MOLECULAR CHARACTERIZATION, ANTIBIOGRAMS AND ANTIBACTERIAL ACTIVITIES OF SELECTED MEDICINAL PLANTS AGAINST SOME ENTERIC PATHOGENS

by

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GENERAL SUMMARY

Infections due to *Escherichia coli* and *Salmonella* spp. are known to account for a substantial degree of morbidity and mortality in different age groups and populations worldwide. These infections may be self-limiting but in some cases antimicrobial therapy are indicated. With vaccine development still in its infancy in most cases and the increasing trend of drug resistance, the continued monitoring and assessment of antibiotics used in the treatment of these infections are paramount. In addition, there is a need to complement orthodox medicine with ethnomedicine for a lead to new drug discovery and for total primary health care coverage of the underserved populations. In this regard, medicinal plants offer great promise and studies to uncover the antimicrobial activities of medicinal plants, including structural elucidation of the active compounds and cytotoxicities of such plants are usually warranted. Furthermore, studies on the molecular landscape of the organisms, especially the various genes coding for virulence and antimicrobial resistance and the phylogenetic relatedness will illuminate knowledge on the pathogenesis and epidemiology of the pathogens.

The thesis, consisting of eight chapters, chronicles the findings on antibiogram profiles of the designated pathogens, inhibitory activities of selected medicinal plants, including their active compounds and cytotoxicity profiles as well as the molecular epidemiology of the pathogens. The first chapter gives a vivid account of the background to the study and the review of the literature.

Chapter 2 is an account of the antimicrobial resistance patterns as well as the molecular basis of resistance in *E. coli* and *Salmonella* isolates from clinical specimens. Isolates were antibiotyped while PCR and sequencing were carried out to identify genes modulating antibiotic resistance. The study showed an increase in multiple-drug resistance and an emerging resistance to penem drugs, an indication of limited choice of drugs in the chemotherapy of enteric disease. An alarmingly high percentage of isolates produced extended-spectrum beta-lactamases (ESBL), with prevalent genotypes being bla_{CMY-2}, bla_{SHV-1}, bla_{TEM-20} and bla_{TEM-1}. In addition, Class I integron was significantly involved in the resistance mechanism among the isolates.

Chapter 3 was designed to investigate the pathotypes and the various virulence factors among *E. coli* and *Salmonella* isolates. Using standard diagnostic methods and molecular analysis, of the 119 *Salmonella* isolates, the results of the serovars were *S. enterica serovar* Choleraesuis (6%), *S. enterica serovar* Enteritidis (4%), *S. enterica serovar* Eppendorf (1%), *S. enterica serovar* Hadar (1%), *S. enterica serovar* Isangi (8%), *S. enterica serovar* Panama (1%), *S. enterica serovar* Typhi (52%), *S. enterica serovar* Typhimurium (25%) and untyped *Salmonella* spp. (2%). The greatest proportion of the 90 diarrhoegenic *E. coli* were enteroaggregative *E. coli* (EAEC) 37 (41.1%), enteropathogenic *E. coli* (EPEC) 21 (23.3%) and enterohemorrhagic *E. coli* (EHEC) 21 (23.3%). Various virulence factors identified amongst the *Salmonella* strains were *flic*-flagellin H1 (12.6%), *invA*-invasion (88.2%), *sefA*- fimbrial antigen (1.7%) while

aggR- transcriptional activator for EAEC aggregative adherence fimbria I expression, *eaeA-E. coli* attaching and effacing and LT- heat-labile enterotoxin were found among the *E. coli* isolates.

In chapter 4, an ethnobotanical survey of medicinal plants used in the treatment of diarrhoea and related diseases in the ORTDM in the Eastern Cape of South Africa was embarked upon. This assisted in documentation of herbal plants and the extent of usage of ethnomedicine in the district municipality with the eventual aim of assessing the efficacies of some of the plants. A total of 32 plant species belonging to 26 families were reportedly used as diarrhoea remedy in the study area. The most predominant families of medicinal plants employed and most frequently recommended were Fabaceae (16.67%), followed by Hyacinthaceae and Hydnoraceae , each accounting for 8.33%. The majority of the rural dwellers depended on ethnomedicine due to the inaccessibility to orthodox medicine. This was due to several factors such as the distance between the communities and the nearest health care centres and financial constraints.

The ethnobotanical survey led to the antibacterial screening of 12 medicinal plants: *Acacia mearnsii, Aloe arborescens*, *Aloe striata, Cyathula uncinulata, Eucomis autumnalis (Mill.) Chitt., E. comosa (Houtt.) Wehrh. Hermbstaedtia odorata, Hydnora africana, Hypoxis latifolia, Pelargonium sidoides, Psidium guajava and, Scilla natalensis* frequently recommended by the traditional healers. A qualitative phytochemical screening and bioassays of the plants' extracts was carried out. Antimicrobial screening was by broth

microdilution and bioautography. The presence of terpenoid and flavonoids in some herbs were inferred from the TLC fingerprints. Most of the tested organisms were sensitive to the crude acetone extracts with MIC values ranging from 0.078–2.5 mg/mℓ. Acetone and methanolic extracts of *Aloe striata, Cyathula uncinulata, E. autumnalis* and *P. guajava* were more active against enteropathogens This preliminary study revealed the promising antibacterial activities of some of the selected herbs used in the treatment of diarrhoea and related diseases and corroborated assertions on their efficacies by traditional healers, as reflected in Chapter 5.

Chapter 6 described the separation of compounds from the bioactive ethyl acetate fractions of *C. uncinulata* by solvent-solvent fractionation. This plant was selected based on its antibacterial activity. The structure of the isolated compound was elucidated by nuclear magnetic resonance spectroscopy and mass spectroscopy methods. The NMR spectra of the isolated compound showed that the compound, with formula $C_{22}H_{38}O_7$ and molecular weight 414.5329 had a long aliphatic chain. The isolated compound was described as Glycosylated oleanolic acid, made up of sugar with fatty acyl moiety. The MIC of fractions of extracts ranged from 0.39 to 2.5 mg/mL. The lowest MIC was observed in ethyl-acetate fraction and for the selected sub-fraction of the ethyl acetate (SSC 1) fractionation MIC was 0.63 mg/mL. The MIC of the final purified compound was 0.34 mg/mL, indicating considerable bioactivity; hence, this may serve as template for new antimicrobial formulations.

The study in chapter 7 was carried out to assess the therapeutic safety of some selected medicinal plants. The cytotoxicity of methanol extracts and fractions of six selected plants was determined using a modified tetrazolium-based colorimetric assay [3-(4, 5-dimethylthiazol)-2, 5-diphenyl tetrazolium bromide (MTT) assay]. The in vitro cytotoxicity assay on human hepatocarcinoma cell line (Huh-7) revealed that the methanol extract of *E. autumnalis* had the strongest cytotoxicity with IC_{50} of 7.8 µg/mL. Ethyl acetate and butanol fractions of *C. uncinulata, H. latifolia, E. autumnalis* and *Lantana camara* had lower cytotoxic effects on the cancer cell lines tested with IC_{50} values ranging from 24.8 µg/mL to 44.1 µg/mL while all the fractions of *A. arborescens* and *A. striata* had insignificant or no cytotoxic effects after 72 h of treatment. The results showed that *E. autumnalis* may serve as anticancer agent while the *Aloe* species were apparently safe within the range studied.

The general conclusions and recommendations of the various parts of the findings were captured in Chapter 8. There was increasing multi-drug resistant and emerging resistance to penem drugs among isolates particularly *Salmonella* strains. Molecular analysis showed that beta-lactamase enzymes and class 1 integron *(Int1*) genes played significant roles in antibiotic resistance among *Salmonella* spp. with diverse virulence genes identified among the *Salmonella* and *E. coli* isolates. In addition, *E. coli* strains from apparently asymptomatic subjects harboured considerable virulence genes making them potential reservoirs of factors important in the spread and acquisition of virulence and

drug resistance genes. Among the plants used in ethnomedicine in ORTDM, *A. arborescens*, *A. striata, C. uncinulata, E. autumnalis* and *P. guajava* were noted as potential plants for antibacterial agents. Further fractionation and purification of bioactive components of the ethyl acetate fraction of *C. uncinulata* yielded an isolated compound - glycosylated oleanolic acid, a sugar with fatty acyl moiety which had considerable antibacterial activities. The evaluation assay for therapeutic safety of selected medicinal plants indicated extensive cytotoxicity and selective anticancer activity of *E. autumnalis*, even though it had strong antibacterial activities.

It was recommended that measures be put in place to curtail the spread of drug resistance, particularly the ESBL-type resistance through improved and standardized laboratory practice. Proper and regular surveillance should be maintained to help guide the rational use of antibiotics in empirical treatment and measures should be in place to discourage the use of broad-spectrum antibiotics. The recognition but cautious use of medicinal plants as an alternative therapy and a probable means to mitigate the emerging resistance problem was recommended.

Overall, improved standard of hygiene both in the hospital settings and the communities is paramount to prevention of infections as ‘prevention is better than cure’.

DECLARATION

I, Mary Adejumoke Bisi-Johnson Student Number 208154760, solemnly declare that this thesis entitled "Molecular characterization, antibiograms and antibacterial activities of selected medicinal plants against some enteric pathogens" constitutes the culmination of original studies undertaken by me and that the report has not been submitted to any other institution. All sources used or quoted in the study have been indicated and acknowledged by way of complete references.

DOCTORAL CANDIDATE: MARY ADEJUMOKE BISI-JOHNSON

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DATE: 18/8/2011

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TABLE OF CONTENTS

	Page
Title	i
General Summary	ii
Declaration	viii
Declaration on plagiarism	ix
Acknowledgments	x
Table of contents	xiii
List of tables	xviii
List of figures	xx
List of appendices	xxii
List of abbreviations	xxiii
List of publications from study	xxv

Chapter 1: General introduction and literature review

1.1. Introduction 1

1.2. Literature survey 6

1.2.1. Diarrhoea as a Disease 6

1.2.2. Forms of Diarrhoea 7

1.2.2.1. Acute Watery Diarrhoea 7

1.2.2.2. Acute Bloody Diarrhoea 8

1.2.2.3. Chronic or Persistent Diarrhoea 8

1.2.3. Causes of Diarrhoea 9

1.2.3.1. Non-infectious agents of diarrhea 10

1.2.3.2. Infectious agents of Diarrhoea 10

I. *Salmonella* 11

II. *Shigella* 17

III. *Escherichia coli* 21

1.2.4. Treatment of Diarrhoea 28

1.2.5. Some considerations in antibiotic therapy of enteric bacteria 35

1.2.6. Mechanism of antibacterial resistance 41

1.2.7. Trends in emergence and spread of antibiotic resistance 46

1.2.8. Consequences of antibiotic resistance..... 48
1.2.8.1. Morbidity and Mortality implications of antibiotic Resistance..... 48
1.2.8.2. Cost implications of antibiotic resistance 49
1.2.9. Prevention of diarrhoea..... 50
1.2.10. Medicinal plants: alternative/complementary diarrhoea therapy..... 53
1.2.10.1. Modes of action of medicinal plants..... 54
1.2.10.2. Further benefits of herbal plants..... 56
1.2.10.3. Safety issues in medicinal herb therapy..... 58
1.3. Objective of the Study..... 62
1.4. Approach..... 63
1.5. References..... 64
Chapter 2: Extended-spectrum beta-lactamases and class I integron mediated resistance in clinical isolates of *Escherichia coli* and *Salmonella*..... 96
2.0 Abstract..... 96
2.1. Introduction..... 97
2.2. Materials and methods..... 101
2.2.1. Data collection and ethical approval..... 101
2.2.2. Bacterial isolates..... 101
2.2.3. Identification and characterization of resistance genes..... 102
2.2.4. DNA extraction and primer synthesis..... 102
2.2.5. DNA amplification..... 102
2.2.6. Sequencing reaction..... 103
2.2.7. Statistical analysis..... 105
2.3. Results..... 105
2.3.1 Demographic features..... 105
2.3.2 Characterization and antibiogram of isolates..... 105
2.3.3 Detection of genes mediating ESBL resistance..... 106

2.4. Discussion..... 110
2.5. Conclusion..... 113
2.6. References..... 114
Chapter 3: Molecular basis of virulence in clinical isolates of *Escherichia coli* and *Salmonella* spp. from a tertiary hospital in the Eastern Cape, South Africa..... 119
3.0 Abstract..... 119
3.1. Introduction..... 120
3.2. Methods..... 124
3.2.1. Specimens' collection and bacterial isolation..... 124
3.2.2. Bacteriological, biochemical analyses and antibiotic susceptibility test 125
3.2.3. DNA extraction..... 126
3.2.4. DNA amplification..... 126
3.2.5. Sequencing reaction..... 126
3.3. Results..... 129
3.3.1. Bacteriological identification and molecular analysis..... 129
3.4. Discussion..... 131
3.5. Conclusion..... 137
3.6. References..... 137
Chapter 4: An ethnobotanical survey of indigenous plants used as diarrhoea remedies in the O.R. Tambo district municipality, Eastern Cape Province, South Africa 146
4.0 Abstract..... 146
4.1. Introduction..... 147
4.1.1. A brief description of O.R. Tambo District unicipality..... 149
4.2. Materials and methods..... 153
4.3. Results..... 154
4.4. Discussion..... 155
4.5. References..... 157
Chapter 5: Phytochemical analysis and antibacterial screening of crude extracts of some herbal plants of the Eastern Cape Province, South 174

Africa.....
5.0 Abstract..... 174
5.1. Introduction..... 175
5.2. Materials and Methods..... 179
5.2.1. Plant material..... 179
5.2.2.1. Preparation and extraction..... 179
5.2.3. TLC analysis of extracts..... 181
5.2.4. Determination of minimum inhibitory concentration..... 182
5.2.5. Bioautographic assay..... 183
5.2.5.1. Preparation of TLC plates for bioautography..... 183
5.2.5.2. Preparation of bacteria..... 184
5.2.5.3. Bioassay method..... 184
5.3. Results 184
5.4. Discussion 189
5.5. Conclusion 193
5.6. References..... 194
Chapter 6: Isolation, characterization and structural elucidation of bioactive fractions of *Cyathula uncinulata* (Schrad.) Schinz..... 199
6.0. Abstract..... 199
6.1. Introduction..... 200
6.2. Materials and methods..... 203
6.2.1. Plant collection..... 203
6.2.2 Extraction of plant material..... 204
6.2.3. Solvent-solvent fractionation of dried plant extract..... 204
6.2.4. TLC fingerprinting of fractions..... 205
6.2.5. Antimicrobial assay of fractions..... 206
6.2.6. Isolation of pure compounds..... 207
6.2.7. Preparation of pure compound for NMR analysis..... 207
6.3. Results..... 207
6.3.1. Solvent-solvent fractionation yield..... 207

6.3.2. TLC fingerprints 208
6.3.3. Minimum inhibitory concentrations of the fractions..... 212
6.3.4. Identification and elucidation of structure of the isolated compound..... 212
6.4. Discussion..... 212
6.5. Conclusion..... 214
6.6. References..... 215
Chapter 7: Cytotoxicity of selected plants used in ethnomedicine in the Eastern Cape Province, South Africa..... 219
7.0. Abstract..... 219
7.1. Introduction..... 220
7.2. Materials and methods..... 222
7.2.1. Plant material, extraction and fractionation..... 222
7.2.2. Antibacterial assay 222
7.2.3. Cytotoxicity assays..... 224
7.3. Results..... 225
7.3.1. Antibacterial activities..... 225
7.3.2. Cytotoxicity of methanol extracts and fractions..... 226
7.4. Discussion..... 228
7.5. Conclusion..... 232
7.6. References..... 232
Chapter 8: General discussion and recommendations. 237
8.1. General conclusions..... 237
8.2. General recommendation..... 239

LIST OF TABLES

Chapter	Title	Page
Chapter 1		
Table 1.1.	Summary of enteric pathogens by group……..	12
Table 1.2.	Summary of diarrhoeagenic *E. coli* pathotypes and clinical manifestations……..	22
Table 1.3.	Some antibiotics that are used in the treatment of enteric infections and their mechanism of action……..	36
Table 1.4.	Some medicinal plants commonly used as diarrhoea remedy in South Africa……..	55
Chapter 2		
Table 2.1.	Primer sets for ESBLs and class I integron and the PCR conditions……..	104
Chapter 3		
Table 3.1.	Primer sets for the pathotypes and virulence genes of *E. coli* and *Salmonella* spp……..	127
Table 3.2.	Distribution of virulence genes among the *E. coli* and *Salmonella* spp……..	130
Chapter 4		
Table 4.1.	Herbal remedies used for the treatment of diarrhoea and associated ailments……..	165
Chapter 5		
Table 5.1.	Names, plant parts and Traditional usage of herbs investigated…	180
Table 5.2.	Average MIC values of plant extracts at 2 h and 24h……..	190

Chapter 6

Table 6.1. Quantity of the fractions extracted from solvent-solvent fractionation of methanolic extract of *C. uncinulata* leaves..... 208

Table 6.2. Average MIC values of the fractions of *C. uncinulata* leaf extracts tested against *E. coli* isolate..... 208

Chapter 7

Table 7.1. Selected plants investigated and their usage..... 223

Table 7.2. Inhibitory concentration, IC_{50} (ug/mℓ) of selected herbs..... 227

LIST OF FIGURES

Chapter	Title	Page
Chapter 2		
Figure 2.1.	PCR products analysis of the genomic DNA from selected *E. coli* and *Salmonella* isolates.....	108
Figure 2.2.	Blast search result for the sequenced integron genes from selected *Salmonella* isolates.....	109
Figure 2.3.	A neighbour-joining phylogenetic relationship of the class 1 integron genes isolated from *Salmonella* spp.....	110
Chapter 3		
Figure 3.1A.	Frequency of the various *Salmonella* isolates.....	129
Figure 3.1B.	Frequency of the diarrhoegenic *E. coli* isolates.....	130
Figure 3.2.	Representative gels for PCR amplification of DNA extracted from selected *E. coli* and *Salmonella* isolates showing the presence of diverse virulence genes.....	132
Chapter 4		
Figure 4.1.	Map of O.R. Tambo District Municipality	163
Figure 4.2.	WHO Cholera endemic zone.....	164
Chapter 5		
Figure 5.1.	A hypothetical TLC plate showing parameters for R_f value.....	183
Figure 5.2.	TLC separation of components of the different plant extracts with BEA as eluent and vanillin-sulphuric acid spray reagent.....	185
Figure 5.3	TLC separation of components of the different plant extracts with CEF as eluent and vanillin-sulphuric acid spray reagent.....	185
Figure 5.4.	TLC separation of components of the different plant extracts	

with EMW as eluent and vanillin-sulphuric acid spray reagent..... 186
Figure 5.5. Bioautograms of components of the different plant extracts with CEF as eluent sprayed with *S. Typhimurium*..... 186
Figure 5.6A. TLC separation of components of the different plant extracts with CEF as eluent viewed under UV 254 nm..... 187
Figure 5.6B. TLC separation of components of the different plant extracts with CEF as eluent viewed under UV 366 nm..... 187

Chapter 6

Figure 6.1A. Leaves of *C. uncinulata* plant (Picture taken from source garden at Flagstaff, Eastern Cape, South Africa)..... 204
Figure 6.1B. *C. uncinulata* showing the plant flowering 204
Figure 6.2. Schematic diagram of solvent-solvent fractionation of *C. uncinulata* 206
Figure 6.3. TLC fingerprints of ethyl acetate fraction of *C. uncinulata*..... 209
Figure 6.4. A-G TLC chromatograms of fractions obtained from open column chromatography of ethyl acetate fraction of the methanol extract of *C. uncinulata*..... 210

Chapter 7

Figure 7.1. Minimum inhibitory concentration of plant extracts and fractions..... 226

LIST OF APPENDICES

Appendix	Title	Page
Appendix 1A.	Volunteer information sheet.....	240
Appendix 1B.	Volunteer information sheet (Xhosa language).....	242
Appendix 2A.	Informed consent.....	244
Appendix 2B.	Informed consent (Xhosa language).....	245
Appendix 3	Survey information from traditional healers on herbal plants used in traditional treatment of diarrhoea.....	246
Appendix 4A.	NMR spectrum for the isolated compound (SSC3-9-10).....	248
Appendix 4B.	Mass spectrum 1 for the isolated compound.....	249
Appendix 4C.	Mass spectrum 2 for the isolated compound.....	250
Appendix 4D.	Structure of the isolated compound.....	251

LIST OF ABBREVIATIONS

Bla	Beta-lactamase gene
CLSI	Clinical and Laboratory Standards Institute (formerly NCCLS)
DAEC	Diffusely adherent *E. coli*
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	Extended-spectrum beta-lactamase
ETEC	Enterotoxigenic *E. coli*
HIV	Human immunodeficiency virus
h*R*f.	100fold retention factor value
IC_{50}	50% inhibitory concentration
INT	*p*-iodonitrotetrazolium salt
Int1	Class 1 integron
L	Litre (s)
MDG	Millennium Development Goals
MIC	Minimum inhibitory concentration
MTT	modified tetrazolium-based colorimetric assay (3-(4, 5-dimethylthiazol)-2, 5-diphenyl tetrazolium bromide assay).
NCCLS	National Committee for Clinical Laboratory Standards (Now CLSI)

NMAHC	Nelson Mandela Academic Hospital Complex
NMEC	Neonatal meningitis *E. coli*
NMR	Nuclear magnetic resonance spectroscopy
ORTDM	Oliver R. Tambo District Municipality
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
R_f value	Retention factor
S	Second (s)
TLC	Thin layer chromatography
UPEC	Uropathogenic *E. coli*
UV	Ultraviolet
WHO	World Health Organization

LIST OF PUBLICATIONS

Publications

Mary A Bisi-Johnson, Chikwelu L Obi, Sandeep D Vasaikar, Kamaldeen A Baba, Toshio Hattori. Molecular basis of virulence in clinical isolates of *Escherichia coli and Salmonella* species from a tertiary hospital in the Eastern Cape, South Africa. BMC Gut Pathogens 2011, 3:9 doi:10.1186/1757-4749-3-9.

Mary A Bisi-Johnson, Chikwelu L Obi, Toshio Hattori, Yoshiteru Oshima, Shenwei Li, Learnmore Kambizi, Jacobus N Eloff, Sandeep D Vasaikar. Evaluation of the antibacterial and anticancer activities of some South African medicinal plants. BMC Complementary and Alternative Medicine, 2011, 11:14 doi:10.1186/1472-6882-11-14.

Bisi-Johnson MA, Obi CL, Kambizi L and Nkomo M. A survey of indigenous herbal diarrhoeal remedies of O.R. Tambo district, Eastern Cape Province, South Africa. Afr J Biotechnol. 2010 9(8): 1245-1254.

Papers Submitted for Publication

Bisi-Johnson MA, Obi CL, Eloff JN, Samuel BB, Hattori T, Oshima Y, Kambizi L, Vasaikar SD. Antibacterial screening of the crude extracts of selected herbal plants used in ethnomedicine in Eastern Cape, South Africa. African Journal of Microbiological Research, 2011.

Mary A Bisi-Johnson, Chikwelu L Obi, Sandeep D Vasaikar, Kamaldeen A Baba, Toshio Hattori. Extended-spectrum beta-lactamases and Class 1 integron mediated resistance in clinical isolates of *Salmonella* species. Annals of Clinical Microbiology and Antimicrobials, 2011.

Conferences and proceedings

2008

1. Bisi-Johnson MA, Obi CL, Kambizi L and Vasaikar S. 2008. A survey and preliminary screening of herbal plants used in the treatment of diarrhoea in Mthatha and environs. First Walter Sisulu University Symposium, 2nd to 4th September 2008, held at Health Resource Centre, Mthatha, Eastern Cape, RSA.

2009

2. Bisi-Johnson MA, Obi CL, Kambizi L and Eloff JN. 2009. Antibacterial properties of some medicinal plants traditionally used to alleviate stomach ailments in the Eastern Cape. Indigenous Plant Use Forum (IPUF) held at Stellenbosch on 6-7 July 2009. p.29.

3. Bisi-Johnson MA, Obi CL, Kambizi L, Eloff JN, Samuel BB, Hattori T, Vasaikar S and Baba K. 2009. Ethnobotanical survey of herbal diarrhoeal remedies and antibacterial activity of some selected plants. 2nd Walter Sisulu University International Conference, East London, South Africa. 25-27 August 2009. p. 15.

4. Bisi-Johnson MA, Obi CL, Vasaikar S, Baba K, Kambizi L. 2009. Can Herbal Remedies be the answer to multidrug resistance? Profile of drug resistance in *Salmonella* species in South Africa. American Society for Microbiology (3rd ASM salmonella Conference), France 3-8 Oct 2009. p. 65.

2010

5. Bisi-Johnson MA, Obi CL, Kambizi L, Eloff JN, Samuel B, Hattori T, Vasaikar S and Baba KA. 2010. Phytochemical and biological screening of crude extracts of selected plants used in the treatment of diarrhoea and related stomach ailments. Japan Society for the Promotion of Science/ National Research Foundation, International symposium on AIDS and Tuberculosis (ISAT 2010) Gonryo Kaikan, Aobaku, Sendai, Japan. January 13-14, 2010. p. 7.

6. Bisi-Johnson MA, Obi CL, Hattori T, Vasaikar S, Baba K. 2010. Diversity of Extended Spectrum Beta-Lactamases in Clinical Isolates of *Salmonella* from the Eastern Cape, South Africa. Walter Sisulu University 3rd International Conference, held at Health Resource Centre, Mthatha, Eastern Cape, South Africa on 18-20 August 2010. p. 55.

7. Bisi-Johnson MA, Obi CL, Hattori T, Oshima Y, Kambizi L, Eloff JN, Li S. 2010. Evaluation of the therapeutic safety of some South African medicinal plants. Walter Sisulu University 3rd International Conference, held at Health Resource Centre, Mthatha, Eastern Cape, South Africa on 18-20 August 2010. p. 213.

8. Bisi-Johnson MA, Obi CL, Hattori T, Oshima Y, Kambizi L, Eloff JN, Li S. 2010. Profiling of South African medicinal plants for their safety. 38th Physiological Society of Southern Africa Conference, East London 27-29 Sept, 2010. p. 32.

CHAPTER 1

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Diarrhoeal diseases remain one of the greatest health problems in many parts of the world. In some cases, the disease is mild and self-limiting; however, the symptoms may be very severe in the elderly and young children (Smith and Cheasty, 1998), as well as in immunocompromised patients such as HIV/AIDS patients. The disease is one of the hallmarks of HIV/AIDS in developing countries and is also a cardinal clinical manifestation of water borne infections (Obi *et al.,* 2007). According to a World Health Organization report (WHO, 2004), diarrhoea was rated second, after respiratory infections out of the eight deadliest diseases worldwide and was responsible for 1.8 million deaths per year. This infectious disease which can cause dehydration is primarily a symptom of gastrointestinal infection but chemical irritation of the gut or non-infectious bowel disease can also result in diarrhoea.

Studies have shown that the predominant causative agents of acute and persistent diarrhoea are Gram-negative rods such as *Escherichia coli, Vibrio cholerae, Aeromonas, Campylobacter, Salmonella species, Shigella species, Plesiomonas shigelloides* and *Yersinia enterocolitica* (Obi *et al.,* 1995, 1998, 2003; Lainson and Silva, 1999; Coker *et al.,* 2002; Oyofo *et al*., 2002). Aside from bacterial agents of diarrhoea, other causative pathogens include protozoa

such as *Giardia lamblia* and *Cryptosporidium parvum;* viruses such as norwalkvirus and rotavirus. Although fungal agents such as *Candida* have been shown to be prevalent in children with diarrhoea (Enweani *et al.* 1994), a more recent study (Forbes *et al.,* 2001), associated higher *Candida* counts with recent antibiotic use. Faecal concentrations of *Candida* were higher in patients with diarrhoea but the study confirmed no association between faecal candida or other yeasts and diarrhoea. Norwalk viruses, calci-like viruses and rotaviruses are the major viral agents of diarrhoea. According to Parashar *et al.,* (2006), rotavirus is the leading cause of diarrhoeal hospitalization among children worldwide, causing 440,000 annual deaths in children under 5 years of age.

Production of disease by enteropathogens often involves specific serotypes of the organisms and special virulence factors. Some arsenals for pathogenicity include ability to adhere to host surfaces, production of special proteins for invasion and colonization. In addition, some pathogens are able to produce virulent factors which incapacitate the host's immune system defenses or those that poison host cells and cause tissue damage. Studies have shown that some of the pathogenic factors of enterobacteria are genetically modulated. The mechanisms of diarrhoea induction and role in the development of diarrhoeal syndrome of several genes associated with pathogenicity islands (PAIS): adhesins, hlyA, hlyB (hemolysin), sfaG (fimbria antigen type S), cnf1 (cytotoxic necrotizing factor 1), estB (heat-stable enterotoxin B), bacterial modulins, cytotonic and cytotoxic enterotoxins, including LT, ST, Shiga-like and

cytolethal toxins have been described (Mavzutov *et al.,* 2007; Zherebtsova *et al*., 2007). Virulence factors of *Salmonella* and *Shigella* species are complex and encoded both on the organism's chromosome and on large (34-120 kd) plasmids. Several *Salmonella* pathogenicity islands have been identified that mediate uptake of the bacteria into epithelial cells (type III secretion system), non-phagocytic cell invasion (*Salmonella* pathogenicity-island 1), and survival and replication within macrophages (*Salmonella* pathogenicity-island 2, phoP/phoQ) (Finlay, 1994; Darwin and Miller, 1999, Kato *et al.,* 2008; Charles *et al.,* 2009). *Shigella* species cause damage by two mechanisms, invasion of the colonic epithelium, which is dependent on a plasmid-mediated virulence factor (ipa protein, invasin), and production of enterotoxin (ShET 1 and ShET 2), secreted by the organism. These have been shown to cause small bowel secretion and watery diarrhoea, which is not essential for colitis but enhances virulence (Wolf and Gianella, 1996; Bernasconi *et al.,* 2004). Verotoxin, which enterohemorrhagic *E coli* and *Shigella* species produce, causes systemic disorders such as seizures and hemolytic-uremic syndrome (HUS) (Trachtman and Christen, 1999).

Increasing resistance to the fluoroquinolones and the third-generation cephalosporins is of major concern among members of the Enterobacteriaceae. The susceptibility patterns of enteric bacteria to various antibiotics as reported in sub-Saharan Africa, (Sinha *et al.,* 2004; Maraki *et al.,* 2005; Yismaw *et al.,* 2006; Obi *et al.,* 2007), showed a slow decline in fluoroquinolone susceptibility. Over

90% of all the organisms isolated from a cohort study in Limpopo (Obi *et al.,* 2007), showed resistance to penicillin, cloxacillin and amoxicillin but conversely, almost all the organisms were sensitive to ciprofloxacin, gentamycin, meropenem and imipenem. On the contrary, decrease in fluoroquinolone susceptibility has been more dramatic in other areas of the world such as Latin America and Southern Europe (Jones *et al.,* 1997). Several mechanisms are involved in the antibiotic resistance of members of the Enterobacteriaceae; these include mutations involved in enzyme productions (Jones *et al.,* 1997; Jones, 2003). Enterobacteriaceae commonly express plasmid-encoded β-lactamases (mostly TEM-1, TEM-2, and SHV-1) that confer resistance to penicillins (Maurine *et al.,* 2002). Another type of resistance affecting third-generation cephalosporins and aztreonam (a monobactam) has been the hyperproduction of an Amp C cephalosporinase by some of the enteric strains (Jones *et al.,* 1997). The resistance to extended-spectrum cephalosporin among the Enterobacteriaceae has become a growing problem (Bradford *et al.,* 2001).

Another factor which confers antibiotic resistance to clinical isolates is class 1 integron (Leverstein-van Hall *et al.,* 2003; Segal *et al.* 2003). Most resistance integrons belong to class 1. Integrons are genetic elements composed of a gene encoding an integrase, gene cassettes and an integration site for the gene cassettes (Fluit and Schmitz, 2004). These DNA elements play important role in the spread of antibiotic-resistance genes. Recently, the discovery of larger integron structures, termed super-integrons has broadened bacterial genome

(Mazel, 2006). Integrons can be broadly divided into two: the resistance integrons and super-integrons. Several reports have linked Class 1 integrons to resistance in various Gram negative genera. This genetic element was detected and characterized in various serotypes of *Salmonella enterica* (Brown *et al.,* 2000; Guerra *et al*., 2001); *Shigella* (Navia *et al.,* 1999; McIver *et al.,* 2002), *Escherichia* (Mazel *et al*., 2000; Zhao *et al.,* 2001). An integron-like drug resistance region and a plasmid-borne class 1 integron was found on the transferable R-plasmid pJA8102-1 from *Aeromonas salmonicida* and an atypical *A. salmonicida* respectively (Inglis *et al.,* 1993; Barnes *et al.,* 1994). Class 2 integrons are embedded in the Tn7 family of transposons and consist of an integrase gene followed by gene cassettes (Radstrom *et al.,* 1994). Integrons belonging to class 2 have also been reported in *Salmonella* (Orman *et al.,* 2000) and *Shigella* (Gonzalez *et al.,* 1998). Class 3 integrons have been described in *Pseudomonas aeruginosa, Serratia marcescens, Alcaligenes xylosoxidans, P. putida* and *Klebsiella pneumoniae* isolates from Japan (Izumiya *et al.,* 2000).

With the emerging multiple antibiotic resistance saga, there has been a paradigm shift to natural products particularly medicinal plants for succor. A large proportion of the populace of developing countries employ the use of traditional medicine, alone, or in combination with Western drugs to treat a wide variety of ailments including diarrhoea (Taylor *et al.,* 2001). In South Africa, traditional medicine is well recognized and different communities use a wide variety of plants to treat gastrointestinal disorders such as diarrhea and infection

by intestinal parasites, which are particularly prevalent in rural areas (McGaw *et al.,* 2000). Many studies have documented the anti-enteric bacterial activities of some medicinal plants, *Herba pogostemonis* extracts (Liu *et al.,* 1999), *Roureopsis obliquifoliolata* and *Epinetrum villosum* (Longaga *et al.,* 2001), *Aegle Marmelos* (Correa) Linn. root extract (Mazumder *et al.,* 2006), *Zornia milneana* (Papilionoideae) (Samie *et al.,* 2007).

The importance of enteric bacteria such *as Salmonella, Shigella* and *E. coli* as agents of diarrhoeal disease is of great significance but, their involvements in diarrhoea cases in this local setting of Eastern Cape Province, South Africa, including associated virulence factors and genes coding for antibiotic resistance in these enteric bacterial species particularly at the molecular level have not been extensively investigated.

1.2. Literature survey

1.2.1. Diarrhoea as a Disease

Of major public health concern are diarrhoeal diseases, particularly with regards to the developing countries where they are the leading cause of childhood morbidity and mortality. Diarrhoea, most commonly caused by gastrointestinal infections occurs world-wide and causes 4% of all deaths and 5% of health loss to disability (WHO, 2000). Annually, there are approximately 4 billion cases of diarrhoea worldwide which kill around 2.2 million people, mostly children in developing countries (WHO and UNICEF, 2000). Diarrhoea goes hand

in hand with hygiene, food and water quality. It has been reported that 88% of diarrhoeal diseases are attributed to unsafe water supply, inadequate sanitation and hygiene (WHO, 2004). In the developing world, sporadic outbreaks of diarrhoea are still a common feature despite advances in health interventions.

Diarrhoea has been defined as the passage of loose, watery stools occurring more than three times in one day or the passage of loose or liquid stools more frequently than is normal for the individual (WHO, 2000). The disease may be mild and lasting a day or two and self-resolves without any special treatment. However, prolonged diarrhoea can be a sign of other problems. Diarrhoea can cause dehydration, which means the body lacks enough fluid to function properly. Dehydration is particularly dangerous in children and the elderly, and it must be treated promptly to avoid serious health problems. Likewise, diarrhoea has been associated with other infections such as malaria, measles, HIV/AIDS, pneumonia, systemic infections and meningitis (Reisinger *et al.,* 2005).

1.2.2. Forms of Diarrhoea

Diarrhoea comes in different forms depending on the type of infection; it may be mucoid, watery or passed with blood.

1.2.2.1. Acute Watery Diarrhoea

Acute diarrrhoea is often a result of gastric flu (gastroenteritis) which is usually of viral origin. The duration of acute diarrhea is usually less than 14 days and its associated symptoms are abdominal pain, nausea, vomiting and fever.

Gastroenteritis is caused in a few cases by bacteria and this is often times more fatal than viral diarrhea. The causative agents of acute watery diarrhea are viruses usually rotavirus in infants under 5 years (Greenberg and Estes, 2009) and norovirus in adults (Patel *et al.,* 2009). Bacterial agents of gastroenteritis include enterotoxigenic *E. coli* and *Vibro cholerae* 01. Acute diarrhea can complicate nutritional status in children leading to malabsorption of nutrients and long-term effects of growth failure and malnutrition (Black *et al*., 1984; Lifshitz, 1989).

1.2.2.2. Acute Bloody Diarrhoea

This is often passage of loose stool with visible blood. It is termed dysentery and is an indication of invasion of bowel tissue. Complications of dysentery include intestinal damage, sepsis, malnutrition and dehydration may also occur. The main causative agents of bloody diarrhea are *Shigella* spp., *Salmonella* spp., *E. coli* 0157, *Campylobacter* spp., and *Entamoeba histolytica* (Goldsmid, 2007). Ten percent of all episodes and 15% of deaths respectively from diarrhoea in children less than 5 years of age worldwide are due to acute bloody diarrhea (Esona *et al.,* 2003).

1.2.2.3. Chronic or Persistent Diarrhoea

Persistent diarrhea is associated with loose or watery stools with or without visible blood lasting more than 14 days. The main complication of this disease is malnutrition and serious non-intestinal infection; dehydration may also occur. Infective diarrhea may persist with no visible symptoms in which case an

individual becomes a carrier, often the case with *Salmonella* infection. Apart from bacterial agents chronic infective diarrhea may be caused by parasites such as *Cryptosporidium, Giardia lamblia,* worms and amoeba. However, some infants between the ages of 6 months to 3 years have chronic diarrhea for which no apparent medical cause can be found (Encyclopedia of Childhood and Adolescence, n.d). Chronic non infective diarrhea usually depicts more severe medical conditions mainly malabsorption in its various forms. These conditions which impede normal digestion include ciliac disease, in which the wheat protein gluten is intolerable to the gut. Others are cystic fibrosis, lactose intolerance, pernicious anemia and fibrosis due to cancer therapy. Antibiotic therapy may favour proliferation of the bacterium, *Clostridium difficile.* This bacterium can produce a toxin that damages the lining of the colon and causes mild to severe diarrhea (Encyclopedia of Childhood and Adolescence, n.d).

1.2.3. Causes of Diarrhoea

Diarrhoea though primarily a symptom of gastrointestinal infection, chemical irritation of the gut or non-infectious bowel disease can also result in this disease. Infectious diarrhoea is caused by a host of bacterial, viral and parasitic organisms most of which can be spread as a result of poor water quality (WHO, 2000). It is more common when there is a shortage of clean water for drinking, cooking and cleaning and basic hygiene is important in prevention. Water contaminated with human faeces such as municipal sewage, septic tanks and latrines is of special concern. Food is another major source of infectious

diarrhoea when it is prepared or stored in unhygienic conditions. Food contamination by irrigation water, fish and seafood from polluted water may also contribute to diarrhea and enteric infections (CDC, 2005b; Newman, 2005; WHO, 2007). Diarrhoea can also spread from person to person, aggravated by poor personal hygiene (WHO, 2000).

1.2.3.1. Non-infectious agents of diarrhea

According to Levy (1988), diarrhoea as a result of non-infectious agents includes:

i. Carbohydrate intolerance:
   a. Primary enzyme deficiencies (e.g. Lactose intolerance)
   b. Glucose-galactose malabsorption (e.g. immune disorders, mucosal diseases)
ii. Gastrointestinal allergies to Cow's milk protein, soy milk or gluten (wheat protein)
iii. Pancreatic insufficiency: cystic fibrosis
iv. Immune deficiencies: hypogamma globulinemia, IgA deficiency
v. Metabolic: Abetalipoproteinemia, familial chloride diarrhoea, acid lipase deficiency.
vi. Anatomic abnormalities: Enteric fistula, short bowel, pseudo-obstruction

1.2.3.2. Infectious agents of Diarrhoea

Enteric pathogens implicated in cases of diarrhoea may be bacterial, viral, fungal, parasitic or protozoal. Another infectious agent which has been

implicated particularly in food-related infection is prion (Mead *et al,* 1999; Collinge, 2001; Smith and Bradley, 2003). Table 1 below is a slight modification of various enteric pathogens with their clinical manifestations as summarized by Goldsmid (2007). However, the scope of this study is bacterial enteric pathogens and rather than providing a general overview of infectious agents of diarrhoea, this section of the review will focus on three enteric bacterial pathogens with vast human morbidity worldwide (Buzby and Roberts, 2009):

- *Salmonella*
- *Shigella*
- *Escherichia coli*

I. *Salmonella*

The salmonellae are actively motile, gram-negative facultative, intracellular anaerobes which do not ferment lactose, but most form H_2S or gas from carbohydrate fermentation. As with the closely related bacterium *Escherichia coli*, salmonellae are potential enteric pathogens and are leading causes of bacterial foodborne illness (Klotchko and Wallace, 2009). *Salmonella* species are also of public health significance worldwide causing a wide spectrum of disease ranging from a gastroenteritis, enteric or typhoid fever (caused by *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi serotypes), bacteremia, osteomyelitis and enterocolitis (primarily *S. enterica* serovar Typhimurium and *S. enteritidis*), to a convalescent lifetime carrier state (Owens, 2009; Klotchko and Wallace, 2009).

Table 1.1. Summary of enteric pathogens by group

PATHOGEN	COMMENTS
Bacteria	
Aeromonas spp.	Can be dysenteric and/or persistent
Bacillus cereus	Food poisoning – often associated with rice
Clostridium perfringens type A	Food poisoning
C. perfringens type C	Enteritis necroticans
C. botulinum	May get diarrhoea in early stages
C. difficile	Antibiotic associated, may be bloody in pseudomembranous colitis
Enteroaaggregative *E. coli* (*EAggEC*)	May cause persistent diarrhoea
Enterohaemorrhagic *E. coli* (*EHEC*)	May cause a bloody diarrhoea; can proceed to haemolytic uraemic syndrome (especially serotype 0157:H7)
Enteroinvasive *E. coli* (*EIEC*)	May cause bloody diarrhoea
Entertoxigenic *S. aureus*	Food poisoning intoxication
Listeria monocytogenes	Occasional cause of diarrhoea
Plesiomonas shigelloides	Can sometimes cause dysenteric illness
Salmonella typhi *S. paratyphi*	Presents initially with constipation but may develop diarrhoea later in infection
Vibrio cholerae 01:0139	Rare in returned travelers
Noncholera vibrios	Some associated with seafood
Yersinia enterocolitica	Mesenteric lymphadenitis; may cause bloody diarrhoea
Virus	
Adenovirus	Causes severe diarrhoea with dehydration
Astroviruses	Often transmitted via food than other viruses
Caliciviruses	Often transmitted via food than other viruses
Hepatitis A, Hepatitis E	Infect the gastrointestinal or respiratory tracts causing a wide range of illness, including diarrhoea, fever, hepatitis, paralysis, meningitis and heart disease Have the human liver as the primary target of replication and give rise to hepatitis or inflammation of the liver
Norovirus	Causes stomach flu, vomiting, diarrhea by inflammation of the stomach and intestines, and some stomach cramping
Norwalk-like viruses	Often transmitted via food than other viruses

Rotavirus	Common cause of severe viral diarrhoea in infants and young children under 5 years old
Protozoa	
Balantidium coli	Zoonotic – causes colitis and dysentery
Blastocystis hominis	If large numbers present in faecal specimen and no other cause of diarrhoea detected
Crptosporidium parvum	Causes enteric illness that has received much attention as an infection of immunocompromised persons
Cyclospora	Emerging as opportunistic pathogens associated with diarrhoea.
Entamoeba histolytica	Mucoid diarrhoea commonly streaked with some amount of blood.
Dientamoeba fragilis	Causes low-grade inflammation coupled with mucous diarrhea and gastrointestinal disturbance
Giardia intestinalis/lamblia	Symptoms include diarrhoea (fatty, yellowish) weakness, weight loss, abdominal pain
Isospora belli	Watery diarrhoea in children and patients with AIDS
Microsporidia	Emerging as opportunistic pathogens associated with diarrhoea
P. falciparum	The initial presentation of complicated malaria may be diarrhoea-like illness
Helminths	
Schistosoma mansoni	Causes intermittent loose stools
S. japonicum	Causes fever, aching, cough, diarrhea, or gland enlargement
Strongyloides stercoralis	Especially in cases of internal autoinfection
Taenia spp.	Beef and pork tapeworms occasionally
Trichuris trichiura	With very heavy worm loads, may be bloody
Trichinella spp.	Diarrhoea in early infection

Salmonella infections may be categorized into four clinical types. Firstly, gastroenteritis (ranging from mild to fulminant diarrhoea, nausea and vomiting) typical in most individuals infected with *S. enterica* serovar Typhimurium (Honish, 2000), secondly, bacteraemia or septicaemia (high spiking fever with positive blood cultures), thirdly, enteric fever (mild fever and diarrhoea) and lastly, a carrier state in persons with previous infections. Symptoms of gastroenteritis appear 4 to 5 days after ingestion of contaminated food or water.

Diarrhoea lasts 3 to 5 days and is accompanied by fever and abdominal pain. *S. enterica* serovars Typhi, Paratyphi A and Paratyphi B can cause enteric fever without any presentation of diarrhoea and are important causes of enteric fever in underdeveloped countries.

Salmonellosis affects both humans and animals but *S. enterica* serovar Typhi is adapted to humans and does not occur in animals while non-typhoidal *Salmonella* serovars (NTS) have a broad vertebrate host range (Gordon, 2008). The dissemination of salmonellosis to various organs depends on the serotype, the size of the inoculum, and the status of the host. NTS display more severe and invasive presentation in immunocompromized individuals especially HIV carriers, including severe and progressive diseases such as chronic granulomatosis disease, blockade of IL-12/ IL-23 /IL-17 and TNF, suppurative foci and bacteremia which may be recurrent (Gordon, 2008). In the small intestine, *Salmonella* ingestion progresses to diffuse mucosal inflammation, edema, and microabscesses (Chatterjee *et al*., 2009). Generally, most NTS do not extend beyond the lamina propria and lymphatics of the gut. Exceptions include *S. enterica* serovar Choleraesuis and *S. enterica* serovar Dublin, which can cause bacteremia with little intestinal involvement (Chiu *et al.,* 2006). *S. enterica* serovar Typhi causes intestinal necrosis which can ulcerate and result in perforation. In addition, this mucosal penetration allows uptake into the draining lymph nodes, contributing to blood stream infections and subsequent invasion of

the liver, spleen, and bone marrow. This process explains the delayed onset of symptoms in *S. enterica* serovar Typhi (Reller, 2008).

Worldwide estimates of NTS range from 200 million to 1.3 billion, with an estimated death toll of 3 million each year (Coburn *et al*., 2007). However, typhoidal salmonellae lead to approximately 2.16 million cases of infections and 216,000 deaths worldwide (Crum *et al*., 2004; Boyle *et al.,* 2007; Sethuraman and Kamat, 2007). In the United States, an annual estimated 1.4 million cases of salmonellosis occur among humans (Mead *et al.,* 1999). Additionally, an estimated 500 people are infected with typhoid *Salmonella* annually (Linam and Gerber, 2007). Typhoid fever is endemic in many developing areas of the world vis-à-vis Africa, Latin America and in particular five Asian countries namely: China, India, Indonesia, Pakistan, and Vietnam (Pickering *et al.,* 2005; Miller *et al.,* 2008).

The genus *Salmonella* is highly polymorphic, occurring in several distinct forms. According to Janda and Abbott (2006), two species of *Salmonella* have been recognized, *S. enterica* and *S. bongori*. Eight subgroups or subspecies have been defined using a variety of molecular methods (Janda and Abbott 1998; Brown e*t al.,* 2002*;* Chan e*t al.,* 2003). However, in 2005, all *Salmonella* species became officially recognized as a single species *S. enterica* based on their close relationship by DNA hybridization studies (Su and Chui, 2007; Reller, 2008). There are currently 2,500 serotypes (serovars) of *Salmonella* (Su and Chui, 2007). The Kauffmann-White scheme for *Salmonella* based on different

combinations of somatic O, surface Vi, and flagellar H antigens have been described (Popoff and Le Minor, 1997; Kenner *et al*., 2000). *S bongori* contains only a single subspecies or subgroup (subgroup V, *bongori*). *S enterica* contains the remaining 7 subgroups, 2 of which contain the clinically relevant salmonellae. *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are the most frequently isolated serovars from food-borne outbreaks throughout the world (Herikstad *et al.*, 2002) particularly from poultry products. Studies in some of the provinces of South Africa have implicated *Salmonella* spp. and other enteropathogens in diarrhoea cases linked especially to water contamination (Obi *et al.,* 2002; 2004).

Whether salmonellosis is confined to the intestinal form or progresses to systemic involvement, the ability of the organism to invade and penetrate intestinal epithelial cells is required (Altier, 2005). This invasion process is not merely a passive consequence of bacterial contact with epithelial cells, but instead requires the active participation of the bacterium, with the expression of numerous bacterial virulence genes. The process of cell invasion requires the production and transport of secreted effector proteins by a type III secretion apparatus encoded in Salmonella pathogenicity island I (SPI-1) (Altier, 2005). In addition, invasion is induced by several factors of the host intestinal milieu. Many invasion regulators have been described, HilA (Lostroh and Lee, 2001), HilC (Schechter *et al.,* 1999), invA gene, a gene associated with the invasive nature of Salmonella (Rahn *et al.,* 1992), InvF (Darwin and Miller, 1999), PhoP/PhoQ

(Bajaj *et al.,* 1996), HilE (Baxter *et al.,* 2003), H-NS (Schechter *et al.,* 2003). PhoP/PhoQ pair is essential to the expression of genes of Salmonella pathogenicity island 2 (SPI-2), which encodes a second type III secretion system. SPI-2 is required for intramacrophage survival, the cell type encountered by Salmonella immediately after the invasion of the epithelium. PhoP/PhoQ also serves to repress SPI-1 genes, a function mediated by hilA (Behlau and Miller, 1993; Bajaj *et al.*, 1996; Fahlen *et al*., 2000). PhoP/PhoQ may thus act as a genetic switch, activating traits required for macrophage survival while repressing those not needed for invasion (Altier, 2005). Reports have shown that mutations in phoP/Q result in a marked decrease in virulence (Fields *et al*., 1989; Miller *et al*., 1993). Other factors involved in Salmonella virulence are the MgtC in *S. enterica* serovar Typhimurium. This is required for growth at low-Mg2+ concentrations and intramacrophage survival. This gene is codified in a conserved region of the *Salmonella* pathogenicity island 3 (SPI-3), and is also present in the chromosome of other *Salmonella* serovars (Retamal *et al.,* 2009).

II. *Shigella*

Shigella, the etiologic agent of bacillary dysentery or shigellosis is a facultative intracellular pathogen. This genus of Gram negative bacteria is composed of four species namely: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei* (also designated as serogroups A to D) (Yang *et al.,* 2005). These are further subdivided into serotypes on the basis of O-specific polysaccharide of the LPS. *Shigella dysenteriae* type 1 produces severe disease

and may be associated with life-threatening complications (Niyogi, 2005). Shigellosis is endemic throughout the world where it is held responsible for some 120 million cases of severe dysentery (Weekly Epidemiological Record, 2005). The annual number of *Shigella* episodes worldwide has been estimated as 165 million, of which more than 100 million occur in the developing world with more than 1 million deaths (Kotloff *et al*., 1999; Dupont, 2005). In developing countries, 69% of shigellosis episodes occur in children under five years of age (Kotloff *et al*., 1999). Although *Shigella* is endemic worldwide, it affects certain populations more than others with varying species predominance in each region. Shigellae are responsible for mortality and or morbidity in high risk populations such as children under five years of age, toddlers in day-care centres, aged individuals, homosexual men and, people in war-ravaged and famine-engulfed region (Thong *et al.,* 2005). The predominant serogroup of *Shigella* circulating in a community appears to be related to the level of socioeconomic development. *S. sonnei* , which has a single serotype, is the causative agent of most shigellosis in industrialized countries where it accounts for 77% of cases (compared to 15% in developing countries), but it also has become predominant in Thailand in recent years (WHO, 2009a). However, *S. flexneri* is endemic in developing countries followed by *S. sonnei.* The frequency of occurrence of *S. dysenteriae* and *S. boydii* are equal. *S. dysenteriae* is seen most often in South Asia and sub-Saharan Africa (Kotloff *et al*., 1999). *S. dysenteriae* type 1, also known as

Shiga bacillus, has been recognized as the major cause of epidemic dysentery for nearly 100 years (WHO, 1999).

Shigella spp. causes dysentery by invading the colonic mucosa and multiplying within colonic epithelial cells ultimately causing mucosal ulceration, inflammation and bleeding. This pathogen is usually transmitted by faecal-oral route via contaminated food and water or through person-to-person contact (WHO, 2009a). Shigellae are considered highly contagious due to the low numbers of infectious inoculum required to cause infection (Dupont *et al.*, 1989). The incubation period for shigellosis is 1 to 4 days but may be up to 8 days in the case of infection due to *S. dysenteria*e (Levine *et al.,* 1973) or last for weeks and months and result in chronic relapsing diarrhoea and a protein-losing enteropathy (Herrington and Taylor, 1991). The initial symptoms of shigellosis are fever, headache, malaise, anorexia, and occasional vomiting. *S. flexneri* and *S. dysenteriae* type 1 infections are usually characterized by frequent passage of small amounts of stool and mucus or blood. Whilst *S. sonnei* and *S. boydii* infections are less severe with watery faeces but little mucus or blood, the watery diarrhoea in *S. dysenteriae* type 1 may progress to typical dysenteric stool. Intestinal complications of shigellosis include toxic megacolon and rectal prolapse. All species of Shigella are known to produce varying quantities of cytotoxins which are biologically similar in properties (Bartlett *et al.*, 1986). However, the term Shiga toxin is reserved for the highly potent protein cytotoxin produced in large quantity by *S. dysenteriae* type 1. Shiga toxin which have been

extensively studied have been shown to consist of a large A subunit and 5 smaller B subunits, a structure similar to those of cholera toxin and heat-labile enterotoxin of *E. coli* (Herrington and Taylor, 1991). Whereas the A subunit is involved in inhibition of protein synthesis, the B subunits are involved in enterotoxic effects and cytotoxicity (Jacewicz *et al.,* 1989). Shiga toxin production is not required for virulence of invasive *S. dysenteriae* strains, but may increase the severity of disease (WHO, 1999). The virulence genes responsible for the pathogenesis of shigellosis may be chromosomal or plasmid-borne. ShET1 and ShET2 are two chromosomal-borne enterotoxin genes which have been incriminated as the putative mediators of the early watery phase of shigellosis (Nataro *et al.,* 1995; Rhee *et al.,* 2001). Other genes responsible for directing epithelial cell penetration by the bacterium and for the modification of host response to infection are ial and ipaH respectively (Hale, 1991; Ménard *et al.,* 1996). Mild infections due to *Shigella* are often self-limiting in healthy individuals but complications may arise in high risk group. Shiga toxin-producing organisms can cause anemia to manifest as hemolytic uremic syndrome in children and as thrombotic thrombocytopenic purpura in adults (Koster *et al.,* 1978). Intestinal perforation is rare but has been reported in association with toxic megacoclon. *Shigella* bacteremia is generally considered an unusual occurrence but has been reported among HIV-infected and other immuno-compromised patients (Batchelor *et al.,* 1996) and may be difficult to eradicate.

III. *Escherichia coli*

In contrast to the essential and beneficial role of most *Escherichia coli* isolates in the human intestine, pathogenic *E. coli* are responsible for a broad spectrum of human diseases. *E. coli* has emerged as an important cause of diarrheal related illnesses, with diverse phenotypes and pathogenic mechanisms. According to the updated report of World Health Organization (2009), numerous types of diarrheagenic *E. coli* have been identified worldwide namely; enteropathogenic (EPEC), enterohaemorragic (EHEC) or Shiga toxin-secreting (STEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), diarrhoea-associated haemolytic (DHEC), entero-aggregative (EAggEC), enteroadherent *E coli* (EAEC) and cytolethal distending toxin-secreting (CDTEC) *E. coli* and diffuse adherent *E. coli* (DAEC) strains. These have been classified on the basis of serological characteristics – O (somatic), H (flagellar), and K (capsular) surface antigen profiles and virulence properties. The prevalence and the burden of diseases of the different pathotypes are uneven (WHO, 2009a). *E. coli,* though a major facultative inhabitant of the large intestine, has become recognized as both a harmless commensal and a versatile pathogen. It is the causative agent of infections ranging from enteric infections, neonatal meningitis, pneumonia, urinary tract infections (UTI), cholecystitis, and bacteremia. Other miscellaneous *E. coli* infections include septic arthritis, endophthalmitis, suppurative thyroiditis, sinusitis, osteomyelitis, endocarditis, and skin and soft-tissue infections (especially in patients with diabetes) (Madappa and Go, 2009). *E. coli* causes 12-

50% of nosocomial infections and 4% of cases of diarrheal diseases. While EAggEC, EAEC and EPEC cause watery *E. coli* childhood diarrhoea which is non-inflammatory and non-bloody, *E coli* dysentery is caused by EIEC or EHEC which is characterized by fever, bloody diarrhea, and dehydration (Madappa and Go, 2009). EPEC has also been described as diarrheagenic *E coli* which produce a characteristic histopathology known as attaching and effacing (A/E) on intestinal cells and that do not produce Shiga, Shiga-like toxins or verotoxins. However, Enterotoxigenic *E coli* (ETEC) strains remain a major cause of infantile diarrhoea

Table 1.2. Summary of diarrheagenic *E.coli* pathotypes and clinical manifestations

E.coli Pathotype	Clinical manifestations
Enterotoxigenic *E coli* (ETEC)	Diarrhea is watery without blood, mucous, or fecal leukocytes and ranges from mild to severe
Enterohemorrhagic *E coli* (EHEC) or Shiga toxin-secreting (STEC)	EHEC infects the large bowel. Disease ranges from mild watery diarrhea to severe hemorrhagic colitis, often accompanied by abdominal cramping and vomiting. Diarrhea becomes bloody in 1-2 days in most patients but is usually not associated with fecal leukocytes. Fever is present in about a third of cases.
Enteroinvasive *E coli* (EIEC)	Causes watery diarrhea, dysentery, fever, vomiting, painful abdominal cramps, and tenesmus. Stools often contain blood and leukocytes.
Enteropathogenic *E coli* (EPEC)	EPEC infects the small intestine, causes watery diarrhea and dysentery. The resultant acute watery diarrhea may cause dehydration or become chronic and lead to failure to thrive.
Enteroaggregative *E coli* (EAEC)	Associated with persistent diarrhea (>14 days), usually watery, secretory and not accompanied by fever or vomiting
Diffusely Adherent E. coli (DAEC)	Majority of patients infected with this pathotype experience vomiting.

in developing countries and of travellers' diarrhoeas in visitors to these countries (WHO, 2009a). Table 1.2 shows a summary of the clinical manifestations of different pathotypes of diarrheagenic *E. coli.*

ETEC are the most frequently isolated bacterial enteropathogen in children below 5 years of age in developing countries, and account for approximately 300 million diarrhoea episodes and 380 000 deaths annually (Black, 1993; Wennerås and Erling, 2004; Steffen *et al.,* 2005). ETEC are the cause in up to 80% of cases of traveller's diarrhoea (Kass, 2005; Peny, 2005). Each year, there are an estimated 10 million cases of LT-related ETEC travellers' diarrhoea worldwide (Steffen *et al.,* 2005) with a median of 42% of travelers' diarrheal episodes in studies in Latin America, 36% in Africa, and 16% in Asia (Black, 1993). Surveillance of hospitalized cases of ETEC diarrhoea has shown that a large proportion of cases also occur in individuals over 10 years of age (WHO, 2009a). ETEC are prevalent in domestic and surface water sources in developing countries. In South Africa, Obi *et al.* (2007) reported a genetic relatedness in *E. coli* from stools of diarrhoeic HIV patients and their household drinking water. Similar result was obtained in Bangladesh by Begum and co-workers (2007) in which case there were antibiotic resistant patterns and clonal similarities between ETEC from the environment and those isolated from the stools of patient. This may explain the endemicity of the disease in these countries. Once attached to the intestinal epithelium, ETEC elaborate both the heat-labile (LT)

and the heat-stable (ST) toxins, which induce the watery diarrhoea (Freytag and Clements, 1999).

