

PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL TESTING OF SELECTED
NAMIBIAN MEDICINAL PLANTS AGAINST LABORATORY AND CLINICAL BACTERIA

THAT CAUSE DIARRHOEA

A THESIS SUBMITTED IN FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

OF

THE UNIVERSITY OF NAMIBIA

BY

A.M.N. IIKASHA

200919563

AUGUST 2016

MAIN SUPERVISOR: DR D. MUMBENGEGWI

CO-SUPERVISORS: DR R. BOCK & PROF I. QUAYE

ABSTRACT

Medicinal plants are rich in a wide variety of bioactive compounds associated with various ailments; including antibacterial activity and some of them have been used worldwide in traditional settings to treat diarrheal diseases. However evidence on safety and efficacy of some of these traditional medicinal plants is lacking, preventing their mainstream use. This study aimed at determining the antibacterial activity of *Boscia albitrunca*, *Ziziphus mucronata*, *Combretum apiculatum*, *Solanum linnaeanum* and *Terminalia sericea* against clinical and laboratory diarrheal pathogens and screen selected plants for the presence of major phytochemical compound. Plant parts (bark, roots and twig) were collected from Kunene region and extracted using aqueous and organic solvents and screened for the presence of triterpenoids, saponins, flavonoids, steroids, coumarins, tannins and alkaloids by thin layer chromatography (TLC). Antibacterial activities of the organic and aqueous extracts were determined against laboratory strains of *Escherichia coli*, *Shigella boydii*, *Staphylococcus aureus* and *Listeria monocytogenes* and clinical strains of *Salmonella*, *E. coli* and *Shigella*, by disc diffusion method at three different concentrations (250, 500 and 1000 µg/ml). The average inhibition zones were determined on Mueller- Hinton agar plates. Phytochemical screening revealed higher presence of antibacterial compounds triterpenoids, saponins, flavonoids, steroids, coumarins and alkaloids in organic extracts and tannins in aqueous extracts. The organic extracts of *T. sericea* showed broad spectrum antibacterial activity against both clinical and laboratory diarrheal pathogens. The highest antibacterial activities at the lowest concentration were observed from organic extracts of *S. linnaeanum* against laboratory *L. monocytogenes* (15.5± mm, n=3) and *C. apiculatum* against clinical *Salmonella* (12 ± mm, n=3) at 250 µg/ml. In aqueous extracts, *C. apiculatum* showed stronger antibacterial activity against clinical *Shigella* (12 ± mm, n=3) and *T. sericea* against

laboratory strains of *E. coli* and *Listeria monocytogenes* ($9 \pm \text{mm}$, $n=3$) at $500 \mu\text{g/ml}$. Ampicillin showed antibacterial activity against laboratory strains of *S. boydii* and *S. typhi* however it showed no antibacterial activities against clinical strains of *Shigella* and *Salmonella*. Organic extracts of *T. sericea* and *C. apiculatum* also showed a lowest Minimum inhibitory concentration (MIC) of $250 \mu\text{g/ml}$ against *E. coli* and *Salmonella* respectively. Minimum inhibitory concentration of $62.5 \mu\text{g/ml}$ was recorded for *T. sericea* and *C. apiculatum* organic extracts against laboratory strains of *S. aureus* and *E. coli* respectively. Organic extracts of *T. sericea* and *C. apiculatum* showed an MIC of $250 \mu\text{g/ml}$ against clinical *E. coli* and *Salmonella* respectively. The study confirmed the possibility of using these plant extracts to develop alternative medicine to treat diarrheal pathogens.

TABLE OF CONTENT

ABSTRACT	ii
ACKNOWLEDGEMENTS	xiv
DECLARATION	xv
DEDICATION	xvi
CHAPTER 1: INTRODUCTION	1
1.1 General introduction: Infectious diseases and diarrheal diseases	1
1.2. Statement of the Problem	2
1.4 Significance of the study	3
1.5 Limitations of the study	3
CHAPTER 2: LITERATURE REVIEW	5
2.1 Microorganisms	5
2.1.1. Acute watery diarrhea	8
2.1.2. Bloody diarrhea (BD)	8
2.1.3. Persistent diarrhea (PD)	9
2.2 Individuals at risk of getting diarrheal diseases	9
2.3 Treatment options and prevention	10
2.3.2. Supplemental zinc therapy	11
2.3.3. Antidiarrheal drugs	11
2.3.4. Antimicrobial therapy	12
2.4 Prevention	14
2.5 Bacterial resistance: a draw back in infectious diarrhea control	14
2.6 The use of Medicinal plants	17
2.7 Phytochemical compounds associated with antidiarrheal properties of medicinal plants	20
2.7.1 Alkaloid	20
2.7.2 Flavonoids	21
2.7.3 Triterpenoids	22
2.7.4 Tannins	23
2.7.5 Saponins	23
2.10 Diarrheal disease causing bacteria	26
2. 10. 1 <i>Shigella boydii</i> (<i>S. boydii</i>)	26
2.10.2. <i>Salmonella typhi</i>	27
2.10.3 <i>Escherichia coli</i> (<i>E. coli</i>)	28

2.10.4 <i>Listeria monocytogenes</i>	28
2.10.5 <i>Staphylococcus aureus</i>	29
2.12 Medicinal plants used in traditional setting in Namibia to treat diarrheal diseases	30
2.12.1 <i>Ziziphus mucronata</i>	30
2.12.2 <i>Combretum apiculatum</i>	31
2.12.3 <i>Terminalia sericea</i>	31
2.12.4 <i>Boscia albitrunca</i>	32
2.12.5 <i>Solanum linnaeanum</i>	33
CHAPTER 3: MATERIALS AND METHODS	34
3.1 Research design	34
3.2. Plants collection and processing	34
3.3 Solvent extraction	35
3.4 Phytochemical screening	36
3.5 Antibacterial assay on Bacterial laboratory pathogen strains	42
3.5.7 Determination of Minimum Inhibitory Concentration by broth dilution method (MIC)	45
3.6 Clinical stool sample collection and bacteria isolation	46
3.7 Media preparation	48
3.11 Data analysis	52
3.12 Research ethics	52
CHAPTER 4: RESULTS	53
4.1 Effect of solvents on percentage yield from plant extracts	53
4.2 Screening for selected phytochemical classes	53
4.3 Antibacterial activity of the plant extracts on laboratory strains of pathogenic bacteria	55
4.4 Clinical description of collected stool samples	64
4.5 Biochemical test confirmation	64
4.6 Antibacterial assay on isolated clinical strains	66
CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS	84
CHAPTER 7: REFERENCES	86
APPENDIX B	107
APPENDIX C	109
APPENDIX D	111

LIST OF ABBREVIATIONS

HIV- Human Immunodeficiency Virus

AIDS- Acquired Immune Deficiency Syndrome

UNICEF- United Nations Children's Fund

WHO- World Health Organization

MoHSS- Ministry of Health and Social Services

TLC- Thin Layer Chromatography

MIC- Minimum Inhibitory Concentration

AID- Acute Infectious Diarrhea

CT- Cholera Toxin

HUS- Hemolytic Uremic Syndrome

VTEC- Vero cytotoxin – producing *Escherichia coli*

BD- Bloody Diarrhea

PD- Persistent Diarrhea

ORT- Oral Rehydration Treatment

ORS- Oral Rehydration Solution

WGO- World Gastro Organization

RDA- Recommended Dietary allowance

TMP-SMX- Trimethoprim-Sulfamethoxazole

STEC- Shiga toxin-producing” *E. coli*

CDC- Center of Disease Control

NCCLS- National Committee Clinical Laboratory Standards

MAC- MacConkey

XLD- Xylose Lysine Desoxycholate agar

NBRI- National Botanical Research Institute of Namibia for scientific identification. Ministry of

MET-Environment and Tourism of Namibia

LIST OF FIGURES

Figure 1. Hilleni Amutenya a traditional knowledge holder from the northern part of Namibia collecting medicinal plant parts of <i>Boscia abitrunca</i> roots and <i>Terminalia seresia</i> leaves.	19
Figure 2: Examples of Alkaloids isolated from plants.	21
Figure 3: Examples of major classes of flavonoids isolated from plants.	22
Figure 4: Example of triterpenoids	22
Figure 5: Example of Tannins from plants.	23
Figure 6: Example of Saponins.....	24
Figure 7: A branch of <i>Ziziphus mucronata</i> . Source: Mazibuko, (2007).	30
Figure 8: A branch of <i>Combretum apiculatum</i> . Source: Masupa & Rampho, (2011).	31
Figure 9: A branch of <i>Terminalia sericea</i> . Source: Masupa (2012).	32
Figure 10: <i>Boscia abitrunca</i> . Source: Protected trees (2013).	33
Figure 10. A branch of <i>Solanum linnaeanum</i> . Source: https://www.google.com/search?q=Solanum+linnaeanum	33
Figure 13. Drying of different plant parts in the shade.	35
Figure 14: Alkaloid screening by TLC using Chloroform: Ethanol (9:1) as a mobile solvent	37
Figure 15: Saponin screening by TLC using Chloroform: methanol and water (7:3:1) as a mobile solvent.	39
Figure 16: Coumarin detection by TLC using Chloroform as a mobile solvent.	41
Figure 17: TLC plate viewed under Uv light at 360 nm.	42
Figure 18: Determination of MICs by broth dilution method from right to left.	46
Figure 19. Rectal swab and stool container used in stool specimen collection.	48
Figure 14. <i>S. boydii</i> growth inhibition by <i>T. sericea</i> plant extract on Muller hinton agar.	56

