et al., 1997; Valero et al., 1999; Dorsey et al., 2004; Smith et al., 2007). *A. haemolyticus* has been associated with endocarditis and verotoxin production; hence, bloody diarrhea (Castellanos et al., 1995).

Both *E. coli* and *Acinetobacter* spp. have been reported to be responsible for increasing incidences of multidrug resistant infections worldwide (Prashanth and Badrinath, 2005; Grotiuz et al., 2006). Antibiotic resistant bacteria have also been introduced into the environment from animal husbandry via liquid and solid manure as well as from human excretions via wastewater or low efficacy treatment of hospital wastewater. This therefore has resulted in increasing concerns about the growing resistance of pathogenic bacteria in the environment and their ecotoxic effects (Reinthaler et al., 2010).

Though, few reports are available on the incidences of *E. coli* O157:H7 in Africa (Browning et al., 1990; Akinyemi et al., 1998; Galane and Roux, 2001; Hayghaimo et al., 2001; Muller et al., 2001; Muller et al., 2003; Presterl et al., 2003), there is paucity of information on the other verotoxin producing *E. coli* pathotypes commonly referred to as non-verotoxic *E. coli* strains, and none at all on *Acinetobacter* spp. The pandemics of antibiotic resistance among these groups of bacteria and the attendant complications arising from treatment of verotoxic infections with antibiotics (Abong'o and Momba, 2009) underline the need to investigate their occurrence in the environment. The inability of commonly prescribed antibiotics to treat some common infections has made the use of traditional medicinal plants popular in Africa, even among urban dwellers. Complications arising from the antibiotic treatment of verotoxic bacteria should be a further inducer to investigate alternative treatment sources, especially from plants.

Curtisia dentata (Cornaceae or dogwood family) or assegai (English common name) is a traditional medicinal plant that has been employed in the treatment of diarrhea and related stomach ailments in South Africa (Notten, 2004). The commonly called assegai (Afrikaans); uSirayi, umGxina (Xhosa), umLahleni (Xhosa, Zulu), uMagunda, uMaginda, umBese, umPhephelelangeni (Zulu), iliNcayi, isiNwati (Stwanee), modula-tshwene (Northern Sotho), and musangwe, mufhefhera (Venda) all in South Africa (Notten, 2004) is an attractive tree with smooth glossy leaves, inconspicuous odourless flowers and small rounded to oval fleshy bitter berries. Medicinally, *C. dentata* is used as a blood strengthener, as an aphrodisiac, in the treatment of heartwater in cattle in the Eastern Cape (South Africa), and for the treatment of pimples (Dold and Cocks, 2001; Shai et al., 2009). Application of *C. dentata* in the treatment of diarrhea makes it a good candidate for the investigation of its potential in controlling, specifically verocytotoxin and other toxin producing bacterial pathogens. Results from this investigation will open up new directions in the search for more effective drugs for the control of MDR verotoxic bacteria. This work was therefore carried out in order to investigate the presence of verotoxin producing *E. coli* other than *E. coli* O157:H7 and verotoxin producing *Acinetobacter* spp. from some wastewater samples and to determine their antibiotic resistance profile as well as the effect of stem bark extracts of *C. dentata* on the verotoxic multidrug resistant bacteria isolates.

MATERIALS AND METHODS

Source of media, antibiotics, chemicals, and plant

Eosin Methylene Blue (EMB), Nutrient Broth (NB), modified Trypton Broth (mTSB), Mueller-Hinton Agar (MHA), and antibiotic discs were all Oxoid grade, and were purchased from Quantum Biotechnologies. Glisa Duopath Verotoxins® (Appendix 5h) test kit, oxidase test strips and all laboratory grade chemicals used in this study were purchased from Merck. *E. coli* polyvalent antisera 2, 3, and 4, and REMEL RapID™ NF plus test kit were purchased from Bioweb, South Africa. All the purchasing companies are based in South Africa. The plant sample of *C. dentata* was authenticated as well as provided by Dr. Charles Laubscher from his plant collections in the Glass House of the Horticulture Department, Cape Peninsula University of Technology, Cape Town South Africa.

Sample collection and preparation of plant material

Thirty two (32) water (18 wastewater samples from a wastewater treatment plant and an abattoir, and 14 river water samples from River Berg (Appendix 4a), River Plankenburg (Appendix 4b)) in Cape Town, South Africa, were collected using the shoreline sampling method as described by Obire et al. (2005). For microbiological analysis, 1 L volume sized sterilized sample bottles were held at the base and dipped downwards below the water surface (20 to 30 cm deep), opened and allowed to fill up, then corked while still under water (Health Protection Agency, 2007). The collected water samples were placed in a cooler box with temperature maintained between 4 to 10°C using ice packs,

and then immediately transported to the Microbiology Laboratory of the Biotechnology Department of the Cape Peninsula University of Technology, Cape Town South Africa, where they were analyzed within 3 to 6 h. For the plant sample, fresh stem barks from *C. dentata* were dried to constant weight in an oven for 6 h at 45°C. The dried stem barks were coarsely grated in a pistil and mortar and then reduced to powdered form using an electric grinder. The powdered plant materials were transferred to brown bottles and were stored at ambient temperature until use.

Cultivation, isolation, and identification of bacteria

For isolation of *E. coli*, water samples were serially diluted up to 10^5 dilution, and 1 ml was inoculated into Brilliance *E. coli*/coliform selective medium (BECSM, Oxoid) by agar dilution method and the plates incubated at 37°C for 24 h. After incubation, discrete colonies were separated and inoculated onto plates of EMB (Oxoid, SA) and incubated at 37°C for 24 h. Isolates were further purified by picking discrete colonies (green metallic sheen) and sub-culturing onto fresh plates of EMB, and once again incubating for 18 to 24 h at 37°C. After incubation, 5 to 10 discrete colonies were characterized using the IMViC (DIFCO, MD, USA) test kit, oxidase test strips, and Erchlich's reagent. Isolates that were indole positive, non motile as well as negative for methyl red, Voges-Proskauer and citrate utilization tests were identified as *E. coli.* Slide agglutination tests were performed on selected 5 to 10 presumptive single colonies using polyvalent *E. coli* antisera 2, 3, and 4 (Bioweb PTY, SA). Differences between colonial isolates were determined by determination of the antibiotic susceptibility of the various isolates. *E. coli* ATCC 25922 was used as control. Serotyped (confirmed) *E. coli* isolates were inoculated onto tryptic soy (TS) slants and were incubated for 24 h at 37°C, and then stored at 4°C (Roy et al., 2004; Tarawneh et al., 2009) until use.