E. coli O157 is sorbitol non-fermenting strain of *E. coli* causing Hemorrhagic uremic syndrome (HUS). The disease symptom is characterized by severe abdominal pain and bloody stools (Griffin *et al.,* 1988; Boyce *et al.,* 1995) and can also present as non-bloody diarrhea (Rodrigue *et al.,* 1995). Of particular concern in pediatric populations estimated to occur in 8% of children, *E coli* 0157:H7 requires a relatively small inoculum for infection and spreads easily from child to child by the fecal-oral route. The incubation period of EHEC is 1-5 days, with illness duration typically 4-10 days. Chronic renal failure develops in as many as 10% of patients with HUS, and HUS kills 3-5% of affected patients (Serna and Boedeker, 2008). *E. coli* O157 is an emerging cause of food-borne illness, particularly in the northern United States and Canada where sources identified in outbreaks have included ground beef, apple juice, and alfalfa sprouts, as well as feacally contaminated drinking water and swimming pools (CDC, 2005a, 2006, 2007, 2008). In the developing countries however, drought, carriage of *E. coli* O157 by cattle, and contamination of surface water were presumed to have been important sources of infection with *E. coli* O157. In a household survey performed during a waterborne outbreak of *E. coli* O157 infections in the United States, bloody stools were reported by only 35% of those with diarrhea (Swerdlow *et al.,* 1992). The report of Effler and his co-worker (2001) provides comprehensive data on the first outbreak of *E. coli* O157

infections from the developing world. Before this outbreak, *E. coli* O157 had been isolated only once before in Southern Africa, from an elderly man undergoing surgery for lower gastrointestinal bleeding in Johannesburg in 1990 (Browning *et al.,* 1990). Thirty-one suspected *E. coli* O157 isolates obtained from clinical and environmental specimens were referred to CDC, and all were confirmed as *E. coli* O157: NM. All isolates had the *uid*A allele specific for *E. coli* O157:H7 and the genes encoding Shiga toxins 1 and 2 (Effler *et al.,* 2001). Two other outbreaks of dysentery have been attributed to *E. coli* O157 elsewhere in Africa, the first in Central African Republic in 1996, and the second in Cameroon beginning in 1997 (Cunin *et al.,* 1999; Germani *et al.,* 1997). Both of these reports have some constraints, however; in the Central African Republic, *E. coli* O157 was not isolated at the outbreak site but only implicated from molecular tests. In the Cameroon outbreak, *E. coli* O157 and *Shigella* spp were each isolated in about half of the specimens tested. Although reported outbreaks of *E. coli* O157 in Africa have been few to date, available information indicates that the pathogen has wide geographic distribution. Since the 1992 outbreak, culture-proven *E. coli* O157 diarrheal illness has been reported from several locations, including Kenya (Sang *et al.,* 1996), Nigeria (Akinyemi *et al.,* 1998), Côte d'Ivoire (Dadie *et al.,* 2000) and Central Africa Republic (Germani *et al.,* 1998). Because *E. coli* O157 is not detected by the usual methods used to isolate and identify traditional enteric bacterial pathogens, microbiology laboratories in many countries in Africa do not routinely test for this pathogen, hence *E. coli* O157

infections may go unrecognized (WHO, 1995; Wittenberg, 1999). Reports on African dysentery outbreaks attributed to *Shigella* spp. sometimes indicate that specimens were not tested until several months into the outbreak or do not describe laboratory methods that are suitable for detecting *E. coli* O157 (Aragon *et al.,* 1995; Malakooti *et al*., 1997). The spectrum of clinical illness resulting from *Shigella* infection overlaps considerably with that of *E. coli* O157 and mixed outbreaks have been reported (Casburn-Jones and Farthing, 2004). Understanding the complex interaction of environmental and behavioral factors that enabled *E. coli* O157 to emerge so intensely in Africa is important for future diarrheal disease control efforts.

The manifestation of clinical symptomology and pathology appears to be closely associated with the possession of certain virulence gene combinations in *E. coli* (Law, 2000; Grauke *et al.,* 2002). Generally, the pathogenicity factors among *E. coli* strains include pili, k-antigen, haemolysin, adhesive factor, enterotoxins, cytotoxins, effacement factors and cytotoxic necrotic factors (Galane and Le Roux, 2001). Various genes encoding virulence markers among *Escherichia coli* were reportedly identified by Obi *et al.* (2004). These included *cnf1* and *cnf2* for necrotoxigenic; *ST* and *LT* for enterotoxigenic; *stx1* and *stx2* for Shiga toxin producing; *bfpA* and *eaeA* for enteropathogenic, and EAE*C* for enteroaggregative *E. coli*. As much as the pathotypes of *E coli* have in common various virulence gene combinations for attachment and elaboration of hemolysins and enterotoxins, there are considerable polymorphisms and

sequence variations in the molecular identities of genes that code for these virulence factors (Nagy and Fekete, 1999; Bertin *et al.*, 2001) while some factors are specific to some strains. For instance, it has been reported that adherence pili, (the bundle-forming pili, Bfp) may be involved in pathogenicity (Hacker, 1992). A PCR method has been developed for the detection of the *bfp* genes of EPEC (Gunzburg *et al.*, 1995) that showed no amplification of DNA from any other bacterial enteropathogens and was 100% specific for EPEC strains which exhibited the characteristic localized-adherence (LA) phenotype. According to Reece *et al.* (2001) variants of the outer membrane protein intimin, expressed by both pathotypes EHEC and EPEC, have been implicated as contributors to tissue tropism. EHEC and EPEC share genetic and phenotypic similarities, most notably the locus of enterocyte effacement (LEE), pathogenicity island (PAI), encoding a type III secretion system (TTSS), and the ability to form attaching and effacing (AE) intestinal lesions and intimate attachment to the host cell (Moon *et al.*, 1983; Nougayrede *et al.*, 2003; Kaper *et al.*, 2004; Caron *et al.*, 2006). The attaching and effacing (A/E) lesions on epithelial cells caused by EPEC or EHEC are mediated by binding of intimin (encoded by the *eae* gene) to its receptor (Tir) on the host cell (Hess *et al.,* 1999). Additional virulence factors in EPEC or EHEC include the expression of a plasmid (pO157)-encoded gene EHEC-*hly* (an enterohaemolysin distinct from the chromosomally encoded a-haemolysin, HlyA), and a b-glucoronidase-encoding *uidA* gene associated with O157:H7 strains (Paton and Paton, 1998). The hair-like filamentous organelles called fimbrial

colonization factor antigens (CFAs), is known to assist ETEC strains in attachment to the small intestine (Raj, 1993). The CFA and enterotoxin genes are generally encoded on plasmids (Nataro and Kaper, 1998). Also in ETEC strains, both LT and ST genes are found and are specifically on transferable plasmids (Levine, 1987); LT is a protein that resembles cholera toxin in structure, function and mechanism of action. STs are monomeric plasmid-mediated toxins made up of an 18 to19 amino-acid polypeptide toxin that causes diarrhoea by binding to and stimulating intestinal -bound guanylate cyclase, leading to an intracellular accumulation of cyclic GMP (Sooka *et al.*, 2004). EIEC strains are similar biochemically and in pathogenesis to *Shigella* spp. This involves epithelial cell penetration, lysis of the endocytic vacuole, intracellular multiplication (Sooka *et al.*, 2004). Ipa A-D genes are located on the large invasive plasmid of all virulent *Shigella* and EIEC organisms and the gene sequences are referred to as the invasion-associated locus (ial). IpaH sequences are present at multiple sites on both the large invasive plasmid and on the chromosomes in Shigellae and EIEC (Sethabutr *et al.*, 1993). EAggEC strains produce a heat-stable enterotoxin (EAST-I) which is non-specific but has also been detected in non-aggregative and non-pathogenic *E. coli* strains. These potential virulence factors are localized on a 60-MDa plasmid common to most EAggEC strains (Czeczulin *et al.*, 1997).

1.2.4. Treatment of Diarrhoea

Enteric infections generally are self-limited conditions. However, early correction of fluid and electrolyte imbalance is the first consideration in diarrhoea

therapy. In many communities of sub-Saharan Africa, modern pharmaceutical agents are a commonly used first line of therapy for the home treatment of diarrhea or they serve as an alternative when traditional remedies fail.

Diarrhoea therapy can broadly be classified as follows: Oral rehydration treatment (ORT), intravenous fluid replacement, anti-intestinal motility (e.g. Diphenoxylate-atropine- reduces duration of diarrhoea, Loperomide), antispasmodic, antimicrobial, antisecretory and probiotics (gut flora balance). However, the WHO case management strategy for acute diarrhea as reported previously (Richards *et al*., 1993) includes:

i. early administration of appropriate fluids at home;
ii. treatment of dehydration with WHO oral rehydration solution;
iii. treatment of severe dehydration with intravenous electrolyte solution;
iv. continued feeding throughout the diarrheal episode;
v. selective use of antibiotics;
vi. non-use of antidiarrheal drugs

Oral rehydration therapy (ORT) in the child with mild-to-moderate dehydration consists of replacement of the child's fluid deficit and ongoing losses and provision of maintenance fluid, electrolyte, and nutritional needs. In severe dehydration or where oral replacement is not possible, intravenous fluids administered in the health care setting may be needed. This should be followed after at least the initial twenty-four hours of glucose electrolyte solution therapy with slow reintroduction of sugar-free fruit drinks, bland food such as vegetables

and then regular feeds (Walker-Smith, 1990). However, there are dangers in the home use of ORT, including inappropriate preparation (electrolyte concentration) or errors of administration (usually insufficient volume) particularly in the developing countries, where literacy level is low. A study in Nigeria found that only 12.7% of people interviewed were able to correctly describe how sugar-salt solution (SSS) is prepared although almost all of them were aware of ORT (Ikpatt and Young, 1992). In Zimbabwe, although nearly half the respondents were able to provide a recipe for SSS, only 12% gave the correct formulation (de Zoysa *et al.*, 1984). The home management of childhood diarrhea with ORT or SSS can be increased positively with public health education (Blum *et al.*, 1990; Touchette *et al.*, 1994).

Patients with diarrhea associated with certain bacterial and protozoal agents may benefit from therapy with an antimicrobial agent (Pickering and Cleary, 2002) In some instances, specific antimicrobial therapy may reduce morbidity and mortality associated with enteric illnesses, may prevent future complications, help to eradicate fecal shedding and to prevent transmission of enteric pathogens but antimicrobial agents should be prescribed with a consideration of their limitations (Pickering, 2003). In viral diarrhoeas for instance, hydration is the mainstay of treatment whether it be oral or parenteral, and specific pharmacologic therapy is not available. Antimotility drugs in some cases often complicate treatment. Pickering (1991) reported that antimotility medications worsen the clinical course in shigellosis and in antimicrobial-

associated colitis and have the risks attendant with overdose. Other conjectured possible side effects include toxic megacolon and colonic hemorrhage (Northrup and Flanigan, 1994) and ileus (Avery and Snyder, 1990).

Salmonella gastroenteritis is usually a self-limiting disease. Fluid and electrolyte replacement may be indicated in severe cases. Antibiotics are not routinely used to treat uncomplicated non-typhoidal *Salmonella* gastroenteritis because they may rather prolong illness, the length of excretion of pathogen or actually prolong the duration of convalescent carriage (Klotchko and Wallace, 2009). However, it has been suggested that infants and the elderly who are at risk for bacteremia or complications due to bacteremia deserve antimicrobial treatment (Cohen *et al*., 1978). Fluoroquinolones such as Ciprofloxacin have been suggested as more effective than standard antibiotics for treating patients with bacteremia due to *Salmonella* (Jacobson *et al.,* 1989) and chronic suppressive therapy may be needed to prevent reoccurrence. The 4-quinolones are not only highly active *in vitro* against a broad range of enteric pathogens but generally also exhibit most of the other properties desirable for the treatment of these infections (Keusch, 1988). They are well tolerated, have good oral absorption and are more rapidly and reliably effective than earlier drugs (WHO, 2005). Nevertheless, fluoroquinolones are not recommended for use in children due to the risk of central nervous system toxicity, cardiovascular toxicity, tendon or articular toxicity, and rarely hepatic toxicity (Nelson *et al.,* 2006) even at therapeutic doses and adverse reactions may manifest during, as well as after

fluoroquinolone therapy has been completed (Saint *et al.,* 2000). Third-generation cephalosporins are widely used in children with serious infections. It has recently been suggested that the specific adverse-effect profile of quinolones must be considered when they are chosen for treatment of bacterial infections because of physiological changes in renal function (Stahlmann and Lode, 2010). The earlier drugs chloramphenicol, ampicillin and amoxicillin and trimethoprim-sulfamethoxazole are used occasionally as alternative therapy for salmonellosis. Presently, apart from the quinolones, and third-generation cephalosporins, macrolide antibiotics are preferred for empiric therapy pending sensitivities. Regrettably, sensitivity to quinolones is declining, and these are no longer fool-proof agents for typhoid fever (WHO, 2005a). A sentry antimicrobial surveillance program for North America (1997-2001) has shown that the level of susceptibility for ciprofloxacin, as the representative of other fluoroquinolones, was equal or slightly inferior to that of representative aminoglycoside (Jones, 2003). According to Ercis *et al.* (2006), nalidixic acid-resistant *Salmonella* strains are suggestive of reduced effectiveness of quinolone therapy in patients. In South Africa, the significantly increasing incidence of extended spectrum betalactamase producing *Salmonella* is set back in the use of extended-spectrum cephalosporins which are the preferred drugs for serious non-typhoidal *Salmonella* infections in children (Kruger *et al.*, 2004).

As in other diarrhoeal diseases, correction of dehydration and maintenance of fluid and electrolyte balance is the first line of therapy in

shigellosis. Antibiotics have reportedly shortened the duration of symptoms in infections due to *Shigella* (Salam and Bennish, 1991; Sack *et al.,* 2001) and can prevent relapse. Ampicillin and trimethoprim-sulfamethoxazole are good choices for sensitive strains of *Shigella.* Nalidixic acid and the newer fluoroquinolones have been shown to be effective against multiply-antibiotic resistant *Shigella* spp. (Rogerie *et al.,* 1986; Ericsson *et al.,* 1987; Pichler *et al.,* 1987). Third generation cephalosporins, such as cefixime and ceftriaxone, have also been proven through clinical trials to be better than ampicillin or TMP-SMZ and safer for use in children (Varsano *et al.*, 1991; Ashkenazi *et al.,* 1993). The WHO recent recommendations for treatment of clinically diagnosed cases of *Shigella* dysentery are ciprofloxacin as first line treatment, and pivmecillinam, ceftriaxone, or azithromycin as second line treatment (WHO 2005b). Treatment of shigellosis with antibiotics should however, be done putting regional differences in sensitivity as well as local antibiotic sensitivity patterns of *Shigella* isolates into consideration (Christopher *et al.*, 2010). In EIEC, treatment of significant symptomatic illness with fever and bloody diarrhoea should follow same course of treatment as *Shigella* diarrhoea (Herrington and Taylor, 1991). *E coli* 0157:H7 are sensitive to most antibiotics *in vitro*, antibiotics have not been shown to limit duration or ameliorate symptoms (Carter *et al.,* 1987). The illness is self limited and treatment is supportive (Herrington and Taylor, 1991). ETEC which is usually a disease of children in the developing countries is treated with ORT and intravenous fluids. Treatment of Traveller's diseases is aimed at reducing

episodes of diarrhoea and number of days of illness. Trimethoprim-sulfamethoxazole can be used both as prophylaxis and as treatment of travelers (DuPont *et al.,* 1982; Steffen *et al.,* 1988).

Manipulation of gut flora to enhance its protective and beneficial role represents a promising field of new therapeutic strategies. Probiotics are non-antibiotic formulations which are considered in diarrhoea therapy. Probiotics are live nonpathogenic microorganisms administered to improve microbial balance, particularly in the gastrointestinal tract. They are originally derived from cultured food, especially dairy products, and consist of *Saccharomyces boulardii,* a yeast, *E. coli* Nissle 1917 (a nonpathogenic *E. coli* strain), or lactic acid bacteria, such as *Lactobacillus* spp., *Lactococcus lactis* and *Bifidobacterium* spp, and are regulated as dietary supplements and foods (Damaskos and Kolios, 2008; Williams, 2010). There has been evidence for the clinical effectiveness of probiotics in the treatment of acute diarrhea, most commonly due to rotavirus, and pouchitis (Heydarian *et al.,* 2010; Williams, 2010). Probiotics appear to be a useful adjunct to rehydration therapy in treating acute, infectious diarrhoea in adults and children (Allen *et al.*, 2003). The mechanisms involved in the remediation effects of probiotics can be that of lowering intestinal pH, decreasing colonization and invasion by pathogenic organisms, or modifying the host immune response. Studies have shown that the mechanisms of action of probiotics include antimicrobial activity and suppression of bacterial growth, immunomodulation and initiation of an immune response, enhancement of

barrier activity, and suppression of human T-cell proliferation (Resta-Lenert and Barrett, 2003; Nerstedt *et al.,* 2007; Peluso *et al.*, 2007). The molecular aspects of probiotic mechanism of action have been shown experimentally, in which they have been found to induce their effect by means of their DNA (Jijon *et al.*, 2007; Rachmilewitz *et al*., 2007). The beneficial effects of probiotics vary from one species or strain to the other.

1.2.5. Some considerations on antibiotic therapy of enteric bacteria

The classifications of antibiotics are based on their mechanisms of action, chemical structure, or spectrum of activity. Antibiotics which generally may be bactericidal (lethal to bacteria) or bacteristatic (prevent bacterial growth) perform their activities mainly by targeting bacterial functions or growth processes (Calderon and Sabundayo, 2007). Bactericidal antibiotics target the bacterial cell wall while bacteriostatic antibiotics target protein synthesis (Finberg *et al.*, 2004). In terms of spectrum of activity antibiotics are also categorized either as "narrow-spectrum" based on their target specificity or “broad-spectrum” if active against a wide range of bacteria group. Table 1.3 gives a summary of some antibiotics that are used in the treatment of enteric infections by their classes and their modes of action.

Table 1.3. Some antibiotics that are used in the treatment of enteric infections and their mechanisms of action.

*CLSI Subclass	Antimicrobial Agent	Mechanism of Action
Aminoglycosides	Amikacin	Interfere with protein biosynthesis by binding to bacterial 30S ribosome subunit, inhibiting the translocation of the peptidyl-tRNA from the A-site to the P-site and also causing misreading of mRNA, leaving the bacterium unable to synthesize proteins vital to its growth.
	Gentamicin	
	Kanamycin	
Aminopenicillins	Ampicillin	Inhibit bacterial cell wall synthesis by disrupting the synthesis of the peptidoglycan layer.
β-Lactamase inhibitor combinations	Amoxicillin-Clavulanic acid	Inhibit bacterial cell wall synthesis by disrupting the synthesis of the peptidoglycan layer.
Carbapenems	Imepenem	Inhibit bacterial cell wall synthesis
	Meropenem	
Cephalosporins (2^{nd} generation) Cephamycins	Cefoxitin	Inhibit bacterial cell wall synthesis by disrupting the synthesis of the peptidoglycan layer.
	Cefuroxime	
Cephalosporins (3^{rd} generation)	Ceftazidime	
	Ceftriaxone	
	Cefotaxime	
Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole	Inhibit bacterial folic acid metabolism
Lincosamides	Clindamycin	Interfere with protein biosynthesis by binding to bacterial 50S ribosome subunit
Macrolides	Azithromycin	Interfere with protein biosynthesis irreversibly to the subunit 50S of the bacterial ribosome, thereby inhibiting translocation of peptidyl tRNA.
	Erythromycin	
Phenicols	Chloramphenicol	interfere with protein biosynthesis including 50S ribosome inhibition
Quinolones	Ciprofloxacin	Bacterial DNA gyrase or topoisomerase IV enzyme inhibitors, thereby inhibiting DNA replication and transcription.
	Nalidixic acid	
Sulfonamides	Sulfisoxazole	Inhibit bacterial folic acid metabolism

Tetracyclines	Tetracycline	Interfere with protein biosynthesis including 30S ribosome inhibition

*CLSI is Clinical and Laboratory Standards Institute formerly NCCLS

The choice of drugs for the treatment of enteric diseases is based on some of the following known therapeutic principles (Keusch, 1988).

i. the agent must be active *in vitro* against the isolate,
ii. the levels must be adequate for treating infections due to invasive pathogens both lumenal and tissue,
iii. the drugs with good enterohepatic circulation may be especially well suited for the treatment of infections due to noninvasive pathogens, and
iv. the drugs active intracellularly may have particular value in therapy for typhoid, especially in reducing the relapse rate.

It is no gainsaying that antimicrobials have saved the lives and eased the suffering of millions of people. By helping to bring many serious infectious diseases under control, these drugs have also contributed to the major gains in life expectancy experienced during the latter part of the last century (WHO, 2002). However, these gains seem to have been eroded as the emergence of drug resistance and multiple drug resistance (MDR) have become a threat in various nations of the world. The effectiveness of most drugs of choice has been jeopardized by many factors such as the incorporation of antibiotics into animal feeds. The authorization for the use of fluoroquinolones in food animals

enhanced the rates of fluoroquinolone-resistant *Salmonella* in animals and food, and then subsequently in human infections (FAO/OIE/WHO, 2003). The total consumption of antimicrobials has been viewed as one of the critical factors in selecting resistance even though the relationship between use and resistance is not a simple correlation (WHO, 2001) so also is under-use through lack of access, inadequate dosing, poor adherence, and substandard antimicrobials. In many developing countries particularly sub-Saharan Africa, some of the contributory factors to acquiring resistance are sources of obtaining drugs. Antimicrobial agents are readily available without a prescription hence, self-prescribing of antimicrobials is common (Dua *et al.*, 1994; Okeke *et al.*, 1999). Some of the potential sources of medications include relatives or friends with unused supplies, locally trained nurses, shopkeepers, market sales people, and unlicensed drug salesmen who buy drugs in major cities and then sell them in rural villages (Bledsoe and Goubaud, 1985; Omotade *et al.*, 1994). Other contributory factors are inadequate and inappropriate use of drugs and lack of education. "Examples of inappropriate uses of antibiotics that exert selective pressure for resistance in various bacteria are: administering them to patients who have viral upper respiratory tract infections; feeding them to farm animals to enhance their growth" (Sack *et al.,* 2001). In addition, antimicrobial agents may be of poor quality, due to degradation, counterfeiting, or lack of bioequivalence in the case of generic drugs. The use of such products results in sub-optimal serum concentrations, which promotes selection for resistance

(Okeke *et al*., 1999). The ease of modern transport systems and frequency of international travels have also contributed greatly to the spread of resistance. Other factors in the spread of resistance are overcrowding and improper sewage disposal which according to Okeke *et al*., (1999) promote the spread of resistant organisms among individuals and provide increased opportunities for genetic exchange among bacteria, facilitating the dissemination of antibiotic resistance determinants.

Emerging drug resistance to ciprofloxacin and second line drugs such as pivmecillinam, ceftriaxone, and azithromycin is increasingly being reported in many parts of the world, as is multiple-drug resistance (Kosek, 2008; Kuo, 2008; Pazhani, 2008). Multi-resistance in *Enterobacteriaceae* is defined as resistance to three or more of the following classes of antibiotics: cephalosporins, carbapenems, quinolones, aminoglycosides and trimethoprim/co-trimoxazole (Rodriguez-Bano *et al*., 2004). MDR is a serious problem for the treatment of shigellosis, particularly those caused by *S. dysenteriae* type 1. Transferable MDR was first described among *Shigella* in Japan in the mid 1950s, (Watanabe, 1963), and has remained a major problem ever since. Multidrug-resistant (MDR) strains of *Salmonella* are also encountered frequently and the rates of multidrug-resistance have increased considerably (WHO, 2005a). The emergence of drug-resistant *Salmonella* is more of response to selective pressure from the use particularly in food animals. A recent global spread of a multidrug-resistant *S. enterica* serovar Typhimurium phage type DT104 in animals and humans showed

that other factors such as major shifts in the occurrence of *Salmonella* serotypes in food animal and humans do occur. In this particular case while antimicrobial usage may have facilitated the spread of DT104, international and national trade of infected animals is thought to play a major role in international spread (WHO, 2005a). MDR strains of enteric bacteria were isolated with increasing frequency from various sub-Saharan nations at various times to date. In Central Africa, MDR strains of *S. dysenteriae* type 1 were isolated during the epidemics between late 1970s and early 1980s (Frost *et al.*, 1981; Ebright *et al.*, 1984). Multiple resistance to antibiotics including trimetoprim-sulfamethazole was also reported in Zambia (Tuttle *et al*., 1995). Data from Nigeria, Ethiopia, Senegal, Kenya, Malawi is suggestive of wide spread and increasing resistance to most commonly used drugs in the sub-Saharan African regions (Lamikanra *et al.*, 1989; Awole *et al.*, 2002; Sow *et al.*, 2007; Onyango *et al.*, 2009; Kingsley *et al.*, 2009). In South Africa, the work of Kruger *et al.* (2004) on non-typhoidal *Salmonella* isolates showed increased resistance to extended-spectrum cephalosporin. Obi *et al.* (2007) reported multiple antibiotic resistance among enteric bacteria obtained from HIV patients and their drinking water and a relationship was established between clinical and environmental strains. A very recent data from South Africa (Keddy *et al.,* 2009), showed that most commonly expressed multiple antibiotic resistance profiles among non-typhoidal *Salmonella* were ACSSuNa and ACSSuTNa, that is, resistance to a combination of 5 or 6 drugs combinations of ampicillin (A), chloramphenicol (C), streptomycin (S), sulfamethoxazole (Su),

tetracycline (T) and nalidixic acid (Na). Also, nalidixic acid-resistant with reduced susceptibility to ciprofloxacin was reported among human isolates of *Salmonella enterica* serovar Typhi from South Africa by Smith *et al.,* (2010). The increasing prevalence of bacterial strains exhibiting multi-resistance to readily available and relatively cheap oral antibiotics constitutes a major obstacle to cost-effective therapy of diarrheas in sub-Saharan Africa.

1.2.6. Mechanisms of antibacterial resistance

Various mechanisms of antimicrobials inactivation have been reported (Neu, 1993; Alekshun and Levy, 2000; Fluit *et al.*, 2001; Sundsfjord *et al.*, 2004) which invariably led to the emergence of multiple drug resistance pathogens. Some of the mechanisms are:

a) enzymatic inactivation of the antimicrobial agent,
b) prevention of access to the target pathogen or,
c) change or mutation in the target site,
d) novel penicillin-binding proteins (PBPs),
e) altered membrane permeability.

Enzymatic inactivation may result either into destruction of antimicrobial agent, such as occurs with the beta-lactamases, or lead to a major modification of the compound so that it does not bind to its target as is seen with the aminoglycosides and chloramphenicol. The second mechanism whereby access to the drug target is prevented may be by substitutions, amplifications or modifications of the drug target reducing the affinity of the drug to the target.

For example, in gram negative organisms the outer membrane proteins may be altered such that antibiotics are unable to cross the cell wall. The other mechanisms of resistance are intrinsic mechanisms not commonly specified by mobile elements such as efflux pumps that expel multiple kinds of antibiotics (Alekshun and Levy, 2007). These may lead to reduced access of the antimicrobial agents to the target by means of permeability barriers. For instance, diarrhoea causing *E. coli, Salmonella* and *Shigella* reportedly possessed plasmids which produce a new dihydrofolate reductase that has a low affinity for trimethoprim and sulfonamides (Nue, 1993). A very recent study on *Salmonella* spp. in South Africa by Smith and co-researchers concluded that amino-acid mutations in GyrA and ParC in combination with active efflux of antibiotic out of the bacterial cell are probable mechanisms conferring quinolone resistance (Smith *et al.,* 2010).

The genes coding for antibiotic resistance and virulence at times share common features of being located in the bacterial chromosome, as well as on plasmids. They are associated in gene clusters to form resistance or pathogenicity islands and are transferred by mobile elements (such as integrons or transposons) or phages (Villa and Carattoli, 2005).

The most appalling resistant issue among the enteric bacteria is that of the declines in susceptibility for the fluoroquinolones and the third-generation cephalosporins. While resistance to the fluoroquinolones often emerges as a result of mutations in the bacterial genome (DNA), resistance to other

antimicrobials often spread by transfer of DNA between bacterial strains (WHO, 2005a). The major mechanism of resistance to beta-lactam antibiotics among enterobacteriaceae involves production of β-lactamases or extended spectrum β-lactamases (ESBLs). ESBLs have traditionally been defined as transmissible beta-lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam. They are a group of enzymes that break down antibiotics belonging to the penicillin and cephalosporin groups and render them ineffective. The ESBLs are generally encoded by mobile genes that can be exchanged between bacteria. The prevalence of ESBL is high among some Enterobacteriaceae such as *E. coli* and *K. pneumoniae* (Winokur *et al.,* 2001). The diverse geographic distributions and remarkably variable substrate affinities of ESBLs can produce confusing susceptibility testing results (Winokur *et al.,* 2001). According to Jones (2003), the average ESBL phenotype rates for a 5 years SENTRY Program (1997-2001) showed the highest occurrence in Latin America, followed by Europe and North America. It has also been noted that when ESBL strains occur they often have co-resistances with the aminoglycosides (gentamicin), tetracyclines, and trimethoprim/ sulfamethoxazole (Winokur *et al.,* 2001).

Effective treatment of human infections has been limited by the emergence of MDR *Salmonella* strains with resistance to fluoroquinolones and third-generation cephalosporins (WHO, 2005a). Regional differences in ESBL type have also been found to reflect in the choice of therapeutic drug against ESBL strains of enteric pathogens. Jones (2003) reported that cefepime, a

fourth-generation cephalosporin, could be used against 88.0% of isolates in North America, but not for strains in the other regions. However, in all the regions of the study by Jones, only the carbapenems consistently inhibited ESBL-producing strains.

Another type of resistance affecting third-generation cephalosporins and aztreonam (a monobactam) has been the hyperproduction of an Amp C cephalosporinase an inducible enzyme by members of Enterobacteriaceae (Jones *et al.*, 1997; Coudron *et al.,* 2000). Amp C cephalosporinase is normally produced in modest amounts and does not significantly destroy the third-generation cephalosporins; however, spontaneous mutations can derepress enzyme production generating enough enzymes to hydrolyze the cephalosporins and aztreonam (Jones *et al.*, 1997). The drugs of choice against Amp C beta-lactamase producing bacteria are the carbapenems while cefepime has been reported to possess physico-chemical characteristics that enable clinical use against the ceftazidime-resistant Enterobacteriaceae (Jones and Varnam, 2002).

The role of integron in the acquisition and dissemination of resistance in enteric bacteria is very crucial. Integrons are DNA elements which contain collections of genes (gene cassettes) that are generally classified according to the sequence of the protein (integrase) that imparts the recombination function (Mazel, 2006). They have the ability to integrate stably into regions of other DNAs where they deliver, in a single exchange, multiple new genes, particularly for drug resistance (Nemergut *et al.*, 2008). Many of the gene cassettes in

resistance integrons which probably originated from super-integrons (larger integron structures with hundreds of accessory genes), encode resistance against newer antibiotics such as cephalosporins and carbapenems (Fluit and Schmitz, 2004). These genetic elements which can be carried by other mobile genetic elements such as plasmids and transposons assist in the rapid dispersion of resistance genes within bacterial species and between different species (Levesque *et al*., 1995; Hall, 1997). Several gene cassettes and 4 distinct classes of integrons have been identified, some of which confer resistance to beta-lactams, aminoglycosides, trimethoprim, chloramphenicol, sulfonamide and quaternary ammonium compounds used as antiseptics and disinfectants (Levesque *et al*., 1995; Hall, 1997; Mazel *et al*., 1998). Plasmid-borne integron have been implicated in MDR *Salmonella enterica* serovar Enteritidis (Brown *et al*., 2000). In 2008, the study by Rayamajhi and his group described a rise in trend of MDR *S. enterica* serovar Typhimurium with clinically important class 1 integron in food animal. This may have a ripple effect of resistance dissemination on the human consumers (Rayamajhi *et al*., 2008). Recently, class 1 integrons for aminoglycoside and trimethoprim resistance were found to be widespread among members of Enterobacteriaceae in China. The study also identified class 2 integron in *Shigella* isolates and believed that these play a role in antibiotic resistance (Gu *et al*., 2008).

1.2.7. Trends in emergence and spread of antibiotic resistance

Antibiotic resistance is a rapidly growing problem in health care systems. It is a global public health concern that is impacted by both human and non-human antimicrobial use. Alexander Fleming selected and described mutants resistant to penicillin soon after he had discovered the antibiotic. In 1946, a statement was credited to Fleming which reads thus "*There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring 'fastness'* [*resistance*]*."* quoted by Alekshun and Levy (2007). More than 6 decades after the introduction of antibiotics into clinical practice, the prescience of Fleming has come to fruition as the infectious disease community has yet to identify an antibiotic that has managed to circumvent the development of resistanc (Alekshun and Levy, 2007). Resistance usually emerge to each new antibiotic used as the drug of choice for treating diarrhoeal disease soon after introduction into clinical practice. In the 1940's which marked the pre-antibiotic era, resistance to penicillin drugs among staphylococci was noted (Barber, 1947). This was mediated by the production of penicillinases in the bacterial pathogens. In the seventies, resistance to ampiciillin, tetracycline and sulfonamides in Gram-negative bacteria was frequent (Willett and Radojcic, 1976; Maselle *et al*., 1980). Resistance to trimethoprim-sulfamethazole quickly emerged soon after it becamce first-line drug of choice and this was soon followed by an explosion of resistance to nalidixic acid (Jesudason and Saaya, 1997). The extensive use of ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole for the treatment of presumed cases of typhoid fever may

have contributed to the high prevalence of resistance to these drugs (Coovadia *et al*., 1992; Morpeth *et al*., 2009). Also, their use in the treatment of non-typhoidal Salmonella infections in sub-Saharan Africa is of limited value since resistance have been reported in many cases (Lepage *et al.,* 1990 ; Enwere *et al*., 2006 ; Gordon *et al*., 2008). In Nigeria, the prevalences of enteric bacterial strains resistant to tetracycline, ampicillin, chloramphenicol, and streptomycin among healthy individuals increased tremendously from between 9% to 35% in 1986 to 100% in 1998 (Okeke *et al.*, 2000). The increasing rate of gentamicin-resistance in Enterobacteriaceae is critical considering the importance of this drug in the treatment of bloodstream infections. Increase in gentamicin-resistance in *E. coli* was reported in Tanzania (Maselle *et al*., 1980; Blomberg *et al*., 2007) and Kenya (Slack and Wamola, 1977; Omari *et al*., 1997). In Bangladesh, the isolation of vibrio strains resistant to antibiotics such as tetracycline, ampicillin, kanamycin, streptomycin and trimethoprim-sulfamethazole became noticeable from 1979 (Glass *et al.,* 1980). In South Africa, gentamicin-resistant strains which have earlier been reported (Dalsgaard *et al*., 2001) have persisted even in environmental samples (Okoh and Igbinosa, 2010). Increasing isolation of enteric pathogens with resistance to beta-lactam drugs have been reported over the years in South Africa (Essack *et al*., 2001; Bell *et al*., 2002; Kruger *et al*., 2004) and to date the status quo seems to have been maintained. Currently, isolation of gram negative bacterial strains resistant to newer cephalosporins and particularly imipenem-resistant *Pseudomonas*

aeruginosa are being reported (Essack *et al*., 2001; Bell *et al*., 2002; Kruger *et al*., 2004). This might be an indication that we have probably entered into the post-antibiotic era, unless drastic intervention strategies are put in place to reverse the trends.

1.2.8. Consequences of antibiotic resistance

Evidences have shown that adverse human health consequences due to the occurrence of resistant microorganisms are grave and may be felt harder in resource-poor settings. These main consequences of antibiotic resistance may be increased frequency of treatment failures and increased severity of infections and can have cost implications, morbidity as well as mortality repercussions.

1.2.8.1. Morbidity and Mortality implications of antibiotic resistance

Whilst some underlying conditions such as old age (>65 years) and immunosuppression may constitute risk factors for colonization and infection with an MDR gram-negative bacterium (Hsu *et al.*, 2005), several studies both retrospective and prospective have shown that infections with MDR gram-negative bacteria are more frequently associated with increased morbidity and mortality than those caused by susceptible pathogens (Helms *et al.*, 2002; Travers and Barza, 2002; Varma *et al.*, 2005). Increased hospitalization and bloodstream infections have been correlated to an increased relative frequency of antimicrobial-resistant *Salmonella* (Varma *et al.,* 2005). Several studies have found similar correlation in other bacteria: ESBL-producing *E. coli* and *Klebsiella* spp., (Lautenbach *et al.,* 2001), imipenem-resistant *P. aeruginosa* (Lautenbach *et*

al., 2010) and other bacteria pathogens (Angulo *et al.*, 2004). In a Canadian study, the burden of illness associated with *Salmonella enterica* serotype Typhimurium infection was identified. Infections by definitive phage type 104 (DT104) and R-type antimicrobial-resistance pattern (AK/CSSuT) were used as risk factor (Martin *et al.*, 2004). The outcome of the study was a significant estimate of hospitalization attributable to the resistance patterns of the infections. Likewise, persons with infections caused by antimicrobial-resistant *Salmonella* have been found to be more likely to have bloodstream infection or die within 90 days following specimen collection than control groups with susceptible infections. The death rate for persons with multi-drug resistant *Salmonella* infections was estimated to be 10 times higher in the two years following specimen collection than for the general population (WHO, 2005a).

1.2.8.2. Cost implications of antibiotic resistance

Besides the human health impacts of resistance to first-line drugs a huge economic cost is incurred in terms of procuring newer effective antibiotics which are usually expensive and may be unavailable to local health centers. Consequentially, inavailability of effective therapy leads further to increased cost due to the length of hospitalization, the cost of the illness, in terms of treating the patient, lost wages for patient and family members and salaries of health care personnel which may be substantial (Sack *et al.,* 2001). The economic implications of drug resistant infections can be of significant value. Antibiotic-resistant infections attributably cost the U.S. healthcare system in excess of $20

Billion annually while the medical costs per patient ranged from $18,588 to $29,069 (Boston and Durham, 2009). In Canada, Salmonellosis has been estimated to cause 627,200 cases of infection and cost Can$846.2 million annually (Todd, 1989). The finding of Broughton *et al.* (2010) was substantially longer stays and higher costs associated with non-typhoidal salmonellosis that was quinolone resistant. The study gave an indication that interventions that decrease quinolone resistant prevalence will lead to significant savings for the health system in the management of hospitalized salmonellosis cases.

1.2.9. Prevention of diarrhoea

The incidence of diarrhoea can be greatly reduced by proper preventive measures. The avoidance of the use of antibiotics, development and use of non-antibiotic drugs such as anti-secretory drugs has been suggested to reduce diarrhea. Some preventive measures are highlighted below.

- Breastfeeding: Exclusively breastfed babies are much less likely to get diarrhoea or to die from it than are babies who are not breastfed or are partially breastfed. Efforts should be made to improve weaning practices (Mahmood *et al.,* 1989).
- Use of safe water: The risk of diarrhoea can be reduced by using the cleanest available water, increased availability and accessibility to, and protecting domestic water from contamination (WHO, 2009b).
- Handwashing: All diarrhoeal disease agents can be spread by hands that have been contaminated by faecal materials. The risk of diarrhoea is

substantially reduced when individuals practice regular handwashing. In addition, the risk of spread of resistant diarrhoeal pathogens, particular in the hospital setting, can greatly be reduced by handwashing of medical personnel (Khan, 1982).

- Food safety: Food contamination by diarrhoeal agents can occur at all stages in the food production chain. These include: during the growing period (by use of human fertilisers), in public places such as markets, from processing plants to kitchens and food service establishments, and when kept without refrigeration after being prepared. Evaluations of improved food handling and storage methods and practices should be done on regular basis. Health education concerning good animal husbandry, good abattoir practice and good hygiene at all stages as well as individual food safety practices should also be emphasized (FAO/WHO, n.d).
- Control of drug resistance through food: Control of drug-resistant enteric pathogens is most efficiently achieved through the reduction of antimicrobial use. Prudent usage in food animals combined with aforementioned food safety and hygiene practices should reduce the numbers of the relevant resistant bacterial strains in food animals and lower the risk of contamination at all stages in the food production chain (WHO, 2005a). This should be complimented with proper surveillance, risk assessment and International collaborative efforts need to be increased.

- Use of latrines and safe disposal of stools: An unsanitary environment contributes to the spread of diarrhoeal agents. Because the pathogens that cause diarrhoea are excreted in the stools of an infected person or animal, proper disposal of faeces can help to interrupt the spread of infection. Faecal matter can contaminate water where children play, where mothers wash clothes, and where they collect water for home use. Every family needs access to a clean, functioning latrine. If one is not available, the family should defecate in a designated place and bury the faeces immediately. Stools of young children are especially likely to contain diarrhoeal pathogens; they should be collected soon after defecation and disposed of in a latrine or buried (WHO, 2009b).
- Immunization: Whenever new, effective vaccines become available for cholera and diseases caused by other enteric pathogens, immunization programs are vital. In 2008, two new vaccines; pneumococcal conjugate and rotavirus vaccines were made available and introduced into clinical practice in South Africa (Harries *et al.*, 2009; Harrison, 2009) as intervention to decrease infant and child mortality from vaccine preventable causes. Measles immunization can substantially reduce the incidence and severity of diarrhoeal diseases. Every infant should be immunized against measles at the recommended age. Evaluation of the success rate of the vaccine should be carried out periodically.

- Health education: The likelihood of eradication of enteric pathogens such as *Salmonella* in domestic animals seems impossible in the foreseeable future (WHO, 2005b). There is a need to educate the general populace on the need to cook animal foods thoroughly prior to consumption so as to keep the incidence of drug-resistant pathogens in foods of animal origin to the barest minimum. Education of food handlers in the principles of safe food handling is an essential step towards reducing the incidence of food-borne diseases resulting from cross-contaminations during processing and preparation of foods (WHO, 2009a). Educating farmers and their families regarding the risks of occupationally acquired infections is also an important step in the control of human infections from food sources.

1.2.10. Medicinal plants: alternative/complementary diarrhoea therapy

The unabated increasing antibiotic resistance has not only led to resistance to several antibiotic groups but has led to selection and development of multidrug-resistant bacterial strains which is a serious worldwide health menace. The need to find lasting solutions to the emerging infections which have become a global concern has necessitated the exploration of natural products in the form of medicinal plants in the treatment of various ailments, diarrhoea inclusive.

In many developing countries particularly in sub-Saharan Africa, healers use a wide variety of plants in the treatment of gastrointestinal ailments. Several studies have reported the use of herbal plants as therapeutic agents in the

treatment of diarrhoea, even though most of the herbs are used in the treatment of various other ailments (Longanga Otshudi *et al*., 2001; Vieira *et al*., 2001; Agunu *et al.,* 2005; Samie *et al*., 2005; Nair and Chanda, 2007; Mbagwu and Adeyemi, 2008). Some of the plants which have been used traditionally in the treatment of diarrhoea and or stomach disorders are listed in Table 4 below.

1.2.10.1. Modes of Action of Medicinal Plants

Medicinal plants generally exhibit multiple non-specific effects which are usually complementary or synergistic. More often than not, they tend to have several broad actions on a number of whole physiological systems at the same time. Investigations have described various modes of action of antidiarrhoeal herbs. These therapeutic agents can have antispasmodic effects, delay intestinal transit, suppress gut motility, inhibit intestinal motility, stimulate water adsorption or reduce electrolyte secretion (Palombo, 2006) and some of these effects have been attributed to the presence of numerous phytochemicals (such as alkaloids, tannins, flavonoids and terpenes). Tannins and flavonoids are believed to exhibit their antidiarrhoeal potentials by increasing colonic water and electrolyte re-absorption (Palombo, 2006). In an animal experimental study by Ojewole and his group, aqueous extract of *Psidium guajava* decreased the

Table 1.4. Some medicinal plants commonly used as diarrhoeal remedy in South Africa

Botanical Names	Active Ingredient	Traditional Usage	Reference

Carpobrotus edulis N.E. Br. (Mesembryanthemaceae)	Malic acid, citric acid, tannins	Antiseptic, anti-diarrhoeal, vasoconstrictive	Van Wyk *et al.* (1997); Wisura and Glen (1993)
Elephantorrhiza elephantina Benth. (Fabaceae)	Entagenic acid, tannins	Antiseptic, anti-diarrhoeal, anti-ulcerative, emetic	Van Wyk *et al.* (1997); Hutchings et *al.* (1996).
Eucomis autumnalis (Mill.) Chit. (family)	Homoisoflavanones, nortriterpenoids	Urinary diseases, stomach ache, diarrhoea, enema for low back pain and healing of fractures	Watt and Breyer-Brandwijk (1962); Hutchings et *al.* (1996), Van Wyk and Gericke (2000)
Pelargonium sidoides DC. (family)	Saponins, tannins	Tuberculosis, diarrhoea (bronchitis, infections of the upper respiratory tract)	Watt and Breyer-Brandwijk (1962); Hutchings et *al.* (1996) ; Van Wyk and Gericke (2000)
Viscum capense L. (Viscaceae)	Viscumside, flavonoids	Anti-diarrhoeal, anti-bronchial, uterotonic, styptic	Van Wyk and Gericke (2000)
Xysmalobium undulatum R. Br. (Asclepiadaceae)	Uzarin	Anti-diarrhoeal, against painful menstrual cramps	Van Wyk and Gericke (2000)

propulsive movement and transit of charcoal meal through the gastrointestinal tract (GIT) (Ojewole *et al*., 2008). The study also showed that the aqueous extract of *P. guajava* at a dose of 50–400 mg/kg, significantly inhibited all the diarrhoeal parameters investigated: onset, frequency and severity of diarrhoea, total number of stools, number of wet stools, and weight of wet stools.

1.2.10.2. Further benefits of herbal plants

Exploring the efficacy of medicinal plants in the amelioration of infectious diseases may not only be a source of succor in abating multiple antibiotic resistance infections but will also make health care affordable and available at the door-step of all. According to Akerele (1978) "If there is to be any real improvement in the health of the under-served populations of the world, then there will have to be full utilization of all available resources, human and material…." In Africa particularly in the rural areas, long waits for medical attention; misgivings about the effectiveness of Western medicine, and cultural dependency on traditional methods have made the people turn orthodox medicine to a last resort. In this instance, traditional medicine especially through the use of herbs has contributed immensely to the primary health care of the people. A study by the Roll Back Malaria Initiative found that in Ghana, Mali, Nigeria and Zambia, herbal medicines were the first line of home treatment for about 60% of children with high fever (Brieger *et al.*, 2005). About 65% of the population in India regularly depends on traditional medicine. The developed countries are not spared in the use of medicinal plants and significant increase in usage over the past decade has been reported (Pagán and Pauly, 2005). In the USA and Australia 42 and 48 % of the populations respectively utilize complementary and alternative medicines (Eisenberg *et al.,* 1998). A WHO estimate states that 70% of Canadians and 75% of people in France have tried complementary or alternative medicine, which often includes herbal remedies (WHO, 2008), and in Japan, 85% of doctors prescribe not only modern medicine

but also the traditional herbal medicine (called Kampo), which is covered by health insurance (Aschwanden, 2001). In San Francisco, London and South Africa, 75% of people living with HIV/AIDS use traditional medicine or complementary and alternative medicines.

Medicinal plants can contribute immensely to the sustainable development of the poor but naturally endowed nations. The assertion of Geldenhuys and Van Wyk (2002) was that natural resources were fundamental in the socioeconomic development of the African continent. Africa is richly endowed with a vast diversity of plants. According to Van Wyk (2008) recent data have shown that a quarter of the total number of higher plants in the world is found in sub-Saharan Africa. Also, a total of 50,136 angiosperm taxa occur in tropical Africa and Southern Africa (Klopper *et al.,* 2006). Medicinal usages in excess of 16,300 have been reported from a list of more than 5400 medicinal plant taxa on the African continent (Neuwinger, 2000). Herbal medicines are also profitable and are highly lucrative. Worldwide, they represent a market value of about US$ 43 billion a year. According to WHO (2008) annual revenues in Western Europe reached US$ 5 billion in 2003-2004. In China, sales of products totaled US$ 14 billion in 2005 and herbal medicine revenue in Brazil was US$ 160 million in 2007. In South Africa, Dold and Cocks (2002) valued annual trade in plant material at approximately R27 million.

Medicinal plants aside from being used directly as teas or in other extracted forms for their natural chemical constituents; they may serve as leads

in the discovery of new drugs. Herbs may be used as agents in the synthesis of drugs or the organic molecules and constituents may be used as models for synthetic drugs. Data have shown that a number of drugs are derived from plant materials (Newman *et al.*, 2003). Twenty-five percent of modern medicines are made from plants first used traditionally. The search for new molecules according to Gurib-Fakim (2006) has taken a slightly different route whereby the science of ethnobotany and ethnopharmacognosy are being used as guides to lead the chemist towards different sources and classes of compounds. Some examples of commonly used pharmaceutical products which are of botanical origin are aspirin from willow bark (*Salix spp.*), digitoxin, digitalin or digoxin from Common foxglove (*Digitalis purpurea*) and quinine from *Cinchona ledgerian* (El-Baz, 2007). A recent example is the endoperoxide-based antimalarial drug Artesunate that originated from artemisinin, first isolated from the Chinese herbal medicine *Artemisia annua* L (Camacho *et al.*, 2000). A number of patented products have been obtained from studies involving medicinal herbs.

1.2.10.3. Safety issues in medicinal herb therapy

The issue of toxicity in natural products applies not only to herbs but also to some ordinary food substances. The constituents of many foods could be regarded as poisonous, such as the alpha gliadin produced by gluten in wheat oats and rye, the cyanogenic glycosides in many fruit seeds, the thiocyanates of the brassica vegetables, alkaloids of the *Solanaceae* and lectins of many pulses including soya and red kidney beans.

Medicinal plants have reportedly cause serious illnesses ranging from allergy to liver or kidney malfunction, to cancer, and even death. The toxicity effects of medicinal plants may be acute or long term. These effects may range from diarrhoea, hypersensitivity reactions, nausea or vomiting, to organ-targeted toxicity, immunotoxicity, embryo/fetal and prenatal toxicity, mutagenicity/ genotoxicity, hepatotoxicity, nephrotoxicity, presence of epileptogenic compounds, cardiac toxins, gastrointestinal toxins to carcinogenicity (Smolinske, 2005). According to US toxicologist, Dr Lois Gold, the carcinogenicity or toxicological potentials of natural plant chemicals are comparable to those of synthetic chemicals (Ames *et al.,* 1990; Gold *et al.,* 1991). Several studies have reported varying level of cytotoxicity of medicinal plants, cytopathic defects and organ dysfunction in animal experiments. The ethanol extracts of *Athrixia phylicoides* DC. (bush tea) which is widely used as a beverage, cough remedy and purgative were relatively toxic (McGaw *et al.*, 2006). Extract and a flavonol glycoside from *Bauhinia galpinii* were cytotoxic to Vero cells (Aderogba *et al.*, 2007; Samie *et al.*, 2010). Pyrrolizidine alkaloids which are not toxic *per se,* but can be converted to toxic metabolites in the liver are reported to be contained in several medicinal plants (Eloff and Rösemann, 2009). Examples are hepatotoxicity of pyrrolizidine-alkaloid-contained in Comfrey (Symphytum), Dryopteris (Male Fern), Viscum (Mistletoe) and Corynanthe (Yohimbe). Michellamine B one of the atropisomeric naphthylisoquinoline alkaloid dimers isolated from *Ancistrocladus korupensis* was inhibitory to several laboratory and

clinical strains of HIV-1, including the AZT resistant strain G910-6 and the pyridinone-resistant strain A17; as well as strains of HIV-2. However, the high toxicity of this compound to several human cell lines prevented its further evaluation (Boyd *et al.*, 1994). In South Africa, mutagenic effects in the *Salmonella* microsome assay have been reported in Crinum *macowanii*, *Chaetacme aristata* Planch. (Celastraceae), *Plumbago auriculata* Lam. (Plumbaginaceae), *Catharanthus roseus* (L.) G.Don. (Apocynaceae) and *Ziziphus mucronata* Willd. (Rhamnaceae) (Elgorashi *et al.*, 2003). In another study, the induction of genotoxicity on bacterial or human cells has been observed (Elgorashi *et al.*, 2002). Comfrey, Lobelia and Sassafras tea has been implicated in liver disease, vomiting, breathing problems, convulsions, cancer, and even death (Snider, 1991). Blindness has also been attributed to the use of herbal medicines. In the United Republic of Tanzania, a study by Yorston and Foster (1994) linked 25% of corneal ulcers to the use of traditional eye medicines, of which many are based on herb extracts. Another study, in Malawi found that herbal medicines were associated with 26% of childhood blindness (Courtright *et al.*, 1994).

Another safety issue is that of contamination problems. Heavy metals, pesticide residues, microbes and microbial metabolites such as aflatoxin contaminants of herbs constitute additional safety problems. Cumin seeds commonly given by Egyptians to their children to relieve coughs, aches, or itching were among five spices purchased at a local market in Egypt and

examined for toxic content. High level of the organophosphate pesticide (profenofos, 0.37g/kg) or nearly twice the residue the WHO and Codex Alimentarius Commission permit in vegetables was detected in the cumin seeds (Ahmed, 2001). According to Roy *et al*. (1988) aflatoxin contamination was detected in 14 out of the 15 common drug plants in India. Contamination of dietary and medicinal plants with exposure to fumonisin B_1 is widespread than expected in the Eastern Cape Province of South Africa (Sewram *et al.*, 2006). Fungal microbes and Fumonisin B_1 contamination of commercial traditional African medicines in South Africa have recently been reported (Katarere *et al*., 2008). A lack of proper sterilization, along with inclusion of components such as urine, saliva, or breast milk in some of medicinal herbs used as eye remedy in Malawi, gives pathogens ample opportunity to thrive in eyes already hard hit by injury or infection (Courtright *et al.*, 1994). Perhaps the biggest problems with herbal medicines are a lack of standardization and of safety regulations. However, a very recent study conducted in Ghana suggested that rather than have heavy metal toxicity effects, the metals found in the plants of study may offer beneficial effect in case of micronutrient deficiency in herb consumers since metals are readily bioavailable (Annan *et al*., 2010).

The foregoing literature review underlines the scale, magnitude and health associated parameters of infective diarrhea, the problems of escalating antibiotic resistance, the forays to uncover the genetic basis of antibiotic resistance and virulence of the designated bacterial pathogens, including the

potentials of the use of medicinal plants and their possible safety related issues. In spite of these challenges, comprehensive studies on updated antibiogram profiles, genetic landscape and activity of medicinal plants against enteropathogens, particularly, *Salmonella* species and *E. coli* have received little attention in South Africa.

1.3. Objective of the study

i. To characterize and determine the antibiograms of *Salmonella* and diarrheogenic *E. coli isolates* from clinical samples of patients. .

ii. To determine extended spectrum beta-lactamases (ESBL's) production and the genetic determinants among the *Salmonella* and *dia*rrheogenic *E. coli isolates*.

iii. To identify the gene encoding virulence factors in *Salmonella* and *E. coli* isolates.

iv. To identify and screen various medicinal plants used by herbalists within the Oliver R. Tambo District Municipality (ORTDM) in the treatment of diarrhea and related diseases for their antimicrobial activities.

v. To isolate pure compounds, elucidate and characterize active ingredients of selected medicinal plants and investigate their cytotoxicity effects.

1.4. Approach

The study involved clinical, field and laboratory studies.

- The first stage of the study was clinical-based and involved gathering demographic information about patients, history of diarrhoea, HIV status (from hospital records and surveillance data), self-medication and the usage of herbal medicine. Clinical specimens analyzed included stool and blood samples which were collected from patients through the help of qualified health care-givers, and from those deposited into the NMAHC laboratory.
- The second stage was the laboratory analysis of clinical samples obtained from the selected human subjects for the isolation of *Salmonella* and *E. coli* using standard microbiological techniques. These included the susceptibility testing of the enterobacteria using standard drugs and the identification of the resistance and virulence factors of the isolates using molecular techniques.
- The third stage of the study involved identification and selection of ethnomedinal plants used in folklore practice in ORTM for diarrhea and associated diseases. This was guided by the information from the interview of traditional healers and informed elders of the communities.
- The last stage of the research involved analysis of the crude extracts of the medicinal plants for their antidiarrhoeal activities and, the isolation and characterization of the active ingredients, including cytotoxicity testing.

1.5. References

Aderogba, M.A., McGaw, L.J., Ogundaini, A.O., Eloff, J.N., 2007. Antioxidant activity and cytotoxicity study of the flavonol glycosides from Bauhinia galpinii. Nat. Prod. Res., 21(7):591-599.

Agunu, A., Yusuf, S., Andrew, G.O., Zezi, A.U., Abdurahman, E.M., 2005. Evaluation of five medicinal plants used in diarrhoea treatment in Nigeria. J. Ethnopharmacol., 101: 27–30.

Ahmed, M.T., 2001. Is baby medicine laced with poison? Bull. Environ. Cont. Toxicol. 66 :421 http://www.newscientist.com/article/mg16922811.700-is-baby-medicine-laced-with-poison.html

Akerele, O., 1987 The best of both worlds: Bringing traditional medicine up to date. Soc. Sc. Med., 24 (2):177-181.

Akinyemi, K.O., Oyefolu, A.O., Opere, B., Otunba-Payne, V.A., Oworu, A.O., 1998. *Escherichia coli* in patients with acute gastroenteritis in Lagos, Nigeria. East. Afr. Med. J., 75:512-515.

Alekshun, M.N., Levy, S.B., 2000. Bacterial drug resistance: response to survival threats. In Bacterial Stress Responses, G. Storz, and R. Hengge-Aronis, eds. (Washington, D.C.: ASM Press), pp. 323-366.

Alekshun, M.N., Levy, S.B., 2007. Molecular Mechanisms of Antibacterial Multidrug Resistance. Cell, 128:1037-1050.

Allen, S.J., Okoko, B., Martinez, E.G., Gregorio, G.V., Dans, L.F., 2003. Probiotics for treating infectious diarrhoea. Cochrane Database of Systematic Reviews; Issue 4. Art. No.: CD003048. DOI: 10.1002/14651858.CD003048.pub2

Altier, C., 2005. Genetic and Environmental Control of Salmonella Invasion. J. Microbiol., 43:85-92 special issue.

Ames, B.N., Profet, M., Gold, L.S., 1990. Nature's chemicals and synthetic chemicals: Comparative toxicology. (carcinogens/mutagens/teratogens/ clastogens/dioxin) Proc. Nati. Acad. Sci. USA, 87: 7782-7786.

Angulo, F.J., Nargund, V.N., Chiller, T.C., 2004. Evidence of an Association Between Use of Anti-microbial Agents in Food Animals and Anti-microbial Resistance Among Bacteria Isolated from Humans and the Human Health Consequences of Such Resistance. J. Vet. Med. B. Infect. Dis. Vet. Public Health, 51(8-9):374-379.

Annan, K., Kojo, A.I., Cindy, A., Samuel, A.N., 2010. Profile of heavy metals in some medicinal plants from Ghana commonly used as components of herbal formulations. Phcog. Res., 2:41-44.

