EFFECT OF STRESS, ANTIBIOTICS AND PHYTOCHEMICALS ON VEROTOXIC ISOLATES OF *ACINETOBACTER HAEMOLYTICUS* AND *ESCHERICHIA COLI* OBTAINED FROM WATER AND WASTEWATER SAMPLES

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DECLARATION

I, **Hamuel James Doughari (Student ID: 208222278)**, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

Water related issues such as water treatment and distribution have become extremely important all over the world due to population growth, growing urbanization, health and environmental pollutions. Contamination of water bodies especially in Africa with antibiotic resistant bacteria strains is a cause for concern. *Escherichia coli* O157 H:7, and various strains of non O157 *E. coli* and *Acinetobacter* spp. are known for antibiotic resistance. Both bacteria are environmental organisms found coexisting together with high potentials of exchange of resistance genes. Despite the stress conditions confronting these bacteria in water, food and the human body, in the form of disinfectants, antibiotics, salts and the innate immunity, they appear to develop adaptive mechanisms that enable them survive and cause infection. This therefore necessitates the need for investigation of effective virulence factor-targeted control measures.

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) for the isolation of *E. coli* and *Acinetobacter* spp. was carried out. Isolates were investigated for virulence factors, antibiotic resistance and transformation of resistance genes. The effect of oxidative stress exerted by 0.3% Crystal violet, 0.3% Bile salt, 4.0% NaCl, and 8% ethanol on some of the multi-drug resistant strains as well as the effect of stem back extracts of *Curtisia dentata* on verotoxin production by the verotoxic strains was also investigated.

Out of the 69 isolates of *E. coli* (including O26:H11, O55, O111:NM, O126, O44, O124, O96:H9, O103:H2, O145:NM and O145:H2.) and 41 isolates of *Acinetobacter* spp. with 26 (53.06%) of the *E. coli* and 6 (14.63%) of the *A. haemolyticus* isolates producing verotoxins, and no *A. lwoffii* isolate produced the toxins. Twenty five - 25(35.23%), 14(20.30%) and 28(40.58%) of the *E. coli* isolates were positive for VTx1&2, Vtx1 and Vtx2 respectively, 49(71.015%), were positive for extended-spectrum beta-lactamases (ESBLs), 7(77.78%) for serum resistance, 57(82.61%) for cell surface hydrophobicity, 48(69.57%) for gelatinase production and 37(53.62%) for haemolysin production. While transformation occurred among the *E. coli* and *Acinetobacter* isolates (transformation frequency: 13.3×10^{-7}- 53.4^{-7}), there was poor curing of the plasmid genes, a confirmation of presence of stable antibiotic resistant genes (DNA concentration between 42.7-123.8 µg) and intra-genetic transfer of multidrug resistant genes among isolates.

Oxidative stress due to chemicals, salts, alcohol or freeze-thawing (blow temperature stress) exerted various degrees of lethality on *E. coli* isolates with some bacterial strains losing their potential to express virulence factors with time. There was however, generally insignificant (*t* test; $P \leq 0.05$) lethal effect against all the *A. haemolyticus* isolates, but crystal violet exerted the highest lethal effect on some individual isolates followed by ethanol, bile salt and NaCl. Isolates from wastewater demonstrated the highest rate of resistance compared to isolates from river water. The cell kill index (CKI) increased as temperature stress (-5; -18; and -28ºC) increased with time. But the rate of loss of expression of virulence factors or viability was slower in isolates from wastewater and abattoir compared to those from river water. Sixty percent of the *E. coli* isolates showed various levels of resistance to different antibiotics

(ampicillin (10 µg), cefuroxime, cephalexin, ceftazidime and tetracycline (30 µg in each case)) (multidrug resistance index (MDRI) values 4.20-5.60%). Relative inhibition zone diameters (RIZD) of *C. dentata* extracts against *E. coli* serotypes ranged between 8-28% (MIC, 100-2500 µg/ml), while against *A. lwoffii* and *A. haemolyiticus*, the RIZD values ranged between 10-28% (MIC, 100-850 µg/ml) and 6-28% (MIC 150-2500 µg/ml) respectively. However, higher MICs (MIC, 70-2500 mg/ml) were recorded for isolates with high MDRI values. Extracts demonstrated inhibitory action against the expression of both Vtx1 and Vtx2 genes in *E. coli*, *A. haemolyticus* and *A. lwoffii*. Saponins, tannins, glycosides, anthraquinones, flavonoids, steroids, phenols quinones, anthocyanins, amines and carboxylic acids were present in *C. dentata*. Ethanol root bark extracts consistently showed the highest DPPH radical scavenging activity (62.43%), total phenol content (TPH) (57.62 26 mg GAE/g) and reducing power (RP) (41.32%), followed by those of the stem bark and leaf extracts with the respective values of 54.68%, 37.77 mg GAE/g and 21.83%. The extracts also induced the leakage of Na^+ and K^+ ions from both test bacteria.

Detection of virulence factors, antibiotic resistance genes and transformation among these isolates is a very significant outcome that will influence approaches to proactive preventive and control measures and future investigations. Resistant verotoxic *A. haemolyticus* could further complicate treatment in verotoxic food-borne or nosocomial infections. Induction of cationic leakage by extracts of *C. dentata* is an indication of one of its mechanism of action on bacterial cells. The plant can therefore be a good source of antibiotic substances for composition as antioxidants or antimicrobials with novel mechanism of action for the treatment of verotoxic bacterial infections.

PAPERS PUBLISHED/FORTHCOMING OR UNDER REVIEW

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12	**James Hamuel Doughari,** Patrick Alois Ndakidemi, Izzane Susan Human and Spinney Bennade. (2009). Shiga toxins (verocytotoxins)	*African Journal of Microbiology Research.* 3(11): 681-693.

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PAPERS PRESENTED/ACCEPTED FOR PRESENTATION AT INTERNATIONAL CONFERENCES

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3	Effect of stem bark extracts of *Curtisia dentata* on multi-drug resistant verotoxic *Escherichia coli* and *Acinetobacter* spp. *Bondo University College and Cape Peninsula University of Technology Joint Workshop on Food Security. 20th – 3rd February, 2011. Bondo, Kenya.*	Presented
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BIOGRAPGHICAL SKETCH

James Hamuel Doughari was born on the 18th of December, 1968 at Yelwa, Mayo-Belwa Local Government Area of Adamawa State Nigeria. He attended Kogin Baba and Gangnai Primary Schools between 1974 to 1982, Government Science Secondary School Ganye between 1982-1986 all in Adamawa State, Nigeria. He later proceeded to College of Education Jalingo, Taraba State, Nigeria between 1986 to 1989 where he obtained N.C.E Bio/Chem. University educational institutions he later attended and the qualifications obtained include - Abubakar Tafawa-Balewa University Bauchi, Nigeria (1993 – 1998): B. Tech Applied Microbiology, (Second Class Upper Division), Federal University of Technology, Yola Nigeria (2002-2005): M. Tech Medical Microbiology. In 2009, he enrolled for his Doctoral degree in Environmental Health with the Cape Peninsula University of Technology, Cape Town South Africa where he graduated in 2012. James H. Doughari has served the Federal Republic of Nigeria as a National Youth Service Corp (NYSC) member with the Federal Medical Center Yenagoa, Bayelsa State, Nigeria in 2000. He has worked at the Microbiology Laboratory unit of the Specialist Hospital Yola, and the Post Primary Schools Management Board Yola, Adamawa State, Nigeria (1991- 2002). James later joined the Department of Microbiology, Federal University of Technology Yola, Nigeria where he is currently a full time Lecturer. He was the Head of Department of Microbiology shortly before moving to South Africa for his Doctoral studies. Professionally, James had taught and supervised over 30 students at the University and has published over 40 peer reviewed publications in various International Journals. He is a member of board of reviewers of over fifteen international Journals and a sectional editor of Intercontinental Journal of Microbiology. He has currently published 8 papers from his Doctoral thesis with 3 under review. His research interests include: emerging and reemerging infectious diseases, environmental health and water quality, microbial enzymes and drug discovery. He is happily married to Momsiri James H. Doughari an Accountant by profession and is blessed with three children: Julia James H. D (girl, 9), El Roi James H. D (boy, 6) and Jessie James H.D (girl, 4). James is a devout Christian and a Christian Leader he worships with the Lutheran Church of Christ in Nigeria (LCCN) Jimeta Cathedral. He enjoys watching football, watching detective films and playing chess.

DEDICATION

To:

- My Lord and Saviour Jesus Christ
- My dear wife, Momsiri James Doughari and
- My children:
    i. Julia James Doughari,
    ii. El Roi James Doughari and
    iii. Jessie James Doughari.

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CHAPTER EIGHT:

LIST OF ABBREVIATIONS

ESBLs = extended spectrum beta-lactamases

MBL- metallo-β-lactamase

OMP - outer membrane protein

HMP - heat modifiable protein

PBP - penicillin-binding protein

MFS - major facilitator superfamily

RND - resistance–nodulation–cell division

MATE - multidrug and toxic compound extrusion

BIMP - bacterial integral membrane proteins.

DW-distill water

DCM-dichloromethane

HX-hexane

CHL-Chloroform

AC-acetone

ET-ethanol.

SBE-stem bark extract

RBE-root bark extracts

LE-leaf extracts

DW-distilled water

DCM-dichloromethane

HX-hexane

CHL-chloroform

Vtx-verotoxin

SXT- sulphomethaxazole/trimethoprim

OFX-ofloxacin

ATM-aztreonam

AMP-ampicillin

TE-tetracycline

AK-amikacin

CAZ-ceftazidim

CL-cephalexin

CRO-ceftriaxone

CXM-cefuroxime

AML-amoxycillin

IMP-impenim

CN-gentamicin

CFM-cefixime

CIP-ciprofloxacin

NA-nalidixic acid

CDD -stem bark extracts of *Curtisia dentata.*

HBSS - Hank's balanced salt solution

CDM - chemically defined medium

ROS-reactive oxygen species

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CHAPTER ONE

1.0 INTRODUCTION

1.1 VEROCYTOTOXIC DIARRHOGENIC BACTERIA AND FOOD AND WATER CONTAMINATION IN DEVELOPING COUNTRIES: A CHALLENGE TO THE SCIENTIFIC AND HEALTH COMMUNITY

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1.1 Verocytotoxic diarrhogenic bacteria and food and water contamination in developing countries: a challenge to the scientific and health community

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1.1.1 ABSTRACT

Water related issues such as water treatment and distribution have become extremely important all over the world due to population growth, growing urbanization, health and environmental pollutions. The majority of drinking water sources in Africa are still the traditional ones including dams, wells, rivers, streams and ponds which might harbor or are prone to contamination with water-borne and vector born disease agents that introduce various toxins most importantly verocytotoxins into the water bodies. These toxins are responsible for various health problems including diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). These conditions often present difficult chemotherapeutic control measures due to physiological complexity development of antimicrobial resistance among the pathogens. Poverty, inadequate potable water supply, unhygienic environments such as slums and refugee camps are predisposing factors abundant in developing countries. There is therefore the need to investigate water bodies for the prevalence of these toxin producing bacteria and their antimicrobial resistance profiles. Medicinal plant sources with potential efficacy in controlling these bacteria should also be investigated with a view to influencing policy and chemotherapy direction for effective control measures.

Key words: Antimicrobial resistance, environmental pollution, *Escherichia coli*, *Acinetobacter haemolyticus*, slums, poverty.

1.1.2 INTRODUCTION: BACKGROUND

An increasing number of countries can be considered water stressed (Hunter, 1997). Of all environmental questions, those related to water are perhaps the most far reaching in their long-term consequences and the most difficult to tackle from the scientific and medical point of view. In many parts of the world there is a widespread scarcity, gradual destruction and increasing pollution of fresh water sources, and many nations face growing problems associated with guaranteeing an adequate drinking water supply. Today in the developing world, one person in three lacks safe drinking water and sanitation, the basic requirement for survival, health and dignity and the prospects for the future do not look any better (Hunter *et al.,* 2003). At the turn of the millennium there were approximately 6.2 billion people alive on this planet (Hunter *et al.,* 2002). By 2025 this number would have risen to 7.9 and 9.1 billion (Hunter, 1997). In the developing countries, the population of people in the urban cities alone stands at 1.9 billion and this figure is expected to increase to 3.3 billion by 2030 (Hunter *et al.,* 2002). The provision of safe drinking water for all these people will be one of the major challenges facing humanity. In addition, most of the world's 6.9 million displaced people and refugees reside in refugee camps or temporary shelters in developing countries (Houmsou *et al.,* 2010). In these often crowded environments, where provision of sanitation, clean water, food and health care services are typically inadequate, where barriers to vectors and animals carrying infectious diseases are usually absent or insufficient, and where person-to-person contact is amplified, diarrheal infections are common and often devastating. In this paper the dearth in information on the occurrence of verocytotoxin diarrhogenic bacteria in unhygienic water, food and environments in developing countries and the need to step up research in this

area with a view to developing proactive preventive measures against widespread outbreaks is highlighted.

1.1.3 Global diarrheal burden and safe drinking water

Safe drinking water or potable water is water that is free of injurious chemicals or microbial contamination (Smith, 1980). Contaminated or polluted water may contain injurious chemicals or microbial waterborne pathogens. Waterborne pathogens represent a serious and growing hazard, and infectious diseases continue to affect populations throughout the world. Other problems such as aging of water treatment infrastructures, and the increasing occurrence or perhaps, the increasing recognition and detection of organisms resistant to conventional disinfection treatments also increases the indices of these infections. Diarrheal illness thus remain the sixth leading cause of death worldwide, responsible for an estimated 2,219,000 deaths in 1998, representing approximately 4.1% of all deaths, mostly among children under the age of five (WHO, 1999; WHO, 2002). Diarrheal illness makes an even greater contribution about 5.3%, to disease burden (WHO, 1999). Most of this burden of illness (Table 1.1) falls on the inhabitants of the underdeveloped or developing world, where it is responsible for 8.1% of the disease burden, ranked second only to respiratory illness (9.1%) (Murray and Lopez, 1996). Diarrheal illness is often attributed to contaminated water (or food) consumption although the percentage specifically due to waterborne pathogens is still unknown. This is because many countries including the most advanced Organization for Economic Cooperation and development (OECD) countries do not have effective surveillance systems in place to detect waterborne disease (Hunter *et al.*, 2003). Even in those countries

with effective surveillance systems, the systems often fail to identify the sources of infection. There is therefore still considerable uncertainty about the proportion of waterborne disease outbreaks detected and the burden of such disease not associated with sporadic diseases.

Protection of drinking and recreational waters from contamination by human or animal waste in sewage, food processing wastes, and storm water runoff is therefore of paramount importance to everyone. Public health concerns include safe water (water that does not contain harmful chemicals or microorganisms in concentrations that could cause illness) and an adequate water supply (one that provides safe water in quantities sufficient for drinking and domestic purposes) (WHO, 2002). Water is unsafe for human consumption when it contains pathogenic, or disease-causing microorganisms. Pathogenic microorganisms (and their associated disease(s)) may include bacteria, such as *Salmonella typhi* (typhoid fever), *Vibrio cholerae* (cholera), *Shigella* (dysentery, shigellosis), viruses, such as poliovirus or Hepatitis A virus and protozoa such as *Giardia lamblia* (giardiasis) or *Cryptosporidium parvum* (cryptosporidiosis). *Giardia* is a protozoan parasite that infects the upper portion of the small intestine of humans and many other species of mammals (Oyeleke and Istifanus, 2008). The usual mode of transmission is from person-to-person through what is termed the "fecal-oral route."

438 Table 1.1. Global burden of diarrheal diseases in children < 5 years

S/No.	Region	Population < 5 (millions)	Snyder & Merson, 1982	Episode per child		
				IOM, 1986	Bern & Glass, 1994	Annual cases (Millions)
1	Africa	89.8	2.2	5	2.5	197-450
2	Asia	351.0	2.2	3	2.3	772-1053
3	Latin America	62.5	2.2	4	3.9	137-250
	Total					1106-1753

439 (Hunter *et al.*, 2003)

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The least common mode of transmission is waterborne. *Cryptosporidium* is a protozoan parasite, like *Giardia,* both humans and animals may serve as sources of environmental contamination and human infection. In 1993-1994, cryptosporidiosis caused by *C. parvum* was the leading cause of illness associated with contaminated drinking water in the United States (Yoder and Beach, 2007). Other disease outbreaks during that time were caused by *G. lamblia*, *Salmonella*, *Shigella*, *Campylobacter jejuni*, and *V. cholerae* (Environmental Protection Agency, 1989).

Acute diarrhea, the second biggest killer of children under 5 years old worldwide, is very high in urban communities where there is lack of sufficient housing, sanitation and clean water (UNESCO, 2003; Thapar and Sanderson, 2004). Of recent concern is the emergence of verocytotoxin (Shiga toxin) producing bacteria that contaminates water and food sources (Grotiuz *et al.*, 2006). Verocytotoxin or Shiga toxin-producing bacterial strains are associated with a broad spectrum of human illnesses throughout the world, ranging from mild diarrhea to hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Ewing, 1999; Guth *et al.*, 2000). Currently, hundreds of distinct serotypes of *E. coli* are recognized as Shiga toxin *Escherichia coli* (STEC) associated with human diseases (Savarino *et al.*, 1996). *E. coli* serotypes such as O157:H7, O111:H8, O26:H11, O103:H2, referred to as enterohemorrhagic *E. coli* (EHEC) and bearing the *eae* gene and the EHEC plasmid, are more frequently related to severe human illnesses (Guth *et al.*, 2000; Guth *et al.*, 2003; Doughari *et al.*, 2009). Verotoxin producing *Acinetobacter haemolyticus* (VAH) is also gaining significance due to increasing reports of multi-drug

476 resistance among various isolates. Unfortunately, despite the lethal nature of these diarrheal
477 infections associated with verocytotoxic bacteria, in both adults and children, little research is
478 done in this area in the developing countries.
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480 Domestic and wild animals are reported as sources of verocytotoxin producing
481 microorganisms; but cattle, sheep and goat are considered as the main reservoirs (Beutin *et al.*,
482 1998). Excreta from these animals frequently contaminate water bodies including drinking and
483 recreational waters especially in Africa. Foods of animal origin, especially ground beef,
484 probably contaminated during the grinding process, have been identified as the main vehicles
485 for transmission of *E.coli* O157:H7 and other non-O157 STEC strains to humans (Cameron *et*
486 *al.*, 1995; Grotiuz *et al.*, 2006). Foods like raw milk, fruits and vegetables, as well as cross
487 contamination due to inadequate food manipulation, and person-to-person transmission have
488 already been associated with human disease (Bergamini *et al.*, 2007). Infected cattle, via
489 contaminated meat and dairy products, elevate the risk that these foodborne pathogens will
490 enter the human food chain (Wells *et al.*, 2001). Food deprivation and transportation to the
491 slaughterhouse stress the cattle's immune systems and induce fecal shedding of the bacteria.
492 Contamination then occurs through interchange of fecal matter between carcasses. Thus water
493 sources, used either for drinking, recreational or domestic purposes can also be affected
494 (Pedersen *et al.*, 2006). Human sewage is also source of fecal contamination and is known to
495 contain pathogenic microorganisms. Direct and indirect exposure to sewage has been
496 associated with illnesses from drinking and recreational water sources (Wade *et al.*, 2006).
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1.1.4 The challenges of supply of potable water and the risk of diarrheal infections

A water supply may come from (i) rain or snow, (ii) surface water (shallow wells, rivers, ponds, lakes and wastewater), (iii) ground water (deep wells and springs). Generally, surface water contains more microbes than do either underground or rain water (Smith *et al.,* 2003). Surface water contains many microbes from the soil, and in the vicinity of cities is often contaminated with sewage bacteria. Generally sources for microbes in water are many - soil, air, decaying bodies and excreta of humans and animals, consequently building up pathogenic populations (ECDGE, 2001). Water related issues such as water treatment and distribution have become extremely important all over the world due to population growth, growing urbanization, health and environmental pollutions. Municipal water supplies are purified or treated to get rid of harmful substances or reduce them to the minimum permissible limit to make them safe and fit for human consumption or suitable for the intended general domestic uses (IOM, 1986). However, the majority of drinking water sources in Africa are still the traditional ones including dams, wells, rivers, streams and ponds which might harbor or are prone to contamination with water-borne and vector born disease agents (IOM, 1986; Zvidzai *et al.*, 2007). In addition to the poor water supply, there is a limited resource for water treatment and distribution and worst still, inadequate sanitation that usually results in the fecal contamination of surface and ground water.

From the global perspective, waterborne disease remains one of the major health problems in the developing world, especially for young children (UNESCO, 2003; Younes and Bartram, 2001; Wright *et al.*, 2004). It is estimated that 80% of all illnesses are linked to use of water of

poor microbiological quality (Snyder and Merson, 1982). The World Health Organization (WHO) currently estimates that 1.1 billion people worldwide lack access to improved water supplies and 2.4 billion do not have access to proper sanitation facilities (WHO, 2000). In Africa, despite the efforts made by some countries, approximately 340 million people are without access to safe drinking water and only 26 countries will reach the water target. The situation of sanitation is even more worrying as 580 million people do not have access to improved sanitation facilities, and only six countries will achieve the 'millennium development goals (MDG)' target for sanitation (WHO/UNCEF, 2008; Abong'o and Momba, 2009) set by most governments.

Under these circumstances, water supplies can become a very efficient means of transmitting enteric infections within and between communities. Several estimates have been made of global pediatric morbidity associated with diarrheal disease. One of the estimates by Bern and Glass (Bern and Glass, 1994), suggests that the number of diarrheal episodes per child per year ranges from 2.3 in Asia to 3.9 in Africa (Table 1.1). The magnitude of the overall disease burden associated with pediatric diarrhea, estimated to be between 1106 and 1753 million cases per year, is staggering, and the proportion of this disease that is directly or indirectly associated with poor water quality and inadequate water quantity is difficult to determine (Snyder and Merson, 1982). Furthermore, it has been reported that 2/3 of diarrheal outbreaks, 1/2 of hospitalized cases, 1/2 of "dysentery" cases and 4/5 of outpatient cases in the developing countries remain undiagnosed further compounding the problem (Sherwood and Gorbach, 2004). A closer examination of data from 22 studies of diarrhea incidence in Africa,

Asia and Latin America indicates that the highest disease rates are in children 6 to 11 months of age (Hunter *et al.,* 2003). This vulnerable time in a child's life is when water and weaning foods are introduced into the child's diet. At this time levels of maternal antibodies are declining as the child's immune system begins to produce its own antibodies. In addition, the child begins to crawl, thus coming into contact with dusty and dirty floors as well as objects that are frequently introduced into the child's mouth. Thus, multiple transmission routes of infectious agents aswell as poor sanitary state of the mother, increase the disease burden in the infants (Hunter *et al.,* 2003).

Water supply in developing countries is bedeviled by several problems as compared to the developed world:

1. There is a wide spectrum of drinking water sources used in developing countries. Many of these water sources are unprotected, often have high levels of fecal contamination, and are used with little or no treatment. The microbiological quality of these water sources can be quite poor. Fecal or thermo tolerant coliform concentrations in drinking water sources have been reported up to 100,000 per 100 ml (Table 1.2). But WHO guidelines for drinking water quality recommend that no thermo tolerant coliform bacteria be detectable in any 100-ml sample (Hunter *et al.,* 2003). Piped water supplies in developing countries are also vulnerable to contamination due to illegal connections and pressure loss.

2. In tropical areas, ambient water temperatures are warmer (typically around 30ºC) than waters in temperate climates. Traditional measures of microbiological water quality such as total or fecal coliform indicator bacteria may not be appropriate for tropical source waters because of higher ambient temperature and nutrient loads in the water.

3. Many households do not have a water tap or pump within the house or compound. Water is collected and transported within a variety of vessels. Transport and storage of water in contaminated vessels have been shown to be a source of water contamination (Roberts, 2001; Wright *et al.*, 2004; Hoque *et al.*, 2006).

4. Fecal pathogens are transmitted by multiple routes due to poor sanitation, food hygiene and personal hygiene. These routes are closely linked to waterborne transmission and make it difficult to assess the risk of disease associated solely with drinking water. Often both inadequate water quality and water quantity contribute to waterborne disease (Hunter *et al.*, 2003).

586 Table 1.2. Reported microbiological quality of domestic water sources in developing countries

S/No	Country	Water source	Fecal coliforms per 100 ml
1	Gambia	Open, hand-dug wells	Up to 100,000
2	Nigeria	Open, hand-dug wells	200-580
3	Philippines	Open dug wells	190[a]
4	Uganda	Hand-dug wells	8-200
5	Tanzania	Open wells	343
6	Tanzania	Protected wells	7
7	Lesotho	Unprotected springs	900
8	Lesotho	Protected springs	200
9	Philippines	Springs	72[a]
10	Philippines	Boreholes	3[a]
11	Philippines	Municipal piped water	3[a]
12	Philippines	Community piped water	188[a]

587 [a] = Geometric mean fecal coliform concentration per 100 ml (Hunter *et al.,* 2003).

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Despite innovations of strategies which include the provision of protected sources such as boreholes, standpipes, protected wells and springs for tackling this problem, the facilities however, are located some distances requiring transportation to homes (Ahmed *et al.,* 1998). During transportation, water gets contaminated with bacteria which grow and proliferate during storage in the homes, consequently posing a risk of infection with water-borne pathogens (Wright *et al.,* 2004; Hoque *et al.,* 2006). Many parts of Africa have been associated with high pit latrine coverage (Taulo *et al.,* 2008). These latrines often collapse because of poor soils (sand) on which they are dug. Leaching of pit latrine contents and flooding of human and animal wastes into drinking or recreational water sources during rainy season could be possible sources of contamination (Mathess *et al.,* 1988). Furthermore, recreational waters can also be contaminated considering that fingers are prone to faecal contamination during toilet use, cross contamination of water bodies is therefore very easy thus promoting occurrence of diarrhoeal disease outbreaks. The potential of water to harbour microbial pathogens and causing subsequent illness is well documented for both developed and developing countries. Dysentery caused by *Shigella* spp. (fecal bacteria) for example is a public health problem in many regions of the world, and is very significant in the developing countries (Luo *et al.,* 2002). Several pathotypes of *E. coli* are also responsible for the rising incidences of infantile diarrhea all over the world and especially in developing countries (Table 1.3). The problem is becoming complicated with the rapid increase in multidrug resistance among pathogenic microbes, rendering most antibiotics currently used for treatment less or ineffective (Levy, 2005). Monitoring of these water bodies for pathogenic microbes and antimicrobial resistance is therefore very important.

620 Table 1.3. Epidemiology of *E. coli* pathotypes causing diarrhea in developed and developing

621 countries

S/No	*E. coli* pathotype	Epidemiology	
		Developed Countries	Developing Countries
1.	Diffusely adherent (DAEC)	? Up to 10% of cases in the UK	?
2.	Enteroaggregative (EAggEC)	Rare - mostly sporadic cases	Common - persistent
3.	Enterohaemorrhagic (EHEC)	Rare epidemics in contaminated food	diarrhea
4	Enteroinvasive (EIEC)	Rare - food borne	Rare
5.	Enteropathogenic (EPEC)	Very rare	Endemic
6.	Enterotoxigenic (ETEC)	Common	Common cause of persistent diarrhea
			Very common

622 (Thapar and Sanderson, 2004)

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Inadequate diagnosis of verocytotoxic bacteria in clinical, food and environmental samples is a widespread problem in Africa. *E. coli* and *Acinetobacter* infections have assumed a very threatening clinical significance especially in Africa, due to the deplorable hygienic conditions, inadequate water supply and over crowding particularly in rural areas. Increasing multi-drug resistance, verocytotoxin production and ability to withstand harsh and unfavorable environments and antibiotic selective pressure in hospital environments has further heightened the prowess of the organisms to cause human infections. Reports on African dysentery outbreaks attributed to *Shigella* spp. sometimes indicate that specimens were not tested for verotoxic bacteria or do not describe laboratory methods that are suitable for detecting EHEC (Wittenberg, 1999; Raji *et al.*, 2006). This is unfortunate because the spectrum of clinical illness resulting from *Shigella* spp infection overlaps considerably with that of *E. coli* and mixed outbreaks have been reported (Wittenberg, 1999).

1.1.5 Challenges of unhygienic environments

Most developing countries are bedeviled with poor and unhygienic conditions. This is predicated largely on poverty and ignorance where people are more concerned and preoccupied with struggling for survival. In addition, most inhabitants live in slums. In such areas, unhygienic practices such as disposal of filled septic tanks directly into gutters, throwing of faeces wrapped in polythene bags directly into streams, rivers or the nearby bush or burying the solid faecal matter in soil, are very common. Most urban centers are also characterized by huge refuse dumps in which faeces, left over and decaying foods, animal carcasses and rotting vegetable parts are left for ages. Such practices and environments facilitate the rapid spread of

gastrointestinal pathogens amongst the population, especially the enteric bacteria. Food and water bodies easily gets contaminated due to runoffs or floods thus serving as ready sources of human infection. Outbreaks of diarrhea and cholera have been reported especially among school children or refugee camps in various developing countries such as Nigeria, Rwanda, Congo, Zimbabwe, Sudan Afghanistan, Chile and Brazil (IOM, 1986; Thapar and Sanderson, 2004; Field, 2003). Urban populations in developing countries are characterized by much higher densities of people, poor housing, inadequate sanitation and solid waste removal, and unsafe drinking water, thus more people are forced to share the same toilets or spaces in rented apartments or slums. In such conditions, diarrheal and other bacterial infections are much more easily transmitted. It has been reported that of the population of the developing countries, 1. 1 billion people do not have access to safe drinking water, and 2· 4 billion are without adequate sanitation (Thapar and Sanderson, 2004). This underlies the need to investigate drinking and recreational water, food, water bodies and wastes for the presence of important diarrhogenic agents including verocytotoxygenic bacteria.

Although occurrence of Shiga toxin producing bacteria in different animal reservoirs have been reported (Giraldi *et al.,* 1990; Griffin and Tauxe, 1991; Saridakis, 1994), data concerning isolation of these microorganisms from drinking and recreational water bodies, especially in Africa, is limited and in most cases absent. In addition, though there are few reports on multi-drug resistant EHEC (Table 4), there is none on *Acinetobacter* spp and reports on research into antimicrobial resistance (including resistance to traditional herbs) amongst verotoxin producing bacteria is completely lacking. Even where Shiga toxin producing *E. coli* were

678 reported, the information was scanty in most regions of Africa, and totally absent in others.
679 The few studies carried out were also concentrated on stool samples, not much research was
680 carried out on water samples. With the prevalence in abundance of predisposing factors such
681 as poor hygiene, poverty, inadequate medical care and potable water, Africa is highly
682 vulnerable to such and other bacterial infections.
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684 *E. coli*, member of the enterobactereaceae family in addition to diarrhea and other
685 gastroenteritis, is also associated with urinary tract and ear infections. Other serotypes also
686 produces Shiga toxin and has been implicated amongst other enteric bacteria in many water
687 and food contaminations. Of recent, *A. haemolyticus* of the Moraxellaceae family has also
688 been reported to produce Shiga toxins and the bacterium is also reported to be a notorious
689 causative agent of multidrug resistant nosocomial infections (Cergole-Novella et al., 2006;
690 Doughari *et al.*, 2009). There is thus the need to investigate drinking and recreational water
691 sources for the occurrence or contamination by members of Enterobactereaceae (*E. coli*) and
692 Moraxellaceae (e.g. *A. haemoliticus)* both known to be causative agents of gastroenteritis and
693 nosocomial infections, for Shiga-toxin production and other virulence and resistance factors
694 including resistance genes and beta lactamase enzymes, and susceptibility of these Shiga-
695 positive isolates to some medicinal plants traditionally used in treating diarrhea-related
696 infections. Such study approach will provide information such as whether:
697 i). there are *A. haemolyticus* and *E. coli* present in some wastewater samples in South Africa;
698 ii). the *A. haemolyticus* and *E. coli* isolates produces extended spectrum betalactamases,
699 verocytotoxins and other virulence factors; iii). there are multidrug antimicrobial resistant

strains among the *A. haemolyticus* and *E. coli* isolates; iv). low-temperature exerts any stress on the viability and virulence of the *A. haemolyticus* and *E. coli* isolates; v). chemical agents, ionic salts and phytochemicals have any impact on the viability and virulence of the *A. haemolyticus* and *E. coli* isolates; and vi). phytochemicals have any effect on the verotoxin and betalactamase producing-multidrug resistant strains of the *A. haemolyticus* and *E. coli* isolates.

1.1.6 Current status of research on verocytotoxic bacteria in Africa

Since the first reported case and description of *E. coli* O157: H7 in the United States of America (USA) in 1982, EHEC has become an important public health problem worldwide. Morbidity and mortality associated with O157:H7 and the threat to public health of infections with EHEC O157 and other EHEC in particular, led the Public Health Laboratories Services (PHLS) to develop interim guidelines for control (CDSC, 1996). Given the magnitude and severity of recent outbreaks of *E. coli* O157: H7 infection, there is an urgent need to reduce the human hazard caused by this pathogen (Raji *et al.*, 2006). Despite the increasing medical significance of these agents, only few reported outbreaks of *E.coli* O157 in Africa have been documented (Table 1.4), and there is relatively no information on the occurrence of *Acinetobacter* and other verocytotoxin producing bacteria.

722 Table 1.4. Status of research on verocytotoxic diarrhogenic *E. coli* and other bacteria in

723 African countries

S/No	Region/Country	Type of Sample Investigated	Method used	Authors
A	**South Africa**			
1	South Africa	Stool	Sorbitol MacConkey agar (SMAC), pulsed field gel electrophoresis patterns.	(Browning *et al.*, 1990)
2	Swaziland, South Africa	Stool, water	Molecular techniques, culture on CT-SMAC agar, chromogenic	(Effer *et al.*, 2001)
3	Gouteng, South Africa	Stool	Rainbow agar O157 medium,	(Galane and Le Roux, 2001)
4	South Africa	Water, sewage	Immunomagnetic separation (IMS), PCR, Immunoassay	(Muller *et al.*, 2003)
B	**East Africa**			
1	Kampala, Uganda	stool (infants, cattle)	Sorbitol MacConkey agar (SMAC)	(Kaddu-Mulindw *et al.*, 2001)
2	Nairobi, Kenya	milk	PCR	(Arimis *et al.*, 2000)
3	Ifaraka, Tanzania	stool	Sorbitol MacConkey agar (SMAC	(Gaswn *et al.*, 2000)
4	Ifaraka, Tanzania	beef	Sorbitol MacConkey agar (SMAC	(Hayghaimo *et al.*, 2001)
C	**West Africa**			
1	Lagos, Nigeria	stool (children and adults)	Sorbitol MacConkey agar (SMAC)	(Akinyemi *et al.*, 1998)
2	Lagos, Nigeria	stool	Sorbitol MacConkey agar (SMAC), colony blot hybridization	(Ogunsanya *et al.*, 1994)
3	Lagos, Nigeria	Stool (children and adults)	Sorbitol MacConkey agar (SMAC)	(Okeke *et al.*, 2000)
4	South Western Nigeria	stool	cytotoxicity in verocells, PCR	(Olorunshola *et al.*, 2000)
5	Lagos, Nigeria	Stool (cattle)	Sorbitol MacConkey agar (SMAC),	(Eduardo *et al.*, 2000)
6	Cote d'Ivoire	Stool (children and adu	PCR	(Dadie *et al.*, 2000)

7	Gabon	Stool (children and adu	Sorbitol MacConkey agar (SMAC), PCR Sorbitol MacConkey agar (SMAC)	(Presterl *et al.*, 2003)
D	**Central Africa**			
1	Zémio, DRC Congo	Stool (bloody diarrhea)	Sorbitol MacConkey agar (SMAC)	(Germanii *et al.*, 1997)
2	Cameroun	Stool (bloody diarrhea)	Sorbitol MacConkey agar (SMAC)	*(Germanii *et al.*, 1998)
E	**North Africa**			
1	Middle Egypt	Stool	Sorbitol MacConkey agar (SMAC)	(Abdul-Raouf *et al.*, 1996)

*only study with antimicrobial susceptibility testing of verocytotoxic bacteria including Enterohaemorrhagic *E. coli* and MDR *S. dysenteriae* type 1 and *S. boydii*

1.1.7 Significance of investigating food and water for verocytotoxic diarrhogenic bacteria in developing countries

Research on water contamination by bacteria producing protein toxins and their antimicrobial resistance profiles is of considerable interest for several reasons. A number of the protein toxins are produced by bacteria and are responsible for severe diseases caused by these organisms (Sandvig and van Deurs, 1994). This is, for instance, the case for diphtheria toxin (although vaccination of the population has helped to control this disease), and for pseudomonas toxin, tetanus toxin, botulinum toxin, and Shiga toxin. Verocytotoxins or Shiga toxins are not only produced by *S. dysenteriae,* which is the infective agent in dysenteries, but also produced by *E. coli* and some species of *Acinetobacter*, giving rise to infections and diseases resulting into serious health problems in several countries (Sandvig and van Deurs, 1994). Knowledge about the toxins and their action on cells is important for the understanding of these diseases. Furthermore, protein toxins have long been used to construct immunotoxins and other toxin conjugates in attempts to find more efficient drugs in the therapy of cancer and other diseases (Olsnes *et al.,* 1989). Also, toxins are very attractive tools in modern cell biology, for instance, with respect to the study of protein translocation across membranes, protein internalization by endocytosis, sorting along the endocytic pathway, and exocytosis. Monitoring organisms for toxins and virulence factors will give more understanding of their physiology for possible development of more effective control measures by research, medical, academic and public health institutions.

Monitoring resistance of emerging and reemerging environmental pathogens is important to detect emerging resistance that may pose a concern for human and animal health and to guide in prescribing decisions. An understanding of the molecular basis of resistance and virulence amongst the Enterobactereaceae and Moraxellaceae will be created, consequently more versatile control measures will be developed. More information on bacterial isolates from water with potential for Shiga toxin production and their antimicrobial resistance profile will also be provided for documentation and policy formulation. There are reports of increasing multi drug resistance of Shiga toxin producing bacteria against antimicrobial agents (Armand *et al.,* 2006; Cergole-Novella *et al.,* 2006). It is also reported that because antimicrobials may cause the lyses of bacterial cell walls, with the liberation of Shiga toxins, and/or the increased expression of the toxin genes *in vivo*, they are not recommended for treating STEC infections (Cergole-Novella *et al.,* 2006). According to Abong'o, and Momba (Abong'o and Momba, 2009), one of the major problems that accompany *E. coli* O157:H7 infection is the danger of treating such patients with antibiotics. This is because treatment of *E. coli* O157:H7 infections with antibiotics may result in the release of Shiga toxins into the blood stream of the infected individuals. It is believed that the release of such toxins affects the kidneys resulting in a condition described as hemolytic uremic syndrome. This presents a great challenge in the treatment approach to be adopted against these pathogens. Africa is richly endowed with medicinal plants, therefore it is important to investigate the potential of these plants in controlling specific verocytotoxin and other toxin producing bacterial pathogens with a view to discover novel mechanisms of action against them for effective control.

1.1.8 CONCLUSION AND RECOMMENDATIONS

First estimates of the global burden of childhood mortality and morbidity became available in the early 1980s. Diarrhoeal illnesses accounted for about 4· 6 million deaths from around 1 billion episodes of diarrhoea every year in children younger than 5 years. The burden of diarrhoeal illness sits firmly in the developing world, both for morbidity (6–7 episodes per child per year compared with 1 or 2 in the developed world) and mortality. Malnutrition and the wholly inadequate provision of safe water, sanitation, and hygiene highlight the stark inequalities that exist within our world. A quarter of children in developing countries are still malnourished, and a reasonable population do not have access to safe drinking water and adequate sanitation (1· 1 billion and 2· 4 billion respectively). Furthermore, urbanization in developing countries has resulted in people migrating from the rural to urban centers with its attendant consequences. Urbanization, a process related to economic and political factors, has a direct bearing on the health of urban dwellers which are already confronted with serious problems such as high population densities with inadequate housing, poor or absent sanitation and water supply, weak health infrastructure, degrading and unhealthy environment with litters around houses. These are well known conditions that favour disease transmission (Houmsou *et al.,* 2010). Water contamination with bacterial agents and their toxins (especially verocytotoxins) undoubtedly accounts for these alarming health problems, especially infant diarrhea. Until improved hygiene and effective control measures are adopted, the goals (MDGs) for sound health by most African governments will continue to remain a challenge. In addition to improved hygiene and provision of clean potable water (Raji *et al.,* 2006), deliberate and vigorous research efforts by research institutions into verocytotoxin producing

bacteria, and their antimicrobial resistance profiles as well as search for effective control agents from the abundant plant resources is one of the important measures to tackle the problem. Important public health measures such as educating the public on the dangers of eating undercooked meat, and drinking unboiled or untreated water, and increasing awareness among clinicians about infections with *A. haemolyticus*, and other *E. coli* pathotypes and mandating case reporting will go a long way in controlling infections associated with these bacteria in Africa.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 SHIGA TOXINS (VEROCYTOTOXINS)

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2.1 Shiga toxins (Verocytotoxins)

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2.1.1 ABSTRACT

Shiga toxins (Stxs) also called Verocytotoxins (Vtxs) and Shiga-like toxins (SLTs), are bacterial toxins produced by some members of the Enterobacteriaceae particulary *Shigella dysenteriae* and *Escherichia coli* O157:H7 as well as *Acinetobacter* spp (Moraxellaceae), *Enterobacter cloacae* and *Aeromonas hymophilus.* The toxin is made of two moieties, the B-moiety that is responsible for its binding to cell surface receptors, and the A-moiety which enters the cytosol and inhibits protein synthesis enzymatically. Their pathological effect in humans is mainly as a result of inhibition of cellular protein synthesis. Shiga toxins are haboured mainly by ruminants principally, cattle as well as sheep, buffaloes, pigs, goats, dogs, cats and pigeons. The two major groups of the toxin, Stx1 and Stx2 are associated with mild or bloody diarrhea to hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) and also, nosocomial infections in humans. Predisposing factors to infection with Shiga toxin producing bacteria include old age, immunosupression, malnutrition, under developed immunity in neonates, poor hygiene, lack of potable water and excreta contamination of existing traditional water sources. Transmission is through consumption of contaminated food and water, person-to-person and animal contact. High rate of antibiotic resistance amongst Stxs-producing bacteria is causing concern all over the world, therefore improved personal and food hygiene and the provision of potable drinking water appears to be the best preventative measure against the infection.

Key Words: Antibiotic resistance, diarrhea, Enterobacteriaceae, Moraxellaceae, nosocomial infection, ruminants, Shiga toxin, transmission.

2.1.2 INTRODUCTION

Shiga toxins (Stx) or Verocytotoxins (Vtxs) are produced by some strains of *Escherichia coli* of the Enterobacteriaceae family or 'coliform group. The genus *Escherichia* is a group of bacteria found as commensal flora inhabiting the gut of humans and animals. The bacteria are acquired by ingestion during the first few days after birth. *E. coli*, the medically important species of the genus, are motile Gram-negative bacilli with or without capsules. They grow over a wide range of temperatures (15-45ºC) and are able to survive under adverse environmental conditions for extended periods of time, thus creating many opportunities for exposure and infection (Cameron *et al.,* 1995; Guth *et al.,* 2002). Most strains of the bacteria are indole positive, and ferments lactose with the production of acids and gas. They can be readily cultivated under laboratory conditions on artificial media. Their colonial morphology vary from smooth colourless (on non-selective media) to pinkish or red (on MacConkey agar) with or without hemolysis on blood agar and colorless, sorbitol-negative colonies on sorbitol MacConkey agar (Koneman *et al.,* 1997).

E. coli are widespread intestinal parasites of mammals and birds and are present wherever there is faecal contamination. Certain strains, however, are pathogens in humans and animals and cause opportunistic infections (Greenwood *et al.,* 1992). Three general clinical syndromes associated with infection from pathogenic *E. coli* strains include: urinary tract infection; sepsis/meningitis; and enteric/diarrhoeal disease (Nataro and Kaper, 1998). Though the organisms are known to cause enteric infections and diarrhea (gastroenteritis), it wasn't more than two decades ago that some strains were identified to produce the toxins: Shiga toxins or

verocytotoxins. These toxins are responsible for lethal bloody diarrhea (haemolytic colitis and haemolytic uremic syndrome) in humans (Karmali *et al.,* 1983; Karch *et al.,* 1999). Recently however, *Salmonella enterica* (Enterobacteriaceae), *Acinetobacter haemolyticus* and *A. baumanni, Aeromonas hydrophila, A. cavia, Citrobacter freundii* and *Enterobacter cloacae* has also been associated with bloody diarrhea and Shiga toxin production (Paton and Paton, 2000; Pedersen *et al.,* 2006). *A. haemolyticus* and *A. baumanni* are aerobic, non motile, catalase positive and oxidase negative Gram-negative coccobacilli that belong to the Moraxellaceae family (Lambert *et al.,* 1993; Bergogne-berezin and Towner, 1996).

Shiga toxin producing *E. coli* (STEC) was first recognized as a human pathogen in 1982, in the USA, during two outbreaks of hemorrhagic colitis (HC) caused by some strains of the serotype O157:H7 (CDC, 1982; Riley *et al.,* 1983; Wells *et al.,* 1983; Karch *et al.,* 1999). In 1983, the association of *E. coli* O157:H7 and several other STEC serotypes with sporadic cases of classical haemorrhagic uremic syndrome (HUS) was first described (Karmali *et al.,* 1983) and subsequently confirmed in a prospective study (Karmali *et al.,* 1985). Since then, epidemiological studies from different parts of the world established STEC as the major cause of bloody diarrhea and HUS in temperate climates, and uncomplicated watery diarrhea in some geographic areas (Huppertz *et al.,* 1996; Karch *et al.,* 1997; Piekrard *et al.,* 1997; Caprioli and Tozzi, 1998; Griffin, 1998; Smith *et al.,* 1998; Spika, 1998). STECs are rated today as one of the most important human pathogens in the developed countries (Reilly, 1998; WHO, 1999; Kaddu-Mulindwa *et al.,* 2001). Research on STEC and associated infections in developing countries however, is at low ebb despite the rising cases of infantile diarrhea. The

understanding of these toxins, their mode of action, predisposing factors, health implications and control measures will be of importance to curtail its threat, particularly in Africa.

2.1.3 Diarrhoeagenic strains of *E. coli*

E. coli associated with diarrhoeal disease are collectively referred to as enterovirulent *E. coli*. The *E. coli* strain O157 also known as the 'hamburger bug' is now recognized as the strain with the propensity to cause several gastrointestinal disease outbreaks (Henderson *et al.*, 2000). Seven groups have been defined based on various virulence factors including toxin production and adhesion (Table 2.1.1; Nataro and Kaper, 1998). The enteroaggregative (EAggEC), enteroinvasive (EIEC), enteropathogenic (EPEC) and enterotoxigenic (ETEC) strains are common in developing countries, with ETEC and EAggEC being the cause of most cases of the dreaded travelers' diarrhea (Lawson, 2004). VTECs or STECs are directly responsible for the hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS) developed by some patients following infection with the bacteria (Cantarelli *et al.*, 2000). Subsets of STEC that are able to cause attaching and effacing (A/E) lesions similar to EPEC organisms are termed enterohemorrhagic *E. coli* (EHEC, or typical EHEC) (Cantarelli *et al.*, 2000). EHEC is rare in the developing countries, but is one of the emerging infectious diseases in the developed countries associated with bloody diarrhea (Lawson, 2004). Even though STEC serotype O157:H7 is the organisms most often implicated in large outbreaks, other serotypes including; O111:H8; O26:H11; and O103:H2, have been reported to cause a considerable number of cases of HUS in many countries (Nataro and Kaper, 1998; Hyatt *et al.*, 2001;

Safarikova and Safarik, 2001; Guth *et al.*, 2003). There is need to investigate the presence of these serotypes and possibly newer ones that might be involved in causing diarrhea in Africa.

Table 2.1.1. Diarrhoeagenic strains of *Eschecrichia coli*

E. coli Strain	Clinical symptoms	Mechanism
Enteropathogenic (EPEC)	Watery diarrhea	Pili, type III secretion
Enterohemorrhagic (EHEC	Bloody diarrhea, HUS	Shiga-like toxin
Enteroinvasive (EIEC)	Dysentry	Cellular invasion and cell-cell spread
Enterotoxigenic (ETEC)	Watery diarrhea,	Colonization factors, heat-labile/-stable toxins
Enteroaggregative (EaggEC)	Watery diarrhea, persistent disease	Fimbriae, heat-stable toxins
Diffusely adherent (DAEC)	Watery diarrhea,	Toxins?
Verocytotoxin producing (VTEC)	persistent disease	
Verocytotoxins (or Shiga toxins)	Bloody diarrhea	

HUS – haemolytic uremic syndrome (Source; Henderson *et al.*, 2000)

2.1.4 Structure and nomenclature of members of Shiga toxin (verocytotoxin) family

Shiga toxins (Stxs) or Verocytotoxins (Vtxs) or Shiga-Like toxins (SLT) are a family of structurally and functionally related exotoxins produced by enteric pathogens (Caldenvood *et al.*, 1996; Pikrard *et al.*, 1997). The discovery of *E. coli* O157: H7 in 1982 as producer of Shiga toxin and causative agent of hemorrhagic colitis (HC) and HUS made it to be considered as an emerging pathogen (Dundas, 1999; Schmitt *et al.*, 1999; O'Brien *et al.*, 2001; Khan *et al.*, 2003). SLTs are produced by *Shigella dysenteriae* (Enterobacteriaceae) serotype 1, the infectious agent associated with epidemic outbreaks of bacillary dysentery (Pikrard *et al.*, 1997; Caprioli *et al.*, 2005).

Stx belongs to a defined protein subfamily, the RNA N-glycosidases that can be classified into two antigenic groups: Shiga toxin 1 (Stx 1) or verocytotoxin 1 (Vtx 1), and Shiga toxin 2 (Stx2) or verocytotoxin 2 (Vtx 2). Stx1 is a rather homologous group with three variants (stx1, stx1c, and stx1d). The Stx2 group is more heterogeneous and comprises several subtypes (Stx2, Stx2c, Stx2d, Stx2e, Stx2f, Stx2g, and activatable Stx2d) (Table 2.1.2; Caldenvood *et al.*, 1996; Pikrard *et al.*, 1997; Collaway, 2004; Caprioli *et al.*, 2005; Grotiuz *et al.*, 2006; Vu-Khac and Cornick, 2008). All the Stxs including those secreted by *S. dysenteriae* (Shiga-like toxin 1) and Stx1 secreted by *E. coli* are identical but differ only in one amino acid (Binnington *et al.*, 2002; Leung *et al.*, 2003). Exposure to antibiotics and other chemical agents might induce mutation in the existing strains. Continued research is required for monitoring of novel genetic strains that might require a different approach to control measures.

Table 2.1.2. Nomenclature of members of Shiga toxin (verocytotoxin) family

Previous nomenclature	Proposed new nomenclature	
	Gene	Protein
Shiga toxin (Stx) or Verocytotoxin (Vtx)	*stx* or *vtx*	Stx or Vtx
Shiga toxin (Stx),Verotoxin 1 (VT1) or Shiga-like toxin I (SLT-I)	*stx1* or *vtx1*	Stx1 or Vtx1
ST2, VT2 or SLT-II	*stx2* or *vtx2*	Stx2 or Vtx2
ST2c, VT2c or SLT-IIc	*stx2c* or *vtx2c*	Stx2c or Vtx2c
ST2e, VT2e or SLT-IIe	*stx2e* or *vtx2e*	Stx2e or Vtx2e

(Source; Paton and Paton, 1998)

2.1.5 Evolution of terms: STEC EHEC and VTEC

Shiga toxin (Stx) was first named after Kioshi Shiga (Shiga, 1898) who initially described the toxin as the agent of epidemic bacterial dysentery. In 1972, it was reported that Stx alone caused fluid accumulation and enteritis in ligated rabbit intestinal segments, the discovery which later lead to the purification of the toxin (Keusch *et al.,* 1972; Olsnes and Eiklid, 1980; Khan *et al.,* 2003). Ten years after discovery, O'Brien *et al.* (1983) established that certain strains of *E. coli* produce a cytotoxin that can be neutralized by anti Stx, an observation that explains the original Shiga-like toxin nomenclature. The *E. coli* strains that produce the Shiga-like toxin (SLT) were named as Shiga toxin producing *E. coli* (STEC) showing that one of the cytotoxins produced by these organisms is essentially identical at the genetic and protein levels to the Stx produced by *S. dysenteriae* 1. Furthermore, Konowalchuk *et al.* (1977) reported that certain diarrhoeagenic *E. coli* strains produce a cytotoxin that can kill the cultured Vero cells (of the green monkey kidney) *in vitro*, hence the name verocytotoxin (Vtx). The *E. coli* strains that produced this type of toxin became known as verotoxigenic *E. coli* or verotoxin producing *E. coli* (VTEC) (Chapman, 1995). It was subsequently shown that Shiga-like toxin and Vero cytotoxin was the same toxin produced by *E. coli* O157: H7 strains. Thus, in 1983 the paths of researches on Stxs and Vtxs merged. Further research unraveled that some *E. coli* strains are capable of producing gastrointestinal lesions and hence are referred to as enterohaemorrhagic *E. coli* (EHEC). Thus, EHEC denotes a subset of STEC which are considered to be pathogens, whereas, not all STEC strains are believed to be pathogenic (Griffin and Tauxe, 1991; Nataro *et al.,* 1998; Khan *et al.,* 2003). Though Stxs, Vtxs and SLT were considered to be the same toxin, it was later realized that EHEC could produce more than one antigenically distinct Stxs.

Thus, a new nomenclature was adopted; Shiga toxin 1 (ST1) or Verocytotoxin 1 (VT1) and Shiga toxin 2 (ST2) or Verocytotoxin 2 (VT2). ST1 is equivalent to VT1 and SLT-1; and ST2 is synonymous with VT2 and SLT-II. SLT-I is identical to ShT, and is not neutralized by antibody to SLT-II. STEC has also been classified based on the host sources; STEC from the animals as ST1(a), VT1(a) or SLT-I(a) and ST2 (a), VT2(a), or SLT-II(a) and those from human sources as ST1(h),VT1(h), or SLT-I(h) and ST2(h), VT2(h), or SLT-II(h) (Fig. 2.1.1; Agbodaze, 1999).

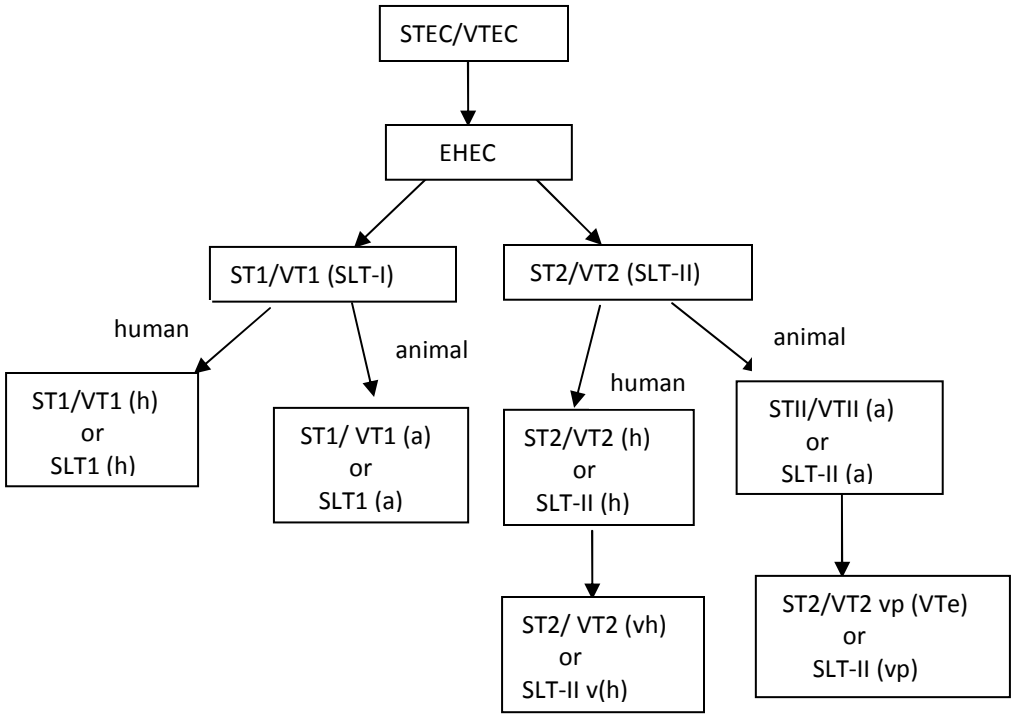

Fig. 2.1.1. Classification of Shiga toxin producing *Escherichia coli* (STEC) according to host source

2.1.6 Structure of Shiga toxins (verocytotoxins)

Structurally, all verotoxins have, in principle the AB_5 toxin structure (including Cholera and Pertusis toxins: Fig. 2.1.2 A and B; Caprioli *et al.*, 2005). One of the moiety of the toxin molecule (the B-moiety) is responsible for binding to cell surface receptors, and the other moiety (the A-moiety) for inhibition of protein synthesis (Figure 2A; Chapman, 1995; Caprioli *et al.*, 2005; Schmitt and Schaffrath, 2005). Cleavage of the A-moiety is achieved via the disulfide bond resulting into 2 fragments (A_1 and A_2) that are linked by a disulfide bond that are responsible for the cytotoxic effect of the toxin (Fig. 2.1.2B).