To isolate and identify *Acinetobacter* spp., 1 ml of 10^5 dilution of the water samples for *E. coli* were inoculated into 9 ml of tubes containing Baumann's enrichment medium (BEM, Appendix 1) instead of EMB. The inoculated BEM was shaken vigorously by vortexing and then incubated at 37°C for 24 h in a shaker incubator with vigorous agitation. After incubation, 2 drops of the BEM culture were further inoculated into modified tryptic soy broth (mTSB) in a test tube and incubated at 37°C for 24 to 48 h. After this, 1 to 2 loopfuls of BEM or mTSB cultures was inoculated onto EMB (Oxoid) or Leeds *Acinetobacter* medium (LAM, Hardy diagnostics USA, Appendix 2) and further incubated at 37°C for 18 to 72 h. After incubation, pink colonies on EMB or pink/purple colonies on LAM were Gram stained to observe for large Gram-negative coccobacilli cells, while 5 to 10 discrete colonies were inoculated into sulfide, indole, and motility (SIM) for motility testing and also subjected to oxidase test using the oxidase test strips (Oxoid, UK) as well as biochemical biotyping using the REMEL RapID^{TM} NF plus (Bioweb, South Africa, Appendix 5g) and antibiotic susceptibility testing. *Acinetobacter* spp. isolates identified with slight colonial variations in the biochemical biotype with REMEL RapID^{TM} NF plus and antibiotic susceptibility pattern were selected (Guardabassi et al., 1999). The strains were further purified by inoculation onto tryptic soy (TS) slants, incubated for 24 h at 37°C, and then stored at 4°C until use (Roy et al., 2004; Tarawneh et al., 2009). *A. haemolyticus* ATCC 19002 was used as control.

Differentiation of verotoxic from non-verotoxic bacteria

All the bacterial isolates were screened for verotoxin production using antibody-based rapid slide agglutination assays with the Duopath kit (Merck, SA Appendix 5h) according to the manufacturer's instructions. The bacterial isolates were first precultured in 1 ml casaminacid yeast extract (CAYE) broth, (Appendix 3) and incubated at 37°C with rotation at 100 rpm for 24 h. After incubation, 10 µl of the precultured broth (approximately 1 × 10^7 cells/ml) was inoculated into fresh CAYE broth and further incubated for 16 h with rotation at 100 rpm at 37°C. The culture was centrifuged at 5000 ×g for 5 min to separate the supernatant and cell pellets. The cell pellets were then washed three times with phosphate buffered saline (PBS, 5 ml), and then was suspended in 0.25 ml of 0.01 M Tris-HCl (pH 7.5). To the pellets, 0.5 ml distilled water containing 50 µg/ml polymyxin B was added and the suspension was incubated at 37°C for 30 min. 200 µl of the culture suspension was then transferred onto the test device using a sterile Pasteur pipette and the result was read after 10 min. The appearance of red bands on the vtx1 or vtx2 bands (Appendix 5h) denoted the presence of either one of or both verotoxins.

Antimicrobial susceptibility testing and determination of MDR index

The disc diffusion method as described by Perilla et al. (2003) was used for the determination of antimicrobial susceptibility testing. MHA plates were inoculated with the test organisms (0.5 McFarland turbidity standard) using a sterile swab stick and the plates were incubated at 37°C for 16 to I8 h for *E. coli* and 20 to 24 h for *Acinetobacter* spp. After incubation, the zone diameters of inhibition (mm) were measured. The bacteria were tested for susceptibility against ampicillin (10 µg), cefuroxime (30 µg), cephalexin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), aztreonam (30 µg), nalidixic acid (30 µg), amikacin (30 µg), tetracycline (30 µg), gentamicin (10 µg), ofloxacin (5 µg), and ciprofloxacin (5 µg) (Oxoid UK) (WHO, 2002) and stem bark extracts of *C. dentata* (12.50 mg/ml). Resistance to more than 4 antibiotics was taken as MDR. MDR index (MDRI) of individual isolates was calculated by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics to which the isolate was exposed (Chandran et al., 2008). Isolates with MDRI values of more than 0.2 or 20% were considered highly resistant.

$$\text{MDRI } (\%) = \frac{\text{Number of antibiotic s resisted}}{\text{Total number of antibiotic s used}} \text{ x } 100$$

Extraction and determination of phytoconstituents from stem bark extracts of *C. dentata*

To extract phytoconstituents from the plant material, 5 g ground plant stem bark was soaked in 200 ml of solvent for 2 h followed by filtration; the procedure was repeated three times. The filtered extracts obtained from extraction with any one solvent were combined, and were dried under laminar flow at 25°C. The percentage yield of the extract was calculated and then used to screen for the presence of phytoconstituents as described by Doughari and Ioryue (2009a).

Determination of antibacterial effects and minimum inhibitory concentration (MIC) of the stem bark extracts of *C. dentata* against *E. coli* and *Acinetobacter* spp.

Antibacterial activity determination was carried out using the filter paper disc diffusion method (Doughari and Obidah, 2008). Dried sterilized filter papers (4 mm in diameter) soaked in different concentrations of extracts (100 to 3000 µg/ml and 2.5 to 200 mg/ml/disc) were placed on MHA plates earlier seeded with the test

Table 1. Characteristics of organisms isolated from the wastewater and water samples investigated.

Sample/Isolate	Morphological characteristics				Biochemical characteristics					*Gelatin liquefaction	*Fermentation reactions						
	EMB	LAM	ShB	Gram reaction	S	I	M	E	O		Gluc	Cit	ADH	URE	EST	IND	NO_3
E. coli	Colonies with green metallic sheen	N/A	N/A	Gram-negative rods	-	+	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
A. lwoffii	Tiny, blue, mucoid colonies	Pink colonies diffused into the medium	-	Gram-negative coccobacilli	-	+	-	N/A	-	-	-	-	-	-	+	-	+
A. haemolyticus	Tiny, blue, mucoid colonies	Pink colonies diffused into the medium	+	Gram-negative coccobacilli	-	+	-	N/A	-	+	+	+	-	+	+	+	+

EMB, Eosin methylene blue; LAM, Leeds *Acinetobacter* medium; ShB, haemolysis on sheep Blood Agar; N/A, not applicable; S, sulphide production; I, Indole production; E , Erchlich's reagent; M, motility; O, oxidase reaction; Gluc, glucose; Cit, citrate; ADH, arginine; URE, urea; EST, triglyceride; IND, tryptophane; NO_3,sodium nitrate. *Some of the tests in Rapid NF plus used mainly for the identification of *Acinetobacter* spp.

organisms (0.5 ml McFarland turbidity standard) and left on the table for 5 min to dry. The plates were then incubated at 37°C for 24 h, after which antibacterial activity was determined by measurement of zone diameter of inhibition (mm) against each test bacteria. The antimicrobial activity (expressed as percentage relative inhibition zone diameter) was calculated by applying the expression:

$$\text{RIZD}\ (\%) = \frac{\text{IZD sample - IZD negative control}}{\text{IZD antibiotic standard}} \text{ x } 100$$

where RIZD is the percentage of relative inhibition zone diameter and IZD is the inhibition zone diameter (mm). The equation compensates the possible effect of the solvent (blank) other than water on the IZD. The test was considered negative (-) when the IZD of the sample is equal to the IZD of the blank (Rojas et al., 2006). Filter paper discs soaked with extracting solvent (ethanol) or 30 µg/ml ampicillin were used as negative and positive controls, respectively.