Aragon, M., Barreto, A., Chambule, J., Noya, A., Tallarico, M., 1995. Shigellosis in Mozambique: the 1993 outbreak rehabilitation-a follow-up study. Trop. Doct., 25:159-162.

Aschwanden, C., 2001. Herbs for health, but how safe are they? Bull. World Health Org., 79(7):691-692 http://www.who.int/bulletin/archives/79(7)691.pdf

Ashkenazi, S., May-Zahav, M., Dinari, G., Gabbay, U., Zilberberg, R., Samra, Z.. 1993. Recent trends in the epidemiology of *Shigella* species in Israel. Clin. Infect. Dis., 17:897–899.

Avery, M.E., Snyder, J.D., 1990. Oral therapy for acute diarrhea. N. Engl. J. Med., 323:891.

Awole, M., Gebre-Selassie, S., Kassa, T., Kibru, G., 2002. Isolation of potential bacterial pathogens from the stool of HIV-infected and HIV-non-infected patients and their antmicrobial susceptibility patterns in Jimma Hospital, south west Ethiopia. Ethiop. Med. J., 40(4):353-364.

Bajaj, V., Lucas, R.L., Hwang, C., Lee, C.A., 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol., 22: 703-714.

Barber, M., 1947. Staphylococci infection due to penicillin-resistant strains. BMJ., 2*:* 863–865.

Barnes, A.C., Hastings, T.S., Amyes, S.G.B., 1994. Amoxycillin resistance in Scottish isolates of *Aeromonas salmonicida*. J. Fish. Dis., 17: 357-363.

Bartlett, A.V. 3rd, Prado, D., Cleary, T.G., Pickering, L.K., 1986. Production of Shiga toxin and other cytotoxins by serogroups of Shigella. J. Infect. Dis., 154(6):996–1002.

Batchelor, B.I., Kimari, J.N., Brindle, R.J., 1996. Microbiology of HIV associated bacteraemia and diarrhoea in adults from Nairobi, Kenya. Epidemiol. Infect., 117:139-144.

Baxter, M.A., Fahlen, T.F., Wilson, R.L., Jones, B.D., HilE interacts with HilD and negatively regulates *hilA* transcription and expression of the *Salmonella enterica*

serovar Typhimurium invasive phenotype. Infect. Immun., 71:1295-1305.

Begum, Y.A., Talukder, K.A., Nair, G.B., Khan, S.I., Svennerholm, A.M., Sack, R.B., et al., 2007. Comparison of enterotoxigenic Escherichia coli isolated from surface water and diarrhoeal stool samples in Bangladesh. Can. J. Microbiol., 53:19-26.

Behlau, I., Miller, S.I., 1993. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol., 175: 4475-4484.

Bell, J.M., Turnidge, J.D., Gales, A.C., Pfaller, M.A., Jones, R.N., 2002. Prevalence of extended spectrum beta-lactamase (ESBL)-producing clinical isolates in the Asia-Pacific region and South Africa: regional results from SENTRY Antimicrobial Surveillance Program (1998–99). Diagn. Microbiol. Infect. Dis., 42:193–198.

Bernasconi, C., Ghiani, M., Daverio, E., Camozzi, M., 2004. Molecular-based methodologies for the assessment of microbiological status of drinking and surface water. EUR 21085 EN. European Communities. A European Commission Joint Research Centre Report.

Bertin, Y., Boukhors, K., Pradel, N., Livrelli, V., Martin, C., 2001. Stx2 subtyping of Shiga toxin-producing *Escherichia coli* isolated from cattle in France: detection of a new Stx2 subtype and correlation with additional virulence factors. J. Clin. Microbiol., 39:3060-3065.

Bisi-Johnson, M.A., Kolawole, D.O., Shittu, A.O., Epidemiological analysis of clinical isolates of *Staphylococcus aureus* in Ile-Ife, Nigeria. Pak. J. Bio. Sci., 8(7): 1016-1020.

Black, R.E., Brown, K.H., Becker, S., 1984. Malnutrition is a determining factor in diarrheal duration, but not incidence, among young children in a longitudinal study in rural Bangladesh. Am. J. Clin. Nutrition, 39: 87-94.

Black, R.E., 1993. Epidemiology of diarrhoeal disease: implications for control by vaccines. Vaccine, 11:100-106.

Bledsoe, C.H., Goubaud, M.F., 1985. The reinterpretation of Western pharmaceuticals among the Mende of Sierra Leone. Soc. Sci. Med., 21:275-282.

Blomberg, B., Manji, K.P., Urassa, W.K., Tamim, B.S., Mwakagile, D.S., Jureen, R., Msangi, V., Tellevik, M.G., Holberg-Petersen, M., Harthug, S., Maselle, S.Y., Langeland, N., 2007. Antimicrobial resistance predicts death in Tanzanian children with bloodstream infections: a prospective cohort study. BMC Infect.

Dis., 22:7:43.

Blomberg, B. 2007. Antimicrobial resistance in bacterial infections in urban and rural Tanzania. Ph.D. University of Bergen. Institute of Medicine Centre for international health University of Bergen Norway.

Blum, D., Emeh, R.N., Huttly, S.R., Dosunmu-Ogunbi, D., Okeke, N., Ajala, M., Okoro, J.I., Akujobi, C., Kirkwood, B.R., Feachem, R.G., 1990. The Imo State (Nigeria) Drinking Water Supply and Sanitation Project,1. Description of the project, evaluation methods, and impact on intervening variables. Trans. R. Soc. Trop. Med. Hyg., 84:309-315.

Boston, M.A., Durham, N.C., 2009. Antibiotic-Resistant Infections Cost the U.S. Healthcare System in Excess of $20 Billion Annually. APUA Oct 19. http://www.tufts.edu/med/apua/Pubs/aam_report.pdf

Boyce, T.G., Swerdlow, D.L., Griffin, P.M., 1995. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. N. Engl. J. Med., 333(6): 364 - 368.

Boyd, M.R., Hallock, Y.F., Cardellina, J.H., Manfredi, K.P., Blint, J.W., McMahon, J.B., Buckheit Jr., R.W., Bringmann, G., Schaeffer, M., 1994. Anti-HIV michellamines from *Ancistrocladus korupensis*. J. Med. Chem., 37(12): 1740-1745.

Boyle, E.C., Bishop, J.L., Grassl, G.A., Finlay, B.B., 2007. Salmonella: from pathogenesis to therapeutics. J. Bacteriol., 189(5):1489-1495.

Bradford, P.A., 2001. Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev., 14: 933–951.

Brieger, W., Unwin, A., Greer, G., Meek, S., 2005. Interventions to Improve the Role of Medicine Sellers in Malaria Case Management for Children in Africa. London, UK and Arlington, Va., USA: the Malaria Consortium and BASICS for the United States Agency for International Development; prepared for Roll Back Malaria's Sub-group for Communication and Training and Malaria Case Management Working Group. http://www.rollbackmalaria.org/partnership/wg/wgmanagement/docs/medsellersRBM mtgsubcommitteereport.pdf

Broughton, E.I., Margaret, I., Coles, C.L., Walker, D.G., 2010. Higher hospital costs and lengths of stay associated with quinolone-resistant *Salmonella enterica* infections in Hong Kong. J. Pub. Health, 32(2):165-172.

Brown, A.W., Rankin, S.C., Platt, D.J., 2000. Detection and characterization of

integrons in Salmonella enterica serotype Enteritidis. FEMS Microbiol. Lett., 191: 145–149.

Brown, E.W., Kotewicz, M.L., Cebula, T.A., 2002. Detection of recombination among Salmonella enterica strains using the incongruence length difference test. Mol. Phylogenet. Evol., 24:102-120.

Browning, N.G., Botha, J.R., Sacho, H., Moore, P.J., 1990. *Escherichia coli* O157:H7 haemorrhagic colitis: report of the first South African case. S. Afr. Med. J., 28:28-29.

Buzby, J.C., Roberts, T., 2009. The economics of enteric infections: human foodborne disease costs. Gastroenterology, 136:1851–1862.

Calderon, C.B., Sabundayo, B.P., 2007. Antimicrobial Classifications: Drugs for Bugs. In Schwalbe R, Steele-Moore L, Goodwin AC. Antimicrobial Susceptibility Testing Protocols. CRC Press. Taylor & Frances group. ISBN 0-8247-4100-5

Camacho, M.R., Croft, S.L., Phillipson, J.D., 2000. Natural products as sources of antiprotozoal drugs. Curr. Opinion Anti-infective Invest. Drugs, 2: 47-62.

Caron, E., Crepin, V.F., Simpson, N., Knutton, S., Garmendia, J., Frankel, G., 2006. Subversion of actin dynamics by EPEC and EHEC. Curr. Opin. Microbiol., 9: 40–45.

Carter, A.O., Borczyk, A.A., Carlson, J.A.K., 1987. A severe outbreak of Escherichia coli O157:H7-associated hemorrhagic colitis in a nursing home. N. Engl. J. Med., 317:1496-1500.

Casburn-Jones, A.C., Farthing, M.J.G., 2004. Management of infectious diarrhoea. Gut, 53:296-305.

CDC, Centers for Disease Control and Prevention, 2005a. Diarrheagenic Escherichia coli. Available at http://www.cdc.gov/ncidod/dbmd/diseaseinfo/diarrecoli_t.htm. Accessed October 1, 2008.

CDC, Centre for Disease Control and Prevention, 2005b. Foodborne Illness. Content source: National Center for Immunization and Respiratory Diseases: Division of Bacterial Diseases. Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30333, U.S.A. October 25, 2005. http://www.cdc.gov/ncidod/dbmd/diseaseinfo/foodborneinfections_g.htm#Top. Accessed May 2010.

CDC, Centers for Disease Control and Prevention, 2006. Ongoing multistate

outbreak of Escherichia coli serotype O157:H7 infections associated with consumption of fresh spinach--United States, September 2006. MMWR Morb. Mortal Wkly. Rep., 55(38):1045-1046.

CDC, Centers for Disease Control and Prevention, 2007. Escherichia coli O157:H7 infection associated with drinking raw milk--Washington and Oregon, November-December. MMWR Morb. Mortal Wkly. Rep., 56(8):165-167.

CDC, Centers for Disease Control and Prevention, 2008. Escherichia coli 0157:H7 infections in children associated with raw milk and raw colostrum from cows--California, 2006. MMWR Morb. Mortal Wkly. Rep., 57(23):625-628.

Chan, K., Baker, S., Kim, C.C., Detweiler, C.S., Dougan, G., Falkow, S., 2003. Genomic comparison of Salmonella enterica serovars and Salmonella bongori by use of an S. enterica serovar Typhimurium DNA microarray. J. Bacteriol., 185:553-563.

Charles, R.C., Harris, J.B., Chase, M.R., Lebrun, L.M., Sheikh, A., LaRocque, R.C., Logvinenko, T., Rollins, S.M., Tarique, A., Hohmann, E.L., Rosenberg, I., Krastins, B., Sarracino, D.A., Qadri, F., Calderwood, S.B., Ryan, E.T., 2009. Comparative Proteomic Analysis of the PhoP Regulon in Salmonella enterica Serovar Typhi Versus Typhimurium. PLoS One, 4(9):e6994.

Chatterjee, A., O'Keefe, C., Cuthill, S.L., Varman, M., 2009. Salmonella Infection. Updated: Apr 27, 2010 [Online] Available at: http://emedicine.medscape.com/article/968672-overview. Accessed 15 August 2010.

Chiu, C.H., Chuang, C.H., Chiu, S., Su, L.H., Lin, T.Y., 2006. Salmonella enterica serotype Choleraesuis infections in pediatric patients. Pediatrics, 117(6):e1193-6.

Christopher, R.H.P., David, K.V., John, S.M., Sankarapandian, V., 2010. Antibiotic therapy for Shigella dysentery. Cochrane Database of Systematic Reviews, Issue 1. Art. No.: CD006784. DOI: 10.1002/14651858.CD006784.pub3.

Coburn, B., Grassl, G.A., Finlay, B.B., 2007. Salmonella, the host and disease: a brief review. Immunol. Cel.l Biol., 85(2):112-118.

Cohen, P.S., O'Brien, T.F., Schoenbaum, S.C., Medeiros, A.A., 1978. The risk of endothelial infection in adults with *Salmonella* bacteremia. Ann. Intern. Med., 89:931-932.

Coker, A.O., Isokpehi, R.D., Bolaji, N.T., Amisu, O.A., Obi, C.L., 2002. Human Campylobacteriosis in developing countries. Emerg. Inf. Dis., 8(3): 237-243.

Collinge, J., 2001. Prion diseases of humans and animals: Their causes and molecular basis Annu. Rev. Neurosci., 24:519–550.

Coovadia, Y.M., Gathiram, V., Bhamjee, A., Garratt, R.M., Mlisana, K., Pillay, N., et al., 1992. An outbreak of multiresistant *Salmonella typhi* in South Africa. Q. J. Med., 82(298):91-100.

Coudron, P.E., Moland, E.S., Thomson, K.S., 2000. Occurrence and Detection of AmpC Beta-Lactamases among Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis Isolates at a Veterans Medical Center. J. Clin. Microbiol., 38: 791-796.

Courtright, P., Lewallen, S., Kanjaloti, S., Divala, D., 1994. Traditional eye medicine use among patients with corneal disease in rural Malawi. British J. Ophthalmol., 74:810-812.

Crump, J.A., Luby, S.P., Mintz, E.D., 2004. The global burden of typhoid fever. Bull. World Health Organ., 82:346–353.

Cunin, P., Tedjouka, E., Germani, Y., Ncharre, C., Bercion, R., Morvan, J., Martin, P.M., 1999. An epidemic of bloody diarrhea: *Escherichia coli* O157 emerging in Cameroon? Emerg. Infect. Dis., 5:285-290.

Czeczulin, J.R., Balepur, S., Hicks, S., Phillips, A., Hall, R., Kothary, M.H., Navarro-García, F., Nataro, J.P., 1997. Aggregative adherence fimbria II, a second fimbrial antigen mediating aggregative adherence in enteroaggregative *Escherichia coli.* Infect. Immun., 65: 4135-4145.

Dadie, A., Karou, T., Adom, N., Kette, A., Dosso, M., 2000. Isolation of enteric pathogeic agents in Côte d'Ivoire: *Escherichia coli* O157:H7 and enteroaggregative *E. coli*. Bull. Soc. Pathol. Exot., 93:95-96.

Dalsgaard, A., Forslund, A., Sandvang, D., Arntzen, L., Keddy, K., 2001. *Vibrio cholerae* O1 outbreak isolates in Mozambique and South Africa in 1998 are multiple-drug resistant, contain the SXT element and the *aadA2* gene located on class 1 integrons. J. Antimicrob. Chemother., 48:827-838.

Damaskos, D., Kolios, G., 2008. Probiotics and prebiotics in inflammatory bowel disease: microflora 'on the scope'. Br. J. Clin. Pharmacol., 65(4):453-467.

Darwin, K.H., Miller, V.L., 1999. Molecular basis of the interaction of Salmonella

with the intestinal mucosa. Clin. Microbiol. Rev., 12(3): 405-428.

de Zoysa, I., Carson, D., Feachem, R., Kirkwood, B., Lindsay-Smith, E., 1984. Home-based oral rehydration therapy in rural Zimbabwe. Trans. R. Soc. Trop. Med. Hyg., 78:102-105.

Dold, A., Cocks, M., 2002. The trade in medicinal plants in the Eastern Cape province, South Africa. S. Afr. J. Sci., 98:589-597.

Dua, V., Kunin, C.M., White, L.V., 1994. The use of antimicrobial drugs in Nagpur, India. A window on medical care in a developing country. Soc. Sci. Med., 38:717–724.

DuPont, H.L., Levine, M.M., Hornick, R.B., Formal, S.B., 1989. Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis., 159:1126-1128.

DuPont, H.L., Reves, R.R., Galinso, E., Sullivan, P.S., Wood, L.V., Mendihola, J.G., 1982. Treatment of travelers' diarrhea with trimethoprim-sulfamethoxazole and with trimethoprim alone. N. Engl. J. Med., 307: 841-844.

Dupont, H.L., 2005. *Shigella* species (Bacillary Dysentery). In: Mandell GL, Bennett JE, Dolin R, Principles and Practice of Infectious Diseases. Elsevier, Philadelphia PA. pp. 2655 - 2661.

Ebright, J.R., Sanborn, W.R., Schaberg, D., Kyle, J., Ishida, K., 1984. Epidemic Shiga bacillus dysentery in Central Africa. Am. J. Trop. Med. Hyg., 33:1192-1197.

Effler, P., Isaäcson, M., Arntzen, L., Heenan, R., Canter, P., Barrett, T., Lee, L., Mambo, C., Levine, W., Zaidi, A., Griffin, P.M., 2001. Factors Contributing to the Emergence of *Escherichia coli* O157 in Africa. Emerg. Infect. Dis., 7(5): 812-819.

Eisenberg, D.M., Davis, R.B., Ettner, S.L., Appel, S., Wilkey, S., van Rompay, M., Kessler, R.C., 1998. 'Trends in alternative medicine use in the United States 1990–1997: Results of a follow up national survey', JAMA., 280: 1569–1575.

El-Baz, M., 2007. Yesterday's Plants - Today's Medicines http://ezinearticles.com/? Yesterdays-Plants---Todays-Medicines&id=484879

Elgorashi, E.E., Taylor, J.L.S., Maes, A., de Kimpe, N., van Staden, J., Verschaeve, L., 2002. The use of plants in traditional medicine: potential genotoxic effects. S. Afr. J. Bot., 68: 408–410.

Elgorashi, E.E., Taylor, J.L.S., Verschaeve, L., Maes, A., van Staden, J., De Kimpe, N., 2003. Screening of medicinal plants used in South African traditional medicine for genotoxic effects. Toxicol. Letters, 143:195–207.

Eloff, J.N., Rosemann, M., 2009. LC-MS-MS Can Distinguish Between Toxic and Non-Toxic Pyrrolizidine Alkaloids in Plants. 21st Scientific Congress of the Austrian Pharmaceutical Society April 16th to April 18th 2009, Vienna, Austria. Conference abstract SL-06 Sci. Pharm., 77: 173. www.scipharm.at Scientia Pharmaceutica 173.

Encyclopedia of Childhood and Adolescence., n.d. Diarrhea. 2005-2006 Thomson Gale. A part of the Thomson Corporation. http://www.bookrags.com/research/diarrhea-geca/

Enweani, B., Obi, C.L., Jokpeyibo, M., 1994. Prevalence of *Candida* species in Nigerian children with diarrhoea. J. Diarrhoeal Dis. Res., 12(2): 133–135.

Enwere, G., Biney, E., Cheung, Y.B., Zaman, S.M., Okoko, B., Oluwalana, C. et al., 2006. Epidemiologic and clinical characteristics of community-acquired invasive bacterial infections in children aged 2–29 months in the Gambia. Pediatr. Infect. Dis. J., 25: 700–705.

Ercis, S., Erdem, B., Hasçelik, G., Gür, D., 2006. Nalidixic acid resistance in Salmonella strains with decreased susceptibility to ciprofloxacin isolated from humans in Turkey. Jpn. J. Infect. Dis., 59(2):117-119.

Ericsson, C.D., Johnson, P.C., DuPont, H.L., Morgan, D.R., Bitsura, J.M., JavierDe La Cabada, F., 1987. Ciprofloxacin or Trimethoprim/sulfamethoxazole as initial therapy for travelers' diarrhea: a placebo-controlled trial. Ann. Intern. Med., 106:216-220.

Esona, M.D., Armah, G.E., Steele, A.D., 2003. Molecular epidemiology of rotavirus infection in western Cameroon. J. Trop. Pediatr., 48:14-17.

Essack, S.Y., Hall, L.M., Pillay, D.G., McFadyen, M.L., Livermore, D.M., 2001. Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum B-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. Antimicrob. Agents Chemother., 45:88–95.

Essack, S.Y., 2000. Treatment options for extended-spectrum beta-lactamase-producers. FEMS Microbiol. Lett., 190(2):181-184.

Fahlen, T.F., Mathur, N., Jones, B.D., 2000. Identification and characterization

of mutants with increased expression of *hilA*, the invasion gene transcriptional activator of *Salmonella typhimurium*. FEMS Immunol. Med. Microbiol., 28: 25-35.

FAO/OIE/WHO. 2003. 1st Joint FAO/OIE/WHO Expert Workshop on Non-human Antimicrobial Usage and Antimicrobial Resistance [pdf 292kb] Scientific assessment, Geneva, 1-5 Dec; pp.4. http://www.who.int/foodsafety/ publications/micro/ en/amr.pdf

FAO/WHO, Food and agriculture Organization of the United Nations and World Health Organization Joint publication (n.d). Assuring food safety and quality: Guidelines for strengthening national food control systems. [Online] Available at: http://www.who.int/foodsafety/publications/capacity/en/english_Guidelines_ Food_conntrol.pdf

Fields, P.I., Groisman, E.A., Heffron, F., 1989. A Salmonella locus that controls resistance to microbicidal proteins from phagocytic cells. Science, 243:1059–1062.

Finberg, R.W., Moellering, R.C., Tally, F.P., Craig, W.A., Pankey, G.A., Dellinger, E.P. *et al.,* 2004. The importance of bactericidal drugs: future directions in infectious disease". Clin. Infect. Dis.,39 (9): 1314–1320.

Finlay, B.B., 1994. Molecular and cellular mechanisms of Salmonella pathogenesis. Curr. Top. Microbiol. Immunol., 192: 163-185.

Fitzgerald, C., Sherwood, R., Gheesling, L.L., Brenner, F.W., Fields, P.I., 2003. Molecular Analysis of the rfb O Antigen Gene Cluster of Salmonella enterica Serogroup O:6,14 and Development of a Serogroup-Specific PCR Assay. Applied Env. Microbiol., 69(10): 6099-6105.

Fluit, A.C., Schmitz, F.J., 2004. Resistance integrons and super-integrons. Clin. Microbiol. Infect., 10: 272–288.

Fluit, A.C., Visser, M.R., Schmitz, F.J., 2001. Molecular detection of antimicrobial resistance. Clin. Microbiol. Rev., 14(4):836-871.

Forbes, D., Ee, L., Camer-Pesci, P., Ward, P.B., 2001. Faecal candida and diarrhoea. Arch. Dis. Child., 84:328–331.

Freytag, L.C., Clements, J.D., 1999. Bacterial toxins as mucosal adjuvants. Curr. Top. Microbiol. Immunol., 236:215-236.

Frost, J.A., Vandepitte, J., Rowe, B., Threlfall, E.J., 1981 Plasmid

characterization in the investigation of an epidemic caused by multiply resistant *Shigella dysenteriae* type 1 in Central Africa. Lancet, ii: 1074-1076.

Galane, P.M., Le Roux, M., 2001. Molecular epidemiology of *Escherichia coli* isolated from young South African children with diarroeal diseases. J. Health. Popul. Nutr., 19 (1) 31-37.

Geldenhuys, C.J., Van Wyk, B-E., 2002. Indigenous biological resources of Africa. In: Baijnath, H., Singh, Y. (Eds.), Rebirth of Science in Africa. Umdaus Press, Hatfield. ISBN 1-919766-23-5.

Germani, Y., Minssart, P., Vohito, M., Yassibanda, S., Glaziou, P., Hocquet, D., et al., 1998. Etiologies of acute, persistent, and dysenteric diarrheas in adults in Bangui, Central African Republic, in relation to human immunodeficiency virus serostatus. Am. J. Trop. Med. Hyg., 59:1008-1014.

Germani, Y., Soro, B., Vohito, M., Morel, O., Morvan, J., 1997. Enterohaemorrhagic *Escherichia coli* in Central African Republic. Lancet, 349:1670.

Glass, R.I,, Huq, I., Alim, A.R., Yunus, M., 1980. Emergence of multiply antibiotic-resistant Vibrio cholerae in Bangladesh. J. Infect. Dis., 142(6):939-942.

Gold, L.S., Slone, T.H., Manley, N.B., Garfinkel, G.B., Hudes, E.S., Rohrbach, L., Ames, B.N., 1991. The Carcinogenic Potency Database: analyses of 4000 chronic animal cancer experiments published in the general literature and by the U.S. National Cancer Institute/National Toxicology Program. Environ. Health Perspect., 96:11–15.

Goldsmid, J.M., Leggat, P.A., 2007. The returned traveler with diarrhoea Aust. Fam. Physician, 36(5):322-327.

Gonzalez, G., Sossa, K., Bello, H., Dominguez, M., Mella, S., Zemelman, R., 1998. Presence of integrons in isolates of different biotypes of *Acinetobacter baumannii* from Chilean hospitals. FEMS Microbiol. Lett., 161: 125–128.

Gordon, M.A., Graham, S.M., Walsh, A.L., Wilson, L., Phiri, A., Molyneux, E.M., Zijlstra, E.E., Heyderman, R.S., Hart, C.A., Molyneux, M.E., 2008. Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clin. Infect. Dis., 46:963–969.

Gordon, M.A., 2008. Salmonella infections in immunocompromised adults. J.

Infect., 56(6):413-422.

Grauke, L.J., Kudva, I.T., Yoon, J.W., Hunt, C.W., Williams, C.J., Hovde, C.L., 2002. Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. Appl. Environ. Microbiol., 68:2269-2277.

Greenberg, H.B., Estes, M.K., 2009. Rotaviruses: from pathogenesis to vaccination. Gastroenterology, 136(6):1939-1951.

Griffin, P.M., Ostroff, S.M., Tauxe, R.V., Greene, K.D., Wells, J.G., Lewis, J.H., Blake, P.A., 1988. Illnesses associated with Escherichia coli O157:H7 infections. A broad clinical spectrum. Ann. Intern. Med., 109:705-712.

Gu, B., Pan, S., Wang, T., Zhao, W., Mei, Y., Huang, P., Tong, M., 2008. Novel cassette arrays of integrons in clinical strains of Enterobacteriaceae in China. Int. J Antimicrob Agents, 32(6):529-33.

Guerra, B., Soto. S.M., Arguelles, J.M., Mendoza, M.C., 2001. Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent Salmonella enterica serotype [4,5,12: i:-]. Antimicrob. Agents Chemother., 45: 1305–1308.

Gunzburg, S.T., Tornieporth, N.G., Riley, L.W., 1995. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. J. Clin. Microbiol., 33:1375-1377.

Gür, D., Gülay, Z., Akan, O.A., Aktaş, Z., Kayacan, C.B., Cakici, O., Eraç, B., Gültekin, M., Oğünç, D., Söyletir, G., Unal, N., Uysal, S., 2008. Resistance to newer beta-lactams and related ESBL types in gram-negative nosocomial isolates in Turkish hospitals: results of the multicentre HITIT study. Mikrobiyol Bul., 42(4):537-544.

Gurib-Fakim, A., 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Mol. Aspects Med., 27:1-93.

Hacker, J., 1992. Role of fimbrial adhesins in the pathogenesis of Escherichia coli infections.Can. J. Microbiol., 38(7):720-727.

Hale, T.L., 1991. Genetic basis of virulence in Shigella species. Microbiol. Rev., 55:206-224.

Hall, R.M., 1997. Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram-negative bacteria. Ciba Found. Symp., 207:192–202.

Harries, J., Moodleya, J., Barone, M.A., Mall, S., Sinanovic, E., 2009. Preparing for HPV vaccination in South Africa: Key challenges and opinions. Vaccine, 27:38–44.

Harrison, D., 2010. An Overview of Health and Health care in South Africa 1994 – 2010. A Discussion Document Commissioned by the Henry J. Kaiser Family Foundation to Help Inform the National Health Leaders' Retreat Muldersdrift, January 24-26, 2010 http://www.doh.gov.za/docs/reports/2010/overview1994-2010.pdf

Helms, M., Vastrup, P., Gerner-Smidt, P., Molbak, K., 2002. Excess mortality associated with antimicrobial drug-resistant *Salmonella* Typhimurium. Emerg. Infect. Dis., 8:490-495.

Herikstad, H., Motarjemi, Y., Tauxe, R. V., 2002. *Salmonella* surveillance: a global survey of public health serotyping. Epidemiol. Infect. 129:1-8.

Herrington, D.A., Taylor, D.N., 1991. Bacterial Enteridities. In Michael Field editor. Diarrheal Diseases. . Elsevier Science Publishing Co., Inc. New York NY 10010. pp. 239-291.

Hess, R.D., Lieske, R., Weber, B., The 2nd International Symposium of the European study Group on Enterohaemorrhagic *Escherichia coli* (EHEC). Emerg. Infect. Dis., 5: 1-9.

Heydarian, F., Kianifar, H.R., Ahanchian, H., Khakshure, A., Seyedi, J., Moshirian, D., 2010. A comparison between traditional yogurt and probiotic yogurt in non-inflammatory acute gastroenteritis. Saudi Med. J., 31(3):280-283.

Honish, L., 2000. Restaurant-associated outbreak of Salmonella typhimurium phage type 1 gastroenteritis--Edmonton, 1999. Can. Commun. Dis. Rep., 26(4):25-28.

Hsu, R., Lin, F., Chen, R.J., Hsueh, P., Wang, S., 2005. Antimicrobial Drug Resistance in Salmonella-Infected Aortic Aneurysms. Ann. Thorac. Surg., 80:530-536.

Huovinen, P., Sundstrom, L., Swedberg, G., Skold, O., 1995. Trimethoprim and sulfonamide resistance. Antimicrob. Agents Chemother., 39:279–289.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A., 1996. Zulu Medicinal Plants. Natal University Press, Pietermaritzburg, ISBN 0-86980-923-7.

Ikpatt, N.W., Young, M.U., 1992. Preliminary study on the attitude of people in

two states of Nigeria on diarrhoeal disease and its management. East African Med. J., 69:219-222.

Inglis, V., Yimer, E., Bacon, E.J., Ferguson, S., 1993. Plasmid-mediated antibiotic resistance in *Aeromonas salmonicida* isolated form Atlantic salmon, *Salmo salar* L., in Scotland. J. Fish. Dis., 16:593-599.

Izumiya, H., Terajima, J., Matsushita, S., Tamura, K., Watanabe, H., 2001. Characterization of multidrug-resistant Salmonella enterica serovar Typhimurium isolated in Japan. J. Clin. Microbiol., 39: 2700–2703.

Jacewicz, M.S., Acheson, D.W.K., Mobassaleh, M., Donohue-Rolfe, A., Balasubramanian, K.A., Keusch, G.T., 1995. Maturational Regulation of Globotriaosylceramide, the Shiga-like Toxin I Receptor, in Cultured Human Gut Epithelial Cells. J. Clin. Invest., 96: 1328-1335.

Jacobson, M.A., Hahn, S.M., Gerberdling, J.L., Sande, M.A., 1989. Ciprofloxacin for Salmonella bacteremai in acquired immunodeficiency syndrome (AIDS). Ann. Intern. Med., 110: 1027-1029.

Janda, J.M., Abbott, S.L., 1998. The Enterobacteria. Lippencott-Raven Publishers, Philadelphia, PA.

Janda, J.M., Abbott, S.L., 2006. The genus Salmonella. In: Janda JM, Abbott SL (eds). The enterobacteria. ASM Press. Washington, DC. pp. 81-103.

Jesudason, M.V., Saaya, R., 1997. Resistance of *Vibrio cholerae* O1 to nalidixic acid. Ind. J. Med. Res., 105:153–154.

Jijon, H., Backer, J., Diaz, H., Yeung, H., Thiel, D., McKaigney, C., De Simone, C., Madsen, K., 2004. DNA from probiotic bacteria modulates murine and human epithelial and immune function. Gastroenterology, 126: 1358–1373.

Jones, R.N., Baquero, F., Privitera, G., Inoue, M., Wiedemann, B., 1997. Inducible β-lactamase-mediated resistance to third-generation cephalosporins. Clin. Microbiol. Infect., 3(suppl 1): S7-S20

Jones, R.N., Varnam, D.J., 2002. Antimicrobial activity of broad-spectrum agents tested against gram-negative bacilli resistant to ceftazidime: report from the SENTRY Antimicrobial Surveillance Program (North America, 2001). Diagn. Microbiol. Infect. Dis., 44:379-382.

Jones, R.N., 2003. Global Epidemiology of Antimicrobial Resistance Among Community-Acquired and Nosocomial Pathogens: A Five-Year Summary From

the SENTRY Antimicrobial Surveillance Program (1997-2001) Semin. Respir. Crit. Care Med., 24(1): 121–133.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol., 2:123–140.

Kass, R., 2005. Traveller's diarrhoea. Aust. Fam. Physician, 34:243–245.

Katerere, D.R., Stockenström, S., Thembo, K.M., Rheeder, J.P., Shephard, G.S., Vismer, H.F., 2008. A preliminary survey of mycological and fumonisin and aflatoxin contamination of African traditional herbal medicines sold in South Africa. Human Exp. Toxicol., 27 (11):793-798.

Kato, A., Groisman, E.A., 2008. Howard Hughes Medical Institute. The PhoQ/PhoP regulatory network of Salmonella enterica. Adv. Exp. Med. Biol., 631:7-21.

Keddy, K.H., Dwarika, S., Crowther, P., Perovic, O., Wadula, J., Hoosen, A., et al., 2009. Genotypic and demographic characterization of invasive isolates of *Salmonella* Typhimurium in HIV co-infected patients in South Africa. J. Infect. Dev. Ctries., 3(8):585-592.

Keusch, G.T., 1988. International Symposium on New Quinolones. Rev. Infect. Dis., 10(1):S199-S205.

Khan, M.V., 1982. Interruption of Shigellosis by handwashing. Trans. Roy. Soc. Trop. Med. Hyg., 76: 174-178.

Kingsley, R.A., Msefula, C.L., Thomson, N.R., Kariuki, S., Holt, K.E., Gordon, M.A., Harris, D., Clarke, L., Whitehead, S., Sangal, V., Marsh, K., Achtman, M., Molyneux, M.E., Cormican, M., Parkhill, J., MacLennan, C.A., Heyderman, R.S., Dougan, G., 2009. Epidemic multiple drug resistant Salmonella Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. Genome Res., 19(12):2279-87.

Klopper, R.R., Chatelain, C., Bänninger, V., Habashi, C., Steyn, H.M., De Wet, B.C., Arnold, T.H., Gautier, L., Smith, G.F., Spichiger, R., 2006. Checklist of the flowering plants of Sub-Saharan Africa. An index of accepted names and synonyms. South African Botanical Diversity Network Report No. 42. SABONET, Pretoria.

Klotchko, A., Wallace, M.R., 2009. Salmonellosis: Treatment and Medication. http://emedicine.medscape.com/article/228174-overview. Updated: Mar 31, 2009.

Kosek, M., Yori, P.P., Pan, W.K., Olortegui, M.P., Gilman, R.H., Perez, J., et al., 2008. Epidemiology of highly endemic multiply antibiotic-resistant shigellosis in children in the Peruvian Amazon. Pediatrics, 122:e541–9.

Koster, F., Levin, J., Walker, L., Trung, K.S.K., Gilman, R.H., Rahaman, M.M., et al., 1978. Hemolytic-uremic syndrome after shigellosis. Relation to endotoxemia and circulating immune complexes. N. Engl. J. Med., 298:927-933.

Kotloff, K.L., Winickoff, J.P., Ivanoff, B., Clemens, J.D., Swerdlow, D.L., Sansonetti, P.J., Adak, G.K., Levine, M.M., 1999. Global burden of Shigella infection: Implications for vaccine development and implementation of control strategies. Bull. WHO, 77, 651-666.

Kruger, T., Szabo, D., Keddy, K.H., Deeley, K., Marsh, J.W., Hujer, A.M., Bonomo, R.A., Paterson, D.L., 2004. Infections with Nontyphoidal *Salmonella* Species Producing TEM-63 or a Novel TEM Enzyme, TEM-131, in South Africa. Antimicrob. Agents Chemother., 48(11):4263–4270.

Kuo, C.Y., Su, L.H., Perera, J., Carlos, C., Tan, B.H., Kumarasinghe, G., et al., 2008. Antimicrobial susceptibility of Shigella isolates in eight Asian countries, 2001-2004. J. Microbiol. Immunol. Infect., 41:107–111.

Lainson, R., Da Silva, B.A.M., 1999. Intestinal parasites of some diarrheic HIV-positive individuals in North Brazil, with particular reference to *Isospora belli* Wenyon, 1923 and *Dientamoeba fragilis* Jepps & Dobell, 1918. Mem. Inst. Oswaldo Cruz., 94 (5): 611-613.

Lamikanra, A., Ako-Nai, A.K., Ola, O., 1989. Incidence of multiple antibiotic resistances in organisms isolated from cases of infantile diarrhoea in a Nigerian oral rehydration therapy clinic. Ann. Trop. Paediatr., 9:256-260.

Lautenbach, E., Patel, J.B., Bilker, W.B., Edelstein, P.H., Fishman, N.O., 2001. Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*: risk factors for infection and impact of resistance on outcomes. Clin. Infect. Dis., 32:1162–1171.

Lautenbach, E., Synnestvedt, M., Weiner, M.G., Bilker, W.B., Jeff Schein, L.V., Kim, M., 2010. Imipenem Resistance in *Pseudomonas aeruginosa:* Emergence, Epidemiology, and Impact on Clinical and Economic Outcomes. Infect. Control Hosp. Epidemiol., 31:47–53.

Law, D., 2000. Virulence factors of *Escherichia coli* O157 and other shiga toxin-producing *E. coli*. J. Appl. Microbiol., 88:729-745.

Lepage, P., Bogaerts, J., Van Goethem, C., Hitimana, D.G., Nsengumuremyi, F., 1990. Multiresistant *Salmonella* Typhimurium systemic infection in Rwanda: clinical features and treatment with cefotaxime. J. Antimicrob. Chemother., 26(Suppl A):53–57.

Leverstein-van Hall, M.A., Blok, H.E.M,, Donders, A.R.T., Paauw, A., Fluit, A.C., Verhoef, J., 2003. Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integrons and is independent of species or isolate origin. J. Infect. Dis., 187: 251-259.

Levesque, C., Piche, L., Larose, C., Roy, P.H., 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother., 39:185–191.

Levine, M.M., DuPont, H.L., Formal, S.B., Hornick, R.B., Takeuchi, A., Gangarosa, E.J., Snyder, M.J., Libonati, J.P., 1973. Pathogenesis of *Shigella dysenteriae* 1 (Shiga) dysentery. J. Infect. Dis., 127:261-270.

Levine, M.M., 1987. *Escherichia coli* that cause diarrhoea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohaemorrhagic and enteroadherent. J. Infect. Dis., 155: 377-389.

Levy, J., 1988. A Practical Approach to Pediatric Gastroenterology. Chicago: Year Book Medical Publishers. 24.

Lifschitz, C.H., Shulman, R.J., 1990. Nutritional therapy for infants with diarrhea. Nutr. Rev., 48(9):329-338.

Linam, W.M., Gerber, M.A., 2007. Changing epidemiology and prevention of Salmonella infections. Pediatr. Infect. Dis. J., 26(8):747-748.

Liu, H., Luo, J., Lai, P., 1999. Studies on the anti-enteropathogenic bacteria action of Herba Pogostemonis extracts. Zhong Yao Cai, 22(8):408-411.

Longanga, O.A., Vercruysse, A., Foriers, A., 2001. Antidiarrhoeal activity of root extracts from Roureopsis obliquifoliolata and Epinetrum villosum. Fitoterapia, 72(3):291-294.

Lostroh, C.P., Lee, C.A., 2001. The Salmonella pathogenicity island-1 type III secretion system. Microbes Infect., 3(14-15): 1281-1291.

Madappa, T., Go, C.H.U., 2009. Escherichia Coli Infections. Emedicine Updated: Feb 19, 2009 http://emedicine.medscape.com/article/217485-overview

Mahmood, D.A., Feaham, R.G., Huttly, R.S.A., 1989. Infants feeding and risks of severe diarrhoea in Basrah city, Iraq: A case control study. Bull. World Health Org., 67: 701-706.

Malakooti, M.A., Alaii, J., Shanks, G.D., Phillips-Howard, P.A., 1997 Epidemic dysentery in western Kenya. Trans. R. Soc. Trop. Med. Hyg., 91:541-543.

Maraki, S., Stylianoudaki, E., Nioti, E., Georgiladakis, A., Samonis, G., Tselentis, Y., 2005. Bacterial pathogens associated with acute diarrhoea on the island of Crete, Greece, and their resistance to antibiotics. Abstract number: 1135_62. 15th European Congress of Clinical Microbiology and Infectious Diseases April 2-5, 2005. Copenhagen / Denmark.

Martin, L.J., Fyfe, M., Dore, K., Buxton, J.A., Pollari, F., Henry, B., Middleton, D., Ahmed, R., Jamieson, F., Ciebin, B., McEwen, S.A., Wilson, J.B., 2004. Increased Burden of Illness Associated with Antimicrobial-Resistant *Salmonella enterica* Serotype Typhimurium Infections. J. Infect. Dis., 189:377–384.

Maselle, S.Y., Ipuge, Y.A., Mhalu, F.S., 1980. Antimicrobial susceptibility patterns of important urinary bacterial pathogens in Muhimbili Medical Centre, Dar es Salaam. *D*ar es Salaam Med. J., 8(1):35-40.

Maurine, A., Leverstein-van Hall, A., Fluit, C., Paauw, A., Box, A.T.A., Brisse, S., Verhoef, J., 2002. Evaluation of the Etest ESBL and the BD Phoenix, VITEK 1, and VITEK 2 Automated Instruments for Detection of Extended-Spectrum Beta-Lactamases in Multiresistant *Escherichia coli* and *Klebsiella* spp. J. Clin. Microbiol., 3703–3711.

Mavzutov, A.R., Bondarenko, V.M., Zherebtsova, N.Y., Valishin, D.A., 2007. Pathogenicity factors of opportunistic enterobacteria and its role in development of diarrhoea. Zh. Mikrobiol. (Moscow), 1: 89-97.

Mazel, D., Dychinco, B., Webb, V.A., Davies, J., 1998. A distinctive class of integron in the *Vibrio cholerae* genome. Science, 280:605–608.

Mazel, D., Dychinco, B., Webb, V.A., Davies, J., 2000. Antibiotic resistance in the ECOR collection: integrons and identification of a novel aad gene. Antimicrob. Agents Chemother., 44:1568–1574.

Mazel, D., 2006. Integrons: agents of bacterial evolution. Nat. Rev. Microbiol., 4: 608-620.

Mazumder, R., Bhattacharya, S., Mazumder, A., Pattnaik, A.K., Tiwary, P.M., Chaudhary, S., 2006. Antidiarrhoeal evaluation of *Aegle Marmelos* (Correa) Linn. root extracts Phytotherapy Res., 20 (1): 82–84.

Mbagwu, H.O.C., Adeyemi, O.O., 2008. Anti-diarrhoeal activity of the aqueous extract of *Mezoneuron benthamianum* Baill (Caesalpinaceae). J. Ethnopharmacol., 116: 16–20.

McGaw, L.J., Jager, A.K., van Steden, J., 2000. Antibacterial, antihelmintic and anti-amoebic activity in South African medicinal plants. J. Ethnopharmacol., 72(1-2):247-263.

McGaw, L.J., Steenkamp, V., Eloff, J.N., 2007. Evaluation of Athrixia bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. J. Ethnopharmacol., 110(1):16-22.

McIver, C.J., White, P.A., Jones, L.A., Karagiannis, T., Harkness, J., Marriott, D., Rawlinson, W.D., 2002. Epidemic strains of Shigella sonnei biotype g carrying integrons. J. Clin. Microbiol., 40:1538–1540.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-Related Illness and Death in the United States Emerg. Infect. Dis., 5(5): 607-625

Ménard, R., Prévost, M.C., Gounon, P., Sansonetti, P., Dehio, C.,1996. The secreted Ipa complex of Shigella flexneri promotes entry into mammalian cells. Proc. Natl. Acad. Sci. USA, 93:1254-1258.

Miller, M.A., Sentz, J., Rabaa, M.A., Mintz, E.D., 2008. Global epidemiology of infections due to *Shigella, Salmonella* serotype Typhi, and enterotoxigenic *Escherichia coli.* Epidemiol. Infect., 136(4):433-435.

Miller, S.I., Loomis, W.P., Alpuche-Aranda, C., Behlau, I., Hohmann, E., 1993. The PhoP virulence regulon and live oral Salmonella vaccines. Vaccine, 11: 122–125.

Moon, H.W., Whipp, S.C., Argenzio, M.M., Levine, M.M., Giannella, R.A., 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun., 41:1340–1351.

Nagy, B., Fekete, P.Z., 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Vet. Res., 30(2-3):259-264.

Nair, R., Chanda, S., 2007. *In-vitro* antimicrobial activity of *Psidium guajava l.* leaf extracts against clinically important pathogenic microbial strains Brazilian J. Microbiol., 38:452-458.

Nataro, J.P., Kaper, J.B., 1998. Diarrhoeagenic *Escherichia coli.* Clin. Microbiol. Rev., 11: 142-201.

Nataro, J.P., Seriwatana, J., Fasano, A., Maneval, D.R., Guers, L.D., Noriega, F., Dubovsky, F., Levine, M.M., Morris, J.G., Jr., 1995. Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. Infect. Immun., 63:4721-4728.

Navia, M.M., Capitano, L., Ruiz, J., Vargas, M., Urassa, H., Schellemberg, D., Gascon, J., Vila, J., 1999. Typing and characterization of mechanisms of resistance of Shigella spp. isolated from feces of children under 5 years of age from Ifakara, Tanzania. J Clin Microbiol 1999; 37: 3113–3117.

Nelson, L.H., Flomenbaum, N., Goldfrank, L.R., Hoffman, R.L., Howland, M.D., Neal, A.L., 2006. Goldfrank's toxicologic emergencies. New York: McGraw-Hill, Medical Pub. Division. http://books.google.com/books?

Nemergut, D.R., Robeson, M.S., Kysela, R.F., Martin, A.P., Schmidt, S.K., Knight, R., 2008. Insights and inferences about integron evolution from genomic data. BMC Genomics, 9:261.

Nerstedt, A., Nilsson, E.C., Ohlson, K., Hakansson, J., Thomas, S.L., Lowenadler, B., Svensson, U.K., Mahlapuu, M., 2007. Administration of Lactobacillus evokes coordinated changes in the intestinal expression profile of genes regulating energy homeostasis and immune phenotype in mice. B.r J. Nutr., 97:1117–1127.

Neu, H.C., 1993. Antibiotic Resistance--Its Impact on a Great Medical Center in the Last 30 Years P&S Medical Review: November 1993, 1:1 http://www.cumc.columbia.edu/news/review/archives/ medrev_v1n1_0002.html

Neuwinger, H.D., 2000. African Traditional Medicine. A Dictionary of Plant Use and Applications. Medpharm Scientific Publishers, Stuttgart, ISBN 3-88763-086-6.

Newman, D.J., Cragg, G.M., Snader, K.M., 2003. Natural Products as sources of new drugs over the period 1981-2002. J. Nat. Prod., 66:1022-1037.

Newman, M.J., 2005. Food Safety: Take life easy; eat, drink and be merry. Luke 12: 19b Ghana Med. J., 39(2): 44–45.

Niyogi, S.K., 2005. Shigellosis. J. Microbiol., 43:133-143.

Northrup, R.S., Flanigan, T.P., 1994. Gastroenteritis. Pediatr. Rev., 15(12):461-472.

Nougayrede, J.P., Fernandes, P.J., Donnenberg, M.S., 2003. Adhesion of enteropathogenic Escherichia coli to host cells, Cell Microbiol., 5:359–372.

Obi, C.L., Coker, A.O., Epoke, E.J., Ndip, R.N., 1998. Distributional patterns of bacterial diarrheagenic agents and antibiograms of isolates from diarrhoeaic and non-diarrhoeaic patients in urban and rural areas of Nigeria. Cent. Afr. J. Med., 44(9):223-229.

Obi, C.L., Coker, A.O., Epoke, J., Ndip, R.N., 1995. *Aeromonas* and *Plesiomonas* species as bacterial agents of diarrhoea in urban and rural areas of Nigeria: Antibiogram of isolates. Cent. Afr. J. Med., 4(12): 397-403.

Obi, C.L., Green, E., Bessong, O.P., de Villiers, B., Hoosen, A.A., Igumbor, E.O., Potgieter, N., 2004. Gene encoding virulence markers among *Escherichia coli* isolates from diarrhoeic stool samples and river sources in rural Venda communities of South Africa. Water SA, 30 (1): 37-42.

Obi, C.L., Potgieter, N., Bessong, P.O., Masebe, T., Mathebula, H., Molobela, P., 2003. In vitro antibacterial activity of Venda medicinal plants. South Afr. J Bot., 69(2): 1-5.

Obi, C.L., Potgieter, N., Bessong, P.O., Matsaung, G., 2002. Assessment of the microbial quality of river water sources in rural Venda communities in South Africa. Water SA, 28 (3): 287-292.

Obi, C.L., Ramalivhana, J., Momba, M.N.B., Onabolu, B., Igumbor, J.O., Lukoto, M., Mulaudzi, T.B., Bessong, P.O., Jansen van Rensburg, E.L., Green, E., Ndou, S. 2007. Antibiotic resistance profiles and relatedness of enteric bacterial pathogens isolated from HIV/AIDS patients with and without diarrhoea and their household drinking water in rural communities in Limpopo Province South Africa. Afr. J. Biotech., 6(8):1035-1047.

Ojewole, J.A.O., Awe, E.O., Chiwororo, W.D.H., 2008. Antidiarrhoeal activity of *Psidium guajava* Linn. (Myrtaceae) leaf aqueous extract in rodents J. Smooth Muscle Res., 44 (6):195–207.

Okeke, I.N., Fayinka, S.T., Lamikanra, A., 2000. Antibiotic resistance in *Escherichia coli* from Nigerian students, 1986-1998. Emerg. Infect. Dis.,

6(4):393-396.

Okeke, I.N., Lamikanra, A., Edelman, R., 1999. Socioeconomic and behavioral factors leading to acquired bacterial resistance to antibiotics in developing countries. Emerg. Infect. Dis., 5:18–27.

Okoh, A.I., Igbinosa, E.O., 2010. Antibiotic susceptibility profiles of some *Vibrio strains* isolated from wastewater final effluents in a rural community of the Eastern Cape Province of South Africa. BMC Microbiology, 10:143 http://www.biomedcentral.com/1471-2180/10/143

Omari, M.A., Malonza, I.M., Bwayo, J.J., Mutere, A.N., Murage, E.M., Mwatha, A.K., Ndinya-Achola, J.O., 1997. Pattern of bacterial infections and antimicrobial susceptibility at the Kenyatta National Hospital, Nairobi, Kenya. East Afr. Med. J., 74(3):134-137.

Omotade, O.O., Kayode, C.M., Dare, O.O., Oladepo, O., Adeyemo, A.A., 1994. Perceptions and first-line home treatment of diarrhoeal diseases in Ona Ara Local Government Area of Oyo State. Nigerian J. Paed., 21 suppl.:80-87.

Onyango, M.D., Ghebremedhin, B., Waindi, E.N., Kakai, R., Rabsch, W., Tietze, E., Konig, W., Konig, B., 2009. Phenotypic and genotypic analysis of clinical isolates Salmonella serovar Typhimurium in western Kenya. J. Infect. Dev. Ctries., 3(9):685-694.

Orman, B.E., Pineiro, S.A., Arduino, S., Galas, M., Melano, R., Caffer, M.I., Sordelli, D.O., Centrón, D., 2002. Evolution of multiresistance in nontyphoid salmonella serovars from 1984 to 1998 in Argentina. Antimicrob. Agents Chemother., 46: 3963–3970.

Owens, M.D., Warren, D.A., 2009. Salmonella Infection. http://emedicine.medscapecom/ article/785774-overview

Oyofo, B.A., Subekti, D., Tjanla, P., Machpud, K., Komalarini, S., Setiawan, B., Simanjuntak, C., Punjabi, N., Corwin, A.L., Wasfy, M., Campbell, J.R., Lesmana, M., 2002. Enteropathogens associated with acute diarrhoea in community and hospital patients in Jakarta, Indonesia. FEMS Immunol. Med. Microbiol., 34(2): 139-146.

Pagán, J.A., Pauly, M.V., 2005. 'Access to conventional medical care and the use of complementary and alternative medicine'. Health Affairs, 24: 255–262.

Palombo, E.A., 2006. Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function

Phytotherapy Res., 20(9):717 – 724.

Parashar, U.D., Gibson, C.J., Bresse, J.S., Glass, R.I., 2006. Rotavirus and severe childhood diarrhea. Emerg. Infect. Dis., 12(2):304-306.

Patel, M.M., Hall, A.J., Vinjé, J., Parashar, U.D., 2009. "Noroviruses: a comprehensive review". J. Clin. Virol., 44 (1): 1–8.

Paton, J.C., Paton, A., 1998. Pathogenesis and diagnosis of shiga toxinproducing *Escherichia coli* infections. Clin. Microbiol. Rev., 11:450-479.

Pazhani, G.P., Niyogi, S.K., Singh, A.K., Sen, B., Taneja, N., Kundu, M., Yamasaki, S., Ramamurthy, T., 2008. Molecular characterization of multidrug-resistant Shigella species isolated from epidemic and endemic cases of shigellosis in India. J. Med. Microbiol., 57:856–863.

Peluso, I., Fina, D., Caruso, R., Stolfi, C., Caprioli, F., Fantini, M.C., Caspani, G., Grossi, E., Di Iorio, L., Paone, F.M., Pallone, F., Monteleone, G., 2007. *Lactobacillus paracasei* subsp: *paracasei* B21060 suppresses human T-cell proliferation. Infect. Immun., 75: 1730–1737.

Penny, M., 2005. Diarrhoeal diseases. In: Eddleston M, et al, editors. Oxford: Handbook of tropical medicine. 2nd ed. Oxford: Oxford University Press, pp. 117–168.

Pichler, H.E.T., Diridl, G., Stickler, K., Wolf, D., 1987. Clinical efficacy of ciprofloxacin compared with placebo in bacterial diarrhea. Am. J. Med., 82 (4A):329-332.

Pickering, L., Baker, C., Long, S., McMillan, J.A., eds. 2006. Salmonella Infections. In: Red Book: Report of the Committee on Infectious Diseases. 27th ed. Elk Grove Village, IL: Am. Acad. Ped.,579-587.

Pickering, L.K., Cleary, T.G., 2002. Therapy for diarrheal illness in children. In Infections of the Gastrointestinal Tract. Blaser MJ, Smith PD, Ravdin JI, Greenburg HB, Guerrant RL, eds. Second edition. New York: Raven Press, pp.1223-1240.

Pickering, L.K., 1991. Diarrhea in children newly enrolled in day care centers in Houston. Pediatr. Infect. Dis. J., 10:282-286.

Pickering, L.K., 2003. Limitations of Antimicrobial Therapy for Enteric Infections. Clin. Update Infect. Dis., 6(3):1-4.

Popoff, M.Y., Le Minor, L., 1997. Antigenic formulas of the *Salmonella* serovars, 7th ed. WHO Collaborating Centre for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France.

Rachmilewitz, D., Katakura, K., Karmeli, F., Hayashi, T., Reinus, C., Rudensky, B., Akira, S., Takeda, K., Lee, J., Takabayashi, K., Raz, E., 2004. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology, 126: 520–528.

Radstrom, P., Skold, O., Swedberg, G., Flensburg, J., Roy, P.H., Sundstrom, L., 1994. Transposon Tn5090 of plasmid R751, which carries an integron, is related to Tn7, Mu, and the retroelements. J. Bacteriol., 176: 3257–3268.

Rahn, K., De Grandis, S.A,, Clarke, R.C., McEwen, S.A., Galán, J.E., Ginocchio, C., Curtiss, R. III, Gyles, C.L., 1992. Amplification of an invA gene sequence of Salmonella typhimurium by polymerase chain reaction as a specific method of detection of Salmonella. Mol. Cell Probes, 6:271-279.

Raj, P., 1993. Pathogenesis and laboratory diagnosis of *Escherichia coli*associated enteritis. Clin. Microbiol. News, 15:89-96.

Rayamajhi, N., Kang, S.G., Kang, M.L., Lee, H.S., Park, K.Y., Yoo, H.S., 2008. Assessment of antibiotic resistance phenotype and integrons in Salmonella enterica serovar Typhimurium isolated from swine. J. Vet. Med. Sci., 70(10):1133-1137.

Reece, S., Simmons, C.P., Fitzhenry, R.J., Matthews, S., Phillips, A.D., Dougan, G., Frankel, G., 2001. Site-directed mutagenesis of intimin alpha modulates intimin-mediated tissue tropism and host specificity. Mol. Microbiol., 40(1):86-98.

Reisinger, E.C., Fritzsche, C., Krause, R., Krej, G.J., 2005. Diarrhea Caused by Primarily Non-Gastrointestinal Infections. Nat. Clin. Pract. Gastroenterol. Hepatol., 2(5):216-222.

Reller, M.E., 2008. Salmonella Species. In: Long A, Pickering, L, Prober C (Editors). Principles and Practices of Pediatric Infectious Diseases Elsevier, Inc. 3rd ed. Orlando, FL: Churchill Livingstone. 812-817.

Resta-Lenert, S., Barrett, K.E., 2003. Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). Gut, 52: 988–997.

Retamal, P., Castillo-Ruiz, M., Mora, G.C., 2009. Characterization of MgtC, a

Virulence Factor of Salmonella enterica Serovar Typhi. PLoS ONE, 4(5): e5551.

Rhee, S.J., Wilson, K.T., Gobert, A.P., Nataro, J.P., Fasano, A., 2001. The enterotoxic activity of Shigella enterotoxin 1 (ShET1) is mediated by inducible nitric oxide synthase activity. J. Pediatr. Gastroenterol. Nutr., 33:400-416.

Richard, L., Claeson, M., Pierce, N.F., 1993. Management of acute diarrhea in children: lessons learned. Pediatr. Infect. Dis. J., 12: 5-9.

Rodrigue, D.C., Mast, E.E., Greene, K.D., Davis, J.P., Hutchinson, M.A., Wells, J.G., Barrett, T.J., Griffin, P.M., 1995. A university outbreak of *Eschericheria coli* O157:H7 infections associated with roast beef and an unusually benign clinical course. J. Infect. Dis., 172:1122-1125.

Rodriguez-Bano, J., Navarro, M.D., Romero, L., Martinez-Martinez, L., Muniain, M.A., Perea, E.J., Perez-Cano, R., Pascual, A., 2004. Epidemiology and clinical features of infections caused by extended-spectrum beta-lactamaseproducing *Escherichia coli* in non-hospitalized patients. J. Clin. Microbiol., 42(3):1089-1094.

Rogerie, F., Ott, D., Vandepitte, J., Verbist, L., Lemmens, P., Habiyaremye, I., 1986. Comparison of norfloxacin and nalidixic acid for treatment of dysentery caused by Shigella dysenteriae type 1 in adults. Antimicrob. Agents Chemother., 29: 883-886.

Roy, A.K., Sinha, K.K., Chourasia. H.K., 1988. Aflatoxin Contamination of Some Common Drug Plants. App. Env. Microbiol., 842-843.

Sack, D.A., Lyke, C., McLaughlin, C., Suwanvanichkij, V., 2001. Antimicrobial resistance in shigellosis, cholera and campylobacteriosis. WHO/CDS/CSR/DRS/ 2001.8

Saint, F., Gueguen, G., Biserte, J., Fontaine, C., Mazeman, E., 2000. Rupture of the patellar ligament one month after treatment with fluoroquinolone. (in French). Rev. Chir. Orthop. Reparatrice Appar. Mot., 86(5):495–497.

Salam, M.A., Bennish, M.L., 1991. Antimicrobial therapy for shigellosis. Rev. Infect. Dis., 13(Suppl 4):S332–S341.

Samie, A., Obi, C.L., Bessong, P.O., Namrita, L., 2005. Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. Afr. J. Biotechnol., 4(12):1443-1451.