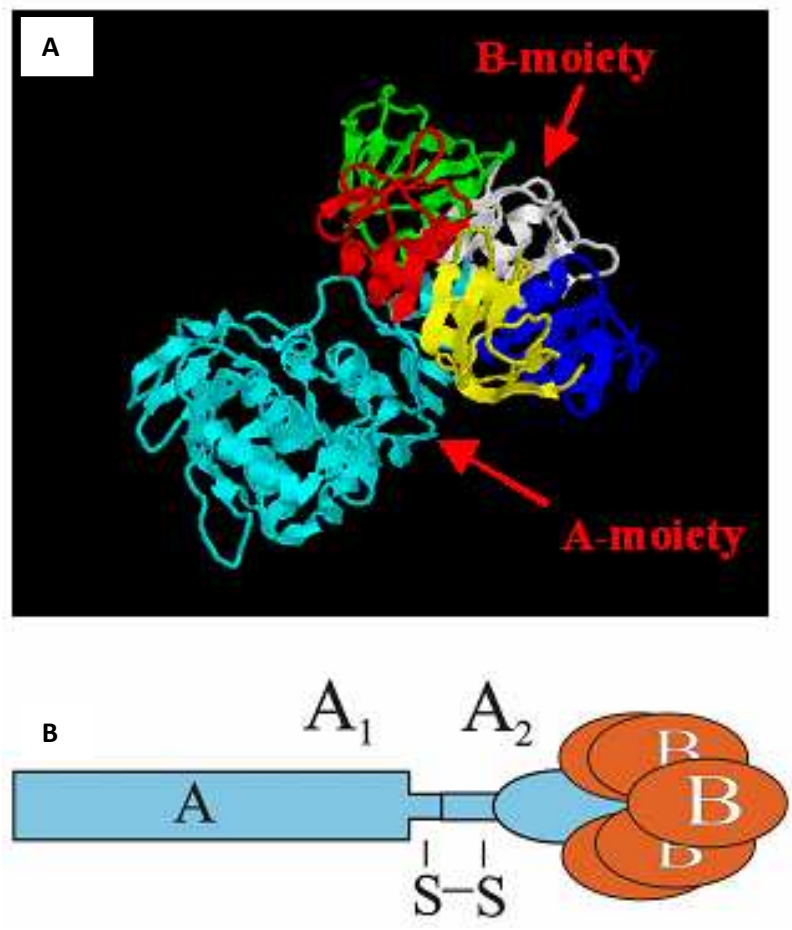

Fig. 2.1.2. Schematic (A) and crystallographic (B) structure of Shiga toxin. As indicated the A fragment of the toxin is cleaved into the A_1 and A_2 fragments (held together by disulphide bonds S-S), and the A_1 fragment can then inactivate ribosomes. The five small B fragments are responsible for binding to Gb_3. (Source; Schmitt, and Schaffrath, 2005).

2.1.7 Epidemiology and pathogenesis of Shiga toxins (verocytotoxins)

Shiga toxin-producing bacteria are ubiquitous microorganisms known to cause infection of varying severity in humans and animals (WHO, 1999; Collaway, 2004). It is now recognized that there is a very broad spectrum of human disease associated with Stx-producing organisms. Stxs are not only causal agents of diarrhea in humans in some geographical areas, but are also significant agents in at least two other life threatening infections, hameolytic colitis (HC) and haemolytic uremic syndrome (HUS) (Parry and Salmon, 1998; Agbodaze, 1999; Pulz and Matussek, 2003). The infections are, therefore, a public health problem of serious concern. HC, also referred to as ischemic colitis', is a distinct clinical syndrome that presents typically with abdominal cramps and watery diarrhoea, followed by a grossly haemorrhagic discharge resembling lower gastrointestinal bleeding. This is accompanied by little or only low grade fever with no inflammatory exudates in the stool. The lack of fever and the absence of inflammatory exudates in the stool differentiate this illness from the dysentery described in shigellosis, campylobacter enteritis, or invasive *E. coli* gastroenteritis. HUS, which was first described as a distinct clinical entity by Gasser *et al*. (1955), is defined by a triad of features: acute renal failure in childhood; thrombotic thrombocytopenia purpura (TTP); and microangiopathic haemolytic anemia (MAHA) or Moschowitz's disease. The last condition is a disease characterized by thrombocytopenia, haemolytic anaemia, bizarre neurological manifestations, azotemia (uremia), fever, and thrombosis of the terminal arterioles and the other capillaries (Agbodaze, 1999; Collaway, 2004; Grotiuz *et al.*, 2006).

Pathogenesis of Shiga toxins is a multistep process, involving a complex interaction between

a range of bacterial and host factors. After oral ingestion of the bacteria through contaminated food or water, the none-invasive bacteria adhere to the intestinal epithelial cells of the distal small bowel and colon (Collaway, 2004). As in most Gram-negative bacteria and the AB toxin system, the pathogens inject their toxins into the cytosol of host cells through bacterial transport machines that function as macro molecular syringes (Fig. 2.1.3). This leads to a rearrangement or modification of the morphology of the cells and initiation of inflammation (Paton and Paton, 1998; Collaway, 2004; Colpoys *et al.*, 2005).

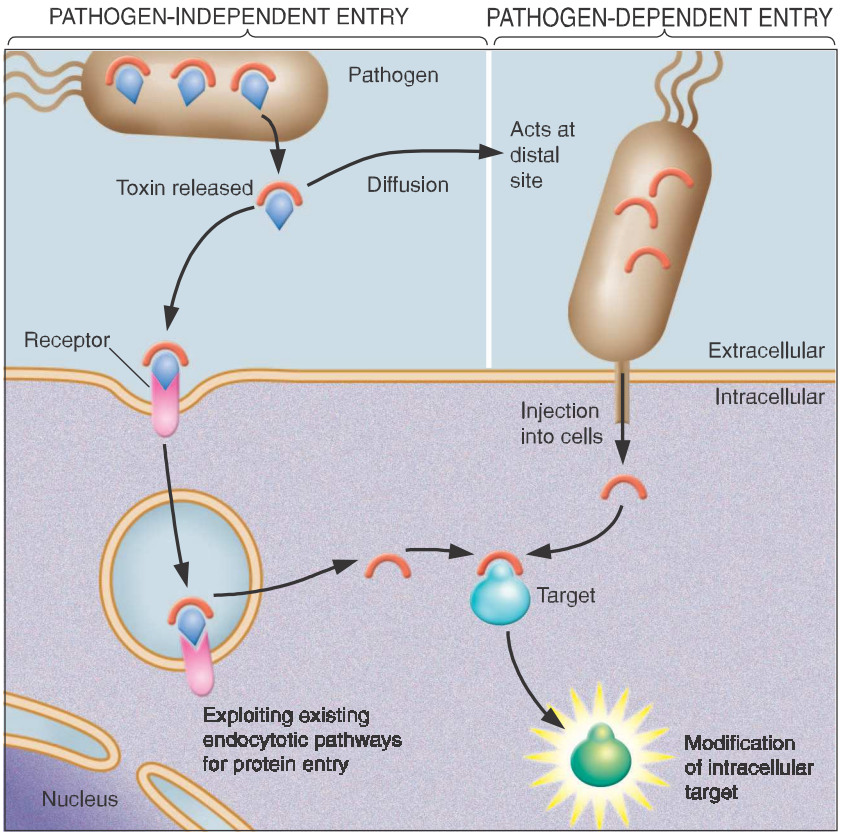

Fig. 2.1.3. Model of toxin entry into the host cell (Source; Blanke, 2006).

The bacteria (often in very low initial doses), has an average incubation period of 3-4 days and must initially survive the harsh (acidic) environment of the stomach and then compete with other gut microorganisms to establish intestinal colonization, as well as release toxins. With the help of bacterial flagellin, the toxins are first absorbed by the intestinal epithelium and then translocated to the bloodstream (Miyamoto *et al.*, 2006).

Shiga toxin can probably reach the circulation because of active transport in these cells and also passively after damage to the intestinal cells (Fig. 2.1. 4A; Acheson *et al.*, 1996). Subsequently, it is transported in the circulation to reach its primary target, the renal endothelium of the kidney. At the renal endothelium, the toxins attach to the specific toxin receptors, the globotriaosylceramide (Gb_3, Pk Antigen, CD77; Fig. 2.1.4B) present on target cell surfaces (the receptors are also found on red blood cells, platelets and B lymphocytes). Consequent of this attachment, the toxin induces both local and systemic effects (Fig. 2.1.4C; Geelen *et al.*, 2007). Gb_3 consists of a ceramide long chain fatty acid embedded in the plasma membrane, and a short extracellular trisaccharide chain terminated by a digalactose residue. The B subunit of Stx (Kd = 0.1 nM) facilitate high affinity binding of the holotoxin to the two terminal binding sites (Site I and Site II) digalactose residue of Gb_3. Stx1 and Stx2c exhibits optimum binding to Gb_3 with a fatty acyl chain lengths of 20 to 22 carbons and 18 respectively (Rivera-Betancourt *et al.*, 2004). Once bound to a target cell membrane, toxin molecules are thought to be internalized by a process of receptor-mediated endocytosis. Internalization involves the formation of a clathrin-coated pit within the cell membrane, which subsequently pinches off to form a sealed-coated vesicle with toxin bound to the internal surface.

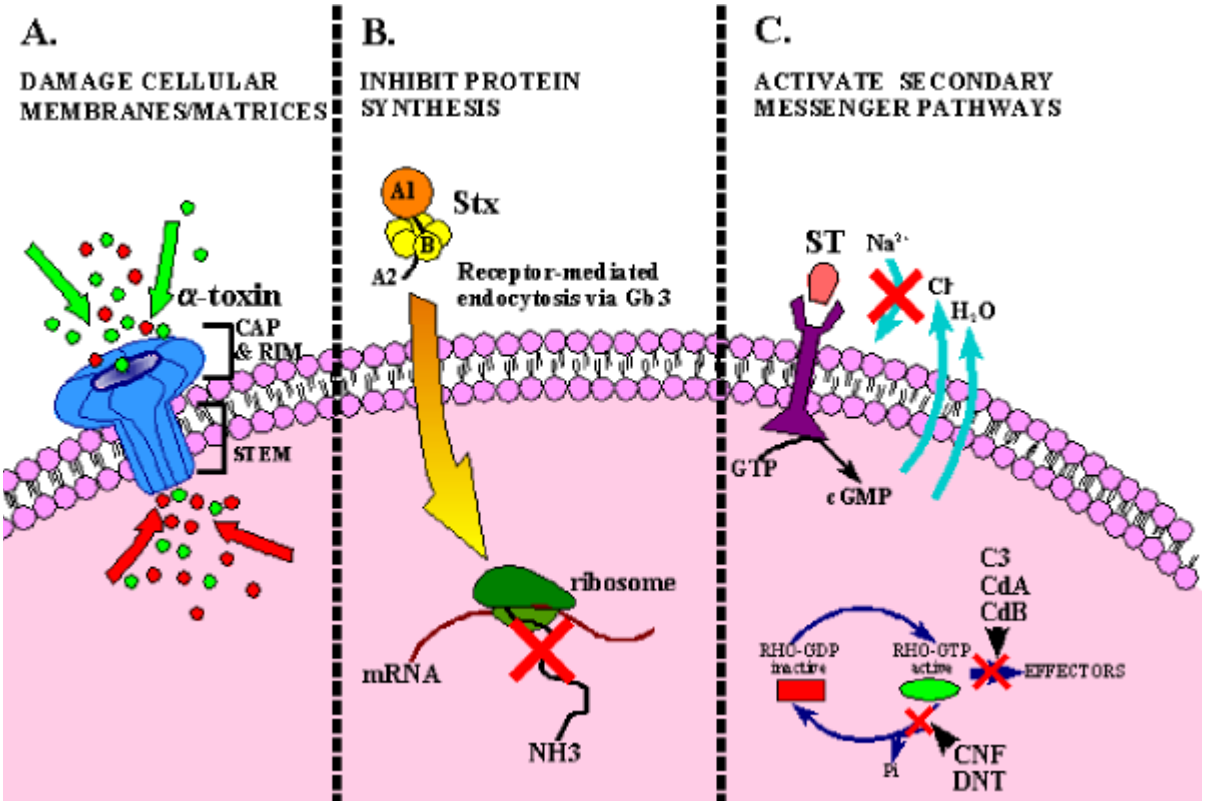

Fig. 2.1.4. Diagrammatic representation of the mode of action of Shiga toxins (Stx) (B) and other bacterial toxins. Stx binds to Gb_3 resulting in inhibition of protein synthesis. Other mechanisms of action commonly used among Stx and other bacterial toxins include: damage to cellular membranes (A) and activation of secondary messenger pathways (C) (Source; Schmitt *et al.*, 1999).

Shiga toxins, which all have one A-fragment and five B-fragments regardless of the source organisms, enter the cytosol of cells and act enzymatically on a cytosolic target (Schmitt and Schaffrath, 2005).

Once inside the cell, the low endosomal pH triggers conformational changes in the toxin molecules. The internalized toxins in some instances are successfully degraded by host lysosomes, while toxins not successfully degraded proceed to cause biological effects (Doyle and Schoeni, 1987; Ge *et al.,* 2002; Rivera-Betancourt *et al.,* 2004). Successfully internalized toxins undergo membrane insertion forming endosomal vesicles that translocates them to the cytosolic side via the endoplasmic reticulum (ER) (Lencer and Tsai, 2003). During this process, the A_1 subunit is nicked at the trypsin-sensitive site near the amino terminus by a membrane bound protease furin, generating two fragments - 28 kDa N-terminal A_1 fragment and a 4 kDa C terminal A_2 fragment (Tesh and O'Brien, 1991). The A_1 terminal fragment (28 kDa N-terminal) is catalytically active and is released from the A_1 skeleton into the cytosol, while the A_1 C terminal remain attached to the B moiety by the disulphide bonds (Fig. 2.1.2). The released catalytically active fragment has RNA N-glycosidase activity and therefore cleaves a specific N-glycosidic bond in the 28S rRNA which mediates peptide bond elongation in cellular protein synthesis. This cleavage prevents elongation factor 1-dependent binding of the aminoacyl-tRNA to the 60S ribosomal subunit, thereby inhibiting the peptide chain elongation step of protein synthesis (Khan *et al.,* 2003).

The resulting disruption of protein synthesis leads to the death of renal endothelial cells, intestinal epithelial cells, Vero cells or Hela cells or any cells which possess the Gb_3 (or Gb_4 for Stx2e) receptor. Subversion of the protein synthesis machinery results in cytotoxicity to the human renal endothelial cells, consequently damage to the glomerular (Calderwood *et al.,* 1987; Lencer and Tsai, 2003; Caprioli *et al.,* 2005; Schmitt and Schaffrath, 2005). There is also occlusion of microvascular function. These pathological effects are characterized by a lowered glomerular filteration, bloody urine and acute renal failure that characterize HUS. In the intestinal mucosa, ulceration of the intestinal walls results in bloody diarrhea, a symptom characterizing HC (Te Loo *et al.,* 2001; Collaway, 2004; Schmitt and Schaffrath, 2005). Although the major extra intestinal target organ is the kidney, virtually any organ can be involved (Amirlak and Amirlak, 2006). Consequently, the binding of toxins to glycolipid receptors on vascular endothelial cells of the central nervous system and the intestinal colon results in neurological complications and hemorrhagic colitis (or bacillary dysentery) respectively. The role of Shiga toxin in the invasion process of *S. dysenteriae* type 1, and SLT-l and SLT-11 in attachment of EHEC to colonic epithelial cells, remains unclear. However, the capacity of the bacteria to invade or adhere to colonic epithelial cells is thought to reduce dilution of the toxins in the gut and allow the toxins to be delivered to the cells in a focal, concentrated manner. Both *in vitro* and *in vivo* experiments with Shiga toxin and the SLTs have demonstrated multiple potent effects. The toxins have been shown to be: (i) directly cytotoxic for certain cell lines; (ii) enterotoxic, mediating fluid accumulation in ligated ileal loops; and (iii) paralytic-lethal when injected intravenously into mice and rabbits (Tesh and O'Brien, 1991).

Other virulence factors may play a role in Shiga toxin pathogenicity, like intimin (encoded by the *eae* A gene), which is required for intimate adherence of these pathogens to tissue culture cells and formation of the attaching and effacing (A/E) lesion (Khan *et al.,* 2003; Collaway, 2004). The formation of A/E lesions is mediated by multiple genes called the Locus of Enterocyte Effacement (LEE). Another virulence factor that contributes to verocytotoxin pathogenicity is the 60-MDa plasmid borne enterohaemolysin A gene (encoded by the E-*hly* A gene). The toxins from *E. coli* of serotype O157 or those that have specific combinations of virulence factors appear to be more virulent in mankind (Khan *et al.,* 2003; Tarawneh *et al.,* 2009).

2.1.8 Structure and organization of Shiga toxin (*Stx*) or verocytotoxin (*Vtx*) genes

The Stx or Vtx genes are invariably chromosomally located. The genes that encode Stx1 and Stx2 are carried chromosomally or by lysogenic bacteriophages. The genes that code for the A and B subunits of Stxs, stxA and stxB, are organized within an operon. The operator region of Stx/Stx1 (but not Stx2) contains a consensus fur box that is responsible for the iron regulation of Stx and Stx1 production (Karmali *et al.,* 1986; Schmitt *et al.,* 1999). The operons of the nucleotide sequences of the genes encoding Stx from *S. dysenteriae,* as well as Stx1 and Stx2 from *E. coli*, have a common structure consisting of a single transcriptional unit, encoding first the A subunit followed by the B subunit. The *stx* B-subunit gene has a stronger ribosome binding site than that of the A-subunit gene, resulting in increased translation of B subunits, thereby satisfying the 1:5 A/B-subunit stoichiometry of the holotoxin. The predicted amino acid sequences were 315, 315, and 318 amino acids long for the A subunits of Stx, Stx1, and

Stx2, respectively, and 89 amino acids for the B subunits of all three toxins (Calderwood *et al.*, 1987; Parry and Salmon, 1998; Bettelheim, 2001; Cherla *et al.*, 2003). Both A and B subunits had hydrophobic N-terminal signal sequences characteristic of secrested proteins, and the predicted *M*r values for the processed A and B subunits were in accordance with previous estimates based on analysis of purified toxins (Parry and Salmon, 1998). Interestingly, a 21-bp region of dyad symmetry spanning the 210 region was found upstream of *stx* and *stx*1, and this motif is thought to be associated with iron regulation of toxin expression (Paton and Paton, 1998). Stx and Stx1 are virtually identical (differing only in a single amino acid in the A subunit) but Stx2 had only 56% identity to the other toxins for both the A and B subunits.

There is also a significant degree of amino acid homology between the A subunits of Stx and the plant toxin ricin (Paton and Paton, 1998). An enzymatically active A subunit is none covalently associated with a binding or B component. The B subunit pentamer directs the binding of the holotoxin to sensitive eukaryotic cells via specific glycolipid receptors. Once internalized, the A polypeptide is cleaved into an enzymatically active A_1 portion and an A_2 portion; these fragments remain associated through a disulfide bond. The A_2 portion serves to link the A_1 fragment and the B pentamer. Other toxins that share this AB structure are the *E. coli* heat-labile toxin, cholera toxin, and pertussis toxin (Schmitt *et al.*, 1999).

2.1.9 Diagnostic Methods

There are a number of difficulties associated with the diagnosis of verocytotoxin infections. Diagnostic procedures are based on detection of the presence of verocytotoxin producing

genes in fecal extracts or fecal cultures, and/or isolation (culture) of the organisms. Other procedures include immunological methods, immunomagnetic separation (IMS), polymerase chain reaction (PCR) and serological methods. These procedures differ in complexity, speed, sensitivity, specificity and cost (Te Loo *et al.*, 2001; Roy *et al.*, 2004).

2.1.10 Culture and isolation of bacterial agent can be carried out on a wide range of specimens including both clinical and environmental samples. Samples such as urine, stool, rectal swabs (Vu-Khac and Cornick, 2008), drag swabs (Tarawneh *et al.*, 2009), food (Ge *et al.*, 2002), blood, meat (Vu-Khac and Cornick, 2008) hides and carcasses (Gilbert *et al.*, 2008), cloacal swabs from pigeons (Pedreson *et al.*, 2006)) water (Heijnen and Medema, 2009), wastewater from treatment plants, animal water troughs and sewage (Luo *et al.*, 2002; Heijnen and Medema, 2006) have been employed. Samples are often cultured on sorbitol MacConkey agar (SMAC) and incubated at 37ºC and examined after 18 to 24 h of incubation for the presence of colorless, sorbitol-negative colonies (Leotta *et al.*, 2006; Pizza and Rappuoli, 2006; Heijnen and Medema, 2009). The test is based on the property of most faecal bacteria not being able to ferment sorbitol, which distinguishes them from the majority of other fecal bacteria especially *E. coli* belonging to other serotypes. The sensitivity of SMAC is limited by the capacity to recognize non-fermenting colonies against the background of other organisms on the plate; this is particularly difficult when the O157 strain forms less than 1% of the flora. Improvements on the isolation rate have been made by supplementing SMAC with cefixime, to inhibit *Proteus* spp. and with cefixime and potassium tellurite (CT-SMAC) (Roy *et al.*, 2004; Tarawneh *et al.*, 2009). Recently, further improvements have been made

especially for the isolation of *E. coli* O157, by the development of a commercial agar medium, Rainbow Agar O157 containing selective agents for *E. coli.* In addition, chromogenic substrates for β-D-glucuronidase and β-galactosidase has become available for the isolation of Shiga toxin producing *E. coli* (STEC). Glucuronidase-negative, galactosidase-positive O157 strains appear as black colonies on this medium, whereas commensal *E. coli* strains are pink (Cherla *et al.*, 2003; Heijnen and Medema, 2006).

2.1.11 Immunomagnetic separation (IMS) techniques have been developed to assist in the isolation of Shiga toxin *E. coli* (principally O157) and other Stx producing bacteria from low-abundance specimens (Chapman *et al.,* 1994). The procedure involves coating magnetic beads (by use of commercial magnetic bead reagent e.g. Dynabeads, Dynal, Oslo, Norway) with anti-lipopolysaccharide (LPS) antibody and mixing them with broth cultures or suspensions of feaces or suspect food homogenates (Karmali *et al.,* 1983; Calderwood *et al.,* 1987; Parry and Salmon, 1998; Bettelheim, 2001; Cherla *et al.,* 2003; Heijnen and Medema, 2006). The beads and bound bacteria are then trapped in a magnetic field, the unbound suspension is decanted, and the beads are washed. After additional binding and washing cycles, the beads are plated and the resultant colonies are tested for reactivity with the appropriate O antiserum and, more importantly, for Shiga toxin production. IMS was reported to be 100 times more sensitive than direct culture on either cefixime-rhamnose SMAC or CT-SMA (Leotta *et al.,* 2006; Pizza and Rappuoli, 2006; Heijnen and Medema, 2009; Sepehriseresht *et al.*, 2009).

For **polymerase chain reaction (PCR)**, specific primers for detection of stx1 and stx2 genes are employed (Te Loo *et al.,* 2001). The mixture for the amplification of stx1 and stx2 genes usually consisted of PCR buffer, salt solution medium, deoxynucleoside triphosphates (dNTPs), primers and Taq DNA polymerase. The amplification conditions consisted of an initial denaturation step (e.g. at 94°C for 4 min), repeated cycles of denaturation (e.g. 30 cycles of 94°C for 1 min), followed by the annealing step (e.g. 55°C for 1 min), and the extension cycle (e.g. 72°C for 1 min, and a final extension at 72°C for 10 min). PCR products were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining with two tubes serving as negative and positive controls (e.g. a tube containing all PCR reaction mixture except template DNA used as negative control and three bacterial standard strains producing Shiga toxins; strain 1 producing Shiga toxin 1, strain 2 producing Shiga toxin 2, and strain 3 producing both of toxins used as positive controls). This then is followed by electrophoresis on agarose gel in TAE buffer (Chapman *et al.,* 1994; Blanco *et al.,* 2003; Zahraei *et al.,* 2007). Various modifications including multiplex-PCR or multiplex-real-time PCR have been employed in detection and identification of the toxin genes in various samples (Te Loo *et al.,* 2001; Zahraei *et al.,* 2007; Sepehriseresht *et al.,* 2009; Tarawneh *et al.,* 2009).

For **serological methods**, the gold standard assay for the presence of toxin in faecal specimens and isolates remains Vero cell culture. However, several commercial toxin kits are now available including two Enzyme Immunoassay (EIA) kits; ProsPecT Shiga Toxin *E. coli* (STEC) Microplate assay and Premier EHEC immunoassay, both of which have been registered by the United States Food and Drug Administration for use on stool specimens

directly or on overnight broth culture. Two other toxin assays, VTEC–RPLA and Duopath Verotoxin (DV) are recommended only for use on colony sweeps or isolates (Park *et al.*, 2003). Neutralisation tests in Vero cell cultures have shown that there is little, if any, cross reactivity between antibodies raised against each of the toxin types (Parry and Salmon, 1998; Cherla *et al.*, 2003; Heijnen and Medema, 2006). The VTEC–RPLA is a reverse-passive latex agglutination assay which differentiates between Stx1 and Stx2 and also quantifies the amount of toxin present. Anti Stx1 and anti Stx2 rabbit antibodies were located on latex particles (Yokoyama *et al.*, 2006). If Shiga toxin 1 or Shiga toxin 2 were present in bacterial supernatant, the toxins gets attached to their specific antibodies and produce a lattice at the end of ELISA microplate wells. But if there were not any of these toxins, lattice would not be formed and the latex precipitated at the end of the wells (Rivera-Betancourt *et al.*, 2004; Sepehriseresht *et al.*, 2009). Duopath Verotoxin (DV) immunochromatographic test was originally intended to confirm STEC isolates from foods (Park *et al.*, 2003). The DV test uses colloidal gold-labelled monoclonal antibodies to "trap" any Stx1 and Stx2 present in samples as they migrate over a membrane. A positive result appears as a red line within 10 minutes. Like the VTEC–RPLA the DV test is recommended for testing colony sweeps or isolates rather than primary faecal broth cultures (Bettelheim, 2001; Pulz and Matussek, 2003). Commercial serological diagnostic reagents ELISAs specific for antibodies to Stx1, Stx2, and O157 LPS have been developed to detect the minutest concentrations of toxins undetected by PCR present in a sample (Sepehriseresht *et al.*, 2009).

2.1.12 Symptoms and Transmission

Many domestic animals particularly ruminants and wildlife carrying verocytotoxin producing bacteria are asymptomatic (McClure, 2000; Collaway, 2004). Certain STEC strains however, are capable of causing diarrhea in cattle, particularly calves, cats and dogs (Anon, 2006a; 2006b). Piglet edema disease is another serious, frequently fatal STEC-related illness characterized by neurological symptoms including ataxia, convulsions, and paralysis; edema is typically present in the eyelids, brain, stomach, intestine, and mesentery of the colon (Paton *et al.*, 2001). This disease is associated with particular STEC serotypes (most commonly O138:K81, O139:K82, and O141:K85) that are not known to infect humans (Rivera-Betancourt *et al.*, 2004; Tarawneh *et al.*, 2009).

In humans, many infected patients initially suffer a watery diarrhea, but in some this progresses within 1 or 2 days to bloody diarrhea and hemolytic colitis (HC) (Table 1). Severe abdominal pain is also frequently reported (Anon, 2006a). In a proportion of patients, infection progresses to hemolytic uremic syndrome (HUS), a life-threatening sequela characterized by a triad of acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia. Some individuals with HUS experience neurological symptoms including lethargy, severe headache, convulsions, and encephalopathy (Tarawneh *et al.*, 2009). Although HUS occurs in all age groups, its incidence is higher in infants, young children, and the elderly. Indeed, it is a major cause of acute renal failure in the pediatric population. The age distribution of HUS may be a consequence of the immunological naivety of young children and declining immune system function in the elderly, although age related differences in receptor expression may contribute.

Healthy individuals can become infected, but some individuals infected with STEC may be completely asymptomatic, in spite of the presence of large numbers of organisms as well as free toxin in the faeces (Wells *et al.,* 2001; Rivera-Betancourt *et al.,* 2004; Ge *et al.,* 2002; Schmitt and Schaffrath, 2005).

Transmission of the pathogen appears to occur via three principal routes including contaminated food and contaminated drinking or swimming water sources (environmental spread), person-to-person transmission and animal contact (zoonotic) (Parry and Salmon, 1998; Tarawneh *et al.,* 2009). Contamination of meat with STEC can occur from bovine faeces during slaughter and meat processing. Not surprisingly, consumption of raw or undercooked meat (particularly ground beef) and unpasteurized milk, are the most commonly implicated foods. Cases have also been linked to the consumption of contaminated cheese, yogurt, cold cuts, lettuce, potatoes, seed sprouts, cooked maize, melon, and fresh-pressed apple juice (McClure, 2000; Vernozy-Rozand, and Roze, 2003). Secondary transmission, which may involve direct hand-to-hand contact (e.g., among children in day care centres) or indirect, e.g., via contaminated water used for swimming is possible through asymptomatic carriers. Massive outbreaks is also likely to occur in fast-food restaurant chains using a common source of ground-beef patties, hamburgers/salad and sub-optimal (unhygienic) cooking and handling procedures (Anon, 2006a). Other sources of infection include foods such as unheated overnight foods, raw or inadequately pasteurized dairy products, fermented or dried meat products such as salami and jerky, and fruit and vegetable products which presumably had come into contact with domestic animal manure at some stage during cultivation or handling.

Stx pathogen was also detected on conveyor belts in beef-processing plants (Rivera-Betancourt *et al.,* 2004) and was implicated in a serious outbreak associated with surface contamination and deficiencies in hygiene and meat handling practices at a supermarket (Banatvala *et al.,* 1996).

2.1.13 Sources and predisposing factors to Shiga toxin infection

Cattle have long been regarded as the principal reservoir of Shiga toxin producing bacterial strains (Hyatt *et al.,* 2001; Rivera-Betancourt *et al.,* 2004; Anon, 2006a; 2006b). Epidemiological surveys have also revealed that gastrointestinal tracts of other domestic animals, including sheep, pigs, goats, dogs, and cats and birds such as pigeons harbor these organisms (Vernozy-Rozand, and Roze, 2003; Anon, 2006a; 2006b; Tarawneh *et al.,* 2009). Soil and water contaminated with animal and human excreta also incubate these bacterial agents.

Environmental risk factors abound in the developing world. For example in Africa, the majority of drinking water sources are still the traditional ones including dams, wells, rivers, streams and ponds which might harbor or are prone to contamination with water-borne and vector born disease agents (Oyeleke and Istifanus, 2008; Zvidzai *et al.,* 2008). River sand and soils are often littered with animal and human excreta and the rivers continue to be playing ground and source of water for nearby inhabitants. Therefore, food and water-related diseases due to faecal contamination continue to be one of the major health problems globally (Younes and Bartram, 2001; UNESCO, 2003; Wright *et al.,* 2004). Faecal matter from human or animal origin often contaminates these drinking or recreational water sources and often present further

health challenges. It is estimated that 80% of all illnesses are linked to use of water of poor microbiological quality (WHO, 2002). One of the strategies for tackling this problem is the provision of protected sources such as boreholes, standpipes, protected wells and springs (Ahmed *et al.,* 1998). Such facilities however, are located some distances requiring transportation to homes. During transportation, water gets contaminated with bacteria which grow and proliferate during storage in the homes, consequently posing a risk of infection with water-borne pathogens, Stx producing bacteria inclusive (Wright *et al.*, 2004; Hoque *et al.*, 2006).

Many parts of Africa have been associated with high pit latrine coverage (Taulo *et al.,* 2008). These latrines often collapse because of poor soils (sand) on which they are dug. Leaching of pit latrine contents and flooding of human and animal wastes into drinking or recreational water sources during rainy seasons could be possible sources of contamination (Mathess *et al.*, 1988). Furthermore, recreational waters can also be contaminated considering that fingers are prone to faecal contamination during toilet use, cross contamination of water bodies is therefore very easy thus promoting occurrence of diarrhoeal disease outbreaks. The potential of water to harbour microbial pathogens and causing subsequent illness is well documented for both developed and developing countries (Taulo *et al.*, 2008). Dysentery caused by *Shigella* spp. (faecal bacteria) for example is a public health problem in many regions of the world, and is very significant in the developing countries (Luo *et al.*, 2002). Most African countries are associated with rearing of cattle which are often housed near settlements, coupled with poor hygiene attitude, food and water sources are easily contaminated with these bacterial agents.

Other general factors include advanced age, immunosuppression, malnutrition and lack of immunity as in neonates.

2.1.14 Antimicrobial resistance and resistance factors among Shiga toxin producing pathogens

Antimicrobial resistance has been a public health concern globally to which for the past few decades policy makers and the academic community are preoccupied to control (Taulo *et al.*, 2008). The evolutionary prowess of microorganisms presents serious challenges to successfully stop the development of antimicrobial resistance (Stephan and Mathew, 2005). Predisposing factors including self medication, over-the-counter sales of antibiotics and flooding the markets with fake and sub standard drugs further aggravates the situation. In recent years, increase of antimicrobial drug resistance among members of the Enterobacterioceae and Moraxellaceae has been observed in several countries (Humphrey, 2000; Cailhol *et al.*, 2006; Grotiuz *et al.*, 2006).

The National Antimicrobial Resistance Monitoring System (NARM) for enteric bacteria began monitoring for resistance to cephalosporins and other drugs among human derived *Salmonella* and *E. coli* O157 isolates in 1996 (NARM , 2004). The increase in resistance to second and third generation cephalosporins among the Enterobacteriaceae is attributed to the acquisition and expression of extended-spectrum β-lactamase (ESBL) enzymes among Enterobactericeae (El Astal and Ramadan, 2008). ESBL producing strains have variable susceptibility rates for floroquinolnes, aminoglycosides and fourth generation cephalorsporins.

The carbapenems are the only class of antibiotics commonly active against ESBL although, ESBLs are known to be multi-drug resistant (Cherla *et al.,* 2003; Alex and Henry, 2005; Heijnen and Medema, 2006). Since their description in the mid-80s, the incidence of ESBL-producing isolates has steadily increased showing variations between geographical areas. They are also involved in nosocomial outbreaks (as well as the Moraxellaceae) conferring multiple drug resistance and resulting in limitation in therapeutic options. ESBLs are derivatives of simple β-lactamase (TEM or SHV) enzymes that are harboured mostly by Gram-negative bacilli. Selective pressure by the use of second and third generation cephalosporins favours the development of mutations that results in conformational changes in the active serine site of amino acid sequence of TEM or SHV enzymes. As a result of these mutational changes these organisms have acquired an extra gene copy that makes them to develop resistance to a wide range of antibiotics to which they were previously susceptible (Wong *et al.,* 2000; Satheesh *et al.,* 2006; Livermore, 2005).

Though it has been reported that antibiotics which inhibit prokaryotic translation (e.g. erythromycin and doxycycline), can be used to effectively control Shiga toxin producing bacteria, the development of high a rate of resistance by this group of organisms to cephalosporins, aminoglycosides and quinolones has significantly reduced treatment options of the resulting infections (Bureau of Epidemiology, 2000; Iroha *et al.,* 2008). Furthermore, the increased secretion of toxin *in vitro* by the bacterial agents as a result of treatment with bacteriophage-inducing antibiotics, including all quinolones, trimethoprim, furazolidone,

fosfomycin and Mitomycin C has made the situation more hopeless (Helms *et al.*, 2002; Jean *et al.*, 2005; Livermore, 2005; Satheesh *et al.*, 2006; Abong'o and Momba, 2009).

2.1.15 CONCLUSION

The direct and indirect costs incurred by infection of humans with Shiga toxin bacteria in terms of morbidity, economic loss and loss of human lives are increasingly becoming enormous. Many cases of diarrhea in the developing countries remain undiagnosed; several of these cases might be as a result of Stx bacteria especially STEC *E. coli.* Therefore, comprehensive microbiological surveillance programs, which would provide early warning and limit the scale of outbreaks, will ultimately be cost effective, as will vaccination programs. Measures to maximize the microbiological safety of foods are also required. Meat products should be made safe by thorough cooking; dairy products and fruit juices by pasteurization; and salad vegetables by adequate irradiation or blanching before consumption. Provision of potable drinking water and improved environmental sanitation by governments and individuals as well as, increased awareness on the benefits and strict observance of personal hygiene by the populace, are the best preventative measures against such bacterial agents in the face of increasing antimicrobial resistance.

The low infectious dose of STEC infections and the consequent illness which is both serious and can lead to death, has made the organisms to be a serious public health issue. This therefore underpins the need for research into the organisms, not only for food-borne cases, but

for environmentally related sources as well. Consequently, specific research approaches should encompass:

i). A better understanding of the epidemiology of the transfer of STEC from animals and the environment to man. For example, research to determine whether the types of STEC which are found in farm animals are the same as those found in human disease. Such research would need to ensure that all routes of human infection are addressed, including farm and wild animals (e.g. deer, rabbits etc.), the environment (water sources, fields etc.), food products and human-to-human transmission;

ii). The agreement, internationally, of objective, standardised techniques and systems for typing the different strains of bacteria, and the use of those methods in the quantification of the toxins and producing bacteria from different environments;

iii). A greater understanding of the pathogen-host interaction between the toxin producing bacteria and man;

iv). Research to determine the risk of transfer of the virulence determinants of the toxins from the producing bacterium to other organisms; and

v). The mystery of Shiga toxins becoming more abundant in medium when exposed to antibiotics needs to be unraveled. Solution to the control of such bacteria might be embedded in novel antibiotic sources from plants with diverse novel mechanisms of action. More investigations into this area are very pertinent now, with higher incidences of Shiga toxin producing bacterial infections in both the developed and the developing countries.

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2.2 The Ecology, Biology and Pathogenesis of *Acinetobacter* spp.: An Overview

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2.2.1 ABSTRACT

Acinetobacter are a major concern because of their rapid development of resistance to a wide range of antimicrobials, and rapid profundity in transformation, surviving desiccation and persisting in the environment for a very long time. The organisms are associated with bacteraemia, pulmonary infections, meningitis, diarrhea and notorious nosocomial infections with mortality rates of 20 to 60%. Transmission is via person-to-person contact, water and food contamination, and contaminated hospital equipment. The increasing virulence and rapid development of multidrug resistance by these organisms highlight the need to search for alternatives for chemotherapy. A poor understanding of the organisms and dearth of information about their occurrence, especially in developing countries, informed the need for this review paper.

Key words: *Acinetobacter*, acinetobactins, biofilms, coccobacilli, ecology, taxonomy

2.2.2 INTRODUCTION

The name “Acinetobacter” originates from the Greek word “akinetos” meaning “unable to move”, as these bacteria are not motile yet they display a twitching kind of motility. Bacteria of the genus *Acinetobacter* have gained increasing attention in recent years; first, as a result of their potential to cause severe nosocomial (Greek *nosos* disease, and *komeion* to take care of) infections (Bergobne-Bérézin and Towner, 1995; Bergogne-Berezin and Towner, 1996; Koneman, 1997; Weinstein, 1998; Vanbroekhoven *et al.*, 2004; Knapp *et al.*, 2006; Towner, 2006; Peleg *et al.*, 2008); second, for their profundity in developing multidrug (MDR) and

extreme drug resistance (XDR) (Bergobne-Bérézin, 1995; Jain and Danziger, 2004; Prashanth and Badrinath, 2005; Peleg *et al.*, 2007; Vallenet *et al.*, 2008); third, for the ability of some strains to produce verotoxins (VA) (Grupper *et al.*, 2007); and forth, for the role members of the genus play in enhanced biological phosphorus removal in wastewater (Nichols and Osborn, 1979; Ghigliazza *et al.*, 1998; Carr *et al.*, 2001). Recently, *Acinetobacter* spp. have demonstrated a hydrocarbon-degrading capability (Margesin *et al.*, 2003; Mandri and Lin, 2007; Zanaroli *et al.,* 2010), that is of interest for soil bioremediation and a specific strain *A.r baylyi* ADP1 has shown remarkable competence for natural transformation irrespective of DNA source, thus making it a potentially important tool for biotechnology (Barbe *et al.,* 2004; Vanbroekhoven *et al.*, 2004; Chen *et al.*, 2008; Vallenet *et al.*, 2008). Possible suggested applications of *Acinetobacter* spp. are summarized in Table 2.2.1.

In addition, since the environment, soil, and animals are their natural habitats, food and water contamination expose humans to infections. The ability of these bacteria to colonize almost any surface and to acquire antibiotic resistance distinguishes them from other infectious bacteria. Despite the huge increase in the frequency of infections caused by MDR *Acinetobacter*, there is still a lack of awareness of the importance of these microorganisms (Doughari *et al.*, 2010). This review therefore gives an overview of the biology, ecology and medical significance of the entire genus *Acinetobacter* in a broad sense with a view to providing basic general information on this group of bacteria for a better understanding and the possible adoption of proactive and effective control measures against infections associated with some of the bacteria.

2220 Table 2.2.1. Possible applications for *Acinetobacter* spp. and their products

Bioremediation of waste waters and effluents	Bioremediation of soils and effluents contaminated with heavy metals	Production of biopolymers and biosurfactant	Biomass production	Clinical uses
i) Phosphate removal	i) Textile or tannery industrial effluent containing heavy metals	i). For prevention of dental plaque	i) Protein production	i) Production of glutaminase-sparaginase
ii) Degradation of petrochemicals	ii) Lead from digested sewage sludge	ii) For use in paper-making and other industries	ii) Manganese leaching from ores	ii) Production of L(−) carnitine
iii) Breakdown of organic pollutants	iii) Chromium-contaminated activated sludge or wastewater	iii) For efficient emulsification of oil waste pollutants	iii) Production c immune adjuvants	
	iv) Silver contaminated photographic wastewater	iv) For incorporation cosmetics, detergents and shampoos		

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2.2.3 Recent taxonomy

The first strain of *Acinetobacter* spp. was isolated from soil and identified as *Micrococcus calcoaceticus* by Beijerinck in 1911 (Bouvet and Joly-Guillou, 2000; Barbe *et al.,* 2004). The *Acinetobacter* group were previously insufficiently defined for a very long time and confusedly classified into more than a dozen different genera (*Achromobacter, Alcaligens, Cytophaga, Diplococcus, Bacterium, Herellea, Lingelsheimia, Mima, Micrococcus, Moraxella* and *Neisseria*) (De Bord, 1939; Piéchaud *et al.*, 1956; Brisou, 1957; Rossau *et al.*, 1991; Barbe *et al.,* 2004).

The genus *Acinetobacter* was first created in 1954 by Brisou and Prevot to separate the non motile from the motile members of the tribe "Achromobactereae" and was composed of non-pigmented Gram-negative saprophytic bacteria comprising both oxidase-negative and oxidase-positive species. In 1957, Brisou identified a typical species named *A. anitratum* (Brisou, 1957). Baumann *et al*. (1968) using distinct nutritional properties later characterized the organisms as oxidase-negative and proposed to classify them under the genus *Acinetobacter*. In 1971, the subcommittee on *Moraxella* and allied bacteria accepted this proposal and the genus was limited to oxidase-negative strains (Lessel, 1971). Three species were initially included in this genus but because of difficulties in distinguishing them based on differences in physiological characteristics, all the species were named *A. calcoaceticus* (Barbe *et al.,* 2004). In fact, *Bergy's Manual of Bacteriology* placed these bacteria in the family Neisseriaceae with only *A. calcoaceticus* as a species and the two subspecies *A. anitratum* and *Acinetobacter lwofii* (59). Several years later, Bouvet and Grimont (Bouvet and Grimont, 1986) identified

more than fifteen genomic species, including *A. baumannii* (formerly *A. calcoaceticus var anitratum* and *A. glucidolytica non liquefaciens*), *A. haemolyticus, A. junii, A. johnsonii* and *A. radioresistens* (De Bord, 1939; Bouvet and Grimont, 1986; Bouvet and Joly-Guillou, 2000).

The species' names have undergone considerable taxonomic changes over the years as molecular methods have advanced understanding of the genetic make-up of this group of organisms (Urban *et al.*, 2003). Recent classifications which seem to have gained wide acceptance among bacterial taxonomists have recognized this group of heterogeneous bacteria as gamma proteobacteria classified in the order *Pseudomonadales* and the family *Moraxellaceae* (Bouvet and Joly-Guillou, 2000). Thus the taxonomical classification is given as; Domain - *Bacteria*, Phylum - *Proteobacteria*, Class – *Gammaproteobacteria*, Order - *Pseudomonadales*, Family - *Moraxellaceae*, Genus - *Acinetobacter* (DNA G+C content 39-47%) and *species* (with *A. baumannii, A. haemolyticus* and *A. calcoaceticus* as species of clinical importance). Recent classifications using cell shape, absence of flagella, G+C content of DNA and nutritional properties, placed these organisms (*A. baumannii, A. haemolyticus* and *A. calcoaceticus* as well as other *Acinetobacters*) in the genus *Moraxella*, now known as *Acinetobacter* (Barbe *et al.,* 2004). Based on DNA-DNA hybridization studies, 32 species of *Acinetobacter* have now been recognized, with 22 assigned valid names and the rest assigned numbers and referred to as a 'genomic group' (Prashanth and Badrinath, 2005; Gerischer, 2008). Among the named species, *A. baumannii* is the main species associated with clinical infections followed by the non-*A. baumannii* species *A. haemolyticus, A. junii, A. johnsonii* and *A. lwofii* (Guardabassi *et al.*, 1999; Barbe *et al.,* 2004). Recently, the emergence of other

species of clinical importance such as *A. ursingii* and *A. schindleri* has been reported (Robinson *et al.*, 2010).

Another difficulty associated with classification is the close resemblance between species such that phenotypic differentiation becomes very difficult. For instance, there is a close relationship between *A. baumannii* and *A. calcoaceticus,* and genomospecies 3 and 13. As a result of the difficulties in distinguishing isolates phenotypically in the former pair, the term *A. baumannii-A. calcoaceticus* complex or *Abc* complex has been used. Furthermore, some authors still report these isolates as *A. calcoaceticus* subspecies *anitratum*. This situation led to contributors to the Manual of Clinical Microbiology to conclude that the majority of species of this group of bacteria cannot be reliably distinguished based on phenotypic tests (Bouvet and Joly-Guillou, 2000; Schreckenberger *et al.*, 2003; Fournier *et al.*, 2006; Richet and Fournier, 2006).

To avoid confusion therefore, a more reliable classification based on combination of the results of DNA-DNA hybridization and on phenotypic characteristics was adopted (Barbe *et al.,* 2004). In clinical practice however, these taxonomic complications have led to the under-recognition and misclassification of the species.

2.2.4 Biology, cultural and biochemical characteristics of the *Acinetobacter* group

The genus *Acinetobacter* consists of strictly aerobic, non-motile, catalase-positive, indole-negative, oxidase-negative, Gram-negative, non-fermentative encapsulated coccobacilli rods

(Bouvet and Joly-Guillou, 2000; Vallenet *et al.,* 2008). Many strains are unable to reduce nitrates to nitrites (Bergogne-Be´re´zin, 2009). The bacteria are strictly aerobic and although they flourish on most laboratory media at temperatures of 20-30^{0}C with the clinical isolates growing at 37 to 42^{0}C, for most strains the optimum temperature is 33-35^{0}C. In the exponential phase of growth, they are bacilli 0.9 to 1.6 µm in diameter and 1.5 to 2.5 µm in length, often in pairs or assembled into longer chains of varying length. *Acinetobacter* spp. are non-fastidious and can be grown on standard laboratory media (Kurcik-Trajkovska, 2009). On blood agar (BA), colonies show typical morphology and size: non-pigmented, white or cream colored, smooth or mucoid (when capsule is present), opaque, 1-2 mm in diameter (after 18-24 h incubation at 37^{0}C) (9), on eosin methylene blue agar (EMB), colonies are bluish to bluish gray, on Herellea agar (HA) they are pale lavender in color (Bergogne-Be´re´zin, 2009), while on Leeds Acinetobacter Medium (LAM) the bacteria are pink on a purple background. In aged cultures the bacteria may be spherical or filamentous. The organisms can be recovered after enrichment culture from virtually all samples obtained from soil or surface water (Peleg *et al.*, 2008). The members of the *Acinetobacter* group are nutritionally versatile chemoheterotrophs and the range of substrates they use as sole carbon and energy sources parallels that of the aerobic pseudomonads.

The cell wall of *Acinetobacter* is typical of that of Gram-negative bacteria, however de-staining is difficult due to a tendency to retain crystal violet and this can lead to incorrect identification as Gram-positive cocci (Allen and Hartman, 2000). The cells of *Acinetobacter* vary in size and arrangement. *Acinetobacter* generally form smooth and sometimes mucoid

colonies on solid media, ranging in color from white to pale yellow or grayish white. Some environmental strains have been reported to produce a diffusible brown pigment (Allen and Hartman, 2000; Peleg *et al.*, 2008). Several clinical isolates show hemolysis on sheep blood agar plates (Peleg *et al.*, 2008).

2.2.5 Pathogenesis, virulence factors and resistance

2.2.5.1 Pathogenesis

In the past, *Acinetobacter* spp. were considered saprophytes of little clinical significance (Bergogne-Berezin and Towner, 1996), but with the introduction of powerful new antibiotics in clinical practice and agriculture and the use of invasive procedures in hospital intensive care units (ICUs), drug resistant-related community and hospital-acquired *Acinetobacter* infections have emerged with increasing frequency (Guardabassi *et al.*, 1999). *A. baumannii* is an important emerging nosocomial pathogen worldwide, followed by *A. lwofii* and *A. haemolyticus.* Conditions attributable to *Acinetobacter* spp. include blood stream infections (BIs), ventilator-associated pneumonia (VAP), bacteremia, meningitis, urinary tract infections, cholangitis, peritonitis, skin and wound infections, ventriculitis, and infective endocarditis (Weinstein, 1998; Berlau *et al.*, 1999; Jain and Danziger, 2004). The bacteria can also colonize the skin and respiratory tract without causing an infection. An infection results if the host's first line of defence is compromised. Studies have, however, revealed that colonization increased with hospital stays (OECD, 2008). In gastroentistenal infections with *A. lwoffıi* and *H. pylori* infections for example, the normal tissue architecture of the gastric epithelium is altered leading to chronic gastritis (Fig. 2.2.1).

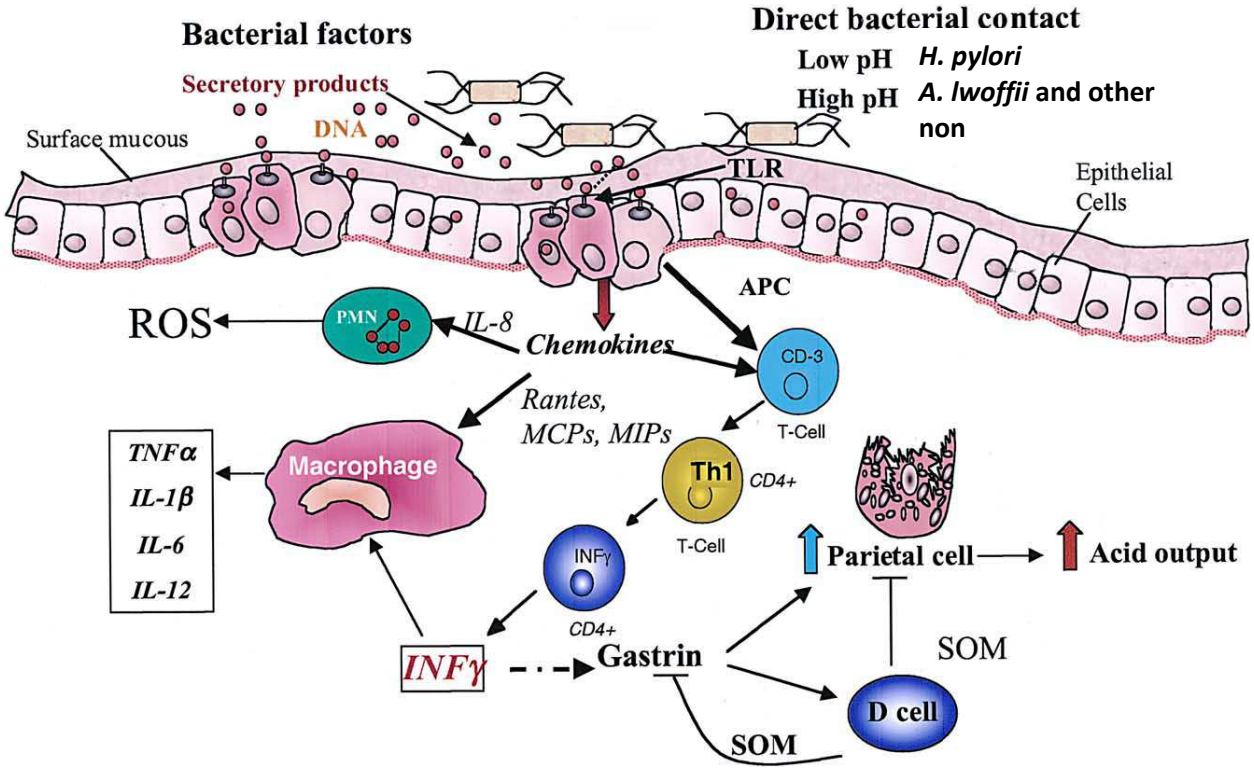

Fig. 2.2.1. Schematic model of bacterial colonization of the gastric mucosa, activation of antigen presenting cells (APC), release of pro-inflammatory cytokines and alteration in the number of gastric epithelial cells involved in acid secretion. MCP, macrophage chemotactic protein; MIP, macrophage inflammatory protein; TLR, Toll-like receptor; SOM, somatostatin; ROS, reactive oxygen species (Poirel *et al.*, 2003). (Reproduced with permission, ©Elsevier).

Infections with *A. lwoffıi* induce production of pro-inflammatory cytokines which increase gastrin levels that, in turn, promote proliferation of the gastric epithelium. Persistent inflammation including the activation of antigen-presenting cells (APCs), release of pro-inflammatory cytokines such as macrophage chemotactic protein (MCP), macrophage inflammatory protein (MIP), Toll-like receptor (TLR), somatostatin (SOM), reactive oxygen species (ROS) involved in acid secretion (Fig. 2.2.1) and changes in the number of gastric epithelial cells can lead to gastritis, peptic ulcers, and more rarely, gastric cancer (Richet and Fournier, 2006). Though colonization occurs more frequently than infections, studies have also shown that lethal infections result from pathogenic strains in immunosuppressed animals with mortality rates of 75 to 100% (Rodríguez-Hernández *et al.*, 2000). The bacteria have also been associated with bacteremia, sepsis in neonatal intensive care units and pediatric oncology units, as well as community acquired meningitis and endophthalmitis (Crawford *et al.*, 1997; Valero *et al.*, 1999; Smith *et al.*, 2007). Other conditions include suppuration; abscesses of the brain, lung, and the thyroid, secondary infections of wounds or surgical trauma, and purulent lesions of the eye. The organisms are ranked 9th after *S. aureus, E. coli, Klebsiella* spp. *P. aerugenosa, C. albicans, Enterococci, Serratia* and *Enterobacter* as agents of nosocomial BIs, and account for 34% of the mortality and 43% of deaths due to hospital-acquired infections (Dorsey *et al.*, 2004). They are the second most commonly isolated nonfermenters in human specimens (Oberoi *et al.*, 2009), after *P. aeruginosa* and their incidence is on the increase and mortality rates are quite high (Jain and Danziger, 2004; Wisplinghoff *et al.*, 2004; Vallenet *et al.*, 2008). *A. baumannii* was found to be associated with a series of fatal cases of community pneumonia (Dorsey *et al.*, 2004) and *A.*

haemolyticus, with endocarditis and verotoxin production, and hence bloody diarrhea (Castellanos *et al.*, 1995; Blanco *et al.*, 2003; Grotiuz *et al.*, 2006).

2.2.5.2 Pathogenic mechanisms

The pathogenic mechanisms of *Acinetobacter* spp. are little understood or studied (Peleg *et al.*, 2009). Though the infective doses of *Acinetobacter* in human infections have yet to be determined, intraperitoneal injections in mice with 40 clinical isolates of *Acinetobacter* showed the LD_{50} to range from 103 to 106 viable cells per mouse (OECD, 2008).

Though *A. baumannii* is the most studied species, the precise mechanisms involved in the establishment and progression of infections by this species are unclear. The organism is not known to produce either diffusible toxins or cytolysins, and few virulence factors have been identified (Gordon and Wareham, 2010). However, comparative genomic studies with *A. baumannii* and the environmental *A. baylyi* have identified genes involved in pilus biogenesis, iron uptake and metabolism, quorum sensing and a type IV secretion system as making up part of the organism's 'virulome' (Valero *et al.*, 1999; Smith *et al.*, 2007). Other authors have also reported common virulence factors among the *Acinetobacters* (which are discussed below). There is a need for microbiologists to further investigate these virulence mechanisms for possible discovery of more effective control measures.