To determine the MIC of the plant extracts against the test bacteria, the organisms were inoculated into test tubes containing varying concentrations of 50 to 3000 µg/ml and 20.0 to 150 mg/ml of plant extract in triplicates. To determine the MIC, a loopful of the test bacteria previously diluted to 0.5 McFarland turbidity standard was introduced into each broth sample. The procedure was repeated on the test organisms in test tubes containing MHB and the standard antibiotic ampicillin (as negative control), or MHB only (as positive control). All the culture tubes were then incubated at 37°C for 24 h. After incubation, they were examined for bacterial growth by observing/measuring of turbidity.

RESULTS

The cultural, morphological, and biochemical characteristics of the bacteria isolated from the wastewater and surface (river) water samples (pH range of 6.4 to 7.2 and temperature range of 17.3 to 17.8°C) are shown in Table 1. *E. coli* on EMB displayed a green metallic sheen colour (Appendix 5a), while *Acinetobacter* spp. appeared as tiny blue and mucoid colonies, and tiny pink on LAM (Appendix 5b). Results of Gram staining revealed that *E. coli* isolates appeared as Gram-negative rods, while *Acinetobacter* spp. appeared as Gram-negative coccobacilli (Appendix 5b). Biochemical characterization revealed that both bacterial species are sulphide negative, oxidase negative, and indole positive as well as non motile, while broth culture containing *E. coli* turned Erlich's reagent red. For the Rapid NF plus test (Appendix 5g), *Acinetobacter* spp. utilized almost all the sugars and amino acids and were also able to liquefy gelatin. Slight variations in the sugar utilization and appearance of haemolysis on sheep's blood agar (ShBA, Appendix 5f) supplemented with 10 mM $CaCl_2$ differentiated the *A. haemolyticus* from the non-hamolytic *Acinetobacter lwoffii* strains.

The *E. coli* serotypes and *Acinetobacter* spp. biotypes isolated from the various samples, their verotoxic status and resistance profiles are shown in Tables 2 and 3. Results showed that, a total of 69 *E. coli* isolates including the serotypes O103:H2, O145:NM, O145:H2, O96:H9, O126, O26:H11, O55, O111:NM, O96:H9, O44, and O124 were isolated from 62 wastewater and water samples (18 wastewater samples each from wastewater treatment plant and an abattoir, respectively and 13 water samples each from River Plankenburg and River Berg, respectively). Results also showed that a total of 41 *Acinetobacter* spp. were isolated comprising 27 *A. lwoffii* and 14 *A. haemolyticus* isolates. Results of

Table 2. Various *E. coli* serotypes, their verotoxin and antibiotic resistance profiles, MDRI (%) and MIC (µg/ml) values against stem bark extract of *C. dentata.*

Sample number (mean pH/Temperature (°C)	Isolate/Serotype	Verotoxin status (Vtx1 and 2)	Resistance pattern	MDRI (%)	RIZD values (%)	MIC (µg/ml) to *C. dentata*
Wastewater (n = 18) (6.4/17.8)	*E. coli* RWW1i O103:H2	Vtx1	ᵡSXT, †OFX, **AMP, *CN,*AK	33	10.00	650.00
	E. coli RWW1ii O86	Vtx1, Vtx2	¶ATM, *AK, CL	20	16.00	250.00
	E. coli RWW1iii O145:H2	Vtx1	@TE, *CN,€CXM, **AMP	27	14.00	350.00
	E. coli RWW1iv O96:H9	Vtx1	**AML	7	8.00	750.00
	E. coli RWW1v O126	Vtx1	@TE,€CL	13.3	14.00	200.00
	E. coli RWW1vi O4	Vtx1	€CFM, €CRO	13.3	16.00	250.00
	E. coli RWW1vii O55	Vtx1, Vtx2	$CIP	7	14.00	400.00
	E. coli RWW1viii O111:NM	Vtx1, Vtx2	†OFX, †NA, **AMP, @TE	27	22.00	150.00
	E. coli RWW2i O96:H9	Vtx2	*CN, @TE	13.3	8.00	1000.00
	E. coli RWW2ii O124	Vtx1	*AK	7	14.00	400.00
	E. coli PSW1i O96:H9	Vtx1	*CN	7	16.00	200.00
	E. coli PSW1ii O145:NM	Vtx2	@TE	7	22.00	150.00
	E. coli PSW1iii O96:H9	Vtx1, Vtx2	**AML	7	16.00	250.00
	E. coli PSW1iv O111:NM	Vtx1, Vtx2	ᵡSXT	7	24.00	150.00
	E. coli PSW2i O86	Vtx1, Vtx2	ᵡSXT	7	14.00	200.00
	E. coli PSW2ii O96:H9	Vtx1, Vtx2	-	20	10.00	550.00
	E. coli PSW2iii O103:H2	Vtx1	**AMP, €CL,CRO, #IPM	13.3	14.00	300.00
	E. coli FEW1i O111:NM	Vtx2	**AML, †NA	13.3	18.00	200.00
	E. coli FEW1ii O103:H2	Vtx1	$NA, €CL	7	14.00	400.00
	E. coli FEW1iii O124	Vtx1	@TE	7	14.00	350.00
	E. coli FEW1iv O44	Vtx2	@TE	7	20.00	200.00
	E. coli FEW2i O124	Vtx2	**AMP	0	20.00	150.00
	E. coli FEW2ii O103:H2	Vtx2	-	0	24.00	100.00
	E. coli FEW2iii O145:NM	Vtx1, Vtx2	-	0	18.00	250.00
	E. coli FEW2iv O145:NM	Vtx1, Vtx2	-	0	14.00	400.00
Abattoir water (n = 18) (6.4/17.8)	*E. coli* PRE1i O4	Vtx2	**AMP, *CN, ᵡ OFX, $NA, @TE,	33	6.00	2000.00
	E. coli PRE1ii O145:H2	Vtx1	*AK	7	6.00	2500.00
	E. coli PRE1iii O111:NM	Vtx1	*CN	7	10.00	600.00
	E. coli PRE1iv O86	Vtx2	@TE,€CL	13.3	8.00	800.00
	E. coli PRE1v O4	Vtx2	€CL	7	16.00	250.00
	E. coli PRE1vi O111:NM	Vtx1, Vtx2	#IPM	0	10.00	500.00
	E. coli PRE2i O103:H2	Vtx1, Vtx2	-	0	28.00	100.00
	E. coli PRE2ii O4	Vtx1, Vtx2	-	0	20.00	250.00

Table 2. Contd.