Figure 15. <i>S. boydii</i> growth inhibition by tetracycline (positive control) on XLD agar.	56
Figure 16: Antibacterial activity of aqueous extracts of <i>T. sericea</i> after 24 hours incubation.....	57
Figure 17: Antibacterial activity of organic extracts of <i>B. albitrunca</i> , <i>Z. mucronata</i> , <i>C. apiculatum</i> , <i>T. sericea</i> and <i>S. linnaeanum</i> against <i>E. coli</i> after 24 hours incubation.	58
Figure 18: Antibacterial activity of organic extracts of <i>B. albitrunca</i> , <i>Z. mucronata</i> , <i>C. apiculatum</i> , <i>T. sericea</i> and <i>S. linnaeanum</i> against <i>S. aureus</i> after 24 hours incubation.	59
Figure 19: Antibacterial activity of organic extracts of <i>B. albitrunca</i> , <i>Z. mucronata</i> , <i>C. apiculatum</i> , <i>T. sericea</i> and <i>S. linnaeanum</i> against <i>L. monocytogenes</i> after 24 hours incubation..	60
Figure 20: Antibacterial activity of organic extracts of <i>B. albitrunca</i> , <i>Z. mucronata</i> , <i>C. apiculatum</i> , <i>T. sericea</i> and <i>S. linnaeanum</i> against <i>S. boydii</i> after 24 hours incubation.	61
Figure 21: Antibacterial activity of organic extracts of <i>S. linnaeanum</i> against <i>S. typhi</i> after 24 hours incubation.....	61
Figure: 22 MAC agar with a) <i>Salmonella</i> lactose negative colonies, b) <i>E. coli</i> lactose fermenting positive colonies, c) <i>Shigella</i> lactose positive colonies.....	64
Figure: 23. XLD agar with a) <i>Shigella</i> clear colonies, b) <i>E. coli</i> yellow colonies c) <i>Salmonella</i> pale-red colonies with black centers.	65
Figure 24: Antibacterial activity of aqueous extract of <i>T. sericea</i> against <i>Shigella</i> after 24 hours incubation.	66
Figure 25: Antibacterial activity of organic extracts of <i>Z. mucronata</i> and <i>C. apiculatum</i> against <i>Shigella</i> after 24 hours incubation	67
Figure; 26. <i>Shigella</i> growth inhibition by a) <i>C. apiculatum</i> organic plant extracts 1000 µg/ml b) Ampicilin (positive control) on Muller hinton agar.....	68

Figure 27 : Antibacterial activity of organic extracts of <i>B. albitrunca</i> , <i>Z. mucronata</i> , <i>C. apiculatum</i> and <i>S. linnaenum</i> against <i>Salmonella</i> after 24 hours incubation.	69
Figure 28: <i>Salmonella</i> growth inhibition by a) <i>C. apiculatum</i> organic plant extracts 250 µg/ml b) Ampicillin (positive control) on Muller hinton agar.....	69
Figure 29: Antibacterial activity of organic extracts of <i>B. albitrunca</i> , <i>Z. mucronata</i> , <i>C. apiculatum</i> and <i>T. sericea</i> against <i>E. coli</i> after 24 hours incubation.	70
Figure: 30. <i>E. coli</i> growth inhibition by a) <i>Z. mucronata</i> organic plant extracts 1000µg/ml b) Tetracycline (positive control) on Muller hinton agar.	70

LIST OF TABLES

Table 1: Causative pathogens for diarrheal diseases (WGO, 2008)	6
Table 2. Top 10 causes of death in Namibia in 2012 (Source (CDC, 2012).....	15
3.2.4.1.1 Antibacterial assay by disc diffusion method	43
3.2.4.1. 2 Preparation of Mueller-Hinton agar.....	43
3.2.4.1. 3 Preparation of plant extracts solution	43
3.2.4.1. 4 Inoculum preparation (growth method).....	44
3.2.4.1. 5 inoculation of test plate and application of discs to inoculated agar plates	44
3.2.7.2. 1 Biochemical test	50
Table 4. Effect of solvents on percentage yield from plant extracts.....	53
Table 5: Phytochemical screening analysis of aqueous plant extracts using TLC	54
Table 6. Phytochemical screening analysis of organic plant extracts using TLC	55
Table 7. Minimum inhibitory concentrations of <i>T. sericea</i> for aqueous extracts against different selected diarrhea causing bacteria.....	63
Table 8. Minimum inhibitory concentrations for organic plant extracts against selected diarrhea causing bacteria.....	63
Table 11 MIC of organic and aqueous plant extracts	73

LIST OF PUBLICATIONS

This work has been partially published in the form of peer-reviewed articles and conference poster presentations.

1. Iikasha, A. M. N., and Mumbengegwi, D. R. (2015). Are ethno medicinal plants viable alternative treatments for diarrheal diseases? Manuscript under review.
2. Iikasha, A. M. N., Eiman, H &. Mumbengegwi, D. R (2015). An investigation into the antimicrobial activity of pigmented secondary metabolites from *Streptomyces* from Windhoek soil. Manuscript under review.
3. Iikasha, A. M. N., Dushimemaria, F., Mbewe, M., Mumbengegwi, D. R., and Eiman, H. (2015). Antimicrobial and antiplasmodial activity displayed by actinomycetes isolated from Kavango and Rehoboth soils. Manuscript under review.

LIST OF CONFERENCES AND POSTERS

1. Iikasha, A. M. N., Mumbengegwi, Quaye, I., & Bock, R. (2014). An investigation into antibacterial activities of medicinal plants against diarrheal causing bacteria. Green medicine symposium. 30-31 July 2015 University of Namibia library auditorium.
2. Iikasha, A. M. N., Mumbengegwi, D. R. Quaye, I., & Bock, R. (2015). Are ethno medicinal plants viable alternative treatments for diarrheal diseases? Multi/ interdisciplinary research first annual conference. 4-6 November 2015. Hilton hotel, Windhoek.
3. Iikasha, A. M. N., Mumbengegwi, D. R. (2015). Ethnoveterinary use of medicinal plants ethanol extracts to combat animal infectious diseases. RUFORUN Conference, 27-29 August 2015. Safari hotel, Windhoek.
4. Iikasha, A. M. N., Eiman, H & Mumbengegwi, D. R (2015). An investigation into the antimicrobial activity of pigmented secondary metabolites from *Streptomyces* from Windhoek soil. National research symposium. National commission for Research Science and Technology (NCRST). 23-25 September 2015. Hilton hotel.

ACKNOWLEDGEMENTS

I wish to acknowledge the following individuals, institutions and programmes for their support and contribution to my studies: First above all I wish to thank God for countless blessings. Dr Davis Ropafadzo Mumbengegwi, a mentor and supporter, for investing time, his patience and energy into my professional growth and for always encouraging me to be the best student. Dr Ronnie Bock who helped shape me professionally by his guidance, patience and encouragement and Prof Isack Quaye for his support, patience and dedication toward shaping and enriching me with the use of advanced technology. I will forever be grateful to Dr Mumbengegwi, Dr Bock and Prof Isack Quaye for their inspirations, encouragements and interests in my studies and wellbeing.

Miss Florence Dushimemaria and who offered statistical guidance and for always being there to help make sense of my data.

Mr Munyaradzi Tambo, Mr Werner Embashu, Ms Iwanette Du Preez and Ms Hatago Stuurmann for their assistance in the laboratory, support and encouragement throughout the study period.

Biomedical research laboratory, Department of Biological sciences and Multidisciplinary Research Center (MRC) for hosting this research in their facilities.

The German Academic Exchange programme (DAAD) for providing me with a scholarship for funding this research.

DECLARATION

I, Albertina Mariina Ndinela Iikasha declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

No part of this report may be reproduced, stored in any retrievable system, or transmitted in any form, or by means (e.g. electronic, mechanical, photocopying, recording or otherwise) without the prior permission of the author, or the University of Namibia in that behalf.

I, Albertina Mariina Ndinela Iikasha grant the University of Namibia the right to reproduce this thesis in whole or in part, in any manner of format, which the University of Namibia may deem fit for any person or institution requiring it for study and research: providing that the University of Namibia shall waive this right in the whole thesis has been or is being published in a manner satisfactory to the University.

..... Date.....

DEDICATION

I wish to dedicate my work to my fellow Namibians, especially those in rural areas. To my family: Hileni Amutenya, Samir Shatri, Rahma Shatri, Tangeni Tulongeni, Lahja Iikasha, Late : Sakaria and Paulus Iikasha and to all those who encouraged me every step of the way.

CHAPTER 1: INTRODUCTION

1.1 General introduction: Infectious diseases and diarrheal diseases

Infectious diseases are illnesses that are caused by biological agents. These agents include viruses, bacteria, parasites and fungi. Infectious diseases can be classified as acute, chronic or lateral infections (National Institute of Allergy and Infectious Diseases, 2009). The following are examples of infectious diseases caused by infectious pathogens: cholera, pneumonia, dysentery, Acquired Immune- Deficiency Syndrome (AIDS), whooping cough, athlete's foot, diarrheal diseases, flu, genital herpes, meningitis, sinusitis, skin diseases, tuberculosis, urinary tract infections and vaginal infections, to name a few. Infectious diseases are the leading causes of human illness and death in human, mostly in developing countries (Vuuren, 2007; Yanling, Xin & Zhiyuan, 2013) and diarrhea is among the top 10 causes of mortality in the world, killing 1.5 million people globally (WHO, 2014). The rate of death from infectious and parasitic diseases is about 14 times higher in developing countries than in developed countries (Population Action International, 2011).

In Namibia, diarrheal diseases are among the top 10 causes of death (Center for disease controls and prevention (CDC), 2012). About ~70% of deaths in children between the ages of 1 – 5 in Namibia are due to pneumonia, diarrhea and HIV/AIDS (Ministry of Health and Social Services (MoHSS), 2008). Furthermore 518 cases of cholera were recorded in the northern part of Namibia (Kunene, Oshana and Omusati regions) with a total of 17 deaths (MoHSS, 2014).

This study was undertaken to investigate the antibacterial activity of selected plants used traditionally for treatment of diarrheal diseases in Namibia. This was done by screening for phytochemical compounds and determining the antibacterial activity of *Boscia albitrunca*, *Ziziphurus mucronata*, *Combretum apiculatus*, *Solanum linnaenum* and *Terminalia serecia*

Namibian plants against laboratory and clinical diarrheal pathogens. This enabled validation of those plants and determination of their effect against antidiarrheal pathogens. Phytochemical screening by thin layer chromatography (TLC) was carried out to create a chemical profile for these plants. Antibacterial screening was carried out to assess the effects of the plant extracts against the laboratory bacteria strains that cause diarrheal diseases. Further antibacterial assays were performed on clinical bacteria strains to determine the effect of the plant extracts against diarrheal pathogens isolated from patient stool samples collected from Katutura state hospitals and Okuryangava clinics in Windhoek. Finally the Minimum inhibitory concentration (MIC) of the five selected plants against diarrheal pathogens was performed to determine the lowest concentration of the plant extracts that inhibited the visible growth of a bacterium after overnight incubation.