Samie, A., Obi, C.L., Dillingham, R., Pinkerton, R.C., Guerrant, R.L., 2007. Enteroaggregative Escherichia coli in Venda, South Africa: distribution of virulence-related genes by multiplex polymerase chain reaction in stool samples of human immunodeficiency virus (HIV)-positive and HIV-negative individuals and primary school children. Am. J. Trop. Med. Hyg., 77(1):142-150.

Samie, A., Obi, C.L., Lall, N., Meyer, J.J.M., 2009. In-vitro cytotoxicity and antimicrobial activities, against clinical isolates of Campylobacter species and Entamoeba histolytica, of local medicinal plants from the Venda region, in South Africa. Ann. Trop. Med. Parasitol., 103 (2):159–170.

Sang, W.K., Saidi, S.M., Yamamoto, H., Ezaki, T., Iida, T., Yoh, M., Honda, T., 1996. Haemorrhagic colitis due to *Escherichia coli* O157:H7 in Kenya. J. Trop. Pediatr., 42:118-119.

Schechter, L.M., Damrauer, S.M., Lee, C.A., 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the *hilA* promoter. Mol. Microbiol., 32: 629-642.

Schechter, L.M., Jain, S., Akbar, S., Lee, C.A., 2003. The small nucleoid-binding proteins H-NS, HU, and Fis affect *hilA* expression in *Salmonella enterica* serovar Typhimurium. Infect. Immun., 71:5432-5435.

Segal, H.R., Thomas, R., Gay Elisha, B., 2003. Characterization of Class 1 integron resistance gene cassettes and the identification of a novel IS-like element in *Acinetobacter baumanii.* Plasmid, 49:16.

Serna, A. 4th, Boedeker, E.C., 2008. Pathogenesis and treatment of Shiga toxin-producing Escherichia coli infections. Curr. Opin. Gastroenterol., 24(1):38-47.

Sethabutr, O., Venkatesan, M., Murphy, G.S., Eampokalap, B., Hoge, C.W., Echeverria, P., 1993. Detection of shigellae and enteroinvasive *Escherichiacoli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. J. Infect. Dis., 167: 458-461.

Sethuraman, U., Kamat, D., 2007. Management of child with fever after international travel. Clin. Pediatr. (Phila), 46(3):222-227.

Sewram, V., Shephard, G.S., van der Merwe, L., Jacobs, T.V., 2006. Mycotoxin Contamination of Dietary and Medicinal Wild Plants in the Eastern Cape Province of South Africa J. Agric. Food Chem., 54(15):5688–5693.

Sinha, S., Shimada, T., Ramamurthy, T., Bhattacharya, S.K., Yamasaki, S., Takeda, Y., Balakrish, N.G., 2004. Prevalence, serotype distribution, antibiotic

susceptibility and genetic profiles of mesophilic Aeromonas species isolated from hospitalized diarrhoeal cases in Kolkata, India. J. Med. Microbiol. 53(6):527-534.

Slack, R.C., Wamola, I.A., 1977. Aminoglycoside resistance in gram-negative rods isolated from clinic specimens in Kenya. East Afr. Med. J., 54(10):552-555.

Smith, A.M., Govender, N., Keddy, K.H., Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA)., 2010. Quinolone-resistant Salmonella Typhi in South Africa, 2003-2007. Epub 2009 Jun 29. Epidemiol. Infect., 138(1):86-90.

Smith, H.R., Cheasty, T., 1998. Diarrhoeal diseases due to *Escherichia coli* and *Aeromonas.* In Topley and Wilson's Microbiology and Microbial Infections: Rev edition. Publisher: Hodder Arnold, vol 3, pp. 513.

Smith, P.G., Bradley, R., 2003. Bovine spongiform encephalopathy (BSE) and its epidemiology. Br. Med. Bull., 66: 185–198.

Smolinske, S.C., 2005. DABAT Herbal Product Contamination and Toxicity. J. Pharm. Pract., 18(3):188-208.

Snider, S., 1991. Beware the unknown brew: herbal teas and toxicity. FDA Consumer,
http://findarticles.com/p/articles/mi_m1370/is_n4_v25/ai_10871081/

Sooka, A., du Plessis, M., Keddy, K., 2004. Enterovirulent *Escherichia coli.* The S African J. Epidemiol. Infect., 19(1):23-33.

Sow, A.G., Wane, A.A., Diallo, M.H., Boye, C.S., Aïdara-Kane, A., 2007. Genotypic characterization of antibiotic-resistant Salmonella enteritidis isolates in Dakar, Senegal. J. Infect. Dev. Ctries.,1(3):284-288.

Stahlmann, R., Lode, H., 2010. Safety considerations of fluoroquinolones in the elderly: an update. Drugs Aging, 27(3):193-209.

Steffen, R., Castelli F, Dieter N.H., Rombo, L., Zuckerman N., 2005. Vaccination against enterotoxigenic Escherichia coli, a cause of travelers' diarrhea. J. Travel Med., 12:102-107.

Steffen, R., Mathewson, J.J., Ericsson, C.D., DuPont, H.L., Helminger, A., Balm, T.K., et al., 1988. Travellers diarrhoea in West Africa and Mexico: Fecal transport systems and liquid bismuth subsalicylate for self-therapy. J. Infect. Dis., 159: 1008-1013.

Su, L.H., Chiu, C.H., 2007. Salmonella: clinical importance and evolution of nomenclature. Chang Gung Med. J., 30(3):210-219.

Sundsfjord, A., Simonsen, G.S., Haldorsen, B.C., Haaheim, H., Hjelmevoll, S.O., Littauer, P., Dahl, K.H., 2004. Genetic methods for detection of antimicrobial resistance. Apmis, 112(11-12):815-837.

Morpeth, S.C., Ramadhani, H.B., Crump, J.A., 2009. Invasive Non-Typhi *Salmonella* Disease in Africa. Clin. Infect. Dis., 49:606–611.

Swerdlow, D.L., Woodruff, B.A., Brady, R.C., Griffin, P.M., Tippen, S., Donnell, J.D., et al., 1992. A waterborne outbreak in Missouri of Escherichia coli O157:H7 associated with bloody diarrhea and death. Ann. Intern. Med., 117:812-819.

Taylor. J.L.S., Rabe, T., McGaw, L.J., Jäger, A.K., van Staden, J., 2001. Towards the scientific validation of traditional medicinal plants. Plant Growth Regulation, 34: 23–37.

Thong, K.L., Hoe, S.L.L., Puthucheary, S.D., Yasin, R., 2005. Detection of virulence genes in Malaysian *Shigella* species by multiplex PCR assay. BMC Infect. Dis., 5:8 http://www.biomedcentral.com/1471-2334/5/8

Todd, E.C.D., 1989. Preliminary estimates of costs of foodborne disease in Canada and costs to reduce salmonellosis. J. Food Prot., 52:586–594.

Touchette, P., Douglass, E., Graeff, E., Monoang, I., Mathe, M., Duke, L.W., 1994. An analysis of homebased oral rehydration therapy in the Kingdom of Lesotho. Soc. Sci. Med., 39:425-432.

Trachtman, H., Christen, E., 1999. Pathogenesis, treatment, and therapeutic trials in hemolytic uremic syndrome. Curr. Opin. Pediatr., 11(2): 162-168.

Travers, K., Barza, M., 2002. Morbidity of infections caused by antimicrobial-resistant bacteria. Clin. Infect. Dis., 34 Suppl 3:S131-S134.

Tuttle, J., Ries, A.A., Chimba, R.M., Perera, C.U., Bean, N.H., Griffin, P.M., 1995. Antimicrobial-resistant epidemic *Shigella dysenteriae* type 1 in Zambia: modes of transmission. J. Infect. Dis., 171:371-375.

UNICEF and WHO, 2000. Diarrhoea: Why children are still dying and what can be done. http://www.childinfo.org/diarrhoea.html. Accessed June 2010.

Van Wyk, B.E., Gericke, N., 2000. People's Plants: A Guide to Useful Plants of

Southern Africa. Briza Publications, Pretoria, ISBN 978-1-875093-19-9.

Van Wyk, B.E., Van Oudtshoorn, B., Gericke, N., 1997. Medicinal Plants of South Africa [2nd improved impression, 2000]. Briza Publications, Pretoria, 1997. ISBN 1- 875093-09-5.

Van Wyk, B.E., 2008. A broad review of commercially important southern African medicinal plants. J. Ethnopharmacol., 119:342–355.

Varma, J.K., Molbak, K., Barrett, T.J., Beebe, J.L., Jones, T.F., Rabatsky-Ehr, T., Smith, K.E., Vugia, D.J., Chang, H.H., Angulo, F.J., 2005. Antimicrobial-resistant nontyphoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. J. Infect. Dis., 191:554-561.

Varsano, I., Eidlitz-Marcus, T., Nussinovitch, M., Elian, I., 1991. Comparative efficacy of ceftriaxone and ampicillin for treatment of severe shigellosis in children. J. Pediatr., 118(4 Pt 1): 627-632.

Vieira, R.H.S., Rodrigues, D., Goncalves, F.A., Menezes, F.G.R., Aragao, J.S., Sousa, O.V., 2001. Microbicidal effect of medicinal plant extracts (*Psidium guajava* Linn. and *Carica papaya* Linn.) upon bacteria isolated from fish muscle and known to induce diarrhea in children. Instituto de Ciencias do Mar-UFC, Ceara, Brazil. Rev. Instit. Med. Trop. de Sao Paulo, 43(3):145-148.

Villa, L., Carattoli, A., 2005. Integrons and Transposons on the *Salmonella enterica* Serovar Typhimurium Virulence Plasmid Antimicrob. Agents. Chemother., 49(3): 1194–1197.

Walker-Smith, J.A., 1990. Management of infantile gastroenteritis. Arch. Dis. Child., 65(9):917–918.

Watanabe, T., 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. 27:87-115.

Watt, J.M., Breyer-Brandwijk, M.G., 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa, 2nd ed. Livingstone, London.

Weekly Epidemiological Record. 2005. Shigellosis: disease burden, epidemiology and case management No. 11, 80, 93–100 http://www.who.int/wer

Wennerås, C., Erling, V., 2004. Prevalence of enterotoxigenic Escherichia coli-associated diarrhoea and carrier state in the developing world. J. Health Popul, Nutr., 22:370-382.

WHO. World Health Organization. 1995. Guidelines for the control of epidemics due to *Shigella dysenteriae* type 1 (WHO/CDR/95.4). Geneva: The Organization; pp. 147.

WHO. World Health Organization. 1999. Basic Food safety for health workers. WHO. Available at: whqlibdoc.who.int/ hq/1999/WHO_SDE_PHE_FOS_99.1.pdf – Accessed May 2010

WHO. World Health Organization. 2000. Global Water Supply and Sanitation Assessment. WHO/UNICEF Joint Monitoring Programme for Water Supply and Sanitation World Health Organization. Geneva. http://www.who.int/ docstore/watersanitation_health/Glob assessment/ GlobalTOC.htm

WHO. World Health Organization. 2001. Global Strategy for Containment of Antimicrobial Resistance. http://whqlibdoc.who.int/hq/2001/WHO_CDS_ CSR_DRS_2001.2. pdf

WHO. World Health Organization. 2004. The global burden of disease: 2004 update. http://www.who.int/healthinfo/global_burden_disease/2004_report _update/en/index.html

WHO. World Health Organization. 2005a. Drug-resistant Salmonella REF 1-6. Fact sheet N°139. Revised April 2005. http://www.who.int/mediacentre/ factsheets/ fs139/en/

WHO. World Health Organization. 2005b. Deptartment of Child and Adolescent Health and Development. Guidelines for the control of Shigellosis, including epidemics due to *Shigella dysenteriae type 1*. Geneva: World Health Organization, whqlibdoc.who.int/publications/2005/9241592330.pdf

WHO. World Health Organization. 2007. Food safety and foodborne illness. 2007. http://www.who.int/mediacentre/factsheets/fs237/en. Accessed May 2010.

WHO. World Health Organization. 2008. Traditional medicine. Fact sheet N°134. http://www.who.int/mediacentre/factsheets/fs134/en/

WHO. World Health Organization. 2009a. Diarrhoeal Diseases: Enterotoxigenic *Escherichia coli* (ETEC). (Updated February 2009) http://www.who.int/ vaccineresearch/ diseases/diarrhoeal/en/ index4.html

Willett, W.C., Radojcic, V., 1976. Urinary tract pathogens and antibiotic sensitivity patterns in Dar es Salaam. East Afr. Med. J., 53(12):685-692.

Williams, N.T., 2010. Probiotics. Am. J. Health Syst. Pharm., 15; 67(6):449-458.

Winokur, P.L., Canton, R., Casellas, J.M., Legakis, N., 2001. Variations in the prevalence of strains expressing an extended-spectrum b-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. Clin. Infect. Dis., 32(2):S94-S103.

Wisura, W., Glen, H.F., 1993. The South African species of *Carpobrotus* (Mesembryanthema, Aizoaceae) Contribution, Boletim Herbage. 15: 76-107.

Wittenberg, D.F., 1999. Emerging and re-emerging diseases-epidemic enterohaemorrhagic infections 100 years after Shiga. S. Afr. Med. J., 89: 750-752.

Wolf, D.C., Gianella, R.A., 1996. Invasive pathogens. Consultations in Gastroenterol., 381-384.

Yang, F., Yang, J., Zhang, X., Chen, L., Jiang, Y., Yan, Y., et al., 2005. Genome dynamics and diversity of *Shigella* species, the etiologic agents of bacillary dysentery. Nucleic Acids Res., 33(19): 6445 - 6458.

Yismaw, G., Negeri, C., Kassu, A., 2006. A five-year antimicrobial resistance pattern observed in *Shigella* species isolated from stool samples in Gondar University Hospital, northwest Ethiopia. Ethiop. J. Health Dev., 20(3):194-198.

Yorston, D., Foster, A., 1994. Traditional eye medicines and corneal ulceration in Tanzania. J. Trop. Med. Hyg., 97:211-214.

Zhao, S., White, D.G., Ge, B., Ayers, S., Friedman, S., English, L., Wagner, D., Gaines, S., Meng, J., 2001. Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing Escherichia coli isolates. Appl. Environ. Microbiol., 67: 1558–1564.

Zherebtsova, N.Y., Baimiev A., Valishin, D.A., Mavzyutov, A.R., 2007. Genetic markers of pathogenicity of conditionally pathogenic Enterobacteria isolated from children with diarrhoeal diseases. Zh. Mikrobiol. (Moscow), 2:3-8.

CHAPTER 2

EXTENDED-SPECTRUM BETA-LACTAMASES AND CLASS 1 INTEGRON MEDIATED RESISTANCE IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* AND *SALMONELLA*

2.0. Abstract

Molecular characterization for the presence of specific antimicrobial resistance mechanisms was carried out on 119 *Salmonella* and 90 *Escherichia* coli isolates from patients with diarrhoea in the Eastern Cape Province, South Africa. PCR and sequencing were used to verify the genetic determinants responsible for extended spectrum beta-lactamases (ESBLs) phenotypes. Of the 119 *Salmonella*

isolates, 61 (51.2 %) were ESBL, positive by genotypic determination. A total of 9 (14.7 %) and 54 (88.5 %) of the 61 isolates were CMY-2 and TEM b-lactamase producers, respectively. Only 1 of the *Salmonella* spp. was CTX-M ESBL's producer. Among the *E. coli* isolates the mediators of resistance were 13 (68.4 %), 9 (47.4 %) and 2 (10.5 %) of blaSHV, bla_{TEM} and bla_{CTX-M} respectively. Class 1 integron *(Int1*) gene occurred significantly among the *Salmonella* species, mostly among isolates expressing TEM genes. This study reports the presence of the bla_{CMY-2}, bla_{SHV-1}, bla_{TEM-20} and bla_{TEM-1} genes and the involvement of class 1 integrons mediated resistance in *Salmonella* and *Escherichia* spp. from the Eastern Cape, South Africa. Results suggest that integrons contribute significantly to antimicrobial resistance among the enteric gram-negative bacilli studied. Molecular genotyping of pathogens is fundamental to tracking disease-associated and drug-resistant strains in various populations.

Key words: ESBLs; *Salmonella*; resistance; phenotypes, genotypes, integrons

2.1. Introduction

Among the agents causing gastroenteritis, *Salmonella, Shigella* and *Escherichia coli* are important bacterial pathogens. *Salmonellae* are regarded as ubiquitous food-borne pathogens causing widely distributed infections with the consumption of especially raw and undercooked foods of animal origin, such as beef, poultry, milk, and eggs. Salmonellae can be differentiated by their somatic (O) antigens, composed of lipopolysaccharides, and the flagellar (H) antigens (Kaye, 1996). More than 2,500 *Salmonella* serotypes have been identified to date (WHO,

2005). Various species of *Salmonella* cause varying types of infectious diseases. While some are host-specific some have a broad range of hosts; *S. enterica* serovar Typhi is adapted to humans and does not occur in animals while non-typhoidal *Salmonella* serovars (NTS) have a broad vertebrate host range (Gordon, 2008). *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are the most frequently isolated serovars from food-borne outbreaks throughout the world (Herikstad *et al.,* 2002) and are the two most important serotypes for salmonellosis transmitted from animals to humans. *Salmonella enterica* serovar Dublin is associated with cattle while *Salmonella enterica* serovar Choleraesuis is linked with pigs (WHO, 2005).

Salmonellosis affects a significant portion of human population causing approximately 2.16 million cases of infections and 216,000 deaths worldwide (Crump *et al.,* 2004; Boyle *et al.,* 2007). Public health concerns involving *Salmonella* include sporadic outbreaks of infections due to the pathogen even in the so called developed countries despite advanced technology and infection controls (Bisi-Johnson *et al.,* 2007). In the US, a recent outbreak of *Salmonella* in ready-to eat sausages linked to spices has been reported (Gifford, 2010). Salmonellosis manifests as diarrhoea, fever, and abdominal cramps between 12 and 72 hours after eating contaminated food. The illness which usually lasts 4–7 days and is self-limiting in most people may spread in vulnerable groups, such as infants, the elderly and immuno-compromised individuals beyond the intestine to the bloodstream and cause a more severe systemic disease.

Escherichia coli is recognized as both a normal intestinal floral with beneficial roles and a versatile human pathogen causing a wide range of infections including diarrhoeal diseases. Based on the strain and pathogenic mechanisms, different pathotypes of diarrheagenic *E. coli* are recognized. These include Enterotoxigenic *E. coli* (ETEC) which is a cause of traveler's diarrhea, Enterohemorrhagic *E. coli* (EHEC) which cause hemorrhagic colitis or hemolytic-uremic syndrome (HUS), Enteropathogenic *E. coli* (EPEC) which cause either a watery or bloody diarrhea, Enteroinvasive *E coli* (EIEC) a cause of *Shigella* -like dysentery, while Enteroaggregative *E. coli* (EAggEC) is often associated with persistent diarrhoea in children in developing countries. The main reason advanced by Chatterjee *et al.* (2009) for the occurrence of many of the strains of diarrheagenic *E. coli* in the developing nations is inadequate sanitary conditions. However, the most common enteropathogen in developing countries is ETEC. This pathotype is reported to account for approximately 210 million diarrhoea episodes and approximately 380,000 deaths (WHO, 2008; Gupta *et al*., 2008).

Antibiotics are often not recommended in the treatment of enteritis. It is believed that antibiotics prolong the carrier state of enteritis due to *Salmonella*. Nevertheless, systemic *Salmonella* infections and infections in the vulnerable groups qualify for antibiotic therapy (DuPont, 2003). Fluoroquinolones are often the drug of choice in the treatment of gastrointestinal infections in humans. Resistance to fluoroquinolones has emerged worldwide during the last decade and is associated with increased illnesses and deaths (Helms *et al.,* 2004).

Extended-spectrum cephalosporins are important for treating persons with severe *Salmonella* infections (Hohmann, 2001). The emergence of *Salmonella* isolates resistant to oxyiminocephalosporins has become a cause for concern due to the fact that cephalosporins are the alternatives to fluoroquinolones in children because of toxicity issues (Aarestrup *et al.*, 2005). The resistant mechanism involves the production of extended-spectrum β-lactamases (ESBLs).

ESBL's have traditionally been defined as transmissible beta-lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam. They are a group of enzymes that break down antibiotics belonging to the penicillin and cephalosporin groups and render them ineffective. The ESBL's are generally encoded by mobile genes that can be exchanged between bacteria. The first plasmid-mediated ampicillin-hydrolyzing beta-lactamases was described in a strain of *E. coli* by Datta and Kontomichalou (1965) in Greece soon after the broad-spectrum penicillin was introduced in the 1960's and was named TEM-1 after the patient, whose name was Temoniera from whom the *E. coli* strain was isolated. This was soon followed by another common plasmid-mediated β-lactamase SHV-1 (for sulphydryl variable) found in *Klebsiella pneumoniae* and *E. coli.*

The first extended-spectrum beta-lactamase (ESBL), SHV-2; capable of hydrolyzing expanded-spectrum β-lactam antibiotics (third-generation cephalosporins) was found in an isolate of *Klebsiella ozaenae* (Kliebe *et al.*, 1985). These enzymes have spread over various regions of the world, across

members of *Enterobacteriaceae* with almost 200 different types identified to date (Bradford, 2001). Other ESBLs are the CTX-M, VEB, PER, GES, OXA groups and the inducible chromosomal class C (AmpC) betalactamases. AmpC beta-lactamases which emerged in *Enterobacter* spp. have also migrated from chromosomal locations to plasmids and are spreading into *E. coli* and *Klebsiella* spp. Hence, it is pertinent to investigate the mechanisms of antibiotic resistance which is one of the arsenals of pathogenicity in enteric pathogens. This study was embarked upon to identify and characterize the array of ESBLs involved in drug resistance in clinical isolates of *E. coli* and *Salmonella* spp.

2.2. Materials and methods

2.2.1. Data collection and ethical approval

Written informed consents were obtained from all patients, parents or guardians as the case may be and questionnaires were administered by trained volunteer health care workers (appendices 1A-1D). The study protocol and data handling were approved by the WSU ethics committee (Protocol No. 0003/08) as well as the Department of Health, Eastern Cape, South Africa.

2.2.2 Bacterial isolates

One hundred and nineteen *Salmonella* spp and 90 *Escherichia coli* isolated from clinical specimens of patients attending the Nelson Mandela Academic Hospital Complex, a tertiary hospital in the Eastern Cape Province, South Africa and satellite clinics were characterized by standard microbiological procedures. Minimum inhibitory concentrations (MICs) of standard antibiotics were determined with an automated antimicrobial susceptibility system according to the manufacturer's instructions (Autoscan-4 antimicrobial susceptibility system; Siemens). Comprehensive antibiograms were determined for the isolates, using a customized antimicrobial panel of 24 antibiotics (Gram Negative combo panel NUC 45). The interpretation of susceptibility was according to the Clinical and Laboratory Standards institute (CLSI) standards for broth microdilution methods (CLSI, 2007).

2.2.3. Identification and characterization of resistance genes

Polymerase chain reaction (PCR) was used to confirm the presence of antibiotic resistance genes in the *E. coli* and *Salmonella* species. Testing for extended-spectrum cephalosporin-resistance mechanisms included susceptibility testing of additional β-lactams, such as ceftazidime and cefotaxime, and molecular characterization of β-lactamases and β-lactamase genes. Isolates were examined for the presence of ESBL enzyme bla_{TEM}, bla_{SHV}, bla_{CMY-2} and bla_{CTX-M}

types (Arlet and Philippon, 1991; Gallego *et al.,* 1990; Gazouli *et al.,* 1988) as well as the involvement of class 1 integron *intI1* genes.

2.2.4. DNA extraction and primer synthesis

DNA template for PCR was obtained from pure overnight bacterial culture using Fungal/Bacterial DNA extraction kit™ (Zymo Research) following manufacturer's instructions. Eluted DNA verification and concentration measurements were carried out using NanoDrop 2000 spectrophotometer (Thermo Scientific). All primers used in amplification and sequencing reactions were synthesized by Inqaba biotechnology Industries Pty. Ltd., Pretoria, South Africa.

2.2.5. DNA amplification

PCR amplifications were performed in a final volume of 25 µL containing: 0.5 to 2 µL of DNA template depending on concentration, 8.5 to 10 µℓ of Nuclease free water, 1 µL of each primer and 12.5 µL Master mix (EconoTaq Green, Fermentas). Amplifications were carried out in a GeneAmp PCR System 9700 Thermocycler (Applied Biosystems). The PCR conditions were 94°C for 5 min followed by 40 cycles of: 94°C for 30 s, 59°C for 1 min, 72°C for 1 min and final 72°C for 7 min for the ESBL primer sets used and 94°C for 5 min followed by 30 cycles of: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final 72°C for 7 min for the integron (*intI1*) gene. Table 2.1 shows the primers for the target genes. Amplification products were separated by electrophoreses on 2 % agarose gel (TopVision TM, Fermentas) in 1X TBE Buffer and ethidium bromide

(5 µL) with a 100-bp ladder (Fermentas) as molecular weight marker. Agarose gels were visualized under UV illumination (TFM-26 Ultraviolet Transilluminator, UVP, Upland, CA) and the images were captured using a digital gel documentation system (DigiDoc-It imaging system, UVP, Upland, CA).

2.2.6. Sequencing reaction

PCR products were sequenced using an Applied Biosystems 3500xL Genetic analyzer (AB Biosystems). Prior to PCR products sequencing, the unincorporated dNTPs were dephosphorylated with a commercial kit from Zymo Research Corporation (Orange, CA). Subsequently, the PCR products were sequenced with the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (AB Biosystems) using the same primers as employed in the PCR reactions. The products were then subjected to the following conditions: 94°C for 2 min, followed by 40 cycles of denaturation at 85°C for 1 s, annealing at 53°C for 10 s and extension at 60°C for 2 min 30 s, with a final extension at 4°C for 0 s.

Table 2.1. Primer sets for ESBLs and class I integron and the PCR conditions.

Target gene	Primer	Nucleotide Sequence (5'- 3')	Amplicon size (bp)	References
bla_{CMY-2}	Amp3 (F) Amp2 (R)	ATGATGAAAAAATCGTTATG CTGC TTATTGCAGCTTTTCAAGAA TGCGCCA	1,145	Pitout *et al.*, 1988
bla_{CTX-M}	CTX-MA CTX-MB	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550	Bell *et al.*, 2002

bla_{SHV}	SHV-1 (F) SHV-3 (R)	ATGCGTTATATTCGCCTGTG GTTAGCGTTGCCAGTGCTCG	846	Fey *et al.*,2000
bla_{TEM}	TEM-1 (F) TEM-4 (R)	ATGAGTATTCAACATTTCCG TG TTACCAATGCTTAATCAGTG AG	840	Fey *et al.*, 2000
intI1	INT1F INT1R	AAGGATCGGGCCTTGATGTT AGCGCATCAAGCGGTGAGC	471	Pongpech *et al.*, 2008

The sequencing reaction products were cleaned up using ZR-96 DNA sequencing clean-up kit™. Thereafter, the ultra-pure products were analyzed on the sequencing machine. Sequences were aligned with known ESBL and integron sequences by a blast search of the National Center for Biotechnology Information (NCBI) data base (www.ncbi.nlm.nih.gov/BLAST/) using Staden package version 1.6.0-beta4 (MRC.WTSI) and comparison and confirmation were also done with known ESBLs (www.lahey.org/studies/) compiled by Jacoby and Bush (n.d).

2.2.7. Statistical analysis

Patients' data were analyzed using Microsoft Excel version 2003. Continuous variables were summarized as mean and standard deviation (SD). Demographic information was obtained from subjects using questionnaire.

2.3. Results

2.3.1. Demographic features

Our subjects were predominated by male patients 165/315 (52.4%) and no age group was excluded, with the youngest patient being 3 months and the oldest 91 years. The median age ± IQR equals 36 ± 22 years where IQR (inter-quartile range) is a measure of the dispersion or how widespread out the values are. In all, there were 55 (17.5%) pediatrics patients. Information obtained from the questionnaires administered indicated that a sizeable number of our subjects depended on domestic and drinking water from unwholesome sources like rivers and streams. Prior to the clinic visit, 13 persons with diarrhea (4.1%) took some form of medicine. Three of the instances were complementary therapy with an instance of antimicrobial active against enteric gram-negative rods (GNRs) and the remaining 10 instances involved the use of concoction of medicinal plants. Most of the persons who took medicinal plants were HIV positive.

2.3.2. Characterization and antibiogram of isolates

Various serovars of *Salmonella* were identified with *S. enterica serovar* Typhi being the highest followed by *S. enterica serovar* Typhimurium. Resistance to 5 or more CLSI antibiotics subclasses was detected in 59/119 (49.6 %) of the *Salmonella* isolates and 18/90 (20 %) of *E. coli.* Eighty-seven (73.1 %) of the *Salmonella* isolates mostly *S. enterica* serovar Typhi were invasive strains. *Salmonella* isolates were resistant to amoxicillin, ampicillin, aztreonam, piperacillin/tazobactam, trimethoprim/ sulfamethoxazole and tetracycline with reduced susceptibility to ciprofloxacin. *E. coli* strains were also identified and showed resistance mostly to ampicillin, ampicillin/sulbactam, cephalothin,

trimethoprim/sulfamethoxazole and tetracycline. No quinolone, carbapenem or gentamicin resistant strain was found among the *E. coli* isolates. Eight out of ten *Salmonella* isolates which were resistant to the quinolone of choice (ciprofloxacin) besides being resistant to the second and third generation cephalosporins tested also exhibited resistance or intermediate resistance to at least 2 or all 3 carberpenems (ertapenem, imipenem and meropenem) drugs tested. Extended spectrum cephalosporins resistant phenotype was exhibited by 25/119 (21.0 %) of the *Salmonella* spp.

2.3.3. Detection of genes mediating ESBL resistance

PCR characterization and sequencing showed that the β-lactam resistance was mediated majorly by bla_{TEM} with the involvement of class 1 integron gene, *intI1*. β-lactam genes bla_{CMY-2} and bla_{SHV} were also expressed but in lower percentages. Of the 119 *Salmonella* isolates, 61 (51.2 %) were ESBL positive by genotypic determination. A total of 54 (88.5 %) and 9 (15.7 %) of the 61 isolates were TEM and CMY-2 (Amp-C) ESBL producers, respectively. Nine (11 %) isolates were positive for both bla_{CMY-2} and bla_{TEM} while only one was positive for bla_{CTX-M} genes. Of the 61 *Salmonella* isolates with one or more β-lactams, 45 (73.8 %) were *S. enterica serovar* Typhi; 9 (14.7 %) were *S. enterica serovar* Typhimurium; and 2 (3.3 %) were *S enterica serovar* Isangi. Out of the 90 *Eshcherichia* isolates, 19 (21.1 %) were ESBL positive by genotypic determination, 13 (68.4 %), 9 (47.4 %) and 2 (10.5 %) of the 19 ESBL positive *E coli* isolates were $bla_{SHV,}$ bla_{TEM} and bla_{CTX-M} genes respectively. Class I

integron genes were expressed in 83 (39.7 %) of all the isolates, 64 (77.1 %) of these were *Salmonella* spp. while 19 (22.9 %) were *E. coli*. Representative positive samples are shown in Figures 2.1a and 2.1b. The alignment and blast search results of representative sequenced products showed in most cases 100% correlation confirming the identity of our isolates, the ESBLs and the integron genes involved in the resistance mechanisms. Among the 4 representative isolates, sequencing for the specific bla_{TEM} genes type revealed the presence of one bla_{TEM-20}, two were bla_{TEM-1} and the fourth was bla_{TEM-4} gene. The SHV genes were of the bla_{SHV-1} type found only in the *Escherichia* spp while the Amp-C was confirmed as bla_{CMY-2}. The designations of the enzymes were as confirmed (http:www.lahey.org/studies/webt.htm). Figure 2.2 shows the results of the blast search of the integron genes among the *Salmonella* isolates. The phylogenetic correlations of the integron gene sequences revealed a monophyletic clustering (Figure 2.3).

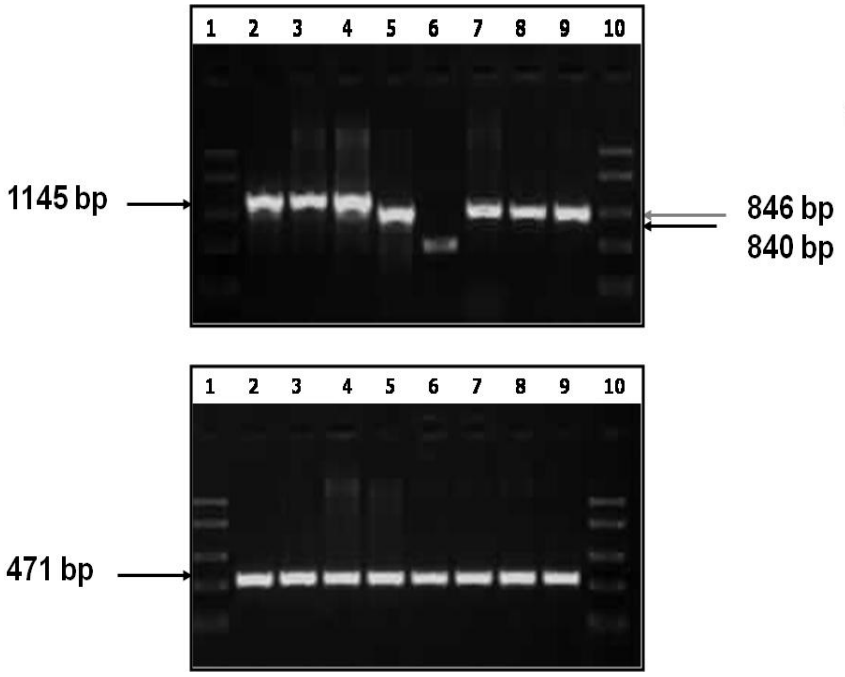


Figure 2.1A

Figure 2.1B

Figure 2.1. PCR products analysis of the genomic DNA from selected *E. coli* and *Salmonella* isolates. Figure 2.1A: 100 bp molecular weight marker (lanes 1 and 10), fragment from bla_{CMY-2} (lanes 2 to 4), bla_{SHV} (lanes 5) and bla_{TEM} (lanes 7 to 9). Figure 2.1B: 100 bp molecular weight marker (lanes 1 and 8) *Int1* (lanes 2 to 9). The relative positions in the gel of predicted size of PCR products are indicated by arrowheads on the right sides.

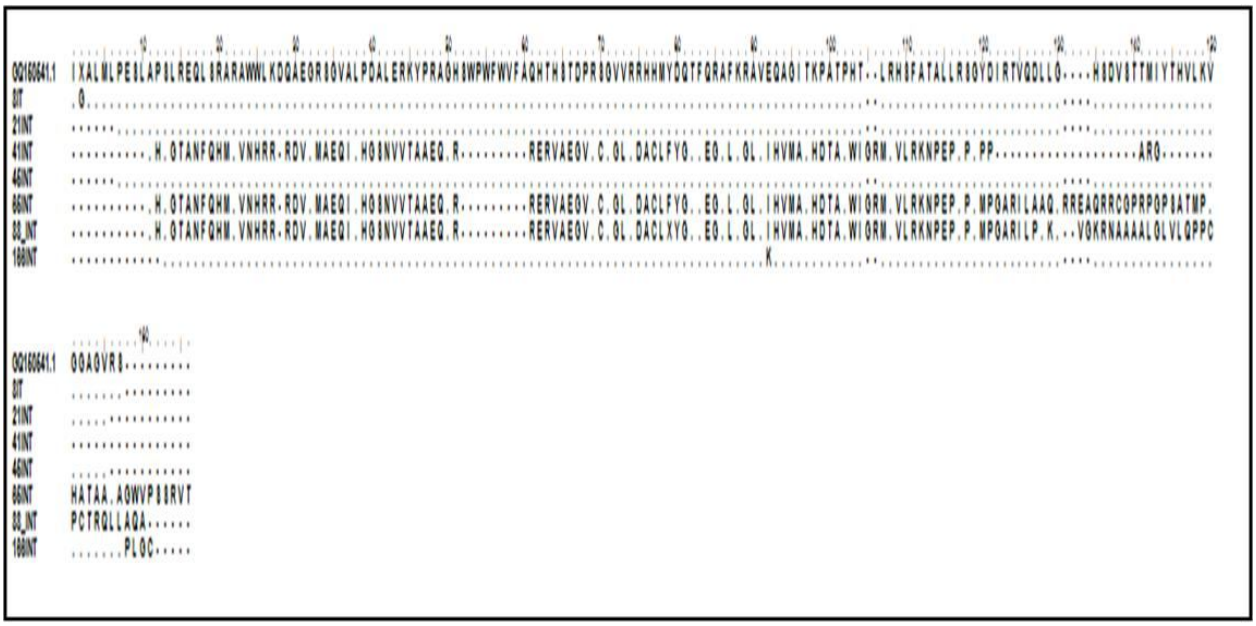


Figure 2.2. Blast search result for the sequenced integron genes from selected *Salmonella* isolates.

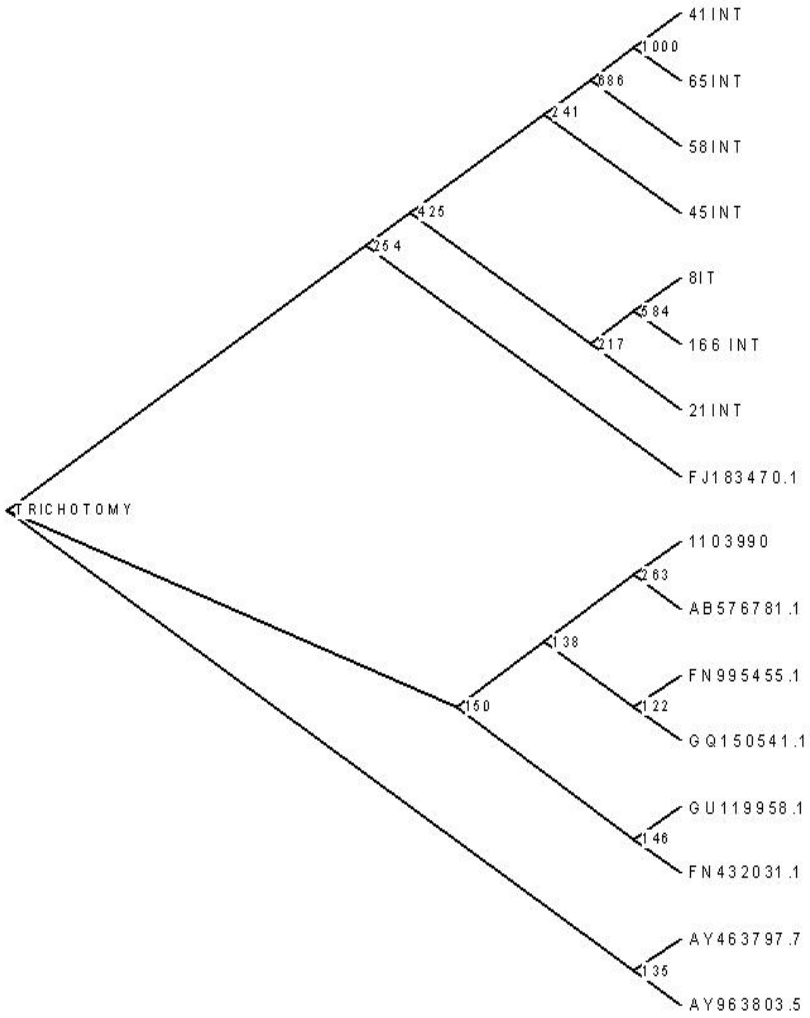


Figure 2.3. A neighbour-joining phylogenetic relationship of the class 1 integron genes isolated from *Salmonella* spp.

2.4. Discussion

The emergence of ESBL producing isolates has become a task which requires drastic interventions. Bacteria strains exhibiting ESBLs are often resistant to a wide variety of commonly used antibiotics. These isolates often are

not phenotypically resistant according to CLSI guidelines with the consequential avoidable treatment failures in patients who received inappropriate antibiotic therapy (Karas *et al.,* 1996; Paterson *et al.,* 1998). For this reason, ESBL producing organisms are to be reported as resistant to all extended-spectrum β-lactam antibiotics with the exception of the cephamycins (NCCLS, 2000; Manickam and Alfa, 2008). Apart from ESBLs, plasmid-mediated AmpC beta-lactamases are also typically associated with broad multidrug resistance (Thompson, 2001). The high frequency of resistance to drugs such as ampicillin, tetracycline and trimethoprim/sulfamethoxazole were not unexpected since, these are some of the oldest drugs used in infectious disease, and some level of resistance would have emerged over time (Salyers and Whitt, 2005).

Often time, clinically relevant ESBL-mediated resistance goes undetected in routine susceptibility tests. Molecular methods, particularly PCR have become widely used techniques for the determination and confirmation of ESBL genes. Our study investigated clinical isolates of *Salmonella* and *E. coli* for the presence of bla_{ampC}, bla_{CTX-M}, bla_{SHV}, bla_{TEM} and class 1 integron (*intI1*) by monoplex PCR. This study found that the percentage of ESBL detected phenotypically were only 21.0 % and 5.5 % for *Salmonella* and *E. coli* respectively, while the percentage detected genotypically were 51.2 % and 21.1 % respectively. AmpC type enzyme was expressed by 11 % of our isolates while bla_{SHV} and bla_{TEM} were noted in 6.2 % and 33.4 % of isolates, respectively. The joint occurrence of AmpC-type enzyme and ESBL in the same strain can mask the expression of ESBL (Bradford

et al., 1997; Moland *et al.*, 2006). This is due to the fact that the AmpC-type beta-lactamase will not be inhibited by clavulanic acid, thus giving high MICs to both the single antimicrobial agent and the clavulanic acid combination. The result of this is that phenotypic confirmatory test may be falsely negative leading to inability to confirm the presence of ESBLs (Moland *et al.*, 2006). In a previous study conducted in South Africa, 15.6% isolates produced SHV or TEM and 1.9% produced CMY-2 ESBLs (Kruger *et al.,* 2004). Comparing this previous study with our results, an increase in the spread or expression of ESBL genes was noted, particularly the TEM and AmpC, also while the previous study failed to identify $bla_{CTX\text{-}M}$ our study observed the expression of this gene in a *Salmonella* isolate identified to be *S. choleraesuis* and 2 *E. coli* isolates. Paterson *et al.* (2003) had shown the significance of $bla_{CTX\text{-}M}$ ESBL type in *Klebsiella pneumoniae* isolates. CTX-M enzymes which predominantly hydrolyze cefotaxime over ceftazidime are reportedly increasing in recent years. Furthermore, more resistance was observed in the *Salmonella* isolates than in the *E. coli* isolates. At the onset, the recognition of β-lactamases conferring resistance to the extended-spectrum cephalosporins, were particularly with resistances among *Klebsiella* species and *Escherichia coli* strains, but other species of the family *Enterobacteriaceae* now also express ESBLs activities (Goldstein *et al.,* 1993; Cheng and Chen. 1994; Mariotte *et al.,* 1994; Philippon *et al.,* 1994; Morosini *et al.,* 1995). The spread of ESBL's genes have been reported to either be via epidemic bacterial strains or by plasmid dissemination between unrelated strains (Ben-Hamouda *et al.,* 2004).

Carbapenems are the drugs of choice against AmpC beta-lactamase producing bacteria (Jones, 2003; Jones and Varnam, 2002).

Int1 genes were expressed in 83 (39.7 %) of all the isolates, 64 (77.1 %) of these were *Salmonella* spp. while 19 (22.9 %) were *E. coli*. There were simultaneous occurrence of bla_{TEM}, bla_{SHV} and class 1 integron in a few isolates. It was also noted that the expression of bla_{TEM} and class 1 integron (*intI1*) seem to co-occur in most positive isolates. Integrons are known to play a vital role in the resistance mechanisms of many pathogens. These mobile gene cassettes are carriers of one or more resistance genes and are inserted into various arrangements between 2 conserved DNA regions, generating various antimicrobial resistance genes (Levesque *et al.,* 1995). As with other mobile DNA elements, integrons assist in the rapid dispersion of resistance genes within bacterial species and between different species (Levesque *et al.,* 1995; Hall, 1997). We may hence attribute a significant role to these genes in the spread and increasing multidrug resistance isolates in our setting.

2.5. Conclusion

The study showed more resistance in *Salmonella* isolates than *E. coli.* The increasing rates of multi-drug resistant strains and particularly the emerging resistance to penem drugs are regrettably leading gradually to fewer options of effective antimicrobials, especially for the treatment of infants and very small children. It is pertinent to increase awareness of clinical laboratories to isolates that exhibit ESBL phenotypes and make adequate diagnostic facilities available.

This may be a serious challenge particularly in the setting of this study where like in other sub-Saharan African countries, such resources are scarce. Molecular genotyping of pathogens is fundamental to tracking disease-associated and drug-resistant strains in various populations.

2.6. References

Aarestrup, F.M., Hasman, H., Jensen, L.B., 2005. Resistant *Salmonella* Virchow in quail products. Emerg. Infect. Dis., 11(12):1984-1985.

Arlet, G., Philippon, A., 1991. Construction by polymerase chain reaction and intragenic DNA probes for three main types of transferable β-lactamases (TEM, SHV, CARB). FEMS Microbiol. Lett., 82:19–26.

Bell, J.M., Turnidge, J.D., Jones, R.N., the SENTRY Asia-Pacific Participants., 2003. Prevalence of extended-spectrum beta-lactamase-producing *Enterobacter cloacae* in the Asia-Pacific region: Results from the SENTRY Antimicrobial Surveillance Program, 1998 to 2001. Antimicrob. Agents Chemother., 47:3989-3993.

Ben-Hamouda, T., Foulon, T., Ben-Mahrez, K., 2004. Involvement of SHV-12 and SHV-2a encoding plasmids in outbreaks of extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* in a Tunisian neonatal ward. Microb. Drug Resist., 10(2):132-138.

Bisi-Johnson, M.A., Momba, M.N.B., Obi, C.L., 2007. Health impact of water-borne pathogens in rural domectic water supply of KwaZulu-Natal province of South Africa on immuno-compromised individuals. Oral Presentation at the First International Conference on Environment Research, Technology and Policy (ERTEP) 16-19 July 2007. Accra, Ghana.

Boyle, E.C., Bishop, J.L., Grassl, G.A., Finlay, B.B., 2007. Salmonella: from pathogenesis to therapeutics. J. Bacteriol., 189(5):1489-1495.

Bradford, P.A., Urban, C., Mariano, N., Projan, S.J., Rahal, J.J., Bush, K., 1997. Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a plasmid-mediated AmpC β-lactamase and the loss of an outer membrane protein. Antimicrob. Agents Chemother., 41:563–569.

Bradford, P.A., 2001. Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev., 14: 933–951.
Chatterjee, A., O'Keefe, C., Cuthil,l S.L., Varman, M., 2009. Salmonella Infection. http://emedicine.medscape.com/article/968672-overview Updated: Apr 27, 2010.

Cheng, Y., Chen, M., 1994. Extended spectrum b-lactamases in clinical isolates of *Enterobacter gergoviae* and *Escherichia coli* in China. Antimicrob. Agents Chemother., 38:2838-2842.

CLSI. Clinical and Laboratory Standards Institute. 2007. Performance standards for antimicrobial susceptibility testing; seventeenth informational supplement. M100-S17. CLSI. Wayne, Pennsylvania.

Crump, J.A., Luby, S.P., Mintz, E.D., 2004. The global burden of typhoid fever. Bull. World Health Organ., 82:346–353.

Datta, N., Kontomichalou, P., 1965. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature, 208(5007):239-241.

DuPont, H.L., 2003. Community-acquired diarrheal disease in Western countries: applications of nonabsorbable oral antibiotic therapy. Adv. Stud. Med., 3(10A): S945-S950.

Fey, P.D., Safranek, T.J., Rupp, M.E., Dunne, E.F., Ribot, E., Iwen, P.C., Bradford, P.A., Angulo, F.J., Hinrichs, S.H., 2000. Ceftriaxone-resistant Salmonella infections acquired by a child from cattle. N. Engl. J. Med., 342: 1242-1249.

Gallego, L., Umaran, A., Garaizar, J., Colom, K., Cisterna, R., 1990. Digoxigenin-labeled DNA probe to detect TEM type β-lactamases. J. Microbiol. Methods, 11:261–267.

Gazouli, M., Tzelepi, E., Sidorenko, S.V., Tzouvelekis, L.S., 1998. Sequence of the gene encoding a plasmid-mediated cefotaxime-hydrolyzing.. Antimicrob. Agents Chemother., 42:1259–1262.

Gifford, D.R., 2010. Spice companies linked to US Salmonella outbreak. NEWS IN BRIEF 04-Feb-2010. [Online] Available at: http://www.foodproductiondaily.com/Quality-Safety/Spice-companies-linked-to-US-Salmonella-outbreak. Accessed 15 May 2010.

Goldstein, F.W., Pean, Y., Rosato, A., Gertner, J., Gutmann, L., the Vigil'Roc Study Group., Characterization of ceftriaxone-resistant Enterobacteriaceae: a multicenter study in 26 French hospitals. J. Antimicrob. Chemother., 32:595–603.

Gordon, M.A., Graham, S.M., Walsh, A.L., Wilson, L., Phiri, A., Molyneux, E. et al., 2008. Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clin. Infect. Dis., 46:963–969.

Gupta, S.K., Keck, J., Ram, P.K., Crump, J.A., Miller, M.A., Mintz, E.D., 2008. Part III. Analysis of data gaps pertaining to enterotoxigenic *Escherichia coli* infections in low and medium human development index countries, 1984-2005. Epidemiol. Infect., 136(6):721-738.

Hall, R.M., 1997. Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram-negative bacteria. Ciba Found. Symp., 207:192–202.

Helms, M., Simonsen, J., Mølbak, K., 2004. Quinolone resistance is associated with increased risk of invasive illness or death during infection with *Salmonella* serotype Typhimurium. J. Infect. Dis., 190:1652–1654.

Herikstad, H., Motarjemi, Y., Tauxe, R.V., 2002. *Salmonella* surveillance: a global survey of public health serotyping. Epidemiol. Infect., 129:1-8.

Hohmann, E.L., 2001. Nontyphoidal salmonellosis. Clin. Infect. Dis., 15: 263-269.

Jacoby, G., Bush, K., Lahey Clinic, (n.d). ß-Lactamase Classification and Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant Enzymes. [Online] Available at:www.lahey.org/studies/ Accessed 15 October 2010.

Jones, R.N., 2003. Global Epidemiology of Antimicrobial Resistance Among Community-Acquired and Nosocomial Pathogens: A Five-Year Summary From the SENTRY Antimicrobial Surveillance Program (1997-2001). Semin. Respir. Crit. Care Med., 24:_121-134.

Jones, R.N., Varnam, D.J., 2002. Antimicrobial activity of broad-spectrum agents tested against gram-negative bacilli resistant to ceftazidime: report from the SENTRY Antimicrobial Surveillance Program (North America, 2001). Diagn. Microbiol. Infect. Dis., 44:379-382.

Karas, J.A., Pillay, D.G., Muckart, D., Sturm, A.W., 1996. Treatment failure due to extended spectrum ß-lactamase. J. Antimicrob. Chemother., 37(1): 203-204.

Kaye, D., 1996. *Salmonella* infections other than typhoid fever. In J.C. Bennet, and F. Plum (Eds.), Cecil textbook of medicine (pp. 1644-1647). Philadelphia, PA: W.B. Saunders.

Kliebe. C., Nies, B.A., Meyer, J.F., Tolxdorff-Neutzling, R.M., Wiedemann, B., 1985. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob. Agents Chemother., 28:302–307.

Kruger, T., Szabo, D., Keddy, K.H., Deeley, K., Marsh, J.W., Hujer, A.M., Bonomo, R.A., Paterson, D.L., 2004. Infections with Nontyphoidal *Salmonella* Species Producing TEM-63 or a Novel TEM Enzyme, TEM-131, in South Africa. Antimicrob. Agents Chemother., 48(11):4263–4270.

Levesque, C., Piche, L., Larose, C., Roy, P.H., 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother., 39:185–191.

Manickam, K., Alfa, M.J., 2008. Extended-Spectrum β-Lactamase (ESBL) producing organisms: What are they and how can we treat infections with these organisms. Micro Notes. Clinical Microbiology Discipline Publication Diagnostic Services of Manitoba DSM September 1, 2008 http://www.dsmanitoba.ca/professionals/microbiology/Micro newsletter 09-01-08.pdf

Mariotte, S., Nordmann, P., Nicolas, M.H., 1994. Extended spectrum β-lactamase in *Proteus mirabilis*. J. Antimicrob. Chemother., 33:925–935.

Moland, E.S., Hanson, N.D., Black, J.A., Hossain, A., Song, W., Thomson, K.S., 2006. Prevalence of newer beta-lactamases in Gram-Negative clinical isolates collected in the United States from 2001 to 2002. J. Clin. Microbiol., 44:3318-3324.

Morosini, M.I., Canton, R., Martinez-Beltran, J., Negri, M.C., Perez-Dia, J.C., Baquero, F., Blazquez, J., 1995. New extended-spectrum TEM-type b-lactamase from *Salmonella enterica* subsp enterica isolated in a nosocomial outbreak. Antimicrob. Agents Chemother. 1995; 39:458–461.

NCCLS. National Committee for Clinical Laboratory Standards. 2000.Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7–A5 and informational supplement M100–S10. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Paterson, D.L., Hujer, K.M., Hujer, A.M., Yeiser, B., Bonomo, M.D., Rice, L.B., Bonomo, R.A., 2003. Extended-spectrum β-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread

prevalence of SHV- and CTX-M-type B-lactamases. Antimicrob. Agents Chemother., 47:3554–3560.

Paterson, D., Ko, W., Von Gottberg, A., Mohapatra, S., Casellas, J., Mulazimoglu, L., Klugman, K.P.,1998. *In vitro* susceptibility and clinical outcomes of bacteremia due to extended-spectrum ß-lactamase (ESBL)-producing *Klebsiella pneumoniae*. Clin. Infect. Dis., 27:956.

Philippon, A., Arlet, G., LaGrange, P.H., 1994. Origin and impact of plasmid mediated extended-spectrum b-lactamases. Eur. J. Clin. Microbiol. Infect. Dis., 1:17–29.

Pitout, J.D.D., Thomson, K.S., Hanson, N.D., Ehrhardt, A.F., Moland, E.S., Sanders, C.C., 1998. ß-Lactamases Responsible for Resistance to Expanded-Spectrum Cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* Isolates Recovered in South Africa. Antimicrob. Agents Chemotherap., 42(6): 1350–1354.

Pongpech, P., Naenna, P., Taipobsakul, Y., Tribuddharat, C., Srifuengfung, S., 2003. Prevalence of Extended-Spectrum Beta-lactamase and Class 1 integron integrase gene *int1* in *Escherichia coli* from Thai patients and healthy adults. South East Asian J. Trop. Med. Pub. Health, 39 (3):425-433.

Salyers, A.A., Whitt, D.D., 2005. Antibiotics that inhibit the synthesis of bacterial proteins, p. 66–82. *In* A. A. Salyers and D. D. Whitt (ed.), Revenge of the microbes: how bacterial resistance is undermining the antibiotic miracle. ASM Press, Washington, DC.

Thomson, K.S., 2001. Controversies about Extended-Spectrum and AmpC Beta-Lactamases. Emerging Infect. Dis., 7(2); 7: 1-4.

WHO. World Health Organization. 2005. Drug-resistant Salmonella REF 1-6 Fact sheet N°139. Revised April 2005. http://www.who.int/mediacentre/factsheets/fs139/en/

WHO. World Health Organization. 2008.Enterotoxigenic *Escherichia coli*. Available on http://www.who.int/vaccine_research/diseases/e_ecoli/en/. Accessed 10 January 2008.

CHAPTER 3

MOLECULAR BASIS OF VIRULENCE IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* AND *SALMONELLA SPECIES* FROM A TERTIARY HOSPITAL IN THE EASTERN CAPE, SOUTH AFRICA

3.0. Abstract

This study investigated the virulence factors of *Escherichia coli* and *Salmonella* species in clinical specimens from patients with diarrhoea presenting to health care centres in Oliver R. Tambo District Municipality, Eastern Cape Province, Republic of South Africa by the use of cultural and molecular techniques. Out of a total of 315 samples screened, *Salmonella* isolates were obtained in 119 (37.8%) of cases and these comprised: *S. typhi* (52%), *S. typhimurium* (25%), *S. isangi* (8%), *S. choleraesuis* (6%), *S. enteritidis* (4%), *S. eppendorf* (1%), *S. hadar* (1%), *S. panama* (1%), and untyped *Salmonella* spp. (2%). Using molecular diagnostic methods, diarrheagenic *E. coli* were detected in 90 cases (28.6%): the greater proportion of this were enteroaggregative *E. coli* (EAEC) 37 (41.1%), enteropathogenic *E. coli* (EPEC) 21 (23.3%) and enterohemorrhagic *E. coli* (EHEC) 21 (23.3%). The virulence factors identified in the *Salmonella* strains were 105 (88.2%) inv, 15 (12.6%) flic, and 2 (1.7%) sef genes. The amino acid identity of the representative genes matched 95 to 100% with corresponding blast searched sequence. This study underlines the importance of understanding the virulence composition and diversity of

pathogens for enhanced clinico-epidemiological monitoring and health care delivery.

Key: *Escherichia coli; Salmonella;* virulence; genes; diarrhoea.

3.1. Introduction

Gastrointestinal infections due to pathogenic *Enterobacteriaceae* in particular *Escherichia* and *Salmonella* species are significant causes of morbidity and mortality worldwide. These infections which usually are self-limiting may be fatal in hosts with debilitating immune systems (Amar *et al.*, 2007). The fatality of infections due to these enteric pathogens depends on their serotypes, the size of the inoculum, and the status of the host (Gordon, 2008). *Escherichia* and *Salmonella* species were reported to have diverged from a common ancestor based on the evolutionary rate estimates from 5S and 16S rRNA sequence analyses while *Shigella* spp. are considered clonal lineages of *Escherichia coli* (Falkow, 1995). *Salmonella* species are mainly pathogenic, with differing host ranges; *S. enterica* serovar Typhi is adapted to humans and does not occur in animals while non-typhoidal *Salmonella* serovars (NTS) have a broad vertebrate host range (Gordon, 2008). Even though *E. coli* is generally known as commensal normal flora of the gut, some *E. coli* strains are the causative agents of neonatal meningitis, urinary tract infections, bacteremia, and infectious diarrhoea.

The major distinguishing factor between pathogenic and non-pathogenic strains of *E. coli* strains is the occurrence of virulence genes, which code for the various known strategies for pathogenecity. Analysis have shown that pathogenic *E. coli* strains from diarrhoea cases and those involved in urinary tract infections are more of a distinct subsets of *E. coli,* rather than a reflection of the random faecal flora (Selander and Musser, 1990). Some of the virulence factors of *E. coli* include ability to adhere, colonize, and invade the hosts' cells. Further to these are the secretion systems, production of cell surface molecules, transport and siderophore formation (Finlay and Falkow, 1997). According to Kaper *et al.* (2004), *E. coli* has been categorized based on the type of virulence factors present and host clinical symptoms basically into the following pathotypes: enteropathogenic *E. coli* (EPEC); enterohemorrhagic *E. coli* (EHEC); enterotoxigenic *E. coli* (ETEC); enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC), a subclass of enteroaggregative *E. coli*; enteroinvasive *E. coli* (EIEC); uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC).