	E. coli FSE1i O113	Vtx2	-	0	20.00	150.00
	E. coli FSE1ii O145:H2	Vtx2	€CFM, €CL, **AMP, $NA,	0	22.00	250.00
	E. coli FSE1iii O86	Vtx2	ɤOFX, ɤ ɤOFX, @TE, *AK,	33	12.00	500.00
	E. coli FSE1iv O111:NM	Vtx2	*CN	13.3	6.00	900.00
	E. coli FSE1v O96:H9	Vtx2	*CN, **AMP	7	8.00	750.00
	E. coli FSE1vi O4	Vtx2	-	13.3	20.00	200.00
	E. coli FSE2i O111:NM	Vtx2	-	0	12.00	500.00
	E. coli FSE2ii O103:H2	Vtx2	-	0	8.00	850.00
	E. coli PST1i O145:H2	Vtx1, Vtx2	-	0	10.00	500.00
	E. coli PST1ii O26:H11	Vtx1, Vtx2	@TE	0	14.00	300.00
	E. coli PST1iii O113	Vtx1, Vtx2	-	7	20.00	150.00
	E. coli PST1iv O4	Vtx2	-	0	10.00	600.00
	E. coli PST1v O96:H9	Vtx2	€CL, €CFM, €CRO	0	4.00	950.00
	E. coli PPST1vi O26:H11	VVtx2	**AML, **AMP	20	18.00	250.00
	E. coli PST2i O124	Vtx1, Vtx2	**AMP, $NA, ɤOFX, @TE, SXT	13.3	24.00	2500.00
	E. coli PST2ii O124	Vtx1, Vtx2	*AK	33	10.00	700.00
	E. coli RBU1i O86	Vtx2	*CN	7	4.00	2500.00
	E. coli RBU2i O113	Vtx2	€CL, €CFM, #IPM	7	12.00	400.00
	E. coli RBU2ii O145:H2	Vtx2	€CRO	13.3	20.00	200.00
	E. coli RBU2iii O113	Vtx2	€CRO	7	12.00	450.00
	E. coli RBD1i O113	Vtx1,	*CN	7	22.00	150.00
	E. coli RBD1ii O4	Vtx1, Vtx2	**AMP	7	16.00	300.00
River Berg (n = 13)	*E. coli* RBD1iii O86	Vtx1, Vtx2	**AML,€CRO, *AK	7	22.00	150.00
(7.2/17.3)	*E. coli* RBI1i O4	Vtx1, Vtx2	-	20	28.00	100.00
	E. coli RBI1ii O103:H2	Vtx1, Vtx2	*CN	0	8.00	750.00
	E. coli RBI2i O124	Vtx2	@TE,#IPM	7	18.00	250.00
	E. coli RBI2ii O86	Vtx2	-	7	22.00	200.00
	E. coli RBI2iii O96:H9	Vtx2	-	0	12.00	350.00
	E. coli RBI2iv O145:H2	Vtx1	-	0	22.00	200.00
	E. coli RBI2v O113	Vtx2	@TE, #IPM	0	14.00	300.00
	E. coli PRK1i O4	Vtx2	**AML, **AMP	7	20.00	150.00
River Plankenberg	*E. coli* PRK1ii O26:H11	Vtx1, Vtx2	€CRO,	13.3	26.00	100.00
(n = 13) (7.2/17.3)	*E. coli* PRK2i O145:H2	Vtx1, Vtx2	**AML, €CRO,	7	16.00	300.00
	E. coli PRK2ii O86	Vtx2	*AK, **AMP, *CN	13.3	24.00	150.00

Table 2. Contd.

E. coli PRK2iii O4	Vtx1, Vtx2	-	13.3	12.00	350.00
E. coli PRK2iii O103:H2	Vtx1, Vtx2	-	0	14.00	450.00

SXT (30 µg),Trimethroprim-Sulfamethaxazole; OFX (5 µg), Ofloxacin; ATM (30 µg), Aztreonam; AMP (10 µg), Ampicillin; TE (10 µg), Tetracycline; AK (30 µg), Amikacin; CAZ (30 µg), Ceftazidime; CL (30 µg), Cefalexin; CRO (30 µg), Ceftriaxone; CXM (30 µg), Cefuroxime; AML (10 µg), Amoxicillin; CN (10 µg), Gentamicin; CFM (5 µg), Cefixime; CIP (5 µg), Ciprofloxacin; IMP, Impenem (30 µg); NA (30 µg), Nalidixic acid. Classes of antibiotics: ᵡSulphonamides; *Aminoglycosides; **Penicillins; €Cephalosporins; @Tetracyclines ; $Quinolones.

Table 3. Various *Acinetobacter* spp. isolates, their verotoxin and antibiotic resistance profiles, MDRI (%) and MIC (µg/ml) values against stem bark extract of *C. dentata.*

Sample number (mean pH/Temperature (°C)	Isolate/Serotype	Verotoxin status (Vtx1 and 2)	Resistance pattern	MDRI (%)	RIZD values (%)	MIC (µgml) to *C. dentata*
Wastewater (n=18) (6.4/17.8)	*A. lwoffii* RWW1i	-	¶ATM, *AK, CL	20	14.00	750.00
	A. lwoffii RWW1ii	-	ᵡSXT, ᵡ OFX, **AMP *CN,*AK	33	10.00	1500.00
	A. haemolyticus RWW1v	-	@TE, *CN,€CXM, **AMP	27	8.00	1000.00
	A. lwoffii RWW1vi	-	**AML	7	24.00	250.00
	A. lwoffiiRWW2i	-	-	0	28.00	100.00
	A. lwoffii RWW2ii	-	€CFM, €CRO	13.3	20.00	350.00
	A. lwoffii PSW1i	-	@TE,€CL, #IPM	13.3	22.00	200.00
	A. lwoffii PSW1ii	-	#IPM	0	26.00	150.00
	A. haemolyticus PSW2i	Vtx1	*AK, €CXM,	13.3	14.00	700.00
	A. haemolyticus PSW2ii	-	ᵡ OFX, ᵡ NA, **AMP, @TE	27	6.00	2000.00
	A. lwoffii FEW1i	-	*CN, @TE	13.3	26.00	250.00
	A. lwoffii FEW2i	-	@TE	7	28.00	150.00
	A. haemolyticus FEW2iv	-	**AML	7	24.00	250.00
Abattoir water (n = 18) (6.4/17.8)	*A. lwoffii* PRE1i	-	ᵡ SXT	7	28.00	150.00
	A. *lwoffii PRE1ii*	-	ᵡ SXT	7	26.00	200.00
	A. lwoffii PRE2i	-	**AMP, €CL,CRO,	20	18.00	450.00
	A. lwoffii PRE2ii	-	$NA, €CL	13.3	22.00	250.00
	A. lwoffii FSE1i	-	@TE	7	24.00	200.00
	A. lwoffii FSE1ii	-	@TE	7	26.00	150.00
	A. lwoffii FSE1iii	-	**AMP	7	28.00	200.00
	A. haemolyticus FSE1iv	Vtx1,Vtx2	-	0	28.00	150.00
	A. haemolyticus FSE1v	Vtx2	**AML, □NA	13.3	28.00	250.00
	A. lwoffii FSE2i	-	#IPM	0	28.00	100.00
	A. lwoffii FSE2ii	-	-	0	26.00	150.00
	A. lwoffii PST1i	-	-	0	22.00	200.00