1.2. Statement of the Problem

There is an increase in cases and deaths due to diarrheal diseases in Namibia especially in rural and resource poor areas; due to poverty and lack of access to available medicine and hospitals (CDC, 2012, Ministry of Health and Social Services (MoHSS), 2014). Therefore, most people in Namibia rely on ethno-medicinal plants used to treat diarrhea, however there is limited sufficient scientific evidence on the phytochemical compositions and antibacterial activities of most plants. This limits the potential use in developing alternative medicine to be used in primary healthcare to treat diarrheal diseases (Chinsemu & Hedimbi, 2010; Auala, Mumbengegwi, Du Preez, & Bock, 2012). Hence, the aim of this study was to screen selected Namibian medicinal plants for phytochemical compounds and antibacterial activities against laboratory and clinical diarrheal causing bacteria. Hence, this study was undertaken to screen selected Namibian medicinal plants

for phytochemical compounds and antibacterial activities against laboratory and clinical diarrheal causing bacteria.

1.3 Objectives of study

1. To identify and document, indigenous plants used medicinally against diarrhea in Kunene region, Namibia
2. To screen for classes of phytochemical compounds present in the selected plants.
3. To evaluate the plants extracts for their antibacterial properties against selected laboratory and clinical diarrheal bacteria.

1.4 Significance of the study

The study served to verify and document knowledge of selected plants use to treat diarrheal diseases in Namibia, by filling the knowledge gap on the use of selected traditional plants for the treatment of diarrheal diseases in Namibia. Furthermore, it serves as evidence to guide policy makers on the use of the selected plants as a primary health care option especially in resource poor settings and in rural communities. Findings of the study can be used to guide future research toward the development of antidiarrheal drugs from selected plants.

1.5 Limitations of the study

Not all the diarrhea pathogens were considered for antibacterial assay since the study only focused on *Salmonella typhi* ATCC unknown, *E. coli* ATCC25922, *S. aureus* ATCC25923, *S. boydii* ATCC0207 and *L. monocytogenes* ATCC13932 on laboratory strains. The plant extracts were only screened for phytochemical compounds such as: saponin, alkaloids, flavonoids,

triterpenoids, tannins, coumarins and steroids that are documented to be associated with antibacterial activity of plants. Furthermore, not all the diarrheal pathogens present in the stool samples were isolated and used in the antibacterial assay, since the study only focused on *Salmonella*, *E. coli* and *Shigella* in clinical stool samples. Finally, the clinical bacterial strains isolated and used for antibacterial assay were only identified to genus level no species identity was obtained.

CHAPTER 2: LITERATURE REVIEW

2.1 Microorganisms

Microbes are ubiquitous microscopic organisms which are found in most environments on the globe including in living organisms such as plants, animals as well as in the human body. Some microbes cause diseases in human, plants and animal, while others are essential for a healthy life and they formed a symbiotic relationship with human, animals and plants. Most microbes belong to one of four major groups: fungi, bacteria, viruses or parasites. Common words for microbes that cause diseases are “germs or pathogens”. Indeed pathogenic microorganisms are responsible for infectious diseases such as diarrhea worldwide (National Institution of Allergy and Infectious Diseases, 2009).

Diarrhea is a condition of the gastrointestinal tract which is generally described as an ailment characterized by having three or more liquid or loose bowel movements per day (Protiva *et al.*, 2012; WHO, 2013). It results in dehydration due to sever water loss. There are two general types of diarrhea: infectious and non-infectious diarrhea. Infectious diarrhea is caused by viruses, bacteria or parasites. It spread quickly from person to person, especially in places where people are exposed to poor sanitation. Non- infectious diarrhea can be caused by toxins produced during food poisoning), chronic diseases such as cystic fibrosis or the use of antibiotics such as ampicillin. Non- infectious diarrhea is not transmissible (Division of Public Health Services Communicable Disease Control Section, 2009). Infectious diarrhea is caused by pathogens such as those listed in Table1 (Salam *et al.*, 2012; World Gastroenterology Organization (WGO, 2008).

Table 1: Causative pathogens for diarrheal diseases (WGO, 2008)

Bacteria	Viruses	Parasites
<i>Vibrio cholera O1</i>	<i>Rotavirus</i>	<i>Blastocystis hominis</i>
<i>Vibrio cholera O139</i>	<i>Norovirus</i> (<i>Calisivirus</i>)	<i>Dientamoeba fragills</i>
<i>Vibrio oarahaemolyticus</i>	<i>Adenovirus</i> (serotype 40/41)	<i>Cyclospora cayetanensis</i>
<i>Escherichia coli</i>	<i>Astrovirus</i>	<i>Isospora belli</i>
<i>Plesiomonas</i>	<i>cytomegalovirus</i>	<i>Entamoeba histolytica</i>
<i>Aeromonas</i>	<i>Coronavirus</i>	<i>Giardia intestinalis</i>
<i>Bacteroides fragills</i>		<i>Cryptosporidium hominis</i>
<i>Campylobacter jejuni</i>		
<i>Campylobacter coli</i>		
<i>Campylobacter upsallensis</i>		
<i>nontyphoidal</i>		
<i>Salmonellae</i>		

<i>Clostridium difficile</i>		
<i>Yersinia enterocolitica</i>		
<i>Yersinia pseudotuberculosis</i>		
<i>Shigella species</i>		

Rotavirus is the most common cause of severe diarrhea and diarrheal mortality in children and it is responsible for about 40% of all hospital admissions (Allen *et al.*, 2010; WHO, 2009). Among all illnesses attributable to food borne transmission, 30% are caused by bacteria, 3% by parasites and 67 % by viruses (Stephanie, MCLaws and Ellis, 2013).

Diarrhea can also be differentiated in to three categories such as: acute, persistent or chronic diarrhea; based on the length of the diarrheal episodes. Acute gastroenteritis is the second to cardiovascular disease in causing death worldwide (Bryan, 2015), in children under four years old, especially in developing countries (Surawicz, 2012). Diarrhea that last for less than two weeks is called acute diarrhea. Diarrhea that last between two and four weeks is called persistent diarrhea. Chronic diarrhea lasts longer than four weeks (Ochoa & Surawicz, 2012; WHO, 2013). Most pathogens that cause diarrhea share a similar mode of transmission (from the stool of one person to the mouth of another person). This is known as fecal- oral transmission (WHO, 2009).

There are three main forms of acute infectious diarrhea (AID), all of which are life threatening and require different treatment courses (WHO, 2009). This includes:

2.1.1. Acute watery diarrhea

Acute watery diarrhea includes cholera and is associated with significant fluid loss and rapid dehydration in an infected individual. It usually last for several hours or days. The pathogens that cause acute watery diarrhea include: *Vibrio cholerae*, *Escherichia coli* and rotavirus. *Vibrio cholerae* affects the transport of water to the small intestine. It does so by secreting a toxin called cholera toxin (CT), which cause osmosis of water from the body into the digestive tract, leading to severe dehydration and ultimately diarrhea (WHO, UNICEF, 2009). Cholera is prevalent in Central and South America, Africa and Asia, though not in United Kingdom as it is usually confined to developing countries. Worldwide, 100 000- 300 000 cases of diarrhea are reported each year with more than 94% cases of cholera in Africa (Symington, 2011). *E. coli O157* is an uncommon cause of infectious gastroenteritis. It is important because it may be severe and sometimes fatal, particularly in infants, young children and elderly people. An important complication is hemolytic uremic syndrome (HUS) (Tidy, 2014). Although *Escherichia coli* strains are common in human intestine, most of them are harmless. However, Vero cytotoxin – producing *Escherichia coli* (VTEC) may cause severe diarrhea (Tidy, 2014).

2.1.2. Bloody diarrhea (BD)

Bloody diarrhea is often referred to as dysentery. It is marked by visible blood in the stools. Bloody diarrhea is associated with intestinal damage and nutrient losses in infected individuals. The most common cause of BD is *Shigella* species. This bacterium cause severe cases of diarrhea (WHO, 2013). Shigellosis (a diarrheal disease caused by *Shigella* species) is a well-known cause of diarrheal deaths (Panda, Patro, Sahoo, Bastia & Dutta, 2012). Another type of

dysentery is amoebic dysentery which is caused by parasitic protozoa called *Entamoeba histolytica*. Amoebic dysentery is transmitted through an oral fecal route (Tilak, 2013). Amoebic dysentery is the second leading cause of parasitic deaths in developing countries (Stanley, 2003).

2.1.3. Persistent diarrhea (PD)

Persistent diarrhea can be an episode with or without blood. It is most common in malnourished children as well as children with Acquired Immune Deficiency Syndrome (AIDS) (WHO, 2013). Persistent diarrhea has a high impact on pediatric morbidity and mortality rates in developing countries causing over 50% of diarrhea-related deaths in developing countries (Andrade & Fagundes-Neto, 2011).

2.2 Individuals at risk of getting diarrheal diseases

Diarrheal disease affects rich and poor, old and young, and those in developed and developing countries (Keusch, Fontaine, Bhargava, Pinto, Bhutta, Gotuzzo *et al.*, 2006) However, a strong relationship exists between poverty, an unhygienic environment. Poverty is associated with poor housing, crowding, dirty floors, lack of access to sufficient clean water or to sanitary disposal of fecal waste, cohabitation with domestic animals that may carry human pathogens and a lack of refrigerated storage for food, all of which increase the frequency of diarrhea (Keusch *et al.*, 2006; Tetteh, 2013). Poverty also restricts the ability to provide age-appropriate, nutritionally balanced diets or to modify diets when diarrhea develops so as to moderate and repair nutrient losses (Nguyen, Van, Hey, Gia & Weintraub, 2006). Worldwide, 780 million individuals lack access to improved drinking water and 2.5 billion people lack improved sanitation. Children who are malnourished and immune-compromised as well as people living with HIV are most at risk of life-threatening diarrhea (WHO, 2013). In Namibia, only 40.6% of people have access to treat the water which (WHO and UNICEF, 2012),

Namibia's most recent Millennium Development Goal report indicates high rates of access to improved water sources in urban areas, at 97 per cent, while in rural areas, 80 per cent of the population have access to an improved water source results (United Nations, 2012) however, diarrheal cases continue to rapidly increase in Namibia (MHOSS, 2008).