The ability of the enteric pathogen to invade and penetrate intestinal epithelial cells is required in salmonellosis whether it is confined as the intestinal form or progresses to systemic involvement (Altier, 2005). The attribute to direct their internalization by the epithelial cells which are not normally phagocytic is a striking Salmonella-host cell interaction. According to Galan and Curtiss (1989) this remarkable phenotype known as invasion allowed for identification and

characterization of invasion genes. The key mechanism involves type III secretion systems which are encoded by pathogenicity island 1 (SPI-1) (McCormick, 2004). *Salmonella* also possess the ability to alter phagocytosis in order to circumvent the process. *S. enetrica serovar* Typhimurium is known to delay significantly the fusion of the phagosome to the lysosome (Buchmeier and Heffron, 1991); thereby hibernating in phagocytic cells and hence adapt to resist the antimicrobial activity of the fused phagolysosome (Pizzaro-Cerda *et al.,* 1997). Bacterial survival in phagocytic cells has been observed as an alternate to invasion in accessing privileged sites in hosts. Rescigno *et al.* (2001) postulated that CD18+ expressing phagocytes are alternate route and these cells have been observed by Vasquez-Torres *et al*. (1999) as vehicles for reaching the spleen in an invasion-independent manner by *S. enterica serovar* Typhimurium.

Molecular analysis is known to give a better picture of epidemiology of infectious diseases. Studies have demonstrated the sensitivities of molecular-based methods to be greater compared to current conventional methods of analysis (Amar *et al.,* 2007). Molecular studies have led to the understanding of the genomic make-up of bacteria which generally consist of stable regions and variable regions, the flexible part that is composed of bacteriophages, plasmids, transposons as well as unstable large regions, called genomic islands. The so-called genomic islands are a gene pool required for encoding virulence factors of pathogenic bacteria and these have been designated “pathogenicity islands” (Hacker *et al.,* 2003). The concept of pathogenicity islands (Pais) was first

identified through the genetic and molecular analysis of virulence genes in uropathogenic *E. coli* and EPEC (Blum *et al.*, 1994; Morschhauser *et al.*, 1994). Pais which are specific regions of chromosomal DNA have been described in more than 30 bacterial species (Hacker *et al.*, 2003). It is a well known concept that bacterial pathogenicity is an organized multifactorial process involving numerous chromosomal and extrachromosomal genes directed by complex regulatory circuits (Mahan *et al*., 1996; Mellata *et al.*, 2010).

There are various shared genetic strategies for pathogenicity in enteric bacilli. Type III secretion is dedicated secretion machinery whose components are coded for by numerous homologous gene sequences shared by enteric pathogens (Falkow, 1995; Galán and Wolf-Watz, 2006). Nevertheless, there has been understanding that the similarities between EPEC virulence attributes and *Salmonella* invasion genes are more than homologous genes associated with secretion (Collazo *et al.*, 1995). Most virulence factors of pathogenic *E. coli, Shigella,* and *Salmonella* strains are plasmid-borne however; one or more of the essential virulence determinants are borne on an extrachromosomal element (Galan and Collmer, 1999; Madden *et al.*, 2001; Naum *et al.*, 2009). In both *E. coli* and *Salmonella* spp fimbriae might play a role in adhesion and invasion (Townsend *et al.*, 2001; Guo *et al*., 2007). The curli fimbriae of these strains were proven to bind to several tissue-matrix proteins as well as plasminogen and its activator t-PA (Falkow, 1995).

Bacteria are emerging with new means of circumventing human efforts at curbing their nefarious schemes and various evolvement patterns and innovations are certainly put in place by these pathogens. A myriad combination of virulence genes against indiscriminate genetic transfer and recombination are required for a successful emergence of pathogen (Musser, 1996; Bansal, 2008). Profiling the expression of these genes will give impetus to understanding the mechanisms by which enteric bacterial pathogens colonize, spread and at times persist in the hosts (Gonzalez-Escobedo *et al.*, 2011). This study investigated the genetic determinants of virulence in *E. coli* and *Salmonella* spp. which are significant pathogens involved in enteric diseases.

3.2. Materials and Methods

3.2.1. Specimens' collection and bacterial isolation

Salmonella isolates deposited at the National Institute of Communicable Diseases (NICD), Johannesburg under the surveillance study of 2005 to 2008 from this tertiary health facility were obtained. For 2009, One hundred and twenty–five fresh stool samples obtained from patients presenting with acute diarrhoea in the tertiary referral facility and surrounding clinics and 75 apparently healthy school pupils in three different schools within OR Tambo District Municipality, Eastern Cape Province, South Africa were collected in sterile stool jars or rectal swabs placed in Cary-Blair transport medium and transported on ice pack to the laboratory where analyses were done within 24 hours. Where this was not possible specimen were kept frozen. Written

informed consents were obtained from all patients, parents or guardians as the case may be and a questionnaire was administered by trained volunteer health care workers (appendices 1A-1D). The study protocol and data handling were approved by the WSU ethics committee (Protocol No. 0003/08) as well as the Department of Health, Eastern Cape, South Africa.

3.2.2. Bacteriological analyses

NICD Isolates: Bacteriological analyses of the specimens for the presence of *E. coli* and *Salmonella* using standard conventional methods and commercial anti-sera (BiowebSA), were carried out at the National Health Laboratory Services, Nelson Mandela Academic Hospital, Mthatha prior to banking at NICD from where isolates were collected for this study.

E. coli: The faecal samples were cultivated on MacConkey agar. After overnight incubation at 37°C, lactose fermenting colonies (LFC) with the typical appearance of *E. coli* were selected for further analysis. Isolates were identified by biochemical assays using Microscan Gram negative combo panel NUC 45 (Siemens/ Dade Behring).

Salmonella: Specimens were cultivated for the isolation of *Salmonella* species on MacConkey agar. After 24 h of incubation at 37°C, suspected colonies with typical characteristics of *Salmonella* were sub-cultured on XLD (xylose lysine deoxycholate) agar for 24 h at 37°C. Confirmation was carried out using Microscan Gram negative combo panel NUC 45 (Siemens/ Dade Behring).

3.2.3. DNA extraction

DNA template for PCR was obtained from pure overnight bacterial culture using Fungal/Bacterial DNA extraction kit™ (Zymo Research) and following manufacturer's instructions. The concentration of the eluted DNA was measured using NanoDrop 2000 spectrophotometer (Thermo Scientific).

3.2.4. DNA amplification

PCR amplifications were performed in a final volume of 25 µL containing: 0.5 µL to 2 µL of DNA template depending on concentration, 8.5 to 10 µLof Nuclease free water, 1 µL of each primer and 12.5 µL Master mix (EconoTaq Green, Fermentas). Amplifications were carried out in a GeneAmp PCR System 9700 Thermocycler (Applied Biosystems). All oligonucleotide primers were synthesized by Inqaba Biotechnology (Pretoria, South Africa) and the sequences are as shown in Table 3.1. The PCR cycling conditions for the *E. coli* strains consisted of 95°C for 5 min while for the *Salmonella* isolates consisted of 95°C for 1 min, which were followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Amplification products were separated by electrophoreses on 2 % agarose gel (TopVision TM, Fermentas) in 1X TBE Buffer and ethidium bromide (5 µL) with a 100-bp ladder (Fermentas) as molecular weight marker.

3.2.5. Sequencing reaction

PCR products were sequenced using an Applied Biosystems 3500xL Genetic analyzer (AB Biosystems). Prior to PCR products sequencing, the unincorporated

Table 3.1. Primer sets for the pathotypes and virulence genes for the *E. coli* and *Salmonella* spp.

Isolate species/ subgroups	Target gene	Primer	Nucleotide Sequence (5'- 3')	Amplicon size (bp)	Reference
E. coli					
EPEC and EHEC	*eaeA*	EAE-a EAE-b	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	248	Wang *et al.*, 2002
EHEC	*stx*1	JMS1-F JMS1-R	GTCACAGTAACAAACCGTAACA TCGTTGACTACTTCTTATCTGGA	95	Jothikumard and Griffiths 2002
ETEC	LT ST	LT-1 LT-2 STa-F STa-R	AGCAGGTTTCCCACCGGATCACCA GTGCTCAGATTCTGGGTCTC GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	132 190	Ito *et al.*, 1992 Franck *et al.*, 1998
EAEC	*aggR* *astA*	AggRks1 aggRkas2 EAST-1S EAST-1AS	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC GCCATCAACACAGTATATCC GAGTGACGGCTTTGTAGTCC	254 106	Ratchtrachenchai *et al.*, 1997 Yatsuyanagi *et al.*, 2002
EIEC	*VirA*	virA-F virA-R	CTGCATTCTGGCAATCTCTTCACA TGATGAGCTAACTTCGTAAGCCCTCC	215	Villalobo and Torres 1998
Salmonella					
	invA	invA 139 invA 141	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284	Rahn *et al.*, 1992
	sefA	S1 S4	GCC GTA CAC GAG CTT ATA GA ACC TAC AGG GGC ACA ATA AC	250	Soumet *et al.*, 1997
	fliC	Fli15 Typ04	CGG TGT TGC CCA GGT TGG TAAT	620	Joys 1985

			ACT GGT AAA GAT GGC T		

KEY: *flic*-flagellin H1; *invA*-invasion; *sefA*- fimbrial antigen; *aggR*- transcriptional activator for EAEC aggregative adherence fimbria I expression; *eaeA-E. coli* attaching and effacing; *astA*–EAEC heat-stable enterotoxin; LT- heat-labile enterotoxin; ST- heat-stable enterotoxin; *VirA*-virulence plasmid.

dNTPs were dephosphorylated with a commercial kit from Zymo Research Corporation (Orange, CA). Subsequently, the PCR products were sequenced with the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (AB Biosystems) using the same primers as employed in the PCR reactions. The products were then subjected to the following conditions: 94°C for 2 min, followed by 40 cycles of denaturation at 85°C for 1 s, annealing at 53°C for 10 s and extension at 60°C for 2 min 30 s, with a final extension at 4°C for 0 s. The sequencing reaction products were cleaned up using ZR-96 DNA sequencing clean-up kit^{TM}. Thereafter, the ultra-pure products were analyzed on the sequencing machine. Sequences were aligned with known *E. coli* and *Salmonella* virulence gene sequences by a blast search of the National Center for Biotechnology Information (NCBI) data base (www.ncbi.nlm.nih.gov/BLAST/) using Staden package version 1.6.0-beta4 (MRC.WTSI).

3.3. Results

3.3.1. Bacteriological identification and molecular analysis

Results showed that *Salmonella* spp. were isolated from 119 (37.8%) of cases while diarrheagenic *E. coli* was found in 90 (28.6%) of the cases. The distributions of the different pathotypes are as shown in Figures 3.1A and 3.1B. Of the *Salmonella* isolates, 87 (74.1%) were invasive. The most common virulence factor detected among the *Salmonella* strains was invA found in 105 *Salmonella* spp. while fliC and sef genes were detected in 15 and 2 *Salmonella* isolates respectively. The predominant virulence gene among the diarrheogenic *E. coli* was 24 EAEC heat-stable enterotoxin *astA* genes. Table 3.2 shows the distribution of the various genes among cases and controls.

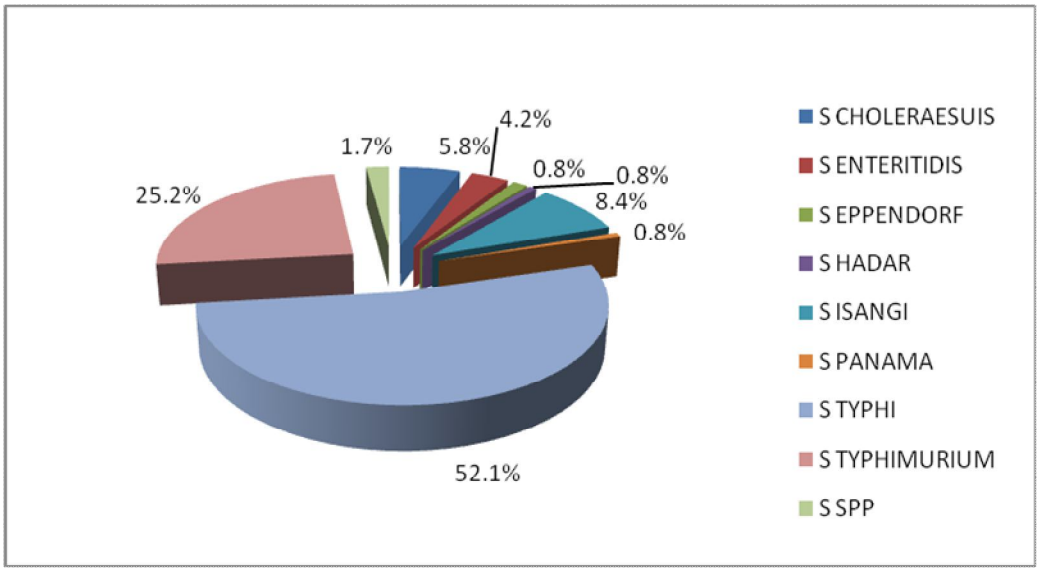


Figure 3.1A. Frequency of the various *Salmonella* isolates

Figure legend: S = *Salmonella*

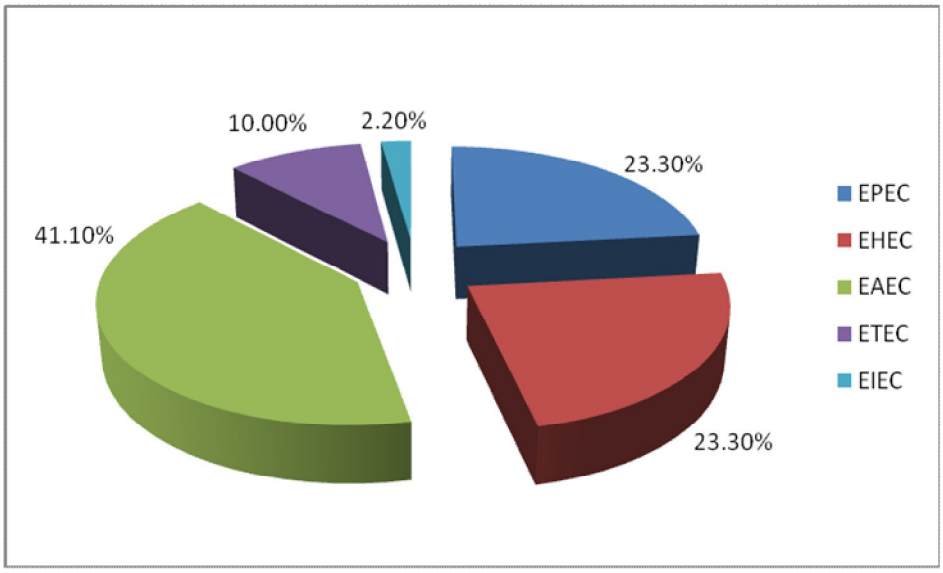


Figure 3.1B. Frequency of the diarrhoegenic *E. coli* isolates

Figure legend: EPEC= Enteropathogenic *Escherichia coli*; EHEC= Enterohemorrhagic *E. coli*: EAEC= Enteroaggregative *E. coli*: Enterotoxigenic *E. coli*: EIEC= Enteroinvasive *E. coli.*

Table 3.2. Distribution of virulence genes among the *E. coli* and *Salmonella* spp.

Bacterial Strain	Number of isolate	Virulence genes								
		fliC	invA	sefA	aggR	eaeA	EAST	LT	ST	virA
Salmonella spp. (case)	119	15	105	O	ND	ND	ND	ND	ND	ND
Salmonella (control)	8	0	7	0	ND	ND	ND	ND	ND	ND
E coli (case)	90	ND	ND	ND	13	21	24	5	0	2
E coli (control)	85	ND	ND	ND	10	11	19	0	0	0

KEY: ND-Not determined.

The representative gels for PCR amplification of DNA extracted from selected *E. coli* and *Salmonella* isolates showing the presence of diverse virulence genes are indicated in Figures 3.2A and 3.2B. One hundred and eighty

isolates were obtained from the 150 control subjects. *E. coli* was the predominant bacterial species being 85 (47.2%) while *Salmonella* spp. was 8 (12.1%). Other recovered bacteria species were *Proteus mirabilis* 45 (25.0%), *Klebsiella pneumonie* 23 (12.8%) and *Enterobacter cloacae* 19 (10.5%). Sequencing analysis of the genes showed 95 to 100% conformation of the various virulence genes with corresponding blast search sequence and confirmed the strain.

3.4. Discussion

Gastroenteritis is a major concern in sub-Saharan Africa as with other developing countries (Mandeville *et al.*, 2009). South African National Burden of Disease study of the year 2000 found that diarrhoea accounted for nearly 3% of all deaths in South Africa (Bradshaw *et al.,* 2004). According to the South African health review of 2007, death due to gastroenteritis among children was put at 15% (Rispel *et al*., 2007) showing increasing mortality. The developed countries are not spared in the global burden of enteric-related diarrhoea. Salmonellosis was considered a major public health problem in the United States (Voetsch *et al.*, 2004). *E. coli* and *Salmonella* are among the bacterial pathogens implicated in gastroenteritis. These enteric pathogens have evolved different strategies for subverting normal host cellular functions (Galán and Sansonetti, 1996).

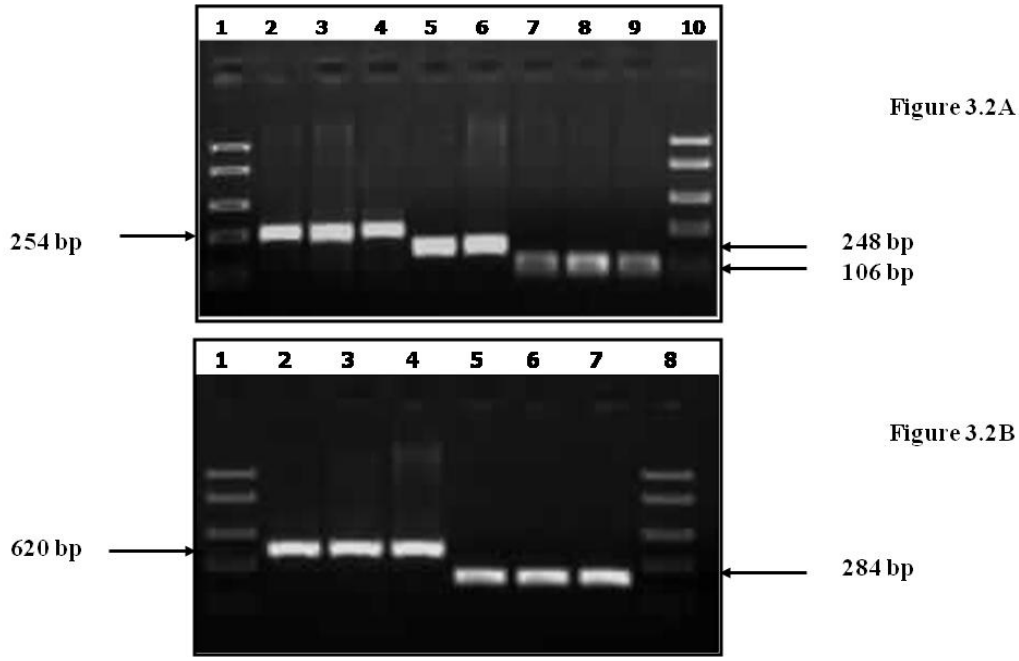


Figure 3.2A

Figure 3.2B

Figure 3.2. Representative gels for PCR amplification of DNA extracted from selected *E. coli* and *Salmonella* isolates showing the presence of diverse virulence genes. Figure 3.2A: 100 bp molecular weight marker (lanes 1 and 10), fragment from *agg*R (lanes 2 to 4), *eaeA* (lanes 5 to 6) and *astA* (lanes 7 to 9). Figure 3.2B: 100 bp molecular weight marker (lanes 1 and 8) *fli*C (lanes 2 to 4) and *inv*A (lanes 5 to 7). The relative positions in the gel of predicted size of PCR products are indicated by arrowheads on the right sides.

These pathogens cause various intestinal and extraintestinal diseases by means of virulence factors that affect a wide range of cellular processes. These virulence induced infections usually involve complex mechanisms with various specific, interdependent interactions between hosts and pathogens (Tang and Holden, 1999). The present study provides information on the pathotypes and some virulence factors associated with local isolates of *E. coli* and *Salmonella* species. *E coli* is more than just a harmless intestinal microflora; it can also be a highly versatile, and frequently deadly, pathogen (Kaper *et al.*, 2004). *E. coli*

strains cause diarrhoea by several distinct pathogenic mechanisms and differ in their epidemiology. Virulence genes were expressed in diarrhoegenic *E. coli* from both cases and controls. EAEC was detected in 37 (41.1%) cases involving diarrhoegenic *E. coli.* Studies conducted in Thailand and Brazil, reported a frequency of 12% and 11% EAEC respectively among children with acute diarrhoea (Souza *et al*., 2001; Ratchtrachenchai *et al*., 2004). Although the prevalence of EAEC is believed to be considerably higher in the developing countries compared to industrially developed countries, a Switzerland study, reported that EAEC was encountered in a significant proportion of diarrhoea cases among children (Pabst *et al.*, 2003). As evident in this study and previous studies, EAEC seems to be endemic within our study population and other locations in sub-Saharan Africa (Geyid *et al.,* 1998; Okeke and Nataro, 2001; Okeke *et al.*, 2003), emerging as a significant diarrheal agent worldwide with the pattern of infection changing from persistent diarrhoea to include acute diarrhoea (Scaletsky *et al.,* 2002).

The second diarrheogenic *E. coli* type detected was EHEC constituting 21 (23.3%) of all diarrheogenic *E. coli*. Enterohaemorrhagic *E. coli* (EHEC) is a subset of Shiga toxin-producing *Escherichia coli* (STEC) which is associated with severe systemic disease as haemorrhagic colitis, haemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura, particularly in infants, young children and in the elderly (Nataro and Kaper, 1998; Paton and Paton, 1998). EHEC infects the large bowel and inflict damage to the colon with infectious dose

estimated to be less than 100 CFU (Griffin and Miner, 1995; Mellies *et al.*, 2007). Of the various virulence factors associated with pathogenicity in the EHEC strain, this study failed to detect both Shiga toxin 1 (*stx1*) and Shiga toxin 2 (*stx2*) but only detected intimin which is encoded by the *eaeA* gene (Dean-Nystrom *et al.,* 1997; Kang *et al.*, 2004). Intimin is known to facilitate the adherence of pathogen to intestinal villi producing attaching and effacing lesions (Holland *et al.*, 1999; Caprioli *et al.*, 2005). Previous studies have implicated EHEC in outbreaks and sporadic infections both in the United States and around the world (Waters *et al.*, 1994; Bell *et al.*, 1994; Dundas *et al.*, 2001).

Intimin (eaeA) gene was also detected in 21 (23.3%) of EPEC. Unlike most studies where ETEC often take the lead in bacterial enteritis due to *E. coli,* a study by Weggerhof (1987) also reported a higher incidence of EPEC from the screening of some pediatric patients with diarrhoea in Mpumalanga Province of South Africa. Another study in 1990 in Iran reported EPEC as the most frequently detected strain of diarrhoegenic *E. coli* (Katouli *et al.*, 1990). More recent studies described the contributions of EPEC to the human disease burden as significant (Kenny, 2002; Clarke *et al.*, 2003; Kaper *et al.*, 2004; Scaletsky *et al.*, 2010). Thus EPEC plays a vital role in acute diarrhoea. EPEC is known to cause illness manifesting as watery diarrhoea with little inflammation of the intestinal mucosa (Koneman *et al.*, 1997). Virulence is initiated in EPEC by the induction of a characteristic ultrastructural lesion in which the bacteria make intimate contact with the apical plasma membrane, causing localized destruction of the intestinal

brush border and distortion of the apical enterocyte membrane (Clarke *et al.*, 2003) as is in the classical attaching and effacing (AE) lesion.

The ETEC and EIEC strains were found only in 9 (10.0%) and 2 (2.2%) of cases with diarrheagenic *E. coli* respectively. Although ETEC strains have been described as a major contributor to infantile diarrhoea in developing countries and of travellers' diarrhoea in visitors to these countries (Ako-Nai *et al.,* 1990; Sooka *et al.*, 2004; WHO, 2009a), our findings were different showing a decline in the involvement of these strains in our setting. ETEC strains cause secretory diarrhoea similar to that of *Vibrio cholerae* by forming plasmid encoded heat-labile (LT) or heat-stable (ST) enterotoxins genes (Levine, 1987; Koneman *et al.*, 1997). ETEC engage strain-specific antigenic, hair-like fimbriae in attachment to specific receptors on the surface of enterocytes in the intestinal lumen (WHO, 2009a). EIEC on the other hand produce dysentery-like diarrhoea similar to that caused by *Shigella* species by invading and multiplying within epithelial cells of the colonic mucosa, resulting in an intense inflammatory response characterized by abscesses and ulcerations that damage the integrity of the epithelial cell lining of the colon (Hale, 1998). EIEC was not a major enteric bacterial pathogen observed in this study, with prevalence similar to that reported in Mexico City by Paniagu *et al.* (2007). This pattern is not consistent with studies in other developing countries where EIEC strains were important causes of pediatric diarrhea and dysentery (Taylor *et al.*, 1986; Pacheco-Gil *et al.*, 2006).

Salmonella species are an important cause of varying food and water-related infections. This study detected *Salmonella* as a major cause of gastroenteritis in our setting. *Salmonella* has previously been described as one of the common causes of gastroenteritis particularly in the developing countries (Guerrant *et al.*, 1990; Galanis *et al.*, 2006). On the contrary, infectious diarrhoea in the developed world is often due to viruses (Jafari *et al.*, 2008). The most common species isolated in this study were *S. enterica* serovar Typhi (52%) and *S. enterica* serovar Typhimurium (25%). This report is consistent with other studies conducted in Iran and South Africa where *S. enterica* serovar Typhi and *S. enterica* serovar Typhimurium were described as major aetiological agents of infectious diarrhoea (Jafari *et al.*, 2008; Keddy *et al.*, 2009). *S. enterica* serovar Isangi was third in ranking of frequency of isolation. Kruger *et al.* (2004) described the increasing importance of this serotype of non-typhoidal Salmonella (NTS) which was a rare serotype in South Africa until 2002. Other species identified were *S. enterica* serovar Choleraesuis, *S. enterica* serovar Enteritidis, *S. enterica* serovar Eppendorf, *S. enterica* serovar Hadar, *S. enterica* serovar Panama and untyped *Salmonella* spp. The virulence factor detected among the majority (105) of the *Salmonella* spp. was *inv*A. This gene which is chromosomally located aids attachment of the pathogen to the epithelial cells (Gallan and Curtiss, 1989). The other detectable virulent gene was fliC detected in 15 isolates. The flagellin gene, fliC is known to aid systemic spread of pathogen and is specific for *S. enterica* serovar Typhimurium (Soumet *et al.*,

1999). Enteric bacteria possessing sefA genes were not encountered in this study. The sefA gene which encodes the SEF14 fimbrial antigen is a virulence plasmid specific for *S. enterica* serovar Enteritidis (Doran *et al.*, 1996).

3.5. Conclusion

This study showed the diversity of virulence gene expression in two major enteric pathogens. It was observed among other things that some diarrhoegenic *E. coli* isolated from apparently asymptomatic subjects expressed some virulence genes at frequency as high as seen in diarrhoegenic cases. This is a pointer to the fact that asymptomatic individuals serve as reservoirs of pathogenic strains of enteric bacteria and may play a role in the spread and acquisition of virulence genes.

3.6. References

Ako-Nai, A.K., Lamikanra, A., Ola, O., Fadero, F.F., 1990. A study of the incidence of enterotoxigenic *Escherichia coli* (ETEC) secreting heat-labile toxin in two communities in Southwestern Nigeria. J. Trop. Med. Hyg., 93: 116-118.

Altier, C., 2005. Genetic and Environmental Control of Salmonella Invasion. J. Microbiol., 43:85-92 special issue.

Amar, C.F., East, C.L,, Gray, J., Iturriza-Gomara, M., Maclure, E.A., McLauchlin, J., 2007. Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993–1996). Eur. J. Clin. Microbiol. Infect. Dis., 26, 311–323

Bansal, A.K., 2008. Role of bioinformatics in the development of new antibacterial therapy Expert Rev. Anti-infective Therap., 6(1): 51-65.

Bell, B.P,, Goldoft, M., Griffin, P.M., Davis, M.A., Gordon, D.C., Tarr, P.I., Bartleson, C.A., Lewis, J.H., Barrett, T.J., Wells, J.G. et al., 1994. A multistate outbreak of Escherichia coli O157:H7-associated bloody diarrhea and hemolytic

uremic syndrome from hamburgers. The Washington experience. JAMA., 272 (17):1349-1353.

Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschape, H., Hacker, J., 1994. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. Infect. Immun., 62: 606–614.

Bradshaw, D., Nannan, N., Laubscher, R., Groenewald, P., Joubert, J., Nojilana, B., Norman, R., Pieterse, D., Schneider, M., 2004. South African National Burden of Disease Study, 2000: Estimates of Provincial Mortality. Tygerberg, SAMRC. South Africa.

Buchmeier, N.A., Heffron, F., 1991. Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. Infect. Immun., 59:2232–2238.

Caprioli, A., Morabito, S., Brugere, H., Oswald, E., 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Vet. Research, 36, 289–311.

Clarke, S.C., Haigh, R.D., Freestone, P.P.E., Williams, P.H., 2003. Virulence of Enteropathogenic *Escherichia coli*, a Global Pathogen. Clin. Microbiol. Rev., 16(3):365–378.

Collazo, C.M., Zierler, M.K., Galan, J.E., 1995. Functional analysis of the *Salmonella typhimurium* invasion genes *invL* and *invJ* and identification of a target of the protein secretion apparatus encoded in the *inv* locus. Mol. Microbiol., 15:25–38.

Dean-Nystrom, E.A., Bosworth, B.T., Cray, W.C., Moon, H.W., 1997. Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. Infect. Immunity, 65: 1842–1848.

Doran, J.L., Collinson, S.K., Clouthier, S.C., Cebula, T.A., Koch, W.H., Burian, J., Banser, P.A., Todd, E.C.D., Kay, W.W., 1996. Diagnostic potential of sefA DNA probes to *Salmonella Enteritidis* and certain other O-serogroup D1 Salmonella serovars. Mol. Cellular Probes, 10, 233-246.

Dundas, S., Todd, W.T., Stewart, A.I., Murdoch, P.S., Chaudhuri, A.K.R., Hutchinson, S.J., 2001. The central Scotland *Escherichia coli* O157:H7 outbreak: risk factors for the hemolytic uremic syndrome and death among hospitalized patients. Clin. Infect. Dis., 33: 923.

Falkow, S., 1995. The evolution of pathogenicity in *Escherichia*, *Shigella*, and *Salmonella*. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd ed. American Society for Microbiology (ASM) Press, Washington, D.C. pp. 2723-2729.

Finlay, B.B., Falkow, S., 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev., 61:136–169.

Franck, S.M., Bosworth, B.T., Moon, H.W., 1998. Multiplex PCR for enterotoxigenic, attaching and effacing, and Shiga toxin-producing *Escherichia coli* strains from calves. J. Clin. Microbiol., 36:1795–1797.

Galan, J.E., Collmer, A., 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science, 284:1322–1328.

Galán, J.E., Curtiss III, R., 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA, 86:6383–6387.

Galan, J.E., Sansonetti, P.J., 1996. Molecular and cellular bases of *Salmonella* and *Shigella* interactions with host cells. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd ed. American Society for Microbiology (ASM) Press, Washington, D.C. pp. 2757–2773.

Galan, J.E., Wolf-Watz, H., 2006. Protein delivery into eukaryotic cells by type III secretion machines. Nature, 444: 567–573

Galanis, E., Lo Fo Wong, D.M., Patrick, M.E., Binsztein, N., Cieslik, A., Chalermchikit, T., Aidara-Kane, A., Ellis, A., Angulo, F.J., Wegener, H.C., 2006. World Health Organization Global Salm-Surv. Web-based surveillance and global *Salmonella* distribution 2000-2002. Emerg. Infect. Dis., 12: 381–388.

Geyid, A., Olsvik, O., Ljungh, A., 1998. Virulence properties of Escherichia coli isolated from Ethiopian patients with acute or persistent diarrhoea. Ethiop. Med. J., 36:123-139.

Gonzalez-Escobedo, G., Marshall, J.M., Gunn, J.S., 2011. Chronic and acute infection of the gall bladder by *Salmonella Typhi*: understanding the carrier state. Nature Rev. Microbiol., 9, 9-14 | doi:10.1038/nrmicro2490.

Gordon, M.A., 2008. *Salmonella* infections in immunocompromised adults. J. Infect., 56: 413-422.

Griffin, M.G., Miner, P.B. Jr., 1995. Conventional drug therapy in inflammatory bowel disease. Gastroenterol. Clin. N. Am., 24: 509-521.

Guerrant, R., Hughes, J., Lima, N., Crane, J., 1990. Diarrhea in developed and developing countries: magnitude, special settings, and etiologies. Rev. Infect. Dis., 1: 41–50.

Guo, A., Lasaro, M.A., Sirard, J., Kraehenbu, J., Schifferli, D.M., 2007. Adhesin-dependent binding and uptake of Salmonella enterica serovar Typhimurium by dendritic cells. Microbiology, 153, 1059–1069.

Hacker, J., Hentschel, U., Dobrindt, U., 2003. Prokaryotic Chromosomes and Disease. Science, 301: 790-793.

Hale, T.L., 1998. Bacillary dysentery. *In* W. J. Hansler and M. Shuman (ed.), Topley and Wilson's microbiology and microbial infections, volume 3. Arnold, London, England. pp. 479-493.

Holland, R.E., Wilson, R.A., Holland, M.S., Yuzbasiyan-GurKan, V., Mullaney, T.P., White, D.G., 1999. Characterization of *eae Escherichia coli* isolated from healthy and diarrheic calves. Vet. Microbiol., 66: 251–263.

Ito, F., Hagino, T., Ito, K.I,, Watanabe, H., 1992. Differentiation and detection of pathogenic determinants among diarrheagenic *Escherichia coli* by polymerase chain reaction using mixed primers. Nihonrinshou, 50: 343–347. (In Japanese).

Jafari, F., Shokrzadeh, L., Hamidian, M., Salmanzadeh-Ahrabi, S., Zali, M.R., 2008. Acute diarrhoea due to enteropathogenic bacteria in patients at hospitals in Tehran. Jpn. J. infect. Dis., 61: 269-273.

Jothikumard, N., Griffiths, M.W., 2002. Rapid detection of *Escherichia coli* O157:H7 with multiplex real-time PCR assays. Appl. Environ. Microbiol., 68:3169–3171.

Joys, T.M., 1985. The covalent structure of the phas-1 flagellar filament protein of *Salmonella* Typhimurium and its comparison to others flagellins. J. Biol. Chem., 260: 15758-15761.

Kang, S.J., Ryu, S.J., Chae, J.S., Eo, S.K., Woo, G.J., Lee, J.H., 2004. Occurrence and characteristics of enterohemorrhagic *Escherichia coli* O157 in calves associated with diarrhoea. Vet. Microbiol., 98: 323–328.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol*.,* 2: 123-140.

Katouli, M., Jaafari, A., Farhoudi-Moghaddam, A.A., Ketabi, G.R., 1990. Aetiological studies of diarrhoeal diseases in infants and young children in Iran. J Tropical Med. Hygiene, 93(1):22–27.

Keddy, K.H., Dwarika, S., Crowther, P., Perovic, O., Wadula, J., Hoosen, A., Sooka, A., Crewe-Brown, H.H., Smith, A.M., 2009. Genotypic and demographic characterization of invasive isolates of Salmonella Typhimurium in HIV co-infected patients in South Africa. J. Infect. Dev. Ctries., 3(8):585-592.

Kenny, B., 2002. Enteropathogenic *Escherichia coli* (EPEC)—a crafty subversive little bug. Microbiology, 148: 1967–1978.

Koneman, .EW., Allen, S.D., Janda, W.M., Schreckenberger, P.C., Winn Jr, W.C., Eds., 1997. Color atlas and textbook of diagnostic microbiology, Fifth edition. Lippincott: Philadelphia, New York, United States of America, pp. 171-199.

Kruger, T., Szabo, D., Keddy, K.H., Deeley, K., Marsh, J.W., Hujer, A.M., Bonomo, R.A., Paterson, D.L., 2004. Infections with Nontyphoidal *Salmonella* Species Producing TEM-63 or a Novel TEM Enzyme, TEM-131, in South Africa. Antimicrob. Agents Chemother., 48(11):4263–4270.

Levine, M.M., 2006. Enteric infections and the vaccines to counter them: future directions. Vaccine, 24: 3865–3873.

Madden, J.C., Ruiz, N., Caparon, M., 2001. Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in Gram-positive bacteria. Cell, 104: 143–152.

Mahan, M.J., Slauch, J.M., Mekalanos, J.J., 1996. Environmental regulation of virulence gene expression in *Escherichia, Salmonella* and *Shigella* spp. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd ed. American Society for Microbiology (ASM) Press, Washington, D.C. pp. 2803–2815.

Mandeville, K.L., Krabshuis, J., Ladep, N.G., Mulder, C.J.J., Quigley, E.M.M., Khan, S.A., 2009. Gastroenterology in developing countries: Issues and advances. World J. Gastroenterol., 15(23): 2839-2854.

McCormick, B.A., 2004. Invasion mechanisms of *Salmonella*. In Richard J. Lamont editor, Advances in Molecular and Cellular Microbiology. Bacterial Invasion of Host Cells. Cambridge University Press. The Edinburgh Building, Cambridge CB2 2RU, UK.pp. 1-24.

Mellata, M., Ameiss, K., Mo, H., Curtiss, R., 2010. Characterization of the Contribution to Virulence of Three Large Plasmids of Avian Pathogenic *Escherichia coli* χ7122 (O78:K80:H9). Infect. Immun., 78(4): 1528–1541.

Mellies, J.L., Barron, A.M.S., Anna, M., 2007. Enteropathogenic and Enterohemorrhagic *Escherichia coli* Virulence Gene Regulation. Infect. Immun., 9 (75): 4199–4210.

Morschhauser, J., Vetter, V., Emody, L., Hacker, J., 1994. Adhesin regulatory genes within large, unstable DNA regions of pathogenic *Escherichia coli:* cross-talk between different adhesin gene clusters. Mol. Microbiol., 11:555–566.

Musser, J.M., 1996. Molecular Population Genetic Analysis of Emerged Bacterial Pathogens: Selected Insights. Emerging Infect. Dis., 2: 1-17.

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev., 11: 142–201.

Naum, M., Brown, E.W., Mason-Gamer, R.J., 2009. Phylogenetic evidence for extensive horizontal gene transfer of type III secretion system genes among enterobacterial plant pathogens Microbiology, 155: 3187–3199.

Okeke, I.N., Ojo, O., Lamikanra, A., Kaper, J.B., 2003. Etiology of Acute Diarrhea in Adults in Southwestern Nigeria. J. Clin. Microbiol., 41(10): 4525–4530.

Okeke, I.N., Nataro, J.P., 2001. Enteroaggregative *Escherichia coli*. Lancet Infect. Dis., 1:304-313.

Pabst, W.L., Altwegg, M., Kind, C., Mirjanic, S., Hardegger, D., Nadal, D., 2003. Prevalence of enteroaggregative *Escherichia coli* among children with and without diarrhea in Switzerland. J. Clin. Microbiol., 41: 2289-2293.

Pacheco-Gil, L., Ochoa, T.J., Flores-Romo, L., DuPont, H.L., Estrada-Garcia, T. 2006. Enteroinvasive *Escherichia coli* severe dysentery complicated by rotavirus gastroenteritis. J. Infect., 53 (5): e211-e213.

Paniagua, G.L., Monroy, E., García-González, O., Alonso, J., Negrete, E., Vaca, S., 2007. Two or more enteropathogens are associated with diarrhoea in Mexican children Annals Clin. Microbiol. Antimicrob., 6:17 doi:10.1186/1476-0711-6-17

Paton, A.W., Paton, J.C., 1998. Detection and characterization of Shiga Toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1, stx2, eaeA*, enterohemorrhagic *E. coli hlyA*, *rfb*O111, and *rfb*O157. J. Clin. Microbiol., 36: 598–602.

Pizarro-Cerdà, J., Moreno, E., Desjardins, M., Gorvel, J.P., 1997. When intracellular pathogens invade the frontiers of cell biology and immunology. Histol. Histopathol., 12 : 1027-1038.

Rahn, K., De Grandis, S.A., Clarks, R.C., McEwen, S.A., Galan, J.E., Ginocchio, C., Curtis III, R., Gyles, C.I., 1992. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella.* Mol. Cell Probes, 6:271–279.

Ratchtrachenchai, O.A., Subpasu, S., Ito, K., 1997. Investigation on enteroaggregative *Escherichia coli* infection by multiplex PCR. Bull. Dept. Med. Sci., 39:211–220.

Ratchtrachenchai, O.A., Subpasu, S., Hayashi, H., Ba-Thein, W., 2004. Prevalence of childhood diarrhoea-associated *Escherichia coli* in Thailand. J. Med. Microbiol., 53(3):237–243.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J.P., Ricciardi-Castagnoli, P., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat. Immunol., 2: 361-367.

Rispel, L., Setswe, G., 2007. Stewardship: Protecting the public's health. In: Harrison S, Bhana R, Ntuli A, editors. South African Health Review. Durban: Health Systems Trust; [Online] Available at: URL: http://www.hst.org.za/ publications/711

Scaletsky, I.C.A., Fabbricotti, S.H., Silva, S.O.G., Morais, M.B., Fagundes-Nieto, U., 2002. HEp-2-adherence Escherichia coli strains associated with acute infantile diarrhea, Sao Paulo, Brazil. Emerging Infect. Dis., 8:855-858.

Scaletsky, S.C.A., Souza, T.B., Aranda, K.R.S., Okeke, I.N., 2010. Genetic elements associated with antimicrobial resistance in enteropathogenic *Escherichia coli* (EPEC) from Brazil. BMC Microbiol., 10: 25.

Selander, R.K., Musser, J.M., 1990. Population genetics of bacterial pathogenesis, p. 11–36. *In* B. H. Iglewski and V. L. Clark, (ed.), Molecular Basis of Bacterial Pathogenesis. Academic Press, Inc.,San Diego, Califonia.

Sooka, A., du Plessis, M., Keddy, K., 2004. Enterovirulent *Escherichia coli.* Southern Afr. J. Epidemiol. Infect., 19(1):23-33.

Soumet, C., Ermel, G., Rose, V., Rose, N., Drouin, P., Salvat, G., Colin, P., 1997. Simultaneous detection by PCR of *Salmonella* Typhimurium and *Salmonella* Enteritidis from environmental samples of poultry houses. In Salmonella *and Salmonellosis Proceedings* ed. Colin, P., Le Goux, J.M. and Clement, G. pp. 53–57. Ploufragan, France: ISPAIA.

Soumet, C., Ermel, G., Rose, V., Rose, N., Drouin, P., Salvat, G., Colin, P., 1999. Identification by a multiplex PCR-based assay of *Salmonella* Typhimurium and *Salmonella* Enteritidis strains from environmental swabs of poultry houses. Let. Applied Microbiol., 29:1-6.

Souza, E.C., Martinez, M.B., Taddei, C.R., Mukai, L., Gilio, A.E., Racz, M.L., Silva, L., Ejzenberg, B., Okay, Y., 2002. Perfil etiologico das diarreias agudas de criancas atendidas em Sao Paulo. Etiologic profile of acute diarrhea in children in Sao Paulo. J. de Pediatria, 78(1):31–38.

Tang, C., Holden, D., 1999. Pathogen virulence genes - implications for vaccines and drug therapy. British Med. Bull., 55(2): 387-400.

Taylor, D.N., Echeverria, P., Pal, T., Sethabutr, O., Saiborisuth, S., Sricharmorn, S., Rowe, B., Cross, J., 1986. The role of *Shigella* spp., enteroinvasive *Escherichia coli*, and other enteropathogens as causes of childhood dysentery in Thailand. J. Infect. Dis., 153:1132-1138.

Townsend, S.M., Kramer, N.E., Edwards, R., Baker, S., Hamlin, N., Simmonds, M., Stevens, K., Maloy, S., Parkhill, J., Dougan, G., Bäumler, A.J., 2001. *Salmonella enterica* Serovar Typhi Possesses a Unique Repertoire of Fimbrial Gene Sequences. Infect. Immun., 69 (5): 2894-2901.

Vasquez-Torres, A., Jones-Carson, J., Baumler, A.J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parkos, W.T., Fang, F.C., 1999. Extraintestinal dissemination of Salmonella by CD-18 expressing phagocytes. Nature, 401: 804-808.

Villalobo, E., Torres, A., 1998. PCR for detection of *Shigella* spp. in mayonnaise. Appl. Environ. Microbiol., 64: 1242–1245.

Voetsch, A.C., Van Gilder, T.J., Angulo, F.J., Farley, M.M., Shallow, S., Marcus, R., Cieslak, P.R., Deneen, V.C., Tauxe, R.V., 2004. FoodNet estimate of the burden

of illness caused by nontyphoidal Salmonella infections in the United States. Clin. Infect. Dis., 38(3): S127-S134.

Wang, G., Clark, C.G., Rodgers, F.G., 2002. Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 Shiga toxin family by multiplex PCR. J. Clin. Microbiol., 40:3613–3619.

Waters, J.R., Sharp, J.C., Dev, V.J., 1994. Infection caused by Escherichia coli O157:H7 in Alberta, Canada, and in Scotland: a five-year review, 1987-1991. Clin. Infect. Dis., 19:834.

Weggerhof, F.O., 1987. The aetiology of gastroenteritis in infants in a rural population. (M.Sc) University of the Witwatersrand, Johannesburg, South Africa.

WHO, World Health Organization, 2009. Diarrhoeal Diseases (Updated February 2009): Enterotoxigenic *Escherichia coli* (ETEC). http://www.who.int/ vaccineresearch/diseases/ diarrhoeal/en/ index4.html

Yatsuyanagi, J., Saito, S., Sato, H., Miyajima, Y., Amano, K.I., Enomoto, K., 2002. Characterization of enteropathogenic and enteroaggregative *Escherichia coli* isolated from diarrheal outbreaks. J. Clin. Microbiol., 40(1): 294-297.

CHAPTER 4

AN ETHNOBOTANICAL SURVEY OF INDIGENOUS PLANTS USED AS DIARRHOEA REMEDY IN O.R.TAMBO DISTRICT, EASTERN CAPE PROVINCE, SOUTH AFRICA

4.0. Abstract

Indigenous health system and the use of herbal plants have been recognized as pivotal in primary health care and a system to reckon with in achieving one of the targets of the Millenial Goals on health. An ethnobotanical survey was conducted to identify indigenous herbal remedies for diarrhoea and associated stomach ailments in rural areas of O.R. Tambo district municipality in the Eastern Cape province of South Africa. The main objective of the study was to gather ethnomedical data on potentially valuable indigenous medicinal plants for the eventual development of new pharmaceuticals and also emphasize the role of ethnomedicine in primary health care. The use of herbal remedies in the treatment of diarrhoea and dysentery was investigated using interviews. The survey was conducted among traditional healers and knowledgeable local elders who use medicinal plants to treat common illnesses. Data from the survey indicated the names of plants commonly used in the treatment of diarrhoea and associated ailments, the methods of preparation, parts used and administration. A total of 32 plant species belonging to 26 families were reportedly used as diarrhoea remedy in the study area. The most predominant families of medicinal plants employed and most frequently recommended were Fabaceae 16.67%, followed by Hyacinthaceae and Hydnoraceae 8.33% each. The most commonly

utilized portions of plants for medicinal purposes included roots and leaves. Other parts were corms, bulbs, tubers, fruits and bark. The methods of preparation often employed were decoctions and infusions whilst medication was frequently administered orally or as enema. Some of the plants were used singly or mixed with other plant(s) while some edible ones are consumed as food. The survey documented a diversity of plants employed as remedy for diarrhoea. Integration of this form of health care system into western medicine is warranted but propagation of such medicinal plants is vital to sustainability of the use of medicinal plants.

Key words: Ethnobotany; Herbal remedy; Indigenous; Diarrhoea, Primary health

4.1. Introduction

One of the targets on Health in the Millennium Development Goals (MDG) declared in 2000 is to provide quality and affordable health for all by the year 2015 (UN, 2000). With the MDG target date only 6 years away, this vision is bound to fail unless further concerted effort is made to improve the health of poor people. According to the World Health Organization (WHO,1978), traditional medicine has been described as one of the surest means to achieve total health care coverage of the world's population, yet this form of health care system has long been relegated to a marginal place. The importance of traditional medicine as a source of primary health care was first officially recognized by the World Health Organization in the Primary Health Care Declaration of Alma Ata (1978)

and has since been given a global attention under Traditional Medicine Programme of the health body. Recently, WHO commitment to the Millennium Declaration has been reaffirmed by its governing bodies (WHO, 2002a, 2002b) and Ministers of Health of the WHO African Region (WHO, 2007) have also made a declaration to recognize the role of traditional medicine in Primary Health Care.

In the rural and remote parts of most African countries, hospitals and clinics are often sparsely located far away from dwellers and where there is accessibility to clinics, other factors such as finance and mobility make orthodox medicine far fetched from these people. It has been estimated that up to 80% of the world's rural populations depend on plants for their primary health care, since western pharmaceuticals are often expensive, inaccessible or unsuitable (Cunningham, 1993; WHO, 1978). Considering the relative ratios of traditional practitioners and university-trained doctors in relation to the whole population in African countries, traditional healers and remedies made from plants play an important role in the health of millions of people. The people are reliant on traditional healers who usually reside among them trusting their ingenuity about the use of herbs and some other cultural and traditional beliefs. These facts thus provide a role for traditional healers among the rural dwellers' trust.

It is becoming increasingly urgent to document the medicinal use of African plants because of the rapid loss of the natural habitat for some of these plants due to anthropogenic activities. The migration factor especially among the youths to urban areas as well as the demise of most of the local practitioners

along with their wealth of knowledge are sources of threat to the future of most of the important cultures including knowledge on the use of plant species (Akerele *et al.*, 1991; Bodeker, 1994; Schlage *et al.*, 1999).

Several studies have been conducted in the Eastern Cape to identify and document biodiversity and ethnomedicinal value of the province (Van Wyk *et al.*, 1997; Grierson and Afolayan, 1999; Dold and Cocks, 2001; Van Wyk and Smith, 2001; Kambizi *et al.*, 2004; Mucina and Rutherford, 2006; Kambizi and Afolayan, 2008). Nevertheless, circumstances still warranted a survey of O.R. Tambo District Municipality (ORTDM). Why is it necessary to conduct the study in this area? What makes this district municipality peculiar? To answer these and some other questions, a grasp of what ORTDM is made up of is necessary.

4.1.1. A brief description of O.R. Tambo District Municipality

O.R. Tambo District Municipality (ORTDM) is located in the east of the Eastern Cape Province along the Indian Ocean coastline of South Africa. It is situated in the former Transkei homeland area of the province which falls within the latitudes 30° 00' and 34° 15' South and longitudes 22° 45' and 30° 15' East. ORTDM is bordered by the Alfred Nzo District to the North, Ukhahlamba district to the Northwest, Chris Hani to the West and Amathole District to the Southwest (Figure 1). From the East to the West, the District measures 170 143 km, North to South 121 725 km and it measures 12857 km^2 in extent (http://isrdp.dplg.gov.za/documents/IDP/ISRDP/OR_Tambo_IDP.pdf). It is more

than 2,700 m above sea level, and descends southward from the great interior plateau to form a relatively narrow coastal plain along the Indian Ocean.

The population of the District Municipality stands at 1.7 million persons and covers an area of 16,617 square km^2. A total of 93% of the District Municipality's population reside in rural areas while an estimated 77% of the population is unemployed (STATSSA, 2001). The mother-tongue of the majority of the dwellers is *isiXhosa*, an Nguni language while the rest of the people speak Afrikaans and English.

The region is predominantly rural with large tracts of arable land, nevertheless, agriculture in ORTDM is inadequately developed and largely subsistence. Traditionally, Eastern Cape is known for rearing livestock which represents 70% of the province's gross agricultural income. Wheat, corn (maize), and sorghum are grown inland with irrigation, while oranges, pineapples, tobacco, and potatoes are cultivated along the coast (Encyclopædia Britannica, 2009). The Langeni Forest and Magwa Tea are the major contributors to the primary sector of the economy with an oncoming Mondi Forests reaching its maturity state soon.

This study area includes moderate and high rainfall areas, principally along its sub-tropical coast, but also in pockets of mountainous areas receiving an annual rainfall of above 800 mm. ORTDM has a diversity of vegetation, from grasslands and thicket to forests and bushveld including coastal and marine habitats. The district is considered to have the richest natural resources and the

most fertile areas in the country, with good soils and climatic condition (http://isrdp.dplg.gov.za/documents/ IDP/ISRDP/ OR_ Tambo _IDP. pdf). The vegetation of our study region has been previously described (Acocks 1988; Dahlgren and Van Wyk 1988; Van Wyk 1990; Hartmann 1991; Cawe *et al.*, 1994; Van Wyk 1994; Low and Rebelo, 1998; Van Wyk and Smith, 2001; Mucina and Rutherford, 2006; Clark et al. 2008).

This district falls within regions (namely-Maputaland, Pondoland and the Drakensberg Alpine) singled out in a world atlas of centres of plant diversity (CPD's) (Pooley, 1998). The area now classified as a Global Hotspot (Steenkamp *et al.*, 2004), a Global Hotspot being defined on the basis of irreplaceability and threat (Myers 1988). Of great significance are the coastal forests, bushveld and grassland of the Pondoland. This area has been identified as a Centre of Plant Endemism, which is floristically species-rich and contain high numbers of species endemic to them (Van Wyk and Smith 2001). It has more than 130 species of plants that occur nowhere else in the world, and including the well-known Pondoland coconut palm (ORTDM, 2009).

However, due to the rich flora of this study area coupled with the disappearing traditional knowledge on medicinally useful plants a lot of grounds still needs to be covered in the documentation of the indigenous knowledge on medicinal plants. Thus, it is critical to preserve the plants and knowledge of their uses.

Diarrhoeal diseases are often associated with low living standard, poor sanitation infrastructure and poor access to potable water sources. This in particular is a prevalent feature in the area of study owing to a high percentage of unemployment as previously reported (STATSSA, 2001). The predominantly rural Eastern Cape Province (ECP) is noted for lack of proper sanitation and piped or clean water (ECDOH, 2009). Hence, water-borne diseases are not far-fetched from ORTDM with its predominant rural dwellers depending largely on spring, pond or river water which are often shared with domestic animals. According to a previous study in South Africa (Obi *et al*., 2007) incidence of diarrhoea could be linked to poor quality of household drinking water. A recent Eastern Cape Department of Health statistics (2009) confirm a significant number of deaths due to diarrhoea among children, with the highest number of deaths reported in the ORTDM. Of grave health consequences is cholera disease within the region. Kwa-Zulu Natal (KZN) Province of South Africa was designated as endemic for cholera (Henninger and Snell, 2002). ORTDM shares border with KZN and as indicted in figure 2, it is worthy of note that the blue arrow indicating direction of spread of cholera is towards ORTDM. A recent outbreak of cholera which ravaged Zimbabwe rapidly spread into South Africa between November 2008 and April 2009 and claimed 65 lives whilst there was >12, 000 case definition of the disease (Archer *et al.,* 2009). With the high incidence of HIV/AIDS and multidrug resistant TB in South Africa (Cohen, 2006), diarrhoea can pose a serious challenge to the public health in terms of burden of diseases.

As a result of the high incidence of diarrhoeal diseases, poor water quality and large dependence on medicinal plants due to the vast rural area, this study aimed to document information on plants commonly prescribed as diarrhoeal remedy in the ethnomedicinal practice of the indigenous people of OR Tambo District Municipality and environs.

4.2. Materials and methods

Ethnobotanical survey for medicinal plants employed in the treatment of diarrhoea and associated ailments was conducted within ORTDM. Areas visited included Port St. Johns, Lusikisiki, Flagstaff, Tabankulu, Bizana, Ugie and Coffee bay from June 2008 to February 2009. The investigation was carried out using interviews among traditional healers and knowledgeable local elders who use medicinal plants to treat common illnesses. Questionniare employed for the interview is shown in Appendix 1C. The survey elicited information on the names of plants commonly used in the treatment of diarrhoea and associated ailments, the methods of preparation, parts used and administration. With the assistance of the local practitioners samples of the plant material used as diarrhoeal remedy were collected from the wild. Scientific identification of samples was aided by staff of the herbarium, Walter Sisulu University, where voucher specimens were deposited. Further characterization of plants and their usage was established by consultation of literatures and monographs (Hutchings *et al.,* 1996; Pooley, 1993, 1998; Van Wyk *et al.*, 1997). For data analysis, plant species were

grouped into their respective families along with local and common names. An inventory of plant species was compiled from this fieldwork.

4.3. Results

Ethnobotanical information obtained from the study area on medicinal plants used in the treatment of diarrhoea has revealed 33 medicinal plants scattered in 26 families. Table 4.1 shows the diverse families of the various indigenous medicinal plants. Among the families, Fabaceae provided the highest proportion of medicinal plants prescribed at 16.67% followed by Hyacinthaceae and Hydnoraceae, 8.33% each.

Most traditional healers claimed never to use cultivated plants but depend on sourcing from the wild. The frequently utilized portions of plants for medicinal decpurposes included roots, corms, leaves, and bark but roots were mostly used for the preparation of medicine in the treatment of diarrhoea, followed by the use of leaves. The methods of preparation varied considerably from one healer to the other. Plant remedies were often utilized in the form of decoctions and infusions. Extraction may be hot by boiling in water or mere soaking plant parts in cold water. Plant part may be eaten raw in some cases whilst medication is frequently administered orally or as enema. Some of these plants were used singly or mixed with other plant(s) or even with western mixtures such as salt, vinegar and in a particular instance Amsphogel (Aluminum hydroxide) was mixed with plant part by the traditional healer. The surveys also revealed that

dosage of plant extracts were not consistent. The analysis has revealed the diversity of indigenous plants used as diarhoeal remedy in OR Tambo. These medicinal plants which are of value in the treatment of diarrhoea are also used to treat different other ailments while some edible ones are consumed as food.

4.4. Discussion

Traditional medicine is the most widely used medical system in the rural setting of ORTDM. Orthodox medicine is costly and often inaccessible. Not only is ethnomedicine popular and acceptable due to their important role in primary health-care delivery systems, but also in many areas, it has been the only system available. Likewise, medicinal plants need more attention because it forms an essential component of the total well-being of humans particularly the rural dwellers whose major sources of food, shelter, energy and medicines are forest plants (Akerele, 1988; Hamayun *et al*., 2003). Furthermore, the emerging global problem of multidrug resistant pathogens (Alekshun and Levy, 2007; Bisi-Johnson *et al*., 2005; Levy 2005; Obi *et al*., 2007) and the need for the discovery of lasting and sustainable therapy to combat diseases such as HIV/AIDs, malaria and cancer which have defied available treatments has led to a paradigm shift to natural herbal product for succor.

This study documented a diverse list of plants used as remedy for diarrhoea in ORTDM. Previous reports have also linked some of the plants encountered in the course of this survey with remedy of diarrhoea, dysentery or

stomach ailments (Watt and Breyer-Brandwijk, 1962; Bigalke, 1967; Hutchings, 1989; Van Wyk and Gericke, 2000; Van Wyk and Wink, 2004;). The parts of the plants commonly used were roots and leaves. The problem of inconsistent dosaging is a critical set back which is crucial in standardization of medicinal plants. Another factor which may impact on the traditional therapy standardization is the work place hygiene and the quality of water used in preparation particularly with cold extraction. Most of the traditional healers boast of treating some ailments that have defied modern medical practice. While some of the healers embrace the idea of both western medicine and traditional medicine complementing each other, a few others do not. Some of the traditional healers tend to hide the information on plants used for different ailments largely for fear of losing patronage to the investigators or interested persons. By a way of mystifying the native trade, the vast majority of plants are collected from the wild and cultivation of the plants is often not encouraged by the traditional healers. Some participants believed that cultivated plants would have been attacked by evil spirit and hence will not be potent for use. This is similar to the findings of Keirungi and Fabricius (2005). Scientific evaluations of the therapeutic claims as well as toxicological data are still underprovided for many of the plant species. This study will form the basis for microbiological and phytochemical research on selected diarrhoeal medicinal plants.