Table 3. Contd.

	A. lwoffii PST1ii	-	*AK, $NA, €CL	20	12.00	850.00
	A. haemolyticus PST1i	Vtx1	**AMP, *CN, ˠ OFX, $NA, @TE,	33	6.00	2500.00
	A. haemolyticus PST2i	-	*CN	7	24.00	200.00
	A. haemolyticus PST2ii	-	@TE,€CL	13.3	22.00	200.00
	A. lwoffii RBU1i	-	€CL	7	26.00	150.00
	A. lwoffii RBU2i	-	€CFM, €CL, **AMP, $NA, †OFX	33	12.00	750.00
	A. lwoffii RBU2ii	Vtx1	-	7	30.00	100.00
	*A.. haemolyticus*RBD1i	-	-	0	26.00	150.00
River Berg (n = 13) (7.2/17.3)	*A. haemolyticus*RBD1ii	-	-	0	24.00	150.00
	*A. haemolyticus*RBD1iii	-	†OFX, $NA	13.3	10.00	900.00
	*A. haemolyticus*RBI1i	-	-	0	28.00	150.00
	A. haemolyticus RBI2i	-	@TE, *AK, $NA	20	12.00	600.00
	A. lwoffii RBI2ii	Vtx1,Vtx2		7	24.00	200.00
	A. lwoffii RBI2iii	-	*CN	13.3	24.00	250.00
River Plankenberg (n = 13) (7.2/17.3)	*A. lwoffii* PRK2i	Vtx2	*CN, **AMP	0	26.00	150.00
	A. lwoffii PRK2ii	-	-	0	28.00	150.00
	A. lwoffii PRK2iii	-	-	0	22.00	200.00

SXT (30 µg), Trimethroprim-Sulfamethaxazole; OFX (5 µg), Ofloxacin; ATM (30 µg), Aztreonam; AMP (10 µg), Ampicillin; TE (10 µg), Tetracycline; AK (30 µg), Amikacin; CAZ (30 µg), Ceftazidime; CL (30 µg), Cefalexin; CRO (30 µg), Ceftriaxone; CXM (30 µg), Cefuroxime; AML (10 µg), Amoxicillin; CN (10 µg), Gentamicin, CFM (5 µg), Cefixime, CIP (5 µg), Ciprofloxacin; IMP, Impenem (30 µg); NA (30 µg), Nalidixic acid. Classes of antibiotics: ˠSulphonamides; * Aminoglycosides; **Penicillins; €Cephalosporins; @Tetracyclines ; $Quinolones.

screening of the isolates for verotoxin production showed that 26 (53.06%) of the 49 *E. coli* isolates obtained produced verotoxins Vtx1, 14 (28.57%) produced Vtx2, and 29 (59.18%) produced Vtx1 (Table 2), while only 6 (14.63%) of the 41 *Acinetobacter* isolates produced verotoxins with 2 (4.88%) producing Vtx1, and 3 (7.32%) producing Vtx2, with none of the *A. lwoffii* isolates producing the toxins (Table 3).

The antimicrobial susceptibility profiles and MDRI of all the bacterial isolates against different classes of antibiotics (beta-lactams, cephalosporins aminoglycoside, quinolones, and carbapenems) are also shown in Tables 2 and 3. Results showed that 50 (72.46%) of the *E. coli* and 31 (71.61%) of the *Acinetobacter* spp. isolates showed resistance to either one or more of the antibiotics tested. Results showed that 13 *E. coli* isolates were resistant to the beta-lactam antibiotic: ampicillin (10 µg); 6 were resistant to the cephalosporins: cefuroxime and cephalexin; ceftazidime (30 µg in each case); 12 isolates were resistant to the aminoglycoside: tetracycline (30 µg); and 5 isolates were resistant to the carbapenem: impenem (30 µg). Results also showed that 5 of the 10 *A. lwoffii* isolates are resistant to the beta-lactam antibiotic: ampicillin (10 µg); 4 were resistant to the quinolones: ofloxacin (5 µg), cefuroxime, and ceftazidime (30 µg); and 4 of the 13 *A. haemolyticus* strains obtained were resistant to the cephalosporins: ceftriaxone, cefuroxime, and nalidixic acid (30 µg); 4 were resistant to the aminoglycosides: amikacin (30 µg) and tetracycline (30 µg); and 3 isolates were resistant to the carbapenem: impenem (30 µg). All the isolates were susceptible to aztreonam (5 µg), gentamicin (10 µg), cefotaxime (30 µg), and ciprofloxacin (5 µg). Results of MDRI showed MDRI values ranging 7 to 33.00% for both *E. coli* and *Acinetobacter* spp. Also, 12 (17.39%) of the *E. coli* isolates and 10 (24.39%) of both *E. coli* and *Acinetobacter* spp.

Table 4. Phytochemical constituents of aqueous stem bark extracts of *C. dentata*.

Extract	Extraction (%)	Phytoconstituents							
		Saponins	Tannins	Alkaloids	Glycosides	Anthraquinones	Flavonoids	Steroids	Phenols
Water extracts	58.82	+	+	-	+	+	+	+	+
Ethanol	38.72	+	+	-	-	+	-	+	+
Dichloromethane	18.73	+	+	-	+	-	+	-	+
Acetone	22.64	+	+	-	-	+	+	+	+

+ = present; - = absent.

Isolates, respectively showed resistance to 3 or more different classes of the antibiotics tested. Tables 2 and 3 also showed the MIC values of each of the isolates against stem bark extracts of *C. dentata.* Results showed that the MIC values ranged from 150 to 300 µg/ml (percentage relative inhibition zone diameter (%RIZD) 4 to 28) for *E. coli isolates* and 150 to 2000 µg/ml (%RIZD 6 to 30) for *Acinetobacter* spp.