2.2.5.3 Virulence factors

Cell surface hydrophobicity and enzymes: *Acinetobacter* spp. have been demonstrated to exhibit cell surface hydrophobicity, an important determinant for bacterial adhesion. For a successful infection to occur, bacteria must successfully adhere to host cells (Costa *et al.*, 2006). The hydrophobicity of a microorganism protects it from being phagocytosed and appears to play an important role in its attachment to various polymers. Hydrophobicity also confers the ability to adhere to plastic surfaces, such as catheters and prostheses. Through this hydrophobicity, *Acinetobacter* spp. coaggregate into flocs in sludge. Non-flocculating *A. johnsonii* S35 displays significant coaggregation with three other bacterial species, *Oligotropha carboxidovorans*, *Microbacterium esteraromaticum*, and *Xanthomonas axonopodis* (Martinez *et al.*, 1998). The degree and mechanism of coaggregation were found to be pair-dependent; and cell surface hydrophobicity was an important factor controlling the coaggregation of *A. johnsonii* S35 and its partner strains (Martinez *et al.*, 1998; Phuong *et al.*, 2009). The hydrophobic properties of bacterial strains depend on their surface structure, which in turn determines the degree of hydrophobicity: the rougher the cell surface, the greater the hydrophobicity and vice versa. In *Acinetobacter,* the presence of protein protrusions on the cell surface confers hydrophobicity. A recent study using scanning electron microscopy showed the presence of blister-like protein protrusions on *A. johnsonii* S35 and *A. junii* S33, these bacterial cells were able to coaggregate efficiently with other bacterial cells compared to a mutant strain *A. johnsonii* IAM1517 with smooth cell surfaces (Phuong *et al.*, 2009) which was unable to form aggregates (Phuong *et al.*, 2009).

Surface hydrophobicity is also mediated by colonization factors, complimentary cell surface receptors, fimbriae and other cell wall components (Kaplan *et al.*, 1985; Braun, 2009) and cell surface enzymes that facilitate the adhesion of bacterial cells to host cells. For example, the urease activity of *Acinetobacter* promotes colonization of the mouse stomach (Costa *et al.*, 2006). Urease also helps *Acinetobacter* spp. colonize the hypochlorhydric or achlorhydric human stomach inducing inflammation (Sauer *et al.,* 2007). Polysaccharide slimes on the bacterial cell surface are reported to confer hydrophobicity (Rossau *et al.*, 1991; Hoštacká, and Klokočníková, 2002; Rathinavelu *et al.,* 2003; King *et al.*, 2009). Other virulence-conferring enzymes secreted by the bacteria include esterases, certain amino-peptidases, and acid phosphatases (Rathinavelu *et al.,* 2003; Towner, 2006). Esterases have strong hydrolyzing activity against short-chain fatty acids, thereby causing damage to lipid tissues. Hydrolytic enzymes usually confer the bacterium with very strong hemolytic activity. The most extensively studied hydrolytic enzymes in *P. aerugenosa* are phospholipases C (PLC)-H, which is encoded by *plcS,* is acidic and has strong hemolytic activity, and PLC-N, which is encoded by *plcN,* is basic, and has no hemolytic activity (Rathinavelu *et al.,* 2003). Recent studies revealed that two copies of the phospholipase C (*plc*) gene with 50% identical to that of *Pseudomonas* are found in *A. baumannii.* It is therefore assumed that these lipases serve a similar function, although this is yet to be elucidated (Vallenet *et al.*, 2008). Hoštacká and Klokočníková (2002) also reported the secretion of phosphotidylethanolamine and sphingomyelin which are all cytotoxic to leucocytes.

Toxic slime polysaccharides: Toxic slime polysaccharides have also been reported among *Acinetobacter* spp. (Hoštacká and Klokočníková, 2002). They are usually produced during the exponential phase of growth and are made up of the glucose building blocks D-glucuronic acid, D-mannose, L-ramnose and D-glucose. The slime polysaccharides are toxic to neutroplils, and inhibit their migration as well as inhibit phagocytosis, but without disrupting the host immune system (Heidelberger *et al.*, 1969; Hoštacká, and Klokočníková, 2002). It is important to understand these structures in order to develop effective control measures. Currently, the authors are focusing on determining the hydrophobicity of *A. haemolyticus* isolates from water and wastewater samples and the effect of stress and phytochemical extracts on this hydrophobicity.

Verotoxins: Grotiuz *et al.* (2006) first reported the production of verotoxins in *Acinetobacter* (from *A. haemolyticus*). Verotoxins are associated with bloody diarrheas and produced by many enteric bacteria including *E. coli* and *S. dysenteriae* (Doughari *et al.*, 2009a). The toxins belong to a particular protein subfamily, the RNA *N*-glycosidases which directly target the cell ribosome machinery, inhibiting protein synthesis. Verotoxins can be classified into 2 antigenic groups, vtx-1 and vtx-2, which include (especially vtx-2) an important number of genotypic variants. The mechanism by which *A. haemolyticus* produces this toxin is, however, not well understood. Lambert *et al.* (1993) speculated that *A. haemolyticus* acquires vtx2-producing activity via horizontal gene transfer in the gut lumen, since it can be rapidly transformed. In any case, the pathogenicity, basic structure, and chemical components of the toxins are the same as those of verotoxins from *E. coli* and other bacteria (Lambert *et al.*, 1993). The

emergence of verotoxin-producing *A. haemolyticus* strains is worrisome given the high transformability of *Acinetobacter* spp. This, therefore, calls for intensive surveillance of these organisms especially in the environment, for the development of proactive control measures. The current work therefore focuses on isolation and identification of *A. haemolyticus* from environmental samples and the determination of their verotoxin production and antimicrobial resistance profiles as well as the effect of phytochemical extracts on verotoxin production by the isolates.

Siderophores: Siderophores are host iron-binding protein structures responsible for iron up take in bacteria. One possible defense mechanism against bacterial infections is the reduction of free extracellular iron concentrations via iron-binding proteins such as lactoferrin or by transfer (Yu *et al.*, 2005; Braun, 2009). The normal concentration of free iron in the body is 10^{-8} M, and the concentration required for bacteria to survive in the human body is 10^{-6} M. Bacteria meet their iron requirement by binding exogenous iron using siderophores or hemophores (Margesin *et al.*, 2003; Yu *et al.*, 2005; King *et al.*, 2009). Bacterial siderophores are called aerobactins. *Acinetobacter* siderophores are called acinetobactins and are chiefly made up of the amine histamine which results from histidin decarboxylation (Mihara *et al.*, 2004). Iron import into the bacterial cell is however regulated by a ferric regulator uptake protein serving as a transcription repressor to induce siderophore synthesis or degradation (Vallenet *et al.*, 2008).

Outer membrane proteins (OMPs): Outer membrane proteins (OMPs) in some Gram-negative bacteria are known to have essential roles in pathogenesis and adaptation in host cells as well as in antibiotic resistance. Several OMPs of the OmpA family have been characterized in various *Acinetobacter* strains (Dijkshoorn *et al.*, 2007; Gordon and Wareham, 2010). Vila *et al.* (2002) reported homology between the genome sequence of OmpA of *A. radioresistens*, *A. baumannii* and *A. junii*. The OmpA proteins induce apoptosis of epithelial cells (Choi *ert al.*, 2005), stimulating gastrin and interlukin B gene expression (Janssen *et al.*, 1997). In a recent study, Vallenet *et al.* (2008) showed that *A. baylyi* OmpA has emulsifying activity and that only one gene in each *Acinetobacter* strain encodes an OmpA protein. In other words, these proteins share more than 89% of their amino acids and thus have the same chromosomal context. The cells of *Acinetobacter* spp. are surrounded by OmpA, a protein to kill host cells (Choi *et al.*, 2008). During an infection, OmpA binds to eukaryotic cells and gets translocated into the nucleus where it causes cell death (Saint *et al.*, 2000; Dijkshoorn *et al.*, 2007; Choi *et al.*, 2008).

2.2.5.4 Resistance to antibiotics and mechanisms of resistance

The major problem with *Acinetobacter* spp. is their resistance to antibiotics (Landman *et al.*, 2002). Savov *et al.* (2002) reported that these organisms are most commonly resistant to ampicillin, cephalothin, carbenicillin, gentamicin, amikacin, chloramphenicol, tetracycline, co-trimoxazole, ciprofloxacin and cefoperazone. Previously ampicillin, second generation cephalosporins, quinolones, minocyline, colistin, amynoglycosides, impenim, sulbactam and gentamicin were used to treat *Acinetobacter* infections. Resistance to these antibiotics has

hindered therapeutic management, causing growing concern the world over (Vila *et al.,* 2002; Prashanth and Badrinath,. 2005; Grotiuz *et al.,* 2006; Perez *et al.,* 2007; Doughari *et al.,* 2009b). *A. baumannii* has been developing resistance to all antibiotics used in treating infections. Currently, most *A. baumannii* strains are resistant to aminoglycosides, tetracyclines, cephalosporins, ampicillins, cefotaximes, chloramphenicols, gentamicins and tobramycins (Prashanth and Badrinath, 2005). The activity of carbapenems is further jeopardized by the emergence of enzymatic and membrane-based mechanisms of resistance (Peleg *et al.,* 2008).

Antimicrobial resistance among *Acinetobacter* is either intrinsic or acquired via transformation. Several mechanisms of resistance including altered penicillin-binding proteins, lowered/decreased permeability of the outer membrane to antibiotics or an increase in the active efflux of the antibiotics, target site mutations, and inactivation via modifying enzymes have been reported (Vila *et al.,* 2002; Jain and Danziger, 2004). Mechanisms of resistance to antibiotics by *Acinetobacter* spp. vary with species, the type of antibiotic and geographical location (Jain and Danziger, 2004). Thus β-lactam antibiotics are inactivated by the production of β-lactamases or alterations of penicillin-binding proteins and decreased permeability of the outer membrane to β-lactams (Poirel *et al.,* 2003); cephalosporins, by chromosomally encoded cephalosporinases and, occasionally, by cell impermeability and aminoglycosides via aminoglycoside-modifying enzymes; and quinolones, by altering the target enzymes DNA gyrase and topoisomerase IV through chromosomal mutations, a decrease in permeability and increase in the active efflux of the drug by the microbial cell (Landman *et al.,* 2002). Several efflux pumps acting against antibiotics have been described for *Acinetobacter* spp. grouped as:

(i) major facilitator superfamilies (MFSs) comprising the Tet (A)- efflux system for tetracycline, Tet (B)- efflux system for tetracycline and minocycline and Caml A-efflux system for chloramphenicol; (ii) resistance-nodulation division (RND) comprising Ade ABC (ATP binding cassettes) – efflux systems against aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracyclines, ethidium bromide, and reduced susceptibility to fluoroquinolones; and (iii) multi drug and toxic compounds extrusion systems (MATEs) comprising of Abe M - efflux system against norfloxacin, ofloxacin, ciprofloxacin, gentamicin, 4, 6-diamino-2-phenylindole (DAPI), triclosan, acriflavin, doxocrubicin, rhodamin 6G and ethidium bromide (Vila *et al.*, 2007). Major mechanisms of resistance to different classes of antibiotics are listed in Table 2.2.2. Owing to this escalation of multidrug resistance, deliberate efforts should be made towards investigating other sources of antibiotics with novel mechanisms of action, with a view to developing effective control measures against the recalcitrant bacteria. Investigations of phytochemicals should be considered since they form a very rich source of antibiotic substances (Doughari *et al.*, 2009b) with potential activity against microbial pathogens.

2558 Table 2.2.2. Major mechanisms of resistance identified for the different classes of antibiotics

Antimicrobial class/ resistance mechanism	Class/family	Variants
β-Lactam		
β-Lactamases	Intrinsic cephalosporinases	AmpC (ADC1–7)
	Class A/high-prevalence $ESBL_A$	VEB-1, -2, PER-1, -2, TEM-92,-116, SHV-12, -5, CTX-M-2, -3
	Class A/low-prevalence $ESBL_A$	SCO-1
	Class D OXA enzymes/$ESBL_{M–D}$	OXA-51-like
Carbapenemases	Class D OXA enzymes/$ESBL_{CARBA-D,}$	OXA-23–27, -37, -40, -58-like, VIM, IMP, SIM
	$MBLs/ESBL_{CARBA-B,}$	
	Class A carbapenemase/$ESBL_{CARBA-A}$	GES-11
OMP loss	CarO, HMP-AB, 33–36 kDa protein, 43 kDa protein	
Efflux pump	AdeABC	
Altered PBP expression		PBP2 downregulation
Tetracyclines		
Efflux pump	MFS, RND	A, TetB, AdeABC
Ribosomal protection		TetM
Glycylcyclines		
Efflux pump	RND	AdeABC
Aminoglycosides		
Enzymatic degradation	Acetyltransferases	AacC1/2, AadA, AadB
	Nucleotidyltransferases	Ant1
	Phosphotransferases	AphA1, AphA6,
16s rDNA methyltransferases		ArmA
Quinolones		
DNA gyrase/topoisomerase mutations		GyrA/ParC
Efflux pumps	RND, MATE, BIMP	AdeABC, AdeM, AbeS
Chloramphenicol		
Efflux pumps	RND	AdeABC, AdeIJK
	MFS	CmlA, CraA
	BIMP	AbeS
Trimethoprim/sulfamethoxazole		
Efflux pump	RND	AdeABC, AdeIJK
Dihydropteroate synthase		FolA
Macrolides		
Efflux pumps		
Polymyxins	MATE, BIMP	AbeM, AbeS
	PmrAB two-component mutation	

2559 MBL- metallo-β-lactamase; OMP - outer membrane protein; HMP - heat modifiable protein; PBP - penicillin-
2560 binding protein; MFS - major facilitator superfamily; RND - resistance–nodulation–cell division; MATE -
2561 multidrug and toxic compound extrusion; BIMP - bacterial integral membrane proteins (Grehn, and von
2562 Graevetnitz, 1978).

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2.2.5.5 Resistance to environmental and host factors

The emergence of nosocomial or community-acquired infections of *Acinetobacter* is a result of high adaptability to adverse environmental conditions, an ability to persist for several days in dry and harsh environments such as the hospital environment, the increased use of broad spectrum antibiotics, the vulnerability of individuals or patients, and the rapid transformation of organisms that results in increased multidrug resistance. To survive and multiply in the host, many bacteria produce a variety of substances that allow them to avoid the defense mechanisms of the host (Joly-Guillou, 2005). *Acinetobacter* spp. are found as natural inhabitants of human skin and repeated isolation may suggest that they are potential pathogens.

Acinetobacter spp. are able to survive on moist and dry surfaces (Wendt *et al.*, 1997) and some strains have been found to be tolerant of soap (Jain and Danziger, 2004; Bergogne-Be´re´zin, 2009). The ability of *Acinetobacter* to persist in dry conditions, on inanimate objects, and in dust for several days and weeks, has been reported. Recent isolates of *A. lwoffii* compared to the isolates from the 1970s are relatively resistant to irradiation. This raises concerns about the persistence of *A. lwoffii* on medical devices that are sterilized by gamma irradiation, especially in intensive care units (Rathinavelu *et al.*, 2003).

Acinetobacter species also survive exposure to the commonly used disinfectants like chlorhexidine, gluconate and phenols, particularly those not used in the appropriate concentrations (Gallego and Towner, 2001). Compared with other genera of Gram-negative

bacilli, *Acinetobacter* is able to survive much better on fingertips or on dry surfaces when tested under simulated environmental conditions (Wendt *et al.*, 1997). Apart from being able to grow at a very broad range of temperatures, they are also able to resist drying. The presence of more electron dense cell walls and nucleic acids are thought to be responsible for the heat (50-75ºC) resistance (Jain and Danziger, 2004). In fact, while *P. aerugenosa* and *E. coli* can only survive heat for a maximum of 24 h, *A. baumannii* can survive for up to 25 days (Joly-Guillou, 2005). Survival for 157 days (*A. radioresistens*), over 30 days (*A. baumannii*) and 21 days (*A. lwoffii*) has been reported (Houang *et al.*, 1998; Jawad *et al.*, 1998; Peterson, 2001). *A. baumannii* has also demonstrated resistance to the killing action of normal human serum (NHS) and the possession of a lipopolysaccharide was thought to be partly responsible (Gerischer, 2008). King *et al.* (2009) also suggested modulation of pathogen interaction with serum by a complement regulator. The complement system is the host innate immune defense comprising a series of serum proteins that initiates the death of the bacterium through either lysis or opsonization. One of the mechanisms by which bacterial cells resist killing by serum compliments is by producing surface proteins that bind human factor H (FH), and thereby inhibit the deposition of complements on the bacterial surface (Koneman , 1997; Garcia *et al.*, 2000). The mechanism by which *Acinetobacter* spp. resist serum compliments is, however, yet to be discovered.

2.2.4. 6 Transfer of resistance among *Acinetobacter* spp.

Resistance to antibiotics is transferred among *Acinetobacter* spp. via plasmids and transposons. While plasmids are DNA elements that carry antibiotic and heavy metal

resistance conferring genes capable of autonomous replication, transposons are sequences of DNA that can move (or transpose) themselves to new positions within the genome of a bacterium (or any other prokaryotic cell). These elements are often present in resistant bacteria and have been reported in clinical isolates of *Acinetobacter* spp. (Gallego and Towner). Plasmids and transposons are easily transferred between bacteria via the process of genetic transformation. Transformation occurs between *Acinetobacter* spp. due to the high frequency and degree of adaptability and transformability among some strains of *Acinetobacter* spp., species capable of colonizing multiple settings can mediate the transfer of novel antibiotic resistance genes from antibiotic-producing environmental species to clinical isolates. For example, an intermediate group including spp. 7, 8, and 9 is capable of adapting to human tissues as well as remaining in the environment, thus serving as effective vehicles for conveying resistance genes between species (Rathinavelu *et al.*, 2003). Gene transfers in *Acinetobacter* spp. also occur via conjugation and transduction. Conjugation in *Acinetobacter* involves a wide host range and chromosomal transfer, while transduction involves a large number of bacteriophages with a restricted host range (Rathinavelu *et al.*, 2003). Owing to the high transformation ability of *Acinetobacters*, the role of genetic elements in the virulence of this group needs to be thoroughly investigated and adequately understood as in the case of *E. coli.* This will no doubt open up more frontiers for more effective control measures and the application of the organisms in biotechnology.

2.2.5 Epidemiology and ecology

Several studies have reported the epidemiology of *A. baumannii* infections in different parts of the world including Europe, the United States and South America (Siau *et al.*, 1999; Kurcik-Trajkovska, 2009). Although these organisms are often associated with nosocomial infections (Weinstein, 1998), community-acquired diarrhoeal outbreaks and pneumonia have been reported with some frequency in tropical regions of the world especially during warm (summer) and humid months (Chen *et al.*, 2001; Jain and Danziger, 2004). An infrequent manifestation of *Acinetobacter* is nosocomial meningitis and these cases have been reported after neurosurgical procedures (Chen *et al.*, 2001; Jain and Danziger, 2004; Joly-Guillou, 2005). The morbidity and mortality rates of *Acinetobacter* infections are comparable to those of methicillin-resistant *Staphylococcus aureus* (MRSA), and the organisms have been termed 'Gram-negative MRSA', manifesting similar epidemiological behavior to MRSA. The impact in terms of morbidity and mortality is probably closer to that of coagulase-negative staphylococci and available data suggest that the mortality rate ranges from 20% to 60% (Joly-Guillou, 2005). Thus, several reports have alerted clinicians to the emergence of a potentially difficult and dangerous organism that is responsible for outbreaks of infection and can cause severe problems (Joly-Guillou, 2005). Owing to the morphological similarity between Acinetobacter and Neisseriaceae (both being Gram-negative diplococci), care should be taken while examining the Gram stain. *Neisseria meningitidis* is, however, far more common as an agent of meningitis. Uncommon conditions involving *Acinetobacter* are contagious osteomyelitis, peritonitis associated with continuous ambulatory peritoneal dialysis, ophthalmic infection, skin and wound infections, abscesses, sepsis, endocarditis and burn

infections. Despite the increasing significance of *Acinetobacter*, there are no significant epidemiological reports on the incidence of infections from many parts of the world, particularly developing countries. Epidemiological investigations of clinical significance on *Acinetobacter* spp. other than *A. baumannii* as well as on the epidemiology of acinetobacteriosis are essential in developing countries.

Acinetobacters are mostly free living saprophytes found ubiquitously in nature (Vallenet *et al.*, 2008). However, different species of the genus are generally associated with different habitats. *Acinetobacter* genomospecies 3 is found in water and soil, on vegetables, and on human skin; *A. johnsonii* and *A. haemolyticus* are found in water, wastewater, soil, on human skin, and in human feces; *A. lwoffii* and *A. radioresistens* are found on human skin; and *Acinetobacter* genomic species 11 is found in water, in soil, and on vegetables as well as the human intestinal tract (Cunha *et al.*, 1980; Berlau *et al.*, 1999).

2.2.5.1 Human and animal body

Acinetobacter spp. are generally considered part of the normal flora of the skin and mucous membranes or the pharynx, human respiratory secretions, urine, rectum (Villegas and Hartstein, 2003) and other human clinical samples (Savov *et al.*, 2002). They are the only group of Gram-negative bacteria that may be natural residents of human skin, with carriage rates of 42.5% in healthy individuals and as high as 75% in hospitalized patients (Savov *et al.*, 2002). In a study conducted by Seifert *et al.* (2007) *Acinetobacter* spp. were isolated from various parts of the human body including the forehead, nose, ear, throat, trachea, conjunctiva

axilla, hand, groin, vagina, perineum and toe web. The organisms are also found in the distal urethra of healthy people (Koneman , 1997). Generally, the species most frequently isolated are *A. johnsonii, A. lwoffii, A. radioresistens, A. baumannii, A. calcoaceticus, A. haemolyticus* and *Acinetobacter* genomospecies 3 and 13. However, colonization of the intestinal tract by *Acinetobacter* spp. is controversial. While some authors suggest that it is an unusual event (Ghigliazza *et al.*, 1998), others report that the gastrointestinal tract is the most important reservoir of resistant strains (Corbella *et al.*, 1996). The difference is probably due to the epidemiological situation i.e. whether there is an epidemic outbreak or not.

Acinetobacter spp. have been isolated from different animal sources including birds; fish and rainbow trout (Berlau *et al.*, 1999; Guardabassi *et al.*, 1999). On several occasions, chicken septicemia (15% death rate), septicemia in turkeys and calves, mastitis and metrititis in cows, abortions in cattle, pigs and horses, keratoconjunctivitis in cattle, omphalitis in calves, ear infections in cats, and respiratory infections and Balanoposthitis in horses have been reported (Francey *et al.*, 2000). *A. beijerinkii* and *A. baumannii* are among the species of *Acinetobacter* implicated in animal diseases (Francey *et al.*, 2000). The organisms have also been isolated from lice collected from homeless people (La Scola and Raoult, 2004).

2.2.5.2 Food contamination

Acinetobacter spp. have been associated with food contamination. Several foods, including vegetables, have long been known to be an important source of contamination with Gram-negative bacteria such as *Escherichia coli* and *Klebsiella* spp. (Peleg *et al.*, 2008).

Acinetobacter spp. have been recovered from vegetables, apples, melons, cabbages, cauliflowers, lettuce, cucumbers, peppers, mushrooms, radishes, carrots as well as tubers such as potatoes and cereals such as sweet corn (Berlau *et al.*, 1999; Peleg *et al.*, 2008). *Acinetobacter* spp. have also been implicated in the spoilage of bacon, chicken, meat, fish and eggs even when stored under refrigeration or after adequate gamma irradiation (Towner, 2006; Peleg *et al.*, 2009). Hospital food could also be a potential source of *A. baumannii* (Berlau *et al.*, 1999; Towner, 2006). Many strains isolated from foods have lipolytic activity and some strains produce diffusible pigments.

2.2.5.3 Soil and wastewaters

Water and soil provide a home to various microorganisms. *Acinetobacter* genomospecies 3, *A. baumannii, A. calcoaceticus* acinetobacters, and *A. calcoaceticus*, *A. johnsonii*, *A. haemolyticus*, and *Acinetobacter* genomic species 11 have been reported to inhabit soil and aquatic environments (Peleg *et al.*, 2007). The organisms have also been isolated from freshwater ecosystems; raw sewage and wastewater treatment plants (Berlau *et al.*, 1999) and activated sludge (Carr *et al.*, 2001; Peleg *et al.*, 2008; Okabe *et al.*, 2010). *A. baylyi*, *A. bouvetii*, *A. grimontii*, *A. tjernbergiae*, *A. towneri*, and *A. tandoii* are commonly found in natural environments but occasionally isolated in activated sludge and have not been found associated with humans (Chen *et al.*, 2008; Peleg *et al.*, 2008). They are able to store phosphate as polyphosphates and may have potential applications in the biological removal of phosphates (Barbe *et al.*, 2004).

2.2.5.4 Biofilms

Biofilms are composed of microorganisms attached to surfaces and encased in a hydrated polymeric matrix made of polysaccharides, proteins and nucleic acids (Vidal *et al.,* 1996; Sauer *et al.,* 2007). Biofilms function in a manner similar to tissues, using a primitive circulatory system to pump fluids and nutrients through channels in the matrix by changing the ionic strength of the extracellular milieu, causing periodic contraction of matrix polymers.

Pilus mediated biofilms (PMBs) are formed by *Acinetobacter* spp. especially *A. baumannii, A. haemolyticus, A. lwoffii* and *A. calcoaceticus* thus forming thin layers of microorganisms on glass, medical devises, metals, ceramics and other inanimate objects (Dijkshoorn *et al.*, 2007; Gaddy and Actis, 2009). The biofilm thus constitutes a colonial niche for the bacteria from where contact with humans will result in infection. The hydrophobic surface polysaccharide and pili on the bacterial cell surface initiates adherence to human epithelial cells thus initiating the infection process (Dijkshoorn *et al.*, 2007).

Life in biofilms provides microbes with protection against assault from the outside world with barriers against penetration by antimicrobial agents, oxygen and nutrients, along with depressed growth rates and an activated adaptive stress response (Saint *et al.*, 2000). It also enables the organisms to resist the immune host response. Vidal *et al*. (2002) and Lee *et al*. (2007) reported the readiness of *A. baumannii* to adhere to both biological and abiotic surfaces, on which it is able to form biofilms thus ensuring its survival (Vanbroekhoven *et al.*, 2004; Lee *et al.*, 2008).

Unlike in other bacteria where the formation of biofilms is facilitated by intrinsic factors such as the presence of type IV pili, flagella, curli and fimbriae, in *Acinetobacter* spp., putative chaperon secretion membrane systems (*csms*) and putative surface adherence protein regions (*sapr*) have been reported to be responsible (Valero *et al.*, 1999; Gaddy and Actis, 2009). The process generally, involves reversible attachment, irreversible attachment, maturation and dispersion. *A. baumannii*-associated infections are often contracted via biofilms on Foley catheters, venous catheters, or cerebrospinal shunts.

2.2.5.5 Hospital environment

A. baumannii is the most important bacterial species associated with nosocomial or hospital-acquired infections. These infections are caused by organisms present in hospitals or other clinical facilities. Most of these infections emerge while the patient is in the hospital but others are not detected until the patient has been discharged. In the early 70s, nosocomial pathogens were predominantly Gram-negative bacilli especially *P. aeruginosa,* and Enterobacteriaceae, while the 90s saw the emergence of Gram-positive methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Vila *et al.*, 2002; Vallenet *et al.*, 2008). In recent times, in addition to MRSA and VRE (mainly in the USA), the introduction of broad spectrum antibiotics in hospitals has resulted in the emergence of multidrug resistant strictly Gram-negative nosocomial bacterial pathogens including *Ps. aeruginosa, Klebsiella pneumonia* and *A. baumannii* (Endimiani *et al.*, 2007). They have been isolated from reusable medical equipments such as ventilator tubings, arterial pressure monitoring devices, humidifiers, washbasins, plastic urinals and respirometers in hospital environments (Cunha *et al.*, 1980;

Horrevorts *et al.*, 1995; Wendt *et al.*, 1997; Vanbroekhoven *et al.*, 2004). The organisms have also been isolated from the skin of healthcare personnel, mattresses, pillows and in all types of ventilator equipment and moist situations such as sinks and tap water (Bergogne-Bérézin, 2001; Jain and Danziger, 2004). The presence of MDR and nosocomial *Acinetobacter* in the hospital environment complicates treatment since such infected patients often need to be isolated (McGowan, 2006; Lee *et al.*, 2007). This underlies the need to fully study these organisms and proffer alternative chemotherapeutic solutions.

Though many *Acinetobacter* spp. are only found in certain habitats, some are distributed widely in nature. *A. calcoaceticus* is found in water and soil and on vegetables (Berlau *et al.*, 1999; Barbe *et al.*, 2004).

2.2.6 Diagnosis

Infection or colonization with *Acinetobacter* is usually diagnosed by the culture of environmental and clinical samples. The environmental samples include wastewater, activated sludge, sewage, aquaculture freshwater habitats, frozen shrimps (Guardabassi *et al.*, 1999) soil (Carr *et al.*, 2001; Vanbroekhoven *et al.*, 2004), vegetables (Berlau *et al.*, 1999), fresh and spoiled meat (Eribo and Jay, 1985) animal droppings and river water while the clinical samples include blood, cerebrospinal fluid, endoctracheal aspirate, pus (Prashanth and Badrinath, 2005), sputum, urine, respiratory secretions (Vila *et al.*, 2002), catheter tips (Costa *et al.*, 2006), wound, stool or sterile body fluid, skin, cordon of newborns, nasal swabs, hand swabs of hospital workers and hospital environments (swabs on surfaces of machines, wash-

hand basins, floors, tables, UV lamps) (Constantiniu *et al.*, 2004). Microbiological cultures can be processed by standard methods on routine media. Antimicrobial susceptibility can be determined by various means, with the agar-dilution method being the goldstandard.

A glance at the literature shows just how non fastidious and versatile *Acinetobacters* are in terms of growth on media. A wide range of media has been employed in cultivating organisms from different sources. For routine clinical and laboratory investigations, traditional methods have used agar (Eribo and Jay, 1985), brain heart infusion agar (Towner, 2006), nutrient agar, tryptic soy agar (Bergogne-Be´re´zin, 2009), Simon's Citrate agar (Dorsey *et al.*, 2004) Violet red bile agar, Luria Bertani agar (Guardabassi *et al.*, 1999) Eosin-methylene blue, MacConkey agar and Holton medium (Eribo and Jay, 1985). For environmental screening, especially where *Acinetobacter* may be in very low numbers, Bauman's' Enrichment Medium has been employed (Guardabassi *et al.*, 1999; Bouvet and Joly-Guillou, 2000) and broths including MacConkey broth, trypton soy broth, Brain Heart Infusion broth (Guardabassi *et al.*, 1999) and Luria broth (Koneman , 1997; Dorsey *et al.*, 2004). Highly contaminated samples are inoculated in a liquid mineral medium containing a single carbon source and ammonium or nitrate salt as the nitrogen source with the final pH of the medium being 5.5 to 5.6 (Koneman, 1997). Shaking during the incubation is employed in order for the strictly aerobic acinetobacters to outgrow pseudomonads. The broths can later be transferred onto Eosin-methylene blue, MacConkey agar or a selective medium such as Herellea agar or Leeds Acinetobacter Medium in which antibiotics have been added to suppress the growth of other bacteria (Holton, 1983; Towner, 2006).

Biochemical typing methods include the use of colorimetric based GN card ID 32 GN, API 20NE, RapID NF Plus and Vitek 2 systems (Chen *et al.*, 2008) all of which are antibody-based agglutination tests. Serological identification has been attempted with the analysis of capsular type and lipopolysaccharide (Russo *et al.*, 2010) molecules as well as protein profiles for taxonomy and epidemiological investigations.

Because of the widespread nature of *Acinetobacter* spp., typing methods are required for genomic characterization (Guardabassi *et al.*, 1999). The differences in antimicrobial efficacy against different species, and the need to select effective chemotherapeutic agents, require the accurate identification of *Acinetobacter* spp. to the species level. Thus, several molecular diagnostic methods, including the polymerase chain reaction (PCR) (Grotiuz *et al.*, 2006), PFGE, RAPD-PCR DNA fingerprinting (Carr *et al.*, 2001; Peleg *et al.*, 2007), fluorescent *in situ* hybridization (FISH) (Vanbroekhoven *et al.*, 2004), 16S rRNA gene restriction analysis (ARDRA) (Nemec *et al.*, 2000), and 16S rRNA gene PCR-DGGE fingerprinting for genetic characterization of *Acinetobacter* spp. from environmental samples have been employed (Vanbroekhoven *et al.*, 2004). A recent diagnostic method which was reported to have high specificity and can discriminate between *Acinetobacter* species is the microsphere-based array technique that combines an allele-specific primer extension assay and microsphere hybridization (Lin *et al.*, 2008). The method was reported to be so efficient that 13 different species of *Acinetobacter* were discriminated in less than 9 h with 90% accuracy and precision (Lin *et al.*, 2008). The use of DNA-DNA hybridization and sequence analysis (Guardabassi *et*

al., 1999; Chen *et al.*, 2008) is considered the gold standard, but the method is labor-intensive and impractical in most clinical laboratories.

Other methods that have been employed in the epidemiological investigation of outbreaks caused by *Acinetobacter* spp. include biotyping, phage typing, cell envelope protein typing, plasmid typing, ribotyping, restriction fragment length polymorphisms and arbitrarily primed PCR (AP-PCR) (Bello *et al.*, 1997). These methods are however too expensive and too technical for use in unequipped laboratories. Exploration of simple laboratory culture procedures will enhance isolation of these organisms, especially in developing countries, where electricity and sophisticated diagnostic procedures, and trained manpower are lacking.

2.2.7 Factors predisposing individuals to acinetobacterioses

Though it is generally agreed that *A. baumannii* is the most medically significant *Acinetobacter* spp., there is an ongoing debate on the clinical impact of the Acinetobacters with controversial views on whether these organisms increase morbidity or mortality. While some researchers are of the opinion that *A. baumannii* infections are responsible for an increase in patient mortality, others are of the opinion that infections occur in critically ill patients and mortality is a result of other underlying diseases (Falagas *et al.*, 2006; Dijkshoorn *et al.*, 2007; Falagas *et al.*, 2007). Whatever the case may be, virtually every study has concluded that there is a detrimental effect (Gordon and Wareham, 2010). The lack of a consensus on the degree of mortality may be due, in part, to the difficulty in distinguishing between colonization and infection, which is compounded by limited information on the

pathogenesis. There is also extensive methodological heterogeneity between studies (prospective versus retrospective), and variation in the definitions of cases versus controls (*A. baumannii* infection versus other infection, polymicrobial versus monomicrobial) (Grupper *et al.*, 2007; Lee *et al.*, 2007). There are also problems with the accurate identification of organisms to the species level as well as assessment of the impact of specific clones, which may differ in virulence potential (Gordon and Wareham, 2010). In addition, the ability of organisms to rapidly develop multidrug resistance and to persist in harsh environmental conditions, calls for the need to take *Acinetobacter* infections seriously.

Individuals vulnerable to *Acinetobacter* infections include those who have recently undergone major surgery, those with malignant diseases or burns, and immunosuppressed patients such as the elderly, neonates with low birth weights, and patients with prolonged illnesses (Torres *et al.*, 1990; Towner, 2006; Peleg *et al.*, 2009). Nosocomial pneumonia occurs in intensive care units with a frequency of 3 to 5% (even higher in patients with mechanical ventilation) and crude mortality rates of 30 to 75% have been reported (Towner, 2006; Peleg *et al.*, 2009). Bacteremia is very common in elderly immunocompromised patients. The main source of bacteremia in these patients is bacterial pneumonia, and the most important predisposing factors are malignant diseases, trauma, prolonged antibiotic treatment, prolonged intravenous lines, post operations, urinary catheterization, renal transplants chest tubes, mechanical ventilation, parenteral nutrition and long hospitalization (OECD, 2008; Kurcik-Trajkovska, 2009). Poor hygienic conditions, and contaminated food and water are common sources of

infection. In addition, it has been reported that lower temperatures and an acidic pH may enhance the ability of *Acinetobacter* spp. to invade dead tissues (Joly-Guillou, 2005).

2.2.8 Treatment, prevention and control

Treatment of *Acinetobacter* infections should be individualized according to susceptibility patterns as the carbapenems, some fluoroquinolones and doxycycline may retain activity. Impenim with an aminoglycoside and β-lactam/β-lactamase inhibitor with an aminoglycoside was found to be synergistic *in vivo*. Quinolone and amikacin synergy was also observed. The treatment of a serious infection with *Acinetobacter* should be combination therapy based on laboratory antimicrobial susceptibility results. Local antimicrobial prescribing habits should be critically guided by the susceptibility results. Suspected hospital outbreaks involving multidrug-resistant *Acinetobacter* infections should not be regarded with akinesis but be tackled ferociously and promptly. The prompt revision of infection control procedures such as hand-washing, patient isolation, ventilator care and good housekeeping is also important. Chemotherapeutic approaches for most antimicrobial-resistant Gram-negative infections, include the use of carbapenems (imipenem and meropenem), but carbapenem-resistant *Acinetobacter* is increasingly reported (Savov *et al.*, 2002, Jain and Danziger, 2004). Resistance to the carbapenem class of antibiotics complicates the treatment of multidrug-resistant *Acinetobacter* infections. However, colistin and polymyxin B have been used to treat highly resistant *Acinetobacter* infections; unfortunately renal toxicity of colistin has made its choice unattractive. *Acinetobacter* isolates resistant to colistin and polymyxin B have also been reported (Giamarellos-Bourboulis *et al.*, 2001). The best approach is combination therapy

where studies have demonstrated *in vitro* susceptibility of multidrug-resistant *Acinetobacter* to various synergistic combinations of antimicrobials including carbapenems, colistin, rifampin, and ampicillin-sulbactam (Landman *et al.*, 2002; Savov *et al.*, 2002; Yu *et al.*, 2005). The clinical utility of these combinations against pan-resistant *Acinetobacter* remains to be determined (Giamarellos-Bourboulis *et al.* 2001; Jain and Danziger, 2004; Yoon *et al.*, 2004).

The costs associated with controlling an outbreak of *Acinetobacter* infections can be staggering, and some institutions have even been forced to close entire units in order to interrupt the transmission of *Acinetobacter* (Siau *et al.*, 1999; Urban *et al.*, 2003; Kurcik-Trajkovska, 2009). Therefore, a compelling need exists to prevent transmission in the healthcare setting and keep the organism from becoming endemic in an institution. It is also important that contamination of the environment, water or food should be guarded against, especially by MDR strains. Careful personal and hand-hygiene should be observed. The disinfection of hands with alcohol-based disinfectants and observation of standard hospital practices cannot be overemphasized. Laboratories should embark on active surveillance to detect cultures and patients who are colonized with multidrug-resistant *Acinetobacter* as well as a community-based surveillance to determine carriage rates. Other measures successful in the control of outbreaks include isolation precautions for infected or colonized patients, cohorting of patients, patients' relatives and staff, environmental disinfection, antimicrobial control, and unit closure (Wendt *et al.*, 1997; Simor *et al.*, 2002). Investigations for novel antibiotic substances with possible activity against *Acinetobacter* spp. from plants and other

natural sources with a view to sourcing alternative treatment, should be seriously considered by both research institutions and pharmaceutical companies.

2.2.9 CONCLUSION

Acinetobacter, previously ignored as low-grade pathogens, have suddenly generated interest. The emergence of multi-resistant strains, some of which are pan-resistant to antibiotics and can cause a sudden outbreak of infection, and the ability of the organism to resist desiccation as well as its rapid transformation potential has overwhelmed scientists worldwide. Despite an exponential rise in *A. baumannii* infections over the past decade, many questions remain unanswered (Gordon and Wareham, 2010). While knowledge of the virulence and particularly the resistance mechanisms is increasing, the populations at risk and the pathogenesis of severe infection are still poorly understood. The association of the organisms with conditions such as bacteraemia or pulmonary infections, diarrhea and nosocomial infections further highlights a major concern. Most available reports are on *A. baumannii* as the most important clinical species, while investigations are concentrated on clinical samples. Other samples, including environmental and food samples, should be investigated for both *A. baumannii* and other species with a view to investigating their health implications. Control measures should not be limited to investigations of the environment (food, water etc.) for microbial characterization and studies of *Acinetobacter* virulence and resistance mechanisms alone, but the use of animal models should also be considered. The dearth of available treatments remains a major concern and although further work on the use and efficacy of combination therapies is warranted, a more urgent priority must be the development of novel therapeutic agents (Gordon and

Wareham, 2010) including studies on the effect of phytochemicals from plant sources (Doughari *et al.,* 2009b).

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2.3 Phytochemicals as Chemotherapeutic agents and Antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria

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2.3.1 ABSTRACT

The interest in plants with antimicrobial properties has been revived due to current problems associated with the use of antibiotics with the increased prevalence of multiple-drug resistant (MDR) bacterial strains. Some emerging species of bacteria such as *Escherichia coli* O517: H7 and *Acinetobacter* species that are verocytotoxin producers presents further chemotherapeutic challenges because of the increased level of toxin production in medium when challenged with antibiotics. The abundant medicinal plant resources and their antioxidant properties and possibly undiscovered novel modes of action can be a solution to the control of multidrug resistant verocytotoxic bacteria.

Key words: Antimicrobial, multi-drug resistance, chemotherapy

2.3.2 INTRODUCTION

Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases. The word ‘phyto-‘ is derived from the Greek *phyto* which means plant (Liu, 2004). The presence of these bioactive components are said to confer them with resistance against bacterial, fungal and pesticidal pathogens. These bioactive components are said to be responsible for the antimicrobial effects of plant extracts *invitro* (Abo et *al.*, 1991; Nweze *et al.*, 2004).

The interest in plants with antimicrobial properties has been revived due to current problems associated with the use of antibiotics with the increased prevalence of multiple-drug resistant (MDR) strains of a number of pathogenic bacteria such as methicillin resistant *Staphylococcus aureus*, *Helicobacter pylori*, and MDR *Klebsiela pneumonia* (Voravuthikunchai and Kitpipit, 2003). On the other hand, infection with *Escherichia coli* O157: H7 involves the risk stimulation of verocytotoxin (VT) production (Yoh *et al.*, 1997 and 1999). Herbal remedies are viewed as a reemerging health aid in a number of countries (UNESCO, 1997). This can be traced to both the increasing cost of prescription drugs, for the maintenance of personal health and antibiotic-resistant strains in the case of infectious diseases (Levy, 1998; Van den Bogaard *et al.*, 2000; Smolinski *et al.*, 2003). In industrialized countries, the extraction and development of many drugs, and cehemotherpeutics from medicinal plants have been increasing (UNESCO, 1998). Complications in the use of antibiotics in the treatment of hemolytic uremic syndrome (HUS), and thrombocytopenic purpura (TTP) encouraged researchers to find effective medicinal plants as effective treatment for *E. coli* O157:H7 and related infections (Sandvig, 2001; Voravuthikunchai *et al.*, 2005; Abong'o and Momba, 2009).

Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential, and that they contained what we would currently characterize as antimicrobial principles, was well accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies. Sanitation and hygiene levels for the majority of people in Africa are not comparable to those of First World countries. This exposes African

people to a wider array of microbial pathogens, which increases their susceptibility to bacterial infections. Local and indigenous plants are often the only available means of treating such infections in situations where commercial drugs are not available or are too expensive (Fennel *et al.,* 2004; McGaw *et al.,* 2005; Yagoub, 2008; Lewu and Afolayan, 2009). For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tea tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Heinrich *et al.,* 2004). Different plant parts and components (roots, leaves, stem barks, flowers or their combinations, essential oils) have been employed in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin (Rojas *et al.*, 2001; R´ıos and Recio, 2005; Adekunle and Adekunle, 2009). Various chemical compounds (phytochemicals) with antimicrobial activity exist in plants. Phytochemicals have been isolated and characterized from fruits such as grapes and apples, vegetables such as broccoli and onion, spices such as turmeric, beverages such as green tea and red wine, as well as many other sources. These compounds are used by the plants as natural defences against bacteria, fungi and pests (Doughari and Obidah, 2008). In general, phenolics have been shown to be the predominant active chemical in plants, with Gram positive bacteria being the most susceptible germs.

Common methods used in the evaluation of the antibacterial and antifungal activities of plant extracts and essential oils, include the agar diffusion method (paper disc and well), the

dilution method (agar and liquid broth) and the turbidimetric and impedimetric monitoring of microbial growth (R´ıos and Recio, 2005). These methods are simple to carry out under laboratory conditions, thus removing any barrier to the possible investigation of more plants for novel antibiotics.

2.3.3 Mechanism of action of phytochemicals

Different mechanisms of action of phytochemicals have been suggested. They either act as antioxidants, or may modulate gene expression and signal transduction pathways (Kris-Etherton *et al.,* 2002; Manson 2003; Surh 2003). They may either be used as chemotherapeutic or chemopreventive agents with chemoprevention referring to the use of agents to inhibit, reverse, or retard tumorigenesis. In this sense chemopreventive phytochemicals are applicable to cancer therapy, since molecular mechanisms may be common to both chemoprevention and cancer therapy (D'Incalci *et al.,* 2005; Sarkar and Li, 2006). Molecular mechanisms of herb–drug interaction have been investigated. The most notable involve the ATP-binding cassette drug transporters such as P-glycoprotein (You and Moris, 2007) and the drug metabolizing enzymes (known as phase I and phase II enzymes), especially cytochrome P450 3A4 (CYP3A4) (Pal and Mitra, 2006; Meijerman *et al.,* 2006). Multiple molecular targets of dietary phytochemicals have been identified, from pro- and anti-apoptotic proteins, cell cycle proteins, cell adhesion molecules, protein kinases, transcription factors to metastasis and cell growth pathways (Awad and Bradford, 2005; Aggarwal and Shishodia, 2006; Choi and Friso, 2006). Polyphenols particularly are among the diverse phytochemicals that have the potential in the inhibition of carcinogenesis (Liu, 2004). The polyphenolic phytochemicals are virtually

ubiquitous in plant materials and may occur at very high levels. Phenolics in plants are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PAL). They are very important to plants and have multiple functions. The most important role of plant phenolics may be in plant defense against pathogens and herbivore predators, and thus are applied in the control of human pathogenic infections (Puupponen-Pimiä *et al.*, 2008). With the discovery of health benefits of plant polyphenols, it has been proposed to optimize the phenolic content of the diet so as to obtain favorable consequences for general health of the population (Parr and Bolwell, 2000). Phytochemicals including plant polyphenols that show health benefits may act via similar or different mechanisms in humans as those functional in plants. This mechanism may be novel to those of synthetic antibiotics for the control antibiotic resistant pathogenic strains. Phytochemicals may also modulate transcription factors (Andreadi *et al.*, 2006), redox-sensitive transcription factors (Surh *et al.*, 2005), redox signaling, and inflammation (Rahman *et al.*, 2006). As an example, nitric oxide (NO), a signaling molecule of importance in inflammation, is modulated by plant polyphenols and other botanical extracts (Chan and Fong, 1999; Shanmugam *et al.*, 2008). Many phytochemicals have been classified as phytoestrogens, with health-promoting effects resulting in the phytochemicals to be marketed as nutraceuticals (Moutsatsou, 2007).

Phytochemicals such as epigallocatechin-3-gallate (EGCG) from green tea, curcumin from turmeric, and resveratrol from red wine tend to aim at a multitude of molecular targets. It is because of these characteristics that definitive mechanisms of action are not available despite decades of research (Francis *et al.*, 2002). The multi-target nature of phytochemicals may be

beneficial in overcoming cancer drug resistance. This multi-faceted mode of action probably hinders the cancer cell's ability to develop resistance to the phytochemicals. It has also been demonstrated that EGCG has inhibitory effects on the extracellular release of VT from *E. coli* O157: H7 (Voravuthikunchai and Kitpipit, 2003). Ethanol pericarp extracts from *Punica granatum* was also reported to inhibited VT production in periplasmic space and cell supernatant. Mechanisms responsible for this are yet to be understood, however the active compounds from the plant are thought to interfere with the transcriptional and translational processes of the bacterial cell (Voravuthikunchai and Kitpipit, 2003). More work is needed to be done in order to establish this assumption.

2.3.4 Safety concerns for phytochemicals

Plants are natural reservoirs of medicinal agents almost free from the side effects normally caused by synthetic chemicals (Fennel *et al.,* 2004). The World Health Organization estimates that herbal medicine is still the main stay of about 75-80% of the world population, mainly in the developing countries for primary health care because of better cultural acceptability, better compatibility with the human body, and lesser side-effects (Kamboj, 2000; Yadav and Dixit, 2008). The over use of synthetic drugs with impurities resulting in higher incidence of adverse drug reactions, has motivated mankind to go back to nature for safer remedies. Due to varied locations where these plants grow, coupled with the problem of different vanacular names, the World Health Organization published standards for herbal safety to minimize adultartion and abuse (WHO, 1999).

A number of modern drugs have been isolated from natural sources and many of these isolations were based on the uses of the agents in traditional medicine (Rizvi *et al.,* 2009). Antimicrobial properties of crude extracts prepared from plants have been described and such reports had attracted the attention of scientists worldwide (Falodun et al. 2006; El- Mahmood and Amey, 2007; El-Mahmood, 2009). Herbs have been used for food and medicinal purposes for centuries and this knowledge have been passed on from generation to generation (Adedapo *et al.*, 2005). This is particularly evident in the rural areas where infectious diseases are endemic and modern health care facilities are few and far thus, compelling the people to nurse their ailments using local herbs. Herbal treatments have been adjudged to be relatively safe (WHO, 1999). For instance, daily oral doses of epigallocatechin-3-gallate (EGCG) for 4 weeks at 800 mg/day in 40 volunteers only caused minor adverse effects (Phillipson, 2007). In a 90-day study of polyphenon E (a formulation of green tea extract with 53% EGCG), the oral no effect level (NOEL) values are 90 mg/kg/day for rats and 600 mg/kg/day for dogs (Boocock *et al.*, 2007). For curcumin, given to cancer patients at 3600 mg/day for 4 months or 800 mg/day for 3 months, only minor adverse effects are seen. For resveratrol, a single oral dose at 5 g in 10 volunteers only causes minor adverse effects (Boocock *et al.,* 2007). Though herbs are relatively safe to use, their combined use with orthodox drugs should be done with extreme caution. Concomitant use of conventional and herbal medicines is reported to lead to clinically relevant herb–drug interactions (Liu *et al.*, 2009). The two may interact either pharmacokinetically or pharmacodynamically resulting into adverse herbal-drug interactions (Izzo, 2005). St John's wort (*Hypericum perforatum*), used for the treatment of mild to moderate depression, interacts with digoxin, HIV inhibitors, theophylline and warfarin. Some

medicinal herbs, when ingested, either affect cytochrome P450 isoenzymes by which drugs are metabolised, or, phosphoglycoprotein transporter systems that affect drug distribution and excretion. Concurrent use of some herbal medicines with other medicines may either lower blood plasma concentrations of medicinal drugs, possibly resulting in suboptimal therapeutic amounts, or lead to toxic concentrations in the blood, sometimes with fatal consequences (Phillipson, 2007).

Despite this observation however, it has been reported that phytochemicals act in synergy with chemotherapeutic drugs in overcoming cancer cell drug resistance and that the application of specific phytochemicals may allow the use of lower concentrations of drugs in cancer treatment with an increased efficacy (Liu, 2004).

Another advantage with phytochemicals is that, among an estimated 10,000 secondary products (natural pesticides), it has been proposed that human ancestors evolved a generalized defense mechanism against low levels of phytochemicals to enable their consumption of many different plant species containing variable levels of natural pesticides (carcinogens) without subsequent ill health (Liu, 2004). Traces of phytochemicals found in fruits and vegetables may potentiate the immune system and help to protect against cancer (Trewavas and Stewart, 2003). Phytochemicals show biphasic dose responses on mammalian cells. Though at high concentrations they can be toxic, sub-toxic doses may induce adaptive stress response (Ames and Gold, 1991). This includes the activation of signaling pathways that result in increased expression of genes encoding cytoprotective proteins. It is therefore suggested that hormetic

mechanisms of action may underlie many of the health benefits of phytochemicals including their action against cancer drug resistance (Mattson, 2008).

Several phytoconstituents also act as antioxidants. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species otherwise called free radicals, such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite which results in oxidative stress leading to cellular damage (Mattson and Cheng, 2006). Natural antioxidants play a key role in health maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischema, carcinogenesis, eurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing (Uddin *et al.,* 2008; Jayasri *et al.,* 2009). The antioxidants act by reacting with free oxygen radicals. The free radicals are metastable chemical species, which tend to trap electrons from the molecules in the immediate surroundings. These radicals if not scavenged effectively in time, they may damage crucial biomolecules like lipids, proteins including those present in all membranes, mitochondria and, the DNA resulting in abnormalities leading to disease conditions (Uddin *et al.,* 2008). Thus, free radicals are involved in a number of diseases including: tumor inflammation, hemorrhagic shock, atherosclerosis, diabetes, infertility, gastrointestinal ulcerogenesis, asthma, rheumatoid arthritis, cardiovascular disorders, cytic fibrosis, neurodegenerative diseases (e.g. parkinsonism, Alzheimer's diseases), AIDS and even early senescence (Chen *et al.,* 2006; Uddin *et al.,* 2008). The human body produces insufficient amounts of antioxidants which are essential for preventing oxidative stress. Free radicals generated in the body can be removed by the body's own natural antioxidant defences

such as glutathione or catalases (Sen, 1995). Therefore, this deficiency had to be compensated by making use of natural exogenous antioxidants, such as vitamin C, vitamin E, flavones, β-carotene and natural products in plants (Madsen and Bertelsen, 1995; Rice-Evans *et al.*, 1997; Diplock *et al.*, 1998).

Plants contain a wide variety of free radicals scavenging molecules including phenols, flavonoids, vitamins, terpenoids hat are rich in antioxidant activity (Madsen and Bertelsen, 1995; Cai and Sun, 2003). Many plants, citrus fruits and leafy vegetables are the source of ascorbic acid, vitamin E, caratenoids, flavanols and phenolics which possess the ability to scavenge the free radicals in human body. Significant antioxidant properties have been recorded in phytochemicals that are necessary for the reduction in the occurrence of many diseases (Hertog and Feskens, 1993; Anderson and Teuber, 2001). Many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C, and thus might contribute significantly to protective effects *in vivo* (Rice-Evans and Miller, 1997; Jayasri *et al.*, 2009). Studies to uncover other novel plant products especially those with potential activity against verocytotoxic bacteria has become very necessary. This is due to the emergence of bacteria producing these toxins and the abundance of predisposing factors ranging from fecal contamination of food and water sources and low level of hygiene and sanitation consciousness in the developing countries. The ready availability of these plants should be a motivating factor in embarking of such a research.

2.3.5 Methods of studying phytochemicals

A successful strategy for investigating plants for biologically active compounds proved to be initial screening followed by bioassay-guided fractionation to aid isolation of active constituents (Perumal *et al.,* 1999; Mattson and Cheng, 2006). Apart from the traditional methods of screening for biological activity using disc diffusion and agar dilution methods, the separation, identification and structure determination of biologically active compounds has been facilitated by continual development of chromatographic and spectroscopic methods of analysis (Bohlin and Bruhn, 1999). These analytical techniques are becoming more and more sophisticated (Hostettmann and Lea, 1987; Philipson, 2007). The NMR techniques are employed for establishing connectivities between neighbouring protons and establishinh C-H bonds. INEPT is also being used for long range heteronuclear correlations over multiple bondings. The application of Thin Layer Chromatography (TLC), High Performance Chromatography (HPLC) and HPLC coupled with Ultraviolate (UV) photodiode array detection, Liquid Chromatography-Ultraviolet (LC-UV), Liquid Chromatography-Mass Spectrophotometry (LCMS), electrospray (ES) and Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR) techniques for the separation and structure determination of antifungal and antibacterial plant compounds is on the increase frequently (Bohlin and Bruhn, 1999; Oleszek and Marston, 2000). Currently available are chromatographic and spectroscopic techniques in new drug discovery from natural products. Currently, computer modelling has also been introduced in spectrum interpretation and the generation of chemical structures meeting the spectral properties of bioactive compounds obtained from plants (Vlietinck, 2000). The computer systems utilise 1H, 13C, 2D-NMR, IR and MS spectral properties (Philipson,

2007). Libraries of spectra can be searched for comparison with complete or partial chemical structures. Hyphenated chromatographic and spectroscopic techniques are powerful analytical tools that are combined with high throughput biological screening in order to avoid re-isolation of known compounds as well as for structure determination of novel compounds. Hyphenated hromatographic and spectroscopic techniques include LC–UV–MS, LC–UV–NMR, LC–UV–ES–MS and GC–MS (Oleszek and Marston, 2000; Philipson, 2007). However, more work is needed in developing simple methods of identification, purification and formulation of bioactive plant components into drugs for the control of verotoxin producing antimicrobial resistant bacteria and other pathogenic bacteria.