2.3 Treatment options and prevention

In the past decade, there have been major improvements in our knowledge base regarding the treatment of infectious diarrhea. Different diarrheal treatments with different modes of action are described in section 2.3.1 to 2.3.4.

2.3.1 Oral rehydration therapy

Oral rehydration therapy has been found to be one of the most important medical advances of the twentieth century (Wardlaw, Salama, Brocklehurst, Chopra and Mason, 2009). Oral rehydration therapy (ORT) is the administration of fluid by mouth. It includes the use of low-osmolality oral rehydration salts (ORS) (WGO, 2008). Oral rehydration solution is the fluid specifically developed for ORT and it helps to prevent dehydration that occurs as a result of diarrhea. ORT is the standard for efficacious and cost-effective management of acute gastroenteritis (World Gastroenterology Organization (WGO), 2008). A more effective, lower-osmolality ORS (with reduced concentrations of sodium and glucose, associated with less vomiting, less stool output, and a reduced need for intravenous infusions in comparison with standard ORS) has been developed for global use (WHO/ UNICEF, 2006). The hypotonic WHO-ORS is also recommended for use in treating adults and children with cholera. ORT consists of: Rehydration through which water and electrolytes are administered to replace lost water and maintenance fluid therapy (along with appropriate nutrition). ORS contains sodium (77mmol/ liter), chloride

(65 mmol/ liter) and glucose anhydrous (75 mmol/ liter), potassium (20 mmol/liter) and citrate (10 mmol/ liter) (WHO/ UNICEF, 2006; WHO, 2013; WGO, 2008).

2.3.2. Supplemental zinc therapy

Supplemental zinc therapy is recommended for all children with diarrhea. Zinc deficiency is widespread among children in developing countries (Asuquo, Georgewill, Nta, Enyidah, Umofia, and Deekae, 2012). Micronutrient supplementation treatment with zinc (20 mg per day until the diarrhea ceases) reduces the duration and severity of diarrheal episodes in children in developing countries. Supplementation with zinc sulfate (2 mg per day for 10–14 days) reduces the incidence of diarrhea for 2–3 months. It helps reduce mortality rates among children with persistent diarrheal illness. Administration of zinc sulfate supplements to children suffering from persistent diarrhea is recommended by the WHO (WGO, 2008). Zinc is involved in many aspects of cell metabolism and it is required for about 100 enzymatic activities. It also plays a great role in protein synthesis, cell division wound healing and DNA synthesis and development during pregnancy and childhood. Furthermore, zinc helps with the development of taste and smell (National institutes of Health, 2016). All children with persistent diarrhea should receive supplementary multivitamins and minerals each day for 2 weeks. These should provide as broad a range of vitamins and minerals as possible, including at least two recommended daily allowances (RDAs) of folate, vitamin A, zinc, magnesium, and copper (WHO, 2013; WGO, 2008).

2.3.3. Antidiarrheal drugs

Anti-motility agents and Anti-secretory agents are two major classes of antidiarrheal agents that are used in reducing the volume of the stool, frequency of diarrheal episodes and abdominal cramps that occur as a result of diarrhea. Anti-motility agents that are commonly used include

loperamide, morphine, codein and a diphenoxylate-atropine combination (Casburn-Jones, and Farthing, 2004). These agents act by increasing intestinal transit time and enhancing the potential for reabsorption of fluid and electrolytes. They have a modest effect on reducing fecal losses. Loperamide is usually the first line treatment in self-therapy. Due to the concern of safety and efficacy, the use of anti-motility agents is discouraged in infants by the WHO and American Academy of Pediatrics due to concerns over safety and efficacy in young children (Li, Grossman, & Cummings, 2007). However, there is limited clinical evidence for this concern. When loperamide is used together with other antibiotics, it was found to be safe and effective in treating bacillary dysentery (Casburn-Jones, and Farthing, 2004).

There is an ongoing search for the ideal anti-secretory agent that is, a drug that will directly inhibit secretory processes within the enterocyte. Intracellular signaling mechanisms were an initial pharmacological target, especially those related to calcium and the calcium binding protein calmodulin. Zaldaride maleate, a calmodulin inhibitor, has been evaluated in phase III randomized controlled trials but their further development into a drug of choice was future discontinued due to the lack of additional benefits when compared to standard anti-diarrheal agents (Ahmed, 2006).

2.3.4. Antimicrobial therapy

There is sufficient evidence to prove the effectiveness of antimicrobial agents in reducing the severity and duration of diarrheal episodes especially in cases of acute infectious diarrhea (Casburn-Jones, and Farthing, 2004). Antimicrobials are also useful in bacterial intestinal infections that cause systemic involvement. There are numerous antibiotics that have been studied in the treatment of infectious diarrhea. The WHO has recommended the use of empirical

antimicrobial therapy in the setting of febrile acute bloody diarrhea in young children (Diniz-Santos, Silva, and Silva, 2006).

Ampicillin and Trimethoprim-Sulfamethoxazole (TMP-SMX) were among the first line drugs that are effective in treating infectious diarrhea; due to their efficacy, safety and affordability (Diniz-Santos, Silva, and Silva, 2006). With the passing of years, outbreaks of infectious diarrhea caused by *Shigella* or *Salmonella* strains resistant to one or both of them have been reported from all continents. Although antibiotics such as tetracycline are cheap and have a broad antimicrobial spectrum; their use in pediatric patients is limited by permanent dental discoloration in children younger than eight years of age. Apart from microbial resistance, chloramphenicol was reported to cause uncomfortable posology and other risky side effects and this have contributed to its displacement as a good drug for the empirical treatment of acute diarrhea (Diniz-Santos, Silva, and Silva, 2006).

The fluoroquinolones have become the drugs of choice for the treatment of multi- drug resistance bacteria that cause acute diarrhea in adults. Their use in pediatrics is prohibited due to numerous side effects that arise as a result of using this drug (American Academy of Pediatrics, 2006). Furthermore, fluoroquinolones also achieve high fecal concentrations, are suitable for oral administration, and have a favorable safety profile in adults (Casburn-Jones, and Farthing, 2004). Cholera is treated with antibiotics, standard therapy is with tetracycline for three days but other agents are equally as effective doxycycline, trimethoprim-sulphamethoxazole, norfloxacin, and ciprofloxacin. Oral azithromycin has been found to be a safe and effective alternative of etiologic agents, and it may be an interesting empirical choice due to its safety, comfortable once-daily posology and high cellular penetration (Casburn-Jones, and Farthing, 2004).

2.4 Prevention

People are encouraged to improve sanitation and hygiene by drinking clean and treated water, practicing hand wash especially after using a toilet, eating well cooked food to kill diarrheal pathogens and to live in clean environments. Mothers are encouraged to exclusively feed their children, take children for vaccination as recommended and to use micronutrient supplements provided at the health care centers (WGO, 2008).

2.5 Bacterial resistance: a draw back in infectious diarrhea control

Although antibiotics are probably one of the most successful forms of chemotherapy in the history of medicine and have saved countless lives; making enormous contribution to the control of infectious diseases since the beginning of antibacterial era (Yanling *et al.*, 2013); Deaths due to diarrheal diseases continue to increase worldwide, with reported death cases in developing countries (Wardlaw *et al.*, 2009). This is due to the increase in cases of microbial resistance to the available medicine with results in re- emerging of diarrheal pathogens with more virulence than before. Studies by Asrat (2008), Mulatu, Beyene, and Zeynudin, 2014 and Mulatu *et al.*, (2014) revealed resistance patterns on different diarrheal pathogens to diarrheal treatments as described below: Bacterial strains of *Shigella* species in Ethiopia were resistant to Erythromycin (100.0%), Tetracycline (97.3%), Cephalothin (86.7%), Ampicillin (78.7%), Chloramphenicol (74.7%) and Sulfonamide (54.7%). Moreover, Yismaw in Gondar also revealed that there was high resistance of *Shigella* species against Ampicillin (79.9%), Tetracycline (86 %) and Cotrimoxazole (73.4%) (Asrat, 2008; Mulatu, Beyene, and Zeynudin, 2014, Mulatu, Beyene, and Zeynudin, 2014). Hence, diarrhea remains the second leading cause of death among children under 5 years old globally. Nearly one in five deaths in this age group accounting for about 1.5 million deaths annually is due to diarrheal diseases (Andrade & Fagundes-Neto, 2011).

According to the WHO (2009), diarrhea killed more children than AIDS, malaria and measles combined, however between 2000 to 2013 the global health community succeeded in decreasing by 54 % the diarrheal deaths in children under five years old (WHO, 2013). Therefore, more have to be done to reduce this number to zero deaths in children under 5 years old.

According to the Namibian demographic survey for 2006-2008, diarrheal diseases attributed to 11% deaths while acute respiratory infections were estimated at 6% (Kazembe & Neema, 2013). Diarrheal diseases (29%), Pneumonia (29%), HIV/AIDS (8%), Tuberculosis (5%) and Malaria (3%) were found to be the top 5 infectious diseases that cause high mortality and morbidity in Namibia (MOHSS, 2008). These same conditions are also amongst the leading causes of child mortality under five, diarrhea causing 35% of the reported deaths and pneumonia and HIV/AIDS causing 24% and 8% deaths respectively (CDC, 2012). Table 2 depicts the top 10 leading causes of death in Namibia (CDC, 2012).

Table 2. Top 10 causes of death in Namibia in 2012

Diseases	Percentage death (%)
HIV	23
Cancer	8
Stroke	7
Lower respiratory infection	5
Diarrheal diseases	5
Tuberculosis	5
Schemic Heart diseases	4
Diabetics	3
Interpersonal violence	3
Malaria	3

Recently, a total of 518 reported cases of cholera have been recorded in the northern part of Namibia (Kunene, Oshana and Omusati regions) with a total of 17 deaths (MoHSS, 2014). In addition, according to the report given in The Namibian newspaper (Tjihenuna, June 2015, pp. 1), at least 300 children under the age of five and more than 270 adults visited the Okuryangava clinic suffering from diarrhea in one month. Furthermore, A total of 17 000 diarrheal cases were reported in Omusati region between 2014- 2015, of which 15 752 were diarrheal cases without blood and 1 513 cases were diarrheal cases with blood (Shivute, The Namibian October 2015 pp. 3). The high prevalence of diarrhea is due to poor sanitation and lack of hygiene practice (MoHSS, 2008).