Table 4 shows results of the phytochemical screening of stem bark (percentage extraction: water (58.82%), ethanol (38.72%), dichloromethane (18.73%), and acetone (22.64%) of *C. dentata*. Results showed the presence of saponins, tannins, glycosides, anthraquinones, flavonoids, steroids, and phenols.

DISCUSSION

Sanitation and absence of clean drinking water remain a major challenge to developing countries. According to the World Bank, as many as 2 billion people lack adequate sanitation facilities to protect them from water-borne disease, while 1 billion lack accesses to clean water altogether, and on the other hand, sewage disposal is increasingly threatening water bodies worldwide. According to the United Nations, 95% of the world's cities, including the developing countries still dump raw sewage into their water supplies (UN Water, 2011). Consequently, 80% of all the health maladies, in developing countries are related to unsanitary water. Amongst the health maladies diarrhea caused by MDR, *E. coli* continues to be the major challenge (WHO, 2002). Isolation of MDR *E. coli* from the wastewater and river water samples in this study further corroborates an already alarming phenomenon globally for which scientists are battling to curtail.

Acinetobacter spp. have recently gained increasingly significant attention due to their ability to develop extreme multi-drug resistance and as causative agents of drug resistant severe nosocomial infections accounting for 34% mortality and 43% deaths (Vila et al., 2002; Dorsey et al., 2004; Vanbroekhoven et al., 2004; Prashanth and Badrinath 2005; Joshi et al., 2006; Robinson et al., 2010). In this study, both the *E. coli* and *A. haemolyticus* isolates were not only MDR; they were also associated with verotoxin production, while none of the *A. lwoffii* isolates produced verotoxins. Grotiuz et al. (2006) first reported verotoxin production by *A. haemolyticus* isolates. Although, *E. coli* O157 is the most reported, none of the O157 verotoxic *E. coli* (*E. coli* O111:H8, O26:H11, and O103:H2) have been associated with severe human disease outbreaks, such as 11 to 15% of cases of traveler's diarrhea in healthy persons visiting developing countries, childhood diarrhea and traveler's diarrhea in Mexico and North Africa as well as other human illnesses (Savarino et al., 1996; Ewing, 1999; Guth et al., 2000, 2003; Galane and Le Roux, 2001; Chandran et al., 2008; Doughari et al., 2009b). Both *E. coli* and *Acinetobacter* spp. disease outbreaks have been linked to contaminated raw ground beef, raw seed sprouts or spinach, raw milk, unpasteurized juice, unpasteurized cheese, and foods contaminated by infected food workers via fecal-oral route (Galane and Le Roux, 2001; Doughari et al., 2009b). In addition the poor state of hygiene and unhygienic handling of foods, especially in the developing countries, are common predisposing factors to infection.

Resistance of both *E. coli* and *Acinetobacter* spp. to three or more classes of antibiotics including carbapenems, with high MDRI (7 to 33.00%) is a cause for concern. Carbapenems are currently the preferred antibiotics effective in the treatment of infections associated with *Acinetobacter* spp. and Enterobacteriaceae (Savov et al., 2002; Gülmez et al., 2008). Previous reports showed that some *E. coli* strains have demonstrated resistance to ampicillin, cefuroxime, cloxacillin, ceftazidime, ofloxacin, nalidixic acid, and amikacin (Santiago-Mercado and Hazen, 1987; Salvadori et al., 2004; Cardonha et al., 2004; Chandran et al., 2008; Haghi et al., 2010). Resistance to carbapenems is simply heightening the already gravely deteriorating chemotherapeutic challenges confronting health workers globally.

The two rivers from which bacteria were isolated (River Plankenburg and River Berg) are located near informal settlements (Kayamanndi and Mbekweni, respectively). The Plankenburg River is one of the three tributaries that combine to make up the Eerste River in Stellenbosch, South Africa. Though, the high level of pollution of this river has been the subject of many workshops, newspaper articles, and even parliamentary caucus

(Nleya and Jonker, 2005), this work is the first documented evidence directed at investigating verotoxicity and multidrug resistance among *E. coli* and *Acinetobacter* spp. in the river. On the other hand, 65% of the Berg River (also called Great Berg River is located just in the north of Cape Town in the Western Cape province of South Africa, approximately 294 km long with a catchment area of 7,715 km² (2979 mi²) and outlets into the Atlantic Ocean area under agriculture. Both Kayamanndi and Mbekweni are located in the upper catchment area of these two rivers, respectively. Kayamandi lies adjacent to the Plankenburg River and according to the Department of Water Affairs and Forestry (2001), measurements in this river show very high levels of *E. coli* and other pollutants in the river. Downstream farmers use the water for irrigation of grape fields. These farmers have complained about the possible impacts of the pollution on their ability to export the grapes, as the polluted irrigation water remains between the grapes, and overseas importers often refuse the grapes because of this. The river also serves as a source of water for downstream users, and poses a health hazard to recreational users (Department of Water Affairs and Forestry, 2001). Contamination of these rivers poses a health risk to the populations in these two informal settlements due to possible contaminated food and drinking water sources. Many informal settlements are confronted with inadequate, broken or open toilet facilities which are prone to leakages that may result in further faecal contamination of the water bodies.

The coexistence of *E. coli* and *Acinetobacter* spp. in all the water samples investigated is also a cause for concern. It has been reported that the rate of adaptative mutations in *E. coli* is on the order of 10^{-5} per genome per generation, which is 1,000 times as high as previous estimates (Imhof and Schlötterer, 2001). The theoretical implication of this is that the exchange of antibiotic resistance factors between *E. coli* and *Acinetobacter* spp. in this mixed culture via mechanisms such as horizontal gene transfer, conjugation or via resistant plasmids (George et al., 1991; Dzidic and Bedekovic,' 2003; Barbe et al., 2004; Chandran et al., 2008; Ishii and Sadowsky, 2008; Willey et al., 2008) can simply mean further spread of antibiotic resistance. Plasmid transfer process to other bacterial species is said to be readily facilitated when *E. coli* is subjected to stress (Aibinu et al., 2007). The use of partially treated wastewaters investigated for irrigation purposes or discharge into rivers may further serve as medium for disseminating these resistant bacteria.

The presence of phytoconstituents in the extracts of *C. dentata* accounts for the demonstration of antibacterial activity against *E. coli, A. haemolyticus* and *A. lwoffii* (Doughari et al., 2008). The low MIC values (150 to 3000 µg/ml, %RIZD 4 to 28 for *E. coli* and 150 to 2000 µg/ml, % RIZD 6 to 30 for *Acinetobacter* spp.) is an indication that the plant extracts contain antibiotic substances which, when purified, will provide very effective alternatives to the treatment of infections caused by these resistant strains of bacteria. To the best of our knowledge, this is the first documented work on the effect of *C. dentata* extracts on MDR verotoxic bacteria.