2.3.6 Future prospects of phytochemicals as sources of antimicrobial chemotherapeutic agents

There are few disadvantages associated with natural products research. These include difficulties in access and supply, complexities of natural product chemistry and inherent slowness of working with natural products. In addition, there are concerns about intellectual property rights, and the hopes associated with the use of collections of compounds prepared by combinatorial chemistry methods. Despite these limitations, over a 100 natural-product-derived compounds are currently undergoing clinical trials and at least 100 similar projects are in preclinical development (Phillipson, 2007). Among these products the highest number are from plant origin (Table 2.3.1). Most are derived from plants and microbial sources. The projects based on natural products are predominantly being studied for use in cancer or as anti-infectives. There is also, a growing interest in the possibility of developing products that

contain mixtures of natural compounds from traditionally used medicines (Charlish, 2008), while, a defined mixture of components extracted from green tea (Veregen TM) has been approved by the US Food and Drug Administration (FDA) and has recently come on the market.

Most of the leads from natural products that are currently in development have come from either plant or microbial sources. Earlier publications have pointed out that relatively little of the world's plant biodiversity has been extensively screened for bioactivity and that very little of the estimated microbial biodiversity has been available for screening (Harvey, 2000; 2008). Hence, more extensive collections of plants (and microbes) could provide many novel chemicals for use in drug discovery assays. With the growing realization that the chemical diversity of natural products is a better match to that of successful drugs than the diversity of collections of synthetic compounds and with the global emergence of multidrug resistant pathogens (Feher and Schmidt, 2003) the interest in applying natural chemical diversity to drug discovery appears to be increasing once again (Galm and Shen, 2007).

3613 Table 2.3.1. Drugs based on natural products at different stages of development

Development stage	Plant	Bacterial	Fungal	Animal	Semi-synthetic	Total
Preclinical	46	12	7	7	27	99
Phase I	14	5	0	3	8	30
Phase II	41	4	0	10	11	66
Phase III	5	4	0	4	13	26
Pre-registration	2	0	0	0	2	4
Total	108	25	7	24	61	225

3614 (Source; Harvey, 2008)

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With advances in fractionation techniques to isolate and purify natural products (e.g. counter-current chromatography (Harvey, 2008) and in analytical techniques to determine structures (Singh and Barrett, 2006), screening of natural product mixtures is now more compatible with the expected timescale of high-throughput screening campaigns. Singh and Barrett (2006) point out that pure bioactive compound can be isolated from fermentation broths in less than 2 weeks and that the structures of more than 90% of new compounds can be elucidated within 2 weeks. With advances in NMR techniques, complex structures can be solved with much less than 1 mg of compound. It has recently been demonstrated that it is possible to prepare a screening library of highly diverse compounds from plants with the compounds being pre-selected from an analysis of the Dictionary of Natural Products to be drug-like in their physicochemical properties (Oleszek and Marston, 2000; Harvey, 2008). It will be interesting to see if such a collection proves to be enriched in bioactive molecules. Several alternative approaches are also being explored in efforts to increase the speed and efficiency with which natural products can be applied to drug discovery. For instance, there is an attraction to screen the mixtures of compounds obtained from extracts of plant material or from microbial broths to select extracts from primary screens that are likely to contain novel compounds with the desired biological activity using the concept of 'differential smart screens'. This approach involves screening extracts of unknown activity against pairs of related receptor sites. By the comparison of the ratios of the binding potencies at the two receptor sites for a known selective ligand and for an extract, it is possible to predict which extract was likely to contain components with the appropriate pharmacological activity (McGaw *et al.,* 2005; Harvey, 2008; Okigbo *et al.*, 2009). Another approach is the use of 'chemical-genetics profiling'

(Harvey, 2008). In this method, by building up a database of the effects of a wide range of known compounds, it is possible to interrogate drugs with unknown mechanisms or mixtures of compounds such as natural product mixtures. The technique highlighted unexpected similarities in molecular effects of unrelated drugs (e.g. amiodarone and tamoxifen) and also revealed potential anti-fungal activity of crude extracts. This activity was confirmed by isolation and testing of defined compounds, stichloroside and theopalauamide (Fig. 2.3.1).

Because these compounds are not structurally similar, they would not have been expected to act via the same biological target, thus providing more chances for a very versatile drug component with high efficacy against antibiotic resistant bacteria. It has been reported that, despite the popularity of chemical drugs, herbal medicine in Africa and the rest of the world continued to be practiced due to richness of certain plants in varieties of secondary metabolites such as alkaloids, flavonoids, tannins and terpenoids (Cowan, 1999; Lewis and Ausubel, 2006; Adekunle and Adekunle, 2009). Stapleton *et al.* (2004) reported that aqueous extracts of tea (*Camellia sinensis*) reversed methicillin resistance in methicillin resistant *S. aureus* (MRSA) and also to some extent reduced penicillin resistance in beta-lactamase-producing *Staphylococcus aureus*. Also, Betoni *et al.* (2006) reported synergistic interactions between extracts of guaco (*Mikania glomerata*), guava (*Psidium guajava*), clove (*Syzyguim aromaticum*), garlic (*Allium sativum*) lemon grass (*Cymbopogon citratus*) ginger (*Zingiber officinale*) cargueja (*Baccharis trimera*), and mint (*Mentha pieria*) and some antibiotics against *S. aureus*. However, these are preliminary investigations and more work is needed to actually determine the active

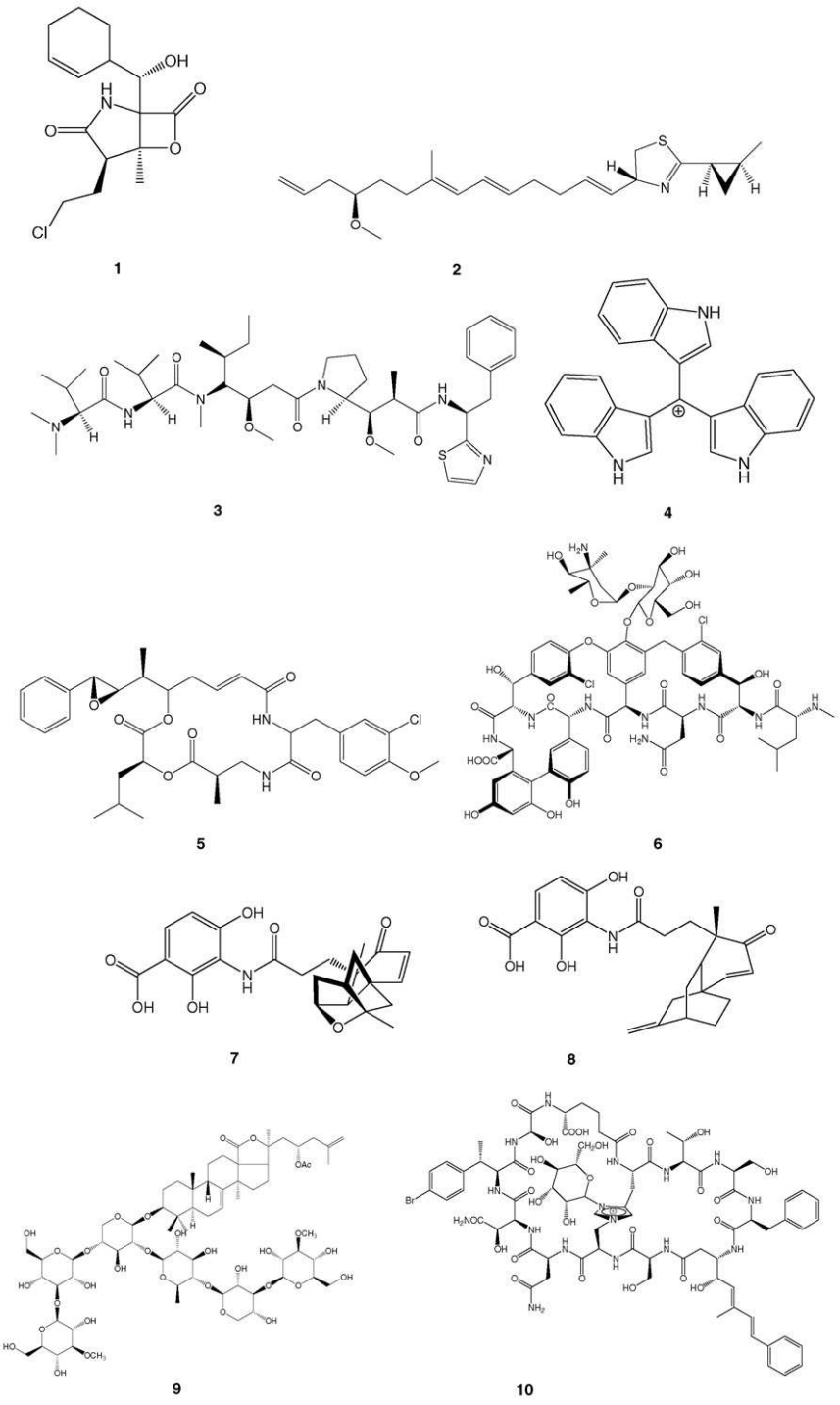

Fig. 2.3.1. Natural products – recently discovered and/or in development. (1) Salinosporamide

A; (2) curacin A; (3) dolastatin 10; (4) turbomycin A; (5) cryptophicin; (6) vancomycin; (7) platensimycin; (8) platencin; (9) stichloroside; (10) theopalauamide (Source; Harvey, 2008).

ingredients in these plants extracts and this may help in improving management of the different infectious diseases that are developing resistance to commonly used antibiotics and possibly to verocytotoxic bacteria. Furthermore, toxicological studies can also be carried out to determine the reliance on these herbs without many side effects.

Researchers have also devised a cluster of chemically related scaffolds which are very useful in guiding the synthesis of new compounds. In an attempt to combine the advantages of virtual screening of chemically diverse natural products and their synthetic analogues (scaffolds) with the rapid availability of physical samples for testing, an academic collaboration has established the Drug Discovery Portal (http://www.ddp.strath.ac.uk/). This brings together a wide variety of compounds from academic laboratories in many different institutions in a database that can be used for virtual screening. Academic biology groups can also propose structures as targets for virtual screening with the Portal's database (and with conventional commercially available databases). Access to the Portal is free for academic groups and the continued expansion of the chemical database means that there is a valuable and growing coverage of chemical space through many novel chemical compounds (Feher and Schmidt, 2003; Galm and Shen, 2007; Harvey, 2008).

Despite all of the advances made by the pharmaceutical industry in the development of novel and highly effective medicines for the treatment of a wide range of diseases, there has been a

marked increase in the use of herbal medicines even including the more affluent countries of the world. Germany has the largest share of the market in Europe and it was reported that the sales of herbal medicinal products (HMPs) in 1997 were US$ 1.8 billion (Barnes *et al.*, 2007). Numerous scientific medical/pharmaceutical books have been published in recent years aiming to provide the general public and healthcare professionals with evidence of the benefits and risks of herbal medicines (Barnes *et al.*, 2007; Phillipson, 2007). The pharmaceutical industry has met the increased demand for herbal medicines by manufacturing a range of HMPs many of which contain standardized amounts of specific natural products. In the 1950s, it would not have been possible to predict that in 50 years' time there would be a thriving industry producing HMPs based on the public demand for herbal medicines. To date, European Pharmacopoeia has even published up to 125 monographs on specific medicinal herbs with another 84 currently in preparation (Mijajlovic *et al.*, 2006; Phillipson, 2007. The monographs are meant to provide up-to-date knowledge of phytochemistry for defining the chemical profiles of medicinal herbs and an understanding of analytical tests for identification of the herbs and for the quantitative assessment of any known active ingredients (Phillipson, 2007). Several regulatory bodies incuding Traditional Medicines Boards (TMBs, in Nigeria and other African Countries), Medicines and Healthcare products Regulatory Agency (MHRA), Herbal Medicines Advisory Committee (HMAC) (Uk) and American Herbal Products Association (AHPA) and several other pharmacopoeia (British, Chinese, German, Japanese) provide guidelines and advice on the safety, quality and utilization of the plant herbal products in several countries (Yadav and Dixit, 2008). Scientific and research communities are currently engaged in phytochemical research, and pharmacognosy, phytomedicine or traditional

medicine are various disciplines in higher institutions of learning that deals specifically with research in herbal medicines. It is estimated that >5000 individual phytochemicals have been identified in fruits, vegetables, and grains, but a large percentage still remain unknown and need to be identified before we can fully understand the health benefits of phytochemicals (Liu, 2004). Despite the increased interest in medicinal plant research worldwide, only rare (Voravuthikunchai *et al.,* 2005) or no publications are found even in the developed countries on efficacy of these plants on verocytotoxic bacteria. Though there are several published data on the efficacy of phytochemicals on *E. coli,* and *Shigella* spp., other Gram-negative bacteria, and antibiotic resistant bacteria (Nascimento *et al.,* 2000; Yagoub, 2008; Okigbo *et al.,* 2009; El-Mahmood, 2009; Aiyegoro *et al.,* 2009), limited data is available on the efficacy of these plants on the verocytotoxin producing *E. coli* O157: H7 and other related bacteria in Africa. Deliberate research drives should be made by researchers especially in the developing world to stockpile beforehand, relevant potential medicinal plant cure against these bacteria. This should be done with a view to developing novel drugs for the chemotherapy of these emerging pathogens.

2.3.7 CONCLUSION

With the increasing interest and so many promising drug candidates in the current development pipeline that are of natural origin, and with the lessening of technical drawbacks associated with natural product research, there are better opportunities to explore the biological activity of previously inaccessible sources of natural products. In addition, the increasing acceptance that the chemical diversity of natural products is well suited to provide the core

scaffolds for future drugs, there will be further developments in the use of novel natural products and chemical libraries based on natural products in drug discovery campaigns. Such array of antimicrobial substances, when discovered, will in no doubt provide prospective alternatives for the control of antimicrobial resistant bacteria in addition to emerging verocytotoxygenic ones.

2.3.8 ACKNOWLEDGEMENT

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2.4 *Curtisia dentata*: ethnopharmacological application

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2.4.1 ABSTRACT

Ethnopharmacology is now being integrated into the mainstream medicine all over the world, including South Africa, due to the increasing popularity of medicinal plants in the treatment of various infections. Several plants have been used in various communities for the treatment of various diseases. *Curtisia dentata*, a medicinal plant, is among the most commonly used medicinal plants in South Africa. The plant is used in the treatment of diarrhea, amongst other diseases. There is dearth in information on its antimicrobial potential as well as phytochemical and toxicological profiles. Investigation of the antimicrobial potentials of *C. dentata* will lead to the discovery of chemical substances that can possibly be used in the development of novel chemotherapeutic agents for the treatment of infections such diarrhea, candidiasis and other microbial infections.

Key words: Ethnopharmacology, South Africa, *Curtisia dentata*, diarrhea, toxicology, medicinal trade market.

2.4.2 INTRODUCTION

Ethnopharmacology, the science of application of indigenous or local medicinal remedies including plants for treatment of diseases (Gurib-Fakim, 2006; Pande *et al.,* 2008) has been the mainstay of traditional medicines throughout the world and is currently beingt integrated into mainstream medicine. Different catalogues, including *De Materia Medica, Historia Plantarum, Species Plantarum,* have been variously published in attempts to provide scientific

information on the medicinal uses of plants (Gurib-Fakim, 2006). A medicinal plant is a plant whose parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains, seeds or roots are employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active. These non-nutrient plant chemical compounds or bioactive components, often referred to as phytochemicals or phytoconstituents, are responsible for protecting the plant against microbial infections or infestations by pests (Pieters and Vlietinck, 2005; Gurib-Fakim, 2006; Doughari *et al.*, 2009). Consequently, medicinal plants are potential sources of new compounds of therapeutic value and as sources of lead compounds in drug development (Matu and van Staden, 2003).

Humans have discovered the secret of exploiting these phytoconstituents in the control of various health ailments through the use of plants for medicinal purposes. The use of traditional medicinal plants dates back since antiquity (Gurib-Fakim, 2006; Egwaikhide and Gimba, 2007) and the types of plants and methods of application vary from locality to locality. It has been reported that 80% of rural dwellers all over the world, especially in Africa, rely on plants as means of treating various diseases (Bodeker and Kronenberg, 2002; Matu and van Staden, 2003; Gurib-Fakim, 2006; Upadhyay *et al.*, 2007). Logistics and beaurocracy associated with modern health, inadequate facilities, limited access to formal and adequate health services, high cost of antibiotics and other drugs, and inaccessibility has forced the underprivileged communities to continue depending on traditional plants as their sources of medicines (Matu and van Staden, 2003). Consequently, a single plant may be used for the treatment of various disease conditions, depending on the community. Several ailments including fever, asthma,

constipation, esophageal cancer and hypertension have been treated with traditional medicinal plants (Cousins and Huffman, 2002; Saganuwan, 2010). The plants are applied in different forms such as poultices, concoctions of different plant mixtures, infusions as teas or tinctures, or as component mixtures in porridges and soups administered in different ways including oral, nasal (smoking, snoffing or steaming), topical (lotions, oils or creams), bathing or rectal (enemas) (Gurib-Fakim, 2006). Despite the widespread application of plants in traditional medicines and their rapidly increasing popularity even among urban dwellers as well as the educated class, scientific analyses of the purported benefits of many plants are still scant. The increasing acceptability is not unconnected with the increasing inefficacy of many modern drugs used for the control of many infections such as typhoid fever, gonorrhea, and tuberculosis. The development of resistance by several bacteria to various antibiotics (WHO, 1996) has forced the scientific, medical, research and the academic community to delve into investigating alternative sources of treatments to these recalcitrant bacteria. In addition, the increase of opportunistic infections especially with Acquired Immune Deficiency Syndrome (AIDS) patients and individuals on immunosuppressive chemotherapy, toxicity of many antifungal and antiviral drugs has further underlined the need of searching for more new drug substances (Maregesi *et al.*, 2008).

Just as in several other parts of the world, medicinal plants are an integral part of African culture. In South Africa, 21st century drug therapy is used side-by-side with traditional African medicines to heal the sick (van Wyk *et al.*, 1997). In their separate studies on six South African urban centers of a total population of over 1.5 million, Hirschowitz and De Castro

(1995) and Mander (1998) reported that 70% of this population visits a traditional healer an average of three times a year. The country is also endowed with a very rich biodiversity of natural plant resources (van Wyk *et al.,* 1997; McGaw and Eloff, 2008; van Wyk, 2008) useful as medicinal plants. These plants span an estimated 147 plant families amongst which the Fabaceae, Asteraceae, Euphorbiaceae, Rubiaceae and Orchidaceae families are the most popular within the Zulu, Sotho and Xhosa ethnomedicine (Hutchings *et al.,* 1996). The most commonly preferred plant part, the stem bark, accounts for approximately 27% of market produce traded annually in KwaZulu–Natal (Mander, 1998). Common medicinal plants employed as local health remedies include *Sutherlandia frutescens* (for flu, as blood purifier and all-purpose tonic) (van Wyk et al., 1997; Mncwangi and Viljoen, 2007), *Bridelia micrantha* (Euphorbiaceae, for diarrhoea, stomach ache, sore eyes), *Combretum molle* (Combretaceae, for fever, abdominal pains, convulsion, worm infections), *Combretum coffrum* (Combretaceae, for conjunctivitis) and *Terminalia sericea* (for cough, diarrhoea, skin and wound infections) (Bessong *et al.,* 2004; McGaw and Eloff, 2008).

Members of the Cornaceae family have been subject of extensive phytochemical and pharmacological research (Lee *et al.,* 2000). There are reports of various compounds including flavonoids, phenolic compounds and terpenoids isolated from one of the Cornaceae family, *Cornus controversa* (Lee *et al.,* 2000). Antimicrobial and antihelminthic activities of some members of this family have also been reported. Dulger and Gonuz (2004) reported activity of the ethanol extracts of *Cornus mas* against *Pseudomonas aeruginosa, Proteus vulgaris* and *Micrococcus luteus*. The genus "*Curtisia"* of the Cornaceae family was first named by the

4084 botanist William Curtis. The species name *"dentata"* is simply coined from the Latin version
4085 "toothed", referring to the slightly serrated margins of its leaves. Previous reports indicate that
4086 the plant is potent against some pathogenic bacteria, fungi and some parasites. Enwerem *et al.*
4087 (2001) has earlier reported the antihelminthic activity of betulinic acid, a chemical compound
4088 isolated from *C. dentata* against *Caenorhabditis elegans*, a free-living nematode, at a
4089 concentration of 500 µg/mℓ after 7 days of incubation. Despite reports on some members of
4090 the Cornaceae family, there is little information on the phytochemical, pharmacological and
4091 biological investigations of *C. dentata*. This paper thus provides botanical information and
4092 highlights the pharmacological potential of *C. dentata.*
4093
4094 **2.4.3 Description and distribution of *C. dentata***
4095 *C. dentata* (Cornaceae or dogwood family) or assegai (English common name) is a traditional
4096 medicinal plant that has been employed in the treatment of diarrhea and related stomach
4097 ailments in South Africa (Notten, 2004). *C. dentata is* locally named in South Africa as - the
4098 Zulu Assegai- because of the 'African spear' traditionally made from this tree's strong wood.
4099 In Southern Africa, the common names include assegaai (Afrikaans.); uSirayi, umGxina
4100 (Xhosa), umLahleni (Xhosa, Zulu), uMagunda, uMaginda, umBese, umPhephelelangeni
4101 (Zulu), iliNcayi, isiNwati (Stwanee), modula-tshwene (Northern Sotho), musangwe,
4102 mufhefhera (Venda) and modula-shtwene (Pede) (Notten, 2004; Shai *et al.*, 2008). Of the 15
4103 plant genera found in the Cornaceae family, only the *Curtisia* genera are found in Africa (Shai
4104 *et al.*, 2008).
4105

The plant is an attractive medium-sized tree with dark bark and fissured in square patches; young branches covered in dense rusty brown hairs. The plant, when young, is velvety to the touch and bronze-gold in colour. The leaves are smooth, glossy and opposite, ovate to broadly elliptic, up to 10 cm in length. The leaf surface is leathery, shiny dark green above, light green and covered in woolly hairs with conspicuous venation below; covered in dense rusty brown hairs with a sharply pointed apex and strongly margined toothing. The flowers are small, inconspicuous and odourless occurring in branched terminal heads, up to 12 cm long, cream, covered in soft, light grey hairs. *C. dentata* fruits are small rounded to oval fleshy bitter berries about 10 mm in diameter, white, turning red when ripe and crowned with the calyx remains. The wood is tough, hard, heavy, fine-grained, dull red, used in the past for furniture, rafters and flooring. *C. dentata* is a very decorative tree, even when small (Fig. 2.4.1) and is usually cultivated from the seed (Notten, 2004).

The plant has a worldwide distribution and especially on mountains, evergreen forests and along the margins of forests and grasslands (Notten, 2004). 'Assegai tree' as it is commonly called, grows in the forests of South Africa and Swaziland, ranging from sea level to 1800 meters elevation, and from Cape Town in the south to Limpopo province in the north. In deep Afromontane forest *C. dentata* grows into a tall tree, but on open mountain slopes and by the coast, the plant remains a small bushy tree. In Southern Africa in general, *C. dentata* is found in Zimbabwe, Mozambique, South Africa (Limpopo, Mpumalanga, KwaZulu-Natal, Eastern and Western Cape Provinces) and Swaziland (Shai *et al.*, 2008). The plant been in decline in some areas, as its bark is highly valued for traditional medicine.

Fig. 2.4.1. *Curtisia dentata* full plant.

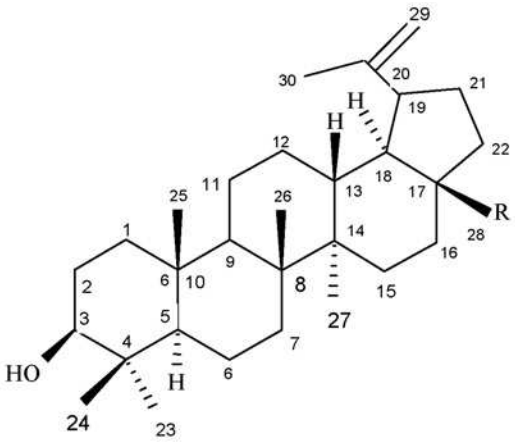
2.4.4 Medicinal properties and antimicrobial potentials of *C. dentata*

C. dentata has been employed in the treatment of various ailments. The stem bark is used by Southern African cultures as an aphrodisiac, a blood purifier and as treatment against various stomach ailments and diarrhea (Pujol, 2000). In the Eastern Cape Province of South Africa, the local populations use the bark to treat heart-water in cattle (Dold and Cocks, 2001). Traditional herbal practitioners use this species in special mixtures because it is scarce and endangered. *C. dentata* is also used for the treatment of pimples (Shai *et al.,* 2009a; Dold and Cocks, 2001). The ethanol and aqueous extracts of the plant have been reported to exhibit antibacterial activity against *Bacillus subtilis* (McGaw *et al.*, 2000). Shai *et al.* (2009a) reported the activity of acetone extracts of leaves, twigs and stem barks of the plant against *E. coli, S. aureus, P. aerugenosa E. faecalis* as well as *C. albicans* as well as inhibition of motility in some parasitic and free living nematodes (Shai *et al.*, 2008; 2009a,b).

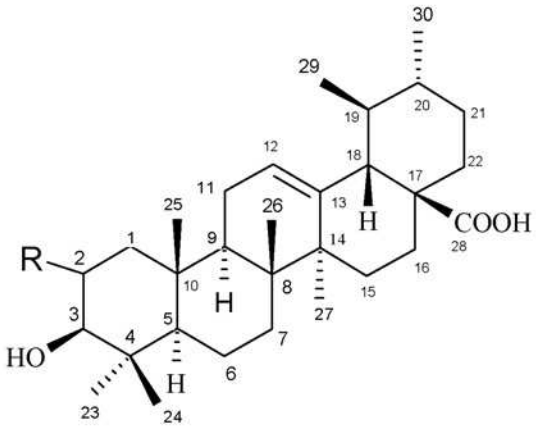
2.4.5 Chemical constituents

There is paucity of reports of phytochemical, pharmacological and biological investigations of *C. dentata,* probably due to its scarce nature. Other members of the Cornaceae family such as *Cornus controversa,* have been subjects of extensive phytochemical and pharmacological research with various flavonoids, phenolic compounds and terpenoids being reported (Lee *et al.*, 2000). Doughari *et al.* (2010a) was the first to report the presence of tannins, flavonoids (Fig. 2.4.2), saponins, anthraquinones, steroids and glycosides in various extracts of the plant (Doughari *et al.,* 2010a). The isolation of four antibacterial and antifungal triterpenoids through bioactivity guided fractionation and bioautogram studies was also (for the first time)

reported by Shai *et al.* (2007; 2008). Their study revealed that the leaf extracts of the plant contained a greater number of antifungal compounds including lupeol, betulinic acid, ursolic acid and 2-alpha-hydroxyursolic acid (Fig. 2.4.2). Several of these compounds displayed common R_f values in thin layer chromatography. Betulinic acid, ursolic acid and 2-alpha-hydroxyursolic acid appreciably inhibited fungal growth with minimum inhibitory concentration (MIC) values ranging from 8-63 µg/ml. The study provided information on the antimicrobial compounds of this species, as well as a preliminary rationale for the use in traditional South African medicine. Another study by Breuer *et al.* (1978) also reported the presence of the fatty acid linolenic acid ($C_{17}H_{31}COOH$) - an unsaturated fatty acid (Fig. 2.4.2), considered essential to the human diet, responsible for cell development and regulation of cellular metabolism. *C. dentata* also contains ellagitannins (complex tannins) (Figs. 2.4.3, 4 and 2.4.5) which are hydrolysable tannins (yielding polyphenols) mainly glucose esters of hexahydroxydiphenic acid and its congeners (Bate-smithi *et al.*, 1975; Barron, 2001).



Lupeol (**1**)	R = CH_3
Betulinic acid (**2**)	R = COOH



Ursolic acid (**3**)	R = H
2α-hydroxyursolic acid (**4**)	R = αOH

Fig. 2.4.2. Structures of some chemical compounds isolated from leaf extracts of *Curtesia dentata* (Shai *et al.*, 2008).

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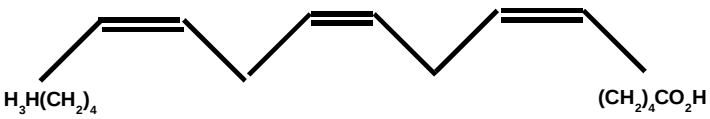

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4184 Fig. 2.4.3. Structure of linolenic acid

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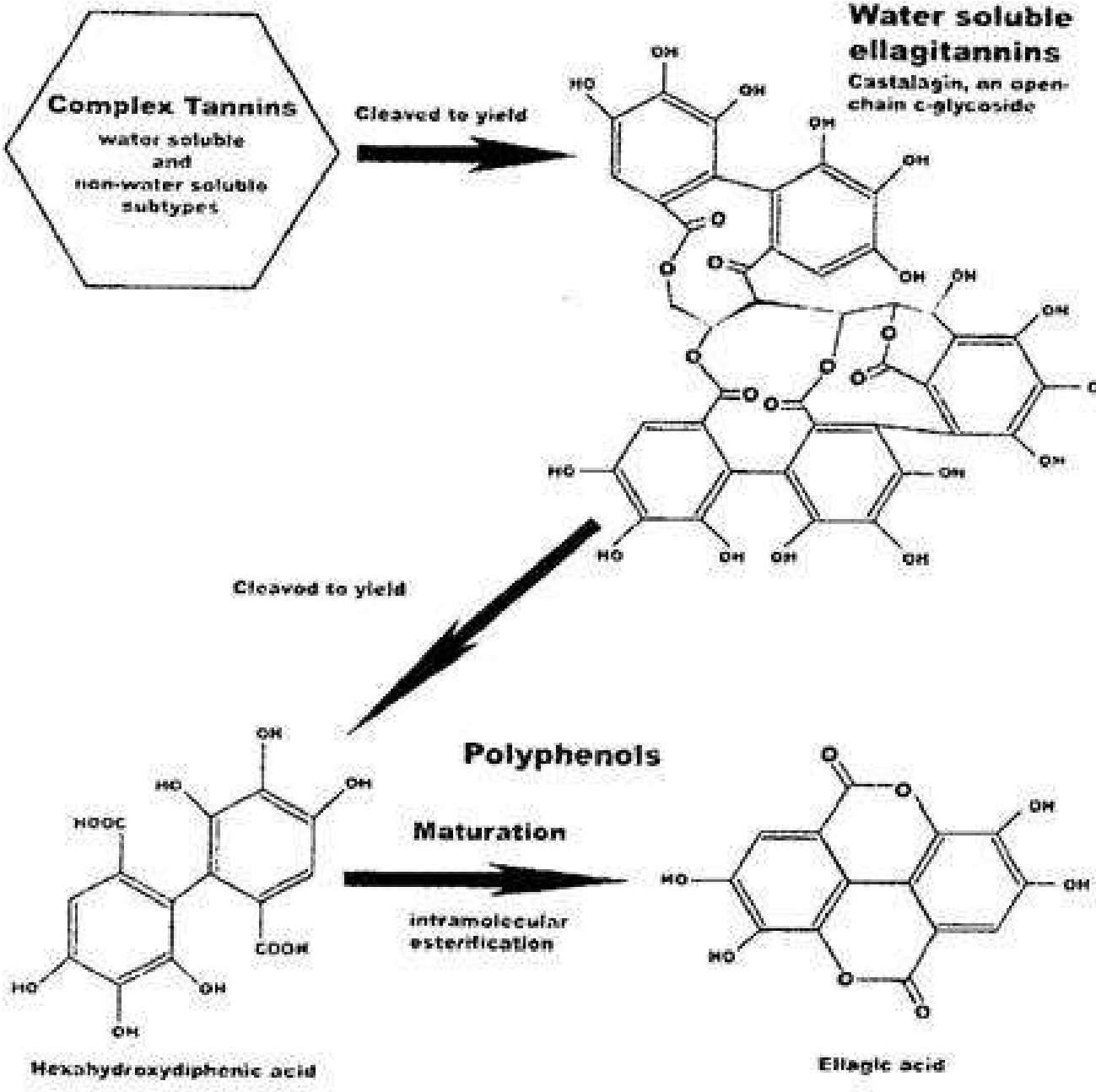

4188
4189 Fig. 2.4.4. Structure of some ellagitannins found in extracts of *Curtisia dentata* (Barron,
4190 2001).

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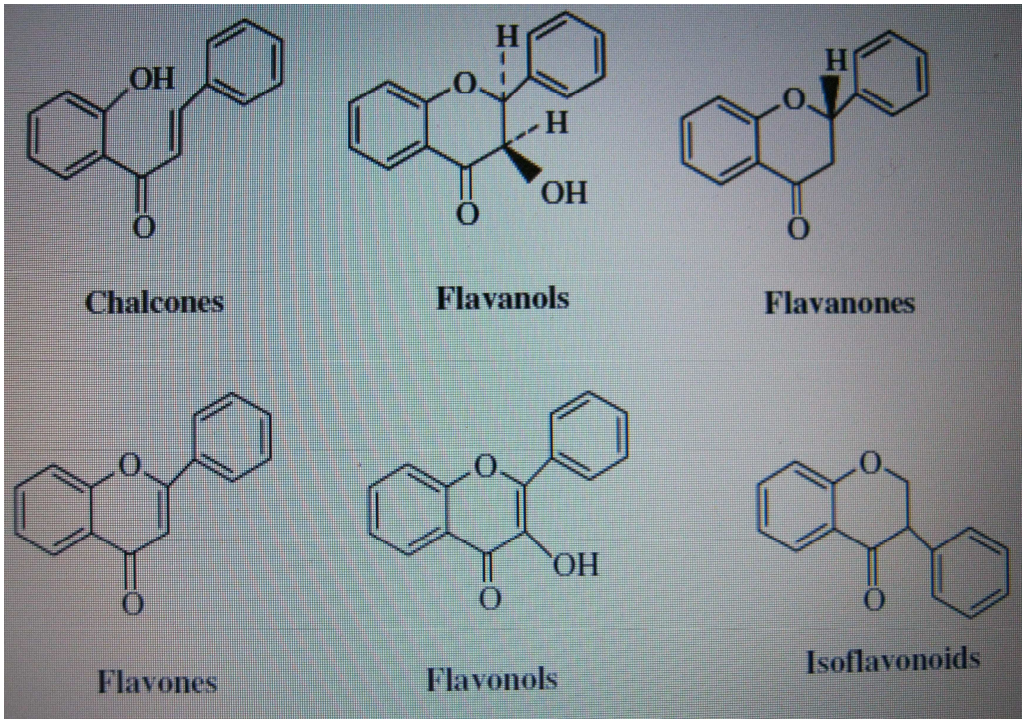

Fig. 2.4.5. Basic structures of some flavonoids.

2.4.6 Challenges of trade and harvesting to *C. dentata* sustainability

In South Africa, an increased demand exists for plant-derived medicines, which has created a trade in indigenous plants estimated to be worth approximately R270 million per annum (Dold and Cocks, 2002). This demand has resulted in more than 700 plant species being actively traded for medicinal purposes throughout the country. Consequently, there is an intensive harvesting of wild material which has posed a serious threat to biodiversity in the region. With the increasing harvesting pressures on traditional supply areas, there is a growing shortage in supply of the popular medicinal plant species (Van Wyk, 2008). One such affected plant just like most African medicinal plants, is *C. dentata*. The plant is in high demand with a high price in South Africa. It is among the ten most commonly sold plant species in the Eastern Cape region of Southern Africa and among the sixty most frequently traded plants. Because the plant is scarce and endangered, traditional medicine practitioners use it in special herbal mixtures (Cunningham, 1988). Due to heavy trading, the plant has become vulnerable, scarce and in decline, therefore making it conservation dependent (Dold and Cocks, 2002). The stem bark of the plant is a common feature in the traditional medicinal trade in South African herbal markets. Because of the need to conserve the plant species, the government enlisted it among the 'nationally protected trees' (Pilot State of the Forest Report, 2005). This therefore calls for stricter management measures to ensure its conservation.

2.4.7 Challenges on research and ethnopharmacological applications of *C. dentata*

African medicinal plant resources are under the threat of extinction. This is as a result of over-exploitation due to excessive utilization, commercialisation, habitat destruction and other

natural and man-made destructive influences. Deliberate targeted conservation measures must be taken in order to ensure their continued availability. The establishment of medicinal botanical gardens, herbarium and farms should be vigorously pursued in this respect. With the increasing relevance the world including researchers and scientists are giving to traditional medicinal plants, and with the global increase in incidence of multidrug resistance by microorganisms to antimicrobial agents, it is very pertinent that medicinal plants should be adequately studied and conserved. The significance of *C. dentata* in the treatment of various infections, especially diarrhea if properly investigated, will go a long way in curbing the high incidences of these infections, which is currently ravaging the African continent. Currently, 2-3 billion incidences of diarrhea and 3-5 million deaths from the disease is said to occur annually in the developing countries (WHO, 1996; Sanchez and Holmgren, 2005; Doughari *et al.*, 2010b), including South Africa (Lin *et al.*, 2002). The fact that the plant is used in the treatment of diarrhea calls for the investigation of its antimicrobial activities against diarrhea-causing bacteria such as *E. coli, S. dysenterie, Salmonella* spp. and other related bacteria. Phytochemical and toxicity studies of this plant should also be carried out, to enable identification of active chemical constituents and cautions to be issued of dangerous practices or its toxic effects.

The high demand of *C. dentata* in the South African medicinal trade market and the dearth in information on its antimicrobial potential as well as phytochemical and toxicological profiles call for more vigorous research as well as stricter conservation measuresin this area.

2.4.8 ACKNOWLEDGEMENT

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CHAPTER THREE

3.0 JUSTIFICATION, AIM AND OBJECTIVES OF STUDY

3.1 JUSTIFICATION

With the continued depletion of water sources due to the increase in human population and urbanization, pressure for water demand results in sharing available water sources between humans and animals, especially in rural areas. Consequenlty, such water bodies are contaminated with pathogenic bacteria. Poor sanitry conditions and poorly constructed pit latrines release their contents into river water or water used for drinking purposes during run-offs. Run-offs also wash animal excreta into water bodies from livestock that are often reared in close proximity with human settlements in typical nomadic set-ups or poor rural communities in Africa. Due to ignorance and poverty, such contaminated water usually ends up being used for either domestic or irrigational purposes with little or no precautionary measures, especially among the rural folks in developing countries. Consequently, pathogenic bacteria including *Escherichia coli*, *Acinetebacter* spp., *Salmonella* and *Shigella* spp. are transmitted to humans.

These pathogenic bacteria, unlike innocuous commensals, alternate between free living and host associated states. In any new environment, they are confronted with conditions foreign to their previous medium which they need to immediately adjust to. The changes in physico-chemical conditions of the medium often exert stress effects on the bacterial cell. Stress is a state of altered homeostasis provoked by a psychological, environmental, or physiological stressor(s) (Peterson *et al.*, 1991). Following exposure of cells to various suboptimal physical and chemical environments, such as cold and warm temperature, low A_w, low hydrostatic pressure, UV light, high salt concentrations, bacteriocins, preservatives, detergents, several

dyes, and antibiotics, many foodbome pathogens and spoilage bacteria are either killed or develop adaptive mechanisms that enable them to survive such conditions. The physico-chemical parameters encountered by the bacteria in these two states are very different and exert different demands and stresses on the bacterial cell (Chowdhury *et al.*, 1996). Consequently, bacteria respond to these environmental changes by trigering some adaptive mechanisms which may involve altering the pattern of gene expression with activation of a set of genes whose products assist in survival and turning off those products that are not necessary in a particular environment. This is particularly done by induction of virulence factors. According to Chowdhury *et al.* (1996), the expression of virulence genes is controlled by regulatory systems in such a manner that the virulence factors are expressed at different stages of the infection process dictated by the changing micro-environment of the host as a consequence of the pathophysiology of infection. Thus, the understanding of the dynamics of bacterial response to various stress conditions, as might be found in water, refrigeration temperatures, laboratory media and disinfectant-utilizing environments is indeed significant in order to develop more proactive control measures.

Verotoxic non O157:H7 *E. coli* and *Acinetobacters* spp. were selected for this study due to their association with various antibiotic resistant diarrhogenic and nosocomial infections. Their presence in a wide range of enviromnments including soil, water, animal bodies and food, as well as their ease in genetic manipulation, makes them ideal for this study.

When introduced into a host or food, bacteria including, *E. coli* and *Acinetobacters* spp. are confronted with an increase or decrease in temperature from that of the environment to that of

the food or the host cell. In the animal body, they are exposed to with either salts, chemicals or biological defence mechanisms such as haemolysins, gelatinases and serum. In food presevation, the organisms are subjected to lower preservation temperatures. In foods or laboratory media, the bacteria are confronted with either salts or chemicals such as sodium chloride, crystal violet, as well as antibiotics. Survival of these bacteria depend on their ability to develop daptive mechanisms. These mechanisms often confer them with resistance to these biophysico-chemical conditions. Resistance factors are genetically mediated and thus are transferable to other bacteria. Contamination of water and food sources with these resistance strains results in further spread of the resistance factors consequently, more recalcitrant infections emerge.

Antibiotic resistant diarrheal and nosocomial infections are often associated with verotoxic *E. coli* and *Acinetobacter* spp. Lack of or inadequate supply of potable water, poor hygiene, and contamination of water sources with agricultural and industrial chemicals and human and animal excreta is a common phenomenon in developing countries. Such contamination introduces these bacteria in the water and subsequently into food due to domestic usage of the water. This explains the alarmingly high incidences of diarrheal infections. For effective control measures to be developed, it is important that the response of the bacteria to the various stress conditions earlier mentioned, be adequately understood.

3.2 Aim of the study

The study was therefore carried out in order to determine the presence and response of antibiotic resistant verotoxic wastewater and river water isolates of *Acinetobacter lwoffii, A. haemolyticus* and non-0157 *E. coli* to oxidative stress and antioxidant phytochemicals of stem bark extracts of *Curtisia dentata* and also to determine transformation amongst some strains of resistant *A. haemolyticus* and *Escherichia coli.*

3.3. Objectives

1. To isolate and identify verocytotoxic *Acinetobacter* spp. and non O157: H7 *E. coli* from wastewater and river water samples;
2. To screen for the presence of virulence factors and antibiotic susceptibility among verotoxic non O157: H7 *E. coli* isolates obtained from water and wastewater samples;
3. To determine multi-drug resistance, verotoxin production and efficacy of crude stem bark extracts of *C. dentata* among *A. haemolyticus, A. lwoffii* and non O157 *E. coli* obtained from water and wastewater samples;
4. To screen for the presence of virulence, resistance genes and transformation amongst environmental isolates of verotoxic non O157*E. coli* and *Acinetobacter* spp.;
5. To determine the effect of oxidative stress on viability and virulence of environmental isolates of non O157 *E. coli;*
6. To determine the effect of oxidative stress on the viability and virulence of environmental *A. haemolyticus* isolates;
7. To determine the effect of plant antioxidants/phytochemicals of *C. dentata* on antibiotic

resistant verotoxin producing *Acinetobacter* spp. and non O157*E. coli* strains; and

8. To screen for the antioxidant, antimicrobial and antiverotoxic potentials of extracts of *C. dentata.*

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CHAPETR FOUR

4.0 VIRULENCE FACTORS AND ANTIBIOTIC SUSCEPTIBILITY AMONG VEROTOXIC NON 0157: H7 *ESCHERICHIA COLI* ISOLATES OBTAINED FROM WATER AND WASTEWATER SAMPLES IN CAPE TOWN, SOUTH AFRICA.

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4.0 Virulence factors and antibiotic susceptibility among verotoxic non 0157: H7 *Escherichia coli* isolates obtained from water and wastewater samples in Cape Town, South Africa

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4.1 ABSTRACT

Forty eight samples (30 wastewater and 18 river water) were collected between July and November, 2010 from different sources in Cape Town, South Africa in order to characterize verotoxic non O157: H7 *Escherichia coli* strains. Samples (1 ml) were inoculated into MacConkey broth (MB, 9 ml) and incubated at 37ºC for 24 h, after which a loopful of the MB was then spread onto Eosin Methylene Blue (EMB) and further incubated for 24 h at 37ºC in order to isolate *E. coli.* The identification of isolates was done using standard biochemical procedures, and confirmed serologically using *E. coli* polyvalent antisera (Bioweb, SA). Isolates were also characterized for virulence factors such as verotoxin, haemolysin, gelatinase, extended spectrum beta lactamases (ESBLs), cell surface hydrophobicity and bacterial serum resistance, as well as susceptibility (using disc diffusion method) to stem bark extracts of *Curtisia dentata.* Results showed the presence of different serotypes of *E. coli* (69 isolates altogether) including O26:H11, O55, O111:NM, O126, O44, O124, O96:H9, O103:H2, O145:NM and O145:H2. Over 60% of the isolates exhibited serum resistance, haemolysin and gelatinase production, 81% exhibited a cell surface hydrophobicity and over 52% produced ESBLs. Results also showed that, while 60% of the isolates showed various levels of resistance to different antibiotics: ampicillin (10 µg), cefuroxime, cephalexin, ceftazidime and tetracycline (30 µg in each case) (multidrug resistance index (MDRI) values 4.20-5.60%), only 28 % were resistant to ethanol stem bark extracts of *C. dentata* (MIC, 70-150 mg/ml). The presence of pathogenic verotoxic antibiotic resistant *E. coli* in these water sources is a threat to water quality and food security and *C. dentata* has a potential for

sourcing novel antibiotic substances for chemotherapy against these resistant pathogenic strains of *E. coli.*

Key words: *Curtisia dentata, Escherichia coli,* haemolysins, cell surface hydrophobicity, gelatinase, plant extracts, verotoxins.

4.2 INTRODUCTION

Escherichia coli (Enterobacteriaceae) are short Gram-negative bacilli, non-spore forming, fimbriate with peritrichous flagellum with capsule or microcapsule often present. The bacteria grow readily on simple culture or synthetic media with glycerol or glucose as the sole carbon source and energy. On solid media, colonies are circular and smooth with a complete edge; some strains produce mucoid colonies (Villaseca *et al.*, 2005). *E. coli* are widespread intestinal parasites of mammals, birds and humans and are present wherever there is faecal contamination (Doughari *et al.*, 2009). *E. coli* is usually considered to be an opportunistic pathogen which constitutes a large portion of the normal intestinal flora of humans. This organism can, however, contaminate, colonize, and subsequently cause infection of extra intestinal sites and is a major cause of septicemia, peritonitis, abscesses, meningitis, and urinary tract infections (UTI) in humans.

E. coli are incredibly diverse bacterial species with the ability to colonize and persist in numerous niches both in the environment and within animal hosts (Wiles *et al.*, 2008). The bacteria are known to cause enteric infections and diarrhea (gastroenteritis) in humans and

animals, and many strains have been identified to produce verotoxins or shiga toxins. These toxins are responsible for lethal acute bloody diarrhea (haemolytic colitis and haemolytic uremic syndrome) in humans (Karmali *et al.,* 1983; Karch *et al.,* 1999). Five classes or virotypes of *E. coli* that are recognized as causative agents of these diarrheal diseases amongst which include enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAggEC) and enterohemorrhagic *E. coli* (EHEC) (Doughari *et al.,* 2009). Each class falls within a serological subgroup and manifests distinct features in pathogenesis.

Diarrheal illness remain the second leading cause of death worldwide, responsible for an estimated 2,219,000 deaths in 1998, representing approximately 4.1% of all deaths, mostly among children under the age of five and accounts for 5.3% of the disease burden in the developing countries (Doughari *et al.,* 2009). Concerns for acute diarrhea have been further heightened since the emergence of verotoxin (shiga toxin) producing bacteria that contaminates water and food sources. *E. coli* O157:H7 or 'hamburger bug' strain was the strain most associated with shiga toxin production (Doughari *et al.,* 2009) and therefore widely studied. However currently, however several other distinct serotypes of *E. coli* other than the O157 are recognized as shiga toxin *E. coli* (STEC) associated with human diseases. These serotypes including *E. coli* O111:H8, O26:H11, O103:H2 have been reported in some parts of developed countries (Guth *et al.,* 2000; 2003; Doughari *et al.,* 2009). The association of various *E. coli* serotypes with disease of varying severity in humans and with sporadic disease or outbreaks has led to the proposal that verotoxin producing *E. coli* be classified into 5

seropathotypes, with seropathotype A comprising of O157:H7 and O157:NM, the serotypes considered to be most virulent. Seropathotype B comprises serotypes O26:H11, O103:H2, O111:NM, O121:H19, and O145:NM, that are similar to the O157 in causing severe disease and outbreaks but occur at lower frequency. Seropathotype C comprises of serotypes that are infrequently implicated in sporadic haemorrhagic uremic syndrome (HUS) but are not associated with outbreaks and include O91:H21 and O113:H21. Seropathotype D is composed of numerous serotypes that have been implicated in sporadic cases of diarrhoea, and seropathotype E comprises of the many verotoxin producing serotypes that have not been implicated in human diseases (Karmali *et al.,* 2003). Though reports abound on *E. coli* O157:H7 outbreaks in developed countries and despite their increasing medical significance, only a handful of reports are available in developing countries especially in Africa, and little or none at all is available on other verotoxic non O157: H7 *E. coli* strains.

Because of its clinical significance, *E. coli* has been the subject of numerous investigations in an attempt to define those virulence factors which allow it to initiate and sustain infections. It is now believed that virulence in *E. coli* is multifactorial (Cavalieri *et al.*, 1984) and attributes such as verotoxin production, production of haemolytic enzymes, gelatinases, cell surface polysaccharides that facilitate adhesion or ability to resist the phagocytic properties of human and animal serum, are associated primarily with virulent strains. These factors often interact in so complicated a manner that the precise mechanisms still remain to be established (Hedge *et al.,* 2008). For the successful development of new therapies and for the effective prevention and control of diarrhea, identification of pathobiologic mechanisms is increasingly important,

since the presence of a microorganism in any sample does not prove a causal relationship to disease (diarrhea) (Cavalieri *et al.*, 1984).

The source of *E. coli* pathogens in most cases is believed to be the host's own intestinal flora, thus transmission is largely via the oral-faecal route through the consumption of food or water contaminated with the organisms. Water or food contamination is often encountered when faeces containing the bacteria gain access to these food and water sources. This phenomenon is an existing threat to food and water safety in the developing countries (WHO, 2002). It is therefore important to investigate food and water sources in order to determine whether pathogenic *E. coli* are present with the view to developing proactive, preventive or control measures. This work was aimed at investigating some water samples in South Africa for the presence of other verotoxic *E. coli* other than *E. coli* O157, their virulence potentials as well as the effect of stem back extracts of *C. dentata* on the isolates.

4.3 MATERIALS AND METHODS

4.3.1 Sample collection

Forty eight water samples (duplicates) were collected from four different sources: Wastewater treatment plant, River Berg, River Plankenberg and Winelands Pork Abattoir, all in Cape Town, South Africa, for a period of five months (July – November, 2010). To collect the water samples, the shoreline sampling method as described by Obire *et al.* (2005) was adopted. In this procedure, 250 ml volume sized sterilized sample bottles were held at the base and dipped downwards below the water surface (20-30 cm deep), opened and allowed to fill up

then corked while still under water (Health Protection Agency, 2007). The collected water samples were maintained in a cooler box with ice packs (4 -10 ºC) and then immediately transported to the University laboratory where they were analyzed within 3-6 h.

4.3.2 Isolation and identification of *E. coli*

For isolation of bacteria, water samples were first filtered using membrane filter, and the filter paper inoculated into MacConkey broth (MB; DIFCO, MD, USA) and incubated at 37°C for 24 h, after which a loopful of the MB was spread onto plates of Eosin Methylene Blue (EMB, Oxoid, SA) and further incubated at 37°C for 24 h. Isolates were further purified by picking discrete colonies (green metallic sheen) and subculturing onto fresh plates of EMB and further incubating for 18-24 h at 37°C. After incubation, 1-2 discrete colonies were inoculated into the presumptive diagnostic medium Sulfide-indole-motility medium (SIM) and incubated at 35ºC for 24 h. Further characterization of isolates was carried out using the IMViC (DIFCO, MD, USA) test kit. Isolates that were indole positive, hydrogen sulfide negative, 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 5-10 presumptive single colonies using polyvalent *E. coli* antisera 2, 3 and 4 (Bioweb PTY, SA). *E. coli* ATCC 25922 was used as control. Serotyped (confirmed) *E. coli* isolates were inoculated onto tryptic soy (TS) slants, incubated for 24 h at 37 °C, and then stored at 4 °C until use (Roy *et al.* 2004; Taraweh *et al.*, 2009).

4.3.3 Detection of virulence factors on the bacterial isolates

4.3.3.1 *Screening of isolates for verotoxin production*

All the bacterial isolates were screened for verotoxin production using antibody-based rapid slide agglutination assays with the Duoperth kit (Merck, SA, Appendix vii h) according to the manufacturer's instructions. The bacterial isolates were first precultured in 1 ml casaminacid yeast extract (CAYE) broth (Appendix iii) and incubated at 37ºC with rotation at 100 rpm for 24 h. After incubation, 10 µl of the precultured broth (approximately 1 x 10^7 cells/ml) was inoculated into fresh CAYE broth and further incubated for 16 h with rotation at 100 rpm at 37ºC. The culture was centrifuged at 5000 x *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 suspended in 0. 25 ml 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 incubated at 37ºC for 30 min. Two hundred microliter (200 µl) of the culture suspension was then transferred onto the test device using a sterile Pasteur pipette and the result read after 10 min. The appearance of red bands on the vtx1 or vtx2 bands denoted the presence of either one of or both verotoxins.

4.3.3.2 *Screening of isolates for haemolysin production*

The plate haemolysis technique as described by Sharma *et al.* (2007) was used to screen for the presence of cytolytic protein toxins known as the alpha haemolysin secreted by most haemolytic bacteria. In this procedure, discrete bacteria colonies (2-3) from nutrient agar (NA) plates were subcultured onto 5% sheep blood agar plates (Appendix vii f) supplemented with

10 mM $CaCl_2$ and incubated at 37ºC for 24 h. After incubation, enterohemolysin production was detected by the appearance of a complete zone of erythrocytes lysis around each bacterial colony on the plates.

4.3.3.3 *Cell surface hydrophobicity test*

The cell surface hydrophobicity of the bacterial isolates was determined using the salt aggregation test (SAT) as described by Raksha *et al.* (2003) and Sharma *et al.* (2007). Briefly, a loopful (10 µl) of bacterial suspension in 1ml of phosphate buffer (pH 6.8) (equivalent to 5 x 10^9 colonies/ml) was mixed with equal volumes of ammonium sulphate solution of different molarities (1.4, 2.0 and 4.0 M) on a glass slide. The suspensions were rotated carefully for 1 min and then microscopically observed for agglutination. The highest dilution of ammonium sulphate solution giving a visible agglutination (Appendix vii *i*) of bacteria was scored as the SAT value. Bacterial suspension clumping at the lowest dilution (1.4 M) was considered autoaggregative, while those with SAT values of $\leq$ 2 M were considered hydrophobic.

4.3.3.4 *Screening of isolates for gelatinase production*

To screen the bacterial isolates for gelatinase production, gelatin agar was inoculated with the test bacteria and then incubated at 37ºC for 24 h; after which the plate was then flooded with mercuric chloride ($HgCl_2$) solution. The development of opacity in the medium and a zone of clearing around the bacterial colonies was considered positive for the presence of gelatinase (Sharma *et al.* (2007).

4.3.3.5 *Bactericidal serum resistance assay*

In this method, bacteria were first grown on blood agar for 18-24 h at 37ºC. The cells were then harvested and suspended in Hank's balanced salt solution (HBSS, Appendix iv). Equal amounts (0.05 ml) of the bacterial suspension and serum was mixed in a test tube and then incubated at 37ºC for 180 min and absorbance read at 600 nm. Viable count (%) was determined by calculating the differences in absorbance value before and after incubation. Resistance of the bacteria to serum bactericidal activity was expressed as the percentage of bacteria survival after 180 min of incubation with serum, in relation to the original count. Bacteria were termed serum sensitive if viable count dropped to 1% of initial value, and resistant if >90% of organisms survived after 180 min of incubation (Sharma *et al.,* 2007).

4.3.3.6 *Screening of isolates for extended spectrum beta-lactamase (ESBL) production*

The screening of isolates for ESBL was carried out using the disc diffusion method according to the criteria recommended by NCCLS (2005). Briefly, two discs (30 µg in each case), ceftazidime and cefotaxime were placed on Muller Hinton agar plates previously seeded with test bacteria and the plates incubated at 37ºC for 18 h. After incubation, ESBLs production was determined by the appearance of zone diameters of inhibition (≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime) against the test bacteria.