Most cases of diarrheal diseases in Namibia are reported in rural areas and remote areas where most people are living in poverty and there is no access to the available antidiarrheal treatments, antimicrobial therapy, or access to clean drinking water (MoHSS, 2014; Shivute, The Namibian October 2015 pp. 3). Hence, these people rely on the use of herbal medicine for the treatment of diarrhea. Furthermore, most bacteria have developed resistance to the available medicine hence, through research, alternative medicine to combat infectious diarrhea can be developed (Panda, Patro, Sahoo, Bastia & Dutta, 2012). For years medicinal plants have been found to be the best alternatives because they are cheap, safe to use and they have been used for centuries in the traditional setting to combat numerous infections (Du Preez and Mumbengegwi, 2012). Hence this study aimed at investigating the antibacterial activity and phytochemical composition of medicinal plants against laboratory strains (standard strains) and clinical strains of diarrheal pathogens isolated from Namibian pediatrics.. This is because most African people still rely heavily on traditional medicine; traditional healers are often the first and last line of defense

against most diseases such as headaches, coughs, and diarrhea, wound healing and skin diseases (Cheikhoussef, Shapi, Matengu, Mu Ashekele, 2011), since they are cheap, available and safe to use.

2.6 The use of Medicinal plants

A medicinal plant is any plant in which one or more of its parts contain substances that can be used for therapeutic purposes on which are precursors for the synthesis of useful medicine (Oloyede, Onocha, Soyinka, Oguntokun and Thonda, 2010). Traditional herbal medicines have been widely used for thousand years in developed and developing countries owing to its natural origin and lesser side effects (Kamboj, 2012; Kubmarawa, Khan and Shuaibu, 2012). Since ancient times, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various illnesses (Sonia et al., 2013). This is because human pathogenic microorganisms have developed resistance in response to the use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases; hence there is a pressing need to develop new and innovative antimicrobial agents (Elumalai, Ramachandran Thirumalai & Vinothkumar, 2011; Wikaningtyas and Sukandar, 2015). Many studies all over the world have shown that different plants have multi-antimicrobial properties. This includes a wide variety of secondary metabolites such as: tannins, terpenoids, alkaloids, flavonoids, phenols essential oils, quinones, saponins, alkaloids, tannins, flavonoids and the phenols (Sonia et al 2013 (Ibtisam, 2010 & Ganiyat *et al.*, 2010; Chinenye *et al.*, 2013; Raveendra *et al.*, 2011; Virgilio *et al.*, 2009; Kariuki & Njoroge, 2011; Ndamene *et al.*, 2013). According to Mohammed (2009) an estimate of 80 % of the world population depends on traditional medicine as means of primary health care, whereby 70 % are African (Du Preez and Mumbengegwi, 2012).

Herbal extracts have been used for several millennia to treat diarrheal diseases (Kisangau, Lyaruu, Hosea and Joseph, 2007). Widely used anti-diarrheal folk remedies include extracts from blackberry roots and bark, *Croton lechleri*, *Galla chinensis*, blueberry leaves and fruits, chamomile leaves, apples, green bananas, wood creosote *Garcinia buchananii* bark extract and *Garcinia* plant bark and fruits (Balemba, Bhattarai, Stenkamp-Strahm, Lesakit and Mawe, 2010).

Antibacterial activity is the ability of a substance to inhibit or kill bacterial cells. Different types of antibiotics and chemotherapeutic agents are being used in the treatment of different ailments. Most of the antibiotics were originally derived from microorganisms while the chemotherapeutic agents are from plants. However, nowadays many antibiotics and chemotherapeutic agents are obtained by various synthetic processes (Sule and Agbabiaka, 2008). Plants are among the potential sources of new agents because, plants have long been investigated and found to contain many bioactive compounds that can be of interest in healing numerous illnesses (Djeussi et al., 2013). However, despite the existence of medicinal plants and continual use over many centuries, the safety and efficacy of many traditional medicines still require sufficient support in order to create awareness for their use in the society. This is due to a lack of adequate or accepted research methodology (Du Preez and Mumbengegwi, 2012).

There are about 2400 traditional medical practitioners in Namibia who are registered with the National Eagle Traditional Healers Association (NETHA), but the actual number practicing could be higher (Cheikhyoussef, Shapi, Matengu, Mu Ashekele, 2011). Traditional medicine is currently the fastest growing medical field with herbal therapies becoming increasingly popular (Kariuki and Njoroge, 2011). The traditional medical practitioners reported that 53 plant species were in demand for medicinal trade in Windhoek (Cheikhyoussef *et al.*, 2011). There is a long tradition of using plants in the treatment of infectious disease in Namibia (Auala, Mumbengegwi,

Du Preez, & Bock, 2012); Different medicinal plants are used traditionally to relieve ailments cause by microbial agents such as malaria, cough, diarrhea (Auala et al, 2012), fever and sore throat (Chinsebu & Hedimbi, 2010). Since 80 % of the world population relies on traditional medicine for therapy (Mahomoodally, 2013; Pan, Litscher, Gao, Zhou, Yu and Chen, et al., 2014), medicinal plants unique to Namibia may be an important resource for drug discovery of new antibacterial compounds.

Figure 1 shows Hilleni Amutenya a traditional knowledge holder from the northern part of Namibia collecting medicinal plant parts of *Boscia abitrunca* roots and *Terminalia seresia* leaves source: Iikasha Albertina. Most plants that showed antidiarrheal activity were found to possess certain classes of secondary metabolites such as: alkaloids, saponins, flavonoids, tannins and triterpenoids (Roberts& Wink, 1998; Tsai *et al.*, 1989; Saxena, Saxena1, Nema, Singh & Gupta, 2013; Prashant, Kumar, Kaur, *et al.*, 2011; Zhang *et al.*, 2013); as described below in section 2.7.



Figure 1. Hilleni Amutenya a traditional knowledge holder from the northern part of Namibia collecting medicinal plant parts of *Boscia abitrunca* roots and *Terminalia sericea* leaves.

2.7 Phytochemical compounds associated with antidiarrheal properties of medicinal plants

Phytochemicals are non-nutritive, secondary metabolites of plant origin (Doughari *et al.*, 2009; Dushimemaria, 2014). Phytochemical screening is a process used to detect classes of chemical compounds found in preparations of ethno medicinal plants (Prashan *et al.*, 2011). Preparation of extracts for phytochemical investigation must be carried out on plant extracts as they are prepared by traditional healthcare practitioners. However, for research and development phytochemical investigation can be carried out using other methods of extraction to determine the most effective method to isolate bioactive compounds.

2.7.1 Alkaloid

Alkaloids exert pharmacological activity in humans of which antibacterial are common, since many of the medicines used today are alkaloids from natural sources. Therapeutic active substances of alkaloids have been isolated since 19th century (Roberts& Wink, 1998). Plants such as *Papaver somniferum* was the first plant from which morphine was derived. Alkaloids have many pharmacological activities, of which antibacterial activities are common. Some alkaloids are used as antiseptics in medicine such as berberine in ophthalmics and sanguinarine in toothpastes (De Wet, 2008). Medicine such as dicentrine from Lauraceae family of plants is found to have antibacterial and antifungal activity (Tsai *et al.*, 1989). Although alkaloids contribute to the antibacterial activity of plants, it is difficult to know to what extent alkaloids give antimicrobial protection in plants (Roberts& Wink, 1998; Tsai *et al.*, 1989; Saxena, Saxena¹, Nema, Singh & Gupta, 2013). Different alkaloids with antimicrobial activity are depicted in figure 2 (Vince, Zoltán, 2011).

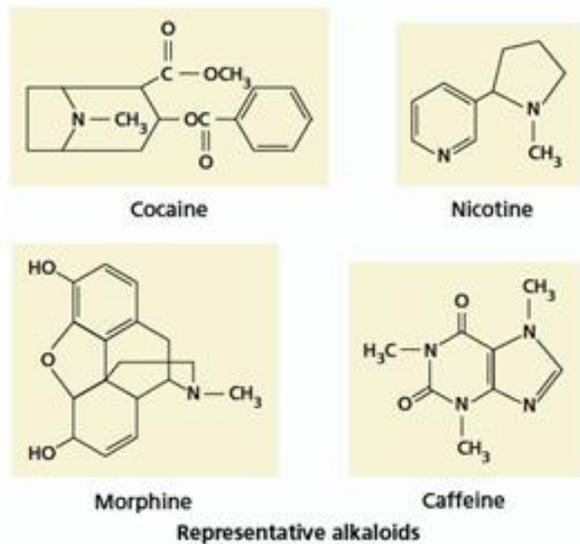


Figure 2: Examples of plant derived Alkaloids with antimicrobial activity.

2.7.2 Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in nature. Flavonoids are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones (Robertson, 2015). More than 4,000 flavonoids have been recognized, many of which occur in vegetables, fruits and beverages like tea, coffee and fruit drinks. The flavonoids appear to have played a major role in successful medical treatments in ancient times, and their use has persisted up to now. Flavonoids have antimicrobial and antidiarrheal activity. They perform their mode of action by forming a complex with cell wall of pathogen and bind to bacteria cell wall by adhesions (Prashant *et al.*, 2011). Figure 3 depict examples of the major classes of Flavonoids (Lakhanpal & Kuma, 2007).