The presence of verotoxin producing MDR *E. coli* other than O157:H7 and *Acinetobacter* spp. in the environment (waste water and surface river waters) as revealed from this study highlights the need to adopt more proactive measures to prevent outbreak of diarrheal diseases and dissemination of MDR strains. The need for proper cooking of food, boiling of drinking water, prevention of cross-contamination, adoption of good hygienic practices, such as wearing of gloves by food workers, institution of health care policies so food industry employees seek treatment when they are ill, pasteurization of juice or dairy products, and proper hand washing requirements must be emphasized. Efficacy of *C. dentata* in this study is an indication that the plant has the potential to provide an alternative source of antimicrobials that can be used in controlling these MDR pathogenic bacteria are currently being investigated. Therefore, the effect of various extracts of *C. dentata* on the virulence as well as verocytotoxin production by the test bacteria is also investigated. The occurrence of verotoxin producing VTEC in other environmental samples, further purification of *C. dentata* extracts, and determination of the most active components as well as toxicological studies should be carried out with the view of utilizing the plant in the development of novel and more effective antibiotics.

ACKNOWLEDGEMENT

This study was supported by Cape Peninsula University of Technology, Cape Town South Africa through the University Research Fund RP03.

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APPENDIX

Appendix 1. Baumann's Enrichment Medium (BEM) Composition.

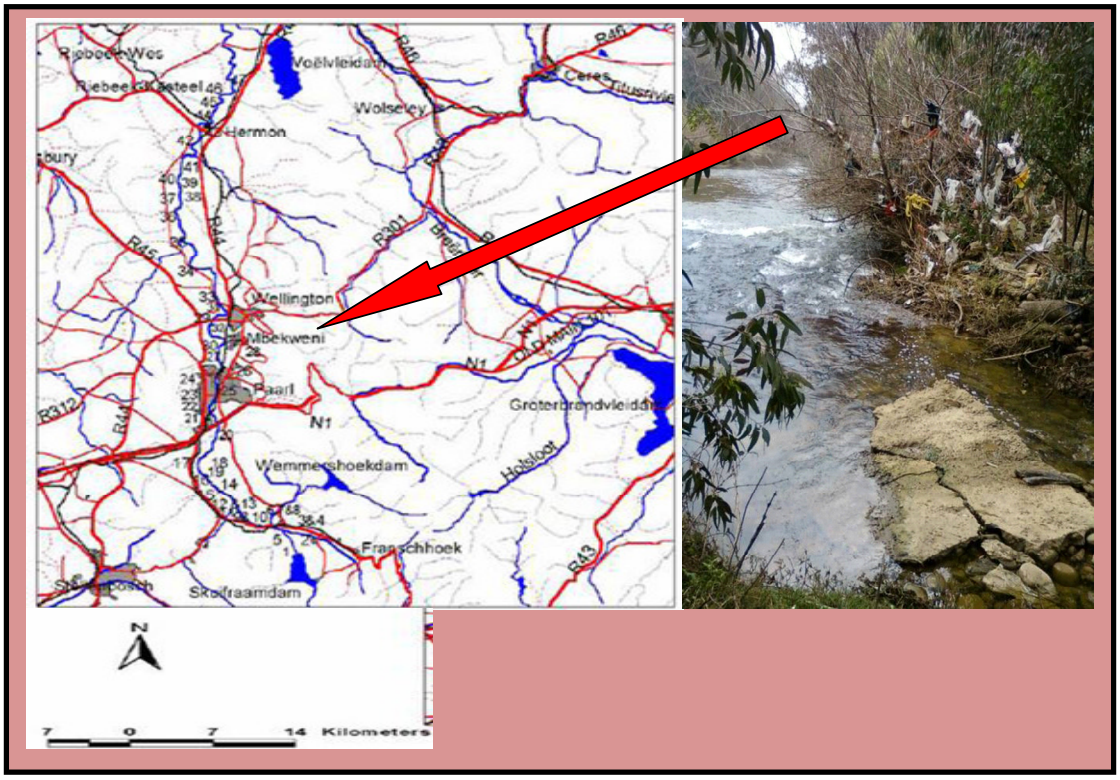
Baumann's Enrichment Medium (BEM) Composition (g/l)	g
Sodium acetate (trihydrate)	2
Potassium Nitrate (KNO3)	2
Magnesium sulphate (MgSO4.7H2O)	0.2
Potassium Dihydrogen Phosphate-	0.04 M
Disodium Hydrogen Phosphate (KH_2PO4-Na_2HPO_4) buffer	pH 6.0
Make up to 1 liter with distilled water.	

Appendix 2. Leeds Acinetobacter Medium (LAM) composition.

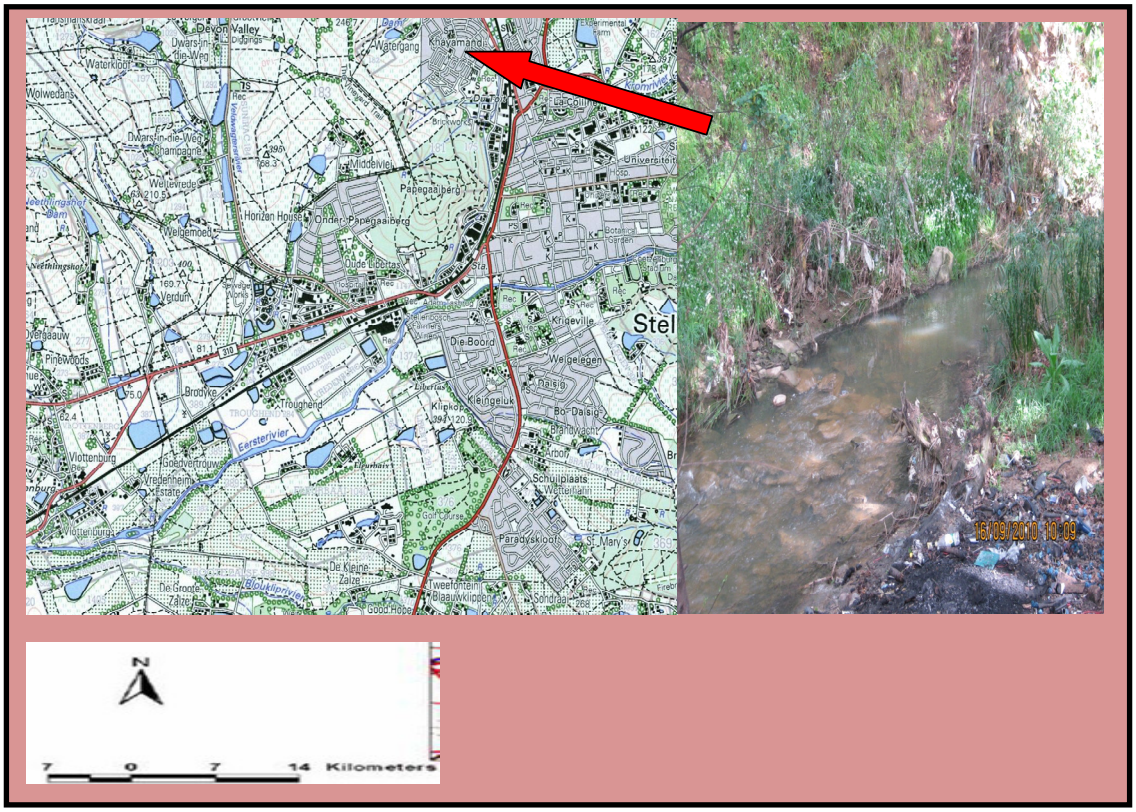
Leeds Acinetobacter Medium (LAM) composition(g/l)	g
Agar	10.00
Acid hydrolyzate of casein	15.00
Soy peptone	5.00
NaCl	5.00
D-fructose	5.00
Succrose	5.00
D-mannitol	5.00
L-phenylalanine	1.0
Iron ammonium citrate	0.4
Phenol red	0.02
Make up to 1 l with distilled water, autoclaved and cool to 50ºC and Add antibiotic solutions of Vancomycin	10.00
Cefsulodin	15.00
Cefradine	50.00