4.3.3.7 *Phenotypic confirmation of ESBLs production*

The Double Disc Synergy Test (DDST) as described by Iroha *et al.* (2008) and Sharma *et al.* (2007) for confirming ESBLs production was used for this purpose. Zero point one milliliter of

each bacterial isolated suspension equivalent to 0.5 McFarland turbidity standard was spread on of Mueller-Hinton agar plates using a sterile swab stick. A combination disc containing (amoxicillin, 20 µg and clavulanic acid 10 µg) was placed at the centre of the Petri-dish and ceftazidime (30 µg) and cefotaxime (30 µg) was placed 15 mm apart center to center on the plates and then incubated at 37ºC for I8 - 24 h. An enhanced zone of inhibition (synergy, regardless of size) between any one of the beta-lactam discs compared to the combined amoxicillin-clavulanic acid disc was considered to be positive for ESBL enzyme production (Iroha *et al.*, 2008).

4.3.4 Susceptibility of test bacteria to antibiotics and stem bark extracts of *C. dentata* and determination of multi-drug resistance index (MDRI)

This test was carried out using the disc diffusion method as described by Perilla *et al.* (2003). Molten Mueller-Hinton agar (MHA) plates were inoculated with the test organisms (0.5 McFarland turbidity standard) using a sterile swab stick and the plates were incubated at 37ºC for I8-24 h. 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), ciprofloxacin (5 µg) (Oxoid UK) (WHO, 2002; NCCLS, 2005) and stem bark extracts of *C. dentata* (250 µg). Resistance to more than 4 antibiotics was taken as multidrug resistance (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.

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

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

To extract phytoconstituents from the plant material, 5 g ground plant stem barks was soaked in 200 ml of solvent (water and ethanol) for 2 h followed by filtration; the procedure was repeated three times. The filtered extracts obtained from extraction with any one solvent was combined, and dried under vacuum 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 (2009).

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

Antibacterial activity determination was carried out using the filter paper disc diffusion method as described by Doughari and Obidah (2008). Filter papers (4 mm in diameter) were cut using a paper punch and then sterilized by autoclaving. The sterilized filter papers were then soaked in different concentrations of extracts (100-3000 µg/ml and 2.5-200 mg/ml/disc) and then allowed to dry. To test for susceptibility, dried extract-soaked filter paper discs were placed on different Mueller Hinton agar plates earlier seeded with different test 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 the antibacterial activity was determined as relative inhibition zone diameters (mm) against each test bacteria. Dried filter paper discs soaked with 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 (1000 µg/ml and 10 to 3000 mg/ml) of plant extract and 1 ml of nutrient broth (NB) added. 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 NB and the standard antibiotic ampicillin (as negative control), or NB 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.

4.4 RESULTS

Results of physical parameters of the water samples (mean pH and temperature values 6.4, 17.8°C (waste water); and 7.4, 17.3°C (river water) respectively), biochemical and cultural characteristics, serotypes, number of resistant isolates and multidrug resistance index values of the *E. coli* isolates obtained from the wastewater and river samples are shown in Table 4.1. Cultural and biochemical characterization of the isolates showed that *E. coli* exhibited a green metallic sheen on Eosin Methylene Blue (EMB) agar with variable haemolysis on sheep's blood agar. Results also showed that out of the 69 non O157 *E. coli* isolates obtained, different

serotypes including O26:H11, O55, O111:NM, O126, O44, O124, O96:H9, O103:H2, O145:NM and O145:H2 were present (Table 4.1). Results of multidrug resistance index (MDRI) showed that the MDRI values ranged between 7.00-33.00% with isolates from wastewater samples exhibiting the highest MDRI values.

Table 4.1. Cultural and biochemical characteristics, percentage resistant to four or more antibiotics and multidrug resistance index (MDRI) values of *Escherichia* serotypes obtained from wastewater and river water samples.

*Water source/Temperature/ pH/Number of samples	*E. coli* serotypes from each water sample	Cultural and Biochemical characteristics of *E. coli* strains isolated from the water samples								Number of isolates	Number (%) of isolates resistant to or more antibiotics	MDRI (%) values range for isolates
		EMB	ShB	G	S	I	M	E	O			
#Wastewater Treatment Plant/17.8/6.4)/18 samples	O103:H2, O86, O145:H2, O96:H9,O126, O4,O55,O111:NM,O124,O44,O124,O44	+	+/-	-	-	+	-	+	+	25	17(68.00)	7.00-33.00
!Abattoir wastewater/ 17.8/6.4/12 samples	O4,O145:H2,O111:NM,O103:H2,O113, O86,O26:H11,O96:H9,O124	+	+/-	-	-	+	-	+	+	24	12(50.00)	7.00-33.00
River Plankenberg/ 17.3/7.2/18 samples	O86,O113,O145:H2,O4, O103:H2,O96:H9	+	+/-	-	-	+	-	+	+	7	0(0.00)	7.00-20.00
River Berg/17.3/7.2/18 sampl	O4,O26:H11,O86,O103:H2	+	+/-	-	-	+	-	+	+	13	4(38.78)	7.00-13.33

EMB = Eosin Methylene Blue, ShB = Haemolysis on Sheep Blood Agar; G = Gram reaction; S = Sulphide Production; I = Indole production; M = motility; E = Erchlich's reagent; O = Oxidase reaction; - = negative; + = positive; +/- = variable haemolysis *48 samples in total; #from Athlone wastewater Treatment Plant; !from Winelands Pork Abattoir.

Table 4.2 shows results of percentage extraction, phytochemical analysis and minimum inhibitory concentration (MIC) values of the aqueous and ethanol stem bark extracts of *C. dentata*. Results showed that the highest amount of extracts (58.82%) was obtained from water compared to that of ethanol (38.72%). For phytochemicals, while saponins, tannins, anthraquinones, steroids and phenols were common to both ethanol and aqueous extracts; only ethanol extracts contained alkaloids and aqueous extracts contained glycosides and flavonoids. Results also revealed that the MIC values of the water extracts ranged between 100-2500 mg/ml and 70-150 mg/ml for ethanol stem bark extracts.

Table 4.2. Phytochemical constituents and mean minimum inhibitory concentration (MIC) values of aqueous and ethanol stem bark extracts of *Curtisia dentata*

Extract		Phytoconstituents								Mean MIC range for *C. dentata* (mg/ml)
	% Extractio	Saponins	Tannins	Alkaloids	Glycosides	Anthraquinones	Flavonoids	Steroids	Phenols	
WE	58.82	+	+	-	+	+	+	+	+	100-2500
EE	38.72	+	+	+	+	+	-	+	+	70-150

WE= aqueous extract; EE = ethanol extract; + = present; - = absent

Fig. 4.1 shows the results of the presence of virulence factors in the various *E. coli* serotypes isolated. Results showed that 47% of the isolates produced verotoxins (both Vtx1 and Vtx2 38%, Vtx1 6% and Vtx2 3%), 81% exhibited cell surface hydrophobicity, over 60% exhibited serum resistance, haemolysin and gelatinase production, while over 52% produced extended spectrum beta-lactamases (ESBLs).

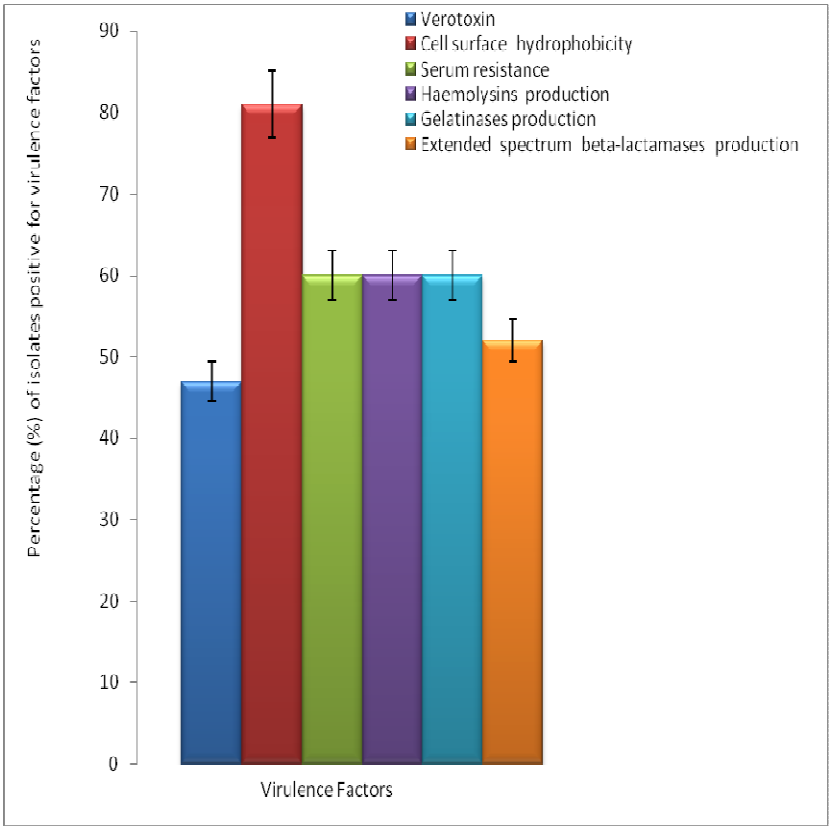

Fig. 4.1. Virulence factors detected on *E. coli* isolates obtained from river and wastewater samples.

Results also showed that, while 60% of the isolates showed various levels of resistance to different antibiotics [ampicillin (10 µg), cefuroxime, cephalexin, ceftazidime and tetracycline (30 µg in each case)] (Fig. 4.2), only 28% showed various resistance to to ethanol stem bark extracts of *C. dentata.*

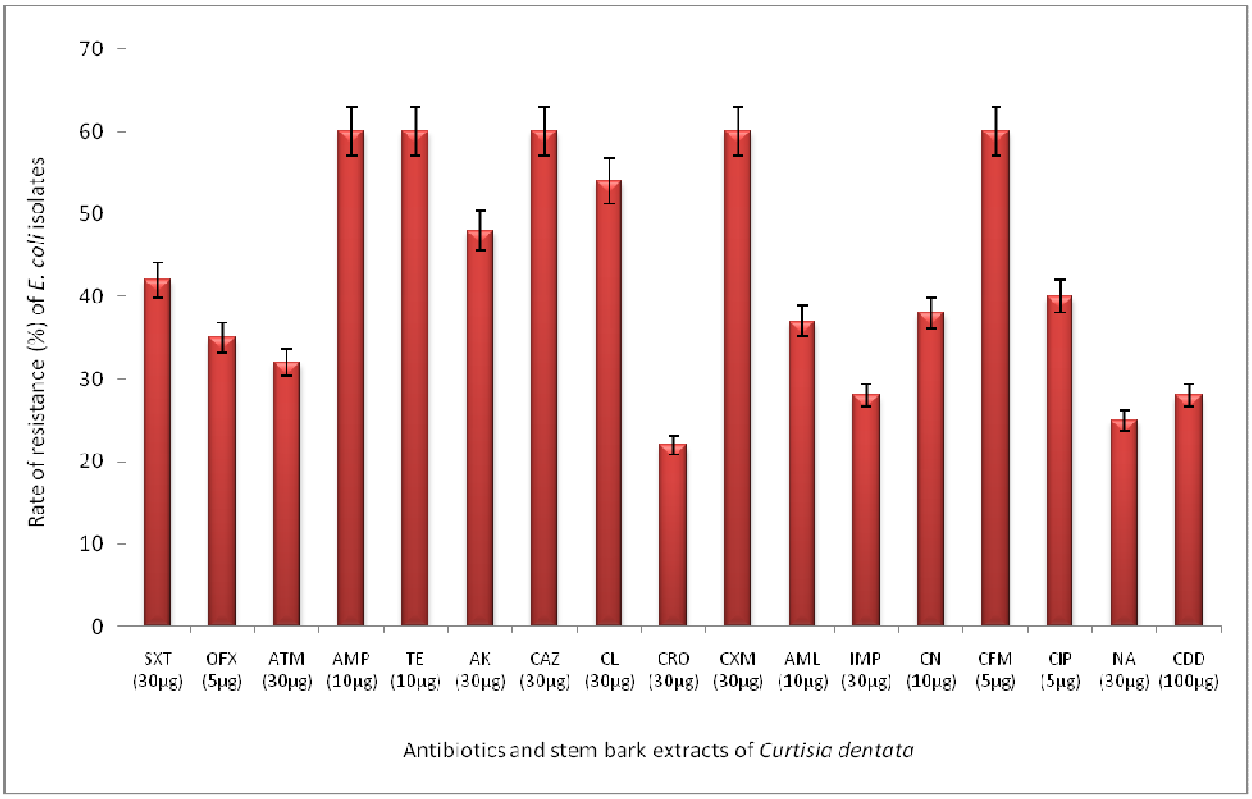

Fig. 4.2. Antibiotic resistance rate amongst, and effect stem bark extracts of *Curtisia dentata* on *Escherichia* isolates obtained from river and waste water samples.(Key: SXT-sulphomethaxazole/trimethoprim; OFX-ofloxacin; ATM-aztreonam; AMP-ampicillin; TE-tetracycline; AK-amikacin; CAZ-ceftazidim; CL-cephalexin; CRO-ceftriazone; CXM-cefuroxime; AML-amoxycillin; IMP-impenim; CN-gentamicin; CFM-cefixime; CIP-ciprofloxacin; NA-nalidixic acid; CDD = stem bark extracts of *Curtisia dentata*).

4.5 DISCUSSION

E. coli is a consistent inhabitant of the human intestinal tract, and it is the predominant facultative organism in the human gastrointestinal tract. The frequency of *E. coli* in the human intestine and faeces has led to its usage amongst other coliform bacteria as an indicator of faecal pollution and water contamination. Thus, the presence of *E. coli* in any given food or water source is an indication of faecal contamination by intestinal parasites of humans. Results of this study revealed the presence of various verotoxin producing non O157 serotypes of *E. coli* (O26:H11, O55, O111:NM, O126, 044, O124, O96:H9, O103:H2, O113 and O145:H2) from the river and wastewater samples investigated. The wastewater samples contained more serotypes of *E. coli* compared to the river samples. The wastewater samples contained mixtures of wastes emanating either from animal or human excreta, industries or the hospitals and these wastes contained a high load of enteric bacteria including *E. coli* which therefore accounts for a higher number of *E. coli* serotypes. The majority of these serotypes exhibited the presence of virulence factors. Virulence factors enable *E. coli* to colonise selectively the mucosal uro-epithelium and to evoke an inflammatory reaction from the host and eventually making the host tissues susceptible to invasion. Therefore the capacity of *E. coli* to produce many virulence factors contributes to its pathogenicity consequently causing a variety of infections such as gastrointestinal and urinary tract infections, soft tissue infections, bacteraemia and neonatal meningitis. These virulence factors enable the bacteria elicit an infection by overcoming the host defence mechanisms. In this study, many of the *E. coli* isolates investigated

exhibited verotoxins, cell surface hydrophobicity, serum resistance, haemolysin, gelatinase production and ESBLs production.

Verotoxin or shiga toxin-producing bacterial strains are associated with a broad spectrum of human illnesses throughout the world, ranging from mild diarrhea to haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Complications arising from antibiotic treatment of verotoxic related human infections have also been reported (Doughari *et al.*, 2010). Though the wastewater samples investigated are not directly consumed, the presence of a significant proportion (47%) of verotoxin producing bacteria from the various water samples investigated is a cause for concern since the water samples often gets discharged into large water bodies or are utilized for irrigation purposes. It is also a common phenomenon for rural dwellers to use river waters for both domestic and drinking purposes as well as for bathing, thus increasing the possibility of contracting these bacteria.

Cell surface hydrophobicity enhances the adherence of bacterial cells to host cell surfaces including mucosal epithelial cells and confers them with resistance to phagocytosis by host cells. In this study,a greater number of the *E. coli* strains demonstrated hydrophobicity. Previous studies on urinary tract infection cases reported high rate of exhibition of cell surface hydrophobicity by some pathogenic strains of *E. coli* (Sunman *et al.*, 2001; Raksha *et al.*, 2003). The presence of hydrophobic strains of *E. coli* in this water sources is an

indication that the water could be a potential source of agents of urinary tract infections or gastroenteritis if consumed.

Haemolysin production as a virulence factor by urinary isolates of *E. coli* has been reported previously (Jhonson, 1991; Raksha *et al.,* 2003). It has been suggested that colonization of the urinary tract with haemolytic strains of *E. coli* is more likely to develop into urinary tract infections. Haemolysis may contribute to tissue injury, survival in renal parenchyma and entry into blood stream and increasing the possibility of establishment of acute pyelonephritis (Raksha *et al.,* 2003). The mode of action of haemolysins involves pore formation on the colonized host cell (Wiles *et al.,* 2008) and their production is associated with pathogenicity of *E. coli*, especially the more severe forms of infection (Jhonson, 1991). The higher rate of haemolysin producing strains isolated from this water samples highlights the presence of invasive *E. coli* strains in this environment.

Serum resistance is the property by which the bacteria resist killing by normal human serum due to the lytic action of complement system. Results of this study also showed a high rate of serum resistance among the *E. coli* isolates. Siegfried *et al.* (1994) and Raksha *et al.* (2003) previously reported serum resistance among *E. coli* isolates obtained from urine samples. Among *E. coli* virulence factors, serum resistance have been shown to have a high correlation with pathogenicity. Chaffer *et al.* (1999) has also reported a relationship between virulence and resistance to compliment in *E. coli* serogroup O2 although additional virulence factors are required for bacteria to successfully cause an infection. The presence

of serum resistant strains of *E. coli* in the water samples studied calls for more proactive measures in the control of potential infections by these bacteria. The wastewater from the waste treatment plant is not often consumed directly but released into farm areas and used for irrigation purposes, consequently if farmers and/or farm workers do not observe very high hygienic standards while using the water, they may be liable to contraction potentially acute bacterial infections. In 2006, an outbreak of *E. coli* O157 H:7 was reported in the United States and Canada and the source was said to be spinach contaminated with irrigation water in California (Ishii and Sadowsky, 2008). Isolation of serum resistant *E. coli* from these wastewater and river water sources has far reaching health implications. For instance, *E. coli* isolates obtained from patients with pyelonephritis, cystitis and bacteraemia were typically serum resistant whereas isolates from patients with asymptomatic bacteriuria were serum sensitive strains (Raksha *et al.*, 2003). In addition, Gram-negative bacteria isolates that showed serum resistance demonstrated a high degree of survival in the blood during bacteraemia (Raksha *et al.,* 2003). A strong correlation between serum resistance bacterial invasion and survival in the human bloodstream amongst Gram-negative has been reported (Siegfried *et al.,* 1994).

Gelatinase is an extracellular metalloendopeptidase capable of hydrolyzing bioactive peptides such as gelatin, pheromone, collagen, casein, fibrinogen, haemoglobin and other bioactive peptides (Makinen and Makinen, 1994). The enzyme is an extracellular zinc endopeptidase that plays a very significant role in bacterial pathogenesis by causing direct or indirect damage to host tissue, thus facilitating microbial invasion and survival in the

host (Alebouyeh *et al.*, 2005; Furumura *et al.*, 2006). The presence of these enzymes on *E. coli* isolates is a further confirmation of their potential to cause infections.

Extended spectrum beta-lactamase (ESBL) production by *E. coli* from this study is high. This might be as a result of selective pressure imposed by extensive use of antimicrobials in animals and agriculture. Animal farming recently involves the use of antibiotics in chemotherapy, while wastewater from the wastewater treatment plant comprised mixtures of water from different sources including hospitals and animal farms where an extensive use of antibiotics in treatment of both animal and human diseases is applied. The indiscriminate use of cephalosporins is responsible for the high rate of selection of ESBL producing microorganisms. Bradford (2001) has earlier reported a high prevalence rate in the production of ESBLs among *E. coli* isolates. ESBLs confer bacteria with resistance to β-lactam antibiotics. However the results of this study are in contrast with those reported by Johnson *et al.* (2003) where a corresponding decrease of ESBLs production with increase in virulence factors among bacteria was reported. Since ESBL production is usually plasmid mediated, it is possible, for one specimen to contain both ESBL producing and non-producing cells and, at the same time, contain other virulent factors.

In this study, most of the *E. coli* isolates have the combination of two or more of the virulent factors (cell surface hydrophobicity, serum resistance, gelatinase or haemolysin production as well as extended beta-lactamase production). The presence of multiple virulence factors increases the virulence of organisms, since such factors function

synergistically in overcoming normal host defences. Thus, bacterial strains with more extensive virulence factors complement are more effective as virulent pathogens. Therefore this implies that isolates from these water sources may be considered as potentially pathogenic.

Studies on the antibiotic susceptibility pattern for all the isolates of *E. coli* obtained, showed resistance to commonly used antibiotics such as ampicillin (10 µg), cefuroxime, cephalexin, ceftazidime and tetracycline (30 µg in each case)). The MDRI of some of the isolates (33.00%) is well above 20% which signifies that the bacteria are highly resistant to available antibiotics. Multi-drug resistance among *E. coli* isolates has been subjects of concern worldwide (Chitnis *et al.*, 2003; Lestari *et al.*, 2008). Dissemination of resistance genes among isolates have been held responsible for rapid and widespread multidrug resistance among not only *E. coli*, but numerous other pathogenic bacteria as well. This calls for strict observance of antibiotic susceptibility test results in the prescription of antibiotics.

The demonstration of antimicrobial activity against many of the *E. coli* isolates by ethanol stem bark extracts of *C. dentata* with a low MIC values (70-100 mg/ml) is an indication that the plant contains bioactive components that are antagonistic to the bacteria. *C. dentata* may therefore play a very important role as source of newer chemical substances that can be used in the development of chemotherapeutic agents for the treatment of diarrhoea, urinary tract infections, bacteraemia and other infections caused by *E. coli.* The high MIC values

(100-2500 mg/ml) recorded for the aqueous extracts compared to the ethanol extracts may not necessarily mean that the extracts do not posses antimicrobial activity, but that the phytoconstituents may be present in very low amounts at the tested concentrations. Antimicrobial activity of the acetone extracts of leaves, twigs and stem barks of *C. dentata* against *Bacillus subtilis, E. coli, S. aureus, P. aerugenosa, E. faecalis* and *C. albicans* as well as inhibition of motility in some parasitic and free living nematodes has earlier been reported (McGaw *et al.,* 2000; Shai *et al.,* 2008; 2009). This, however, is the first report on the activity of *C. dentata* on verotoxic bacteria. Though water yielded the highest amount of extracts, the ethanol extracts showed higher activity against the test bacteria compared to the aqueous extracts. Solvents are known to have different degrees of extraction depending on their polarity (Doughari and Ioryue, 2009). The presence of phytoconstituents such as saponins, tannins, alkaloids, anthraquinones, steroids and phenols further confirms the potential application of the plant in sourcing antibiotic substances for a possible development of novel chemotherapeutic agents.

4.6 CONCLUSION

Though the *E. coli* strains studied in this paper were isolated from water samples, the bacteria have demonstrated the capacity to adapt and survive in different tissues, by producing virulent factors and by developing a drug resistance. The isolates strains obtained from the water sources therefore are potential agents of human infections such as diarrhoea, urinary tract and ear infections, depending on the site of colonization. The rise in incidences of drug resistance amongst pathogenic *E. coli* strains has been demonstrated from the

results of this study. This calls for proper selection of antibiotics for treatment, based on an adequate detection of bacteria resistant to drugs through the results of antibiotic susceptibility test as well as the judicious use of antibiotics in humans and animals. Good antibiotic policy is also required in order to limit the emergence and spread of antibiotic resistance in bacteria. Farmers should further be informed on the need to maintain personal hygiene especially while handling wastewater for irrigation purposes. People should be educated on the need for boiling of river water before drinking, as well as maintenance of food hygiene, and also personal hygiene amongst food handlers.

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CHAPTER FIVE

5.0 MULTI-DRUG RESISTANCE, VEROTOXIN PRODUCTION AND EFFICACY OF CRUDE STEM BARK EXTRACTS OF *CURTISIA DENTATA* AMONG *ESCHERICHIA COLI* (NON-O157) AND *ACINETOBACTER* SPP. ISOLATES OBTAINED FROM WATER AND WASTEWATER SAMPLES

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

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5.1 ABSTRACT

Drug resistant diarrhea and nosocomial infections caused by verotoxic *Escherichia coli* and some *Acinetobacter* spp. has posed serious therapeutic challenges especially in developing countries. The aim of this work was 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 Methylin 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 *A. haemolyticus* isolates producing verotoxins, and no *A. lwoffii* isolate produced the toxins. Multi-drug resistance index (MDRI) values of isolates ranged between 7-33.00% 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 MIC values of 150-300 µg/ml for *E. coli* and 150-2000 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 to 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.

5.2 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 (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 *et al.*, 2006). These strains have also been linked to outbreaks of food poisoning (Duffy and Garvey, 2000).

Members of the genus *Acinetobacter* (Gram-negative cocco-bacilli) 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 *A. baumannii, A. johnsonnii, A. haemolyticus, A. junii,* and *Acinetobacter* genomospecies 3, and 13. A part from MDR nosocomial infections, these bacteria are implicated in endocarditis, bacteremia, sepsis in neonatal intensive care units and paediatric onchology 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 (Vila *et al.*, 2002; Barbe *et al.*, 2004; 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) underlines 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 assegaai (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.

5.3 MATERIALS AND METHODS

5.3.1 Source of media, antibiotics, chemicals and plant material

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 vii h) test kit, Oxidase test strips and all laboratory grade chemicals used in this study were purchased from Merck. *E. coli* polyvalent antisera 2, 3 & 4, and REMEL RapID™ NF plus test kit was purchased from Bioweb, South Africa. All the purchasing companies are based in South Africa. The plant sample *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.

5.3.2 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, River Plankenburg) 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 (20-30 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 4-10°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 3-6 h. For the plant sample, fresh stem barks from *C. dentata* were dried to constant weight in an oven for 6 h at 45ºC. 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 stored at ambient temperature until use.

5.3.3 Cultivation, isolation and identification of bacteria

For isolation of *E. coli*, water samples were serially diluted up to 10^5 dilution and 1 ml 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 Eosin Methylene Blue (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 18-24 h at 37°C. After incubation, 5-10 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 5-10 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 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 i) instead of EMB. The inoculated BEM was shaken vigorously by vortexing and then incubated at 37ºC 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 24-48 h. After this, 1-2 loopfuls of BEM or mTSB cultures was inoculated onto EMB (Oxoid) or Leeds Acinetobacter medium (LAM, Hardy diagnostics USA, Appendix ii) 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 5-10 discrete colonies were inoculated into 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 vii g) 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.

5.3.4 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 vii h) according to the manufacturer's instructions. The bacterial isolates were first precultured in 1 ml casaminacid yeast extract (CAYE) broth, (Appendix iii) and incubated at 37ºC with rotation at 100 rpm for 24 h. After incubation, 10 µl of the precultured broth (approximately 1 x 10^7 cells/ml) was inoculated into fresh CAYE broth and further incubated for 16 h with rotation at 100 rpm at 37ºC. The culture was centrifuged at 5000 x *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 suspended in 0. 25 ml 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 incubated at 37ºC for 30 min. Two hundred microliter (200 µl) of the culture suspension was then transferred onto the test device using a sterile Pasteur pipette and the result read after 10 min. The appearance of red bands on the vtx1 or vtx2 bands (Appendix vii h) denoted the presence of either one of or both verotoxins.

5.3.5 Antimicrobial susceptibility testing and determination of multidrug resistant (MDR) index

The disc diffusion method as described by Perilla *et al.* (2003) was used for the determination of antimicrobial susceptibility testing. Molten Mueller-Hinton agar (MHA) plates were inoculated with the test organisms (0.5 McFarland turbidity standard) using a sterile swab stick and the plates were incubated at 37ºC for 16-I8 h for *E. coli* and 20-24 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), œfotaxime (30 µg), ceftriaxone (30 µg), aztreonam (30 µg), nalidixic acid (30 µg), amikacin (30 µg), tetracycline (30 µg), gentamicin (10 µg), ofloxacin (5 µg), 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 multidrug resistance (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.

$$\% \text{ MDRI} = \frac{\text{Number of antibiotics resisted}}{\text{Total number of antibiotics used}} \times 100$$

5.3.6 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 was combined, and 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 (2009).

5.3.7 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 (100-3000 µg/ml and 2.5-200 mg/ml/disc) were placed on Mueller Hinton agar (MHA) plates earlier seeded with the test 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.

5.4 RESULTS

The cultural, morphological and biochemical characteristics of the bacteria isolated from the wastewater and surface (river) water samples (pH range 6.4-7.2 and temperature range 17.3-17.8°C) are shown in Table 5.1. *E. coli* on Eosin Methylene Blue (EMB) displayed a green metallic sheen colour (Appendix vii a) while *Acinetobacter* spp. appeared as tiny blue and mucoid colonies, and tiny pink on Leeds Acinetobacter Medium (LAM) (Appendix vii d). Results of Gram staining revealed that *E. coli* isolates appeared as Gram-negative rods, while *Acinetobacter* spp. appeared as Gram-negative coccobacilli. 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 vii g), *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 vii f) supplemented with 10 mM $CaCl_2$ differentiated the *A. haemolyticus* from the non-hamolytic *A. lwoffii* strains.

5543 Table 5. 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
Escherichia col		N/A	N/A	Gram-negative rod	-	+	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Acinetobacter lwoffii	Colonies with green metallic sheen	Pink colonies diffused into th	-	Gram-negative coccobacilli	-	+	-	N/A	-	-	-	-	-	-	+	-	+
Acinetobacter haemolyticus	Tiny, blue, mucc colonies	Pink colonies diffused into th	+	Gram-negative coccobacilli	-	+	-	N/A	-	+	+	+	-	+	+	+	+
	Tiny, blue, mucc colonies																

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5545 EMB - Eosin Methylene Blue, Leeds Acinetobacter Medium; ShB -Haemolysis on Sheep Blood Agar; N/A - not applicable; S - Sulphide Production; I -
5546 Indole production; E - Erchlich's reagent; M-motility, O - Oxidase reaction; Gluc - glucose; Cit – citrate; ADH - Arginine; URE - urea; EST - Triglyceride;
5547 IND – Tryptophane; NO_3 sodium nitrate*some of the tests in Rapid NF plus used mainly for the identification of *Acinetobacter* spp.
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The *E. coli* serotypes and *Acinetobacter* spp. biotypes isolated from the various samples, their verotoxic status and resistance profiles are shown in Tables 5.2 and 5.3. 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 of 27 *A. lwoffii* and 14 *A. haemolyticus* isolates. Results of 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 5.3).

The antimicrobial susceptibility profiles and multidrug resistant indexes (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 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, nalidixic acid (30 µg), 4 were resistant to the aminoglycosides - amikacin (30 µg), 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 multi-drug resistance index (MDRI) showed MDRI values ranging between 7-33.00% 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. 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 between 150-300 µg/ml (percentage relative inhibition zone diameter (%RIZD) 4-28) for *E. coli isolates* and 150-2000 µg/ml (%RIZD 6-30) for *Acinetobacter* spp.

5596 Table 5.2. Various *Escherichia coli* serotypes, their verotoxin and antibiotic resistance profiles, multidrug
5597 resistance index (MDRI) (%) and minimum inhibitory concentration (MIC) (µg/ml) values against stem bark
5598 extract of *Curtisia dentata*.

Sample number (mean pH/Temp. ^{o}C)	Isolate/ serotype	Verotoxin status (Vtx1&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	$^{\text{ɤ}}$SXT, †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		27	22.00	150.00
	E. coli RWW2i O96:H9	Vtx2	†OFX, †NA, **AMP, $^{@}$TE	13.3	8.00	1000.00
	E. coli RWW2ii O124	Vtx1	*CN, $^{@}$TE	7	14.00	400.00
	E. coli PSW1i O96:H9	Vtx1	*AK	7	16.00	200.00
	E. coli PSW1ii O145:NM	Vtx2	*CN	7	22.00	150.00
	E. coli PSW1iii O96:H9	Vtx1, Vtx2	$^{@}$TE	7	16.00	250.00
	E. coli PSW1iv O111:NM	Vtx1, Vtx2	**AML	7	24.00	150.00
	E. coli PSW2i O86	Vtx1, Vtx2	$^{\text{ɤ}}$SXT	7	14.00	200.00
	E. coli PSW2ii O96:H9	Vtx1, Vtx2	$^{\text{ɤ}}$SXT	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		33	6.00	2000.00
	E. coli PRE1ii O145:H2	Vtx1	**AMP, *CN, $^{\square}$OFX, $^{\$}$NA, $^{@}$TE, *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
	E. coli FSE1i O113	Vtx2	-	0	20.00	150.00
	E. coli FSE1ii O145:H2	Vtx2	$^{€}$CFM, $^{€}$CL, **AMP, $^{\$}$NA, $^{\square}$OFX,	0	22.00	250.00
	E. coli FSE1iii O86	Vtx2	$^{\square}$OFX, $^{@}$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, $^{\square}$OFX, $^{@}$TE, SXT	13.3	24.00	2500.00
	E. coli PST2ii O124	Vtx1, Vtx2	*AK	33	10.00	700.00

River Berg (n = 13) (7.2/17.3)	*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
	E. coli RBD1iii O86	Vtx1, Vtx2	**AML, $^{€}$CRO, *AK	7	22.00	150.00
	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
River Plankenberg (n = 13) (7.2/17.3)	*E. coli* PRK1i O4	Vtx2	**AML, **AMP	7	20.00	150.00
	E. coli PRK1ii O26:H11	Vtx1, Vtx2	$^{€}$CRO,	13.3	26.00	100.00
	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
	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

^{ϒ} = sulphonamides; * = Aminoglycosides; ** = Penicillins; $^{€}$ = Cephalosporins; $^{@}$ = Tetracyclines ; $^{\$}$ = Quinolones

Table 5.3. Various *Acinetobacter* spp. isolates, their verotoxin and antibiotic resistance profiles, multidrug resistance index (MDRI) (%) and minimum inhibitory concentration (MIC) (µg/ml) values against stem bark extract of *Curtisia dentata*.

Sample number (mean pH/Temp. °C)	Isolate/ serotype	Verotoxin status (Vtx1&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	-	□SXT, □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	-	□OFX, □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	-	□SXT	7	28.00	150.00
	A. *lwoffii PRE1ii*	-	□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
	A. lwoffii PST1ii	-	*AK, $NA, €CL	20	12.00	850.00
	A. haemolyticus PST1i	Vtx1	**AMP, *CN, □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
River Berg (n = 13) (7.2/17.3)	*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	-		7	30.00	100.00
	*A.. haemolyticus*RBD1i	Vtx1	-	0	26.00	150.00
	*A. haemolyticus*RBD1ii	-	-	0	24.00	150.00
	*A. haemolyticus*RBD1iii	-	-	13.3	10.00	900.00
	*A. haemolyticus*RBI1i	-	†OFX, $NA	0	28.00	150.00
	A. haemolyticus RBI2i	-	-	20	12.00	600.00
	A. lwoffii RBI2ii	-	@TE, *AK, $NA	7	24.00	200.00
	A. lwoffii RBI2iii	Vtx1,Vtx2	*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: □ = sulphonamides; * = Aminoglycosides; ** = Penicillins; € = Cephalosporins; @ = Tetracyclines ; $ = Quinolones

Table 5.4 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.

Table 5.4. Phytochemical constituents of aqueous stem bark extracts of *Curtisia dentata*

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

+ = present; - = absent

5.5 DISCUSSION

Sanitation and absence of clean drinking water remain a major challenge to deveoping 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 access to clean water altogether, and on the other hand, sewage disposal is increasingly threatening water bodies worldwide. According to the United Nations, 95 percent of the world's cities including the developing countries still dump raw sewage into their water supplies (UN Water, 2011). Consequently, 80 percent of all the health maladies, in developing countries are related to unsanitary water. Amongst the health maladies diarrhea caused by multidrug resistant (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; Barbe *et al.*, 2004; 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 multi-drug resistant; 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 O157 verotoxic *E. coli* (*E. coli* O111:H8, O26:H11, O103:H2) have been associated with severe human disease outbreaks such as 11-15% 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; Galane and Le Roux 2001; Guth *et al.,* 2003; Chandran *et al.,* 2008; Doughari *et al.,* 2009). 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.,* 2009). 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 multi-drug resistant indices (MDRI) (7- 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* species in the river. On the other hand, 65% of the Berg river (also called Great Berg River - located just 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, andposes 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 contamination 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 fractors 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; Chandran *et al,.* 2008; Ishii and Sadowsky, 2008; Willey *et al.,* 2008; Robinson *et al.,* 2010) 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 and Obidah, 2008). The low MIC values (150-3000 µg/ml, % RIZD 4-28 for *E. coli* and 150-2000 µg/ml, % RIZD 6-30 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 multidrug resistant *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 multi-drug resistant 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. The occurrence of verotoxin producing VTEC in other environmental samples, further purification *C. dentata* extracts and determination of the most active components as

well as toxicological studies should be carried out with the view to utilizing the plant in the development of novel and more effective antibiotics.

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CHAPTER SIX

6.0 VIRULENCE, RESISTANCE GENES AND TRANSFORMATION AMONGST ENVIRONMENTAL ISOLATES OF *ESCHERICHIA COLI* AND *ACINETOBACTER* SPP.

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6.0 Virulence, resistance genes and transformation amongst environmental isolates of *Escherichia coli* and *Acinetobacter* spp.

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Right running head: Resistance genes amongst *E. coli* and *Acinetobacter* spp.

6.1. ABSTRACT

The association of verotoxic *E. coli* and *Acinetobacters* spp. with various antibiotic resistant diarrhogenic and nosocomial infections has been a cause for concern worldwide. *E. coli* and *A. haemolyitcus* isolated on various selective media were screened for virulence factors, antibiotic resistance and transformation of resistance genes. Out of 69 *E. coli* isolates obtained, 25(35.23%), 14(20.30%) and 28(40.58%) were positive for VTx1&2, Vtx1 and Vtx2 respectively, 49(71.015%), were positive for extended-spectrum beta-lactamases (ESBLs), 7(77.78%) for serum resistance, 57(82.61%) for cell surface hydrophobicity, 48(69.57%) for gelatinase production and 37(53.62%) for haemolysin production. For the 14 *A. haemolyticus* isolates, only 2(14.29%) in each case from all the samples investigated were positive for Vtx1, Vtx2 and Vtx1&2 respectively, 8(57.14%) for ESBLs, 7(50.00%) for serum resistance, 11(78.57%) for cell surface hydrophobicity, 4(28.57%) for gelatinase production and 8(57.14%) for haemolysin production. While transformation occurred among the *E. coli* and *Acinetobacter* isolates (transformation frequency: 13.3 x 10^{-7}- 53.4^{-7}), there was poor curing of the plasmid genes, a confirmation of presence of stable antibiotic resistant genes (DNA concentration between 42.7-123.8 µg) and intra-genetic transfer of multidrug resistant genes among isolates. Isolates are potentially virulent and contain potentially transferable antibiotic resistance genes. Detection of virulence factors, antibiotic resistance genes and transformation among these isolates is a very significant outcome that will influence approaches to proactive and preventative and control measures as well as future investigations. However, continued surveillance for drug resistance among these

bacteria and further investigation of the mechanism of action of their virulence factors are a necessity.

Keywords: Cell surface hydrophobicity, extended-spectrum beta-lactamases, gelatinases, haemolysins, resistance genes, transformation, virulence factors.

6.2 INTRODUCTION

Pathogenic bacteria utilize a number of mechanisms to cause disease in human hosts (Wilson *et al.*, 2002). These mechanisms are often expressed in a wide range of molecules that enable adhesion of bacteria to host cell targets in order to initiate the infection process and as a result trigering a variety of different host responses (Sharma *et al.*, 2007). The virulence factors are of two main types; those produced on the surface of the cell and those produced within the cell and then exported to the site of action. Those on the surface include different sorts of fimbriae (s, p, or type F 1 or curli fimbriae), certain other mannose-resistant adhesins, K capsules or cell surface lipopolysaccharides (LPS) that have a role in adhesion to the surface of host cells but may also have additional roles such as tissue invasion, biofilm formation or cytokine induction (Emo˝dy *et al.*, 2003). While virulence factors secreted within the cell and exported into the medium or host cell protoplast include enzymes such as haemolysins, gelatinases and beta-lactamases. Just as in other bacteria *Escherichia coli* and *Acinetobacter haemolyticus* depend largely on several virulence factors for survival in host tissues and for pathogenicity. Verotoxic *E. coli* (VTEC) has been a source of public concern, responsible for outbreaks of bloody and traveler's diarrhea (WHO, 2002), while

Acinetobacter spp. that were considered nonpathogenic are now associated with notorious multi-drug resistant nosocomial infections as well as bloody diarrhea (Grotiuz *et al.,* 2006). Though *E. coli* O157:H7 is responsible for approximately half of all confirmed VTEC infections in Europe, there is growing concern about the risk of non-O157 VTEC serotypes to human (Cross *et al.,* 1986). The mechanisms involved in VTEC adherence to epithelial cells and colonization is yet to be understood (Bolton, 2011) A key to fighting these bacterial pathogens is the identification and characterisation of all the virulent factors that makes them so potent. This is crucial for effective diagnosis of the infection, surveillance of animal reservoirs, assessment of public health risks, and the development of control interventions. There are growing concerns for the increasing significance of both *Acinetobacter* spp. and *E. coli,* as causative agents of notorious antibiotic resistant infections, in both hospital and community setups. Recently, there was a diarrheal outbreak associated with *E. coli* infections in Germany and other parts of the world (CDC, 2011). Therefore, the need exists for surveillance of the presence of these organisms, characterization of their virulent potentials and determination of their potential to transfer resistant genes to other bacteria, especially in the developing countries. Thus study is the first report on virulence factors, antibiotic resistance genes and potential for transfer of resistance amongst environmental isolates of verotoxic strains of *E. coli* and *Acinetobacter* spp. isolated from waste- and river water samples in South Africa.

6.3 MATERIALS AND METHODS

6.3.1 Source of media, antibiotics, chemicals and plant material

Eosin Methylene Blue (EMB), Nutrient Broth (NB), modified Trypton Broth (mTSB), Mueller Hinton Agar (MHA) and antibiotic discs were all Oxoid grade, and were obtained from Quantum Biotechnologies, South Africa (SA). Glisa Duopath Verotoxins® (Merck, Germany, Appendix vii h) test kit, Oxidase test strips and all laboratory grade chemicals used in this study were obtained from Merck, SA. *E. coli* polyvalent antisera 2, 3 & 4, and REMEL RapID™ NF (Appendix vii g) plus test kit was obtained from Bioweb, SA.

6.3.2 Sample collection

Sixty two water (18 wastewater samples each - from a wastewater treatment plant and an abattoir, and 14 river water samples - each from River Berg, River Plankenburg) all in Cape Town South Africa, were collected using the shoreline sampling method (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 (20-30 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 4-10°C using ice packs. The samples were then immediately transported to the Microbiology Laboratory of the Biotechnology Department of Cape Peninsula University of Technology, Cape Town South Africa where they were analyzed within 3-6 h.

6.3.3 Cultivation, isolation and identification of bacteria

For isolation of *E. coli*, water samples were serially diluted up to 10^5, and 1 ml 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 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 18-24 h at 37°C. After incubation, 5-10 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 5-10 presumptive single colonies using polyvalent *E. coli* antisera 2, 3 and 4 (Bioweb PTY, SA). Differences between colonial isolates were determined by determination of their antibiotic susceptibility profiles. *E. coli* ATCC 25922 was used as control. Serotyped (confirmed) *E. coli* isolates were inoculated onto tryptic soy (TS) slants and incubated for 24 h at 37°C, and then stored at 4°C until use (Roy *et al.*, 2004; Tarawneh *et al.*, 2009)

To isolate and identify *Acinetobacter* spp., 1 ml of 10^5 dilution of the water samples as for *E. coli* were inoculated into 9 ml of tubes containing Baumann's enrichment medium (BEM, Appendix i) instead of EMB. The inoculated BEM was shaken vigorously by vortexing and then incubated at 37ºC for 24 h in a shaker incubator with vigorous agitation. After incubation, 2 drops of the BEM culture were further inoculated into mTSB in a test tube and

incubated at 37°C for 24-48 h. After this, 1-2 loopfuls of BEM or mTSB cultures was inoculated onto EMB (Oxoid) or Leeds Acinetobacter medium (LAM, Hardy diagnostics USA, Appendix ii) and further incubated at 37°C for 18 to 72 h. After incubation, pink colonies on EMB or pink/purple colonies on LAM (Appendix vii c and d) were Gram stained to observe for large Gram-negative coccobacilli cells, while 5-10 discrete colonies were inoculated into SIM for motility testing medium, and also subjected to oxidase test using the oxidase test strips (Oxoid, UK). The isolates were also subjected to biochemical biotyping using the REMEL RapID^{TM} NF plus (Bioweb, South Africa, Appendix vii g). The strains were further purified by inoculation onto TS slants, incubated for 24 h at 37°C, and then stored at 4°C until use (Guardabassi *et al.*, 1999; Raksha *et al.*, 2003; Russo *et al.*, 2010). *A. haemolyticus* ATCC 19002 was used as control.

6.3.4 Detection of virulence factors on the bacterial isolates

6.3.4.1 *Screening of isolates for verotoxin production*

All the bacterial isolates were screened for verotoxin production using antibody-based rapid slide agglutination assays with the Duoperth kit (Merck, SA, Appendix vii h) according to the manufacturer's instructions. The bacterial isolates were first precultured in 1 ml casaminacid yeast extract (CAYE) broth (Appendix iii) and incubated at 37ºC with rotation at 100 rpm for 24 h. After incubation, 10 µl of the pre-cultured broth (approximately 1 x 10^7 cells/ml) was inoculated into fresh CAYE broth, and further incubated for 16 h with rotation at 100 rpm at 37ºC. The culture was centrifuged at 5000 rpm 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 suspended in 0. 25 ml 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 incubated at 37ºC for 30 min. Two hundred microliter (200 µl) of the culture suspension was then transferred onto the test device using a sterile Pasteur pipette, and the result read after 10 min. The appearance of red bands on the vtx1 or vtx2 bands denoted the presence of either one of or both verotoxins.

6.3.4.2 *Screening of isolates for haemolysin production*

The plate haemolysis technique was used to screen for the presence of cytolytic protein toxins known as the alpha haemolysin secreted by most haemolytic bacteria (Sharma *et al.*, 2007). In this procedure, discrete bacteria colonies (2-3) from nutrient agar (NA) plates were subcultured onto 5% sheep blood agar plates (supplemented with 10 mM $CaCl_2$ for *A. haemolyticus*), and incubated at 37ºC for 24 h. After incubation, enterohemolysin production was detected by the appearance of zone of complete lysis of the erythrocytes around each bacterial colony on the plates, and clearing of the medium.

6.3.4.3 *Cell surface hydrophobicity test*

The cell surface hydrophobicity of the bacterial isolates was determined using the salt aggregation test (SAT) (Raksha *et al.*, 2003; Sharma *et al.*, 2007). Briefly, a loopful (10 µl) of bacterial suspension in 1ml of phosphate buffer (pH 6.8) (equivalent to 5 x 10^9 colonies/ml) was mixed with equal volumes of ammonium sulphate solution of different molarities (1.4, 2.0 and 4.0 M) on a glass slide. The suspensions were rotated carefully for 1

min, and then microscopically observed for agglutination. The highest dilution of ammonium sulphate solution giving a visible agglutination of bacteria (Appendix vii *i*) was scored as the SAT value. Bacterial suspension clumping at the lowest dilution (1.4 M) was considered auto-aggregative, while those with SAT values of ≤2 M were considered hydrophobic.

6.3.4.4 *Gelatinase test*

This was carried out in order to screen the bacterial isolates for gelatinase production (Park *et al.,* 2007; Roy *et al.,* 2004). Gelatin agar was inoculated with the test bacteria, and then incubated at 37ºC for 24 h, after which the plate was then flooded with mercuric chloride ($HgCl_2$) solution. The development of opacity in the medium, and zone of clearing around the bacterial colonies was considered positive for the presence of gelatinase.

6.3.4.5 *Bactericidal serum resistance assay*

In this method, bacteria were first grown on blood agar for 18-24 h at 37ºC. The cells were then harvested and suspended in Hank's balanced salt solution (HBSS) (Appendix iv). Equal amounts (0.05 ml) of the bacterial suspension and serum was mixed in a test tube and then incubated at 37ºC for 180 min. After incubation, 10 µl of the mixture was withdrawn and spread-inoculated onto blood agar plates, and once again incubated at 37ºC for 18 h, and the viable count determined. Resistance of the bacteria to serum bactericidal activity was expressed as the percentage of bacteria surviving after 180 min of incubation with serum, in relation to the original count. Bacteria were termed serum sensitive if viable count dropped

to 1% of initial value, and resistant if >90% of organisms survived after 180 min of incubation (Ranjan *et al.,* 2010).

6.3.4.6 *Screening of isolates for extended spectrum beta-lactamase (ESBL) production*

The screening of isolates for ESBL was carried out using the disc diffusion method according to recommended criteria (NCCLS, 2005). Briefly, two discs (30 µg in each case), ceftazidime and cefotaxime were placed on Muller Hinton agar plates previously seeded with test bacteria, and the plates incubated at 37ºC for 18 h. After incubation, ESBLs production was determined by the appearance of zone diameters of inhibition (≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime) against the test bacteria.

6.3.4.7 *Phenotypic confirmation of ESBLs production*

The Double Disc Synergy Test (DDST) for confirming ESBLs production was used (Sharma *et al.,* 2007; Iroha *et al.,* 2008). Zero point one milliliter (0.1 ml) of each bacterial isolate suspension equivalent to 0.5 MacFarland turbidity standard was inoculated on the surface of Mueller-Hinton agar plates using a sterile swab stick. A combination disc containing (amoxicillin, 20 µg and clavulanic acid 10 µg) was placed at the centre of the Petri-dish, and ceftaxidime (30 µg) and cefotaxime (30 µg) was placed 15 mm apart center to center on the plates. The culture plates were then incubated at 37ºC for I8 - 24 h. An enhanced zone of inhibition (synergy, regardless of size) between any one of the beta-lactam discs compared to the combined amoxicillin-clavulanic acid disc was considered to be positive for ESBL enzyme production (Iroha *et al.,* 2008).

6.3.5 Determination of antimicrobial susceptibility pattern of isolates

The disc diffusion method as described was used for this purpse (Guardabassi *et al.,* 1999; Perilla *et al.,* 2003). Molten Mueller-Hinton agar (MHA) plates were inoculated with the test organisms (0.5 McFarland turbidity standard) using a sterile swab stick and the plates were incubated at 37ºC for I8 h. After incubation susceptibility to antibiotic was determined by measurement of zone diameters of inhibition (mm) against test bacteria. Antibiotics tested included 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), ciprofloxacin (5 µg), impenim (5 µg) (Oxoid UK). Antibiotics were selected based on recommended criteria for surveillance of antibiotic resistance in Enterobacteriaceae and *Acinetobacter* spp (Seifert *et al.,* 1993; NNIS, 2000; Wilson *et al.*, 2002; NARMS, 2004; Jones *et al.*, 2005). Isolates that were resistant to 3-7 antibiotics were selected and used for further studies.

6.3.6 Extraction and quantification of bacterial DNA

Extraction of plasmid DNA was carried out as earlier described (Davis *et al.*, 1986; Podar *et al.*, 2007). Briefly, bacterial cultures (2 ml) grown at 37°C for 24 h in MHB was transferred into a microcentrifuge and centrifuged at 5,000 rpm for 10 min, and the supernatant decanted and discarded. The cell pellets were mixed with universal DNA extraction buffer (Promerga, USA) and 2 µl proteinase K (20 $mgml^{-1}$) before shake-incubating for 30 min at 37°C. A 300 µl of 20% SDS (IBD, UK) was added and mixture was incubated for 2 h at 65°C before being centrifuged at 13,000 rpm for 3 min. This was followed by the addition of an equal

amount of chloroformamyl alcohol (24:1) (Merck, Germany) and the suspension was properly mixed. The aqueous part was transferred to a new tube, and isopropanol and 70% ethanol (Merck, Germany) were added to wash the pellet obtained. Ten microliters (100 µl) of TE buffer (Sigma, USA) was then added to dissolve DNA. Concentration of the extracted DNA was measured using UV visible spectrophotometer (Spekol 1300 analytik jena, United Scientific SA) at 260 nm.

6.3.7 Transformation test

Equal volumes (50 ml) of bacterial broth culture and lysed cells or DNA isolated from either *Acinetobacter* sp. or *E. coli* was mixed in an Eppendorf tube and then spread onto a nitrocellulose filter (Millipore). The nitrocellulose filter was then placed on Luria Bethany agar (LBA) plate supplemented with ampicillin and rifampin, and incubated at 30°C for 18 h. The DNA used was either purified bacterial DNA at concentrations of 0.1, 1, 10, and 50 mg per 50 ml of broth or cell lysates at concentrations of 1, 10, and 100 ml per 50 ml of broth. After incubation, the overgrown filter was transferred to a 50 ml Falcon tube and vortexed with 2 ml of a solution containing 0.85% NaCl and 50 ml of DNase I (5 mg ml^{-1}). Tenfold dilutions were plated onto LBA plates supplemented with ampicillin and impenim (recipient counts), and ampicillin, impenim, amikacin, ceftriaxone (transformant counts), and colony forming unit (CFU) counts were determined after incubation of the plates at 30°C for 72 h. Plates obtained from filters containing either culture suspension in 50 ml of water (for occurrence of spontaneous impenim mutants and bacterial contamination), only DNA (10 ml) or 100 ml of lysate (to check for sterility) were used as controls. Transformation

frequencies (presented as mean triplicate values) are given as the number of *Acinetobacter* sp. or *E. coli* colonies growing on transformant-selective LBA plates divided by the number of colonies on recipient-selective plates after the filter transformations (Davis *et al.*, 1986; Podar *et al.*, 2007).

6.3.8 Transfer of resistance genes between *E. coli* and *Acinetobacter* spp.

Transfer of antibiotic resistant genes between *E. coli* strains susceptible to 3-7 antibiotics and strains resistant to up to 7 antibiotics as well as between resistant *E. coli* strains and susceptible *A. lwoffii* and *A. haemolyticus* was tested. Mueller Hinton broth MHB (10 ml) was dispensed in four different sets of flasks and used for each of the bacterial isolates. The first test tube was inoculated with 0.5 McFarland standard antibiotic susceptible *E. coli* strains (*E. coli S*), the second flask was inoculated with antibiotic resistant *E. coli* strains (*E. coli R*), resistant to the seven antibiotics (AMP-AMK-GEN-CEP-CRO-IMP-CXM) and the third flask was inoculated with both *E. coli S* and *E. coli R* strains to determine transfer of resistance between *E. coli* strains. In the forth flask, either resistant *A. lwoffii* or *A. haemolyticus* was inoculated instead of *E. coli R* in order to determine transfer of resistance between *E. coli* and *Acinetobacter* spp. and all the flasks were then incubated at ambient temperature for 48 h. After incubation, a loopful of each broth culture was spread inoculated onto plates of MHA and susceptibility pattern determined as earlier described (Bakar *et al.*, 2010) in order to determine recombinant isolates that have acquired resistance genes.

6.3.9 Plasmid curing test

Tube containing 10 ml peptone water supplemented with 20 mg/ml acridine orange was inoculated with 0.1 ml of overnight broth culture and incubated at 37ºC for 24 h. Appropriate dilutions of the culture were inoculated on MHA to obtain single colony isolates. After overnight incubation at 37ºC, resulting colonies were tested for loss of antibiotic resistance on MHA plates containing appropriate concentration of antibiotics (Ramteke and Tewari, 2007). Transformation of cured isolates was carried out using the $CaCl_2$ protocol described elsewhere with cured cultures serving as competent recipients. To make the cured cultures competent, 0.1 ml of 2-4 h LB broth (grown at 37ºC) was chilled in ice bath, and centrifuged for 5 min. at 3000 rpm and the pellet suspended in 4 ml ice-cold 50 mM $CaCl_2$ and incubated for 10 min on ice. The cells were centrifuged at 3000 rpm for 5 min, re-suspended in 2 ml of ice-cold 50 mM $CaCl_2$ and then incubated for 5 min on ice. The cells were again centrifuged at 3000 rpm for 5 min, and 125 µL fresh LB broth was added to the pellets. To 50 µL of this competent cells 5 µL of plasmid DNA was added in a vial and properly mixed, and the vial was placed on ice for 3 min. The ice-cold vial was then transferred to a water bath containing water heated at 42ºC for 3 min. To this mixture, 500 µL of fresh LB medium was added and the broth culture now incubated at 37ºC for 2 h. After incubation, 100 µL of competent-plasmid DNA treated culture and competent cells with no plasmid DNA (negative control) were spread-inoculated onto antibiotic-containing MHA plates and incubated for 18 h at 37ºC.

6.3.10 Statistics

The significance in transformation and difference between the percentage values of antibiotic-resistance among bacterial strains was evaluated using the Student *t* test ($P \leq 0.05$) of the SIGMATPLOT statistical package.