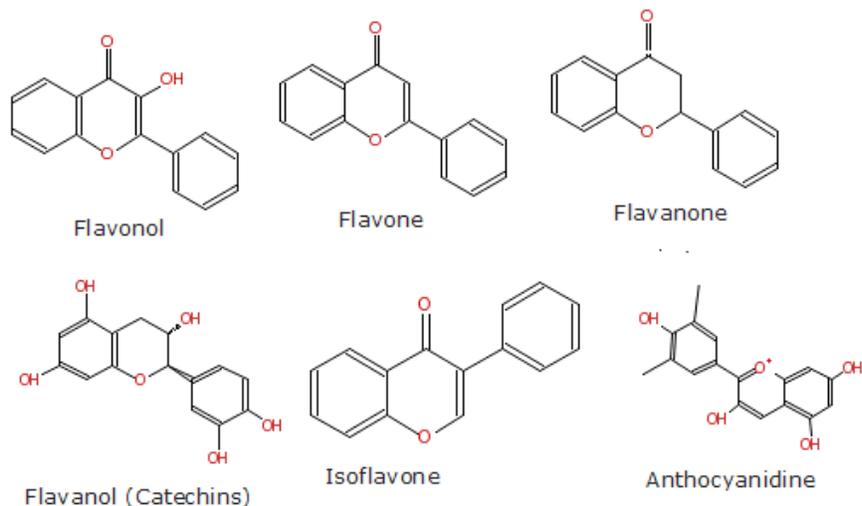


Figure 3: Examples of major classes of flavonoids isolated from plants.

2.7.3 Triterpenoids

Triterpenoids are terpenoids that consists of six isoprene units e.g. Lanosterol and squalene found in wheat germ, and olives. Triterpenes have been shown to confer a range of pharmacological properties which has led to investigations of resinous plant material such as frankincense, for anti-inflammatory, asthma, bowel disease, brain tumors antimicrobial and other pharmacological activity (Zhang *et al.*, 2013). Triterpenoids such as glycyrrhizin are known to have antimicrobial activity figure 4.

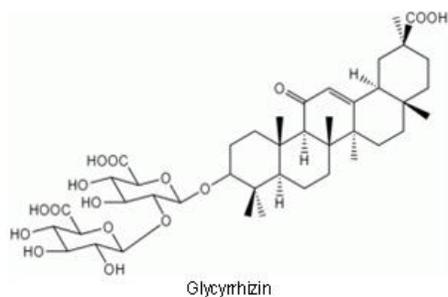


Figure 4: Example of triterpenoids

2.7.4 Tannins

Tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin, etc.), In medicine, the tannin-containing plant extracts are used against diarrhea (Saxena *et al.*, 2013). They complete their mechanism of action by forming complexes with the bacteria cell wall and disrupting the membrane (Prashant *et al.*, 2011). Figure 5 depicts examples of tannins from plants (Natural Chemistry Research Group, 2014):

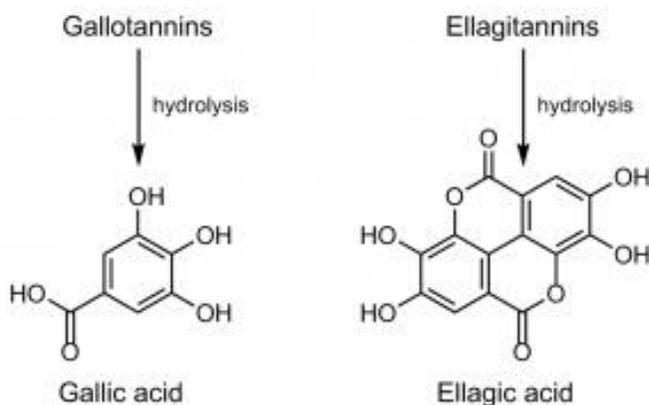


Figure 5: Example of Tannins from plants.

2.7.5 Saponins

Saponins are a group of secondary metabolites found widely distributed in the plant kingdom. Saponins form a stable foam in aqueous solutions such as soap, hence the name “saponin”. Many saponins are known to be antimicrobial, to inhibit microbial growth. Saponins may be considered a part of plants’ defense systems, and as such have been included in a large group of protective molecules found in plants named phytoanticipins or phytoprotectants (Saxena *et al.*, 2013). Figure 6 shows examples of saponins with antimicrobial activity.

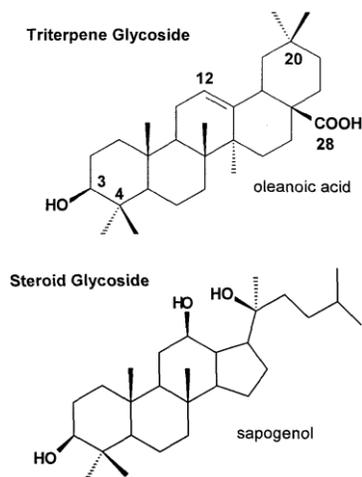


Figure 6: Example of Saponins

2.8 Different method used to screen for the presence of phytochemical compounds

There are different methods used to screen for the presence of phytochemical compounds. This includes: Solvent extraction (that use different solvents to extract different phyto-constituents), Supercritical fluid extraction (that involve the use of gases such as Carbon dioxide), Microwave-Assisted extraction, Solid phase extraction (involves sorption of solutes from a liquid medium onto a solid adsorbent by the same mechanisms by which molecules are retained on chromatographic stationary phases) and Chromatographic fingerprinting and marker compound analysis (which is a chromatographic pattern of the extract of some common chemical components of pharmacologically active and chemical characteristics (Doughari, 2012). High performance Thin layer Chromatography (HPTLC) that belongs to the group of Chromatographic fingerprinting and marker compound analysis was selected as a method of phytochemical screening in this thesis because it have the ability to analyze several samples at the same time using a small quantity of mobile phase. This reduces time and cost of analysis and this diminishes exposure risks and significantly reduces disposal problems of toxic organic

effluents, thereby reducing possibilities of environment pollution. HPTLC also facilitates repeated detection of chromatogram with same or different parameters (Tambe, Kulkarni and Bhise, 2013).

2.9 Indigenous knowledge, Documentation and validation of traditional knowledge

Indigenous knowledge is the knowledge that people in a given community has developed over time, and continues to develop. It is based on experience, it is often tested over centuries of use and it is adapted to local culture and environment (Traditional Knowledge Sector Paper, 1999; Mutema, 2013, Du Preez & Mumbengegwi, 2012). This knowledge may be useful in the search for new medicine and the development of ethno medicines from plants that are affordable and accessible to local people. However, this knowledge is only known to the indigenous people, and is passed on verbally from generation to generation (Nyota & Mapara, 2008). As Traditional Knowledge is transmitted verbally, it is exposed to change and valuable information is lost during this mode of communication, hence documentation of Traditional Knowledge is very important as it provides a platform for validation and promotes the use of Traditional medicine (Dupreez & Mumbengegwi, 2012).

Validation is significant because it enables the use of traditional medicine to be accepted as a mainstream alternative to orthodox medicine. Furthermore, it enables clear understanding on the safety and efficacy of the use of medicinal plants. Validation also helps to investigate the chemical composition and the biological compositions of plant extracts by means of phytochemical screening (Dupreez & Mumbengegwi, 2012).

In some cases, the crude extract of medicinal plants may be used as the treatment or alternatively, the active compounds are isolated and identified for the elucidation of the mechanism of action of the compounds. Hence, research on both the mixture of traditional medicine and single active compounds is very important (Pan *et al.*, 2014). Aspects such as the toxicity and the efficacy of traditional medicines must also be evaluated as they provide basic information for future

development of traditional medicines (Mosihuzzaman, and Choudhary, 2008). Medicinal plants have become the new option sources of bacterial treatment with different modes of action to routinely used antibiotics for which resistance may occur. Medicinal plants may also provide chemical entities with antibacterial activity (Wikaningtyas and Sukandar, 2015).

2.10 Diarrheal disease causing bacteria

Many different bacteria can cause gastroenteritis. From studies of stool cultures however, *Campylobacter*, *Salmonella*, *Shigella* and *Escherichia coli* are always associated with diarrheal diseases (National Digestive Diseases, 2011). This study focused on *Salmonella*, *Shigella* and *Escherichia coli* as common diarrheal pathogens. Furthermore *Listeria monocytogenes* and *Staphylococcus aureus* are also part of this study since they are commonly associated with diarrheal diseases and are responsible of many cases on food poisoning (Argudín, Mendoza and Rodicio, 2010).

2. 10. 1 *Shigella boydii* (*S. boydii*)

S. boydii is a Gram-negative bacterium of the genus *Shigella*. Like other members of the genus, *S. boydii* is a non-motile, non-spore forming, rod-shaped (James and George, 2004). *S. boydii* is the most genetically divergent species of the *Shigella* genus (Feng, 2004), with a total of 19 serotypes (Yang, 2005). *S. boydii* are among the most important foodborne pathogens. Ingestion of food contaminated with these bacteria causes shigellosis within 12 - 48 hours. Fever, aches, fatigue and loss of appetite are the initial symptoms, which may be associated with watery diarrhea that, in turn, may develop into bloody stools or dysentery. A fatal hemolytic-uremic syndrome (HUS), due to the production of Shiga toxin, may also develop in certain severe cases (Yang, 2005). More than 140 million cases of Shigellosis diarrhea have been reported worldwide

with 600 000 deaths annually worldwide with 60 % in children under the age of five years (Panda, Patro, Sahoo, Bastia & Dutta, 2012).

2.10.2. *Salmonella typhi*

Salmonella are Gram-negative, non-spore forming rod-shaped bacteria and are members of the family Enterobacteriaceae (Jay *et al.* 2003). It belongs to the group of species: *Salmonella enterica* (comprising six subspecies). Over 99% of human *Salmonella spp.* infections are caused by *S. enterica* (Bell and Kyriakides, 2002). *Salmonella typhi* in humans are host specific. This means that the severity and nature of *Salmonella* infection varies from host to host (Morgan, Campbell, Rowe, Bispham, Stevens, Bowen, Barrow, Maskell, Wallis, 2004). Hence, species such as: *Salmonella pullorum* and *S. gallinarum* only cause diarrhea in chicken and birds but not in human; while *S. typhi* and *S. paratyphi* cause diarrhea in human and not in birds. Hence this host specificity, enable human to be carriers of *S. pullorum* and *S. gallinarum* and birds to be carriers of *S. typhi* and *S. paratyphi* without them getting diarrhea. Within human, infection severity may vary by the resistance of each individual and the immune system as well as the virulence of the *Salmonella* isolates (Andino and Hanning 2015). These organisms are etiological agents of diarrheal and systemic infections in humans, causing a disease called Salmonellosis, which is one of the most commonly, reported enteric illnesses worldwide (Center for Food Security and Public Health, 2005). The prevalence of *Salmonella* infection varies depending on the water supply, waste disposal, food preparation practices and climate. Outbreaks attributed to *Salmonella spp.* have predominantly been associated with animal products such as eggs, poultry, raw meat, milk and dairy products, but also include fresh produce, salad dressing, fruit juice, peanut butter and chocolate (Jay *et al.* 2003).