An observation in the course of this study which poses a sort of concern is some of the methods used in harvesting these medicinal plants. For instance,

root excavation and bark striping of plants pose a threat to the continued existence of such plants. These two methods have been reported as most harmful harvesting methods for plants (Akerele 1991; Cunningham *et al*., 2002). The tendency for extinction of scavenged species is obvious going by the unsustainable handling and the habit of not cultivating medicinally valuable species. For conservation to be effected, planting of designated valuable herbal plants in small gardens in the homesteads is strongly recommended. Large scale farming of commercially viable plants should be encouraged whilst scientist are implored to undertake studies on various factors affecting growth of the plants such as soil conditions, temperature, seasonal variations and disseminate best propagation methods. Conclusively, it is pertinent for scientists to urgently salvage this cheap and alternative health care system from extinction, help preserve indigenous knowledge and conserve nature.

4.5. References

Archer, B.N., Cengimbo, A., De Jong, G.M., Keddy, K.H., Smith, A.M., Sooka, A., Ntshoe, G., Blumberg, L., 2009. Cholera outbreak in South Africa: preliminary descriptive epidemiology on laboratory-confirmed cases, 15 November 2008 to 30 April 2009. Communicable Dis. Surveill. Bull. 7(2). Available from www.nicd.ac.za/pubs/survbull/2009/CommDis BullMar09.pdf

Acocks, J.P.H., 1988. Veld types of South Africa (3rd edn). Memoirs of the Botanical Survey of South Africa No 28. Government printer, Pretoria.

Akerele, O., 1988. Medicinal plants and primary healthcare: an agenda for action. Fitoterapia, 59: 355–363.

Akerele, O., Heywood, V., Synge, H., 1991. The conservation of medicinal plans; Proc. International consultation 21 - 27 March 1988; Chiang Mai, Thailand. Cambridge University Press.

Alekshun, M.N., Levy, S.B., 2007. Molecular Mechanisms of Antibacterial Multidrug Resistance Cell, 128: 1037-1050.

Batten, A., Bokelman, H., 1996. Wild Flowers of the Eastern Cape. Books of Africa, Cape Town.

Bhat, R.B., Jacobs, T.V., 1995. Traditional herbal medicine in Transkei. J. Ethnopharmacol., 48: 7-12.

Bigalke, E.H., 1967. A list of plants used medicinally in the following districts of Transkei: Bizana, Flagstaff, Libode, Lusikisiki, Qumbu: East London Museum Unpublished. .

Bisi-Johnson, M.A., Kolawole, D.O., Shittu, A.O., 2005. Epidemiological analysis of clinical isolates of *Staphylococcus aureus* in Ile-Ife, Nigeria. Pakistan J. Biol. Sci., 8 (7): 1016-1020.

Bodeker, G., 1994. Traditional health knowledge and Public policy. Nature Res., 30(2): 5-16.

Bryant, A.T., 1996. Zulu Medicine-men. C. Struik, Cape Town (originally published in 1909 in the Annals of the Natal Museum.

Cawe, S.G., Moll, E.J., Mckenzie, B., 1994. An evaluation of the phytochronological classification of the forests of Transkei. In: Seyani, J.A. and A.C. Chikuni (eds.) Proc. XIIIth Plenary Meeting AETFAT, Malawi. 2:1043-1059.

Cohen, J., 2006. Infectious disease. Extensively drug-resistant TB gets foothold in South Africa. Science, 314(5798):419-420.

Cunningham, A.B., 1988. An Investigation of the Herbal Medicine Trade in Natal/KwaZulu. Institute of Natural Resources Investigational Report 29. University of Natal, Pietermaritzburg.

Cunningham, A.B., 1993. Ethics, ethnobiological research and biodervisty. WWF-World Wide Fund for Nature (formerly World Wildlife Fund), Gland, Switzerland.

Cunningham, A.B., Ayuk, E., Franzel, S., Duguma, B., Asanga, C., 2002. An economic evaluation of medicinal tree cultivation: *Prunus africana* in Cameroon. Peoples and Plants Working Paper 10, UNESCO, Paris.

Dahlgren, R., van Wyk, A.E., 1988. Structures and relationships of families endemic to or centered in southern Africa, Monogr. Syst. Bot. Missouri Bot. Gard., 25: 1-94.

Dold, A.P., Cocks, M.L., 2001. Traditional veterinary medicine in the Alice district of the Eastern Cape Province, South Africa. S. Afr. J. Sci., 97: 375-379.

Encyclopædia Britannica. 2009. Eastern Cape. from Encyclopedia Britannica Online: http://www.britannica.com/EBchecked/topic/ 176933/ Eastern-Cape. Retrieved March 16, 2009.

Eastern Cape Department of Health (ECDOH) Large drop in Eastern Cape diarrheoa statistics. 2009. Available from http://www.ecdoh.gov.za/press _releases/509/Large_drop in_Eastern_Cape_diarrheoa_statistics/ 29_January 2009.

Gerstner, J., 1939. A preliminary checklist of Zulu names of plants with short notes. Bantu Stud. 13(1) 47-64, (2) 131-149, (4) 307-326.

Grierson, D.S., Afolayan, A.J., 1999. An ethnobotanical study of plants used for the treatment of wounds in the Eastern Cape, South Africa. J. Ethnopharmacol., 66: 103-106.

Hamayun, M., Khan, M.A., Begum, S., 2003. Marketing of medicinal plants of Utror-Gabral Valleys, Swat, Pakistan. Ethnobotanical Leaflets, SIUC, USA. Available at: http:www.siu.edu/~ebl/leaflets/market.htm

Hartmann, H.E.K., 1991. Mesembryanthema in Pondo land. Plant Life, 5: 19-20.

Henninger, N., Snell, M., 2002. Where Are the Poor? Experiences with the Development and Use of Poverty Maps. Washington, D.C.: World Resources Institute and UNEP/GRID-Arendal.

Hutchings, A., 1989. Observations on plant usage in Xhosa and Zulu medicine. Bothalia, 19, 225-235.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A.B., 1996. Zulu Medicinal Plants. An Inventory. (1st edn). University of Natal Press, Private Bag X01, Scottsville 3209.

Iwu, M.M., 1993.Handbook of African Medicinal Plants CRC Press, Inc.

Jacot Guillardmod, A.m 1971. Flora of Lesotho. Cramer, Lehre.

Kambizi, L., Sultana, N., Afolayan, A.J., 2004. Bioactive compounds isolated from *Aloe ferox*: A plant traditionally used for the treatment of sexually transmitted infections in the Eastern Cape, South Africa. J. Pharm. Biol., 42 (8): 636-639.

Kambizi, L., Afolayan, A.J., 2008. Extracts from *Aloe ferox* and *Withania somnifera* inhibit *Candida albicans* and *Neisseria gonorrhoea.* Afr. J. Biotechnol., 7 (1):012-015.

Keirungi, J., Fabricius, C., 2005. Selecting medicinal plants for cultivation at Nqabara on the Eastern Cape Wild Coast, South Africa. Research in Action. S. Afr. J. Sci., 101, 497-501.

Kokwaro, J.O., 1976. Medicinal Plants of East Africa. East African Literature bureau, Nairobi.

Levy, S.B., 2005. Antibiotic resistance-the problem intensifies. Adv. Drug Delivery Rev., 57:1446– 1450.

Low, A.B., Rebelo, A.G., 1998. Vegetation of South Africa, Lesotho and Swaziland. Department of Environmental Affairs and Tourism, Pretoria.
Mucina, L., Rutherford, M.C. (Eds.). 2006. The Vegetation of South Africa, Lesotho and Swaziland. Strelitzia, vol. 19. South African National Biodiversity Institute, Pretoria.

Myers, N., 1988. Threatened Biotas: 'Hotspots' in Tropical Forests. The Environmentalist, 10: 243–256.

Obi, C.L., Ramalivhana, J., Momba, M.N.B., Onabolu, B., Igumbor, J.O., Lukoto, M., Mulaudzi, T.B., Bessong, P.O., Jansen van Rensburg, E.L., Green, E., Ndou, S., 2007. Antibiotic resistance profiles and relatedness of enteric bacterial pathogens isolated from HIV/AIDS patients with and without diarrhoea and their household drinking water in rural communities in Limpopo Province South Africa. Afr. J. Biotechnol., 6; 8: 1035-1047.

ORTDM, 2009. The OR. Tambo District Municipality IDP and Budget review 2008/2009. (http://isrdp.dplg.gov.za/documents/IDP/ISRDP/OR_Tambo_IDP.pdf) Accessed 19 March 2009.

Pooley, E., 1993. The Complete Field Guide to Trees of Natal Zululand and Transkei. (1st edn). Natal Flora Publication Trust. C/o Natal Herbarium Botanic Gardens Road, Durban 4001. pp. 512.

Pooley, E., 1998. A Field Guide to Wild Flowers Kwazulu-Natal and the Eastern Region.(1st edn). Natal Flora Publication Trust. C/o Natal Herbarium Botanic Gardens Road, Durban 4001.

Pujol, J., 1990. Nature Africa: The Herbalist handbook. Jean Pujol Natural Healers Foundation, Durban.

Roberts, M., 1983. Book of Herbs. Jonathan Ball, Bergvlei, Cape Town.

Roberts, M., 1990. Indigenous Healing Plants. Southern Book Publishers, Halfway House.

Rood, B., 1994. Uit die veldapteek. Tafelberg Publishers, Cape Town.

Schlage, C., Mabula, C., Mahunnah, R.L.A., Heinrich, M., 2000. Medicinal Plants of the Washambaa (Tanzania): Documentation and Ethnopharmacological Evaluation. Plant Biol., 2: 83 - 92.

Silayo, A., Ngadjui, B.T., Abegaz, B.M., 1999. Homoisoflavonoids and stilbenes from the bulbs of *Scilla nervosa* subsp. *rigidifolia.* Phytochem., 52 (5): 947-955.

Singh, Y., 1999. Hypoxis: yellow stars of horticulture, folk remedies and conventional medicine. Veld & Flora. 85: 123-125.

STATSSA. Statistics South Africa. 2001. Census 2001: Census in brief / Statistics South Africa. Pretoria: Statistics South Africa, 2003 Previous title: The people of South Africa: Population census 1996: Census in Brief, 1998 108p. [Report No: 03-02-03(2001)] 2001.(http://www.statssa.gov.za/census01/html/default.asp) Accessed 16 March 2008.

Steenkamp, Y., Van Wyk, A.E., Victor, J.E., Hoare, D.B., Dold, A.P., Smith, G.F., Cowling, R.M., 2004. Maputaland-Pondoland-Albany Hotspot. In: Mittermeier, R.A., Gil, P.R., Hoffmann, M., Pilgrim, J., Brooks, T., Mittermeier, C.G., Lamoreux, J. & Fonseca, G.A.B. da (eds.) *Hotspots revisited.* EMEX, pp.218–229. ISBN 968-6397-77-9.

Temu, R.P.C., Chihongo, A., 1998. Field survey of wild and underutilized edible plants of Ruvuma Region, Tanzania. COSTECH Dar es Salaam. Pp.46.

UN, United Nations, 2009. United Nations Millennium Declaration. New York, NY, United Nations, 2000 (A/RES/55/2; http://www.un-ngls.org/MDG/A-RES-55-2.pdf) 2000. Accessed 27 April 2009.

Van Wyk, B.E., 1990. A new species of *Leucadendron* (Proteaceae) from Pondoland, with a discussion of its biogeography. S. Afr. J. Bot., 56: 458-466.

Van Wyk, B.E., 1994. Maputaland-Pondoland region. In S.D. Davis, V.H. Heywood and A.C. Hamilton (eds.) *Centres of plant diversity. A guide and strategy for their conservation.*Volume 1, pp. 227-235. IUCN Publications Unit, Cambridge.

Van Wyk, B.E., Gericke, N., 2000. People's Plants: A Guide to Useful Plants of Southern Africa. Briza Publications, Pretoria.

Van Wyk, B.E., Smith, G.F. 2001. Regions of floristic endemism in southern Africa. Umdaus press, Hatfield.

Van Wyk, B.E., Van Oudtshoorn, B., Gericke, N., 1997. Medicinal plants of South Africa. Briza, Pretoria, pp. 1-304.

Van Wyk, B.E., Wink, M., 2004. Medicinal Plants of the World. Briza Publications, Pretoria, ISBN 1-875093-44-3.

Watt, J.M., Breyer-Brandwijk, M.G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa, 2nd ed. Livingstone, London.

WHO. World Health Organization. 1978.The Promotion and Development of Traditional Medicine. Technical Report No. 622. Geneva.

WHO. World Health Organization. 2002a. Resolution EB109.R3. WHO's contribution to achievement of the development goals of the United Nations Millennium Declaration. In: *109th Session of the Executive Board, Geneva, 14-21 January 2002. Resolutions and decisions*. Geneva, World Health Organization, (EB109.R3; http://www.who.int/gb/ebwha/pdf_files/EB109/eeb109r3. pdf, Accessed 27 April 2009.

WHO. World Health Organization. 2002b. WHO's contribution to achievement of the development goals of the United Nations Millennium Declaration. Note by the Director-General. In: *Fifty-fifth World Health Assembly, Geneva, 13-18 May 2002.* Geneva, World Health Organization, (A55/6; http://www.who.int/ gb/ebwha/pdf_files/WHA55/ ea556.pdf) Accessed 27 April 2009.

WHO. World Health Organization. 2007. Declaration on Traditional Medicine being an undertaking by Ministers of Health attending the 57th session of the WHO Regional Committee for Africa. Accessed 27 April 2009). (http://www.afro.who.int/trm/declaration_on_traditional_medicine.pdf)

Figure 4.1. Map of O.R. Tambo District Municipality

(Source: http://isrdp.dplg.gov.za/documents/IDP/ISRDP/OR_Tambo_IDP.pdf).

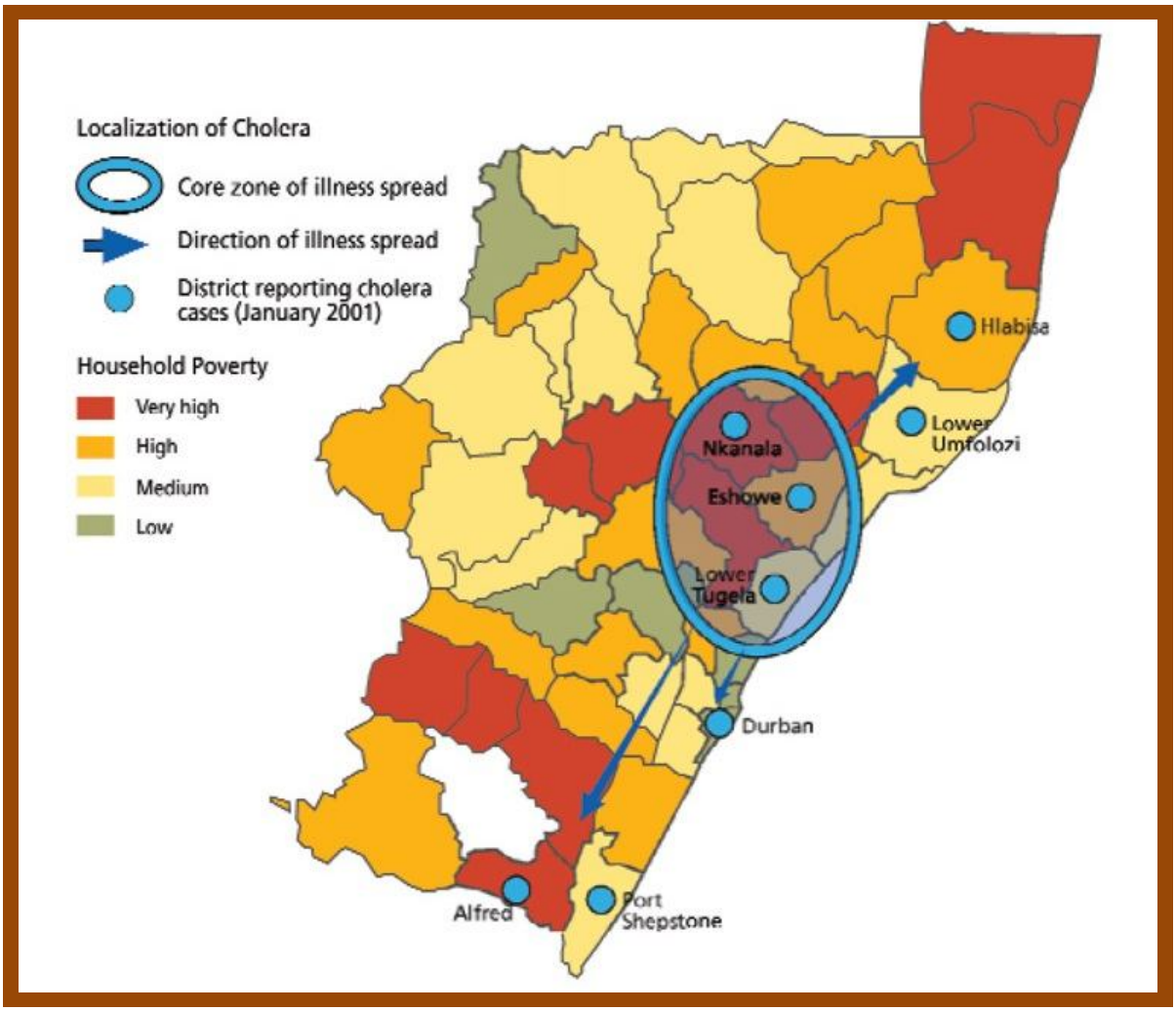


Figure 4.2. WHO Cholera endemic zone
(Source: www.who.int/entity/heli/tools/en/gridmap2.jpg; Henninger and Snell, 2002).

Table 4.1. Herbal remedies used for the treatment of diarrhoea and associated ailments.

FAMILY/SCIENTIFIC NAMES COMMON NAME	LOCAL NAMES	PLANT PART	USAGE & PREPARATION	OTHER USAGE
ALLIACEAE *Tulbaghia alliacea L.f.* Wild Garlic	Umwelela (X) ivimba-mpunzi (X) Sikwa (Z)	Bulb	Stomach ailments, bulb infusion taken orally to treat fever.	Remedy for tuberculosis (TB) and influenza, as an antihypertensive or to expel intestinal worms (Watt & Breyer-Brandwijk, 1962). As a medicated bath to treat paralysis, rheumatism and reduce fever temperature (Watt & Breyer-Brandwijk, 1962).
AMARANTHACEAE *Hermbstaedtia odorata* Wild Cockscomb Rooi-aarbossie	Ubuphuphu (X,Z)	Leaves	Leaves eaten as food and infusion for diarrhoea.	Root cleansing stomach wash alone or with *Acaccia xanthophloea* and *Cappa* (Hutchings *et al.*, 1996).
AMARYLLIDACCEAE *Scadoxus puniceus* (=*Hemanthus magnificus, H. natalensis*) Bloody Lily, Snake Lily	Umphompho – wezinja, Isiphompo, umgola (Z)	Bulb & roots	Stomach ailments, diarrhoea, nausea.	Bulb poisonous, use for poultices (Batten & Bokelmann, 1966). Coughs (Bryant, 1966), headaches, poisoning antidote (Hutchings *et al.*, 1996).

ANACARDIACEAE *Protorhus longifolia* (Bernh. ex C. Krauss) Engl. Red Beech	Izintlwa, ikubalo, umkupati (X)	Bark	Bark dried powdered + guava + Qangazani boiled & taken orally for diarrhoea and bloody stool.	Heart burn and stomach bleeding (Hutchings *et al.*, 1996).
APOCYNACEAE *Acokanthera oppositifolia* (Lam.) Codd= *A. venenata* Common Poison-bush	inHlungunyembe Intlungunyembe (X, Z)	Leaves	Leaf decoction for stomach ache, diarrhoea, antihelmintic	Treat snakebite (Gerstner, 1939). Spider bite, aches, intestinal worms, cold (Pooley, 1993). Powder from dried roots as snuff for headache (Bhat and Jacobs, 1995).
ASCLEPIADACEAE *Xysmalobium undulatum* (L.) W.T.Aiton Milkwort, Uzura	Ishongwe (X,Z), Itsongwe	Roots	Boil root for diarrhoea, stomach ailments or pain.	Dysentery, Headaches (Pujol, 1990). Flower and seed decoction for colic, poison antidote (Watt & Breyer-Brandwijk, 1962)
ASPHODELACEAE *Aloe candelabrum* Berger. Candelabrum Aloe *Bulbine abyssinica,* Bushy Bulbine,	Ikhalana Inkalane (X) Uphondonde (Z) Utswelana Intelezi (X) Ibhucu (Z),	Leaves Leaves, tubers	Leaf decoction for diarrhoea. Boil leaves for vomiting, diarrhoea, TB.	 Treat bilharzias, dysentery, cracked lips, skin ailments, urinary

	Incelwane (X);			complaints, rheumatism, as a charm (Pooley, 1998). Tuber decoctions-antispasmodic to quell vomiting (Hutchings *et al.*, 1996).
ASTERACEAE *Bidens bipinnata* Spanish-black jack, Spanish needle	Uvelemampondweni uvelegoli	Leaves	Leaves edible, infusion for diarrhoea.	Rheumatism (Watt & Breyer-Brandwijk, 1962). Hemorrhage, reduce cancer, flu, cold and fever (Pooley, 1998).
CORNACEAE *Curtisia dentata* (Burm.f.) C.A.Sm. = *C. faginea* Assegaai	Umlahleni (X,Z), Unsirayi (X), Umgxina, Umlahleni (Sefile), Uzintlwa	Bark, Root	Diarrhoea, stomach ailments.	Stomach ailments including diarrhoea, blood strengthener, aphrodisiac (Pajol, 1990).
EUPHORBIACEAE *Euphorbia cooperi* N.E.Br. ex. Berger Euphorbia or Milkweed, Spurge, Transvaal Candelabra Tree	Umhlonhlo (X)	Bark of root	Bark of root ground dry boiled, then mix with a sachet epsom salt, cool & add 2 spoons vinegar for diarrhoea, stomach disorders. For infants, mix equal portion of decoction with Amsphojel to flavour milk.	

FABACEAE subfamily MIMOSACEAE *Acacia mearnsii* De Wild Blackwood,Black Wattle	Ublekwana (X) Udywabasi (X, Z) Indywabasi	Bark	Bark infusion taken orally for diarrhoea, dysentery	Sore throat, coughs, children fever, tooth ache (Hutchings *et al.*, 1996).
FABACEAE (LEGUMINOSAE) *Elephantorrhiza elephantina* (Burch.) Skeels Elephant's Root	Intolwane (X,Z)	Root, stem	Boil equal part plant + *Acokanthera oblongifolia* for diarrhoea, stomach ailment. Infusion of ground stem alone for diarrhoea and menstrual disorder.	Syphilis, stop bleeding (Jacot Guillardmod, 1971), chest complaints, heart conditions (Watt & Breyer-Brandwijk, 1962). Fever, ulcers, dysentery, diarrhoea, dysmenorrhoea (Bryant, 1966).
GERANIACEAE *Pelargonium sidoides* Rose-scented Pelargonium	Umsongelo (X)	Leaves, roots	Infusion of leaves or root enema for diarrhoea, dysentery and vomiting.	Bruised leaves soothes skin rashes, in tea to treat kidney & bladder ailments, nausea, gonorrhoea, root decoction severe diarrhoea in Transkei (Hutchings *et al*., 1996). Leaf paste for wound, powdered root mixed with food for dysentery (Watt & Breyer-Brandwijk, 1962).
P. luridum Wild geranum	Umsongelo, ishwaqa	Leaves	Eaten raw as vegetable, treat dysentery, nausea, vomiting, fever	

HYACINTHACEAE *Eucomis autumnalis* (Mill.) Chitt. Common Pineapple Flower	Ubuhlungu becanti Isithithibala (X) Umathunga (Z)	Bulb	Boil bulb for abdominal problems.	Decoctions of bulb and roots for coli, flatulence (Cunningham, 1993). Syphilis (Watt & Breyer-Brandwijk, 1962). Treat urinary & pulmonary ailments, fever (Hutchings *et al.*, 1996; Roberts, 1990).
Scilla nervosa (Burch.) Jessop White Scilla	Umagaqana, magaqana (X), Imbizankulu ingema (Z)	Bulb	All purpose herb. For diarrhoea, TB, various diseases, cleans blood.	Purgative, sprain, fracture, cancers (Watt & Breyer-Brandwijk, 1962).
Scilla sp.	Umasixabane (X)	Root	Infusion of ground tuber + *H. africana* + *Curtisia dentate* for diarrhoea	Rheumatic fever, dysentery (Rood, 1994; Silayo *et al.*, 1999).
HYDNORACEAE *Hydnora africana* Warty Jackal Food, Jakkalskos Kanip	Ubuklunga (X) Umavumbuka(Z), Umafumbuka (X)	Fruits, tuber, leaves	Boil handful of ground dried tubers + blackwood + peach for diarrhea	Parasitic on *Euphorbia* roots. Fruit pulp like potato eaten by people birds jackal, plant dried ground raw for dysentery, amenorrhoea. Swollen glands or inflamed throat (Watt & Breyer-Brandwijk, 1962).
HYPOXIDACEAE				

Hypoxis latifolia Hook. African potato (Eng.) Small Yellow star-flower *H. hemerocallidea = H. rooperi* Star-flower	Inongwe Ilabateka (X) Inongwe Ilabateka (X)	Tuber	Boil ground dried tuber for diarrhoea.	Treat benign prostrate (van Staden, 1981). Headaches, dizziness, mental disorders, inflammation, HIV (Singh, 1999; van Wyk, 2000).
IRIDACEAE *Gladiolus sericeo-villosus* Hook.f. *forma sericeo-villosus* Natal Lily, parrot's beak gladiolus	Umnunge (X)	Corm	Corm's decoction for cold and dysentery, TB (use with care)	Corms for dysentery oral and enema (Hutchings *et al.*, 1996). Impotence (Watt & Breyer-Brandwijk, 1962). Menstrual pain (Bryant, 1966).
MESEMBRYANTHEMACEAE *Carprobotus edulis* Hottentot's fig, ghaukum, rankvy	Ikhambi-lamabulawo, umgongozi, Igcuthuma Unomatyumtyum, igcukuma	Leaves	Various diseases, diarrhoea	Allergies, diabetes, sore throats (Hutchings *et al*., 1996). Juice from pounded leaves as gargle for sore throats, thrush, diphtheria, treat digestive troubles, diarrhea & dysentery (Watt & Breyer-Brandwijk, 1962).
MYRTACEAE *Psidium guajava* L. Guava	Ugwava (X,Z)	Leaves	Leaves boiled for alone or mixed with other plants for diarrhea.	Infusion of leaves for bloody diarrhea (Hutchings *et al.*, 1996). Roots for veneral disease by Vhavenda (Mabogo,

				1990) The Ethnobotany of the Vhavenda. Unpublished Master of Science Thesis, University of Pretoria).
OLEACEAE *Olea europaea* subsp. *Africana* Wild Olive	Uzintlwa (X)	Fruit	Infusion for diarrhoea and bloody Stool	Leaves for urinary and bladder infections (Roberts, 1983; Pooley, 1993). Immature fruits as astringents against diarrhea (Iwu, 1993).
POLYGONACEAE *Rumex obtusifolius* Dock	Idololenkonyane (X,Z)	Leaves	Leaf extract for diarrhea	Infertility in women (Watt & Breyer-Brandwijk, 1962). Leaf decoction for worms (Watt & Breyer-Brandwijk, 1962). Scabies, powdered root as gargle for laryngitis
ROSACEAE *Prunus africana (*Hook.f.) Kalkman Red Stinkwood, Bitter Almond, Peach	Umkhakhazi (X), Umkakase (X)	Roots	Root of peach + bark of blackwood + leaves of guava + roots of umswaninge for diarrhoea, abdominal ailments.	The bark extracts have become popular in Europe for the treatment of benign prostate hypertrophy (Van Wyk *et al.* 1997)
RUBIACEAE *Pentanisia prunelloides*	Icishamlilo, Icimamilo	Roots, leaves,	Boil grated dried bulb,	A range of ailments, root

(Klotzsch ex Eckl. & Zeyh) Walp Broad-leaved Pentanisia	(X,Z)	bulb	a spoon taken orally to stop vomiting, diarrhoea in children. For adult, a wine shot 3 times daily. Expose face to steam from boiling herb fro pimples. Rub leaves to soothe swollen body.	as enema for stomach pain (Hutchings *et al.*, 1996). Hemorrhoids, snakebite, rheumatism (Bryant, 1966; Gerstner, 1941).
RUBICEAE Pavetta – Bride's Bush *Psychotria capensis* (Eckl.) Vatke Black Bird-berry	Isithitibala (Z), UmGono-gono (X)	Fruits	For diarrhoea and vomiting.	Unspecified part for tuberculosis (Batten & Bokelmann, 1966). Leave paste for wound. For gastric complaints and root infusions are taken to cause vomiting (Hutchings *et al.*, 1996).
SAPINDACEAE Atalaya – Krantz Ashes *Hippobromus pauciflorus* (L.f.) Radilk. False Horsewood	Ulwathile (X)	Bark, root, leaves	Diarrhoea, dysentery	Coughs, catarrh related headaches (Bryant, 1966). Eye problems (Watt & Breyer-Brandwijk, 1962).
SCOPHULARIACEAE *Physalis peruviana* Cape gooseberry	Igquzu (X)	Leaves	Leaf edible, stomach disorders.	Leaf infusion as enema to relieve abdominal ailment in children (Watt & Breyer-Brandwijk, 1962). Treat high blood pressure,

				diabetes (Watt & Breyer-Brandwijk, 1962).
SOLANACEAE *Solanum aculeastrum* Dun Apple of Sodom, poison apple, Goat apple	Umthuma (X,Z)	Fruits, roots, leaves.	Fruit decoction orally for haemorrhoids & dysentery, fruit as enema for diarrhoea.	Fruit pulp as enema. Rheumatism, ringworm in cattle (Pooley, 1993). Root & leaves for coughs, fever, sore throats, colic, indigestion, abdominal pain, venereal diseases (Watt & Breyer-Brandwijk, 1962; Kokwaro, 1976).
VERBENACEAE *Clerodendrum glabrum* E.mey = *C. rehmannii* Cat's Whiskers or Verbena Tree or Tinderwood	Umqangazani Uqangazana (X), iNunkisiqaqa(X) Umqangazane	Leaves	Bloody stool, chest infections.	Snakebite (Roberts, 1990). Leaf infusion for intestinal parasites (Watt & Breyer-Brandwijk, 1962). Leaf + root of *Cymbopogon marginata* (Steud.) for roundworms, threadworms, cough, fevers (Hutchings *et al.*, 1996).

KEY: X = Xhosa; Z = Zulu.

CHAPTER 5

PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL SCREENING OF CRUDE EXTRACTS OF SOME HERBAL PLANTS OF THE EASTERN CAPE PROVINCE, SOUTH AFRICA

5.0. Abstract

This study was conducted to identify and evaluate the antibacterial activity of indigenous herbal remedies for diarrhoea and associated stomach ailments in rural areas of O.R. Tambo district municipality in the Eastern Cape Province, South Africa. The crude aqueous, methanolic and acetone extracts of twelve selected medicinal plants were screened against clinical isolates of *Salmonella isangi, S. typhi, S. Typhimurium, Shigella flexneri* type 1b and *Sh. sonnei* phase II. The herbs which were selected based on high frequency of prescriptions by the traditional healers were *Acacia mearnsii, Aloe arborescens*, *Aloe striata, Cyathula uncinulata, Eucomis autumnalis (Mill.) Chitt., E. comosa (Houtt.) Wehrh. Hermbstaedtia odorata, Hydnora africana, Hypoxis latifolia, Pelargonium sidoides, Psidium guajava, Scilla natalensis.* A qualitative phytochemical screening of the plants extracts was by thin layer chromatography. Plants extracts were screened for antibacterial activity using two types of bioassays: serial dilution microplate technique and bioautography. The preliminary screening revealed that most of the tested organisms were sensitive to the crude acetone extracts with MIC values ranging from 0.078–2.5 mg/mL. Acetone and methanolic extracts of *Aloe striata, Cyathula uncinulata, E. autumnalis* and *P. guajava* were more active against enteropathogens matching up to gentamicin in

terms of the MIC. Water extracts showed minimal or no activity in most cases. The presence of terpenoid and flavonoids in some herbs were inferred from the TLC fingerprint. The bioautograms revealed different fractions of the extract mainly responsible for the antibacterial activity. *S. enteric serovar* Typhi was the most sensitive of the tested organisms to the crude extracts. This preliminary study has revealed the antibacterial activities of some of the selected herbs used in the treatment of diarrhoea and related diseases and corroborates assertions by traditional healers on their efficacies.

Key words: Herbal remedy; Indigenous; Diarrhoea; Antibacterial

5.1. Introduction

Diarrhoea is a disease which remains a problem worldwide, leading to nearly 19% of the 10 million under-5 deaths that occurred in the world in 2004 (World Health Statistics, 2006). Studies have shown that diarrhoea, HIV/AIDS, water and food-borne diseases account for a high percentage of morbidity and mortality in different age groups but mostly in children 0-5 years (WHO, 2003; Bryce *et al*., 2005). Every individual has at least an episode or more of diarrhoea in a lifetime. Children may have up to 10 episodes before their fifth birthday. Alarmingly, a child dies every 15 seconds from diarrhoea, caused largely by unsafe water and inadequate sanitation (WHO, 2005). Despite advancement in technology in the industrialized nations, diarrhoea continues to be an important cause of morbidity and incurs substantial health-care costs (World

Gastroenterology Organisation, 2008). The greatest burden of the disease however, is in sub-Saharan Africa and Asia (Morris *et al.*, 2003).

Treatment failures of enteric diseases, particularly, the emerging multidrug resistant enteric bacteria is a big challenge. Multiple antibiotic resistances is on the increase among clinical isolates of bacteria (Bisi-Johnson *et al.*, 2005; Obi *et al.*, 2007) and the emergence of resistance to the 3rd and 4th generation beta-lactam drugs has made therapy more complicated. The burden of resistance to extended-spectrum cephalosporin and other beta-lactam drugs among the *Enterobacteriaceae* is enormous both in the hospital and community (Bradford, 2001). Pharmaceutical industries have produced a number of new drugs in the last three decades. Currently, an estimated 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Fabricant and Farnsworth, 2001). Some of these include Ephedrine (bronchodilator) derived from *Ephedra sinica,* Quinine (antimalarial) from *Cinchona ledgerian* (El-Baz, 2007) and recently is the antimalarial compound Artemisin, derived from *Artemisia annua* L (Camacho *et al.*, 2000). Despite these big strides, resistance to antimicrobials by microorganisms is still on the increase due mainly to the remarkable genetic plasticity of the microorganisms (Kunin, 2009), inappropriate use: high selective pressures of use or under use through inaccessibility, poor quality drugs, inadequate dosing, poor adherence, and the increased mobility of the world population (Okeke *et al.*, 2007). In addition, the incorporation of antimicrobials as growth promoting additives in animal feed is also a contributory

factor to the emergence of drug-resistant bacteria (WHO, 2005; Foley and Lynne, 2007). The sub-therapeutic use leads to bacterial exposure to sublethal concentration of drugs over a period of time leading to selection of resistance strains (Burt and Reinders, 2003). The rate at which new drugs are developed is way behind compared to the changing virulence and drug resistant patterns of microbes. According to Kunin (2009) review of the book by Owen and Lautenbach (2008), antimicrobial resistance is the inevitable result of Darwinian evolution — natural selection and survival of the fittest. While there is a continuous modification of strategies by microbes in the 'no victor no vanquish' fight for survival, drug resistance and development according to Ridley (2001) takes 10 to 15 years and hence the quest for new drugs should be a continuous process (Hostettmann *et al.,* 2000).

Medicinal plants have been acknowledged as potential sources of new compounds of therapeutic value and as sources of lead compounds for drug design and development (Newman *et al.,* 2003). The use of traditional herbs in the treatment of various ailments has been as long as the history of man even though the mechanisms of actions of many herbs are unknown. The Fossil date of plant use as medicines is put at approximately 60, 000 years ago (Fabricant and Farnsworth, 2001). Traditional medicine has been reaffirmed by the WHO (2007) as one of the surest means to achieve total health care coverage of the world's population. Various studies have documented the use of medicinal plants in various parts of the world including developed countries (Van Wyk *et al.* 1997;

Aschwanden, 2001; WHO, 2008) and more than 80% of the populace are reportedly dependent on traditional remedies (WHO, 1978). In South Africa, a sizeable number of both the rural and urban dwellers rely on traditional medicine for their primary health care (Fennell *et al*., 2004). In most cases, a plant is often used for a variety of ailments which may then suggest a concerted or wholesome therapy. This postulation is corroborated by the review of Gurib-Fakim (2006) where medicinal plants are said to be made of mixture of chemical components which may act individually, additively or synergistically. Some of the documented use of plants in stomach related ailments includes that of Iwu (1993) in which immature fruits of *Olea europaea* subsp*. Africana* (Wild Olive), was reported to be used as astringents against diarrhoea. *Pentanisia prunelloides* (Klotzsch ex Eckl. & Zeyh) Walp is being used for a range of ailments and the root serves as enema for stomach pain (Hutchings *et al.,* 1996). However, many herbs are yet to be explored scientifically and moreover, the need to find a lasting solution to the problem of infectious diseases with lingering treatment failures necessitated further exploration of natural products to uncover new grounds in drug production. This study screened and evaluated the antibacterial activity of selected medicinal plants used in ethnomedicine in the Oliver R. Tambo municipality of Eastern Cape South Africa against some enteropathogenic bacteria.

5.2. Materials and Methods

All reagents and chemical used in this study were of technical grade and were obtained from Merck Pty Ltd, and Biorad Pty Ltd, South Africa except otherwise stated. Standard aseptic techniques were observed in all cases.

5.2.1. Plant material

Various plant parts used in the treatment of diarrhoea and related diseases in Oliver R. Tambo District municipality in the Eastern Cape province of South Africa were collected over a period of 12 months (June 2008 to July 2009). These were corms, bulb, tuber, leaves and bark of *Acacia mearnsii, Aloe arborescens*, *Aloe striata, Cyathula uncinulata, Eucomis autumnalis (Mill.)* Chitt*., E. comosa (Houtt.)* Wehrh*. Hermbstaedtia odorata, Hydnora africana, Hypoxis latifolia, Pelargonium sidoides, Psidium guajava, Scilla natalensis.* The common names and ethnomedical usage of the selected plants are listed in Table 5.1.

5.2.2.1. Preparation and extraction

Plant parts were washed with distilled water, air-dried and milled into a fine powder with a Jankel and künkel Model A10 mill. Fifty gram portion of ground dried material macerated in 500 ml of each of acetone, methanol and water on an orbital shaker for 2-3 h. The samples were suction-filtered through a Whatman No 1 filter paper. The filtrate was concentrated at 45°C using a Rotavapor (Eyela N-1100, Rikakikai, China). The reduced extract was transferred to a pre-weighed glass vial and sunction-dried at room temperature with a vacuum pump (ULVAC KIKO, Tokyo). Working stock solutions of extracts were

Table 5.1. Names, plant parts and Traditional usage of herbs investigated

Plant Name	Common name	Plant part	Traditional usage	References
Acacia mearnsii De Wild. Family: Fabaceae	Blackwood Black Wattle	Bark	Diarrhoea, dysentery, sore throat, coughs, children fever, tooth ache	Hutchings *et al.*, 1996
Aloe arborescens Family: Asphodelaceae	Aloe	Leaves	Vomiting, Skin ailments, diarrhoea, urinary complaints, rheumatism, tuberculosis	Castleman, 1991; Hutchings *et al.*, 1996.
Aloe striata Family: Asphodelaceae	Aloe	Leaves	Treatment of constipation	Haller, 1990
Cyathula uncinulata (Schrad.) Schinz Family: Amaranthaceae	NA	Leaves	Antidiarrhoea, philter or medicine for love	Tomani *et al.*, 2008; Yamada, 1999.
Eucomis autumnalis (Mill.) Chitt. Family:Hyacinthaceae	Common Pineapple Flower	Bulb	Decoctions of bulb and roots for colic, flatulence	Cunningham, 1993.
Eucomis comosa (Houtt.) Wehrh. Family:Hyacinthaceae	Pineapple lily	Bulb	Help teething in children and to treat rheumatism	Watt and Breyer-Brandwijk ,1962
Hermbstaedtia odorata Wild Cockscomb Family: Amaranthaceae	Rooi-aarbossie	Leaves	Cleansing stomach wash alone or with *Acaccia xanthophloea* and *Cappa*	Hutchings *et al.*, 1996
Hydnora africana Thunb. Family: Hydnoraceae	Warty Jackal Food, Jakkalskos Kanip	Tuber	Diarrhoea, plant dried ground raw for dysentery, amenorrhoea, swollen glands	Watt and Breyer-Brandwijk ,1962

Hypoxis latifolia Hook. *Family:* Hypoxidaceae	African potato	Tuber	Headaches, dizziness, mental disorders, to treat cancers, inflammation, HIV, diarrhoea	Singh, 1999; van Wyk, 2000
Pelargonium sidoides Family: Geraniaceae	Rose-scented Pelargonium	Root	Gonorrhoea, diarrhoea, dysentery, root decoction severe diarrhoea, stomach ailment in children	Hutchings *et al.*, 1996
Psidium guajava Family: Myrtaceae	Guava	Leaves	Leaves used for diarrohea, Infusion of leaves for bloody diarrhoea, infusion as enema for severe diarrhoea	Hutchings *et al.*, 1996
Scilla nervosa (Burch.) Jessop Family:Hyacinthaceae	White Scilla	Corms	Rheumatic fever, dysentery. All purpose herb.	Rood, 1994; Silayo *et al.*, 1999

Key: NA= Not available

obtained by re-dissolving in acetone or methanol as the case may be to yield 10 mg/ml solutions. Plant materials were kept in air-tight containers while extracts were kept at 4°C in the dark.

5.2.3. TLC analysis of extracts

Thin layer chromatography (TLC) was used to determine the composition of extracts. The TLC plates were prepared in duplicate and developed in different solvent systems according to Eloff (1998): Benzene: ethanol: ammonium hydroxide (BEA) (36:4:0.4); Ethylacetate: methanol: water (EMW) (40:5.4:4);

Chloroform: ethylacetate: formic acid (CEF) (20:16:4). An aliquot of 10 µℓ of extract was separated by TLC (Merck, Kieselgel 60 F_{254}) in a closed, saturated TLC tank. Chromatograms were visualized under visible and ultraviolet light (254 nm and 360 nm, Camac Universal UV lamp TL-600). One TLC plate per solvent system was then sprayed with Vanillin-sulphuric acid (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) for the detection of higher alcohols, phenols, steroids and essential oils (Stahl, 1988). The plates were heated at 105°C until the colours of chromatograms were completely developed.

For the qualitative evaluation of a given substance, the R_f value (retention factor) on TLC was used as the parameter for comparison. R_f values of substances = ratio of distance start – substance zone to distance start – solvent front and specific chemical reactions. An hypothetical TLC plate showing this is seen in figure 5.1.

$$R_f \text{ values of substances} = \frac{\text{distance starting line – middle of spot}}{\text{distance starting line – solvent front}} = \frac{b}{a}$$

A further qualitative assignment is by the visualisation of native fluorescence of separated substances which is excited by UV (Ultraviolet) light at 254 nm and 366 nm but mostly long-wave UV.

5.2.4. Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) values of plant extracts against the enteric pathogens were determined in 96 well microtiter plates (Eloff, 1998). The test organisms used were *Escherichia coli, Enterococcus faecalis, Pseudomonas auruginosa, Staphylococcus aureus, S. typhi, S. enterica* serovar

Typhimurium, *Shigella flexneri* type 1b and *Sh. sonnei* phase II. A McFarland No1 standard suspension of bacteria inoculum was prepared in Mueller-Hinton Broth. After filling each well with 100 µL sterile distilled water and a series of two-fold dilutions of extract (10 mg/mL), 100 µL of the inoculum was added to the well to make a final volume of 300 µl. Plates were incubated at 37°C for 18 h. An hour before the end of incubation, 40 µL of 0.2 mg/mL INT (*p*-iodonitrotetrazolium salt) solution was added to each well. The lowest concentration where growth was inhibited was recorded as the minimum inhibitory concentration (MIC). This was indicated by a clear well after incubation with INT as against pink colouration.

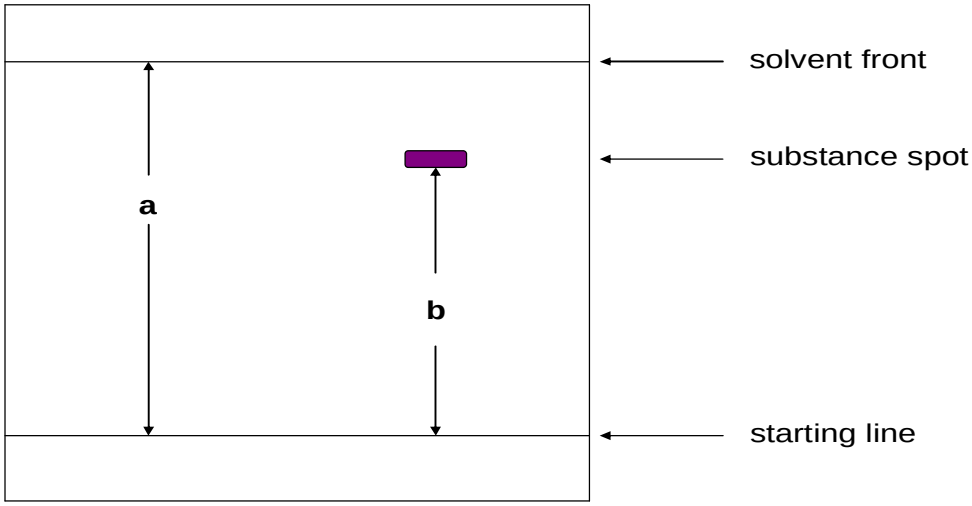


Figure 5.1. A hypothetical TLC plate showing parameters for R_f value

5.2.5. Bioautographic assay

5.2.5.1. Preparation of TLC plates for bioautography

The bioautography procedure as described by Begue and Kline (1972) was used to identify bioactive chromatograms of plant extracts. The duplicate of TLC

plates prepared in section 2.3 were dried overnight under a stream of air to remove residual TLC solvents which may be harmful to bacteria.

5.2.5.2. Preparation of bacteria

A 10 ml of overnight broth culture of test bacteria in Mueller Hinton broth (Merck) was centrifuged at 5300 x g for 20 minutes. The supernatant was discarded and the pellet was re-suspended in 2– 4 ml of fresh broth.

5.2.5.3. Bioassay method

The dried chromatographic plates were sprayed with the test bacteria until they were completely wet. The plates were incubated overnight at 37°C in a clean chamber at 100% relative humidity. After overnight incubation, plates were sprayed with a 2 mg/mL solution of INT (*p*-iodonitrotetrazolium violet, Sigma Chemicals). Plates were returned into incubator and monitored for colour development. Inhibition of growth was indicated by clear or yellow zones on chromatogram.

5.3. Results

Acetone and methanol extracts of plants yielded upon separation more compounds than the aqueous extracts; hence, water was not a good eluting solvent. This is similar to the observation of Eloff *et al.* (2005). The chromatograms of most extracts were best eluted by the medium polar (CEF) and polar extractants (EMW) separating compounds in extracts into bands based

on polarities. The TLC fingerprints of the different chromatograms with the various solvent systems are shown in figures 5.2, 5.3, 5.4

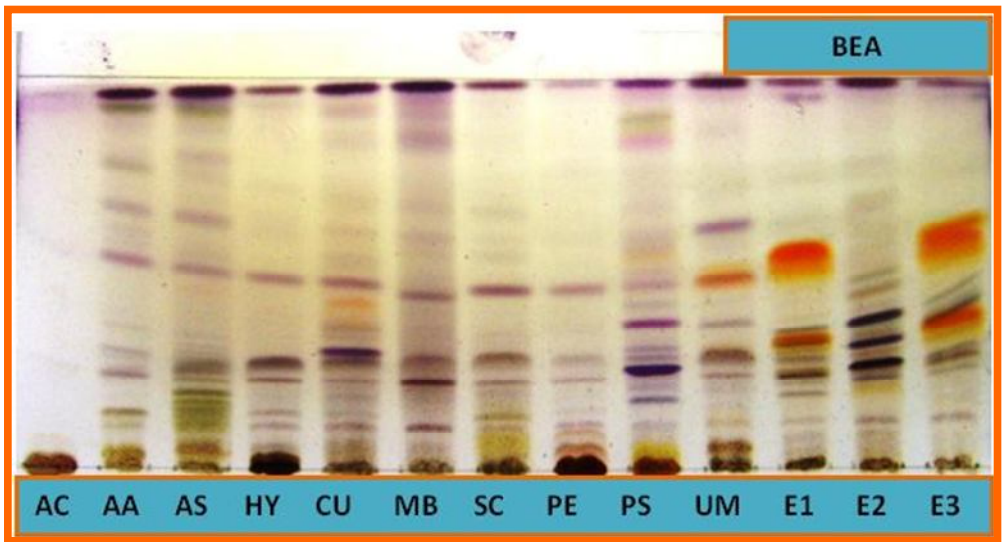


Figure 5. 2. TLC separation of components of the different plant extracts with BEA as eluent and vanillin-sulphuric acid spray reagent. For name of extracts lanes from left to right see list of abbreviations.

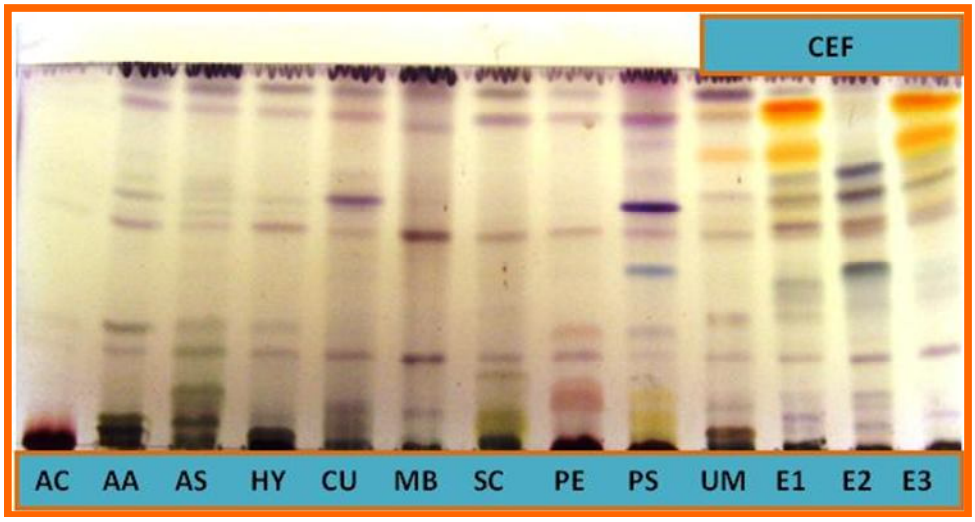


Figure 5. 3. TLC separation of components of the different plant extracts with CEF as eluent and vanillin-sulphuric acid spray reagent. For name of extracts lanes from left to right see list of abbreviations.

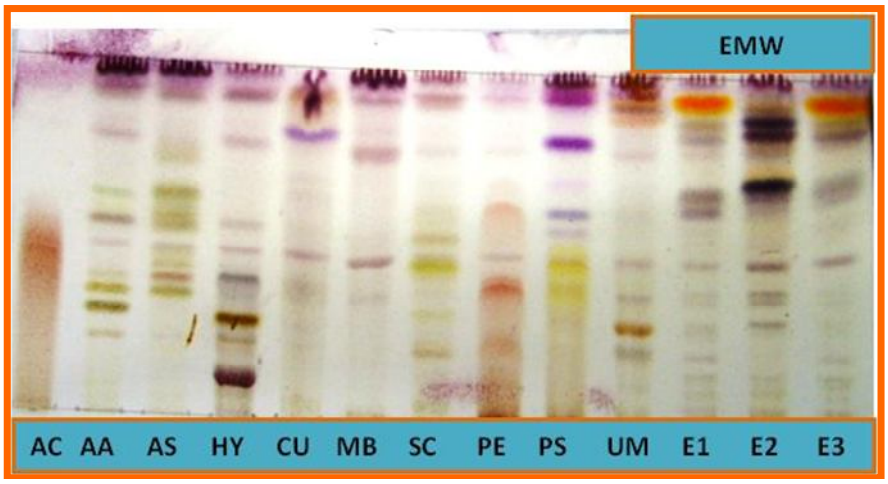


Figure 5. 4. TLC separation of components of the different plant extracts with EMW as eluent and vanillin-sulphuric acid spray reagent. For name of extracts lanes from left to right see list of abbreviations.

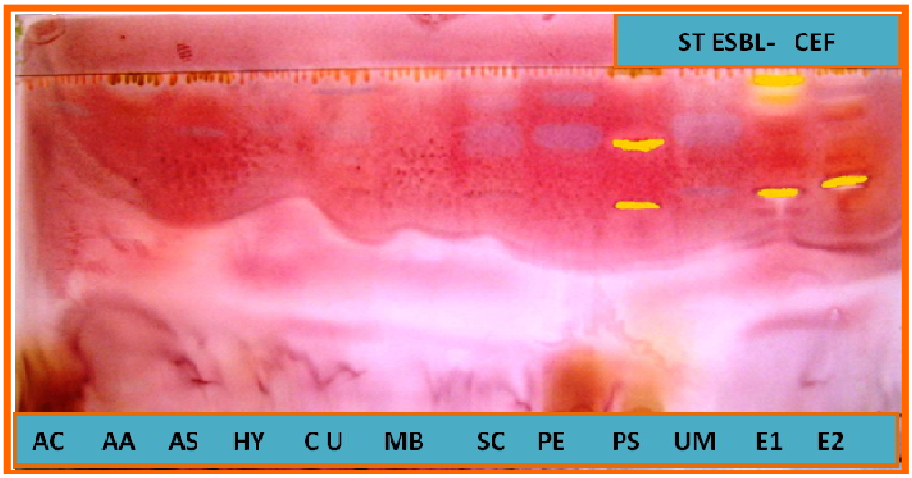


Figure 5.5. Bioautograms of components of the different plant extracts with CEF as eluent sprayed with S. Typhimurium. For name of extracts lanes from left to right see list of abbreviations.

The TLC plates as captured under short and long-wave UV were as shown in figures 5.6A and 5.6B respectively. The characteristic green and blue fluorescence in lanes 5 to 8 and 10 under long-wave UV light (366 nm)

presumptively indicated presence of flavonoids according to the TLC evaluation scheme of Wagner and Bladt (1996), whereas spots of quenching of the TLC

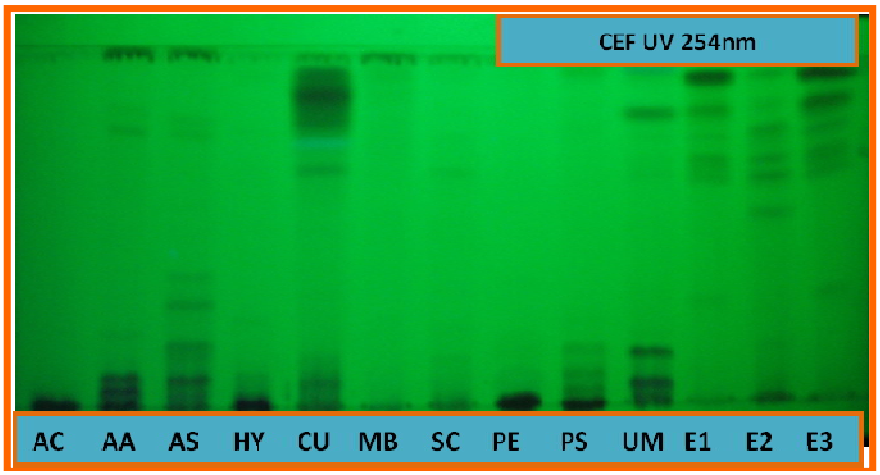


Figure 5. 6A. TLC separation of components of the different plant extracts with CEF as eluent viewed under UV 254 nm before vanillin-sulphuric acid reagent spray and heat treatment. Lanes from left to right: AC-*Acacia mearnsii*, AA, AS, HY, CU, MB, SC, PE, PS, UM, E1, E2, E3.

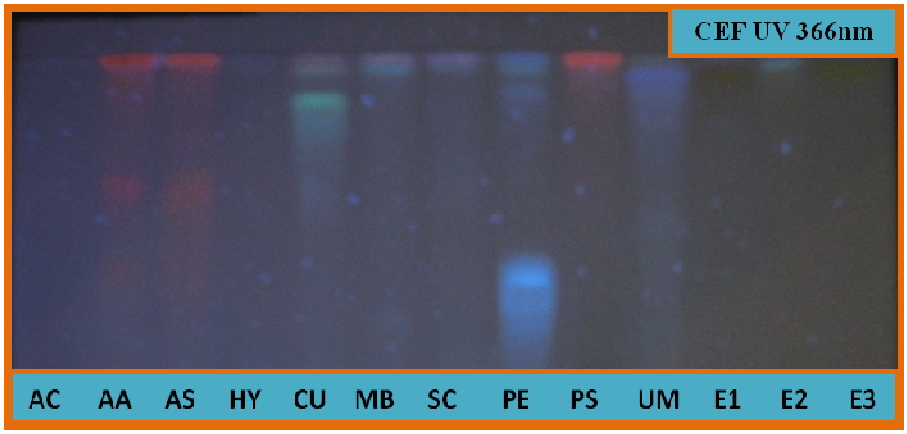


Figure 5. 6B. TLC separation of components of the different plant extracts with CEF as eluent viewed under UV 366 nm before vanillin-sulphuric acid reagent spray and heat treatment. Lanes from left to right: AC-*Acacia mearnsii*, AA, AS, HY, CU, MB, SC, PE, PS, UM, E1, E2, E3.

fluorophor appeared as dark zones against light green fluorescent background with short-wavelength UV light (254 nm). These evaluation methods were further augmented with the non-specific post-chromatographic charring

detection technique (spraying with vanillin-sulphuric acid and heat treatment) (Jork *et al.*, 1989). Based on the chosen visualization spray reagent, most solvents extracted at least seven compounds with the exception of extract of *Acacia mearnsii.* The R_f values of major compounds eluted with CEF (Figure 5.3) were AA (0.59, 0.67, 0.89), AS (0.57, 0.89), HY (0.59, 0.88), CU (0.26, 0.65, 0.86), MB (0.18, 0.48, 0.84), SC (0.18, 0.24, 0.48, 0.86), PE (0.26, 0.30, 0.57, 0.88), PS (0.18, 0.47, 0.57, 0.64, 0.88), UM (0.18, 0.56, 0.77, 0.84, 0.88), E1 and E3 (0.24, 0.59, 0.65, 0.71, 0.79, 0.89), E2 (0.26, 0.50, 0.60, 0.68, 0.71).

The bioautography results revealed different fractions of the extracts which were responsible for the antibacterial activity. EA, EC and *P. guajava* gave one or two major bands of bioactive compounds (Figure 5.5). There were two major antibacterial compounds from PG with R_f values of 0.64 and 0.79, two from EA with R_f values of 0.65 and 0.88, and one from EC with R_f values of 0.68 which inhibited the growth of *S. enterica* serovar Typhimurium with CEF used as the solvent system. Active antibacterial compound was shown as clear or yellowish spot of inhibition of the growth of test organism by bioautogram. The pinkish contrasting area of bacterial growth was indicative of non-cleavage of the tetrazolium salt to yield the pinkish or purplish formazan product seen in the background (Begue and Kline, 1972). Little or no correlation existed between the MIC values and bioautography of some plant extracts because many of the components extracted did not show area of antibacterial activity, even though there were several chromatograms shown with the chemical detection. For

instance *A. arborescens* and *A. striata* had impressive MIC values but no bioactive bioautogram was shown for these plant species. This observation may be attributable to volatility, decomposition or instability of bioactive components during the course of the bioassay. For the extracts showing bioactive compounds (PS, E1 and E2) the antibacterial activity resided mostly in non-polar compounds.