6.4 RESULTS

Identification of *E. coli isolates and* detection of virulence factors

E. coli colonies on eosin methylene blue (EMB) were tiny with green metallic sheen, negative for motility test, sulphide and oxidase production and for Erclich's reagent, but positive for indole production. The various serotypes identified are shown in Table 6.3. For verotoxin production genes, results showed that out of the 69 *E. coli* isolates obtained, 25(35.23%) produced VTx1&2, 28(40.58%) produced Vtx1 and 14(20.30%) produced Vtx2 (Table 6.1). The highest rate (40%) of production of Vtx1&2 was obtained from river water islates, followed by isolates from treatment plant wastewater (36%) and abattoir wastewater (33%). Results also showed that 49(71.015%) were positive for extended-spectrum beta-lacatamases (ESBLs), 7(77.78%) for serum resistance, 57(82.61%) for cell surface hydrophobicity, 48(69.57%) for gelatinase production, and 37(53.62%) for haemolysin production. Result also showed that 19 of the 25 (76.00%) isolates from treatment plant waste water, 13 (54.17%) of the 24 from abattoir wastewater and 17(80%) of the 20 isolates from river water produced the ESBLs enzymes. This shows that the highest percentage of isolates that produced the enzymes were from the river water, followed by isolates from treatment plant wastewater and abattoir wastewater. The highest rate of haemolysin

production was obtained in isolates from treatment plant wastewater samples with 19(76%) out of the 24 isolates producing haemolysins, followed by isolates from the river water, 10(50.00%) and abattoir wastewater, 8(33.33%). Results also showed that all the isolates from all the water sources tested were positive for cell surface hydrophobicity with the percentage occurrence of 19(76.00%) (treatment plant wastewater), 10(50.00%) (river water), and 8(33.33%) (abattoir wastewater). Results for gelatinase test, showed that 12(48.00%) out of the 25 isolates from treatment plant wastewater produced gelatinase followed by 18(75.00%) out of 24 and 18(90.00%) out of 20 isolates from the abattoir wastewater and river waters respectively. For bactericidal serum resistance assay, the highest percentage resistance of 16(64.00%) out of 25 isolates was recorded from treatment plant wastewater, followed by 9(37.50%) of the 24 isolates from abattoir wastewater, and 6(30.00%) of the 20 isolates from river water samples examined.

Table 6.1. Virulence characteristics of *_Escherichia coli_* isolates obtained from water samples

Sample source	No. of isolates	Number (%) positive for virulence factor					
		Verotoxin	ESBLs	Serum Resistance	Cell surface hydrophobicity	Gelatinase	Haemolysin
Wastewater	25	Vtx1/ 10(40.00) Vtx2/ 6(24.00) Vtx1&2 9(36.00)	19(76.00)	16(64.00)	22(88.00)	12(48.00)	19(76.00)
Abattoir waste	24	Vtx1/ 2(8.33) Vtx2/ 12(50) Vtx1&2/ 8(33.33)	13 (54.17)	9(37.50)	21(87.5)	18(75.00)	8(33.33)
River water	20	Vtx1/ 2(10.00) Vtx2/ 10(50.00) Vtx1&2/ 8(40.00)	17 (85.00)	6(30.00)	14(70.00)	18(90.00)	10(50.00)

ESBLs = extended spectrum beta-lactamases

*green metallic sheen on eosin methylene blue (EMB), negative for motility test, sulphide and oxidase production and for Erclich's reagen, but positive for indole production

6.4.1 Identification of *Acinetobacter* spp and detection of virulence factors

Colonies of *Acinetobacter* spp. were tiny blue mucoid colonies on EMB (Appendix vii d), pink defused on Leeds *Acinetobacter* medium (LAM, Appendix vii e) and Gram-negative coccoballi. They also fermented glucose, urea, citrate, nrite and tryptophane (Table 6.2). Results for the detection of virulence factors in *A. haemolyticus* isolates obtained from the various water samples shows that out of the 14 *A. haemolyticus* isolates obtained, only 2(14.29%) isolates for each of the samples were positive for Vtx1, Vtx2 and Vtx1&2 verotoxic genes, 8(57.14%) were positive for ESBLs, 7(50.00%) for serum resistance, 11(78.57%) for cell surface hydrophobicity, 4(28.57%) for gelatinase production and 8(57.14%) for haemolysin production (Table 2). But for the absence of haemolysin in 5 of the river water isolates and gelatinase in 4 of the wastewater isolates, all other virulent factors were present on isolates from all the water samples investigated. Results also showed that the most prevalent virulent factor among the isolates is cell surface hydrophobicity which was present in all the 4(100%) isolates from the wastewater, 4(80.00%) of the 5 isolates from abattoir wastewater, and in 3(75.00%) of the 5 isolates from the river water samples. Furthermore, results revealed that 3(75%) out of the 4 isolates from the wastewater samples were positive for both ESBLs and serum resistance factors.

Table 6.2. Virulence characteristics of **_Acinetobacter haemolyticus_ isolates obtained from water samples.

Sample source	No. of isolates	Number (%) positive for virulence factor					
		Verotoxin	ESBLs	Serum Resistance	Cell surface hydrophobicity	Gelatinase	Haemolysin
Wastewater	4	Vtx1 1(25.00) Vtx2 0(0.00) Vtx1&2 0(0.00)	3(75.00)	3(75.00)	4(100.00)	0(0.00)	4(100.00)
Abattoir waste	5	Vtx1 1(20.00) Vtx2 1(20.00) Vtx1&2 1(20.00)	2 (40.00)	3(60.00)	4(80.00)	3(60.00)	4(80.00)
River water	5	Vtx1 0 (60.00) Vtx2 1(20.00) Vtx1&2 1(20.00)	3(75.00)	1(20.00)	3(75.00)	1(20.00)	0(0.00)

ESBLs = extended spectrum beta-lactamases

**Colonies of *Acinetobacter* spp were tiny blue mucoid colonies on EMB, pink colonies difused into the medium on LAM, Gram-negative coccoballi and fermented glucose, urea, citrate, nrite and tryptophane.

6.4.2 Determination of antimicrobial susceptibility pattern of isolates

Results of antimicrobial susceptibility (Tables 6.3 and 6.4) showed that majority of the isolates were resistant to between 4-10 antibiotics. Only 4 *E. coli* isolates (*E. coli* FEW O124, *E. coli* FEW O103:H2, *E. coli* FEW2iii O145:NM), and 3 *Acinetobacter* species (*A. lwoffii* RWW2i and *A. haemolyticus* PST2i *A. haemolyticus* PST2i) were susceptible to all the antibiotics tested.

6.4.3 Extraction and quantification of bacterial DNA, resistance genes and transformation rates among bacterial isolates

Quantification of extracted bacterial DNA showed that there was an increase in DNA concentration in all the transformed bacterial samples compared to the untransformed cells with transformation frequencies ranging between 13.3 – 53.4 x 10^{-7} (Table 6.3). Results showed a significant transformation frequency ($P \leq 0.05$) among all the isolates irrespective of sample source. Results also showed that resistance genes among *E. coli* and *Acinetobacter* spp. isolates included amongst others, resistant genes against ampicillin, gentimicin, nalidixic acid, ceftriaxone, tetracycline, amoxicillin, ofloxacin and ciprofloxacin.

6445 Table 6.3. Resistance pattern and DNA concentrations of normal and transformed *E. coli* and

6446 *Acinetobacter* spp. obtained from river water and wastewater samples.

Bacterial isolate	Resistance pattern untransformed/ normal cells	DNA Quantity (µg/ml) of normal cells	Resistance pattern of transformed cells	DNA Quantity (µg/ml of transformed cells	Transformation frequency
E. coli FEW O124	-	98.4	SXT, OFX, AMP, CN, AK, NA,CXM	102.7	46.7 x10-^{7}
E. coli FEW O103:H2	-	108.3	ATM,AK,CL,IPM,TE	110.3	33.3 x10-^{7}
E. coli FEW2iii O145:NM	-	86.0	TE,CN,CXM,CIP,AMP,NA,CRO	92.4	46.7 x10-^{7}
E. coli RWW 1iv O96:H9	AML	112.0	AML,OFX,TE,CN,AMP	115.2	33.3 x10-^{7}
E. coli RWW1v O126	TE,CL	78.8	TE,CL,AMP,CIP,CXM	81.4	33.3 x10-^{7}
E. coli RWW1vi O4	CFM,CRO	52.6	CFM,CRO	56.0	13.3 x10-^{7}
E. coli RWW1vii O55	CIP	90.4	CIP,OFX,NA,AMP,TE	91.4	33.3 x10-^{7}
E. coli PSW2ii O96:H9	CN,TE	68.7	AMP,CL,CRO,IPM,TE,CFM,CIP,SX	73.4	53.4 x10-^{8}
E. coli PRE1i O4	CL	42.7	CL,AMP,CN,OFX,NA,TE,AML,AK	45.7	53.4 x10-^{7}
E. coli FSE1ii O145:H2	AK	73.0	AK,CL,AMP,TE	83.1	26.7 x10-^{7}
E. coli PST1v O96:H9	CN,AMP	68.2	CL,CFM,CRO,CXM,AMP,AK	74.0	40.0 x10-^{7}
E. coli PST2ii O124	AK	93.2	AK,AMP,NA,OFX,TE,SXT,IMP,CN	95.6	53.3 x10-^{7}
E. coli RBD1iii O86	AML,CRO,AK	67.4	CFM,CL,AMP,NA,SXT,TE	77.2	40.0 x10-^{6}
E. coli PRK2ii O86	AK,AMP,CN	86.3	AK,AMP,CN,AML,TE,	88.1	40 x10-^{7}
A. lwoffii RWW2i	-	67.5	ATM,AK,CL,SXT,OFX	67.8	33.3 x10-^{7}
A. lwoffii PSW1ii	IMP	103.7	CL,AMP,CRO	111.6	33.3 x10-^{7}
A. haemolyticus PST2i	-	87.0	OFX,CN,CXM	92.3	20.0 x10-^{7}
A. lwoffii PSW1i	TE,CL,IPM	56.0	ATM,AK,CL,IPM,TE	62.4	33.3 x10-^{7}
A. haemolyticus FEW2iv	AML	123.5	AML,TE	123.8	13.3 x10-^{7}
A. lwoffii PRE2i	AMP,CL,CRO	87.4	AML,OFX,TE,CN,AMP	89.4	33.3 x10-^{7}
A. haemolyticus PST2i	-	94.2	TE,CL,AMP,CIP,CXM,AML	98.4	33.3 x10-^{7}
A. haemolyticus PST2i	CL	119.3	CFM,CRO,CIP,TE,NA	120.4	40.0 x10-^{7}
A. lwoffii RBI2ii	TE,AK,NA	67.8	OFX,NA,AMP,TE	68.2	40.0 x10-^{7}

6447 SXT (30 µg) - Trimethroprim-Sulfamethaxazole; OFX (5 µg) - Ofloxacin; ATM (30 µg) - Aztreonam;
6448 AMP (10 µg) - Ampicillin; TE (10 µg) - Tetracycline; AK (30 µg) - Amikacin, CAZ (30 µg) - Ceftazidime, CL
6449 (30 µg) - Cefalexin; CRO (30 µg) - Ceftriaxone; CXM (30 µg) - Cefuroxime; AML (10 µg) - Amoxicillin; CN
6450 (10 µg) - Gentamicin, CFM (5 µg) - Cefixime, CIP (5 µg) - Ciprofloxacin; IMP – Impenem (30 µg); NA (30
6451 µg) -Nalidixic acid; - = susceptible.

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6.4.4 Plasmid curing among *E. coli* and *Acinetobacter* spp. isolates

Results of plasmid cure for the *E. coli* and *Acinetobacter* spp. showed that out of 14 multi-drug resistant *E. coli* isolates, 7 were cured of resistance against ampicillin, cephalexin, trimethroprim-sulfamethaxazole, ciprofloxacin and nalidixic acid only (Table 6.4). For the 9 *Acinetobacter* spp., 6 were cured of ampicillin, amoxicillin, amykacin, tertracyclin, cefuroxime and ceftaxidime. The spectrum of antibiotics cured was however narrow compared to the total number of antibiotics tested.

Table 6.4. Resistance pattern of plasmid cured cells of *E. coli* and *Acinetobacter* spp. isolates obtained from river water and wastewater samples.

Bacterial isolate	Resistance pattern before plasmid curin	Resistance pattern after plasmid curin	Reistance Markers cured
E. coli FEW O124	SXT, OFX, AMP, CN, AK, NA,CXM	OFX, CN, AK, NA,CXM	SXT, AMP
E. coli FEW O103:H2	ATM,AK,CL,IPM,TE	AK,IPM,TE,ATM	CL
E. coli FEW2iii O145:NM	TE,CN,CXM,CIP,AMP,NA,CRO	TE,CN,CXM,CIP,AMP,NA,CRO	-
E. coli RWW 1iv O96:H9	AML,OFX,TE,CN,AMP	AML, OFX,TE,CN,AMP	-
E. coli RWW1v O126	TE,CL,AMP,CIP,CXM	TE, CXM	CL,AMP,CIP
E. coli RWW1vi O4	CFM,CRO	CFM,CRO, CFM,CRO	-
E. coli RWW1vii O55	CIP,OFX,NA,AMP,TE	CIP,OFX,NA,TE	AMP
E. coli PSW2ii O96:H9	AMP,CL,CRO,IPM,TE,CFM,CIP,SXT	AMP,CL,CRO,IPM,TE,CFM,CIP,SXT	-
E. coli PRE1i O4	CL,AMP,CN,OFX,NA,TE,AML,AK	CL,AMP,CN,OFX,NA,TE,AML,AK	-
E. coli FSE1ii O145:H2	AK,CL,AMP,TE	AK,CL,AMP,TE	CL,CFM, AMP
E. coli PST1v O96:H9	CL,CFM,CRO,CXM,AMP,AK	AK,CRO,CXM	SXT,AK
E. coli PST2ii O124	AK,AMP,NA,OFX,TE,SXT,IMP,CN	IMP,CN	NA
E. coli RBD1iii O86	CFM,CL,AMP,NA,SXT,TE	AMP,OFX,TE	-
E. coli PRK2ii O86	AK,AMP,CN,AML,TE,	AK,AMP,CN,AML,TE	-
A. lwoffii RWW2i	ATM,AK,CL,SXT,OFX	ATM, AK,AML,TE,	AMP,CRO
A. lwoffii PSW1ii	CL,AMP,CRO	CL	CN,CXM
A. haemolyticus PST2i	OFX,CN,CXM	OFX	CN,CXM
A. lwoffii PSW1i	ATM,AK,CL,IPM,TE	ATM, TE,CL,IPM	AK
A. haemolyticus FEW2iv	AML,TE	-	AML,TE
A. lwoffii PRE2i	AML,OFX,TE,CN,AMP	AML,OFX,TE,CN	AMP
A. haemolyticus PST2i	TE,CL,AMP,CIP,CXM,AML	TE,CL,AML,CIP,CXMAML,	-
A. haemolyticus PST2i	CFM,CRO,CIP,TE,NA	CFM, CIP,TE,NA	-
A. lwoffii RBI2ii	OFX,NA,AMP,TE	OFX,AMP,TE,NA	-

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. - = no resistance marker.

6.4.5 Transfer of resistance genes between *E. coli* and *Acinetobacter* spp.

Table 6.5 shows results for transformation of some *Acinetobacter* spp. using resistant *E. coli* (resistant against trimethroprim-Sulfamethaxazole (SXT), ofloxacin (OFX), ampicillin (AMP), gentamicin (CN), amikacin (AK), nalidixic acid (NA), cefuroxime (CXM)) as donor. Results showed that the *A. lwoffii* and all the *A. haemolyticus* tested acquired resistance genes from the *E. coli.* Resistance genes acquired by *A. lwoffii* include AK, and ampicillin, while those acquired by *A. haemolyticus* isolates include ampicillin, gentamicin, and ofloxacin.

Table 6.5. Transformation of *Acinetobacter* spp by resistant *E. coli* (*E. coli R* resistant to SXT, OFX, AMP, CN, AK, NA and CXM)

Bacterial isolate	Resistance pattern before transformation (with *E. coli*)	Resistance pattern after transformation with *E. coli*	Resistance genes acquired
E. coli R	SXT, OFX, AMP, CN, AK NA,CXM	N/T	N/T
A. lwoffii PSW1ii	CL,AMP,CRO	CL,AMP,CRO,AK,AMP	AK,AMP
A. haemolyticus PST2i	OFX,CN,CXM	OFX,CN,CXM, CN	CN
A. haemolyticus FEW2iv	AML,TE	AML,TE,AMP,CN,OFX	AMP,CN,OFX
A. haemolyticus PST2i	-	OFX, CN	OFX, CN

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; N/T – not tested.

6.5 DISCUSSION

Virulence of bacterial pathogens including *E. coli* and *A. haemolyticus* largely depends on the presence of virulent factors. These factors enable the bacteria to cause infection by overcoming the host defence mechanisms resulting in a variety of infections such as urinary tract infection, soft tissue infections, bacteraemia and neonatal meningitis. In this study, *E. coli* and a number of the *A. hameolyticus* isolates produced verotoxins. The verotoxins, also called shiga toxins act by production of cytotoxins that inhibit protein synthesis (Bolton, 2011). The toxins cause hemorrhagic colitis and potentially fatal systemic sequelae in humans and are the leading cause of acute renal failure in children (Bolton, 2011). Presence of verotoxins in most of the isolates obtained from this study calls for more effective surveillance and control measures. ESBLs confer bacteria with resistance to beta-lactam antibiotics. Incidence of the increase in incidence of ESBL producing strains of *E. coli* among clinical isolates has been steadily increasing over the past few years resulting in limitation of therapeutic options (Russo *et al.*, 2010). The extensive use of antimicrobials and the indiscriminate use of cephalosporins are responsible for the high rate of selection of ESBL producing microorganisms (Raksha *et al.*, 2003; Shukla *et al.*, 2004). Alpha-hemolysin is one of the very few proteins produced by members of the family Enterobacteriaceae that is released extracellularly. *E. coli* alpha-hemolysin is a protein that causes *in vitro* lysis of erythrocytes and subsequent invasion of the cells from several species of animals (Konig *et al.*, 1986). Haemolysis, though not essential for establishment of acute pyelonephritis, may contribute to tissue injury, survival in renal parenchyma and entry into blood stream. In this study, both *E. coli* and *A. haemolyitcus* were associated with

haemolysin production, an indication that they are potentially invasive if they colonize any cell. Gelatinase, a zinc-metallo-protease is thought to contribute to virulence through degradation of host proteins such as collagen, fibrinogen, fibrin, and complement components (Park *et al.,* 2007; Thurlow *et al.*, 2010). The enzyme is also known to contribute to biofilm formation (Obire *et al.,* 2005). Cell surface hydrophobicity protects the bacterium from phagocytosis by host cells and helps to adhere to surfaces. Enzymes such as esterases, amino-peptidases, and acid phosphatases and lipo-polysaccharide slimes on the bacterial cell surface are reported to confer the organisms with surface hydrophobicity (Russo *et al.*, 2010). Serum resistance is the ability of a bacterial cell to resist the lytic effects of serum *in vitro* and to invade and survive the human blood stream (Russo *et al.*, 2010). This resistance to killing has been associated with the presence of capsular polysaccharides in *E. coli* (Cross *et al.*, 1986).

The presence of various resistance genes among the bacterial isolates confirms that the antibiotic resistant genes are located on plasmids. The presence of resistant transformants also confirms that the resistance genes are carried on plasmids and is also an indication that the antibiotic resistant plasmid genes carry multidrug resistance marker(s). This can also mean that intrageneric resistance transfer is actively taking place among the *E. coli* isolates coexisting in the water samples. This was further confirmed by the exchange of antibiotic resistance between *E. coli* and *Acinetobacter* spp. The plasmid curing experiments showed a limited curing process among the *Acinetobacter* isolates which is an indication of stability among the multidrug resistant genes. To the best of our knowledge this is the first

documented work on exchange of resistance genes between *E. coli* and *A. lwoffii* and *A. haemolyticus.*

Demonstration of virulence factors and multidrug resistance plasmid genes among the bacteria in this study leads us to conclude that *E. coli* and *Acinetobacter* spp. isolates from these water samples are developing high level of resistance and virulence. Their spread into drinking water or food sources, if not controlled, could have disastrous consequences. The recent emergence of a very virulent strain of *E. coli* outbreak in Europe (CDC, 2011) shows how rapidly these bacteria are transforming. The results for the study emphasize the need for continued monitoring processes in both the developed and developing countries to enable development of more proactive control and prevention measures.

6.6 ACKNOWLEDGEMENT

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CHAPTER SEVEN

7.0 EFFECT OF OXIDATIVE AND TEMPERATURE STRESS ON VIABILITY AND TOXIN PRODUCTION OF ENVIRONMENTAL ISOLATES OF *ESCHERICHIA COLI*

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7.0 Effect of oxidative and temperature stress on viability and toxin production of environmental isolates of *Escherichia coli*

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7.1 ABSTRACT

The effect of oxidative stress on expression of virulence factors and the effect of low temperature stress on toxin production among *Escherichia coli* serotypes isolated from wastewater and river water samples were determined. Oxidative stress due to chemicals, salts, alcohol or low temperature stress has exerted various degrees of lethality to the bacterial cells with bacterial strains losing their potential to express virulence factors with time. The cell kill index (CKI) increases as temperature stress (-5; -18; and -28ºC) increases with time. However, the rate of loss of expression of virulence factors or viability was slower in isolates from wastewater and abattoir compared to those from river water. Contamination of food or drinking water sources with these strains should be prevented to avoid human infection with disease conditions such as diarrhea, urinary tract infections and gastroenteritis.

Key words: cell kill index, cell surface hydrophobicity, contamination, foodborne pathogens, stress

7.2 INTRODUCTION

Contamination and proliferation of bacterial pathogens in food and water are of great concern for food and water safety and public health. Many environmental factors such as temperature, pH, moisture content, antimicrobial agents, and water activity affect the growth of bacteria in nature (FDA, 2001). The food industry has a long history of manipulating these factors to control food-borne pathogens during food processing. Among these factors,

temperature control is one of the most effective to reduce or minimize populations of *Escherichia coli* in foods (Yuk and Marshall, 2003). In addition, many chemical agents including salts, have also been used for preservation purposes. Several studies have reported increasing cases of disease outbreaks due to *E. coli* (Aksoy *et al.,* 2007) and many other food-borne pathogens especially in the developing world (Yuk and Marshall, 2003; WHO, 2005; Doughari *et al.,* 2010). Pathogenecity by these food pathogens is made possible due to the possession of virulence factors. Common virulence factors include surface hydrophobicity, colonization factor, capsular polysaccharides, siderophores, serum resistance, and resistance to phagocytosis, haemolysins, and enterotoxins (Raksha *et al.,* 2003; Hedge *et al.,* 2009). The virulence factors of *E. coli* are multiple and usually complex, affecting pathogenicity in combination with one another (Hedge *et al.,* 2009).

For successful infection, bacterial pathogens must overcome the host innate immunity (Davies *et al.,* 2011). Phagocytic leukocytes, especially neutrophils, play a critical role in innate immune responses against bacteria, fungi, and other pathogens (Witko-Sarsat *et al.,* 2000). Neutrophil-mediated bacterial killing can involve both oxygen-independent and oxygen-dependent processes (Mydel *et al.,* 2006). While oxygen-independent bacterial killing involve the use of bactericidal peptides, proteins, and protease fibres to to trap, entangle and efficiently kill invading bacteria, oxygen-dependent processes involved exertion of oxidative stress (OS) by reactive oxygen species (ROS). ROS such as phagocytic cells (neutrophils and macrophages) and superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$), generated as by-products of endogenous metabolism

(Soutourina *et al.*, 2010) destroy the invading bacterial pathogens via oxidative burst. The mechanism by which bacteria overcome these factors to successfully establish infection is said to be complex and poorly understood (Khanduja *et al.*, 1998; Hedge *et al.*, 2009). To develop effective virulent-factor-targeted control measures, it is important to understand the response of pathogenic bacteria to these stress factors. This study investigated the effect of oxidative stress exerted by various physicochemical parameters (crystal violet, bile salt, and sodium chloride) and effect of low temperature stress (freeze-thaw) on the virulence factors and toxin production among environmental isolates of *E. coli.* Attempts were also made to investigate the association of antibiotic resistance to stress resistance among isolates resistant to more than three classes of antibiotics.

7.3 MATERIALS AND METHODS

7.3.1 Source of bacterial strains and blood sample

Nineteen stock cultures of *E. coli* serotypes: *E. coli* RWW1i O103:H2; *E. coli* RWW1ii O86; *E. coli* RWW1iii O145:H2; *E. coli* PSW1iii O111: NM; *E. coli* PSW2ii O96:H9; *E. coli* PSW1iv O111:NM (from treatment plant wastewater); *E. coli* PRE1i O4; *E. coli* FSE1ii O145:H2; *E. coli* FSE1iii O86; *E. coli* PST1v O96:H9; *E. coli* PST2i O124; *E. coli* PRE1vi O111:NM; *E. coli* PST1iii O113; and *E. coli* PST1iv O4 (from abattoir wastewater) and *E. coli* RBU2i O113; *E. coli* RBD1iii O86; *E. coli* RBI2iii O96:H9; and *E. coli* PRK2ii O86 (from river) were used for this study. The strains were previously isolated from the various water sources, characterized using standard methods to be positive for verotoxins, cell surface hydrophobicity, serum resistance and haemolysin production and maintained them as

stock cultures in the Microbiology Laboratory, Department of Biotechnology, Faculty of Applied Sciences Cape Peninsula University of Technology, Cape Town South Africa (Doughari *et al.*, 2011). The non pathogenic strain *E. coli* ATCC 25922 was used as control. All the bacteria were subcultured into tryptic Soy Slants (TAS) and incubated at 37ºC for 18 h before use. Sheep blood erythrocyte was purchased fresh from the National Research Council (NRC) Cape Town South Africa. The blood was used without further treatment as a source of serum complement.

7.3.2 Antimicrobial susceptibility testing

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), ciprofloxacin (5 µg) (Oxoid UK) (WHO 2002) using the disc diffusion method (Aksoy *et al.*, 2007). Antibiotic discs were placed on Molten Mueller-Hinton agar (MHA) plates, earlier preseeded with the test bacteria (0.5 McFarland turbidity standard), using a sterile swab stick and the plates were incubated at 37ºC for I8 h, after which antimicrobial activity was determined by measurement of zone diameters of inhibition (mm) against each bacterial strain.

7.3.3 Effect of stress on bacterial viability and virulence

7.3.3.1 Effect of oxidative stress on surface hydrophobicity of bacterial cells

To screen isolates for the effect of oxidative stress on bacterial viability, bacterial strains from TAS were subcultured twice in chemically defined medium (CDM) of Snyder and Koch as described by Hedge *et al.* (2009) and each time incubated at 37ºC for 24 h. After the final subculture, the cells were washed thrice in sterile physiological saline (0.85% w/v NaCl) and finally suspended in saline to get a solution of OD_{600} 0.1 (equivalent to 0.5 McFarland turbidity standard or 10^{-8} cells/ml).

To determine the effect of oxidative stress exerted by H_2O_2, CDM (10 ml) containing three different molar concentrations of H_2O_2 (0.1, 0.2 and 0.3 M) were prepared and dispensed aseptically in three sets of 100 ml Erlenmeyer flasks. The bacterial suspensions were inoculated into each of the flasks to obtain a cell inoculum of approximately 10^{-6} cells/ml, and a forth flask containing *E. coli* ATCC 25922 suspended in CDM with no H_2O_2 was used as control. The flasks were then incubated at 37ºC in a rotary water bath at 160 rpm for 24 h, centrifuged at 3000 rpm for 10 min and the supernatants discarded and packed cells retained. The packed cells were washed with sterile phosphate buffered saline (PBS, 10 ml) and then suspended in PBS to get a density of 0.3 at OD_{600} (OD Initial). From this bacterial suspension, 3 ml was withdrawn and mixed with 0.3 ml *p*-xylene and votexed for 1 min, then left for 30 min at ambient conditions and the final OD_{600} (OD Fl) determined. Degree of retention hydrophobicity was determined by calculating the percent hydrophobicity index (HI).

$$HI = \frac{OD\ I - OD\ F}{OD\ I} x100$$

7.3.3.2 Effect of oxidative stress on bacterial haemolysin production

This was carried out using the quantitative α-haemolysin assay. The H_2O_2 treated (0.3 M) bacterial suspension earlier grown in the Erlenmeyer flasks (10 ml), was transferred into sets of tubes and centrifuged at 3000 rpm for 10 min. The supernatant was collected and diluted in 0.8% calcium chloride solution (10 ml), 1 ml withdrawn and mixed with 1% (v/v) sheep erythrocyte suspension and incubated at 37ºC for 1 h. After the incubation, 2 ml of 0.8% NaCl saline was added to each tube exhibiting partial haemolysis and the bacterial suspensions centrifuged at 1500 rpm for 10 min to pellet the unlysed erythrocytes. The supernatant fluid was separated and the OD_{540} determined. Fifty percent (50%) haemolysis standard prepared by mixing 1 ml of 1% (v/v) sheep erythrocyte suspension and 3 ml of diluent was used as control. Inverse of the dilution which caused 50% lysis was recorded as HU 50 (50% haemolysis units) (Hedge *et al.,* 2009). A tube containing non-H_2O_2 treated bacterial suspension (*E. coli* ATCC 25922) suspended in CDM was used as control.

$$\text{HU } 50 = \frac{\text{ODs - ODt}}{\text{ODs}} x100$$

Where OD_s = 50% haemolysis standard, OD_{ex} = final haemolysis measured, HU 50 = 50% haemeolysis rate

7.3.3.3 Effect of oxidative stress on bacterial serum resistance

This was carried out using the quantitative serum bactericidal assay (Hughes *et al.,* 1982). H_2O_2 treated (0.3 M) bacterial suspension (0.5 ml) was mixed with 1.5 ml of fresh undiluted serum and incubated at 37ºC. Cell viability was determined turbidiometrically at 600 nm

after 3, 6 and 18 h. Serum resistance index (SRI) of bacteria was calculated using the formula:

$$\text{SRI } (\%) = \frac{\text{ODI - ODF}}{\text{ODI}} x100$$

Where SRI = serum resistance index, ODI = initial turbidiometric reading, ODF = Final turbidiometric reading. A tube containing non-H_2O_2 treated bacterial suspension (*E. coli* ATCC 25922) suspended in CDM was used as control.

7.3.4 Effect of temperature stress

The effect of low temperature treatment on viability of the bacterial isolates was determined as described in literture (Chou and Cheng, 2000). A 1 ml aliquot of bacterial suspension (initial density 10^8 CFU/ml) grown at 37ºC was inoculated into 9 ml trypton soy broth (TSB) in two different sets of test tubes and thoroughly mixed. The tubes were then stored at -5; -18; and -28ºC for a period of 21 days. After every 5 days of storage, the tubes were removed from the freezers and the contents thawed under running tab water for 5 min and then returned to the freezer. After the 21 days storage, the cultures were removed and 2-3 loopfuls inoculated into tryptic soy broth (TSB), incubated for 18 h at 37ºC and the viable cell index (VCI) determined by taking the OD_{600} values first at 0 min (OD Initial) then at 10 min interval for 1 h. Viable cell counts on the scale of 100% was calculated using the formula:

$$\text{VCI} = \frac{\text{OD I - OD F}}{\text{OD I}} x100$$

To confirm that viable bacteria were in a culturable state, viable counts were made by making serial dilutions of 2-3 loofuls of bacterial culture in 10 ml TSB and surface spread

inoculating onto NA plates, incubating h at 37°C and determining percentage survival after 18 h. Bacterial suspension containing non-H_2O_2 treated and non-freeze-thawed bacterial (*E. coli* ATCC 25922) suspension in CDM was used as control.

7.3.5 Effect of ionic salt concentrations and other chemicals on cell viability and verotoxin production

Zero point twom milliliters of 0.3 M H_2O_2 stressed bacterial culture suspension was inoculated into 10 ml of solution containing either 0.03% crystal violet, 0.3% bile salt, 4% NaCl, 8% ethanol and incubated at ambient conditions for 1 h (Chou and Cheng, 2000). Bacterial culture not subjected to H_2O_2 stress was (*E. coli* 25922), or alcohol and salts was used as control. After incubation, each of the samples was serially diluted in Butterfield's phosphate diluents and 0.1 ml surface plated on trypton soy agar (TSA) and incubated at 37ºC at 10 (initial) and 30 min (final) after which, the percent killed cells was determined. Percent kill index (CKI, %) after incubation calculated as follows:

$$\text{CKI } \% = \frac{\text{Initial Population - Final Population}}{\text{Initial Population}} x100$$

For effect of H_2O_2 (0.3 M) stress and tempreture freeze thawing on verotoxin production, the Duoperth kit (Merck, SA) antibody-based rapid slide agglutination assay was employed according to the manufacturer's instructions. The H_2O_2 stressed or freeze thawed bacterial isolates (-5, -18, and -28ºC) were first precultured in 1 ml casaminacid yeast extract (CAYE) broth (20 g of casamino acid, 6 g of yeast extract, 2.5 g of NaCl, 8.71 g of KH_2PO_4, and 1 ml of trace salt solution - 0.5% $MgSO_4$, 0.5% $MnCl_2$ and 0.5% $FeCl_3$ dissolved in 0.0005 M

H_2SO_4) accordingly and incubated at 37ºC with rotation at 100 rpm for 24 h. After incubation, 10 µl of the pre-cultured broth (approximately 1 x 10^7 cells/ml) was inoculated into fresh CAYE broth and further incubated for 16 h with rotation at 100 rpm at 37ºC. The culture was centrifuged at 5000 x *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 suspended in 0. 25 ml 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 incubated at 37ºC for 30 min. Two hundred microliter (200 µl) of the culture suspension was then transferred onto the test device using a sterile Pasteur pipette and the result read after 10 min. The appearance of red bands on the vtx1 or vtx2 bands denoted the presence of either one of or both verotoxins.

7.3.5 Statistical analysis

The student t test of SIGMAPLOT 8.0 statistical software package was used to relate the effect of oxidative stress on viability of bacteria at $P \leq 0.05$ and also to calculate the standard deviation and standard error of mean of values obtained.

7.4 RESULTS

Table 7.1 shows the effect of various concentrations of crystal violet, bile salt, sodium chloride and ethanol on virulence and effect of freeze thawing on toxin production of *E. coli* isolates obtained from wastewater and river water sources. Results showed that 4% NaCl exhibited the highest lethal effect against *E. coli* PRK2ii 086 (initial cell population – ICP/ml 8000) and *E. coli* RBU2i O113 (ICP/ml 3840) with cell kill index (CKI) values of 52.48

(final cell population-ICP/ml 6600) and 45.83% (FCP/ml 3200) respectively (Table 7.1), followed by 8% ethanol and 0.3% bile salt with the highest effects against *E. coli* RBD1iii O86 and *E. coli* PRK2ii O86 (CKI values of 40.00 and 38.16% respectively). The highest CKI values of 25.20 (crystal violet), 38.16 (bile salt), 40.00 (ethanol) and 52.48% (NaCl) was recorded for *E. coli* PRE1i O4 (from abattoir wastewater), *E. coli* PRK2ii O86 (from river water), *E. coli* RBD1iii O86 (River water), and *E. coli* PRK2iiO86 (river water). *E. coli* isolates from river water samples recorded the highest CKI values (4.25- 52.48) followed by those from abattoir (CKI values, 2.2-32.83%) and wastewater (CKI values, 1.34-22.86%). The CKI values of *E. coli* ATCC 25922 (control) ranged between (1.00-5.67%) with ethanol recording the lowest (1.00). Among all the 19 isolates, the wastewater isolates *E. coli* RWW1ii O86 from wastewater treatment plant and *E. coli* PST2i O124 from abattoir wastewater recorded the lowest CKI values of 0.00 each for bile salt and crystal violet respectively.

6952 Table 7.1. Cell Kill Index (CKI) (%) of *Escherichia coli* serotypes isolated from wastewater and river
6953 water samples.

E. coli serotypes	Cell Kill Index (CKI, %) of *E. coli* serotypes											
	Crystal violet (0.03%)			Bile salt (0.3%)			NaCl (4%)			Ethanol (8%)		
	ICP/100ml min)	FCP/100ml min)	CK	ICP/100ml min)	FCP/100ml min)	CKI	ICP/100ml min)	FCP/100ml min	CKI	ICP/100ml min)	FCP/100ml min)	CK
Waste water isolates												
E. coli RWW1i O103:H2	6700	6610	1.34	4000	3780	5.50	4000	3840	4.00	5200	3840	26.15
E. coli RWW1ii O86	8000	7660	4.25	4000	4000	0.00	7000	7340	2.13	6500	6300	3.17
E. coli RWW1iii O145:H2	4400	4300	2.27	3940	3720	5.58	4840	6200	11.30	5000	4400	12.00
E. coli PSW1iii O111: NM	4000	3600	10.00	3800	2100	17.89	4000	3600	10.00	4000	3200	20.00
E. coli PSW2ii O96 : H9	3500	3200	8.57	3300	3120	5.45	3500	2700	22.86	4400	4320	1.82
E. coli PSW1iv O111:NM	4600	3850	6.40	3000	2920	4.36	3200	3000	3.78	4800	4100	2.68
E. coli RBI2iii O96:H9	3600	3100	4.76	3700	3120	6.72	3000	2630	4.54	4600	4000	3.03
E. coli PRE1i O4	5000	3740	25.20	6000	4030	32.83	5700	4020	29.47	5000	4100	18.00
E. coli PRE1vi O111:NM	4700	4400	9.26	5620	4800	18.47	5300	4780	11.68	4800	4620	19.76
E. coli FSE1ii O145: H2	4200	4200	0.00	4000	3750	6.25	4000	3700	7.50	6000	5670	5.50
E. coli FSE1iii O86	6500	5960	8.31	6000	5600	6.67	6500	6040	7.10	4000	3800	5.00
E. coli PST1iii O113	4350	4000	1.87	4000	3700	5.82	4700	4620	4.86	3800	3700	4.67
E. coli PST1iv O4	4800	4550	4.82	4440	4000	10.56	5020	4800	6.80	4100	3780	6.72
E. coli PST1v O96: H9	4500	4400	2.20	3000	2820	6.00	4000	3800	5.00	5200	4580	11.92
E. coli PST2i O124	4600	4600	0.00	4400	4000	9.10	6000	5300	11.67	6000	5200	13.33
River water isolates												
E. coli RBU2i O113	3840	3200	16.67	4000	3900	2.50	2400	1300	45.83	3000	2300	23.33
E. coli RBD1iii O86	3400	3200	5.88	4600	3240	29.57	3400	2600	23.23	4500	2700	40.00
E. coli PRK2ii O86	8000	6600	17.50	7600	4700	38.16	7500	2300	52.48	6500	4340	33.23
Control												
E. coli ATCC 25922	3000	2900	3.30	3000	2830	5.67	3000	2950	1.67	3000	2870	1.00

6954 ICP – initial cell population at 3h incubation; FCP – final cell population at 18 h incubation

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Results of antimicrobial susceptibility profile and the effect of temperature stress on verotoxin production (Table 7.2) shows that, while the majority of the isolates were resistant to more than 3 antibiotics, *E. coli* PSW1iv O111:NM (Vtx1, Vtx2) *E. coli* PRE1vi O111:NM (Vtx1,Vtx2) were only resistant to one antibiotic each (amoxicillin and impenim respectively) and *E. coli* RBI2iii O96:H9, *E. coli* PST1iii O113 and *E. coli* PST1iv O4 were not resistant to any one antibiotic. The control strain was resistant to tetracycline and gentamicin. Results also showed that while *E. coli* RBU2i O113 (from river water), *E. coli* PRK2ii O86 (from river water), *E. coli* PST1v O96: H9 (from abattoir wastewater), *E. coli* PRE1i O4 (from river water) lost the ability to produce Vtx1, only *E. coli* RWW1i O103:H2 (from wastewater) lost ability to produce Vtx2 at all the temperatures tested. At -28°C however, *E. coli* FSE1ii O145: H2 (from abattoir wastewater) completely lost the ability to produce any of the verotoxins. However, H_2O_2 (0.3 M) stress however, did not show any significant effect on verotoxin production among the bacterial isolates at the tested concentration.

6991 Table 7.2. Effect of H_2O_2stress and temperature freeze-thawing son verotoxin production among multi-
6992 drug resistant environmental *Escherichia coli* serotypes

Escherichia coli serotype	Antibiotic resistance pattern	Temperature (°C) stress treatment/verotoxin status									
		37 (non treated)		0.3 M H_2O_2		-5		-18		-28	
		Vtx1	Vtx2	Vtx1	Vtx2	Vtx1	Vtx2	Vtx1	Vtx2	Vtx1	Vtx2
Wastewater isolates											
E. coli RWW1i O103:H2	SXT, OFX, AMP, CN, AK	+	-	+	+	+	-	+	-	+	-
E. coli RWW1ii O86	ATM, AK, CL	+	+	+	+	+	+	+	+	+	+
E. coli RWW1iii O145:H2	TE, CN, CXM, AMP	+	+	+	+	+	+	+	+	-	+
E. coli PSW1iii O111: NM	OFX, NA, AMP, TE	+	+	+	+	+	+	+	+	+	+
E. coli PSW2ii O96: H9	AMP, CL, CRO, IPM	+	+	+	+	+	+	+	+	+	+
E. coli PSW1iv O111:NM	AML	+	+	+	+	+	-	-	-	-	-
E. coli RBI2iii O96:H9	-	-	+	-	-	+	-	-	-	-	-
E. coli PRE1i O4	AMP, CN, OFX, NA, TE	-	+	-	+	-	+	-	+	-	+
E. coli PRE1vi O111:NM	IPM	+	+	+	+						-
E. coli FSE1ii O145: H2	CFM, CL, AMP, NA	+	+	+	+	-	+	-	+	-	-
E. coli FSE1iii O86	OFX, TE, AK	-	+	-	+	-	+	-	+	-	+
E. coli PST1iii O113	-	+	+	+	+	-	-	-	-	-	-
E. coli PST1iv O4	-	-	+	-	-	-	+	-	-	-	-
E. coli PST1v O96: H9	CL, FM, CRO	-	+	-	+	-	+	-	+	-	+
E. coli PST2i O124	AMP, NA, OFX, TE, SXT	+	+	+	+	+	+	+	+	+	+
River water isolates											
E. coli RBU2i O113	CL, CFM, IPM	-	+	-	+	-	+	-	+	-	+
E. coli RBD1iii O86	AML, CRO, AK	+	+	+	+	+	+	+	+	+	+
E. coli PRK2ii O86	AK, AMP, CN	-	+	+	+	-	+	-	+	-	+
Control		-									
E. coli ATCC 25922	TE, CN		-	+	+	-	-	-	-	-	-

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6994 SXT-Sulphomethaxazole/Trimethoprim; AMP-Ampicillin; ATM- aztreonam; TE-Tetracycline; AK-Amikacin; OFX-
6995 Ofloxacin;
6996 CAZ-Ceftazidim; CL-Cephalexin; CRO-Ceftriazone; CXM-Cefuroxime; AML-Amoxycillin; CN-Gentamicin; CFM-
6997 Cefixime; CIP-Ciprofloxacin; NA-Nalidixic acid; IPM-impenim; + = positive; - = negative
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The hydrophobicity index values (HI) for various *E. coli* isolates from wastewater and river water samples are shown in Fig. 7.1. Results showed that oxidative stress exerted by H_2O_2 had significant effect on surface hydrophobicity of *E. coli.* The HI values indicating loss of surface hydrophobicity were in the order 23.12, 49.442, 49.917 and 43.967% at 0.1 M H_2O_2 for the *E. coli* serotypes RWW1i O103:H2, RWW1iii O145:H2 and PSW2ii O96:9 (all from treatment plant wastewater), and *E. coli* PRK2ii O86 (from abattoir wastewater) respectively. Results also showed that while *E. coli* PSW1iii O111:NM, *E. coli* PSW2ii O96:H9, *E. coli* FSE1ii O145:H2, *E. coli* PST1iii O113 lost their HI at 0.3M H_2O_2, strains *E. coli* FSE1iii O86, *E. coli* PST2i O124 (both from abattoir wastewater) and *E. coli* RBU2i (from river water) completely lost their hydrophobicity at all the tested concentrations. Isolates from wastewater sources (RWW, PSW, FSE and PST) retained their HI more than isolates from river water (RBU and PRK). *E. coli* ATCC 25922 (control) which was not subjected to treatment with H_2O_2 retained its hydrophobicity.

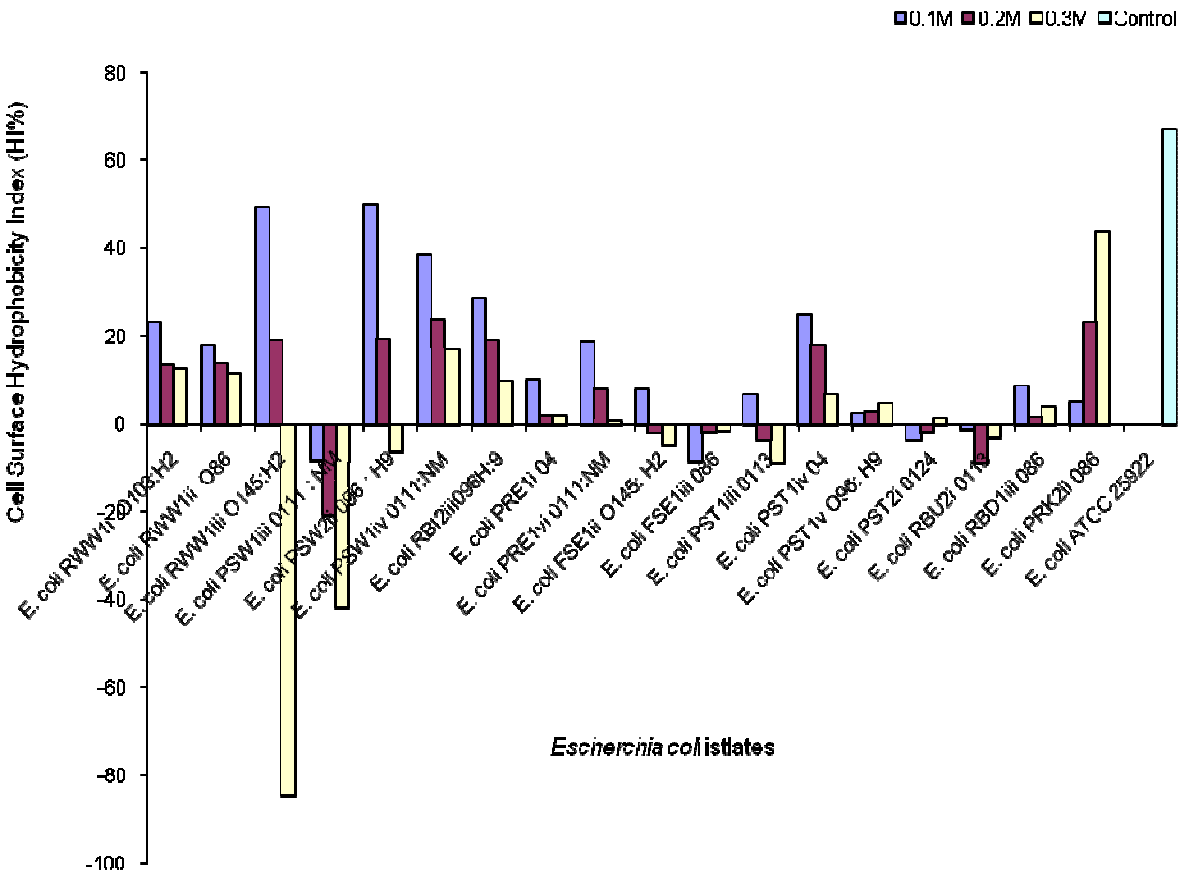

Fig. 7.1. Effect of various concentrations of H_2O_2 on cell surface hydrophobicity (HI) values for temperature stressed *Escherichia coli* serotypes isolated from wastewater and river water sources.

Fig. 7.2 shows the result of effect of oxidative stress on haemolysin production among the *E. coli* serotypes. Out of the 19 isolates, 12(63.16%) retained their potential for haemolysin production after being subjected to stress conditions with HU values ranging between 49.6-74.04%. *E. coli* ATCC 25922 (control) also was positive (HU 61.336%) for haemeolysin.

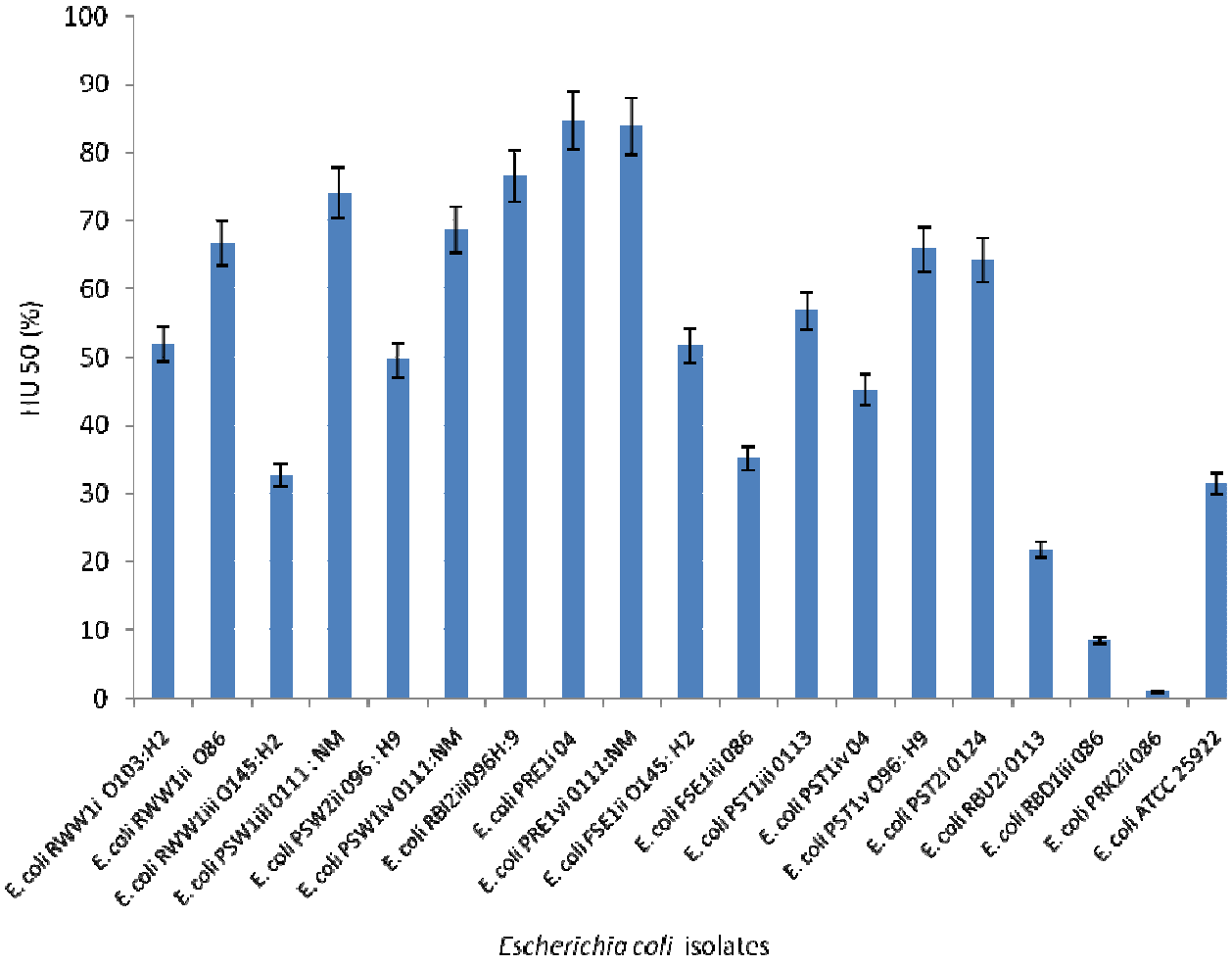

Fig. 7.2. Effect of oxidative stress on haemolysin production of temperature-stressed *Escherichia coli* serotypes isolated from wastewater and river water sources.

For bacterial serum resistance, results showed that while the erythrocytes were lethal to most of the isolates with increase in time of exposure, isolates from wastewater samples were less susceptible compared to those from river and the control (Fig. 7.3). For wastewater isolates, *E. coli* PST1v O96:H9 (from abattoir) was the most resistant with only a very insignificant reduction in the SRI values with time (1.72, 1.69, 1.62 and 1.51% at 0, 3, 6 and 12 h respectively). Isolates from river water showed the least SRI values (1.02, 0.38, 0.05 and -.61% at 0, 3, 6 and 18 h respectively) compared to all other isolates, including the control.

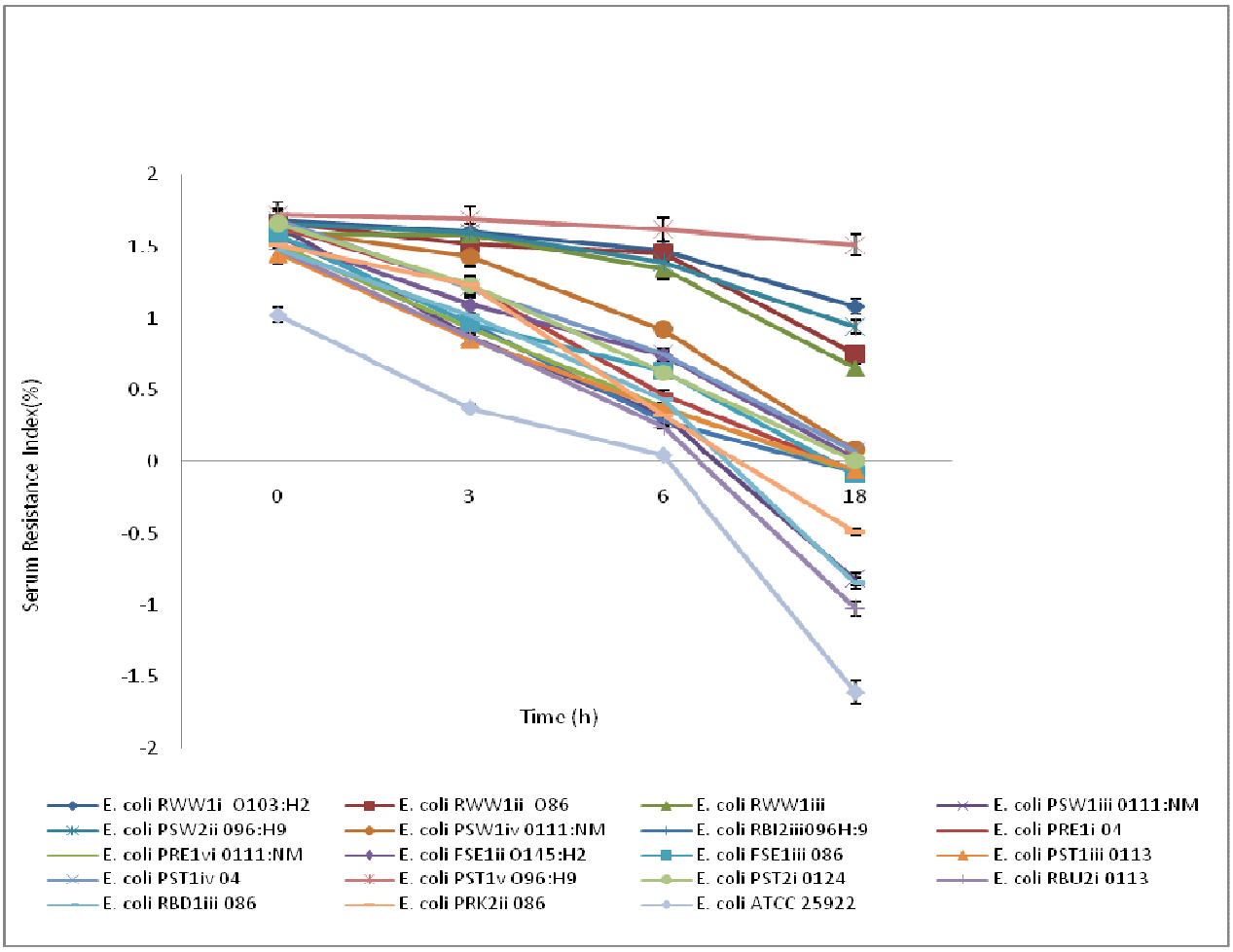

Fig. 7.3. Effect of oxidative stress on serum resistance of H_2O_2 (0.3 M) stressed *Escherichia coli* serotypes isolated from wastewater and river water sources.

Fig. 7.4 (a-c) shows results of viability of *E. coli* serotypes under different low temperature conditions (-5; -18; and -28°C). Generally, results showed that while the growth of temperature-stressed cells begins to decline after incubation for 6 h, those of non-freeze-thawed cells only declined between incubation for 10 to 12 h. For instance, at -5°C, the viable cell indexes of *E. coli* RWW1i O103:H2 (from treatment plant wastewater) increased from 0.36% at 0 h to 0.56% after 8 h incubation, and this value decreased to 0.03% after 12 h of incubation at 37°C. For *E. coli* ATCC 25922 (control), the VCI increased from 0.343 to 0.87% from 0 to 6 h of incubation, and after 12 h the VCI had increased to 0.905% (Fig. 7.4a). A similar trend was observed for all the isolates at the various temperature stress conditions (-18; and -25°C). The VCI values for isolates from river water samples were generally higher than those from wastewater samples and at 12 h incubation, about 50% of all the temperature stressed cells at -28°C recorded negative VCI values, but at this temperature (-28°C) however, the control bacteria significantly ($P \leq 0.05$) recorded the highest VCI value (Fig. 7.4c).