2.10.3 *Escherichia coli* (*E. coli*)

E. coli is a gram negative bacterium that normally lives in the intestines of humans and animals. Although most types of these bacteria are harmless, several produce toxins that cause illness. Some strains of *E. coli*, including *E. coli* O157:H7, produce toxins known as Shiga toxins and are called “Shiga toxin-producing” *E. coli* (STEC). These may cause severe diarrhea. There are various different VTEC strains; the most important one associated with human diarrhea is called VTEC O157 produced by *E. coli* O157 (World Gastroenterology Organization, 2012). *E. coli* O157 causes approximately 73,000 illnesses, 2,000 hospitalizations, and 50-60 deaths each year worldwide (World Gastroenterology Organization, 2012). Up to 63% of children with persistent diarrhea in low and middle income countries have tested positive for *E. coli* strains (Stephanie *et al.*, 2013).

2.10.4 *Listeria monocytogenes*

Listeria monocytogenes is a gram positive, none-spore forming rod- shaped bacterium. It belongs to the genus of *Listeria* along with *L. ivanovii*, *L. innocua* and *L. grayi*. There are thirteen known serotypes of *L. monocytogenes* (anonymous, 2013). It has been known for a long time that many patients experience diarrhea antecedent to the development of bacteremia or meningoencephalitis due to *Listeria monocytogenes*, but it was only recently that convincing evidence was obtained that this organism can cause acute, self-limited, febrile gastroenteritis in healthy persons (Ooi and Lorbe, 2004). At least 7 outbreaks of foodborne gastro-enteritis due to *L. monocytogenes* have been reported. Illness typically occurs 24 h after ingestion of a large inoculum of bacteria and usually lasts 2 days. *Listeria* is transmitted through eating raw food from animal sources such as beef, pork and poultry or by eating unwashed vegetables as well as by eating food contaminated with faeces of birds, fish and other animals (CDC, 2016). Common symptoms

include fever, watery diarrhea, nausea, headache, and pains in joints and muscles (Ooi and Lorbe, 2004; Pichler *et al* 2009). *Salmonella*, *Listeria*, *Toxoplasma* and rotavirus are responsible for more than 75% of the deaths related to unknown causes of food borne illnesses each year (Stephanie *et al.*, 2013)

2.10. 5 *Staphylococcus aureus*

Staphylococcus aureus is a Gram - positive bacterium found in the nose and on the skin of about 25 percent of healthy people and animals. It is a common cause of food poisoning; in fact, it is capable of producing seven different enterotoxins that frequently cause food poisoning (Healthline Networks, 2016; Lima, Loiko, Casarin and Tondo, 2013). It is most commonly transferred to food products such as milk and cheese through contact with contaminated food workers. Symptoms of *Staphylococcus aureus* food poisoning (SFP) are similar to that of gastroenteritis such as diarrhea, vomiting, and abdominal pain (Healthline Networks, 2016). In the last two decades, *S. aureus* has been identified as the second most frequent agent of foodborne illnesses (Lima *et al.*, 2013).

2.11 Comparing laboratory and clinical specimens

Laboratory and clinical strains differs in such a way that, the genetic sequence for laboratory strains is determined and known while the genetic sequence of clinical isolated strain have to be determined only upon isolation. The human body is exposed to the use of different antibiotics and different environmental factors; hence pathogens have a high chance of developing resistance due to the use of different antibiotics to treat different ailments. Laboratory strains on the other hand, after packaging, they are stored at low temperature in media and they are not exposed to any external environmental changes or to the use of antibiotics, hence they have

limited chances of developing resistance. Most studies conducted in antibacterial assays involved the use of laboratory strains only or clinical strains only, however, there are no studies that tried to compare whether the effect of the antibiotics and plant extracts are similar or differs when the assay is done on both clinical and laboratory strains. This will provide useful information when deciding on performing further analysis toward drug discovery, Hence in this study, antibacterial activity was determined for both laboratory and clinical diarrheal strains, to compare the effects of the antibiotics and plant extracts against thee pathogens.

2.12 Medicinal plants used in traditional setting in Namibia to treat diarrheal diseases

2.12.1 *Ziziphus mucronata*

Ziziphus mucronata belong to a family of Rhamnaceae. It is locally referred to as *Mokgalo*, *N#a*, *#arosor* or Buffalo thorn. Its roots and leaves are used traditionally to treat pain, dysentery, fever, diarrhea and malaria (Roodt, 1998; Mannheimer & Curtis, 2009; Van den Eynden *et al.*, 1999; Van Koenen, 2001; Burke, 2007). Olajuyigbe and Afolayan (2013) reported on the antibacterial activity of *Z. mucronata* against clinically important bacteria. Figure 7 shows *Ziziphus mucronata* branch.



Figure 7: A branch of *Ziziphus mucronata*. Source: Mazibuko, (2007).

2.12.2 *Combretum apiculatum*

Combretum apiculatum belongs to a family of Combretaceae and it is locally referred to as Kudu-bush or *omumbuti*. Its roots and leaves are used traditionally to treat Diarrhea (Chinsemu & Hedimbi, 2010; Mannheimer & Curtis, 2009). Aderogba, Kgate, McGaw and Eloff (2011) reported on antioxidant activity of *C. apiculatum*. Figure 8 shows a picture of *Combretum apiculatum* branch.



Figure 8: A branch of *Combretum apiculatum*. Source: Masupa & Rampho, (2011).

2.12.3 *Terminalia sericea*

Terminalia sericea belong to a family of Combretaceae and it is locally referred to as *Za''ò*; *Ndjao* or *Silver terminalia*. Its roots, bark, leaves and twig are used to treat Persistent coughing, diarrhea, dysentery and abdominal pain (Leffers, 2003; Mannheimer & Curtis, 2009; Du Preez and Mumbengegwi, 2012). Vuuren et al (2015) reported on the antidiarrheal activity of *T. sericea* against diarrheal pathogens such as *Bacillus cereus*, *E. coli*, *Proteus vulgaris*, *Salmonella typhi*, *Shigella flexneri* and *Staphylococcus aureus* laboratory standards. Figure 9 shows a picture of *Terminalia sericea* branch.



Figure 9: A branch of *Terminalia sericea*. Source: Masupa (2012).

2.12.4 *Boscia albitrunca*

Boscia albitrunca belong to the Capparidaceae family. It is locally referred to as Shepherd's Tree, White-stem Tree, and Coffee Tree (English); *Witgat*, *Witstamboom* or *omutenderereti*. Its barks and leaves are used to treat bacterial infections. Roots are used to ferment milk (Herrmann & Colleen, 2003). Pendota *et al* (2016) reported on the germination, seedling growth requirements and antibacterial activity of *B. albitrunca* against *B. subtilis*, *S. aureus*, *E. coli* and *K. pneumonia* laboratory standards. Figure 10 depicts a flowering branch of *Boscia albitrunca*.



Figure 10: *Boscia abitrunca*. Source: Protected trees (2013).

2.12.5 *Solanum linnaeanum*

Solanum linnaeanum belong to a family of Solanaceae family, It is also referred to as; Afghan thistle, apple of Sodom, apple-of-Sodom, bitter apple, black-spined nightshade, Dead Sea apple, devil's apple, poison apple, poison bush, poison weed, Sodom's apple or Sodom-apple. *S.*

linnaeanum is a good source of solamargine and solasonine, with are glycoalkaloids. solasodine with medicinal properties. Solasodine is also used as a precursor of steroidal drugs (Gürbüz et al., 2015). Figure 11 shows a branch of *Solanum linnaeanu*.



Figure 11. A branch of *Solanum linnaeanum*. Source:

<https://www.google.com/search?q=Solanum+linnaeanum>

CHAPTER 3: MATERIALS AND METHODS

3.1 Research design

The flow chart in figure 12 gives an outline of the experimental design that was used to investigate the antibacterial activities of medicinal plants against diarrheal pathogens.