The MIC values of the plants extracts ranged from 0.039 mg/mL and 2.5 mg/mL after 24 h of incubation. The average MIC values vary for the different bacterial species with the lowest in *S. typhi* (Table 5.2.). Of all the crude plant extracts evaluated, *A. arborescence, C. uncinulata, E autumnalis* and *P. guajava* showed considerable antibacterial activities having minimal MIC between 0.039 mg/mL and 0.078 mg/mL (red highlights, Table 5.2.) which were comparable to that of the reference antibiotic (gentamicin).

5.4. Discussion

It is a known fact that non polar bioactive compounds may not be extracted with water, however in traditional practice, water is used as extractant in most preparations hence water was also included as extraction solvent for the selected plants. However, water was not a good eluting solvent compared with acetone or methanol which yielded upon separation more compounds than the aqueous extracts. This is similar to the observation of Eloff *et al.* (2005). Acetone and methanol extract of these plants also possessed better bioactivity than the aqueous extracts. The very limited activity of the aqueous extracts agreed with

Table 5.2. Average MIC values of plant extracts at 2 h and 24 h

BACTERIA	PLANT EXTRACTS AND ANTIBIOTIC CODES												
CODES	AC	AA	AS	E1	E2	HY	IQ	MB	SC	PE	PS	UM	
EC 2H	1.25	2.5	2.5	0.625	2.5	2.5	2.5	2.5	2.5	1.25	0.156	2.5	0.039
EC 24H	1.25	0.625	0.625	0.312	0.625	2.5	1.25	2.5	2.5	1.25	0.312	1.25	0.078
EF 2H	0.625	1.25	2.5	0.312	2.5	2.5	1.25	2.5	2.5	0.312	0.078	2.5	0.625
EF 24H	0.625	0.039	0.039	0.156	0.078	2.5	0.625	2.5	0.312	0.625	0.156	0.312	0.625
PA 2H	0.312	1.25	1.25	0.312	0.312	2.5	0.312	1.25	0.078	0.078	0.039	0.078	2.5
PA 24H	1.25	0.312	0.625	0.312	0.625	1.25	1.25	2.5	0.078	1.25	0.312	1.25	2.5
SA 2H	0.312	2.5	2.5	0.078	0.312	1.25	0.625	2.5	2.5	0.156	0.078	2.5	0.078
SA 24H	0.312	0.018	0.018	0.312	0.156	0.312	0.156	0.078	0.156	0.312	0.156	0.078	0.078
SI 2H	1.25	0.156	0.312	0.156	0.312	2.5	0.625	1.25	1.25	1.25	0.312	1.25	0.625
SI 24H	1.25	1.25	0.625	1.25	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
St 2H	0.039	0.078	0.312	0.078	0.078	0.625	0.625	1.25	0.312	0.312	0.156	0.625	0.156
St 24H	0.312	0.156	1.25	0.625	0.312	2.5	1.25	2.5	1.25	0.312	0.312	1.25	1.25
STE- 2H	1.25	2.5	2.5	0.156	0.625	2.5	1.25	2.5	1.25	1.25	0.312	1.25	2.5
STE- 24H	1.25	0.078	0.078	0.156	0.625	1.25	1.25	2.5	0.625	1.25	0.312	0.625	2.5
STE+ 2H	1.25	1.25	2.5	0.156	0.312	2.5	0.625	1.25	1.25	1.25	0.078	1.25	0.078
STE+ 24H	1.25	0.078	0.312	0.312	0.625	2.5	1.25	2.5	1.25	1.25	0.312	0.625	0.156
SHF 2H	0.312	0.078	0.156	0.625	0.625	0.312	0.625	1.25	1.25	0.625	0.078	1.25	0.078
SHF 24H	0.625	0.018	0.078	0.078	0.312	1.25	1.25	2.5	0.625	0.625	0.312	0.312	0.078
SHS 2H	0.625	0.156	0.312	0.312	1.25	2.5	0.625	1.25	2.5	1.25	0.156	2.5	0.156
SHS 24H	0.625	0.039	0.039	0.039	0.312	1.25	0.312	1.25	0.625	0.625	0.312	0.312	0.156

Legend 1

EC 2H	*Escherichia coli* at 2h
EC 24H	*E. coli* at 24h
EF 2H	*Enterococcus faecalis* at 2h
EF 24H	*E. faecalis* at 24h
PA 2H	*P. aeruginosa* at 2h
PA 24H	*P. aeruginos* at 24h
SA 2H	*Staphylococcus aureus* at 2h
SA 24H	*S. aureus* at 24h
SI 2H	*Salmonella isangi* at 2h
SI 24H	*S .isangi* at 24h
St 2H	*S. typhi* at 2h
St 24H	*S. typhi* at 24h
STE- 2H	*S. Typhimurium* ESBL-ve at 2h
STE- 24H	*S. Typhimurium* ESBL-ve at 24h
STE+ 2H	*S. Typhimurium* ESBL+ve at 2h
STE+ 24H	*S. Typhimurium* ESBL+ve at 24h
SHF 2H	*Shigella flexneri* at 2h
SHF 24H	*Sh. flexneri* at 24h
SHS 2H	*Sh. sonnei* at 2h
SHS 24H	*Sh. sonnei* at 24h

Legend 2

AC	Extract of *Acacia mearnsii*
AA	Extract of *Aloe arborescence*
AS	Extract of *Aloe striata*
CU	Extract of *Cyathula uncunilata*
E1	Extract of *Eucomis autumnalis* (A)
E2	Extract of *Eucomis comosa*
E3	Extract of *Eucomis autumnalis* (B)
HY	Extract of *Hypoxis latifolia*
MB	Extract of *Hermbstaedtia odorata*
SC	Extract of *Scilla nervosa*
PE	Extract of *Pelargonium sidoides*
PS	Extract of *Psidium guajava*
UM	Extract of *Hydnora Africana*
GENT	Gentamycin

the findings of Alanis *et al.* (2005) where aqueous extracts activity was minimal compared with other extracts. The separation and qualitative analysis of crude extracts of plants were based on the microanalytical separation TLC method. The principle of TLC as an analytical tool is known for more than 100 years now and was enhanced by the pioneering work of Egon Stahl (1988). For the qualitative evaluation by TLC, the parameter used is not only the the *R*f value (retention factor) but also the 100fold value h*R*f. The *R*f values are between 0 and 1 but best between 0.1 and 0.8 (i.e. 10 – 80 for h*R*f). The *R*f values of most of the separated compounds ranged between 0.18 and 0.8 except for a few compounds, hence these values were within the stipulated best Rf value range (Nagel, n.d.).

The presence of compounds such as flavonoids and triterpenoids as indicated by the TLC plates are in accordance with some other previous studies (Heller and Tamm, 1981; Della Loggia *et al.*, 1989; Amschler *et al.,* 1996; Koorbanally *et al.,* 2006). A total of seven homoisoflavonoids of varying sub-classes including a novel benzylidene type and two spirocyclic nortriterpenoids were isolated from three species of *Eucomis* by Koorbanally *et al.,* (2006). The occurrence of homoisoflavonoids (homoisoflavanones or 3-benzyl-4-chromanones) is said to be restricted largely to the Hyacinthaceae. Several reports on the biological activity of homoisoflavonoids indicated their anti-inflammatory, antibacterial, antihistaminic, antimutagenic and angioprotective qualities, and value as potent phosphodiesterase inhibitors (Heller and Tamm,

1981; Della Loggia *et al.*, 1989; Amschler *et al.,* 1996). *Aloe* species are known to elaborate various phenolic compounds, including anthrone-C-glycosides, phenylpyrone derivatives and chromones (Reynolds, 1985). Of the root samples of 172 species of *Aloe*, aloesaponarin I, aloesaponarin II and laccaic acid o-methyl ester, together with their corresponding pre-anthraquinones were detected in 129 species (van wyk *et al*., 1985). Furthermore, the study revealed that 1,8 - Dihydroxyanthraquinones (chrysophanol and asphodelin) were in all the tested species except three. *A. striata* Haw was positive for the presence of chrysophanol and asphodelin while *A. arborescens* Mill was positive for the presence of chrysophanol, asphodelin, laccaic acid D-methyl ester and aloesaponol 1 (van wyk *et al*., 1985). The laxative effects of Aloe are said to be due primarily to the 1, 8-dihydroxyanthracene glycosides, aloin A and B (formerly known as barbaloin) (Tyler *et al*., 1988; Tyler, 1994). Aloe emodin, chrysophanol and aloin have been reported from *Aloe ferox* (Kambizi *et al.*, (2004) and these compounds were shown to possess antibacterial activities.

In most cases plant extracts were reportedly more active against Gram-positive (GP) pathogens (Vlietinck *et al.,* 1995), a similar observation was found in this study but in addition, most of the extracts had substantial activity against the selected Gram-negative (GN) enteric bacteria. *P. sidoides* gave a moderate antibacterial activity in particular against *E. faecalis, S. aureus* and *Shigella* species. Similarly, anti-infective properties of the commercially important *Pelargonium* have been investigated (Lalli *et al.,* 2008). *H. latifolia* did not show

good antibacterial activity against most of the tested bacteria and this is in harmony with the findings on *H. decumbens* (Chiappeta *et al.*, 1984). Even though the sterols in *Hypoxis* spp. had been reported to reduce plasma viral loads and stabilized CD4 cell counts in HIV positive patients (Bouic *et al.*, 1999), while an aqueous extract of *Hypoxis hemerocallidea (H. rooperi)* (62.5 µg/ml) inhibited some microorganisms, the inhibitory concentration was less potent than ciprofloxacin, the reference antibiotic (Steenkamp *et al.,* 2006).

5.5. Conclusion

This preliminary screening showed that the crude extracts of some of the herbs used in traditional medicine in our area of study have potentials as antibacterial agents. *A. arborescens*, *A. striata, C. uncinulata, E. autumnalis* and *P. guajava* are particularly promising in the context of this study since the activities of the crude extracts compared reasonably with gentamicin. This gives scientific credence to the use of these plants. Further studies on the active compounds and evaluation of the safety of the plants and or their components are warranted. The need for continued screening of indigenous plants is inevitable because of the dynamic nature of infectious agents and their continued development of resistance over time.

5.6. References

Amschler, G., Frahm, A.W., Hatzelmann, A., Killian, U., Müller-Doblies, D., Müller-Doblies, U., 1996. Constituents of Veltheimia viridifolia; I. Homoisoflavanones of the bulbs. Planta Medica, 62(6): 534-539.

Aschwanden, C., 2001. Herbs for health, but how safe are they? Bull World Health Org. 79(7):691-692. http://www.who.int/bulletin/archives/79(7)691.pdf

Begue, W.J., Kline, R.M., 1972. The use of tetrazolium salts in bioautographic procedures. J. Chromatography, 64: 182 – 184.

Bisi-Johnson, M.A., Kolawole, D.O., Shittu, A.O., 2005. Epidemiological analysis of clinical isolates of *Staphylococcus aureus* in Ile-Ife, Nigeria. Pakistan J. Biol. Sci., 8 (7): 1016-1020.

Bouic, P.J.D., Clark, A., Lamprecht, J., 1999. The Effects of B-Sitosterol (BSS) and BSitosterol Glucoside (BSSG) Mixture on Selected Immune Parameters of Marathon Runners: Inhibition of Post Marathon Immune Suppression and Inflammation. Int. J. Sports Med., 20:258-262.

Bradford, P.A., 2001. Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev., 14, 933–951.

Bryant, A.T., 1996. Zulu Medicine-men. C. Struik, Cape Town (originally published in 1909 in the Annals of the Natal Museum.

Bryce, J., Boschi-Pinto, C., Shibuya, K., Black, R.E., 2005. WHO Child Health Epidemiology Reference Group. WHO estimates of the causes of death in children. Lancet, 365, 1147–1152.

Burt, S.A., Reinders, R.D., 2003. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Lett. Appl. Microbiol., 36(3):162-167.

Castleman, M., 1991. The healing herbs. Emmaus, PA, Rodale Press. 42–44.

Cunningham, A.B., 1991. Ethics, ethnobiological research and biodervisty. WWF-World Wide Fund for Nature (formerly World Wildlife Fund), Gland, Switzerland.

Della Logia, R., Del Negro, P., Tubaro, A., Barone, G., Parilli, M., 1989. Homoisoflavanones as anti-inflammatory principles of Muscari comosum. Planta Medica, 55: 587–588.

El-Baz, M., 2007. Yesterday's Plants - Today's Medicines [Online] Available at: http://ezinearticles.com/?Yesterdays-Plants---Todays-Medicines&id=484879. Accessed 15 August 2010.

Eloff, J.N., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica, 64, 711-714.

Eloff, J.N., Famakin, J.O., Katarere, D.R.P., 2005. Combretum woodii Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive Bacteria. Afr. J. Biotechnol., 4 (10): 1161-1166.

Fabricant, T.S., Farnsworth, N.R., 2001. The value of plant used in traditional medicine for drug discovery. Env. Health Perspective, 109: 69-75.

Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M., van Staden, J., 2004. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. J. Ethnopharmacol., 94: 205–217.

Foley, S.L., Lynne, A.M., 2007. Food animal-associated Salmonella challenges: Pathogenicity and antimicrobial resistance. J. Anim. Sci.,86:E173-E187. doi: 10.2527/jas.2007-0447.

Gurib-Fakim, A., 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Mol. Aspects Med., 27:1-93.

Haller, J.S., 1990. A drug for all seasons, medical and pharmacological history of aloe. Bull. New York Acad. Med., 66:647–659.

Heller, W., Tamm, C., 1981. Homoisoflavanones and biogenetically related compounds. Forschritte der Chemie Organischer Naturstoffe, 40: 105-152.

Hostettmann, K., Wolfender, J., Terreaux, C., 2001. Modern Screening Techniques for Plant Extracts. Pharmaceutical Biol., 39 (1), 18 – 32.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A., 1996. Zulu Medicinal Plants. Natal University Press, Pietermaritzburg, ISBN 0-86980-923-7.

Iwu, M.M., 1993. Handbook of African Medicinal Plants CRC Press, Inc.

Jork, H., Funk, W., Fischer, W., Winner, H., 1989. DünnschichtChromatographie, VCH Verlagsgesellschaft.

Kambizi, L., Sultana, N., Afolayan, A.J., 2004. Bioactive compounds isolated from *Aloe ferox*: a plant traditionally used for the treatment of sexually transmitted infections in the Eastern Cape, South Africa. Pharmaceutical Biol., 42: 636–639.

Koorbanally, C., Crouch, N.R., Mulholland, D.A., 2006. The phytochemistry and ethnobotany of the southern African genus *Eucomis* (Hyacinthaceae: Hyacinthoideae) Phytochemistry: Adv. Res., 69-85.

Kunin, C.M., 2009. Antimicrobial Resistance: Problem Pathogens and Clinical Countermeasures. N. Engl. J. Med., 360:550-551.

Lalli, J.Y.Y., Van Zyl, R.L., Van Vuuren, S.F., Viljoen, A.M. 2008.*In vitro* biological activities of South African *Pelargonium* (Geraniaceae) species. S. Afr. J. Bot., 74: 153–157.

Morris, S.S., Black, R.E., Tomaskovic, L., 2003. Predicting the distribution of under-five deaths by cause in countries without adequate vital registration systems. Int. J. Epidemiol., 32: 1041-1051 doi: 10.1093/ije/dyg241 pmid: 14681271.

Newman, D.J., Cragg, G.M., Snader, K.M., 2003. Natural Products as Sources of New Drugs over the Period 1981-2002. J. Nat. Prod., 66: 1022-1037.

Obi, C.L., Ramalivhana, J., Momba, M.N.B., Onabolu, B., Igumbor, J.O., Lukoto, M., Mulaudzi, T.B., Bessong, P.O., Jansen van Rensburg, E.L., Green, E., Ndou, S. 2007. Antibiotic resistance profiles and relatedness of enteric bacterial pathogens isolated from HIV/AIDS patients with and without diarrhoea and their household drinking water in rural communities in Limpopo Province South Africa. Afr. J. Biotechnol., 6 (8): 1035-1047.

Okeke, I.N., Aboderin, O.A., Byarugaba, D.K., Ojo, K.K., Opintan, J.A., 2007. Growing problem of multidrug-resistant enteric pathogens in Africa. Emerging Infect. Dis., 13(11) [cited 17 Feb 2011]. Available from http://www.cdc.gov/ EID/content/13/11/1640.htm

Owens, R.C., Lautenbach, E., Editors, 2008. Antimicrobial Resistance: Problem Pathogens and Clinical Countermeasures (Infectious Disease and Therapy. 48.) 492 pp., illustrated. New York, Informa Healthcare, ISBN: 978-0-8247-2941-7.

Reynolds, T., 1985. The compound in Aloe leaves exudates, a review. Bot. J. Linn. Soc., 90: 157-177.

Ridley, R., 2001. Malaria Drug Development. Malaria in Africa. Emerging prevention and control strategies. Report from a symposium held at the 2001

AAAS Annual Meeting Feb 17, 2001, San Fransisco. http://www.aaas.org/international/africa/malaria/ridley.html

Rood, B., 1994. Uit die veldapteek. Tafelberg Publishers, Cape Town.

Silayo, A., Ngadjui, B.T., Abegaz, B.M., 1999. Homoisoflavonoids and stilbenes from the bulbs of *Scilla nervosa* subsp. *rigidifolia.* Phytochem., 52 (5): 947-955.

Stahl, E., 1967. Thin Layer Chromatography. Springer-Verlag, Berlin 1967, Heidenberg, New York. 2nd edition. Reprint 1988.

Steenkamp, V., Gouws, M.C., Gulumian, M., Elgorashi, E.E., Van Staden, J., 2006. Studies on Antibacterial, Anti-inflammatory and Antioxidant Activity of Herbal Remedies Used in the Treatment of Benign Prostatic Hyperplasia and Prostatitis. J. Ethnopharmacol., 103:71-75.

Tomani, J.C., Nkurunziza, J.P., Mukazayire, M.J., Kanezeza, R., Baziruwitonda, E., Duez, P., 2008. Antidiarrhea activity and preliminary phytochemical screening of *Indigofera arrecta, Cyathula uncinulata, Persea americana and Cupressus lusitanica.* 7th Joint Meeting of AFERP, ASP, GA, PSE & SIF on Natural Products with pharmaceutical, nutraceutical, cosmetic and agrochemical interest. 3-8 August 2008. Athenaeum Intercontinental, Athens Greece.

Tyler, V.E., Bradley, L.R., Robbers, J.E., eds., 1988. *Pharmacognosy*, 9th ed. Philadelphia, Lea & Febiger, 1988:62–63.

Tyler, V.E., 1994. Herbs of choice. New York, Pharmaceutical Products Press, 155–157.

Van Wyk, B.E., Van Oudtshoorn, B., Gericke, N., 1997. Medicinal Plants of South Africa [2nd improved impression, 2000]. Briza Publications, Pretoria, ISBN 1-875093-09-5.

Van Wyk, B.E., Yenesew, A., Dagne, E., 1995. Chemotaxonomic survey of Anthraquinones and Pre-anthraquinones in Roots of *Aloe* species. Biochem. System Ecol., 23(3): 267-275.

Vlietinck, A.J., Van Hoof, L., Totte, J., Lasure, A., Vanden Berghe, D., Rwangabo, P.C., Mvukiyumwami, J., 1995. Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. J. Ethnopharmcol., 46, 31-47.

Wagner, H., Bladt, S., Rickl, V., 1996. Plant drug analysis: a thin layer chromatography atlas, 2nd edn. Springer Publications. Springer-Verlag, Berlin, pp. 204.

Watt, J.M., Breyer-Brandwijk, M.G., 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa, 2nd ed. Livingstone, London.

WGO. World Gastroenterology Organisation. 2008. World Gastroenterology Organisation practice guideline: Acute diarrhea. [ONLINE] http://www.omge.org/assets/ downloads/en/pdf/guidelines/01_acutediarrhea.pdf. Accessed 17 Feb 2010.

WHO. World Health Organization. 1978. The Promotion and Development of Traditional Medicine. Technical Report No. 622. Geneva.

WHO. World Health Organization. 2003. Emerging issues in water and infectious disease. Geneva, World Health Organization. 2003. [ONLINE] http://www.who.int/water sanitation health/emerging/en/emerging.pdf. Accessed October 22, 2009.

WHO. World Health Organization. 2005. Health and the Millennium Development Goals. MDGs, Health and Development Policy World Health Organization Publications of the World Health Organization can be obtained from WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

WHO. World Health Organization. 2007. Declaration on Traditional Medicine being an undertaking by Ministers of Health attending the 57th session of the WHO Regional Committee for Africa. [ONLINE] http://www.afro.who.int/trm/declaration_on_traditional_medicine.pdf. Accessed 27 April 2009).

WHO. World Health Organization. 2008. Traditional medicine. Fact sheet N°134. December 2008 http://www.who.int/mediacentre/factsheets/fs134/en/ accessed 21 September 2010.

WHO. World Health Statistics. 2006. 1. Health status indicators. 2. World health. 3.Health services - statistics. 4. Mortality. 5. Life expectancy. 6. Demography. 7. Statistics. [ONLINE] http://www.who.int/whosis/whostat 2006.pdf. World Health Organization. WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

Yamada, T., 1999. A report on the ethnobotany of the Nyindu of the Eastern part of the former Zaire. Afr. Study Monographs, 20(1):1-72.

CHAPTER 6

ISOLATION, CHARACTERIZATION AND STRUCTURAL ELUCIDATION OF BIOACTIVE FRACTIONS OF *CYATHULA UNCINULATA (*Schrad.) Schinz

6.0. Abstract

Cyathula uncinulata (Schrad.) Schinz is used in ethnomedine in various parts of the world. This study isolated and characterized bioactive compounds from *C. uncinulata* (Schrad.) Schinz based on its antibacterial activities from the previous plant screening. Separation of compounds from the bioactive ethyl acetate fractions of the plant was by solvent-solvent fractionation. The structure of the compounds was elucidated by nuclear magnetic resonance spectroscopy and mass spectroscopy methods. The NMR and EI-MS spectra of the isolated compound showed that the compound with formula $C_{22}H_{38}O_7$ and molecular weight 414.5329, has a long aliphatic chain and is said to be made up of sugar and fatty acyl moiety. The MIC of fractions of extracts ranged from 0.39 mg/mL to 2.5 mg/mL, with the lowest MIC observed in ethyl-acetate fraction. The MIC of the selected sub-fraction of the ethyl acetate fractionation (SSC 1) was 0.63 mg/mL. The MIC of the final purified compound was 0.34 mg/mL, indicating considerable bioactivity. This study isolated a glycosylated oleanolic acid from *C. uncinulata* (Schrad.) Schinz. The compound possessed considerable bioactivity in comparison with the crude extract and the control antibiotic. This is an indication that this plant may be a candidate template for new antimicrobial.

Key words: *Cyathula uncinulata,* extract, fraction, MIC, bioactive, compound

6.1. Introduction

Cyathula uncinulata (Schrad.) Schinz is a large genus of sub-shrubs of about 27 species distributed in Asia, Pacific Islands, Africa, and North and South America belonging to the family *Amaranthaceae.* The stems are erect or ascending and leaves are opposite, petiolate with entire margin. Flowers clustered in cymose partial inflorescences, 1-3 in each cluster, hermaphroditic and partly accompanied by sterile ones. The flowers are bracts ovate, membranous and often spiny. The seeds of *C. uncinulata* are oblong or ellipsoid.

In South Africa, *C. uncinulata* (CU) was recently described as one of the flora of 1195 species of the botanically least known regions, the Sneeuberg mountain complex (Eastern Cape). This region where the plant is referred to locally as *Isinama* (Dold and Cocks, 1999); comprises one of the most prominent sections of the Great Escarpment in Southern Africa (Clark *et al.*, 2009). Previous studies on CU have reported its ethnobotanical study (Jeruto *et al*., 2008a), antidiarrheal activity and phytochemical screening (Tomani *et al*., 2008). In the Eastern part of former Zaire, CU is used by men as a philter or medicine for love (Yamada, 1999). Furthermore, an Ethiopian study reported a considerable consensus among traditional healers in the treatment of *Rissaa* (an ailment) by drinking a mixture of CU and leaves of *Croton macrostachyus* crushed fresh and mixed with water (Yineger *et al.,* 2008).

Various reports on the ethnomedicine and ethnobiology of the genus *Cyathula* exist. A phylogenetic analysis found that *Cyathula*, some other members of the families *Amaranthaceae* and *Chenopodiaceae* constituted a monophyletic group and hence supported the call for *Chenopodiaceae* to be merged with *Amaranthaceae* (Ogundipe and Chase, 2009). *Cyathula officinalis* Kuan (COK) was shown to contain polysaccharides which could increase mouse red cell immunity function by strengthening red cell immune adherence and cleanup of circulating immune complex (Li *et al.*, 1999). COK whose root is a commonly-used traditional Chinese herbal medicine with a wide range of pharmacological activities (Pharmacopoeia Commission of People's Republic of China, 2000) has also demonstrated anti-tumor activity. Components of COK were able to suppress the growth of mouse S180 tumor, H22 liver cancer cells (Chen and Liu, 2001). In an antiviral experiment, sulfated derivative of RCP, a kind of polysaccharide extracted from COK was adjudged to express comparable antiviral activity with the commonly used antiviral drug ACV (0.5 mg/mL) at concentrations of 2.0 mg/mL and 4.0 mg/mL.

In their bid to reinvestigate phytoecdysteroids of the *Cyathula* species, Okuzumi and co-investigators (2005) isolated two cyasterone stereoisomers and a known cyasterone reported to possesses antitumor-promoting activity (Takasaki *et al.,* 1999). Several C_{29}-phytoecdysteroids such as cyasterone (Takemoto *et al*., 1967; Slama *et al*., 1993), capitasterone (Takemoto *et al.*, 1968) and makisterone C (Imai *et al*., 1968) possess potent insect molting

hormone activity. In a clinical experiment, a traditional Chinese medicine: concentrated herbal extracts of cooked *Cyathula* and concoction of 9 other ingredients restored ovarian function effectively and promptly in a patient with premature ovarian failure (POF) and 8-year secondary amenorrhea, thus the combination may offer another option for treating infertility in patients with POF (Chao *et al*., 2003). *Cyathula* is a component of some Chinese formulations acclaimed to be therapeutic against endometriosis and have severe blood stasis effects (Dharmananda, 2002).

The shoots of *C. prostrata* (L.) Bl. was reported to be used in India ethnotherapy of dysentery, skin diseases and as an appetizer (Kala 2005). In Kenya, the leaves and roots of *C. schimperiana* non Moq are used as decoction (internal) for malaria, as antidiarrhoea, against fungal infections while the root of *C. cylindrica* Moq roots is used as decoction (internal) for malaria, purgative and as emetic (Jeruto *et al*., 2008b). Among the native Ecuadorian, the Quichua people apply the flowers and shredded leaves of *C. achyranthoides* to dog bites (Lescure *et al.*, 1987) while the Shuar eat the raw young leaves to relieve headaches (Russo, 1992).

Previous studies have isolated bioactive compounds from some members of this Genus. Glycoside and oleanic acids were isolated and tested for their inhibitory activities by Zhou and his co-worker (2005). Fructans which exist as a wide range of oligo- and polysaccharides in many species of bacteria, fungi, and plants were isolated from COK by Chen and Tian (2003). Bioassays showed that

the graminans-type fructan that is comprised of a β-D-fructofuranosyl backbone could inhibit growth of Lewis pulmonary carcinoma implanted in mice (Chen and Tian, 2003). Recently, a new heterocyclic compound, named 5, 5′-diisobutoxy-2, 2′-bifuran was isolated for the first time from COK (Liu *et al.* 2010). From our preliminary screening, *C. uncinulata* was among the plants which gave promising MIC values. However, based on the literature, studies on bioactive compounds from this plant species are very rare. This study explores the active components of *C. uncinulata* and their biological effects.

6.2. Materials and methods

6.2.1. Plant collection

The leaves of *C. uncinulata* were collected in the Eastern Cape province of South Africa. *C. uncinulata was* identified by the Kei Herbarium curator, Dr. Immelman in the Department of Botany, Walter Sisulu University, South Africa where voucher specimen has been deposited. South Africa. The photographic images of CU are shown in Figure 6.1. The plant materials were air-dried at room temperature. Dried plant materials were ground to powder and stored in the dark at room temperature for further analysis.

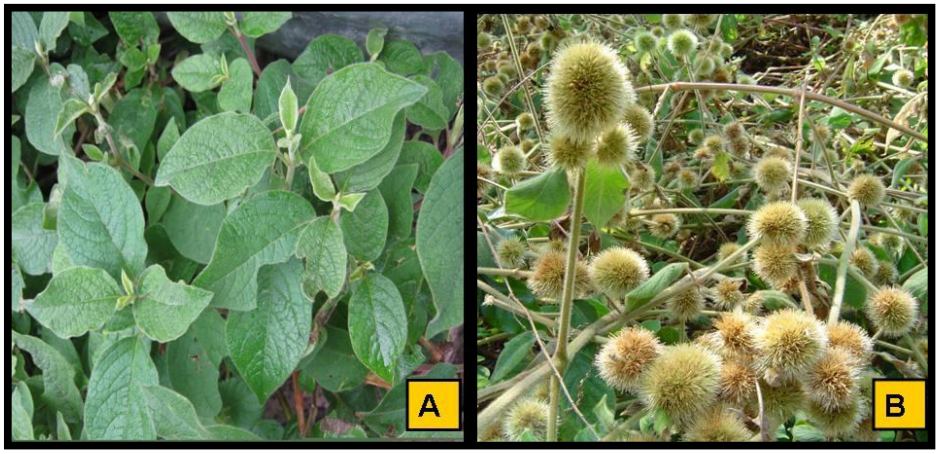


Figure 6.1A. Leaves of *C. uncunilata* plant (Picture taken from source garden at Flagstaff, Eastern Cape, South Africa). Figure 6.1B. *C. uncunilata* showing the plant flowering. Source http://www.metafro.be/prelude/view_symptom?si=V(027)

6.2.2 Extraction of plant material

Powdered plant materials were exhaustively extracted with methanol (MeOH). The supernatant was filtered through cotton wool and Whatman No. 1 filter paper using a Buchner funnel. The extract was concentrated at 45 °C using a Rotavapor (Eyela N-1100, Rikakikai, China). The reduced extract was transferred to a pre-weighed glass vial and sunction-dried at room temperature with a vacuum pump (ULVAC KIKO, Tokyo). The quantity extracted was then determined to be 2g.

6.2.3. Solvent-solvent fractionation of dried plant extract

The dried methanol extract of *Cyathula* leaves was subjected to solvent-solvent fractionation as previously described by Oshima *et al.* (1986). The MeOH extract was partitioned sequentially in ethyl acetate (EtoAc), n-butanol and water. The EtoAc fraction (1 g) was subjected to column chromatography over 100 g silica gel 60 (70- 230 mesh) eluting with *n*-hexane–ethly acetate (10:1 →

4:1 → 1:1 → 1:4, v/v) and ethyl acetate–MeOH (10:0 → 9:1→ 4:1 → 1:1, v/v) to yield fractions designated as SSC1-1 to SSC1-8. Fraction SSC1-5 (20 mg) denoted SSC2 was further subjected to column chromatography over a lipophilic Sephadex (bead size 25-100 µL; Sigma-Aldrich) eluting sequentially with Chloroform and Chloroform-MeOH (20:1 → 9:1). The sub-fractions obtained were combined based on their TLC fingerprint pattern to yield 4 sub-fractions SSC2-1-2, SSC2-3 (3.6 mg); SSC2-4-6 (14.3 mg) and SSC2-7-10 (1.1 mg). A final column chromatography was performed with sub-fraction SSC2-4-6 now denoted as SSC3 using a finer silica gel 60 (40 – 50 µm Cica-Reagent, Kantochemical, Japan). The solvent flow of eluent was n-hexane- acetone (8:1 → 4:1 → 2:1) and acetone only. The flow through with similar R_f values from the TLC fingerprint were pooled together to yield four sub-fractions designated SSC3-1-8; SSC3-9-10; SSC3-11-14 and SSC3-15. A schematic pictograph of the steps involved in the fractionation of extract and fractions is shown in figure 6.2.

6.2.4. TLC fingerprinting of fractions

The dried fractions were dissolved to a concentration of 10 mg/mL in methanol and TLC fingerprinting was done as described previously (Stahl, 1988). Several mobile systems were used. For the TLC of SSC1 fractions the mobile systems were H: E 4:1, H: E 1:1, C: M 20:1, C: M 8:1, C: M 2:1 and C: M: W: A 1:1:0.1:0.05. For the TLC of SSC2, the mobile systems were H: A 2:1 and C: M 20:1. Finally for the TLC of SSC3, the mobile system was C: M 20:1. The detecting agent for chromatograms was anisaldehyde.

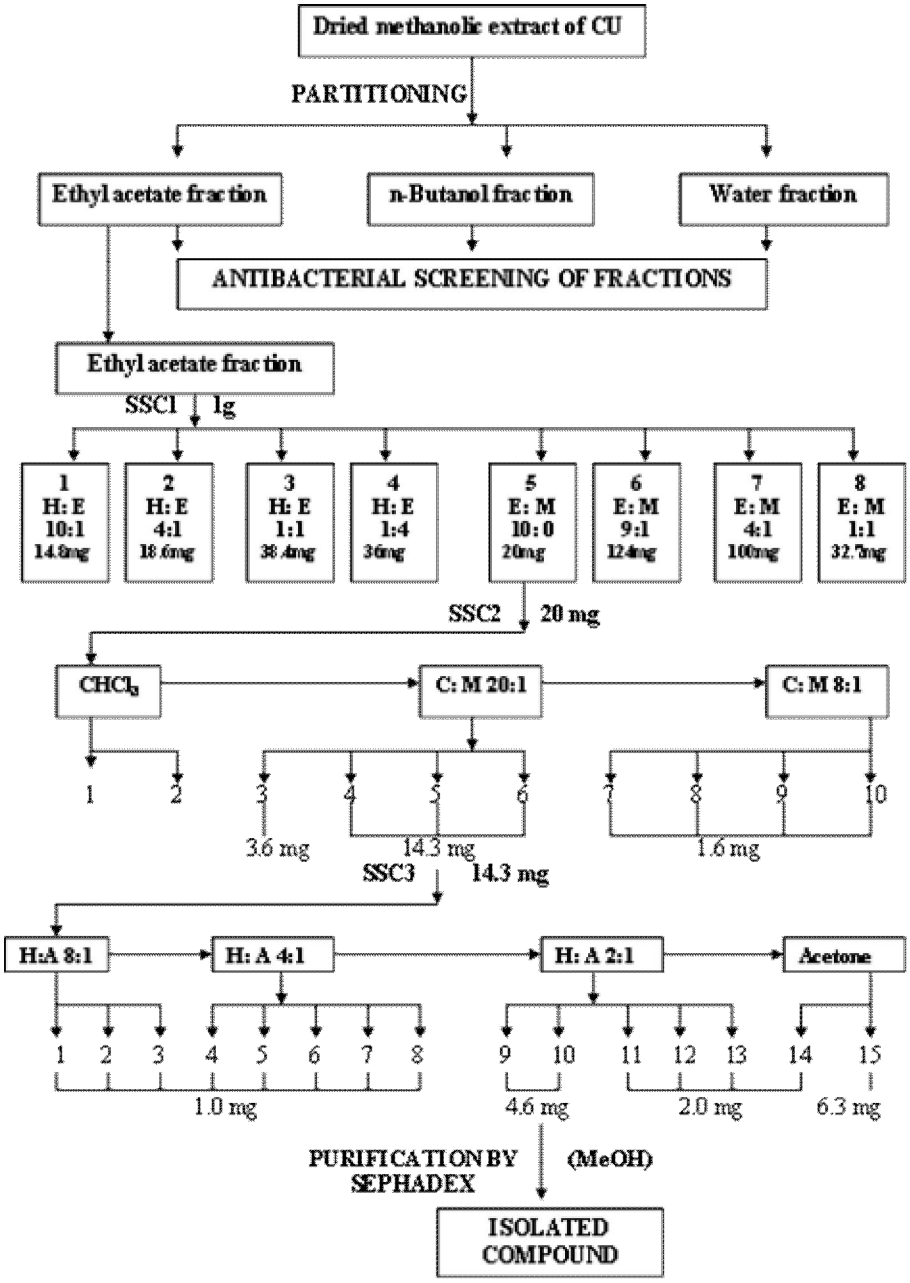


Figure 6.2. Schematic diagram of Solvent-Solvent fractionation of *C. uncinulata*

6.2.5. Antimicrobial assay of fractions

The microplate dilution assay as previously described (Eloff, 1998) was used to determine the minimum inhibitory concentration (MIC) values of

fractions of *C. uncinulata* extracts against a kanamycin sensitive *Escherichia coli* HB101 strain (Lacks and Greenberg, 1977).

6.2.6. Isolation of pure compounds

Based on TLC analysis and the bioassay-guided fractionation, sub-fraction SSC3-9-10 (Figure 6.2) was selected for purification and further characterization. SSC3-9-10 was purified by a lipophilic Sephadex LH-20 (bead size 25-100 µℓ; Sigma-Aldrich) using MeOH (Oshima *et al.*, 1995). The final pure compound was then subjected to NMR analysis and EI-MS.

6.2.7. Preparation of pure compound for NMR and MS analysis

NMR analysis was as previously described (Oshima *et al.*, 1995). The vacuumed concentrated isolated pure compound was added to an NMR capillary tube and was dissolved by adding Chloroform-d for NMR (ACROS ORGANICS) and 99.8+% D, stab. with silver foil, 0.03 v/v % TMS (Merck) to a depth of 4 cm. The NMR capillary tube was then fitted in place for the 13C and 1H NMR analysis in a JEOL-AL 400 FT NMR system 400 MHz (JEOL FT NMR System AL400). Electro impact mass spectra (EI-MS) were recorded on a JMS DX-303 mass spectrometer (Guo *et al.*, 2006).

6.3. Results

6.3.1. Solvent-solvent fractionation yield

The solvent-solvent fractionation yielded four fractions. The result of fraction yield is tabulated below (Table 6.1). The highest quantity of plant

material remained in the water fraction; the quantity of plant material extracted in ethyl- acetate fraction was 300.7 mg while the lowest quantity was obtained in the methanol extract.

Table 6.1 Quantity of the fractions extracted from solvent-solvent fractionation of methanolic extract of *Cyathula uncinulata* leaves

Fractions	Quantity extracted (mg)
Methanol	100.1
Ethyl acetate	300.7
n-butanol	100.7
Water	739.9
Total of extract fractions	1242.4

Table 6.2 Average MIC values of the fractions of *Cyathula uncinulata* leaf extracts tested against *E. coli*

Fractions	MIC of fractions against *E. coli* (mg/ml)
Methanol	1.09
Ethyl acetate	0.39
n-butanol	2.34
Water	2.50
Kanamycin	0.19

In total, 1242.4 mg (approximately 1.2 g) was extracted from the 2 g starting methanol extract of *C. uncinulata* giving a 60 % yield.

6.3.2. TLC fingerprints

The TLC fingerprints of *C. uncinulata* after bulk extraction are shown below (Figure. 6.3). The solvent systems were Hexane and Ethyl acetate ratio one to one with 4 times capillary application of extract on TLC (H:E 1:1x 4); Hexane and Ethyl acetate ratio one to one with one capillary application of extract on TLC (H:E 1:1x1); Hexane and Ethyl acetate ratio four to one with 4 times capillary application on TLC (H:E 4:1x 4); Hexane and Ethyl acetate ratio

four to one with one capillary application of extract on TLC (H:E 4:1x1); Chloroform and Methanol ratio two to one with 4 capillary application of extract on TLC (C : M 2:1x 4); Chloroform and Methanol ratio eight to one with one capillary application of extract on TLC (C:M 8:1x1); Chloroform and Methanol ratio twenty to one with one capillary application of extract on TLC = C:M 20:1x1);

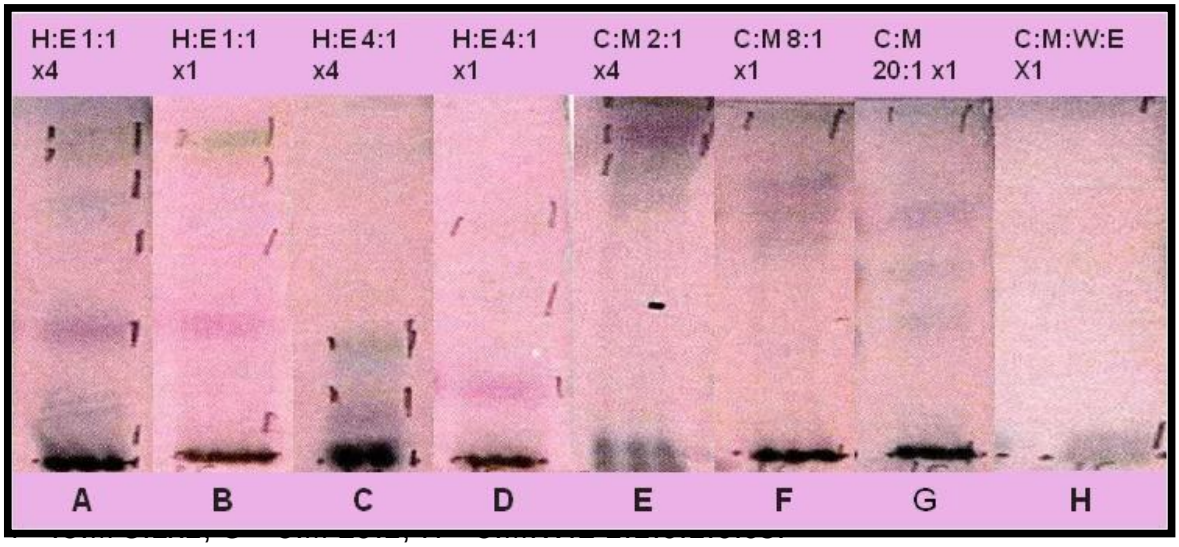
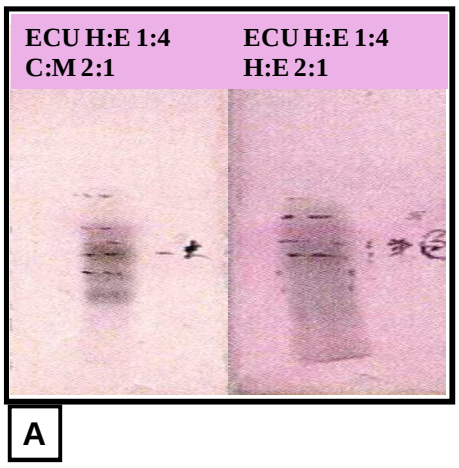


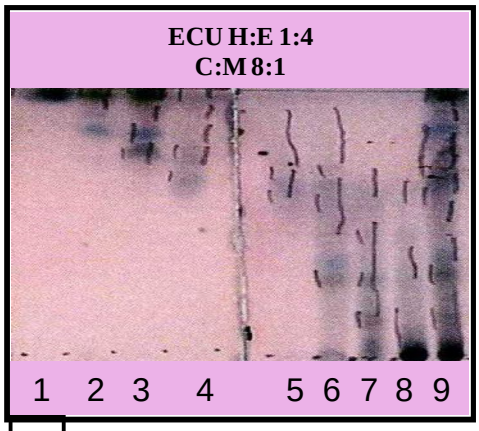
Figure. 6.3. TLC fingerprints of *C. uncinulata* after bulk extraction

Chloroform, Methanol, water and Ethyl acetate with one capillary application of extract on TLC (C:M:W:A 1:1:0.1:0.05). A better separation of plant extract components was obtained with the hexane-ethyl acetate combinations compared to chloroform-methanol. The TLC plates of the further sequential fractions developed in anisaldehyde are shown in figures 6.4A to 6.4G. Lanes having

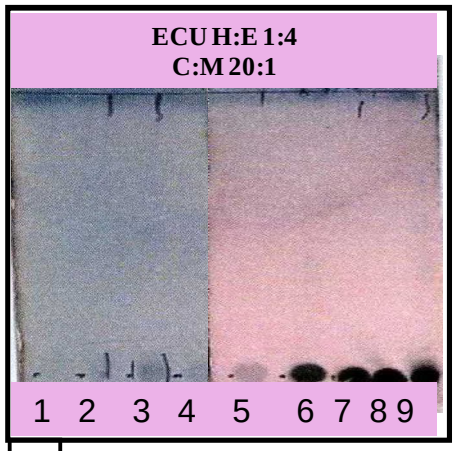
compounds with the same R_f values were pooled together. These pooling together of fractions in lanes 9 and 10 (Figure 6.4G) led finally to the isolation of a pure compound with R_f of 0.38.



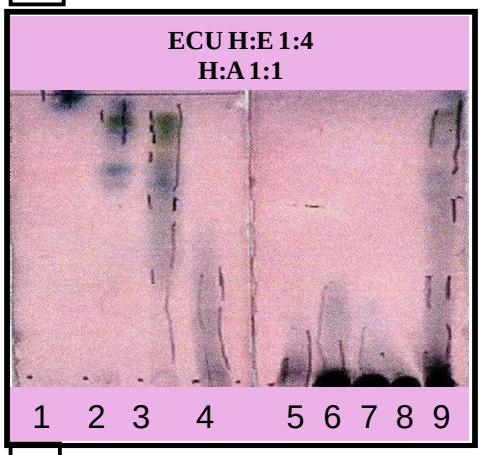
A



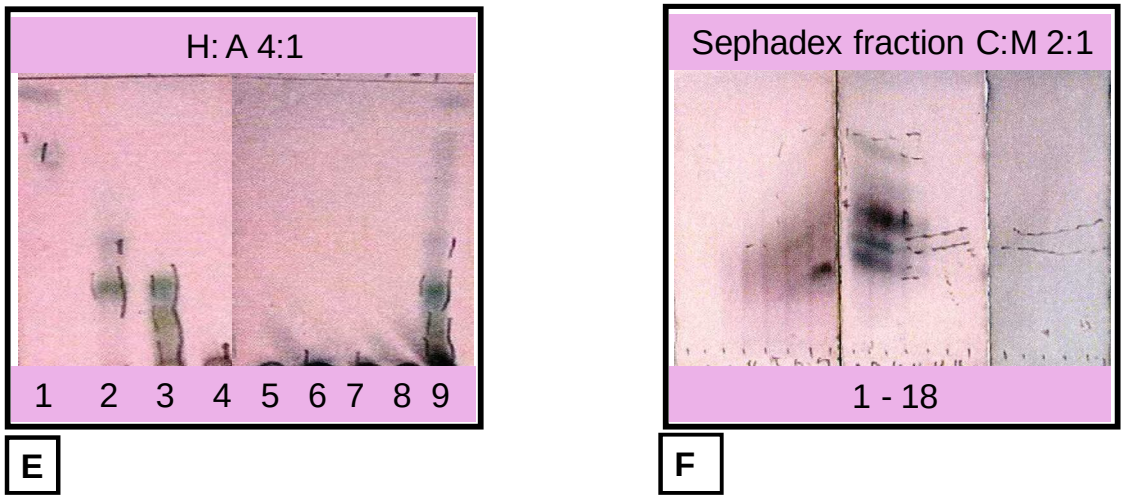
B



C



D



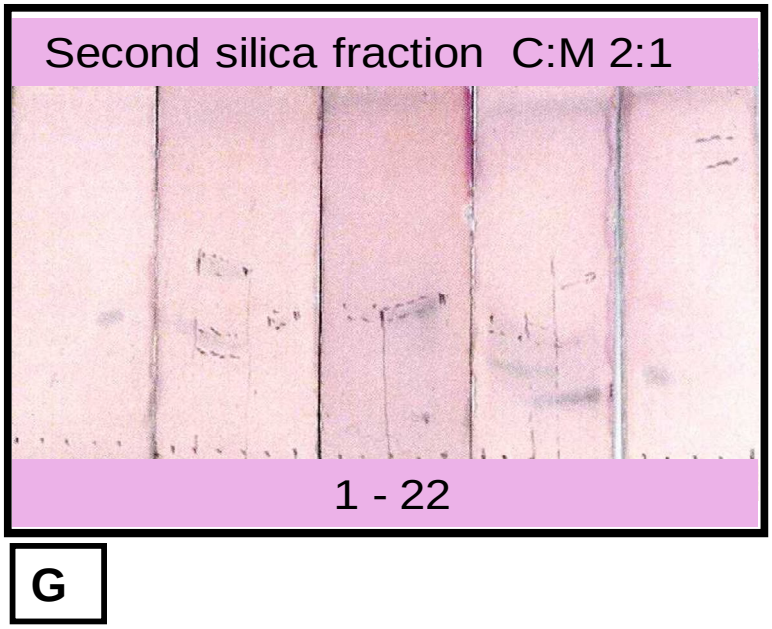


Figure 6.4. TLC chromatogram showing fractions obtained from open column chromatography of ethyl acetate fraction of the methanol extract of *C. uncinulata* leaves. A- TLC for SSC1; the mobile systems were H: E 2:1 and C: M 20:1. B, C, D, E-TLC for SSC2; the mobile systems were C: M 8:1, C: M 20:1, H: A 1:1, H: A 1: 4 respectively. F, G- TLC for SSC3; mobile system was C: M 2:1. All TLC plates were detected with anisaldehyde. (A= Acetone; H=Hexane; E=Ethyl acetate; C=Chloroform and M= Methanol).

6.3.3. Minimum inhibitory concentrations of the fractions

The MICs of the fractions of *C. uncinulata* against *E. coli* are provided in Table 6.2. The MIC ranged from 0.39 mg/mL to 2.5 mg/mL whereas the MIC of Kanamycin, the positive control was 0.19 mg/mL. The lowest MIC was observed in ethyl-acetate fraction while the highest MIC values were recorded in n-butanol and water fractions. The MIC of the selected sub-fraction of the ethyl acetate (SSC 1) fractionation was 0.63 mg/mL while for the final purified compound; the MIC was 0.34 mg/mL

6.3.4. Identification and elucidation of structure of the isolated compound

The NMR and the EI MS spectra of the isolated compound are as shown in appendices 4A to 4C. The NMR spectra of the isolated compound showed that the compound has a long aliphatic chain. The proposed structure for the isolated compound as determined by 1H NMR spectrum is shown in appendix 4D. The formula of the compound is $C_{22}H_{38}O_7$ while the molecular weight is 414.5329.

6.4. Discussion

Solvent-solvent fractionation and column chromatography were employed to isolate the pure compound. The structure of this compound was elucidated predominantly by nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy methods. NMR essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different

groups (as –CH2, -, -CH3-, -CHO, -NH2-, -CHOH-, etc.) and the NMR spectrum provides a record of the number of hydrogen or carbon atoms in the different locations. The NMR proton and EI MS spectra of the fraction yielding the pure compound are shown in Appendix 4A-C. The isolated compound shown in appendix 4D is made up of sugar and fatty acyl moiety composed of hydrocarbon tails. A new glycoside, 3-O-[α-L-rhamnopyranosyl-(1→3)-(n-butyl-β-D-glucopyranosiduronate)]-28-O-β-D-glucopyranosyloleanolic acid together with several other glycosides were isolated from the roots of *C. officinalis* by Zhou and co-workers (2005a). Glycosides of fatty acids or glycolipids are found in bacteria, yeasts and fungi but also in several plants. The functions and potential applications of this compound have been reviewed by Kitamoto and co-worker (2002). Sugar-based esters of fatty acids are nonionic surfactants of great potential applications. They are nontoxic, odourless, tasteless and biodegradable and because of their multi-functional properties and safety, this group of compounds has attracted attention from cosmetics, pharmaceutical, surfactant and nutrition scientists (Kitamoto *et al.,* 2002).

The aqueous fraction of the extract of *C. uncinulata* yielded the highest quantity of plant components while the ethyl acetate fraction extracted the least (Table 6.1). However, the least antibacterial activity was recorded in this fraction as opposed to the ethyl acetate fraction which had the least MIC values of 0.39 mg/mℓ (Table 6.2) hence the choice of ethyl acetate fraction for further fractionation and isolation of bioactive compound. The final compound had an

average MIC of 0.34 mg/mL which was very close to 0.19 mg/mL of kanamycin, the positive control, hence possessing a considerable antibacterial activity.

Researchers have reported the isolation and biological activities of various compounds from members of the genus *Cyathula*. Four new compounds 4-[(1-ethoxy-2-hydroxy) ethyl]phenol (1), 2,3-isopropylidene cyasterone (2), 24-hydroxycyasterone (3) and 2,3-isopropylidene isocyasterone (4), together with fourteen known compounds were isolated from the *C. Officinalis* Kuan (Zhou *et al*., 2005b). *C officinalis* Kuan seems to be the most widely studied of the genus whereas reports on isolation of bioactive compounds from *C. uncinulata* are scarce. Other studies on the genus *Cyathula* reported mainly on biological activities and cytotoxicity effect of extracts of *C. achyranthoides* and *C. prostata* (Sowemimo *et al*., 2009). This study is a report on a bioactive compound from *C. uncinulata.* Further work will be required to confirm the nomenclature of this compound but going by literature (Zhou *et al*., 2005a), this bioactive compound may be described as a glycosylated oleanolic acid.

6.5. Conclusion

This study isolated a glycosylated oleanolic acid from *C. uncinulata* (Schrad.) Schinz. The compound possessed considerable bioactivity in comparison with the crude extract and the control antibiotic. This is an indication that this plant may be a candidate or serve as template for new antimicrobial. The main focus of several studies are the isolation and identification of bioactive

compounds from plants, nonetheless, it is imperative to recognize the complexity of plants and that a single compound may not be responsible for the observed activity but rather a combination of compounds acting by synergism or as complements. This may further explain why bioactivity is lost in some cases in the course of isolation of active components.

6.6. References

Chao, S.L., Huang, L.W., Yen, H.R., 2003. Pregnancy in premature ovarian failure after therapy using Chinese herbal medicine. Chang Gung Med. J.. 26(6):449-452.

Chen, H., Liu, Y-P., 2001. The elementary anti-tumour research of *Cyathula officinalis* Kuan polysaccharide. J. Chengdu Univ. Tradit. Chin. Med.. 24: 49-50. (in Chinese with English abstract).

Chen, X.M., Tian, G.Y., 2003. Structural elucidation and antitumor activity of a fructan from *Cythula officinalis* Kuan. Carbohydrate Res., 338: 1235–1241.

Clark, V.R., Barker, N.P., Mucina, L., 2009. The Sneeuberg: A new centre of floristic endemism on the Great Escarpment, South Africa. S. Afr. J. Bot., 75 (2): 196-238.

Dharmananda, S., 2002. Chinese Herbal Therapy for Endometriosis. Internet J Institute Trad Med Prev Health Care. ITM Online. Retrieved August 11, 2010 from:
http://www.itmonline.org/journal/arts/endometriosis.htm

Dold, A.P., Cocks, M.L., 1999. Preliminary list of Xhosa plant names from the Eastern Cape, South Africa. Bothalia, 29(2): 267-292.

Eloff, J.N., 1998. A sensitive and quick method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica, 64: 711-713.

Guo, y., Xu, J., Li, Y., Watanabe, R., Oshima, Y., Yamakuni, T., Ohizumi, Y., 2006). Ridoids and sesquisterpenoids with NGF-potentiating activity from the rhizomes and roots of *Valeriana faurieri.* Chem. Parm. Bull., 54(1): 123-125.

Imai, S,, Hori, M., Fujioka, S., Murata, E., Goto, M., Nakanishi, K., 1968. Isolation of four new phytoecdysones, makisterone A, B,C,D, and the structure of makisterone A, a cm steroid, Tetrahedron Lett., 9: 3883–3886.

Jeruto, P., Lukhoba, C., Ouma, G., Otieno, D., Mutai, C., 2008. An ethnobotanical study of medicinal plants used by the Nandi people in Kenya. J. Ethnopharmacol., 116 (2): 370-376.

Jeruto, P., Lukhoba, C., Ouma, G., Otieno, D., Mutai, C., 2008. Herbal treatments in Aldai and Kaptumo divisions in Nandi district, Rift Valley province, Kenya. Afr. J. Trad. CAM., 5(1):103 – 105.

Kala, C.P., 2005. Ethnomedicinal botany of the Apatani in the Eastern Himalayan region of India J. Ethnobiol. Ethnomed.,1:11 doi:10.1186/1746-4269-1-11

Kitamoto, D., Isoda, H., Nakahara, T., 2002. Functions and potential applications of glycolipid biosurfactants: from energy saving materials to gene delivery carriers. J. Biosci. Bioeng., 94: 187-201.

Lacks, S., Greenberg, B., 1977. Complementary specificity of restriction endonucleases of *Diplococcus pneumoniae* with respect to DNA methylation. J. Mol. Biol., 114:153-168.

Lescure, J.P., Baslev, H., Alarcon, R., 1987. Plantas Utiles de la Amazona Ecuatoriana. ORSTOM, Quito, Ecuador. pp. 407.

Li, Z.L., Shi, S.H., Chen, H., Liu, Y.P., 1999. The Promoting red cell immunity function of*Cyathula officinalis*Kuan polysaccharide. Chin. Tradit, Med, Pharmacol, Clin., 15: 26-27. (in Chinese with English abstract).

Liu, J., Xu, J., Zhao, X.J., Gao, W.Y., Zhang, S.Z., Guo, Y.Q., 2010. A new heterocyclic compound from *Cyathula officinalis* Kuan. Chinese Chem. Lett., 21(1): 70-72.

Ogundipe. O.T., Chase, M., 2009. Phylogenetic Analyses of Amaranthaceae Based on matK DNA Sequence Data with Emphasis on West African Species. Turk. J. Bot., 33:153-161.

Okuzumi, K., Hara, N., Uekusa, H., Fujimoto, Y., 2005. Structure elucidation of cyasterone stereoisomers isolated from *Cyathula officinali.* Org. Biomol. Chem., 7; 3(7):1227-1232.

Oshima, Y., Cordell, G.A., Fong, H.H.S., 1986. Studies on Zoapatle, III. Flavonoid Glycosides from Montanoa tomentosa ssp. Tomentosa. J. Nat. Prod., 49(3):552–553.

Oshima, Y., Namao, K., Kamijou, A., Matsuoka, S., Nakano, M., Terao, K., Ohizumi, Y., 1995. Powerful hepatoprotective and hepatotoxic plant oligostilbenes, isolated from the Oriental medicinal plant Vitis coignetiae (Vitaceae), Cell. Mol. Life Sci., 51(1): 63-66.

Pharmacopoeia Commission of People's Republic of China. 2000. Pharmacopoeia of the People's Republic of China. Compiled by the State Pharmacopoeia Commission of the People's Republic of China. English ed. Beijing, China: Chemical Industry Press, http://catalogue.nla.gov.au/Record/3107557

Russo, E.B., 1992. Headache treatments by native peoples of the Ecuadorian Amazon: a preliminary cross-disciplinary assessment. J. Ethnopharmacol. 36(3):193-206.

Sláma, K., Abubakirov, N.K., Gorovits, M.B., Baltaev, U.A., Saatov, Z., 1993. Hormonal activity of ecdysteroids from certain Asiatic plants. Insect Biochem. Mol. Biol., 23: 181–185.

Sowemimo, A., van de Venter, M., Baatjies, L., Koekemoer, T., 2009. Cytotoxicity activity of selected Nigerian plants. Afr. J. Trad. CAM., 6(4): 526 – 528.

Takasaki, M., Tokuda, H., Nishino, H., Konoshima, T., 1999. Cancer chemopreventive agents (antitumor-promoters) from Ajuga decumbens. J. Nat, Prod., 62: 972–975.

Takemoto, T., Hikino, Y., Nomoto, K., Hikino, H., 1967. Structure of cyasterone, a novel C29 insect-moulting substance from *Cyathula capitata.* Tetrahedron Lett., 8: 3191–3194.