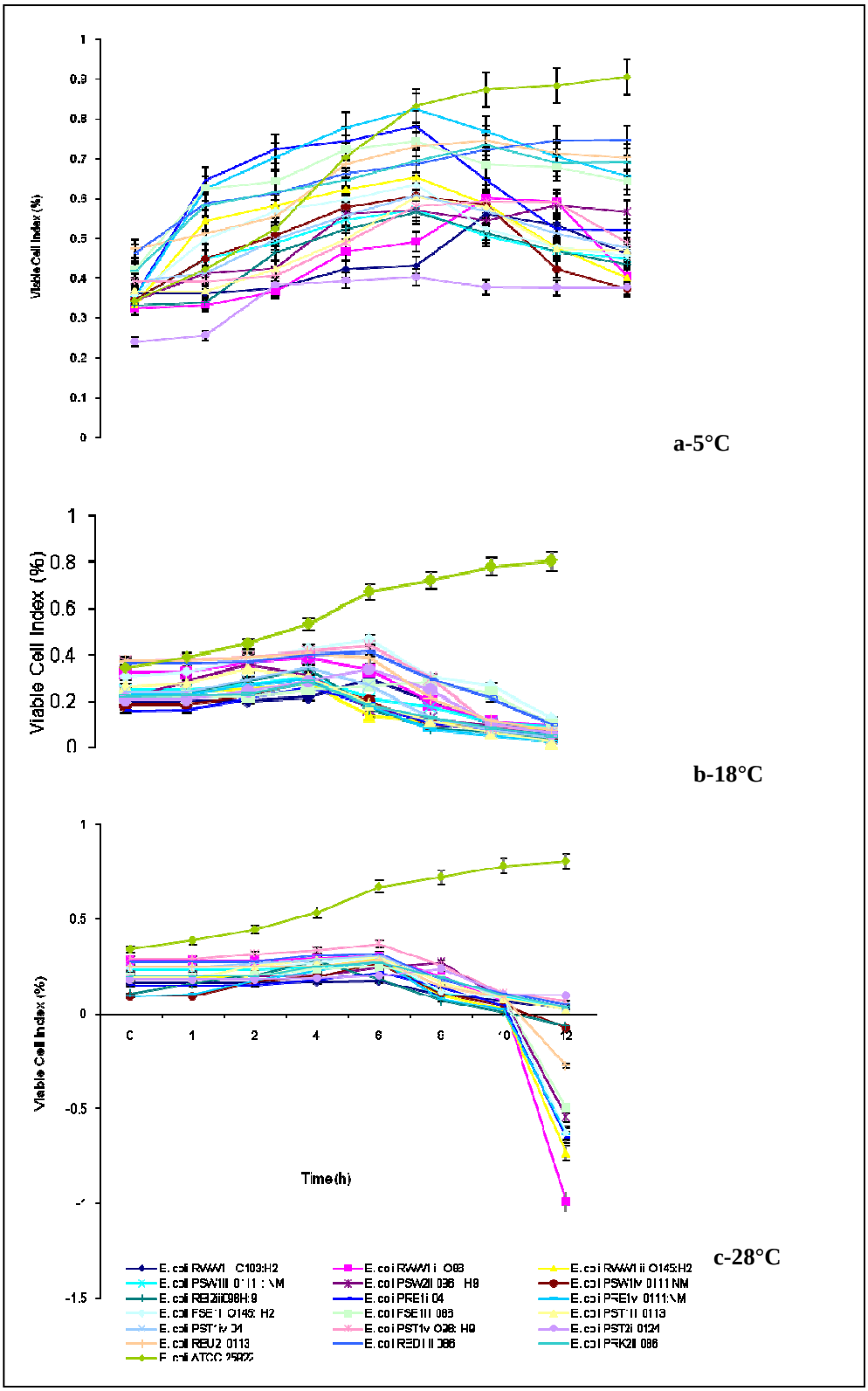

Fig. 4. Effect of temperature stress (a -5°C; b-18°C; c -28°C) on viability of *Escherichia coli* serotypes isolated from wastewater and river water sources.

7.5 DISCUSSION

After exposure to various concentrations of chemicals and sodium chloride, the majority of the isolates retained their surface hydrophobicity, and haemolysin production potential. Most isolates from the abattoir wastewater lost their ability to produce verotoxins but those of water treatment plant and rivers still produced the toxins and the majority of the isolates from wastewater retained high serum resistance. A higher CKI value is an indication of high susceptibility (and vice vasa) to chemicals tested. Resistance among wastewater samples could be as a result of adaptation to a mixture of chemicals, salts and antibiotics of varying concentrations in the wastewaters. Exposure of isolates to harsh conditions provided by higher concentrations of these chemicals might have induced the cells to develop resistance.

The study showed that loss of verotoxin expression occurred more in the non-antibiotic resistant isolates or those that showed resistance to only one antibiotic. Though verotoxin and antibiotic resistance genes are genetically mediated and are often carried within bacterial nuclei, previous studies has not established any association between antibiotic resistance and verotoxin production in bacteria (Aksoy *et al.,* 2007). However, many of the isolates lost the ability to express the Vtx1 gene compared to the Vtx2 gene. Vtx1 and Vtx2 genes are responsible for expression of toxin production in bacteria (European Food Safety Authority, 2007). No difference in the chemical nature of Vtx1 and Vtx2 has been reported and therefore reasons for the difference observed in this study are not immediately discernible. It will be interesting to determine the responses of these genes individually to various physicochemical parameters. At lower temperatures, isolates from abattoir wastewater

completely lost the ability to express the verotoxin genes. A possible explanation is the fact the freeze-thawing provided by the low temperature stress might have compromised the integrity of the various bacterial cell walls and membranes resulting in the alteration of the protoplasmic content, conformational changes and structural damages, consequently affecting their ability to withstand stress. According to Yuk and Marshall (2003), low-temperature bacterial growth decreases heat resistance of cells due to increase in membrane unsaturated fatty acids, which increases membrane fluidity consequently interrupting the selective permeability function of the cell membrane (Yuk and Marshall, 2003). The population of temperature-stressed *E. coli* serotypes in this study was affected by various chemicals and salts with the effect increasing with time of exposure. Susceptibility was as a result of increased permeability in the cell walls of the bacterial cells caused by the freeze thawing process. The increased susceptibility of *Listeria monocytogens* to salt after freezing has earlier been reported (Golden *et al.,* 1988). There was a slight difference in cell viability between the test and control cultures in this study, with the control showing a slightly higher ($p \leq 0.05$) viable cell index (VCI). This is an indication that their cell walls were less permeable since they were not subjected to temperature stress prior to exposure and hence have a reduced absorption rate of the salts or chemicals tested. The various *E. coli* strains responded differently in terms of production of virulent factors and cell viability to the salts of chemicals tested. This is possibly due to differences in physiological adaptability or resistance amongst the bacterial cells. The wastewater samples showed higher serum resistance compared to isolates from water samples. The exposure of this isolates to different

environmental conditions might have induced the test bacteria to develop some degree of resistance to these chemicals.

Sodium chloride is one of the most important food adjuncts used for food preservation. During the handling of food and food ingredients from the farm to table, foodborne bacteria are exposed to different suboptimal physical and chemical environments. This can enable foodborne pathogens and spoilage bacteria, as well as beneficial bacteria, to develop characteristics that are different from those of normal cells. Most foodborne pathogens (especially the enteric pathogens) and spoilage bacteria (especially Gram-negative) are susceptible to low pH and die off rapidly in high-acid foods (pH 4.5) during storage. If they are first acid-adapted, they become relatively resistant to lower pH and other treatments at minimal levels and survive in food. Acid-adapted pathogenic strains surviving low pH and low heat treatment have recently been associated with outbreak of foodbome diseases from the consumption of fruit juices, fermented sausages, and acidified foods containing viable *Salmonella, E. coli* O157:H7, and *L. monocytogenes* (Ray and Bhunia, 2008). Crystal violet and bile salt are commonly used in the selective inhibition of bacteria in selective bacteriological media such as MacConkey Sorbitol agar, while ethanol is used as a common food preservative as well as disinfectant. Resistance of *E. coli* to these chemicals will further pose a challenge to their laboratory cultivation, which will necessitate the search for alternative culture media. Resistance of *E. coli* to disinfectants has been a source of concern in healthcare settings (Guimarães *et al.*, 2000).

Results showed differences in the rate of loss of hydrophobicity among the various serotypes. For instance, *E. coli* O86 from river water (*E. coli* PRK2ii O86) showed higher HI than those from wastewater (*E. coli* RWW1ii O86). While surface hydrophobicity is one of the virulence factors which contributes to the adherence of microorganisms to host tissue (Wojnicz and Jankowski, 2007; Hedge *et al.,* 2009), previous studies have shown correlation between this factor and bacterial adherence to epithelia (Jahnn *et al.,* 1981; Hedge *et al.,* 2009). Strains retaining their surface hydrophobicity in this study might likely adhere more to epithelial cells if faced with similar concentrations of salt.

All the 3 isolates from river water samples lost their ability to produce haemolysins, while serum resistance was highest among the wastewater isolates compared to those from river water and the control isolates. *E. coli* haemolysins are proteins that cause *in vitro* lysis of erythrocytes from several species of animals (Kŏnig *et al.,* 1986). Serum resistance also confers Gram-negative bacteria with the ability to resist the lytic effects of serum and to invade and survive in the human bloodstream. While bacterial haemolysins are found complexed with lipopolysaccharides, serum resistance in *E. coli* is imparted by capsular polysaccharides (CPS) and membrane proteins (La Regione and Woodward, 2002). The relative contributions of these polysaccharides and proteins to the virulence factors remains poorly understood (Cross *et al.,* 1986). The higher serum resistance rate observed among the wastewater samples might not be unconnected with previous exposure to blood cells from the abattoir or treatment plant. The wastewater samples contain mixed wastes from both animal and human excreta, hospital and industrial environments where blood cells and

antibiotics are part of the mixtures. For isolates from wastewater, prior exposure to animal blood (abattoir wastewater) and harsh pH, antibiotics and high salt concentrations (wastewater treatment plant) might have conferred them with adaptive potentials and consequently development of resistance mechanisms against erythrocytes. For instance, it has been reported that exposure of cells for an extended period to mild acidic environment (e.g. pH 5.0-5.8) enables them to develop resistance to subsequent exposure to pH ~ 2.5 (acid resistance of acid adaptation) and a brief exposure of cells to mild acidic environment enables them to survive subsequent exposure to pH 2.4-4.0 developing what is termed acid tolerance or acid tolerance response (ATR) (Ray and Bhunia, 2008). Absence of blood and less concentration of antibiotics in the river water might have posed a less challenging survival task to the bacteria.

Howver, a casual glance at the results showed that the antibiotic resistant (resistant to more than 3 antibiotics) isolates were slightly more tolerant to the various chemicals and salts. It is therefore is possible that antibiotic resistance confers the bacteria with the advantage to withstand other chemical agents. Cooke *et al.* (2010) recently found equal distribution of virulence factors between susceptible and multidrug resistant (MDR) nosocomial and community blood stream *E. coli* isolates, but whether the degree of multidrug resistance is proportional to virulence is yet to be established.

In general, as the temperature of frozen storage increased, the percentage of surviving cells decreased. This could be due to injury to the cells during storage, especially because the cells

were freeze-thawed intermittently. The conformational changes to the bacterial cell wall as a result of the abrupt change in temperature during the freeze-thawing process limits the supply of essential nutrients such as iron, while oxidative stress results from increased levels of superoxide anion and H_2O_2 which leads to oxidative bursts, suboptimal pH conditions, osmotic stress, swelling and bursting of cell in hypotonic environments or in plasmolysis and dehydration in hypertonic conditions (Hedge *et al.,* 2009). Although Shen *et al.* (2010) did not study the effect of low temperature stress; incubation of *V. parhaemolyticus* at -18°C for 15-30 days inactivated the bacteria. The expression of virulent genes in bacteria however, is highly regulated and responds differently to environmental stimuli, such as temperature, pH and nutrient availability (Clarkem *et al.,* 2003; Hedge *et al.,* 2008). This explains the various responses obtained by the various *E. coli* serotypes to the different stress conditions in this study. Furthermore, results showed that the CKI values of cells stored at -5°C were higher than those of -18 and -28°C. This is an indication that *E. coli* are less susceptible at -5°C. Even the control isolate which was not multidrug resistant responded differently with each virulent factor under different oxidative stress conditions.

7.6 CONCLUSION

In this study, retention of virulence by some strains of the *E. coli* serotypes, even after subjection to oxidative stress, is a cause for concern. This is because contamination of food and drinking water with these strains might mean the proliferation of more virulent strains. *E. coli* are associated with various human diseases including urinary tract infections gastroenteritis and severe diarrhea. Diarrhea is of particular concern in developing countries

with over 5 million cases and over 3 million deaths annually. The fact that virulent factors are regulated by virulence genes and their subsequent expression are in response to changes in environmental conditions (Hedge *et al.*, 2008) the search for more effective control measures against diarrheogenic agents should take into consideration the manipulation of these factors.

7.7 ACKNOWLEDGEMENT

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CHAPTER EIGHT

8.0 EFFECT OF OXIDATIVE STRESS ON VIABILITY AND VIRULENCE OF ENVIRONMENTAL *ACINETOBACTER HAEMOLYTICUS* ISOLATES

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8.0 Effect of oxidative stress on viability and virulence of environmental *Acinetobacter haemolyticus* isolates

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8.1 ABSTRACT

The Effect of oxidative stress exerted by 0.03% crystal violet, 0.3% bile salt, 4.0% NaCl and 8% ethanol on the survival and production of virulence factors among *A. haemolyticus* isolates obtained from wastewater and river water samples was investigated. Though generally there was insignificant lethal effect against all the isolates, crystal violet exerted the highest lethal effect followed by ethanol and bile salt, NaCl exerted the least effects compared to crystal violet and ethanol. Isolates from wastewater demonstrated the highest rate of resistance compared to isolates from river water. Presence of resistant verotoxic *A. haemolyticus* in the environments investigated is a cause for concern. Resistance exhibited by the bacteria means that the efficacy of these salts, chemicals and temperature conditions frequently employed as control/preservation agents in hospitals and food industries may be undermined.

Key words: cell viability, hydrophobicity, contamination, food-borne pathogens, oxidative stress, virulence

8.2 INTRODUCTION

The emergence of multidrug resistant nosocomial or community-acquired infections of *Acinetobacter* spp. is a result of high adaptability to adverse environmental conditions, ability to persist in harsh environments (e.g. hospital environment), increased use of broad spectrum antibiotics, vulnerability of individuals or patients, and rapid transformation. Contamination of food and water sources with these bacterial agents results in outbreak of

various forms of infections. *Acinetobacter* spp. has been recovered from vegetables and fruits and has also been implicated in the spoilage of bacon, chicken, meat, fish, eggs and hospital foods. Food safety and public health, has preoccupied many governments, including those of developing countries (FDA, 2001; WHO, 2005). *A. haemolyticus* and other *Acinebacter* spp. have been causative agents of resistant nosocomial infections and costs associated with controlling such infections are staggering (Kurcik-Trajkovska, 2009), forcing some institutions to close entire units as a control measure. The major problem with *Acinetobacter* spp. is their resistance to antibiotics. It has been reported that the organisms are most commonly resistant to ampicillin, cephalothin, carbenicillin, gentamicin, amikacin, chloramphenicol, tetracycline, co-trimoxazole, ciprofloxacin and cefoperazone. Resistance to these antibiotics has hindered therapeutic management, causing growing concern the world over (Doughari *et al.* 2011b). Verotoxins also present treatment challenges. Abong' o and Momba (2009) reported complications arising from antibiotic treatment of verotoxic bacteria. The toxins are released into the medium as the bacterial cells are lysed by the antibiotics causing further health complications such as the bloody diarrhea (Abong' o and Momba, 2009) and sometimes kidney complications. This informed the need to investigate the efficacy of various salts, chemicals and temperature conditions frequently employed as control agents in hospitals and food industries with a view to developing more effective control measures. Thus the study reports the effect of oxidative stress exerted by low temperature stress, crystal violet, bile salt, and sodium chloride on some virulence factors of environmental isolates of *A. haemolyticus.*

8.3 MATERIALS AND METHODS

8.3.1 Source of bacterial strains and blood sample

From Athlone wastewater Treatment Plant): *A. haemolyticus* RWW1v, *A. haemolyticus* PSW2i, *A. haemolyticus* PSW2ii and *A. haemolyticus* FEW2iv. From Winelands Pork abattoir wastewater: *A. haemolyticus* FSE1iv, *A. haemolyticus* FSE1v, *A. haemolyticus* PST1i, *A. haemolyticus* PST2i and *A. haemolyticus* PST2ii and from River Berg: *A. haemolyticus* RBD1i, *A. haemolyticus* RBD1ii, *A. haemolyticus* RBD1iii, *A. haemolyticus* RBI1i and *A. haemolyticus* RBI2i (from River Berg) were used for this study. All sample sites were located in Cape Town South Africa.

8.3.2 Confirmation of isolates

Before use, the bacteria were reconfirmed by culture on Eosin methylene blue (EMB, Oxoid SA) and Leeds Acinetobacter medium (LAM, Hardy Diagnostics USA) and characterized using biochemical reagents and Rapid NF plus identification kits (Merck, SA) (Table 1) and confirmed (Doughari *et al.*, 2011a) as positive for verotoxins, cell surface hydrophobicity, serum resistance and haemolysin production using standard methods (Chou and Cheng, 2000; Hedge *et al.*, 2009). The cultures were maintained on trypton soy agar slants (TSAS, Oxoid, SA) in the Microbiology Laboratory, Department of Biotechnology, Faculty of Applied Sciences Cape Peninsula University of Technology, Cape Town South Africa. To screen for virulence factors, bacteria were subcultured on chemically defined medium (CDM) as described by Hedge *et al.* (2009). The non pathogenic strain *A. haemolyticus* 19002 (static culture) was used as control. For each experiment, bacteria were sub-cultured

onto trypton soy agar (TSA, Oxoid, SA) and incubated at 37ºC for 18 h before use. Sheep blood erythrocyte was purchased fresh from the National Research Council (NRC, Cape Town, South Africa). The blood was used without further treatment as a source of serum complement.

8.3.3 Antimicrobial susceptibility testing

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), ciprofloxacin (5 µg) (Oxoid UK) (WHO 2002) using the disc diffusion method (Aksoy *et al.*, 2007). All antibiotics were obtained from Quantum Biotechnologies, South Africa.

8.3.4 Effect of stress on bacterial viability and virulence

8.3.4.1 Effect of oxidative stress on surface hydrophobicity of bacterial cells

Bacterial strains from TSA were sub-cultured twice in shake flasks with CDM (Hedge *et al.*, 2009) and each time incubated at 37ºC for 24 h. After the final subculture, the cells were washed thrice in sterile physiological saline (0.85% w/v NaCl) and finally suspended in saline to get a solution of OD_{600} 0.1 (0.5 McFarland turbidity standard). CDM (10 ml) containing various concentrations (0.1, 0.2 and 0.3 M) of H_2O_2 were dispensed aseptically in three sets of 100 ml Erlenmeyer flasks. The bacterial suspensions were inoculated into each of the flasks to obtain cell concentration of approximately 10^6 cells/ml. A flask containing *A.*

haemolyticus ATCC 19002 suspended in CDM without H_2O_2 was used as control. The flasks were then incubated at 37ºC in rotary water bath at 160 rpm for 24 h, centrifuged at 3000 rpm/10 min and retained packed cells were washed with sterile phosphate buffered saline (PBS, 10 ml) then suspended in PBS to get a density of 0.3 at OD_{600} (OD Initial). To this bacterial suspension (3 ml), *p*-xylene 0.3 ml, was added and vortexed for 1 min, left for 30 min at ambient conditions and final OD_{600} (OD Final) determined. Degree of hydrophobicity retention was calculated as percent hydrophobicity index (HI) (Equation 1).

$$HI = \frac{OD\ I - OD\ F}{OD\ I} x100$$

Equation 1. HI = hydrophobicity index, ODI = initial optical density, ODF = final optical density.

8. 3.4.2 Effect of oxidative stress on bacterial haemolysin production

This was carried out using the quantitative α-haemolysin assay. The H_2O_2 treated (0.3 M) bacterial suspension earlier grown in the Erlenmeyer flasks (10 ml), was transferred into sets of tubes and centrifuged at 3000 rpm for 10 min. The supernatant was collected and diluted in 0.8% calcium chloride solution (10 ml), 1 ml withdrawn and mixed with 1% (v/v) sheep erythrocyte suspension and incubated at 37ºC for 1 h. After the incubation, 2 ml of 0.8% NaCl saline was added to each tube exhibiting partial haemolysis and the bacterial suspensions centrifuged at 1500 rpm for 10 min to pellet the unlysed erythrocytes. The supernatant fluid was separated and the OD_{540} determined. Fifty percent (50%) haemolysis standard prepared by mixing 1 ml of 1% (v/v) sheep erythrocyte suspension and 3 ml of

diluent was used as control. Inverse of the dilution which caused 50% lysis was recorded as HU 50 (50% haemolysis units) (Hedge *et al.,* 2009). A tube containing non-H_2O_2 treated bacterial suspension (*A. haemolyticus* ATCC 19002) suspended in CDM was used as control (Equation 2).

$$\text{HU } 50 = \frac{\text{ODs - ODt}}{\text{ODs}} x100$$

Equation 2. OD_s = 50% haemolysis standard, OD_{ex} = final haemolysis measured, HU 50 = 50% haemeolysis

8. 3.4.3 Effect of oxidative stress on bacterial serum resistance

The effect of oxidative stress on bacterial serum resistance was determined using the quantitative serum bactericidal assay (Hughes *et al.,* 1982). H_2O_2 treated (0.3 M) bacterial suspension (0.5 ml) was mixed with 1.5 ml of fresh undiluted serum (from blood) and incubated at 37ºC. Cell viability was determined turbidiometrically at 600 nm after 3, 6 and 18 h. Serum resistance index (SRI) of bacteria was calculated (Equation 3).

$$\text{SRI } (\%) = \frac{\text{ODI - ODF}}{\text{ODI}} x100$$

Equation 3. SRI = serum resistance index, ODI = initial turbidiometric reading, ODF = Final turbidiometric reading. A tube containing non-H_2O_2 treated bacterial suspension (*A. haemolyticus* ATCC 19002) suspended in CDM was used as control.

8.3.5 Effect of low temperature stress

A 1 ml aliquot of bacterial suspension (initial density 10^8 CFU/ml) grown at 37ºC was inoculated into 9 ml trypton soy broth (TSB) in two different sets of test tubes, thoroughly mixed and then stored at -5, -18 and -28ºC for 21 days. The tubes were removed from the freezers after every 5 days, and thawed under running tab water for 5 min then returned to the freezer. After 21 days, the cultures were removed and 2-3 loopfuls inoculated into TSB, incubated for 18 h at 37ºC and the viable cell index (VCI) determined by taking the OD_{600} values first at 0 min (OD Initial) then at 10 min interval for 1 h (Chou and Cheng, 2000) and VCI on the scale of 100% calculated as follows (Equation 4):

$$VCI = \frac{OD\ I - OD\ F}{OD\ I} x100$$

To confirm that viable bacteria were in a culturable state, viable counts were made by making serial dilutions of 2-3 loopfuls of bacterial culture in 10 ml TSB and surface spread by inoculating onto NA plates, incubating at 37°C and determining percentage survival after 18 h. Bacterial suspension containing non-H_2O_2 treated and non-freeze-thawed bacterial (*A. haemolyticus* ATCC 19002) suspension in CDM was used as control.

8.3.6 Effect of ionic salt concentrations and other chemicals on cell viability and verotoxin production

Low-temperature stressed culture suspension (0.2 ml) was inoculated into solution (10 ml) containing either 0.03% crystal violet, 0.3% bile salt, 4% NaCl, or 8% ethanol and incubated at ambient conditions for 1 h. Bacterial culture (*A. haemolyticus* ATCC 19002) not subjected

to H_2O_2 stress, low temperature storage, or alcohol and salts was used as control. After incubation, each of the samples was serially diluted in Butterfield's phosphate diluents (USFDA, 2001) (appendix iv) and 0.1 ml surface plated on TSA and incubated at 37ºC for 10 (initial) and 30 min (final) after which, the percent cell kill index (CKI, %) was determined (Chou and Cheng, 2000) (Equation 5):

$$\text{CKI } \% = \frac{\text{Initial Population - Final Population}}{\text{Initial Population}} x100$$

For effect of H_2O_2 (0.3 M) stress and temperature freeze thawing on verotoxin production, the Duoperth kit (Merck, SA) antibody-based rapid slide agglutination assay was employed according to the manufacturer's instructions. The H_2O_2 stressed or freeze thawed bacterial isolates from three different sets of TSA (-5, -18, and -28ºC isolates) were first cultured in 1 ml casaminoacid yeast extract (CAYE) broth and incubated at 37ºC at 100 rpm for 24 h. After incubation, 10 µl of the broth culture (approximately 1 x 10^7 cells/ml) was inoculated into fresh CAYE broth and further incubated for 16 h at 100 rpm at 37ºC, then centrifuged at 5000 rpm/5 min, cell pellets retained, washed thrice with phosphate buffered saline (PBS, 5 ml), then suspended in 0. 25 ml 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 incubated at 37ºC for 30 min. Culture suspension (200 µl) was then transferred onto the test device using a sterile Pasteur pipette and result read after 10 min. The appearance of red bands on the vtx1 or vtx2 bands denoted the presence of either one of or both verotoxins.

8.4 STATISTICAL ANALYSIS

Triplicates of values obtained were recorded as ±SEM using the SIGMAPLOT 8.0 statistical software at $p \leq 0.05$.

8.5 RESULTS

8.5.1 Confirmation of isolates

The isolates were Gram-negative coccobacilli, tiny, blue, mucoid colonies on EMB, pink colonies diffused into the medium on LAM and all were haemolytic on sheep blood agar. All fermented triglyceride (EST), glucose (Gluc), citrate (Cit), arginine (ADH), urea (URE), tryptophane (IND) and produced nitrates (NO_3). They were oxidase (O) negative, sulphide negative and indole positive (Table 8.1 footnotes).

8.5.2 Effect of ionic salt concentrations and other chemicals on bacterial viability and production of virulence factors

Results showed that majority of the isolates did not show any significant ($p>0.05$) CKI values except for one isolate from treatment plant waste water; *A. haemolyticus* FEW2iv (CKI 42.86±0.032% against 8% ethanol) one isolate from abattoir wastewater; *A. haemolyticus* FSE1iv (CKI 53.85±0.001% against 0.03% crystal violet), and two isolates from river Berg; *A. haemolyticus* RBD1i, and *A. haemolyticus* RBI1i with respective CKI values of 56.06±0.052, and 62.86±0.005 against 0.03% crystal violet. *A. haemolyticus* RBI2i also showed a significant ($p>0.05$) CKI value (42.25±0.000) against 8% ethanol All the control isolates (*A. haemolyticus* 19002) showed significant ($p>0.05$) CKI values of

34.75±0.022 (against 0.03% crystal violet), 34.88±0.000 against 8% ethanol, 43.13±0.041 (against 4% NaCl) and 57.69±0.009% (against 0.3% bile salt) (Table 1).

Table 8.1. Cell Kill Index (CKI) (%) of *A. haemolyticus* from wastewater and river water samples

[#]*Acinetobacter* isolates	Cell Kill Index (CKI) (%)			
	Crystal violet (0.03%)	Bile salt (0.3%	NaCl (4%)	Ethanol (8%)
Treatment plant wastewater				
A. haemolyticus RWW1v	5.77±0.023	9.80±0.000	2.44±0.000	19.05±0.012
A. haemolyticus PSW2i	2.02±0.000	1.33±0.000	15.00±0.000	9.80±0.121
A. haemolyticus PSW2ii	3.85±0.031	11.1±0.017	8.37±0.000	12.7±0.301
A. haemolyticus FEW2iv	4.56±0.011	7.96±0.044	4.76±0.000	42.86±0.032*
Abattoir wastewater				
A. haemolyticus FSE1iv	53.85±0.001*	7.88±0.037	16.67±0.000	8.00±0.000
A. haemolyticus FSE1v	33.02±0.036	7.14±0.033	6.90±0.000	2.40±0.000
A. haemolyticus PST1i	3.23±0.001	3.70±0.000	23.33±0.034	8.62±0.130
A. haemolyticus PST2i	7.00±0.000	7.00±0.000	2.44±0.027	6.76±0.068
A. haemolyticus PST2ii	31.67±0.021	6.90±0.000	8.89±0.026	10.00±0.032
River Berg water				
A. haemolyticus RBD1i	56.06±0.052*	9.76±0.071	6.67±0.0022	5.33±0.111
A. haemolyticus RBD1ii	30.60±0.000	10.83±0.034	8.50±0.000	6.31±0.023
A. haemolyticus RBD1iii	10.64±0.010	12.50±0.000	6.00±0.000	15.91±0.047
A. haemolyticus RBI1i	62.86±0.005*	15.32±0.005	3.23±0.038	11.04±0.000
A. haemolyticus RBI2i	20.00±0.000	17.86±0.016	27.27±0.021	42.25±0.000*
Control				
A. haemolyticus 19002	34.75±0.022*	57.69±0.009*	43.13±0.041*	34.88±0.000*

[#]Isolates were non motile, negative for sulphide production, oxidase reaction and arginine fermentation but positive for indole production, and fermented triglyceride, glucose, citrate, urea, tryptophane, as well as oxidation of sodium nitrate; Gram-negative coccobacilli and tiny, blue, mucoid colonies on Eosin Methylene Blue (EMB); Pink colonies diffused into the medium on Leeds Acinetobacter Medium (LAM) and all were haemolytic on Sheep blood agar.*significant at (P>0.05)

8.5.3 Antimicrobial susceptibility and effect of low temperature stress on verotoxin production and bacterial viability

Results showed that most of the isolates from waste water sources were resistant to between 3-5 antibiotics including tetracycline (TE), gentamicin (CN), cefuroxime (CXM), ampicillin (AMP), nalidixic acid (NA), and ofloxacin (OFX) (Table 2).

Low temperature treatments (-5, -18 and -28°C) did not have any significant (P>0.05) effect on either verotoxin production. Results also showed that the low temperature freeze-thaw stress conditions applied had no effect on the viability of the isolates as there was no significant differences in the initial cell population (ICP) and the final cell population (FCP) with the waste water samples consistently maintaining significantly (P>0.05) higher population of viable cells compared to the river water samples (Table 8.2).

7709 Table 8.2 Efect of low temperature stress on verotoxin production among environmental *A. haemolyticus* isolates

Acinetobacter isolates	Antibiotic resistance patte	Temperature (°C) stress treatment/ verotoxin status and viability											
		37 (non treated)			-5			-18			-28		
		Vtx1	Vtx2	ICP/ml	Vtx1	Vtx2	FCP/ml	Vtx1	Vtx2	FCP/ml	Vtx1	Vtx2	FCP/ml
Treatment plant wastewater													
A. haemolyticus RWW1v	TE, CN, CXM, AMP	-	-	5200.00	-	-	5200.00	-	-	5200.00	-	-	5100.00
A. haemolyticus PSW2i	AK, CXM,	+	-	6300.00	+	-	6300.00	+	-	6200.00	+	-	6200.00
A. haemolyticus PSW2ii	OFX, NA, AMP,TE	-	-	5200.00	-	-	5200.00	-	-	5000.00	-	-	5000.00
A. haemolyticus FEW2iv	AML	-	-	4820.00	-	-	4820.00	-	-	4700.00	-	-	4700.00
Abattoir wastewater													
A. haemolyticus FSE1iv	-	+	+	2600.00	+	+	600.00	+	+	2400.00	+	+	2600.00
A. haemolyticus FSE1v	AML, NA	-	+	5300.00	-	+	5300.00	-	+	5100.00	-	+	5300.00
A. haemolyticus PST1i	AMP, CN, OFX,NA,CN	+	-	6200.00	+	-	6200.00	+	-	6120.00	+	-	6000.00
A. haemolyticus PST2i	TE, CL	-	-	4000.00	-	-	4000.00	-	-	3820.00	-	-	4600.00
A. haemolyticus PST2ii	-	-	-	6000.00	-	-	6000.00	-	-	6000.00	-	-	5820.00
River Bergwater.	-												
*A. haemolyticus*RBD1i	-	+	-	3300.00	+	-	3300.00	+	-	3300.00	+	-	3100.00
*A. haemolyticus*RBD1ii	-	-	-	6340.00	-	-	6340.00	-	-	6240.00	-	-	6200.00
*A. haemolyticus*RBD1iii	OFX, NA	-	-	4700.00	-	-	4700.00	-	-	4500.00	-	-	4400.00
*A. haemolyticus*RBI1i	-	-	-	7000.00	-	-	7000.00	-	-	6700.00	-	-	7800.00
A. haemolyticus RBI2i		-	-	2000.00	-	-	2000.00	-	-	1800.00	-	-	2820.00
Control	TE												
A. haemolyticus 19002		-	-	4000.00	-	-	4000.00	-	-	3860.00	-	-	3800.00

7710 SXT-sulphomethaxazole/trimethoprim; AMP-ampicillin; ATM-aztreonam; TE-tetracycline; AK-amikacin; OFX-ofloxacin; CAZ-ceftazidim; CL-cephalexin;
7711 CRO-ceftriazone; CXM-cefuroxime; AML-amoxycillin; CN-gentamicin; CFM-cefixime; CIP-ciprofloxacin; NA-nalidixic acid) +(positive); - (negative); ICP-
7712 Inicial cell population; FCP-final cell population.

8.5.4 Effect of oxidative stress on surface hydrophobicity, haemolysin production and serum resistance of bacterial isolates

Results (Table 3) showed that at 0.3M H_2O_2 the least HI value (0.009±21,) was exhibited by *A. haemolyticus* PST2ii (from abattoir wastewater) and the highest value (0.789±31) was exhibited by *A. haemolyticus* PSW2ii (from Athlone Treatment Plant wastewater). At 0.1M and 0.3 M H_2O_2, the respective HI values of 0.651±12 and 0.526±25 for *A. haemolyticus* RWW1v (from Athlone wastewater) was recorded compared to 0.172±00 (0.1M H_2O_2) and 0.023±37% (0.3 M H_2O_2) of *A. haemolyticus* RBI2i (from river water). The highest SRI values of 85.23±23, 76.42±67 and 73.36±27% were recorded for *A. haemolyticus* RWW1v, *A. haemolyticus* PSW2i and *A. haemolyticus* FSE1iv from treatment plant and abattoir waste waters compared to 67.60±01% for *A. haemolyticus* RBD1i from river Berg. Similarly for HU 50%, *A. haemolyticus* PSW2i and *A. haemolyticus* PST2i from waste water and abattoir water recorded the highest values of 83.21±13 and 78.45±31% respectively compared to the highest value of 58.12±01% for *A. haemolyticus* RBD1iii from river Berg. The control isolate *A. haemolyticus* 19002 consistently showed the least values for HI, SRI, HU and CVI (Table 8.3). Results showed that the wastewater isolates were less affected by H_2O_2, sheep blood erythrocytes/serum compared to river water isolates.

Table 8.3. Haemolysin unit (HU), Serum resistance index (SRI), and effect of hydrogen peroxide cell surface hydrophobicity (HI) and low temperature stress on *A. haemolyticus*

A. haemolyticus isolates	Hydrophobicity index (HI, %) /Mc concentrations (M) of H_2O_2			SRI (%)	HU 50%
	0.1M	0.2M	0.3M		
Treatment plant wastewater					
A. haemolyticus RWW1v	0.651±12	0.633±10	0.526±25	85.23±23	67.23±43
A. haemolyticus PSW2i	0.833±00	0.796±02	0.788±00	76.42±67	83.21±13
A. haemolyticus PSW2ii	0.906±04	0.183±00	0.789±31	43.76±23	65.21±00
A. haemolyticus FEW2iv	0.753.00	0.602.23	0.585.00	19.96±00	23.44±00
Abattoir wastewater					
A. haemolyticus FSE1iv	0.418±11	0.306±00	0.204±00	73.36±27	57.68±06
A. haemolyticus FSE1v	0.511±00	0.504±31	0.489±23	58.48±11	64.51±04
A. haemolyticus PST1i	0.669±23	0.632±01	0.602±43	68.66±34	45.87±11
A. haemolyticus PST2i	0.774±41	0.731±00	0.625±01	34.74±00	78.45±31
A. haemolyticus PST2ii	0.136±03	0.003±00	0.009±21	48.78±00	22.67±22
River Berg water					
*A. haemolyticus*RBD1i	0.462±32	0.372±12	0.152±00	67.60±01	24.66±00
*A. haemolyticus*RBD1ii	0.227±25	0.172±14	0.031±00	48.46±34	38.33±00
*A. haemolyticus*RBD1iii	0.127±23	0.087±01	0.067±23	24.98±18	58.12±01
*A. haemolyticus*RBI1i	0.439±22	0.282±00	0.131±21	33.47±00	26.87±21
A. haemolyticus RBI2i	0.172±00	0.08±15	0.023±37	48.36±00	12.56±34
Control					
A. haemolyticus 19002	0.472.±00	0.183±13	0.114±00	22.56±09	10.83±22

8.6 DISCUSSION

Cell kill index (CKI) values give the degree of lethality of chemical agents and salts against the bacteria tested in this study; high CKI values indicate susceptibility, low CKI values indicates resistance to the chemicals tested. Though generally there was insignificant lethal effect against all the isolates, crystal violet exerted the highest lethal effect followed by ethanol and bile salt, NaCl exerted the least effects compared to crystal violet and ethanol. Also, wastewater isolates demonstrated significantly the highest rate of resistance (low CKI values, $P>0.05$) compared to river water isolates. The demonstration of less sensitivity by *A. haemolyticus* to various stress conditions indicates potential to survive or rapidly adapt to harsh environmental conditions and chemical agents. This is the first report of presence of and resistance (to antibiotics and oxidative stress) among environmental isolates of *A. haemolyticus* in South Africa. Yuk and Marshall, (2003) reported that stress due to change in salts or chemical concentrations and freeze-thawing can compromise the integrity of bacterial cell walls and membranes resulting in the alteration of the protoplasmic content, conformational changes and structural damages. However, absence of any significant change in the cell population and physiological functions of bacteria in this study indicates minimal or absence of damage on their cell walls. Sodium chloride is one of the most important food adjuncts used for food preservation.

Resistance to antibiotics among bacterial pathogens especially multidrug resistant nosocomial infections among *Acinetobacter* spp. is of current global concern (Lee*et al.* 2007). Savov *et al.* (2002) reported resistance among *A. baumanni, A. lwoffii, A. junii* and *A.*

johnsonii to various antibiotics including ampicillin, cephalothin, carbenicillin, gentamicin, amikacin, chloramphenicol, tetracycline, co-trimoxazole, ciprofloxacin and cefoperazone. In this study, *A. haemolyticus* demonstrated the potential to be multidrug resistant.

Verotoxin production among *A. haemolyticus* isolates was first reported by Grotiuz *et al.* (2006). This is the first report on verotoxic *A. haemolyticus* isolates in South Africa and to our knowledge there is no report on effect of stress on these bacteria. In this study, low temperature stress had no effect on both bacterial population and verotoxin production. There was no significant difference between the initial viability index of isolates at -5°C compared to the final viability at -28°C, an indication of little or no effect of freeze-thaw temperatures on the bacterial cell population. During the handling of food and food ingredients from the farm to table, food-borne bacteria are exposed to different suboptimal physical and chemical environments including low temperature storage. Resistance to oxidative stress, antibiotics or suboptimal temperatures as demonstrated in this study might lead chemotherapeutic challenges in situations where the bacteria become sources of outbreaks (Ray and Bhunia, 2008). Resistance demonstrated by verotoxin producing *A. haemolyticus* in this study is worrisome because of the potential dangers associated with verotoxic resistant bacteria especially in environments with poor sanitation and inadequate potable water such as obtainable in developing countries. Outbreak of infections associated with this class of bacteria will have very serious health implications.

While surface hydrophobicity is one of the virulence factors which contribute to the adherence of microorganisms to host tissue (Wojnicz and Jankowski, 2007; Hedge *et al.*, 2009), previous studies have shown correlation between this factor and bacterial adherence to epithelia (Jahnn *et al.* 1981; Hedge *et al.*, 2009). The retention of surface hydrophobicity - demonstrated by low hydrophobicity index (HI%) values - by most isolates from this study means that the isolates are likely to adhere more to epithelial cells even if challenged with similar concentrations of salts or chemicals. Serum resistance on the other hand confers Gram-negative bacteria with the ability to resist the lytic effects of serum and to invade and survive in the human bloodstream (La Regione and Woodward, 2002). Surface hydrophobicity and serum resistance is mediated by cell surface polysaccharides and proteins respectively whose specific roles remains poorly understood (Cross *et al.*, 1986). In this study, isolates demonstrated high SRI and HU 50% values, an indication of the potential to resist host defense mechanisms.

The control cultures showed low HU 50% and SRI values indicating that they were more susceptible to stress than the environmental isolates. This might be due to the fact that the control isolates were not exposed to similar physiochemical conditions as the isolates from the wastewater or river waters investigated. Control isolates however also demonstrated low HI values and indication that they also have surface adherence potentials.

8.7 CONCLUSION

A. haemolyticus in this study exhibited resistance to oxidative stress conditions and some antibiotics as well as freeze-thawing. Contamination of food and drinking water with these strains might mean the proliferation of more virulent and resistant nosocomial infections including severe bloody diarrhea. Diarrhea is of particular concern to developing countries with over 5 million cases and over 3 million deaths annually. More effective environmental monitoring and risk assessment studies of environmental and hospital wastes and survey of resistant strains of these bacteria should be considered. .

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CHAPTER NINE

9.0 ANTIOXIDANT, ANTIMICROBIAL AND ANTIVEROTOXIC POTENTIALS OF EXTRACTS OF *CURTISIA DENTATA*

Under review with the Journal of Ethnopharmacology

9.0 Antioxidant, antimicrobial and antiverotoxic potentials of extracts of *Curtisia dentata*

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9.1 ABSTRACT

The potential of *Curtisia dentata* as antimicrobial, antioxidant and antiverotoxin against environmental isolates of *E. coli* and *Acinetobacter* spp. as well as the presence of phytochemicals and some organic compounds, was determined. Phytochemical analysis using standard methods revealed the presence of anthraquinones, alkaloids, essential oils, glycosides, phenols, steroids, saponins and tannins and the organic compounds quinones, anthocyanins, amines and carboxylic acids. Extracts demonstrated high antimicrobial activity and low minimum inhibitory concentrations as well as inhibitory action against the expression of both Vtx1 and Vtx2 genes in *E. coli*, *A. haemolyticus* and *A. lwoffii*. Ethanol root bark extracts consistently showed the highest DPPH radical scavenging activity (62.43%), total phenol content (TPH) (57.62 26 mg GAE/g) and reducing power (RP) (41.32%), followed by those of the stem bark and leaf extracts with the respective values of 54.68%, 37.77 mg GAE/g and 21.83%. The extracts induced the leakage of Na^+ and K^+ ions from both test bacteria. *C. dentata* can be used to source novel antimicrobial agents for the treatment of verotoxic bacterial infections. *C. dentata* is a very effective source of antioxidant and a possible alternative to sourcing antiverotoxic antibiotics with novel mechanism of action.

Key words: DPPH radical scavenging activity, antioxidant, relative zone diameter of inhibition, organic compounds.

9.2 INTRODUCTION

Antioxidant supplements are vital to combat oxidative damage by free radicals in many oxidative stress-mediated disease conditions such as cancer, atherosclerosis, diabetes, inflammation and aging. Recently, natural antioxidants are in high demand for application as nutraceuticals and as food additives (Tawaha *et al.,* 2007; Jayasri *et al.,* 2009; Kalim *et al.,* 2010). Exertion of oxidative stress on human cells by free radicals which seek stability through electronpairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells cause protein and DNA damage along with lipid peroxidation resulting in pathological processes (Niki *et al.,* 1994; Maxwell 1995; Braca *et al.,* 2002; Hazra *et al.,* 2008). While plants serve as rich, natural, and safer sources of antimicrobials, the rapid incidences of increased resistance to available antibiotics worldwide have turned the attention of researchers and the pharmaceutical industries to plants in search of viable alternatives. Recent outbreaks due to verotoxic bacteria (Eaton *et al.* 2008; CDC, 2011) and further complications arising from the use of antibiotics in the chemotherapy of verotoxic infections calls for more investigations into alternative, more effective agents (Doughari *et al.,* 2009).

Curtisia dentata (Cornaceae or dogwood family) or assegai (English common name) is a traditional medicinal plant that has been employed in the treatment of diarrhoea and related stomach ailments in South Africa (Notten, 2004). In South Africa and other parts of Southern Africa, the common names include: assegaai (Afrikaans), uSirayi, umGxina (Xhosa), umLahleni (Xhosa, Zulu), uMagunda, uMaginda, umBese, umPhephelelangeni (Zulu), iliNcayi, isiNwati (Stwanee), modula-tshwene (Northern Sotho), musangwe,

mufhefhera (Venda) and modula-shtwene (Pede) (Notten, 2004; Shai *et al,*. 2008). Of the 15 plant genera found in the Cornaceae family, only the *Curtisia* genera are found in Africa (Shai *et al.,* 2008).

Traditionally, the plant concoction is used as an aphrodisiac, a blood purifier and for treatment of heart-water in cattle, various stomach ailments, pimples and diarrhea (Pujol, 2000; Dold and Cocks 2001; Shai *et al.,* 2008). The ethanol and aqueous extracts of the plant have been reported to exhibit antibacterial activity against *Bacillus subtilis, E. coli, S. aureus, P. aerugenosa E. faecalis* as well as *C. albicans* (McGaw *et al,*. 2000; Shai *et al.,* 2009). Shai *et al.* (2008) also reported its inhibition of motility in some parasitic and free living nematodes. Despite the medicinal potentials of *C. dentata,* there is paucity of reports of phytochemical, pharmacological and biological investigations of the plant. This study reports on the antioxidant potential of the roots, stem bark and leaves, and the antimicrobial and antiverotoxic potentials of stem bark extracts of *C. dentata* against *E. coli* and *Acinetobacter* spp.

9.3 MATERIALS AND METHODS

9.3.1 Collection and processing of plant sample

C. dentata was donated by Prof. Charles Laubscher of the Horticulture Department, Faculty of Applied Sciences of the Cape Peninsula University of Technology, Cape Town, South Africa. The fresh parts (stem bark, leaves and roots) were dried to a constant weight in the oven at 45ºC for 24-48 h, grated and reduced to powder and then stored in amber-coloured

bottles at ambient conditions until use (Doughari and Obidah, 2008). For this work, all three plant parts were used for the determination of antioxidant activity, total phenolic content as well as reducing power, while only stem bark extract was used in the determination of antiverotoxic and antimicrobial activity.

9.3.2 Extraction and determination of phytoconstituents

Plant parts were exhaustively extracted by sonicating 5 g ground plant parts for 30 min in 200 ml of solvent (dichloromethane, hexane, acetone and ethanol in this order), alongside aqueous extraction using distilled water followed by filtration; this procedure was repeated three times per extractant by replacing the solvent after each extraction. The filtrates from any one solvent was combined, and dried under vacuum at 25ºC and percentage yield of the extracts obtained [hexane (42.68%w/w) dichloromethane (18.73%w/w), acetone (22.64%w/w) ethanol (38.72%w/w) and water extracts (58.82%w/w)] used to screen for the presence of phytoconstiuents (Doughari and Ioryue, 2009) and some organic compounds.

9.3.2.1 Test for saponins

Two grammes (2 g) of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. To the filtered sample (10 ml), distilled water (5 ml) was added, shaken vigorously and observed for a stable persistent frothing. The frothing suspension was mixed with 3 drops of olive oil and shaken vigorously and observed for the formation of emulsion.

9.3.2.2 Test for Tannins and phenolics

Dried powdered sample (0.5 g) was boiled in water (20 ml) in a test tube and then filtered. 1 ml of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

9.3.2.3 Test for alkaloids

Aqueous extracts (1 ml) was mixed with picric acid solution (2 ml) in a test tube and observed for the formation of orange coloration.

9.3.2.4 Test for glycosides

To coarse plant material (1 g) 5 ml each of dil. H_2SO_4 or water was added in two sets of beakers, heated for 3 min and filtered. To the filtrates, 1 ml of $NaOH_{(aq)}$ was added, heated with 5 ml of Fehling's solution for 3 min and observed for the appearance of a reddish-brown precipitate.

9.3.2.5 Test for anthraquinones

Powdered plant (3 g) was soaked into benzene (10 ml) in a conical flask and allowed to stand for 10 min then filtered. To the filtrate, 5 ml of 10% ammonia solution was added, shaken for 30 sec, and observed for the appearance of a pink, red or violet colour in the ammonia phase.

9.3.2.6 Test for flavonoids

Equal volumes (5 ml) of dil. $NH_{3(aq)}$ and the aqueous extract filtrate were mixed with 2-3 drops of conc. H_2SO_4. The formation of a yellow coloration indicated the presence of flavonoids.

9.3.2.7 Test for steroids

Acetic anhydride (2 ml) was added to 0.5 g of extracts followed 2 ml dil. H_2SO_4. Colour change from violet to blue or green showed the presence of steroids.

9.3.2.8 Determination of amines

Phenolphthalein (1 drop) was added to 20 ml each of 4 M HCl solution and plant extract in a conical flask and shaken to mix until a pink to brown colour was formed. The presence of an offensive (cartbolic) odour signified the presence of amines (Kenner and Obrien, 1997).

9.3.2.9 Determination of carboxylic acids

Phenolphthalein (1 drop) was added to 25 ml each of plant extract, and standard solution of $K_3Mn_5O_7$ in a conical flask. The appearance of a faint pink colour which disappeared after 30 seconds indicated the presence of carboxylic acids (Kenner and Obrien, 1997).

9.3.2.10 Determination of phenols

To 20 ml each of plant extract and 2 M sodium hydroxide (NaOH) solution in a conical flask, phenolphthalein (1 drop) was added, and the mixture gradually shaken to mix and observed for the appearance of a purple colour within 30 seconds (Kenner and Obrien, 1997).

9.3.2.11 Determination of Anthocyanins

Briefly, 1 ml of boiling water, 0.5 ml of 37% HCl to 10 mg of dry extract weree mixed in a test tube and mixture heated at 100°C, cooled and 0.4 ml of amylic alcohol added and observed for colour change to dark blue (Rojas *et al.,* 2006).

9.3.2.12 Determination of Quinones

Quinones were identified by extracting 10 ml of the aqueous extract with dichloromethane, evaporating the organic phase, and adding 5 ml of ethanol, 1 ml of 5% H_2O_2 and 1 ml of 50% H_2SO_4. The mixture was heated, cooled, extracted with benzene and 1 ml of NH_4OH added. The quinone extracts was then separated from the benzene and NH_3 phase by careful decantation (Rojas *et al.,* 2006).

9.3.3 Effect of plant extracts on bacterial beta-lactamase and verocytotoxin production

For the purpose of this study, 5 ml trypton soy broth (TSB) culture of the bacteria was centrifuged at 2000 rpm for 10 min. The supernatant was decanted and the sediment (bacterial cells) was twice washed with normal saline by centrifuging at 2000 rpm for 10 min

and the cells made up to 10 ml with normal saline. After standardizing the cells to 0.5 McFarland standard (equivalent to 10^8 cfu/ml), equal volume (5 ml) was mixed with 30 mg/ml crude extract, adequately shaken to mix and held at room temperature (28 ± 2°C) for 6 h and then incubated at 37°C for 18 h. After incubation, a loopful of bacterial culture from the surviving bacteria after exposure to extracts was inoculated onto trypton soy agar (TSA) and further incubated at 37°C for 18 h. A loopful of surviving bacteria was then suspended in sterile distilled water and 1 ml inoculated into TSB and then incubated for 18–20 h at 37°C while shaking at 120-150 rpm to allow for toxin secretion into broth medium. Bacterial suspension was then centrifuged for 20 min at 4,000 rpm and 4°C. Supernatant was transferred to new tubes and then screened for verotoxin production using Duopath® verotoxin latex reagent (Merck, SA, Appendix vii*h*) as described by the manufacturer. The non-pathogenic strains *E. coli* ATCC 25922 and *A. haemolyitcus* 19002 were used as controls. To test for beta-lactamase production, 1 ml of the supernatant was inoculated into 5 ml of Muller Hinton broth (MHB) and incubated for 6 h at 37°C then subcultured onto Muller Hinton agar (MHA) plates onto which two discs, ceftazidime and cefotaxime (30 µg in each case) were then placed. The culture plates were incubated at 37ºC for 18 h and extended beta-lactamase production (ESBL) production was determined by the appearance of zone diameters of inhibition (≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime) against the test bacterial growths.

9.3.4 Quantification of extract-induced cationic leakage from bacterial cell wall

The cation (Na^+ and K^+) leage assay was used for this purpose. Na^+ and K^+ leakage was determined from 5 strains of *E. coli* and 4 each of *A. lwoffii* and *A. haemolyticus* after exposure to 30 mg/ml of the crude ethanol plant extracts for 1 h. The bacteria were first exposed to salt solutions of Na^+ and K^+ separately by mixing equal volumes (5 ml) each of 25 ppm each of NaCl and KCl with a broth culture (5 ml) of the test bacteria (0.5 McFarland standard) and incubating at intervals of 0; 10; 20; and 30 min at 37°C. The cells were then centrifuged at 2000 for 10 min, the supernatant decanted and the sediment washed twice in distilled water by centrifuging at 2000 rpm for 10 min. To 1 ml of this washed bacterial test suspension, 1 ml of the 30 mg/ml of crude extract was added in different sets of sterilized curvets and incubated at ambient conditions for 1 h. Curvets containing test bacteria, extract or 25 ppm Na^+ or K^+ only were used as controls. The non-pathogenic strains *E. coli* ATCC 25922 and *A. haemolyitcus* 19002 were used as controls, while salt-treated *A. lwoffii* RWW1i unexposed to extract was used as control for *A. lwoffii* strains. Presence of Na^+ or K^+ were determined spectrophometrically from each cell suspension and the controls according to their respective incubation periods by placing the curvets in an atomizer orifice and taking readings at 266 nm.

9.3.5 Determination of antioxidant activity using the DPPH radical scavenging system

The hydrogen or electrons donation ability of the extracts was measured from bleaching of purple methanol solution of 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) free radical (Changwei *et al.,* 2008). A 2-ml aliquot of a suspension of the ethanol extracts was mixed with 1 ml of

0.5 mM DPPH solution and 2 ml of 0.1 M sodium acetate buffer (pH 5.5), properly shaken and incubated at ambient temperature in the dark for 30 min, following which the absorbance was measured at 517 nm using a UV-160A spectrometer. Ethanol was used as negative control. Radical scavenging activity expressed as the inhibition percentage was calculated as described by Abe *et al.* (1998) using the formula:

$$\% \text{ radical scavenging activity} = [(A_{control} - A_{test})/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution plus antioxidant).

9.3.6 Determination of Reducing Power of extracts

Reaction mixture containing plant extract at different concentrations (10-100 µl) in phosphate buffer (0.2 M, pH 6.6) and equal amounts of 1% (w/v) potassium ferricyanide, was incubated at 50ºC for 20 min. The reaction was terminated by the addition of equal volumes of 10% (w/v) tricarboxyllic acid (TCA) solution and the mixture centrifuged at 3000 rpm for 20 min. The supernatant was mixed with equal volume of distilled water and 0.1 % (w/v) ferric chloride solution and the absorbance measured at 700 nm. Increased absorbance of the mixture with concentration indicated the reducing power of the extract.

9.3.7 Determination of total phenolic content

Stock solution (0.5 mg/ml) of plant extracts was prepared and further diluted to five different concentrations 0.4; 0.3; 0.2; 0.1; and 0.05 mg/ml). Zero point one milliliter (0.1 ml) each of test concentration and Folin-Ciocalteu reagent (Sigma-Aldrich) was added to the extracts in different sets of test tubes, shaken thoroughly, and left to stand for 1 min. Two point eight milliliters of 10% $NaHCO_3$ was then added and the mixture once again allowed to stand for 30 min. after which the absorbance (725 nm) was measured spectrophotometrically and the total phenolic content (TPH) was expressed as mg equivalent of Gallic acid (mg GAE) (0.05-0.5 mg/ml as control/blank) per gram dry weight of the extract (Djeridane *et al.,* 2006).