Appendix 3. Casaminacid yeast extract (CAYE) broth composition.

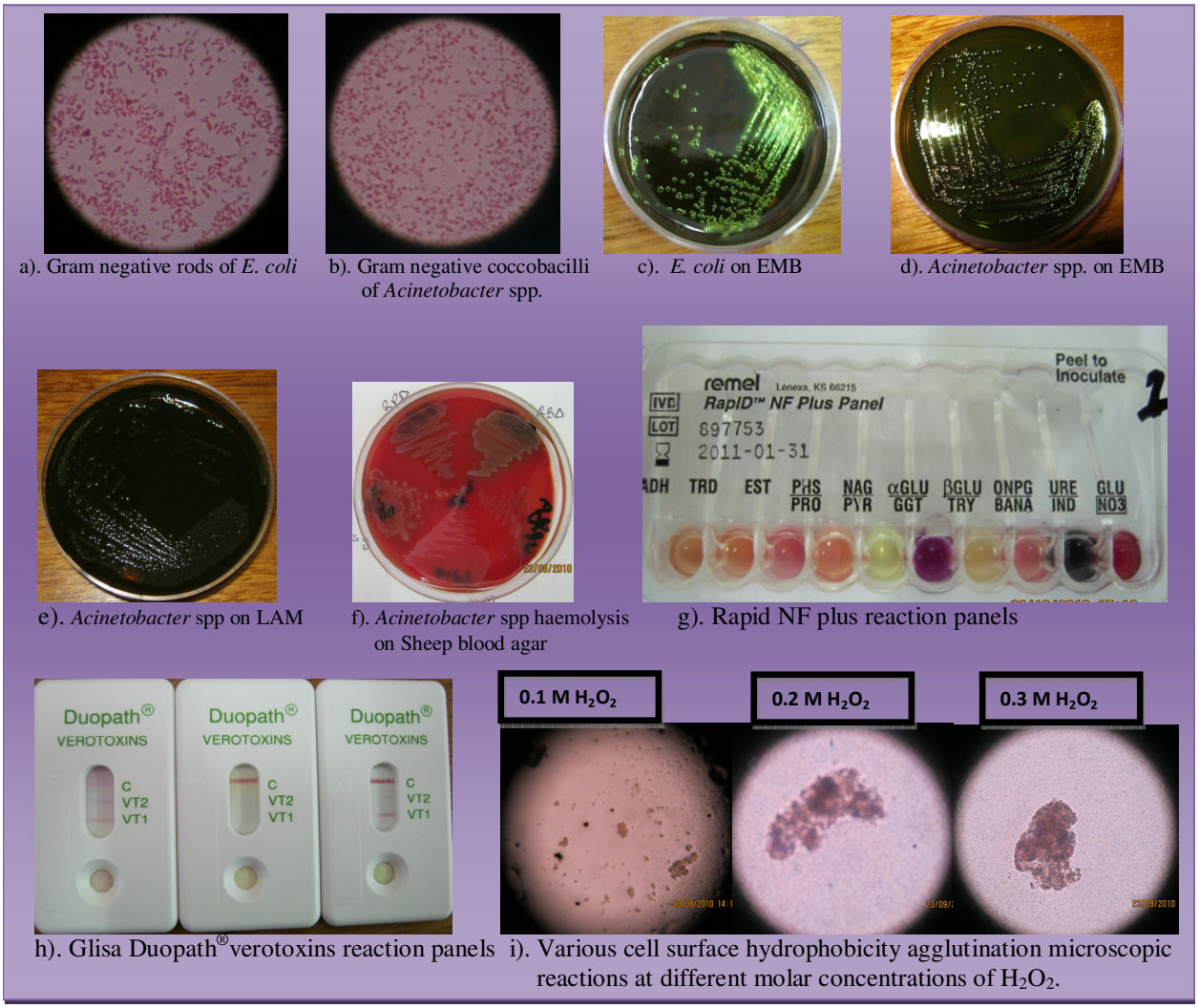
Casaminacid yeast extract (CAYE) broth composition(g/l)	g
Casamino acid	20.00
Yeast extract	6.00
NaCl	2.50
KH_2PO_4	8.71
Trace salt solution – (0.5% $MgSO_4$, 0.5% $MnCl_2$ and 0.5% $FeCl_3$ dissolved in 0.0005 M H_2SO_4)	1.00 ml



Appendix 4a. River Berg sampling site at Mbekweni Cape Town, South Africa.



Appendix 4b. River plankenburg sampling site at Kayamandi Cape Town, South Africa.



a). Gram negative rods of *E. coli*

b). Gram negative coccobacilli of *Acinetobacter* spp.

c). *E. coli* on EMB

d). *Acinetobacter* spp. on EMB

e). *Acinetobacter* spp on LAM

f). *Acinetobacter* spp haemolysis on Sheep blood agar

g). Rapid NF plus reaction panels

h). Glisa Duopath® verotoxins reaction panels

i). Various cell surface hydrophobicity agglutination microscopic reactions at different molar concentrations of H_2O_2.

Appendix 5 (a-i). Cultural and biochemical properties and, Gram reaction of *Acinetobacter* spp. and *Escherichia coli* isolates.