Takemoto, T., Nomoto, K., Hikino, Y., Hikino, H., 1968. Structure of Capitasterone, a Novel C29 Insect-Moulting Sub- stance from *Cyathula capitata.* Tetrahedron Lett., 9: 4929–4932.

Tomani, J.C., Nkurunziza, J.P., Mukazayire, M.J., Kanezeza, R., Baziruwitonda, E., Duez, P. 2008. Antidiarrhea activity and preliminary phytochemical screening of *Indigofera arrecta, Cyathula uncinulata, Persea americana and Cupressus lusitanica.* 7th Joint Meeting of AFERP, ASP, GA, PSE & SIF on Natural Products with pharmaceutical, nutraceutical, cosmetic and agrochemical interest. 3-8 August 2008. Athenaeum Intercontinental, Athens Greece.

Yamada, T., 1999. A report on the ethnobotany of the Nyindu of the Eastern part of the former Zaire. Afr, Study Monographs, 20(1):1-72.

Yineger, H., Yewhalaw, D., Teketay, D., 2008. Ethnomedicinal plant knowledge and practice of the Oromo ethnic group in southwestern Ethiopia. J. Ethnobiol. Ethnomed., 4:11 doi:10.1186/1746-4269-4-11

Zhou, R., Li, B-G., Zhang, G-L., 2005. Glycosides from roots of *Cyathula officinalis* Kuan. J. Integrat. Plant Biol., 47 (3):368 – 374.

Zhou, R., Li, B-G,, Zhang, G-L., 2005. Chemical study on *Cyathula officinalis* Kuan. J. Asian Nat. Prod. Res., 7(3): 245-252.

CHAPTER 7

CYTOTOXICITY OF SELECTED PLANTS USED IN ETHNOMEDICINE IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA

7.0. Abstract

Several herbs are traditionally used in the treatment of various ailments particularly in the rural areas of South Africa where herbal medicine is often the only source of health care system. Many of these herbs have not been assessed for safety or toxicity to tissues or organs of the mammalian recipients. This study evaluated the safety of some medicinal plants used inter alia in the treatment of diarrhoea, and stomach disorders. The cytotoxicity of methanol extracts and fractions of six selected plants was determined by using a modified tetrazolium-based colorimetric assay (3-(4, 5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay). The in vitro cytotoxicity assay on human hepatocarcinoma cell line (Huh-7) revealed that the methanol extract of *Eucomis autumnalis* had the strongest cytotoxicity with IC_{50} of 7.8 µg/mL. Ethyl acetate and butanol fractions of *Cyathula uncinulata, Hypoxis latifolia, E. autumnalis* and *Lantana camara* had lower cytotoxic effects on the cancer cell lines tested with IC_{50} values ranging from 24.8 µg/mL to 44.1 µg/mL while all the fractions of *Aloe arborescens* and *A. striata* had insignificant or no cytotoxic effects after 72 h of treatment. Our results indicate that the methanol fraction of *E. autumnalis* had a profound cytotoxic effect which puts a query on its safety and hence a call for caution in its usage.

Key words: Herbs; extracts; fractions; safety; cytotoxicity; profile; treatment

7.1. Introduction

Various plants are used in the ethnomedicine of enteric diseases. Several studies have documented reports on some herbs used in ethnotherapy of diarrhea, dysentery, vomiting, stomach cramps and other associated ailments (McGaw *et al*., 2000; Vieira *et al*., 2001; Samie *et al*., 2005). Contrary to the belief of a large proportion of the populace that anything natural is safe, many commonly used herbs cause acute toxic effects and in the long term may be toxic. The toxic effects may range from diarrhoea, hypersensitivity reactions, nausea or vomiting, to organ-targeted toxicity; immunotoxicity, embryo/foetal and prenatal toxicity, mutagenicity/genotoxicity, hepatotoxicity, nephrotoxicity, presence of epileptogenic compounds, cardiac toxins, gastrointestinal toxins to carcinogenicity (Smolinske, 2005). Other adverse effects of herbal medicines may include cardiovascular, hepatic, renal, neurological and dermatologic toxic effects. In the review by Luyckx and Naicker (2008), it was stated that 'drug-induced nephrotoxicity reportedly contributes to up to 26% of cases of hospital-acquired acute kidney injury (AKI) and 18% of cases of community-acquired AKI globally, and folk remedies account for up to 35% of cases of AKI in the developing world.'

Many of the plants widely acclaimed to be of therapeutic values have not enjoyed vigorous assessment of their safety profiles. A number of complications arising after the administration of medicinal herbs have been reported. Foyaca-Sibat *et al*. (2001) described case reports of two patients with neuromyotonia not linked to malignancies. The patients were reported to have developed acute

renal failure while under treatment with herbal medications by their traditional healer in the former Transkei region of South Africa.

Other researchers have also identified medicinal plants with potential toxicity such as the extracts of *Athrixia phylicoides* DC. (Bush tea) (McGaw *et al.*, 2006); extracts and a flavonol glycoside from *Bauhinia galpinii* (Aderogba *et al.*, 2007). Genotoxicity and mutagenic effects in the *Salmonella* microsome assay respectively have been reported in *Crinum macowanii*, *Chaetacme aristata* Planch. (Celastraceae), *Plumbago auriculata* Lam. (Plumbaginaceae), *Catharanthus roseus* (L.) G.Don. (Apocynaceae) and *Ziziphus mucronata* Willd. (Rhamnaceae) (Elgorashi *et al.*, 2003). Additionally, Michellamine B-an alkaloid dimers isolated from *Ancistrocladus korupensis* was inhibitory to several laboratory and clinical strains of HIV-1, including the AZT resistant strain G910-6 and the pyridinone-resistant strain A17; as well as strains of HIV-2. However, the high toxicity of this compound to several human cell lines prevented its further evaluation (Boyd *et al.*, 1994). Data on the cytotoxic assessments of herbs are very few compared with the huge number of plants acclaimed to have therapeutic values (Oshima *et al.,* 1986; Neuwinger, 2000). This study investigated the cytotoxic effects of *Aloe arborescens, A. striata, Cyathula uncinulata, Eucomis autumnalis, Hypoxis latifolia* and *Lantana camara* commonly used in the treatment of gastrointestinal infections in the Oliver R. Tambo District Municipality (ORTDM), Eastern Cape Province, South Africa.

7.2. Materials and methods

7.2.1. Plant material, extraction and fractionation

Fresh plant parts were collected in ORTDM, Eastern Cape Province, South Africa between July 2008 and February 2010. The plants were identified in the Kei herbarium, Walter Sisulu University, South Africa where voucher specimens have been deposited. Information on the selected herbs is presented in Table 7.1 (van Staden, 1981; Cunningham, 1993; Pooley, 1993; Inada *et al.* 1995; Dold and Cocks, 2000; Van Wyk and Gericke, 2000; Abdulla *et al.,* 2009; Bisi-Johnson *et al.,* 2010). The air-dried plant parts were extracted three times with methanol (Merck, Japan) and filtered using a Buchner funnel and Whatman No. 1 filter paper. The extracts were concentrated under reduced pressure at a temperature of 40^{o}C using a rotating evaporator to yield methanol extract. The methanol extract was then suspended in deionised water and partitioned sequentially with Ethyl acetate and n-butanol. The fractions were concentrated under reduced pressure to yield the corresponding fractions and the remaining water fraction.

7.2.2. Antibacterial assay

The antibacterial assays involved the determination of the minimum inhibitory concentrations (MICs) of plant extracts against test organisms. The broth dilution method was carried out in 96-well microtitre plates using ampicillin-resistant and

Table 7.1. Selected plants investigated and their usage

Herb	Local name	Plant part	Uses and Refences
Aloe arborescens Mill	Ikhala Inkalane	Leaves	Leaf decoction for diarrhoea (Bisi-Johnson *et al*. 2009), effective

ASHODELACEAE	encane (Z)		burn treatment (Pooley, 1993)
Aloe striata Ham ASHODELACEAE	Inkalana (X) Intelezi (Ng)	Leaves	Leaf decoction for diarrhoea ((Bisi-Johnson *et al*. 2009), purgative, expels worms
Cyathula uncinulata AMARANTHACEAE	Isinama (X)	Leaves	Leaf decoction for HIV treatment (Bisi-Johnson *et al*. 2009), stomach ailment
Eucomis autumnalis HYACINTHACEAE	Ubhulungu becanti (X) Umathunga (Z)	Root	Decoctions of bulb and roots for coli, flatulence (Cunningham, 1993)
Hypoxis latifolia HYPOXIDACEAE	Ilabatheka (X,Z)	Root	Treat benign prostrate (van Staden , 1981); Headaches, dizziness, mental disorders, HIV inflammation (Singh, 1999; van Wyk, 2000); Boil ground dried tuber for diarrhoea.
Lantana camara VERBENACEAE	Ubuhobe besikhiwa (N) Mbarapati (S) Iqunube, utywala bentaka (X)	Leaves Flower	Leaf decoction for HIV treatment (this study); antivirus (Inada *et al* 1995); rheumatism, sexually transmitted diseases (Dold and Cocks, 2000); treatment of wound (Abdulla *et al* 2009)

Key: N – Ndebele; Ng – Nguni; S – Shona; X – Xhosa; Z – Zulu

kanamycin-resistant strains of *Escherichia coli* as the test organisms. A McFarland No1 standard suspension of bacteria inoculum was prepared in sterile Mueller Hinton Broth (MHB). Triplicate tests were performed in a series of two-fold dilutions of extract (10 mg/mL) as previously described (Eloff, 1998). Kanamycin was used as the positive control for ampicillin-resistant *E. coli* strain while ampicillin was used in the case of kanamycin-resistant *E. coli* strain. Plates were incubated at 37^{o}C for 18 h and an hour before the end of incubation, 40 μL of 0.2 mg/mL INT (*p*-iodonitrotetrazolium salt) solution was added to each well.

The lowest concentration indicating inhibition of growth was recorded as the MIC. This was indicated by the clear well after further incubation with INT as opposed to the pinkish colouration in growth wells.

7.2.3. Cytotoxicity assays

The *in vitro* cytotoxicities of the selected herbs and solvent-solvent fractions on a human hepatoma cell line, (Huh-7), which was established from a hepatocellular carcinoma were examined using a modified MTT assay (Plumb *et al*., 1989). The Huh-7 was maintained at – 80^{o}C in Dulbecco's Modified Eagle Medium (DMEM) and recovered from preservative by centrifugation. The pellet was re-suspended in fresh DMEM and cultured in a humidified atmosphere at 37^{o}C using RPMI 1640 supplemented with 10% foetal bovine serum, 100 U/mL penicillin G and 100ug/mL streptomycin and L-glutamine (Gibco BRL) in 5% CO_2 incubator (Thermo Fischer Scientific, Wakenyaku Co. Ltd, Japan). Cells were sub-cultured every 2 days after confluent growth was observed.

The MTT assay was carried out as follows. Briefly, the cells at a density of 1×10^4 per cell were seeded in each well of a flat bottom 96-well plate containing 100 µL of the growth medium. Cells were permitted to adhere for 24 h, and then treated with various fractions at concentrations 0, 1, 10 and 100 µg/mL for 72 h. After that, 20 µL of 5 mg/mL MTT in phosphate buffered saline (PBS) was added to each well and the plate was incubated for an additional 2 h. The medium was discarded and the formazan blue, which formed in the cells, were dissolved with 100 µL MTT stop solution (Triton-X100-20 mL; Isopropyl alcohol-500 mL; HCl-2

mL). After incubation at 37^{o}C for 10min, the absorbance of the dissolved solutions was detected at 490 nm on a microplate ELISA reader (Thermo Labsystems, Japan). Cytotoxicity was expressed as the concentration of extract or fraction inhibiting cell growth by 50% (IC_{50}) and was analysed using MS Excel 2007. All tests and analyses were run in triplicate.

7.3. Results

7.3.1. Antibacterial activities

The average MIC values of the plant extracts ranged from 0.27 mg/mL and 2.5 mg/mL after 24 h of incubation (Figure 7.1). The Ethyl acetate fraction of each type of plant was the most active fraction against the test bacteria. *E. autumnalis* had the least MIC (0.27 mg/mL) followed by *C. uncinulata* (0.39 mg/mL) and thus demonstrated good antibacterial activities. The methanol extract of *H. latifolia*, water fraction of *A. arborescens, C. uncinulata, E. autumnalis, and L. camara* had the highest value of MIC (2.5 mg/mL).

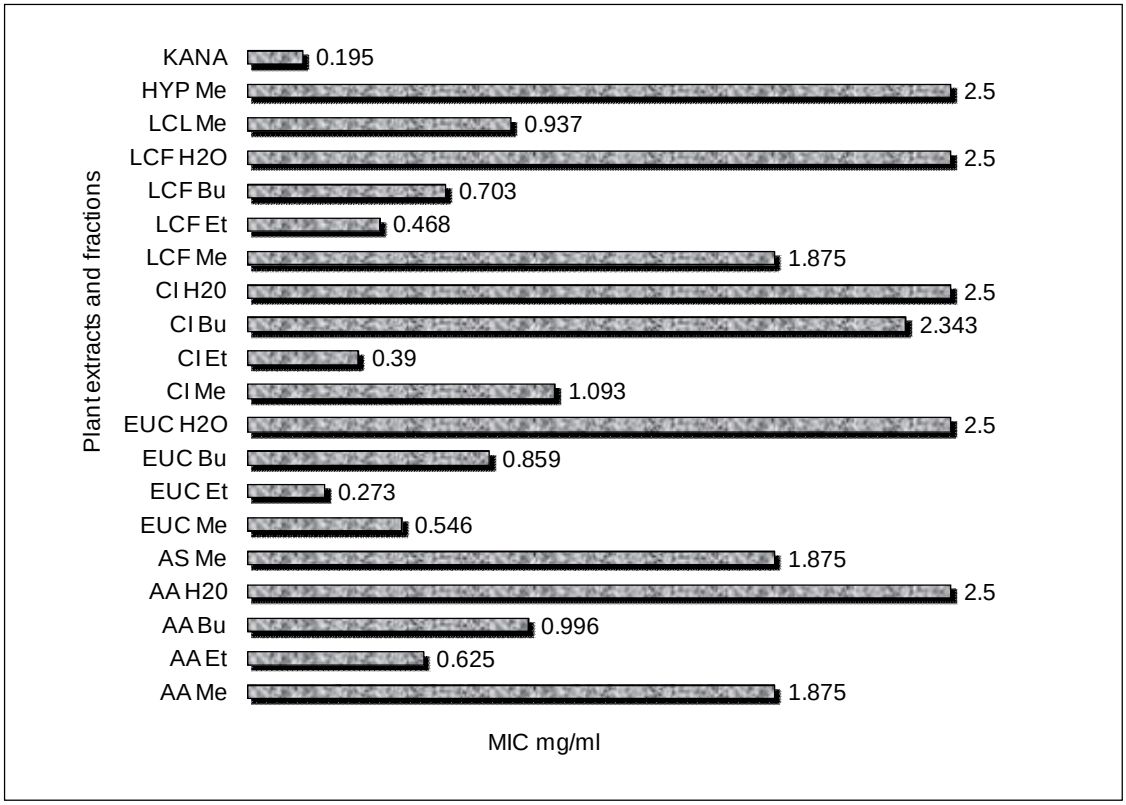


Figure 7.1. Minimum inhibitory concentrations of plant extracts and fractions

Figure legend:
AA Me = Methanol fraction of *A. arborescens* (AA); AA Et = Ethyl acetate fraction of AA; AA Bu = n-Butanol fraction of AA; AA H_2O = Water fraction of AA; AS Me = Methanol fraction of *A. striata* (AS); EUC Me = Methanol fraction of *E. autumnalis* (EA); EUC Et = Ethyl acetate fraction of *EA*; EUC Bu = n-Butanol fraction of *EA*; EUC H_2O = Water fraction of *EA*; CI Me = Methanol fraction of *C. uncinulata* (CU); Et = Ethyl acetate fraction of *CU*; CI Bu = n-Butanol fraction of *CU*; CI H_2O = Water fraction of *CU*; LCF Me = Methanol fraction of *L. camara* fruit/flower (LCF*)*; LCF Et = Ethyl acetate fraction of LCF ; LCF Bu = n-Butanol fraction of LCF; LCF H_2O = LCF Water fraction of LCF; LCL Me = Methanol fraction of *L. camara* leaves; HYP Me = Methanol fraction of *Hypoxis latifolia.*

7.3.2. Cytotoxicities of methanol extracts and fractions.

The results of cytotoxicities of the extracts and fractions of the investigated plants on human hepatic cell line (Huh-7) are shown in Table 7.2. The methanol extract of *E. autumnalis* (IC_{50} 7.8 µg/mL) was more cytotoxic than

the control toxic substance, berberine (IC_{50} 9.8 µg/mL), other plant extracts and solvent fractions.

Table 7.2. Inhibitory concentration, IC_{50} (ug/ml) of selected herbs

Code S/No	Sample code	Extract fractions	IC_{50} (ug/ml)
1	BUB Me	AA Methanol fraction	>1000
2	BUB Et	AA Ethyl Acetate fraction	>1000
3	BUB Bu	AA n-Butanol fraction	>1000
4	BUB H2O	AA Water fraction	>1000
5	BUS Me	AS Methanol fraction	1000
6	EUC Me	EA Methanol fraction	7.8
7	EUC Et	EA Ethyl Acetate fraction	28.5
8	EUC Bu	EA n-Butanol fraction	39.3
9	EUC H2O	EA Water fraction	379.0
10	IS Me	CU Methanol fraction	24.8
11	IS Et	CU Ethyl Acetate fraction	36.3
12	IS Bu	CU n-Butanol fraction	30.0
13	IS H2O	CU Water fraction	714.0
14	LCF Me	LCF Methanol fraction	169.0
15	LCF Et	LCF Ethyl Acetate fraction	44.1
16	LCF Bu	LCF n-Butanol fraction	150.0
17	LCF H2O	LCF Water fraction	1000
18	LCL Me	LCL Methanol fraction	161.0
19	HYP Me	HYP Methanol fraction	24.4
PC	Positive control	Berberine	9.8

Key: AA – *Aloe arborescens;* AS – *Aloe striata*; EA – *Eucomis autumnalis*; CI – *Cyathula uncinulata*; LCF – *Lantana camara (*flower/fruits); LCL – *Lantana camara* (leaves); HYP – *Hypoxis latifolia.*

Of the solvent fractions, the Ethyl acetate and butanol fractions of *C. uncinulata, H. latifolia, E. autumnalis* and *L. camara* had moderate cytotoxic effects on the cancer cell lines tested with IC_{50} values ranging from 24.8 µg/mL to 44.1 µg/mL while all the fractions of *A. arborescens* and *A. striata* had insignificant or no cytotoxic effects after 72 h of treatment (IC_{50} 1000 to >1000 µg/mL).

7.4. Discussion

This study evaluated the cytotoxicity and antibacterial activities of methanol extracts and solvent fractions of *A. arborescens, A. striata, C. uncinulata, E. autumnalis, H. latifolia* and *L. camara*. Among all the samples, the methanol extract of *E. autumnalis* exhibited greatest cytotoxicity on the cell line tested with IC_{50} of 7.8 ug/mL). However, the water fraction of *E. autumnalis* and that of other plants showed markedly less cytotoxicity on the cell line, compared with the polar solvent fractions (Table 7.2).

The Ethyl acetate fraction of *E. autumnalis* had the least MIC (0.27 mg/mL) followed by *C. uncinulata* (0.39 mg/mL) against the test bacteria and thus demonstrated good antibacterial activities. Various biological activities of *Eucomis* were reported (Du Toit *et al*., 2005; Gaidamashvili and van Staden, 2006). *E. autumnalis* is known for its anti-inflammatory and antispasmodic activities and these have been attributed to components such as homoisoflavones and flavonoids (Notten and Kirstenbosch, 2002). *E. autumnalis* also contains some steroidal triterpenoids which are known to be beneficial in

wound therapy (Notten and Kirstenbosch, 2002). However, the bulb was reported to be toxic (Notten and Kirstenbosch, 2002), in agreement with our findings.

Eucosterol glycoside, a lanosterol oligosaccharides isolated from *E. bicolor* have demonstrated antitumor activity by causing 44% inhibition of TPA-stimulated ^{32}P incorporation into phospholipids of HeLa. This activity has been suggested to probably relate to the use of bulb decoctions of *E. autumnalis* to relieve abdominal distensions and abdominal pain by the Tswana and Pedi tribes of South Africa (Watt and Breyer-Brandwijk, 1962). According to Koorbanally *et al.* (2006), sheep drenched with fresh bulbs of *E. autumnalis* in an animal feeding trial presented with listlessness, anorexia, foaming at the mouth, tympanites, an inactive rumen and a strong pulse leading eventually to death within twenty-four hours. Despite these submissions *Eucomis* is one of the most traded genera of plants in South Africa (Dold and Cocks, 2002), hence the need for caution in their usage.

The *Hypoxis latifolia* fraction also exhibited a considerable toxicity level (24.4 ug/mL). The antimycobacterial activity of hypoxoside has been attributed in part to the high cytotoxicity of this compound derived from *Hypoxis* species and this activity is presumed to be comparable to the cytotoxic effects demonstrated by Hypoxoside against melanoma cancer cells *in vitro* (Albrech *et al*., 1995). *Lantana camara* which is known to have several uses in ethnomedicine possessed moderate cytotoxic effects. The folk medicine uses includes

the treatment of cancers, chicken pox, measles, asthma, ulcers, swellings, eczema and tumors (Day *et al*., 2003). Some of the biological antagonistic or potential biocides usage includes the capability of its aqueous leachate at 1–3% to kill water hyacinth, a troublesome weed in many tropical countries even though its application as a weedicide depends on the size of the water bodies being treated and the cost of extraction of the leachate (Day *et al*., 2003). Some taxa of the widely variable *L. camara* complex are toxic to small ruminants and this effect has been associated with the types and relative amounts of some triterpene ester metabolites (Ghisalberti, 2000). Verbascoside, a bioactive compound from *L. camara,* was reported to possess antimicrobial, immunosuppressive and antitumor activities (Day *et al*. 2003). Other compounds isolated from *L. camara*: lantanoside, linaroside and camarinic acid have been investigated as potential nematocides (Begum *et al*. 2003).

Cytotoxicity assay is a rapid inexpensive determination of significant quantities of biologically harmful contents of product measured as functions of fundamental biochemical pathways leading to cell death (Mosmann, 1983). Toxicity testing of ethnomedical preparations is essential because herbal products may contain undesirable or misidentified ingredients, sometimes toxic such as a toxic plant taken as a desired nontoxic plant or presence of heavy metals, radioactive particles, and microbes including pathogens, mycotoxins, endotoxins and pesticides (WHO, 2007). In addition, safety assessment of herbal products is necessary for the safety of the recipients since herbs are generally heterogeneous, may produce multiple effects, and may affect multiple organs systems, including the nervous, cardiovascular, gastrointestinal, hepatic, renal, and hematologic systems (Barrueto and Hirshon, 2009). Furthermore,

cytotoxicity assay is a means of forestalling financial loss due to failure of a drug making it through the developmental processes. Some reasons for the failures are due to unacceptable toxicity levels (Ricerca, 1998). Testing hence prevent unacceptable compound going through unnecessary costly developmental stages.

Various methods are employed and these include using cell line assays and in vivo tests (use of animals). In addition, the brine shrimp (BS) mortality assay is widely accepted as a convenient probe for potential pharmacological activity in plants (Meyer *et al.*, 1982), however some known toxic plants have exhibited non-toxic effect in brine shrimp (McGaw and Eloff, 2005) generating a misgiving on the capability of BS detection of plant toxicity, hence the preference of cell-line cytotoxicity assay. Another approach to evaluate the safety of healthcare products is Bioequivalence studies in terms of toxicity effect. Two drugs are considered bioequivalent when there is no significant difference between them in terms of their absorption rates (Tu and Koh, 2010). Considering the body weight of each of two mice versus time for oral administration of two herbal products (Tyrel^{TM} and Rumbion^{TM}), Tu and Koh (2010) were able to conclude that there was no significant difference between the toxicity effect of Tyrel^{TM} and Rumbion^{TM} at 5000 mg kg-1 body weight. For the determination of mycotoxins in herbs, HPLC-based technique using a monoclonal antibody immunoaffinity column is recommended (Farmacopea Argentina, 2003).

7.5. Conclusion

In conclusion, the results obtained indicated that the methanol extract of *E. autumnalis* exhibited much greater cytotoxicity than the methanol extract and solvent fractions of all other plants investigated even though it had strong antibacterial activities. *E. autumnalis* showed selective anticancer activity against the human hepatoma cell line, whereas the two *Aloe* spp. were non toxic on the cell line. In addition, the study showed that *Aloe arborescens, A. striata* and *C. uncinulata* may be candidate plants for eventual drug design. Medicinal plants are natural products and may have therapeutic potentials; however, being natural does not make them automatically safe.

7.6. References

Abdulla, M.A., Hassandarvish, P., Ali, H.M., Noor, S.M., Mahmoud, F.H., Bashah, N.S.A,, et al., 2009. Acceleration of wound healing potential by *Lantana camara* leaf extract in experimental rats. Res. J. Med. Sc., 3(2): 75-79.

Aderogba, M.A., McGaw, L.J., Ogundaini, A.O., Eloff, J.N., 2007. Antioxidant activity and cytotoxicity study of the flavonol glycosides from *Bauhinia galpinii*. Nat. Prod. Res., 21(7):591-599.

Albrech, C., Kruger, P., Theron, E., 1995. Morphological characterization of the cell-growth inhibition by Rooperol and pharmacokinetic aspects of Hypoxoside as a prodrug of cancer therapy. South Afr. Med. J., 85(9): 853-860.

Barrueto, F. Jr, Hirshon, J.M., 2009. Plant Poisoning, Herbs Updated: Feb 12, 2009. Accessed March 2011. http://emedicine.medscape.com/article/817427-overview.

Begum, S., Wahab, A., Siddiqui, B.S., 2003. Pentacyclic triterpenoids from the aerial parts of *Lantana camara.* Chem. Pharm. Bull., 51:134–137.

Bisi-Johnson, M.A., Obi, C.L., Kambizi, L., Nkomo, M., 2010. A survey of indigenous herbal diarrhoeal remedies of O.R. Tambo district, Eastern Cape Province, South Africa. Afr. J. Biotechnol., 9(8): 1245-1254.

Boyd, M.R., Hallock, Y.F., Cardellina, J.H., Manfredi, K.P., Blint, J.W., McMahon, J.B., Buckheit, Jr. R.W., Bringmann, G., Schaeffer, M., 1994. Anti-HIV michellamines from *Ancistrocladus korupensis*. J. Med. Chem., 37(12): 1740-1745.

Cunningham, A.B., 1993. Ethics, ethnobiological research and biodiversity. WWF-World Wide Fund for Nature (formerly World Wildlife Fund), Gland, Switzerland.

Day, M.D., Wiley, C.J., Playford, J., Zalucki, M.P., 2003. *Lantana*: current management status and future prospects. Australian Centre forInternational Agricultural Research Canberra. ACIAR Monogr., 102: 1-128.

Dold, A.P., Cocks, M.L., 2000. The medicinal uses of some weeds, problem and alien plants in the Grahamstown and Peddie districts of the Eastern Cape, South Africa. S. Afr. J. Sci., 96: 467-473.

Dold, A.P., Cocks, M.L., 2002. The trade in medicinal plants in the Eastern Cape Province, South Africa. S. Afr. J. Sci., 98:589-597.

Du Toit, K., Elgorashi, E., Malan, S.F., Drewes, S.E., van Staden, J., Crouch, N.R., Mulholland, D.A., 2005. Anti-inflammatory activity and QSAR studies of compounds isolated from Hyacinthaceae species and Tachiadenus longiflorus Griseb. (Gentianaceae). J. Bioorganic. Med. Chem., 13:2561–2568.

Elgorashi, E.E., Taylor, J.L.S., Verschaeve, L., Maes, A., van Staden, J., De Kimpe, N., 2003. Screening of medicinal plants used in South African traditional medicine for genotoxic effects. Toxicol. Letters, 143:195–207.

Farmacopea Argentina, 2003. Vol. 1, 7th ed. Buenos Aires, Ministry of Health,

Foyaca-Sibat, H., Ibañéz, V., Awotedu, A.A., 2001. Acute renal failure due to herbal medicine intoxication in acquired neuromyotonia. [Online] Available at:http://www.uninet.edu/cin2001-old/paper/ ibanez/foyaca.html. Accessed 10 July 2010.

Gaidamashvili, M., van Staden, J., 2006. Prostaglandin inhibitory activity by lectin-like proteins from South African medicinal plants. S. Afr. J. Bot.,, 72: 661–663.

Ghisalberti, E.L., 2000. *Lantana camara* L. (Verbenaceae). Fitoterapia, 71 (5): 467-486.

Inada, A., Nakanish, T., Tokuda, H., Nishino, H., Iwasma, A., Sharma, O.P., 1995. Inhibitory effects of lantadenes and related triterpenoids on Epstein-Barr virus activation. Planta Med., 61: 558-559.

Kaplan, G., Post, F.A., Moreira, A.L., Wainwright, H., Kreiswirth ,N., Tanverdi, M., Mathema, B., Ramaswamy, S.V., Walther, G., 2003. Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity. Infect. Immun., 71(12): 7099-7108.

Koorbanally, C., Crouch, N.R., Mulholland, D.A., 2006. The phytochemistry and ethnobotany of the southern African genus *Eucomis* (Hyacinthaceae: Hyacinthoideae) Phytochemistry: Advances in Res., 69-85.

Luyckx, V.A., Naicker, S., 2008. Acute kidney injury associated with the use of traditional medicines. Nat. Rev. Nephrol., 4: 664-671.

McGaw, L.J., Eloff, J.N., 2005. Screening of 16 poisonous plants for antibacterial, antihelminthic and cytotoxicity activity in vitro. S. Afr. J. Botany, 71: 302-306.

McGaw, L.J., Jager, A.K., van Steden, J., 2000. Antibacterial, antihelmintic and anti-amoebic activity in South African medicinal plants. J. Ethnopharmacol., 72(1-2):247-263.

McGaw, L.J., Steenkamp, V., Eloff, J.N., 2007. Evaluation of *Athrixia* bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. Ethnopharmacol., 110(1):16-22.

McGaw, L.J., Van der Merwe, D., Eloff, J.N., 2007. In vitro anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African ethnoveterinary medicine. Vet. Journal, 173:366–372.

Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E., McLaughlin, J.L., 1982. Brine shrimp: a convenient general bioassay for active plant constituents. J. Med. Plant Res., 45: 31–34.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.

Neuwinger, H.D., 2000. African Traditional Medicine. A Dictionary of Plant Use and Applications. Medpharm Scientific Publishers, Stuttgart, ISBN 3-88763-086-6.

Notten, A., Kirstenbosch, N.B.G., 2002. *Eucomis autumnalis* (Mill.) Chitt Family: Hyacinthaceae (Hyacinth Family) [Online] Available at:http://www.plantzafrica.com/ frames/plantsfram.htm

Oshima, Y., Cordell, G.A., Fong, H.S., 1986. Studies on Zoapatle, III. Flavonoid Glycosides from *Montanoa tomentosa* ssp. Tomentosa. J. Nat. Prod., 49 (3):552–553.

Plumb, J.A., Milroy, R., Kaye, S.B., 1989. Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. Cancer Res., 49:4435-4440.

Pooley, E., 1993. The Complete Field Guide to Trees of Natal Zululand and Transkei. (1st edn). Natal Flora Publication Trust. C/o Natal Herbarium Botanic Gardens Road, Durban 4001. pp. 512.

Samie, A., Obi, C.L., Bessong, P.O., Namrita, L., 2005. Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. Afr. J. Biotechnol., 4(12):1443-1451.

Smolinske, S.C., 2005. DABAT Herbal Product Contamination and Toxicity J. Pharm. Pract., 18(3):188-208.

Tu, C., Koh, W.Y., 2010. Using Bioequivalence Approach to Assess Toxicity Effect of Herbal Products, Tyrel™ and Rumbion™. Am. J. Pharmacol. Toxicol., 5 (2): 109-111.

van Staden, J., 1981. Constituents of *Hypoxis rooperi*, a valuable medicinal plant in South Africa. Deutsche Apotheke Zeitung, 33: 460-464.

Van Wyk, B.E., Gericke, N., 2000. People's Plants: A Guide to Useful Plants of Southern Africa. Briza Publications, Pretoria, ISBN 978-1-875093-19-9.

Vieira, R.H.S., Rodrigues, D., Goncalves, F.A., Menezes, F.G.R., Aragao, J.S., Sousa, O.V., 2001. Microbicidal effect of medicinal plant extracts (*Psidium guajava* Linn. and *Carica papaya* Linn.) upon bacteria isolated from fish muscle and known to induce diarrhea in children. Rev. do Instituto de Med Tropical de Sao Paulo. 43(3):145-148.

Watt, J.M., Breyer-Brandwijk, M.G., 1962. The medicinal and poisonous plants of southern and eastern Africa, E.& S. Livingstone Ltd., Edinburgh and London.

WHO, World health Organization, 2007. WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues. World Health Organization Publications, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland. pp. 118.

CHAPTER 8

GENERAL CONCLUSIONS AND RECOMMENDATIONS

8.1. General conclusions

Based on the results of this study, it is concluded that

- The increasing rates of multi-drug resistant strains and emerging resistance of *E. coli* and *Salmonella* spp. to penem drugs and other antibiotics, regrettably may lead to fewer options of effective antimicrobials in the management of infections caused by the pathogens.
- Molecular analysis showed that beta-lactamase enzymes ($bla_{CMY-2,}$ $bla_{SHV-1,}$ bla_{TEM-20} and bla_{TEM-1} genes) and class 1 integron *(Int1*) genes occurred significantly among the *Salmonella* spp.
- Virulence of *Salmonella* spp. may be attributed to the virulence genes identified among the *Salmonella* strains; namely *flic*-flagellin H1, *invA*-invasion, *sefA*- fimbrial antigen while *aggR*- transcriptional activator for EAEC aggregative adherence, fimbria I expression, *eaeA-E. coli* attaching and effacing and LT- heat-labile enterotoxin found among the *E. coli* isolates were the main indices of virulence.
- Some of the diarrhoegenic *E. coli* isolated from apparently asymptomatic subjects expressed some virulence genes at frequency as high as seen in cases, wh*ich was* an indication that asymptomatic individuals could serve as reservoirs of pathogenic strains of enteric

bacteria and may play a role in the spread and acquisition of virulence genes.

- Ethnobotanical survey showed that traditional medicine was a popular and widely used system in the rural setting of ORTDM and was the only system available in many parts of the study areas.
- Among the commonly used plants in traditional medicine, *A. arborescens*, *A. striata, C. uncinulata, E. autumnalis* and *P. guajava* exhibited antimicrobial properties and antibacterial activity was higher in the ethyl acetate fractions of the methanol extracts than other solvents employed.
- Further fractionation and purification of bioactive components of the ethyl acetate fraction of *C. uncinulata* yielded an isolated compound, glycosylated oleanolic acid, a sugar with fatty acyl moiety, which had considerable antibacterial activities.
- Cytotoxicity testing of the medicinal plants indicated that the methanol extracts of *E. autumnalis* were more cytotoxic than the methanol extracts and solvent fractions of other plants investigated, even though it had strong antibacterial activities. The use of *E. autumnalis* may therefore be fraught with risk of disagreeable side effects. The *Aloe* spp. studied were non toxic to cell lines, indicating strong potentials for drug development.

8.2. General recommendations

The under-listed recommendations are made against the backdrop of the findings of the study.

- Periodic studies on antimicrobial susceptibility or resistance patterns of E*. coli* and *Salmonella* spp. should be undertaken to provide updated information for empiric management of patients.
- Public awareness campaigns or educational programs for traditional health care practitioners on the cytotoxic effects of some plants should be fore-grounded.
- Extensive studies on the structural elucidation of the active compounds of the medicinal plants, including their effects on physiological, immunological and haematological parameters are necessary to further gauge their safety and potentials as candidate templates for drug design and discovery.
- The administration of antibiotics, without results, on susceptibility testing may be fraught with risk due to increasing resistance profiles of *E. coli* and *Salmonella* spp. Therefore, antimicrobial therapy should be predicated on laboratory results of antibiograms.
- Phylogenetic studies of *E. coli* and *Salmonella* spp. from different environmental and clinical sources, including correlation with virulence markers/ genes are recommended for a better understanding of their molecular epidemiology and pathogenicity.

APPENDICES

APPENDIX 1A

Faculty of Health Sciences

Department of Medical Microbiology,
Nelson Mandela Drive,
Private Bag X1, Mthatha 5117
Tel/Fax: 047 502 2301

VOLUNTEER INFORMATION SHEET

TITLE OF PROJECT: "*MOLECULAR CHARACTERIZATION, ANTIBIOGRAMS AND ANTIBACTERIAL ACTIVITIES OF SELECTED MEDICINAL PLANTS AGAINST SOME ENTERIC PATHOGENS"*

Hello! My name is Mary Bisi-Johnson and I would like to ask you for a little of your time to explain something to you, and ask you to please assist us in a research work that we are doing. As we discuss the information below please feel free to ask any questions.

In South Africa, a substantial number of deaths due to diarrhoeal diseases have been reported. Some studies have implicated some of these enteric pathogens we are studying in cases of diarrhoea in people across ages. By doing this study, we will be able to know the germs that is making people sick of diarrhoea. We are at present doing a study that will be looking at the use of certain antibiotics/medicines and local herb in the community and how it affects the germs that infect people. We want to see if there is an increase in germs that will not be treated by antibiotics/medicines that we normally use and see if the local herb will be able to help. This will help in the control and prevention of these diseases.

We would like to make sure that all the information we have about you is correct and then ask a few more questions about use of antibiotics. We will keep this information confidential, no one else will know that it is about you and all summaries or publications will only refer to group data.

You can make the decision entirely on you own and none of us can force you to take part. If you decide to answer some of these questions, you may also change your mind at any time.

You do not have to agree, and if you decide not to be involved it will not change the way you are treated in the hospital, and your doctor will not do anything differently.

Thank you for your time. Once you have asked any questions you may have, there is a form you need to sign if you agree to take part.

Investigators details:

Mrs. Mary Adejumoke Bisi-Johnson	Contact No: 0766813494
Prof. CL. Obi	Contact No: 047 502 2260/4

Date:

APPENDIX 1B

Faculty of Health Sciences

Department of Medical Microbiology,
Nelson Mandela Drive,
Private Bag X1, Mthatha 5117
Tel/Fax: 047 502 2301

VOLUNTEER INFORMATION SHEET

TITLE OF PROJECT: "*MOLECULAR CHARACTERIZATION, ANTIBIOGRAMS AND ANTIBACTERIAL ACTIVITIES OF SELECTED MEDICINAL PLANTS AGAINST SOME ENTERIC PATHOGENS"*

Molo!, Igama lam ndingu Mary Bisi-Johnson, ndicela okokuba undiphe imizuzu embalwa kwixesha lakho onalo, ndinqwenela ukukucacisela nto ithile, ndikwakucela ukuba usincede kweli phulo siliqulunqayo. Xa sicacisa lo mba ongezantsi, uvumelekile ukuba ungabuza imibuzo.

Emzantsi Afrika, Isibalo esiphezulu sabantu abasweleke ngenxa yesifo Sotyatyazo sithe sabikwa. Ezinye izifundo zithe zaveza ukuba ezintsholongwane esizifundayo zolutyatyazo zikho kubo bonke abantu, ukususela komncinci ukuyakutsho komdala. Ngokwenza ezizifundo, sizakuyazi intsholongwane ebangela ukuba abantu bagule sesisifo sotyatyazo.

Okwangoku senza izifundo ezizakuthi zijonge ukuba leliphi ichiza/iyeza okanye nokuba sesiphi isityalo sasekuhlaleni nokuba liyichaphazela kanjani lentsholongwane esulela abantu. Sifuna ukubona ukuba iintsholongwane ezingenakunyangeka ngalamayeza ziyenyuka kusini na?, okanye Isityalo sasekuhlaleni singanceda na!!. Oku kunganceda Ekulawuleni nasekunqandeni ezi zifo.

Singacela ukuqinisekisa ukuba zonke inkcukacha esinazo ngawe ziyinyaniso, siphinde sibuze eminye imibuzo ngokusetyenziswa kwamayeza/kwamachiza. Sizakuzigcina ezikcukacha ziyimfihlelo, Akukho namnye umntu ozakuyazi ukuba ezinkcukacha zingawe, kwaye wonke umpapasho uzakwenziwa ngesininzi.

Isigqibo sesakho wedwa sokuba uyafuna ukuthabatha inxaxheba kwezi ngxoxo. Ukuba ufuna ukuphendula inxalenye yalemibuzo, naleyo ikuwe, isigqibo sesakho.

Akunyanzelekanga ukuba uvume, kwaye ukuba awufuni ukubandakanyeka lonto ayizukutshintsha impatho oyifumana apha esibhedlele, noGqirha wakho akazukwenza tshintsho ngokwempatheko yokho.

Siyabulela ngexesha osiphe lona, Xa ubuzile imibuzo onganayo, kukho isivumelwano okufuneka usityikitye, ukuba uyavuma ukuthabatha inxaxheba.

Iingcukacha zabaphandi:

Mrs. Mary Adejumoke Bisi-Johnson	0766813494
Prof. CL. Obi	047 502 2260/4

UMHLA: ……..

APPENDIX 2A

Faculty of Health Sciences

Department of Medical Microbiology,
Nelson Mandela Drive,
Private Bag X1, Mthatha 5117
Tel/Fax: 047 502 2301

INFORMED CONSENT

TITLE OF PROJECT: *"MOLECULAR CHARACTERIZATION, ANTIBIOGRAMS AND ANTIBACTERIAL ACTIVITIES OF SELECTED MEDICINAL PLANTS AGAINST SOME ENTERIC PATHOGENS*"

Investigators:

Department of Medical Microbiology, WSU, Mthatha
Mrs. Mary Adejumoke Bisi-Johnson Contact No: 0766813494
Directorate of Academic Affairs & Research, WSU, Mthatha
Prof. CL. Obi Contact No: 047 502 2260/4

Hello! My name is Mary Bisi-Johnson and I would like to ask you some information. I will need to explain what it is we would like to do. You can help us a little if you listen and tell us what you think.

We want to ask you or your mother/father/guardian some questions about you. We will ask questions about recent history of diarrhoea and about what medicines you took. We want to try and see if the germs we are studying (*Salmonella* and *E. coli*) are found in your stool and know the antimicrobial profile and the virulence factors. This is to enable us know whether the germs are causing more sickness in South Africa and if they are becoming more difficult to treat.

Would it be alright for us to ask the above questions? When we work with this information we would not require your name.

I have read and/or I understand the contents of the information sheet and understand that I have been invited to participate, that my agreeing is fully voluntary, and that I can withdraw at any time.

Consent given:	Date:
Witness: A	Date:

APPENDIX 2B

Faculty of Health Sciences

Department of Medical Microbiology,
Nelson Mandela Drive,
Private Bag X1, Mthatha 5117
Tel/Fax: 047 502 2301

INFORMED CONSENT

TITLE OF PROJECT: *"MOLECULAR CHARACTERIZATION, ANTIBIOGRAMS AND ANTIBACTERIAL ACTIVITIES OF SELECTED MEDICINAL PLANTS AGAINST SOME ENTERIC PATHOGENS*"

Investigators:

Department of Medical Microbiology, WSU, Mthatha
Mrs. Mary Adejumoke Bisi-Johnson Contact No: 0766813494
Directorate of Academic Affairs & Research, WSU, Mthatha
Prof. CL. Obi Contact No: 047 502 2260/4

Molo!, Igama lam ndingu Mary Bisi-Johnson, ndicela ukukubuza Imibuzo nje embalwa. Kuzakunyanzeleka ndikucacisele ukuba yintoni na le ndifuna siyenze. Ungasinceda ngokuba simamelane kwaye uyitsho into oyicingayo.

Sicela ukubuza, wena No-Mama/No-Tata/Umniki Ncedo kwimpilo(Unomakhaya) wakho imibuzo ngempilo yakho nesigulo eso onaso. Sizakubuza imibuzo ngesigulo onaso nangamayeza obuwathabatha phambi kokuba uze Esibhedlele. Sifuna ukuzama, sibone ukuba intsholongwane le Siyifundayo/Siyihlolayo ayinguye na unobangela wezigulo esinazo apha emzantsi Afrika.

Ingaba sivumelekile ukukubuza lemibuzo sele ndiyikhankanyile apha ngasentla, Xa sisebenza ngezinkcukaca asizukulibiza/ukulisebenzisa igama lakho.
Ungaxhalabi xa uqonda ukuba kukho umbuzo ongacacelanga ukuwuphendula, akukho nento ezakujika ngempatho oyifumana kugqirha.

Ndiyifundile/Ndiyawuqonda umxholo oqulethwe yile-ncwadilkwaye ndiyavuma ukuba ndimenyiwe ndithabathe inxaxheba, kwaye ukuvuma kwam sisigqibo endizithabathele sona ngokokwam kwaye ndingarhoxa nangaliphi ixesha ndithanda.

IMVUME:	UMHLA:
INGQINA:	UMHLA:

APPENDIX 3

SURVEY INFORMATION FROM TRADITIONAL HEALERS ON HERBAL PLANTS IN TRADITIONAL TREATMENT OF DIARRHOEA

DATE OF VISIT: PLACE OF VISIT:
NAME OF TRADITIONAL HEALER:
CONTACT ADDRESS & NO:

Department of Medical Microbiology, WSU, Mthatha
Mrs. Mary Adejumoke Bisi-Johnson Contact No: 0766813494

i) Do you treat Diarrhea? Yes ☐ No ☐

ii) Do you treat some of other diseases linked to diarrhea? ☐ Yes ☐ No

iii) Do you treat both males and females? Yes ☐ No ☐

iv) Which age group do you treat for diarrhoea?

Children	Young adult	Adult

v) Do patients try self-treatment of diarrhea before consulting you? _____

vi) How effective is your treatment of diarrhea?_____

_____

_____

vii) Do you experience cases where patients show adverse reactions to your treatment? Yes ☐ No ☐

viii) How do you feel about patients going to hospitals or clinics for further or (alternative) treatment?_____

_____

ix) Which plant/plants do you use for the treatment of diarrhea?

_____

x) Do you use cultivated plants for the treatment of diarrhea? Yes ☐ No ☐

xi) Which plant parts do you use?

Leaves	Stem	Tree bark	Whole plant	Root	Tuber	Combination

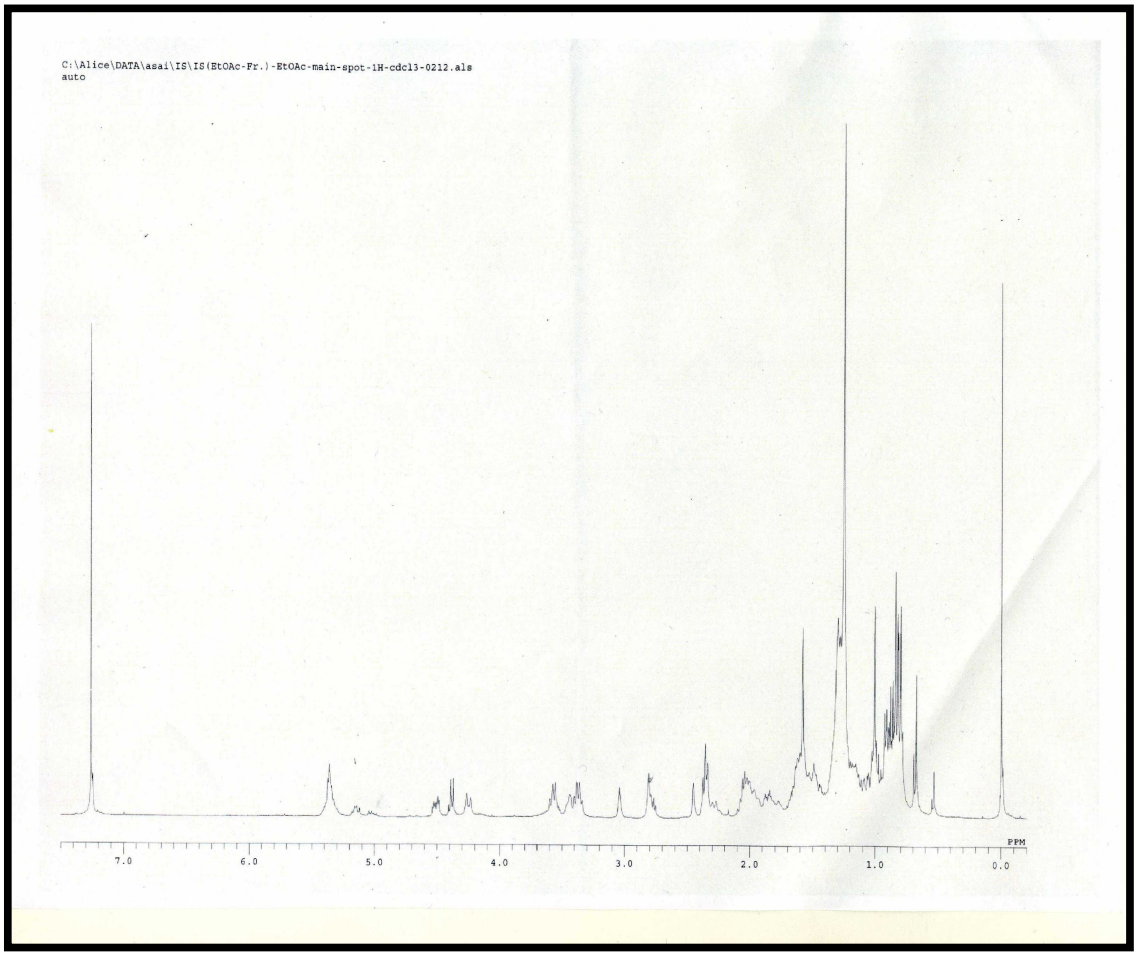
xii) How did you learn about the plant? From parent or during training_____

xiii) What dosage/quantity do you give you patient?_____

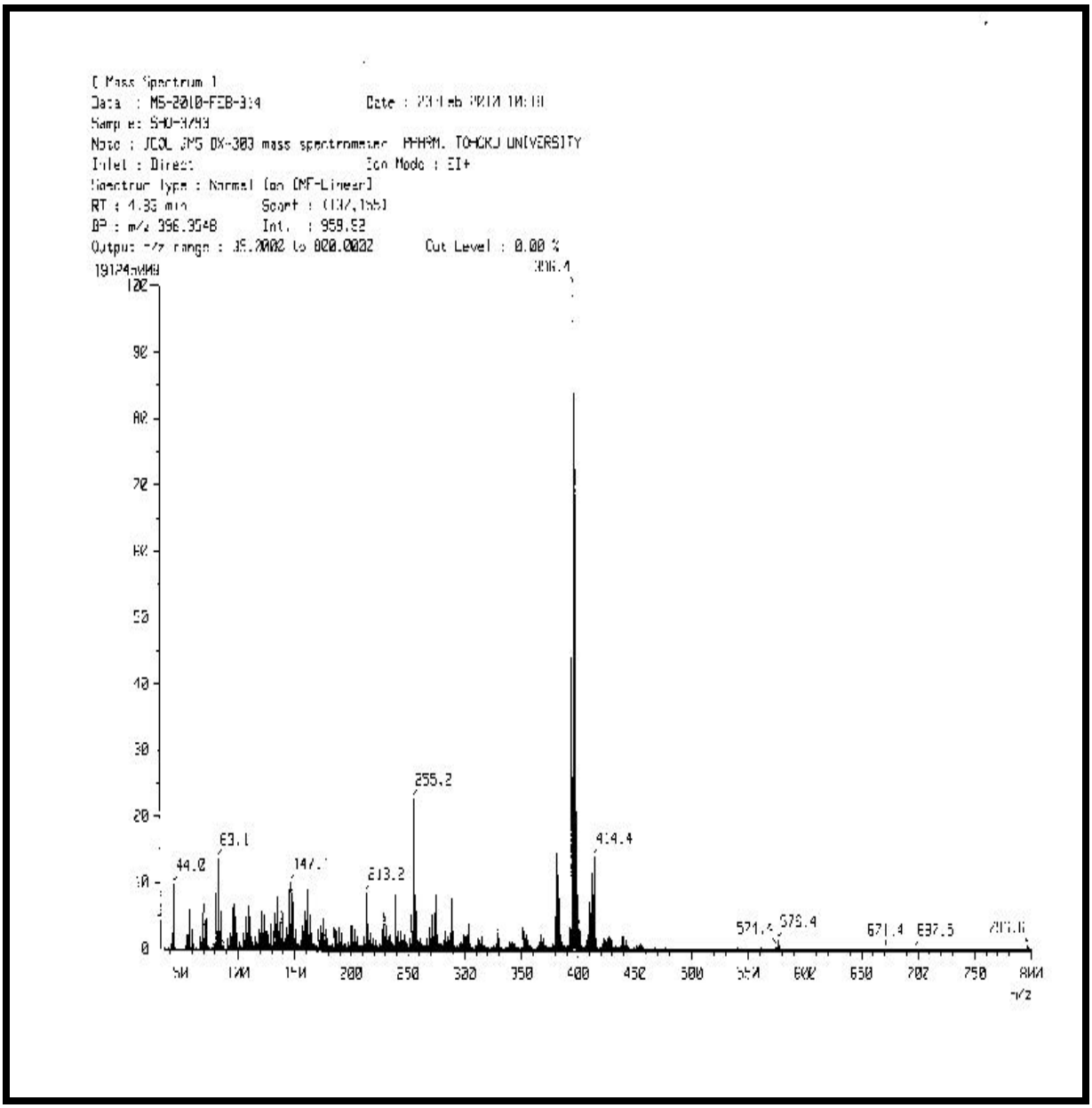
xiv) For how many days do they take the herb?_____

xv) How do you prepare your medication?

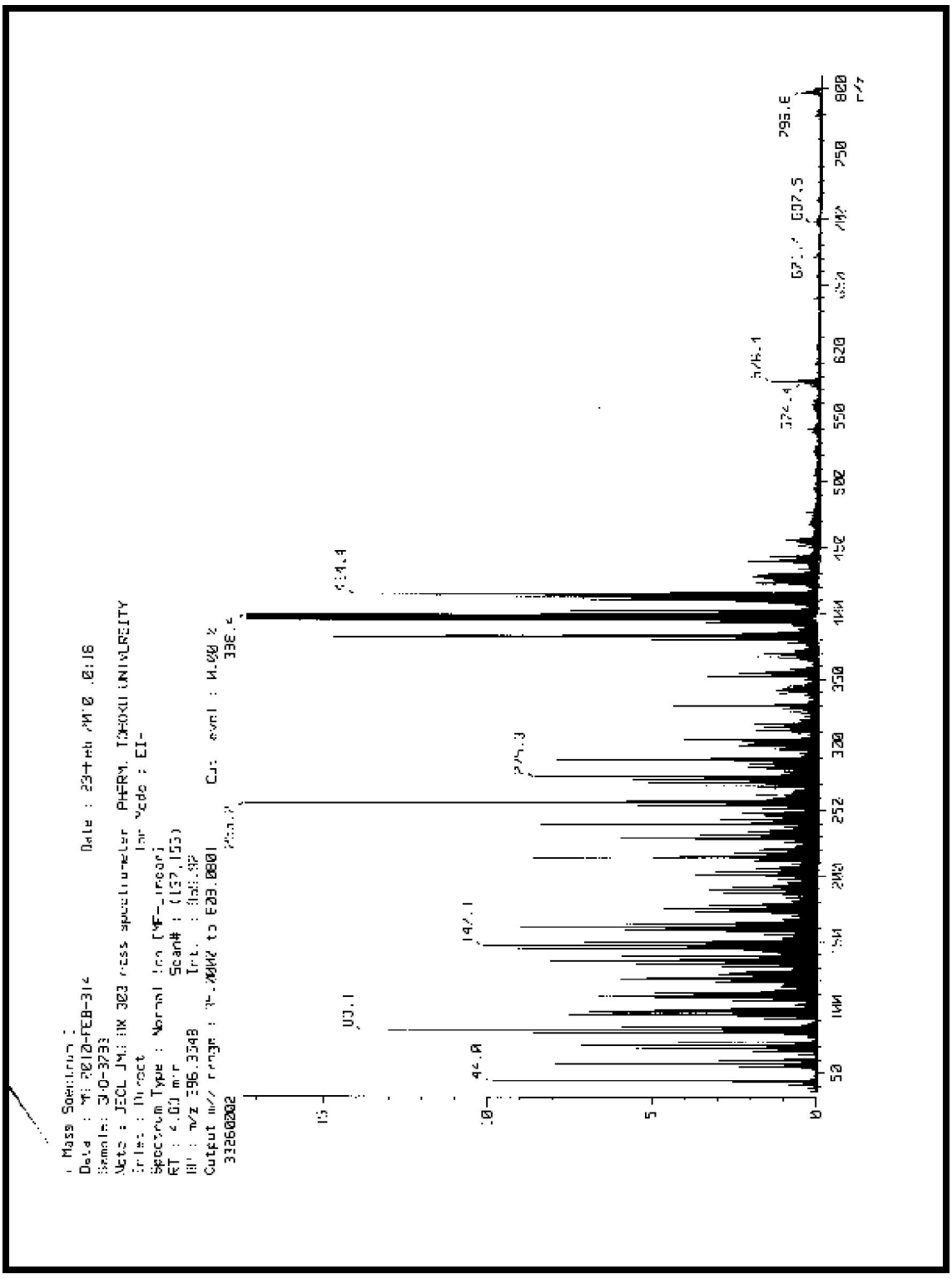
Appendix 4A. NMR spectrum for the isolated compound (SSC3-9-10).



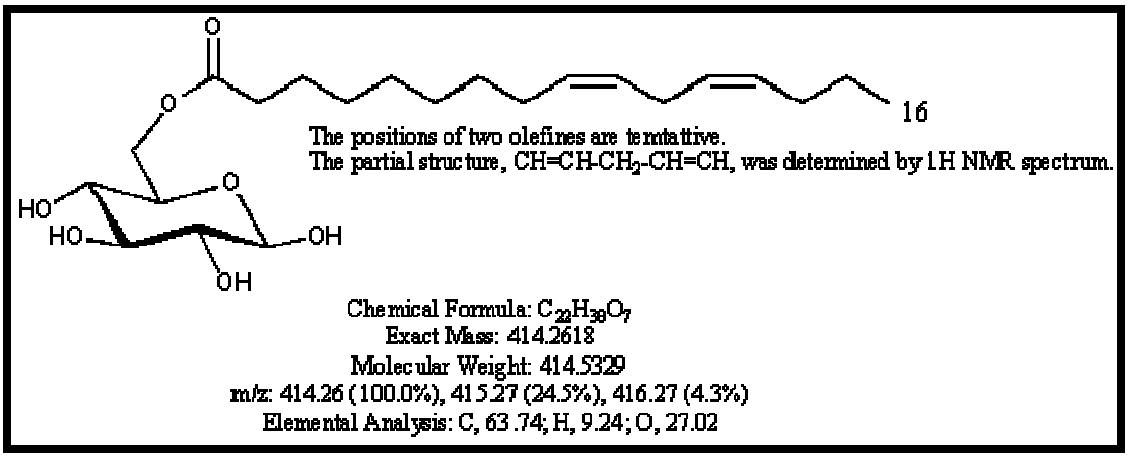
Appendix 4B. Mass spectrum 1 for the isolated compound



Appendix 4C. Mass spectrum 2 for the isolated compound



Appendix 4D. Structure of the isolated compound



The positions of two olefines are temtattive.
The partial structure, CH=CH-CH_2-CH=CH, was determined by 1H NMR spectrum.

Chemical Formula: $C_{22}H_{38}O_7$
Exact Mass: 414.2618
Molecular Weight: 414.5329
m/z: 414.26 (100.0%), 415.27 (24.5%), 416.27 (4.3%)
Elemental Analysis: C, 63.74; H, 9.24; O, 27.02