9.3.8 Antimicrobial susceptibility test of plant extracts

Briefly, 0.5 ml McFarland turbidity standard of test bacteria was seeded on to sterile MHA plates, spread out using sterile glass rod in order to achieve confluent growth and the plates left on the table for 5 min to dry. Sterile filter paper discs (4 mm in diameter) soaked in the extract solution at different concentrations (5.0; 10.0; to 300 mg/ml/disc) were placed on the different MHA plates preseeded with different test organisms and the plates were then incubated at 37ºC for 24 h. Filter papers soaked in ethanol and ampicillin (10 µg/ml) were used as negative and positive controls respectively. Antibacterial activity was determined by measurement of zone diameter of inhibition (mm) against each test bacteria (Doughari and Obidah, 2008). 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 equaled to the IZD of the controls (Rojas *et al.*, 2006).

9.3.9 Determination of minmum inhibitory concentration (MIC) plant extracts

The MIC was carried out on extracts that showed antimicrobial activity (RIZD % of 1 and above) using the broth dilution method. The organisms were inoculated into test tubes containing varying concentrations (100-3000 µg/ml and 2.5-200 mg/ml/disc) of plant extract and 1 ml of nutrient broth (NB) added. 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 NB only and the standard antibiotic ampicillin (10 µg) as negative and positive controls respectively. 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. The MICs for verotoxin inhibition at these same extract concentrations were also determined as earlier described.

9.3.10 Bacterial strains

Sixty nine stock cultures of *Acinetobacter haemolyticus, A. lwoffii iand Escherichia coli* serotypes obatined variously from treatment plant wastewater, abattoir wastewater and from

Rivers Berg and Plankenberg all in Cape Town, South Africa. We previously isolated these strains from the various water sources and characterized them using standard methods for verotoxins in the Microbiology Laboratory, Department of Biotechnology, Faculty of Applied Sciences Cape Peninsula University of Technology, Cape Town, South Africa. The veropositive isolates were used to test for antiverotoxic actitivity of the plant extracts. The non-pathogenic strains of *E. coli* ATCC 25922 and *A. haemolyitcus* 19002 were used as control. The bacteria were previously maintained on TSA slants at 4°C were subcultured onto plates of Muiller hinton agar (MHA) and incubated at 37ºC for 18 h before use.

9.4 STATISTICAL ANALYSIS

Results are given as mean ± SEM values while relationships between antibacterial activity and test bacteria and plant extracts were determined using the student *t* test of the SIGMAPLOT at $P \leq 0.05$.

9.5 RESULTS

Phytochemical and other organic compounds analysis of *C. dentata* showed that the solvent extracts of stem bark contain differing classes of compounds. Ethanol extracts (ET) contain the highest classes, followed by dichloromethane (DCM), acetone (AC) and hexane (HX). Distilled water (DW) extracts contained the lowest group of phytochemicals in all the plant parts. Phytochemicals and organic compounds detected include anthraquinones, alkaloids, essential oils, glycosides, phenols, steroids, saponins and tannins and the organic compounds quinones, anthocyanins, amines and carboxylic acids (Table 9.1).

8204 Table 9.1. Phytochemicals and Organic compounds present in extracts of *Curtisia dentata* parts

Phytochemical	Plant part/solvent														
	Root bark extract					Stem bark extract					Leaf extract				
	DW	DCM	HX	AC	ET	DW	DCM	HX	AC	ET	DW	DCM	HX	AC	ET
Anthraquinones	-	-	-	+	+	+	-	-	+	+	-	-	-	+	+
Alkaloids	-	-	+	+	+	-	-	-	-	+	-	-	+	-	+
Essential oils	-	-	-	-	+	-	-	-	-	+	-	+	-	-	+
Flavonoids	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-
Glycosides	+	+	-	-	-	+	+	-	-	+	+	+	-	+	-
Phenols	+	+	-	-	+	+	+	+	-	+	+	+	-	-	+
Steroids	-	-	-	-	+	-	-	-	-	+	-	-	+	-	+
Saponins	-	+	-	+	+	+	+	-	+	+	-	+	-	+	+
Tannins	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+
Quinones	-	+	-	+	+	-	+	-	-	+	-	+	-	-	+
Anthocyanins	+	+	-	+	+	+	+	-	+	+	+	-	-	+	+
Amines	-	+	+	-	+	-	+	+	-	+	-	-	+	-	+
Carboxyllic acids	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+

8205 Solvents: DW-distill water, DCM-dichloromethane, HX-hexane, AC-acetone, ET-ethanol.

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Results of antimicrobial potentials of *C. dentata* against *E. coli* serotypes as relative inhibition zone diameters (RIZD) of the extracts ranged between 8-28% (MIC, 100-2500 µg/ml) against *A. lwoffii* and *A. haemolyiticus,* 10-28% (MIC, 100-850 µg/ml) against *A. lwoffii* and 6-28% (MIC 150-2500 µg/ml) against *A. haemolyticus* (Table 9.3). Results of antiverotoxic activity showed that the extracts demonstrated inhibitory activity against both Vtx1 and Vtx2 production. The ethanol extracts (ET) demonstrated the highest inhibitory action against 82.61% of the verotoxic *E. coli* serotypes, followed by the dichloromethane extracts (DCM, 71.01%), hexane (HX, 44.93%), chloroform (CHL, 34.78%) and acetone (AC, 27.54%). Water extracts did not show any antiverotoxic activity against the test bacteria. Extracts also inhibited the vtx gene expression for verotoxin production at MIC range of 100-2500 µg/ml for both *E. coli* (Table 9.2) and *Acinetobacter* spp. (Table 9.3).

8240 Table 9.2. Relative inhibition zone diameters (%), minimum inhibitory concentration (MIC) (µg/ml) and antiverotoxic effect of stem bark ethanol extracts of
8241 *Curtisia dentata* on various environmental *Escherichia coli* serotypes

Sample number (mean pH/Temp. °C)	Isolate/ serotype	RIZD values (%)	MIC (µg/ml) to *C. dentate*	Vtx & ESBL status before treatment	Vtx & ESBL status after treatment					
					DW	DCM	HX	CHL	AC	ET
Wastewater (n=18)	*E. coli* RWW1i O103:H2	10.00	650.00	Vtx1	Vtx1	-	Vtx1	Vtx1	Vtx1	-
(6.4/17.8)	*E. coli* RWW1ii O86	16.00	250.00	Vtx1, Vtx2	Vtx1, Vtx2	- Vtx2	- Vtx2	Vtx2	-	-
	E. coli RWW1iii O145:H2	14.00	350.00	Vtx1	Vtx1	-	-	Vtx1	Vtx1	-
	E. coli RWW1iv O96:H9	8.00	750.00	Vtx1	Vtx1	-	-	Vtx1	Vtx1	-
	E. coli RWW1v O126	14.00	200.00	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1
	E. coli RWW1vi O4	16.00	250.00	Vtx1	Vtx1	-	-	-	Vtx1	-
	E. coli RWW1vii O55	14.00	400.00	Vtx1, Vtx2	Vtx1, Vtx2	- Vtx2	- Vtx2	- Vtx2	- Vtx2	-
	E. coli RWW1viii O111:NM	22.00	150.00	Vtx1, Vtx2	Vtx1, Vtx2	-	-	-, Vtx2	-	-
	E. coli RWW2i O96:H9	8.00	1000.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli RWW2ii O124	14.00	400.00	Vtx1	Vtx1	-	-	-	-	-
	E. coli PSW1i O96:H9	16.00	200.00	Vtx1	Vtx1	-	Vtx1	Vtx1	Vtx1	-
	E. coli PSW1ii O145:NM	22.00	150.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli PSW1iii O96:H9	16.00	250.00	Vtx1, Vtx2	Vtx1, Vtx2	-, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-	-
	E. coli PSW1iv O111:NM	24.00	150.00	Vtx1, Vtx2	Vtx1, Vtx2	-	-	-	-	-
	E. coli PSW2i O86	14.00	200.00	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, -	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, -
	E. coli PSW2ii O96:H9	10.00	550.00	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, -	-,Vtx2	-	Vtx1, Vtx2	-
	E. coli PSW2iii O103:H2	14.00	300.00	Vtx1	Vtx1	-	-	-	-	-
	E. coli FEW1i O111:NM	18.00	200.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli FEW1ii O103:H2	14.00	400.00	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1	-
	E. coli FEW1iii O124	14.00	350.00	Vtx1	Vtx1	-	-	Vtx1	-	-
	E. coli FEW1iv O44	20.00	200.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2
	E. coli FEW2i O124	20.00	150.00	Vtx2	Vtx2	-	Vtx2	Vtx2	Vtx2	-
	E. coli FEW2ii O103:H2	24.00	100.00	Vtx2	Vtx2	-	-	-	Vtx2	-
	E. coli FEW2iii O145:NM	18.00	250.00	Vtx1, Vtx2	Vtx1, Vtx2	- Vtx2	- Vtx2	- Vtx2	- Vtx2	-
	E. coli FEW2iv O145:NM	14.00	400.00	Vtx1, Vtx2	Vtx1, Vtx2	- Vtx2	- Vtx2	- Vtx2	- Vtx2	-
Abattoir water (n = 18	*E. coli* PRE1i O4	6.00	2000.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2
6.4/17.8)	*E. coli* PRE1ii O145:H2	6.00	2500.00	Vtx1	Vtx1	-	Vtx1	-	Vtx1	-
	E. coli PRE1iii O111:NM	10.00	600.00	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1
	E. coli PRE1iv O86	8.00	800.00	Vtx2	Vtx2	-	-	Vtx1	Vtx2	-
	E. coli PRE1v O4	16.00	250.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli PRE1vi O111:NM	10.00	500.00	Vtx1, Vtx2	Vtx1, Vtx2	- Vtx2	- Vtx2	- Vtx2	- Vtx2	- Vtx2
	E. coli PRE2i O103:H2	28.00	100.00	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-
	E. coli PRE2ii O4	20.00	250.00	Vtx1, Vtx2	Vtx1, Vtx2	-	-	-	-	-
	E. coli FSE1i O113	20.00	150.00	Vtx2	Vtx2	-	Vtx2	Vtx2	Vtx2	-
	E. coli FSE1ii O145:H2	22.00	250.00	Vtx2	Vtx2	-	Vtx2	Vtx2	Vtx2	-
	E. coli FSE1iii O86	12.00	500.00	Vtx2	Vtx2	Vtx2	-	Vtx2	Vtx2	Vtx2
	E. coli FSE1iv O111:NM	6.00	900.00	Vtx2	Vtx2	-	Vtx2	Vtx2	Vtx2	-
	E. coli FSE1v O96:H9	8.00	750.00	Vtx2	Vtx2	Vtx2	Vtx2	-	Vtx2	-
	E. coli FSE1vi O4	20.00	200.00	Vtx2	Vtx2	-	-	-	-	-
	E. coli FSE2i O111:NM	12.00	500.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli FSE2ii O103:H2	8.00	850.00	Vtx2	Vtx2	-	Vtx2	Vtx2	Vtx2	-

	E. coli PST1i O145:H2	10.00	500.00	Vtx1, Vtx2	Vtx1, Vtx2	-, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-
	E. coli PST1ii O26:H11	14.00	300.00	Vtx1, Vtx2	Vtx1, Vtx2	-	-, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-
	E. coli PST1iii O113	20.00	150.00	Vtx1, Vtx2	Vtx1, Vtx2	-	-	-	-	-
	E. coli PST1iv O4	10.00	600.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2
	E. coli PST1v O96:H9	4.00	950.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli PST1vi O26:H11	18.00	250.00	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, -	Vtx1, Vtx2	Vtx1, Vtx2	-	-
	E. coli PST2i O124	24.00	2500.00	Vtx1, Vtx2	Vtx1, Vtx2	-	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-
	E. coli PST2ii O124	10.00	700.00	Vtx1, Vtx2	Vtx1, Vtx2	-	-	-	-	-
River Berg (n = 13) (7.2/17.3)	*E. coli* RBU1i O86	4.00	2500.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli RBU2i O113	12.00	400.00	Vtx2	Vtx2	-	Vtx2	-	Vtx2	-
	E. coli RBU2ii O145:H2	20.00	200.00	Vtx2	Vtx2	-	-	Vtx2	Vtx2	-
	E. coli RBU2iii O113	12.00	450.00	Vtx2	Vtx2	-	-	Vtx2	Vtx2	-
	E. coli RBD1i O113	22.00	150.00	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1,	-
	E. coli RBD1ii O4	16.00	300.00	Vtx1, Vtx2	Vtx1, Vtx2	-	-	-	Vtx1, Vtx2	Vtx1,-
	E. coli RBD1iii O86	22.00	150.00	Vtx1, Vtx2	Vtx1, Vtx2	-, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-
	E. coli RBI1i O4	28.00	100.00	Vtx1, Vtx2	Vtx1, Vtx2	-, Vtx2	Vtx1,-	-	Vtx1, Vtx2	-
	E. coli RBI1ii O103:H2	8.00	750.00	Vtx1, Vtx2	Vtx1, Vtx2	-	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-
	E. coli RBI2i O124	18.00	250.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli RBI2ii O86	22.00	200.00	Vtx2	Vtx2	-	-	Vtx2	Vtx2	-
	E. coli RBI2iii O96:H9	12.00	350.00	Vtx2	Vtx2	Vtx2	Vtx2	-	Vtx2	Vtx2
	E. coli RBI2iv O145:H2	22.00	200.00	Vtx1	Vtx1	-	-	Vtx2	Vtx2	-
	E. coli RBI2v O113	14.00	300.00	Vtx2	Vtx2	-	Vtx2	Vtx2	Vtx2	-
River Plankenberg (n = 13) (7.2/17.3)	*E. coli* PRK1i O4	20.00	150.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2
	E. coli PRK1ii O26:H11	26.00	100.00	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, -	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-
	E. coli PRK2i O145:H2	16.00	300.00	Vtx1, Vtx2	Vtx1, Vtx2	- Vtx2	- Vtx2	-	-	- Vtx2
	E. coli PRK2ii O86	24.00	150.00	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	Vtx2	-
	E. coli PRK2iii O4	12.00	350.00	Vtx1, Vtx2	Vtx1, Vtx2	-	Vtx1, Vtx2	Vtx1, Vtx2	Vtx1, Vtx2	-
	E. coli PRK2iii O103:H2	14.00	450.00	Vtx1, Vtx2	Vtx1, Vtx2	-	-	-	-	-
	E. coli ATCC 25922	18.00	250.00	-	-	-	-	-	-	-

DW-distilled water extract, DCM-dichloromethane extract, HX-hexane extract, CHL-chloroform extract, AC-acetone extract, ET-ethanol extract, ESBL-extended spectrum beta lactamase, Vtx-veortoxin.

Table 9.3. Relative inhibition zone diameters (%), minimum inhibitory concentration (MIC) (µg/ml) and antiverotoxic effect of stem bark ethanol extracts *Curtisia dentata* on various environmental isolates of *Acinetbacter* spp.

Sample number (mean pH/Temp. °C)	Isolate/ serotype	RIZD values (%)	MIC (µg/ml) to *Curtisia dentata*	Vtx & ESBL status before treatment	Verotoxin status after treatment					
					DW	DCM	HX	CHL	AC	ET
Wastewater (n=18) (6.4/17.8)	*A. lwoffii* RWW1i	14.00	750.00	-	-	-	-	-	-	-
	A. lwoffii RWW1ii	10.00	1500.00	-	-	-	-	-	-	-
	A. haemolyticus RWW1v	8.00	1000.00	-	-	-	-	-	-	-
	A. lwoffii RWW1vi	24.00	250.00	-	-	-	-	-	-	-
	A. lwoffiiRWW2i	28.00	100.00	-	-	-	-	-	-	-
	A. lwoffii RWW2ii	20.00	350.00	-	-	-	-	-	-	-
	A. lwoffii PSW1i	22.00	200.00	-	-	-	-	-	-	-
	A. lwoffii PSW1ii	26.00	150.00	-	-	-	-	-	-	-
	A. haemolyticus PSW2i	14.00	700.00	Vtx1	-	Vtx1	Vtx1	Vtx1	Vtx1	-
	A. haemolyticus PSW2ii	6.00	2000.00	-	-	-	-	-	-	-
	A. lwoffii FEW1i	26.00	250.00	-	-	-	-	-	-	-
	A. lwoffii FEW2i	28.00	150.00	-	-	-	-	-	-	-
	A. haemolyticus FEW2iv	24.00	250.00	-	-	-	-	-	-	-
Abattoir water (n = 18) (6.4/17.8)	*A. lwoffii* PRE1i	28.00	150.00	-	-	-	-	-	-	-
	A. *lwoffii* PRE1ii	26.00	200.00	-	-	-	-	-	-	-
	A. lwoffii PRE2i	18.00	450.00	-	-	-	-	-	-	-
	A. lwoffii PRE2ii	22.00	250.00	-	-	-	-	-	-	-
	A. lwoffii FSE1i	24.00	200.00	-	-	-	-	-	-	-
	A. lwoffii FSE1ii	26.00	150.00	-	-	-	-	-	-	-
	A. lwoffii FSE1iii	28.00	200.00	-	-	-	-	-	-	-
	A. haemolyticus FSE1iv	28.00	150.00	Vtx1,Vtx2	Vyx1	-	-,Vtx2	Vtx1,Vt	Vtx1,Vt	-
	A. haemolyticus FSE1v	28.00	250.00	Vtx2	VTx2	-	Vtx2	Vtx2	Vtx2	-
	A. lwoffii FSE2i	28.00	100.00	-	Vtx2	-	-	-	-	-
	A. lwoffii FSE2ii	26.00	150.00	-	-	-	-	-	-	-
	A. lwoffii PST1i	22.00	200.00	-	-	-	-	-	-	-
	A. lwoffii PST1ii	12.00	850.00	-	-	-	-	-	-	-
	A. haemolyticus PST1i	6.00	2500.00	Vtx1	-	Vtx1	Vtx1	Vtx1	Vtx1	-
	A. haemolyticus PST2i	24.00	200.00	-	-	-	-	-	-	-
	A. haemolyticus PST2ii	22.00	200.00	-	-	-	-	-	-	-
River Berg (n = 13) (7.2/17.3)	*A. lwoffii* RBU1i	26.00	150.00	-	-	-	-	-	-	-
	A. lwoffii RBU2i	12.00	750.00	-	-	-	-	-	-	-
	A. lwoffii RBU2ii	30.00	100.00	-	-	-	-	-	-	-
	*A.. haemolyticus*RBD1i	26.00	150.00	Vtx1	Vtx1	-	-	-	-	-
	*A. haemolyticus*RBD1ii	24.00	150.00	-	-	-	-	-	-	-
	*A. haemolyticus*RBD1iii	10.00	900.00	-	-	-	-	-	-	-
	*A. haemolyticus*RBI1i	28.00	150.00	-	-	-	-	-	-	-
	A. haemolyticus RBI2i	12.00	600.00	-	-	-	-	-	-	-
	A. lwoffii RBI2ii	24.00	200.00	-	-	-	-	-	-	-
	A. lwoffii RBI2iii	24.00	250.00	Vtx1,Vtx2	-Vtx2	Vtx1	Vtx1	Vtx1	Vtx1	Vtx1
River Plankenberg (n = 13) (7.2/17.3)	*A. lwoffii* PRK2i	26.00	150.00	-	-	-	-	-	-	-
	A. lwoffii PRK2ii	28.00	150.00	-	-	-	-	-	-	-
	A. lwoffii PRK2iii	22.00	200.00	-	-	-	-	-	-	-
	A.haemolyticus 19002	20.00	200.00	-	-	-	-	-	-	-

DW-distilled water extract, DCM-dichloromethane extract, HX-hexane extract, CHL-chloroform extract, AC-acetone extract, ET-ethanol extract, ESBL-extended spectrum beta lactamase, Vtx-veortoxin.

Results of effect of extracts on bacterial cell wall revealed the presence of sodium and potassium cations in the tested medium. While isolates incubated for 30 min generally showed higher ODs, *A. haemolyticus* isolates showed lower OD values (0.172-54.44 for Na^+ and 0.572-102.78 for K^+) (Figure 3) compared to the *A. lwoffi* (0.432-184.45 for Na^+ and 0.76-367.27 for K^+) (Fig. 9.2) and *E. coli* (12.06-334.67 for Na^+ and 22.36-596.55 for K^+) isolates (Fig. 9.1).

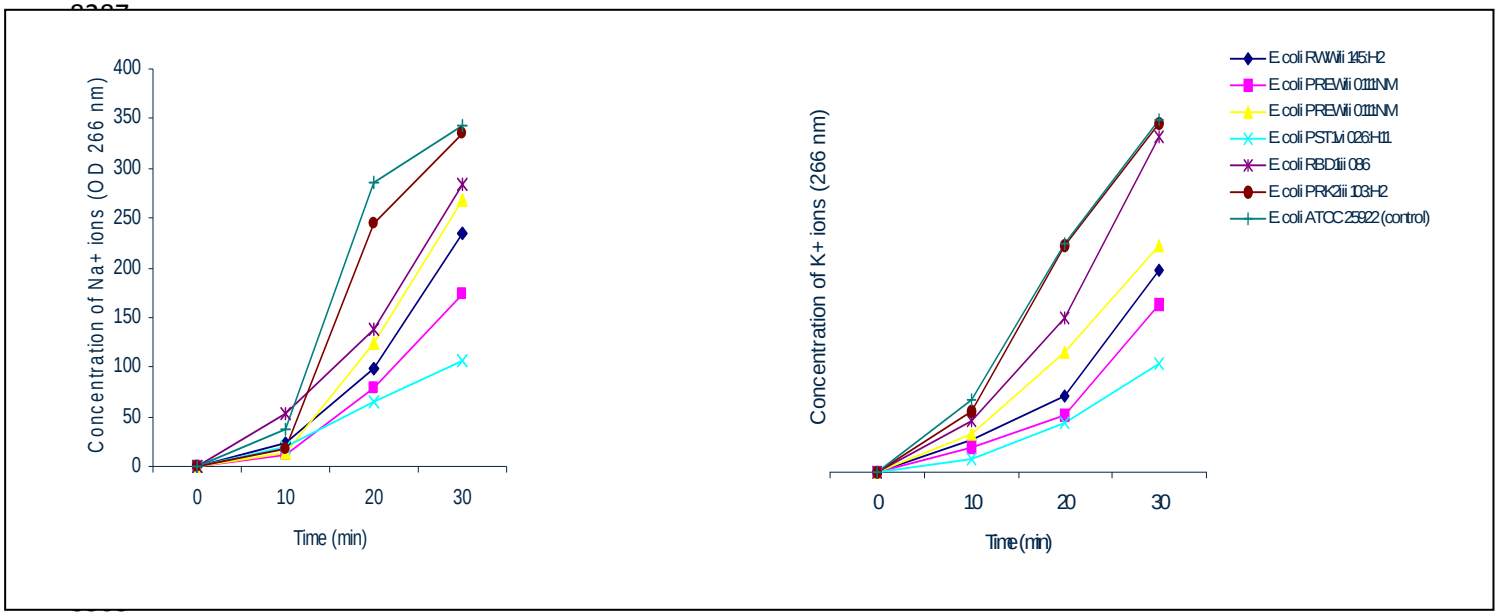

Figure. 9.1 Leakage of $Na^+$and K^+ ions from *E. coli* isolates by stem bark ethanol extracts of *Curtisia dentata*

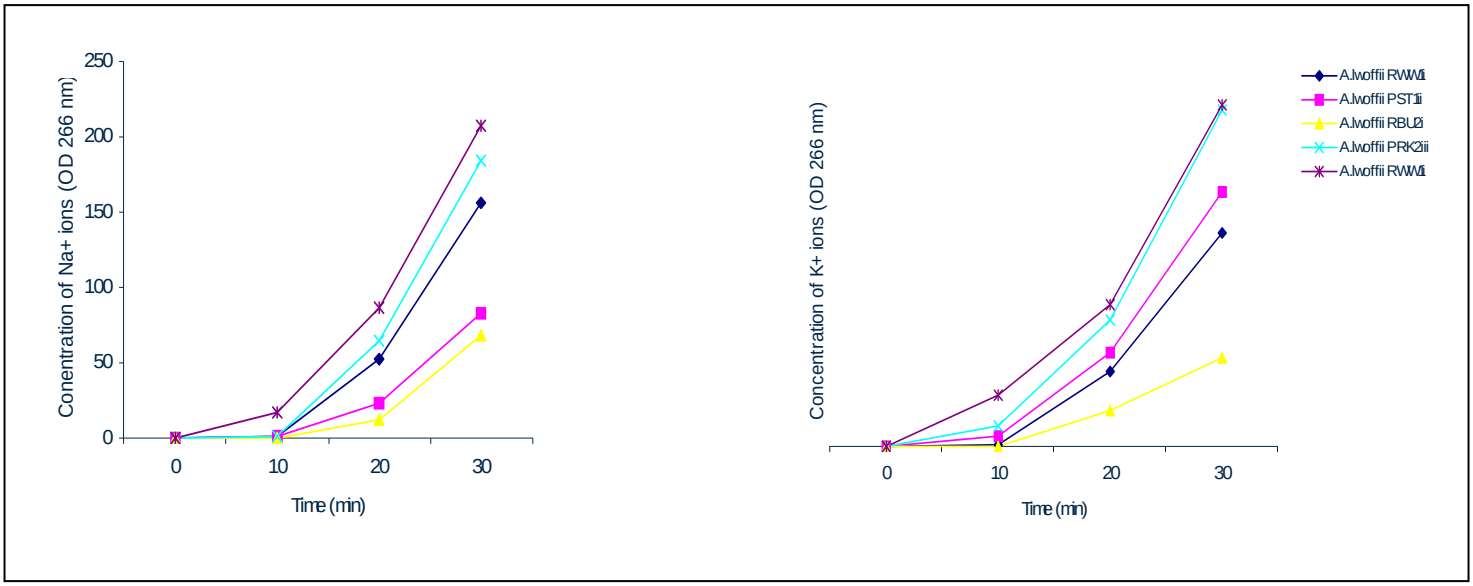

Figure.9.2. Leakage of $Na^+$and K^+ ions from *A.lwoffii* isolates by stem bark ethanol extracts of *Curtisia dentata*

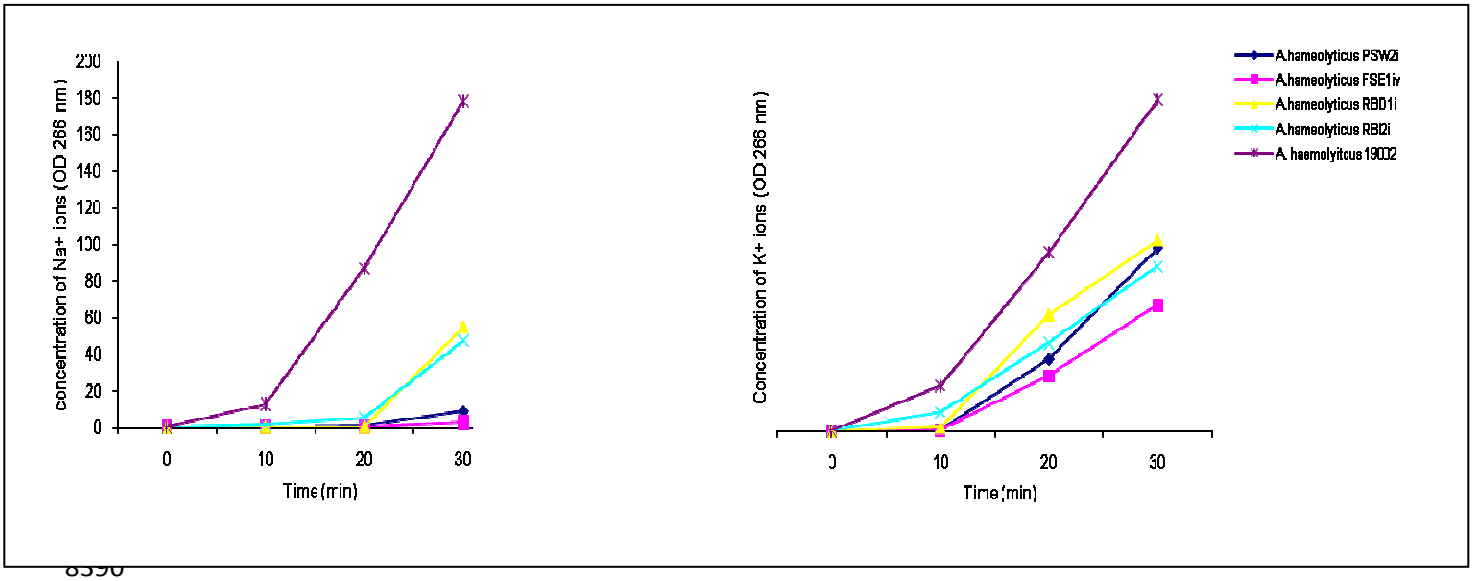

Figure 9.3. Leakage of $Na^+$and K^+ ions from *A.haemolyticus* isolates by stem bark ethanol extracts of *Curtisia dentata*

The DPPH radical scavenging activities, total phenolic content (TPH) and reducing power (RP) of the different extracts are shown in Table 9.4. The ethanol root bark extracts consistently showed the highest DPPH radical scavenging activity (62.43%), TPH (57.62 mg GAE/g) and RP (41.32%) followed by those of the stem bark extracts with the respective values of 54.68%, 37.77 mg GAE/g and 21.83%. Water extracts (DW) showed the least values in all the test results. Among the solvents, ethanol demonstrated the highest values for both DPPH, TPH and RP followed by DCM, HX, AC and DW in this order.

Table 9.4. Antioxidant activity, total phenol content and reducing power of extracts of *Curtisia dentata*

SBE-stem bark extract, RBE-root bark extracts, LE-leaf extracts, DW-distilled water,

Plant pa	DPPH (% at 0.1mg/ml, 517nm)						Total Phenolic content (TPH) (725nm)						Reducing Power (RP)					
	DW	DCM	HX	CHL	AC	ET	DW	DCM	HX	CHL	AC	ET	DW	DCM	HX	CHL	AC	ET
Control	12.68	12.68	12.68	12.68	12.68	12.68	14.52	14.52	14.52	14.52	14.52	14.52	3.27	3.27	3.27	3.27	3.27	3.27
SBE	14.68	50.34	41.47	38.55	26.28	54.68	11.32	18.22	16.61	30.22	24.25	37.77	11.83	21.15	16.18	14.21	12.26	21.83
RBE	32.43	56.67	42.82	38.52	24.51	62.43	17.44	42.36	28.71	51.12	21.32	57.62	22.32	32.74	36.42	27.21	21.28	41.32
LE	18.45	40.34	38.45	28.37	18.26	44.56	8.64	18.83	15.18	12.58	8.84	24.73	1.62	3.61	3.84	3.22	1.07	4.62

DCM-dichloromethane, HX-hexane, CHL-chloroform, AC-acetone, ET-ethanol.

9.6 DISCUSSION

The presence of phytocosntiuents in various parts of *C. dentata* confirms its potential as source of antimicrobial substances. Generally, anthraquinones, alkaloids, essential oils, glycosides, phenols, steroids, saponins and tannins observed in this study are reported to confer innate defence mechanisms against invading bacteria, fungi, pests and diseases (Fink-Gremmels, 2010). Individually, alkaloids have been variously employed therapeutically as antimicrobials, analgesics/narcotics, mydriatics, miotics, hypertensives, hypotensives, bronchodilators, stimulants or antileukemic agents (Pengelly, 2004). Anthraquinones as laxatives for the treatment of constipation and their antiseptic effects deter the growth of enteric pathogens. Some anthraquinones and napthaquinones significantly inhibit Epstein-Barr virus early antigen activation at low doses. Essential oils (or volatile oils) have stimulant, decongestant, antiviral, antitumour, antimicrobial, antiseptic, tonifying, spasmolytic, anti-inflammatory or antiviral potential (Pengelly 2004). Tannins exert astringent activity via precipitation of proteins, thereby protecting the underlying tissue leading to improvement of wound healing (Tyler *et al.,* 1998; Madziga *et al.,* 2010). Awosika (1991) also reported that tannins inhibit microbial proliferation by denaturation of enzymes involved in microbial metabolism and their potential as antiviral, antibacterial, antiparasitic and anticancer effects have also been reported (Akiyama *et al*., 2001). Saponins have been associated with anaesthetic or CNS stimulant potentials and thus have been applied as local analgesics and as antimalarials. Steroids on the other hand have been

observed to promote nitrogen retention in osteoporosis and in animals with wasting illness, inhibit growth of tumours and to reduce blood cholesterol (Pengelly, 2004; Aliu and Nwude, 1982). Therapeutic effects of flavonoids such as the antiallergic, antioxidant, antiviral, hepatoprotective, antiatheromatous, anti-inflammatory, anti-microbial and anti-cancer activity and antihypertensive have been widely reported (Yamamato and Gaynor 2002; Pengelly 2004; Stauth 2007). Cardiac glycosides have been used in the treatment of congestive heart failure, constipation, edema and microbial infections (Robinson, 1967; Franstisk, 1991). Saponins have expectorant and antibacterial properties and have been employed in the treatment of upper respiratory tract and other microbial infections (Birk and Petri, 1980; Trease and Evans, 1984). Presence of these various phytochemicals in the extracts of *C. dentata* and demonstration of activity of these extracts against various *E. coli* and *Acinetobacter* spp. provides the possibility of sourcing a wide range drugs and antibacterial substances against these various ailments and infections associated with these bacteria.

The organic chemical components such as quinones, anthocyanins, amines and carboxylic acids, have formed bases for many synthetic antibiotics including ubiquinol and pycnogenol (Pengelly, 2004). Quinones form an important component of the electron-transport system in plants and mammals. Ubiquinol, the reduced form of coenzyme Q10, and menaquinone (vitamin K) have significant antioxidant properties, playing a major role in protecting cells

from free-radical damage. Pycnogenol® is the proprietary name for oligomeric procyanidins (OPCs) extracted commercially from grape seeds and pine bark, and are responsible for many of the benefits associated with red wines, including treatment of cardiovascular and cerebrovascular diseases. Amines and carboxylic acids are used in the hydrolytic synthesis of amide drugs such as acetaminophen, a well-known anti-inflammatory drug - a simple amide formed from 4-hydroxyphenylamine and acetic acid. Such amide functional groups so formed, are quite resistant to hydrolysis, and amide linkages between amino acids and peptides are essential to the stability of proteins. The presence of these organic compounds in *C. dentata* offers very promising sources of chemical backbones for antioxidant therapeutic drugs.

There were differences in concentration of the chemical components on different parts of the plant as observed from this study. Mountousis *et al.* (2006) had earlier reported differences in chemical component concentration from one plant part to the other, depending on their degree of maturity. Care must therefore be taken in the choice of plant part in medicinal plant drug research. The MICs were generally low, and since the plant extracts were in crude form, this outcome is promising. Low MIC values indicate potentially high efficacy of the extracts as antimicrobial agents (Doughari *et al.*, 2008; Sharma *et al.*, 2010). Also, higher antiverotoxic potentials against the test bacteria demonstrated by ethanol extracts might be as a result of higher concentration of phytoconstituents in this solvent compared to the other

solvents used. Absence of antiverotoxic activity from aqueous extracts however does not rule out the presence of such activity, but the phytoconstituents may be occurring in very low ineffective concentrations. The inhibition of the expression of both Vtx1 and Vtx2 genes in both *E. coli* and *Acinetobacter* spp. is a very significant finding as it provides a gateway for the development of very effective antiverotoxic drugs. Currently, antibiotic treatment induces the release of more of the toxins into the protoplasm resulting in further complications. Recently *E. coli* O104:H4 was implicated in a fatal foodborne illness resulting in 882 people contracting hemolytic uremic syndrome (HUS) with 32 deaths in Europe and 1 death in America within just 2 months (CDC, 2011). This, in addition to the emergence of some verotoxic strains of *Acinetobacter* spp. underline the significance of findings of this study and the need to continue searching for potential control agents. Though this study did not establish the toxic effect of this plant to human cells, the plant has demonstrated potential as source of novel antimicrobial agents for the treatment of verotoxic bacterial infections. Furthermore, the study represents the first report of antiverotoxic activity of *C. dentata* extracts against various Vtx genes from bacteria. Future research work to determine the possible impact on human cells should be carried out.

The presence of Na^+ and K^+ ions in the medium indicates leakage of these ions through the bacterial cell walls. Therefore, this is an indication that the extracts are capable of causing damage to bacterial cell walls, thereby causing leakage of protoplasmic contents - one of

several mechanisms of actions of antimicrobials. The differences in Na^+ and K^+ ion leakage rates might be due to differences in ionic sizes of the two metal ions. Though both have an equivalent number of charges, the greater leakage rate observed for K^+ might be as a result of its higher molecular size and atomic mass compared to that of Na^+ ions. However, this does not imply that cell wall leakage is the sole mechanism of action of this plant given the variety of compound classes present in the crude extract. Although actual microscopic damage to the bacterial cell walls was not determined, results of the studyindicate thatdamage to bacterial cell wall could be one of several mechanisms of action of the plant extract. *A. haemolyticus* isolates showed low OD values compared to *A. lwoffi* and *E. coli* isolates. Extract impurity might be accountable for this low activity in addition to possible innate immunity to antibiotic-like compounds.

There was a correlation between total phenolic content and high antioxidant activity as well as reducing power of extracts. Tawaha *et al.* (2007) reported a similar correlation between phenolic content with antioxidant activity from plant extracts. Among natural antioxidants, plant polyphenols are especially important (Kalim *et al.* 2010). The exhibition of high DPPH radical scavenging activity, total phenol content and reducing power by the extracts is an indication that drugs useful as antioxidants can be sourced from *C. dentata.* Results also showed differences in extraction efficiency by the various solvents with highest DPPH, TPH and RP values followed by dichloromethane, hexane, acetone and distilled water.

Differences in antioxidant activity between the various solvents may due to variation in polyphenol concentration extracted. Different solvents have different degrees of solubility depending on their polarity (Doughari, 2006). DPPH assay has been commonly employed in screening antioxidant activity of plant extracts. Radical scavenging activity potential of *C. dentata* observed in this study is a promising outcome for possible control of many oxidative stress-related diseases. Recently, much attention has been directed towards the development of ethnomedicines with strong antioxidant properties but low cytotoxicity. It has been estimated that approximately two-thirds of anticancer drugs approved worldwide up to 1994 were derived from plant sources (Kalim *et al.,* 2010). The demonstration of antioxidant activity by extracts of *C. dentata* is an indication that the plant can serve as a useful source for chemical substances for development of novel drugs.

9.7 Conclusion

The study revealed the presence of a wide range of phytochemicals in *C. dentata* extracts, as well as the possession of antioxidant, antimicrobial and antiverotoxic activity anagainst strains of *E. coli* and *Acinetobacter* spp. The study also revealed the possible damaging effect of the ethanol extracts on the bacterial cell walls an indication of the possible mechanism of action of the plant. Toxilogical studies and further purification of *C. dentata* extracts for possible structural illucidation of the phytochemical compounds to enable possible sourcing of antibiotic substances should be carried out. This might serve as a

8563 milestone for the development of novel antibiotic substances for treatment of verotoxic as
8564 well as nosocomial infections associated with these bacterial starins.
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8571 **9.9 REFERENCES**
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CHAPTER TEN

10.0 GENERAL CONCLUSION AND RECOMMENDATIONS

10.0 GENERAL CONCLUSION AND RECOMMENDATIONS

10.1 GENERAL CONCLUSION

Significant discoveries have been made from this study. *E. coli*, *A. haemolyticus* and *A. lwoffii* isolated from the wastewater and river water samples investigated demonstrated the capacity to adapt and survive in different tissues, by producing virulent factors and by developing a drug resistance. They are therefore potential agents of human infections such as diarrhoea, urinary tract and ear infections, depending on the site of colonization. Demonstration of antibiotic resistance among the isolates further confirms the prevalence of drug resistance amongst pathogenic *E. coli* strains as well as *Acinetobacter* spp. Verotoxins were expressed by both Vtx1 and Vtx 2 genes from all the *E. coli* and some of the *A. haemolyticus* isolates. Association of non O157 *E. coli* and *A. haemolyticus* isolates with verotoxin production in the waste water and surface river waters investigated in this study highlights the need to adopt more proactive measures to prevent and/or protect against outbreak of diarrheal diseases and further dissemination of MDR strains. The isolates demonstrated high transformation rates for antibiotic resistance and since virulence factors are gene-borne just as multidrug resistance factors are plasmid-borne, dissemination of these multidrug virulent verotoxic isolates into the drinking water and food sources might have serious health consequences. Stable mulitidrug resistant genes and active exchange of resistance genes between *E. coli* and *A. lwoffii* and *A. haemolyticus* occurred in this study,

stressing the need for appropriate chemotherapeutic culture based on accurate laboratory sucsepetibility diagnostic results.

Many of the isolates retained their virulence even after subjection to oxidative stress with chemicals, salts, phytochemicals and low temperatures. Therefore, contamination of food and

drinking water with these strains might mean the proliferation of more virulent strains, hence more severe drug resistant infectons associated with these bacteria (example, nosocomial infections, urinary tract infections gastroenteritis and severe diarrhea). Though most *Acinetobacter* related nosocomial infections are more commonly associated with *A. baumannii,* this study revealed the presence of potentially virulent strains of verotoxin-producing *A. haemolyticus.* This is the first report of verotoxin producing *A. haemolyticus* on African soil.

C. denata demonstrated activity against multidrug resistant verotoxin producing isolates of both *E. coli* and *Acinetobacter* spp. by *C. dentata*. This is an indication of the potential of the plant to provide alternative sources of antimicrobials that can be used in controlling notorious nosocomial infections, verotoxin related infections and infections caused by multidrug resistant pathogenic bacteria. The efficacy of the plant against vertoxin production provides hope for sourcing novel antiverotoxic agents, possibly to be used as an alternative to toxin-inducing antibiotics. The study furthermore revealed that *C. dentata* extracts

induced the leakage of both potassium and sodium ions from the cell membranes of *E. coli* and *Acinetobacter* spp. this discovery provides a significant insight into the mechanism of action of *C. dentata* as a potential source of novel antibiotics. The plant also inhibited verotoxin production. Which gives the indication that verotoxin producing bacteria can be effectively controlled by chemotherapeutics drugs that could be sourced from this plant without the risk of releasing the bacterial toxins into the host cell medium.

10.2. RECOMMENDATIONS

The co-existence of verotoxic, multidrug resistant and virulent isolates of *E. coli* and *Acinetobacter* spp in the water samples investigated means that there is an indication of the possible emergence of more drug resistant strains. The recent emergence of a very virulent strain of *E. coli* during an outbreak in Europe shows how rapidly these bacteria are transforming. This therefore calls for:

- continued monitoring processes for water and other environmental samples in both the developed and developing countries to enable development of more proactive control and prevention measures;
- more effective environmental monitoring and risk assessment studies of environmental and hospital wastes and survey of resistant strains of these bacteria especially in developing countries;

- Adequate disposal of hospital wastes as against refuse dumping is a common practice in some developing countries, which should be outrightly banned;
- The use of primarily treated water for irrigation purposes should be discouraged among farmers;
- foods and meat products should be made safe by thorough cooking; dairy products and fruit juices by pasteurization and salad vegetables by adequate irradiation or blanching before consumption;
- provision of potable drinking water and improved environmental sanitation by governments and individuals and, over all, increased awareness on the benefits and strict observance of personal hygiene by the populace which the best preventive measures against such bacterial agents in the face of increasing antimicrobial resistance;
- good antibiotic policyand proper selection of antibiotics for treatment, based on adequate detection of bacteria resistant to drugs through the results of antibiotic susceptibility tests as well as the judicious use of antibiotics in humans and animals in order to limit the emergence and spread of antibiotic resistant bacteria;
- farmers should further be enlightened on the need to maintain personal hygiene, especially while handling wastewater for irrigation purposes;

- People should be educated on the importance for boiling of river water before drinking as well as maintenance of food hygiene and also personal hygiene amongst food handlers.

10.3. LIMITATIONS OF THE STUDY AND FUTURE RESEARCH DIRECTION

The study scope did not include the establishment of a direct molecular link (and characterization) between the environmental isolates with active human disease conditions, as well as epidemiological and risk assesement studies. Very important chemical groups were also detected from the plant extracts in this study and needs to be further investigated. Furture research should therefore be focused on:

- Molecular studies to determine the genome of the isolates for more adequate understanding of their nature;
- Epidiomological studies to determine whether the types of STEC which are found in the wastewaters, (and farm animals) are the same as those found in human disease;
- Epidemological survey of all routes of human infection, including farm and wild animals (e.g. deer, rabbits etc.), the environment (water sources, fields etc.), food products and human-to-human transmission,;
- Risk assessement studies to determine the potential of transfer of the virulence determinants of the toxins from the producing bacterium to other organisms;

- Further structural elucidation of the chemical components of *C. dentata* detected in this study;
- Identification of specific antiverotoxic active chemical constituents of the plant; and
- Toxicological studies using *in vivo* animal models in order to determine their level of safety for possible human consumption.

11.0 LIST OF APPENDIXES

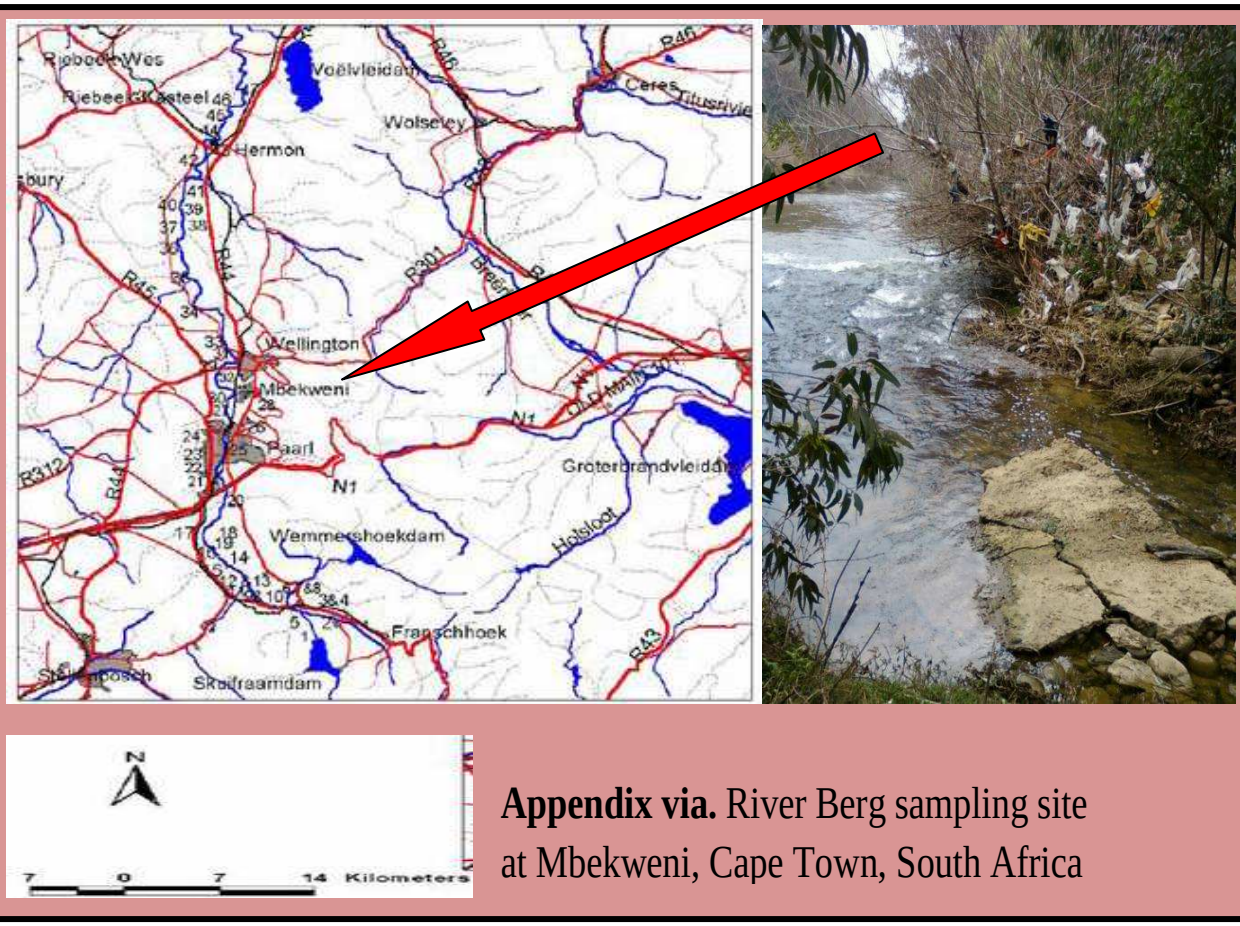
Appendix i. Baumann's Enrichment Medium (BEM) Compositin	**(g/l)**
Sodium acetate (trihydrate)	2.0
Potasium Nitrate (KNO_3)	2.0
Magnesium sulphate ($MgSO_4.7H_2O$)	0.2g
Potassium dihydrogen Sulphate-Disodium Hydrogen Phosphate (KH_2PO_4-Na_2HPO_4)	0.04M
Buffer	(pH 6.0)
Make up to 1 liter with distilled water.	

Appendix ii. Leeds Acinetobacter Medium (LAM) composition	**(g/l)**
Agar	10
Acid hydrolyzate of casein	15
Soy peptone	5.0
NaCl	5.0
D-fructose,	5.0 Sucrose
	5.0
D-mannitol	5.0
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 50°C and	
Add antibiotic solutions of Vancomycin	10.0
Cefsulodin	15.0
Cefradine	50.0 mg/l

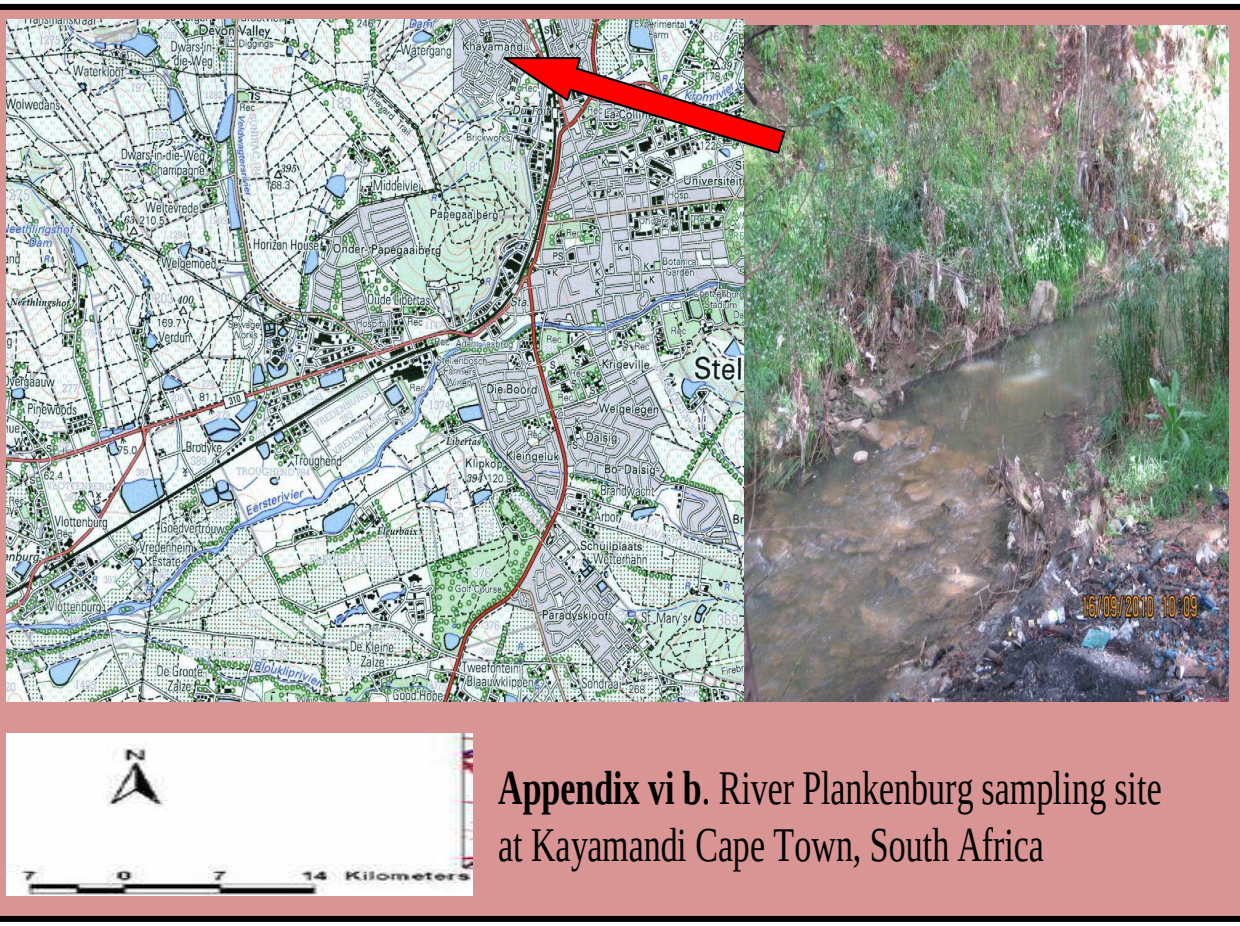
Appendix iii. Casaminacid yeast extract (CAYE) broth composition	**(g/l)**
Casamino acid	20
Yeast extract	6.0
NaCl	2.5
KH_2PO_4	8.71
Trace salt solution – (0.5% $MgSO_4$, 0.5% $MnCl_2$ and 0.5% $FeCl_3$	
dissolved in 0.0005 M H_2SO_4)	1.0 ml

Appendix iv. Hank's balanced salt solution (HBSS) composition	**(g/ml)**
NaCl	8.0
KCl	0.4
KH_2PO_4	0.06
$NaH_2PO_4.7H_2O$	0.048
$MgSO_4.7H_2O$	0.098
$CaCl_{2.2}H_2O$	0.14
$NaHCO_3$	0.035
Glucose	1.0
Phenol Red, Na Salt	0.001

Appendix v. Butterfield's phosphate diluent	**(g/l)**
KH_2PO_4	34 g
$MgCl_2$ (81.1g MgCl2•6H2O per liter of purified water)	5 ml
Adjust to pH 7.2 using 1*N* NaOH	175 ml
Make up to 1 l with distilled water	
Sterilize by autoclaving at 121°C for 15 minutes.	



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



Appendix vi b. River Plankenburg sampling site at Kayamandi Cape Town, South Africa

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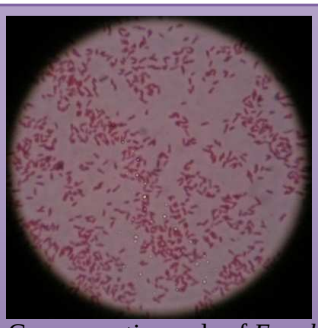
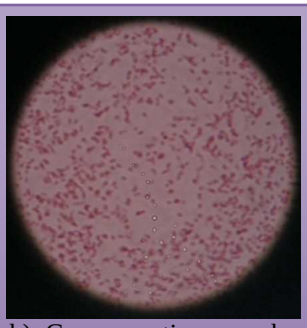


a). Gram negative rods of *E. coli*



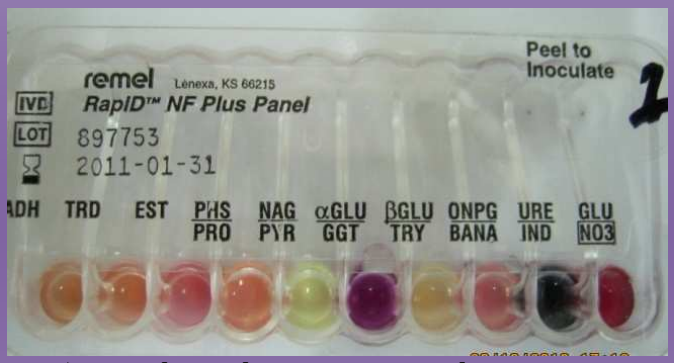
b). Gram negative coccobacilli of *Acinetobacter* spp.

c). *E. coli* on EMB

d). *Acinetobacter* spp. on EMB

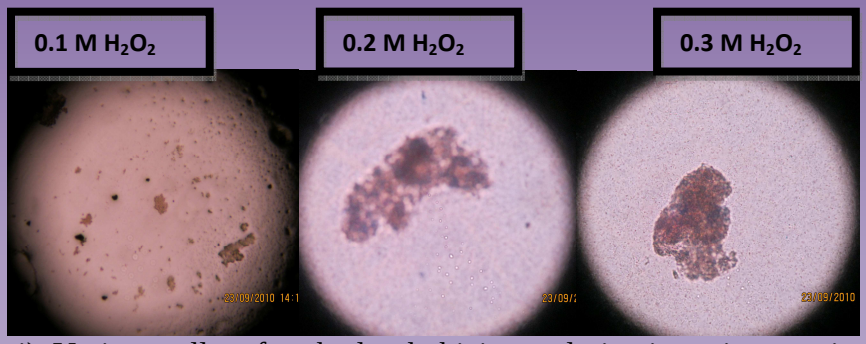
e). *Acinetobacter* spp on LAM

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 vii. Cultural and biochemical properties and, Gram reaction of *Acinetobacter* spp. and *Escherichia coli* isolates