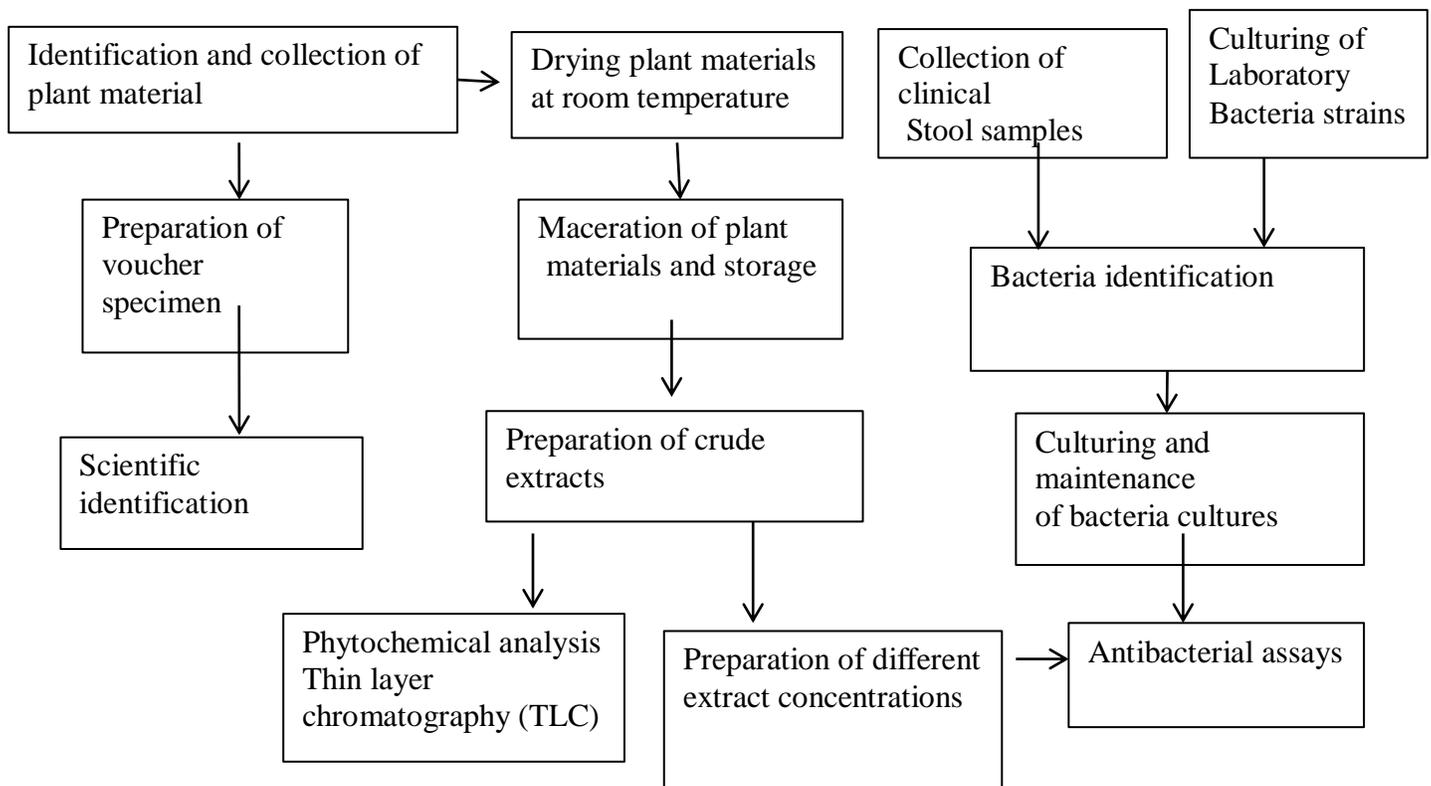


Figure 12: Flow chart showing the investigation of antibacterial activity of medicinal plants against diarrheal pathogens used in this study.

3.2. Plants collection and processing

Plants specimens for *B. albitrunca*, *Z. mucronata*, *C. apiculatum*, *S. linnaeanum* and *T. sericea* were collected from different areas of Kunene region in Namibia in 2011. Plant materials (bark, roots and leaves) were collected with the help of the botanists and voucher specimens were prepared upon plant collection. The collected specimens were identified and authenticated by the National Herbarium at the National Botanical Research Institute in Namibia. The collected plant

materials (bark, roots and leaves) were air dried for 4 weeks at ambient temperature, and powdered using an industrial blender. The powder was then stored in capped bottles at -20 degrees Celsius till further use. Figure 13 depicts process of plant drying (Albertina Iikasha, 2015).



Figure 13. Drying of different plant parts in the shade.

3.3 Solvent extraction

Plant extract (aqueous and organic extracts) preparation was performed based on the protocol by Bayoub *et al* (2010) with a few adjustments.

Twenty grams of the powdered plant material was added to a conical flask containing 200 ml of distilled water for aqueous extraction and 90 % ethanol for organic extraction. The flasks were shaken for 5 minutes to allow mixing and the conical flasks were then covered with parafilm. The conical flasks containing the prepared mixtures were stored at room temperature for 24 hours. After 24 hours the mixtures were filtered using Whatman no 1 filter papers with a

diameter of 110 millimetres (mm) and the filtrate was dried using rotary evaporation at pressure of 30 mbar and boiling temperature of 60 °C. The aqueous plant extracts were further dried using a freeze dryer. Dry extracts were scraped out of the round bottom flasks and weighed. The mass of the dry plant extracts was recorded in grams (g). The percentage yield was calculated by dividing the mass of dry extract with the original mass of plant material used for extraction purposes multiplied by a 100. The dried plant extracts were then stored at -4 °C degree till further use.

3.4 Phytochemical screening

Freshly prepared organic and aqueous plant extracts were screened for the presence of flavonoids, alkaloids, tannins, steroids and coumarins by Thin layer Chromatography (TLC). Plant extracts (organic and aqueous) were prepared by dissolving 32 mg plant extract in 4 ml 90 % ethanol for organic extracts and 32 mg plant extracts in 4 ml distilled water for aqueous extracts. The extracts were then vortexed for 5 minutes.

The presence of coumarins, alkaloids, tannins, steroids, flavonoids and triterpenoids in the plant extracts (organic and aqueous extracts) of *B. albitrunca*, *Z. mucronata*, *S. linnaeanum*, *C. apiculatum* and *T. sericea* was determined by using methods adapted from Harborne (1998). The preparation of solvents used in screening of each bioactive compound is described below:

3.4.1 Screening of Alkaloids

Chloroforms: ethanol in a ratio of 9:1 was used as the solvent system. Dragendorff reagent was used as a chromogenic reagent for screening of alkaloids in different plant extracts. Quinine was used as a positive control. Approximately 10 µl of each the plant extracts was applied on a TLC plate using capillary tubes. The plant extracts were applied in sequential application while allowing drying of the spots. The spots were made 2 cm from left to right edge of the TLC plate

with 1 cm between each spot. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of Chloroform: Ethanol (9:1) solvent. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank as depicted in figure 14. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with Dragendorff reagent, the plates were viewed under UV light at 360 nm. The presence of the orange and blue fluorescence at 360nm represented the presence of alkaloids. The R_f value for each spot was calculated.

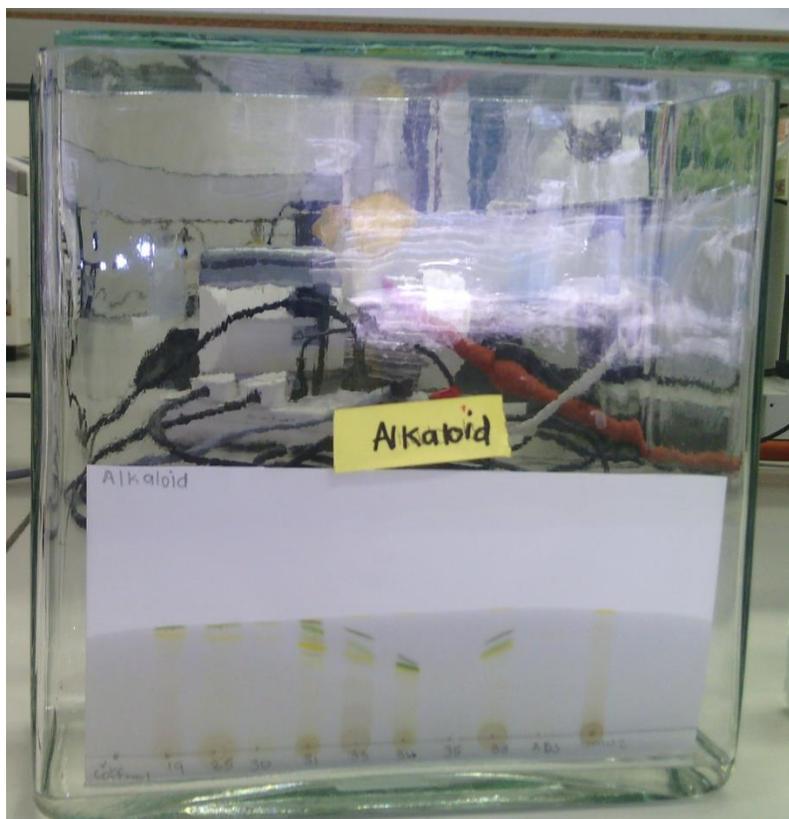


Figure 14: Alkaloid screening by TLC using Chloroform: Ethanol (9:1) as a mobile solvent

3.4.2 Flavanoids

Butanol: acetic acid: Water in a ratio of 4:1:5 were used as the solvent system and 10% Antimony chloride in chloroform was used as a chromogenic reagent. Quercetin was used as a

positive control. A 1 cm line was measured and drawn with a soft pencil on a TLC plate from the bottom of the TLC plate. Approximately 10 μ l of each the plant extracts was applied on a TLC plate using capillary tubes. The spots were made 2 cm from left to right edge of the TLC plate with 1 cm between each spot. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of Butanol: acetic acid: Water in a ratio of 4:1:5 solvent. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with the chromogenic reagent the plates were viewed under UV light at 360 nm and examined for the presence of the yellow orange and blue florescence spots. The Rf value for each spot was calculated.

3.4.3 Saponins

Chloroform: methanol and water in a ratio of 7:3:1 was used as the solvent system and Vanilin, ethanol, sulfuric acid were used to prepare the chromogenic reagent. Saponin was used as a positive control. A 1 cm line was drawn on a TLC plate using a soft pencil from the bottom of the TLC plate. Approximately 10 μ l of each plant extracts was applied on a TLC plate using capillary tubes. The spots were made 2 cm from left to right edge of the TLC plate with 1 cm between each spot. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of Vanillin, ethanol, sulfuric acid solvent as depicted in figure 15. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with the chromogenic reagent the plates were viewed under UV light at 360 nm and examined for the presence of the blue florescence. The Rf value for each spot was calculated.

the TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with, Chromogenic reagent A and B, the TLC plates were viewed under UV light at 360 nm and examined for the presence of the dark spots upon heating at 120 °C. The Rf value for each spot was calculated.

3.4.5 Tannins

1 % Potassium hydroxide in Methanol was used as the solvent system and 1 % ferric chloride in 50 % aqueous methanol was used as the chromogenic reagent. B- sitosterol was used as a positive control. A 1 cm line was drawn from the bottom of the TLC plate. Approximately 10 µl of each the plant extracts was applied on a TLC plate using capillary tubes. Spots that are 2 cm apart were drawn on a TLC plate and it was on this spots were the extracts were applied. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of 1 % Potassium hydroxide in Methanol. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with the chromogenic reagent the TLC plates were viewed under UV light at 360 nm and examined for the presence of purple and orange florescence. The Rf value for each spot was calculated.

3.4.6 Coumarins

Chloroform was used as the solvent system and Copper sulphate, sodium citrate, anhydrous sodium carbonate were used to prepare a chromogenic reagent. Coumarins was used as a positive control. Approximately 10 µl of each the plant extracts was applied on a TLC plate using capillary tubes. The spots were made 2 cm apart on a TLC plate and it was on this spots were the extracts were applied. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of chloroform. The mobile phase was allowed to run up the TLC plate and the TLC

plates were removed from the tank as depicted in figure 16. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with the chromogenic reagent, the TLC plates were viewed under Uv light at 360 nm and examined for the presence of blue long wave length florescence. The Rf value for each spot was measured.

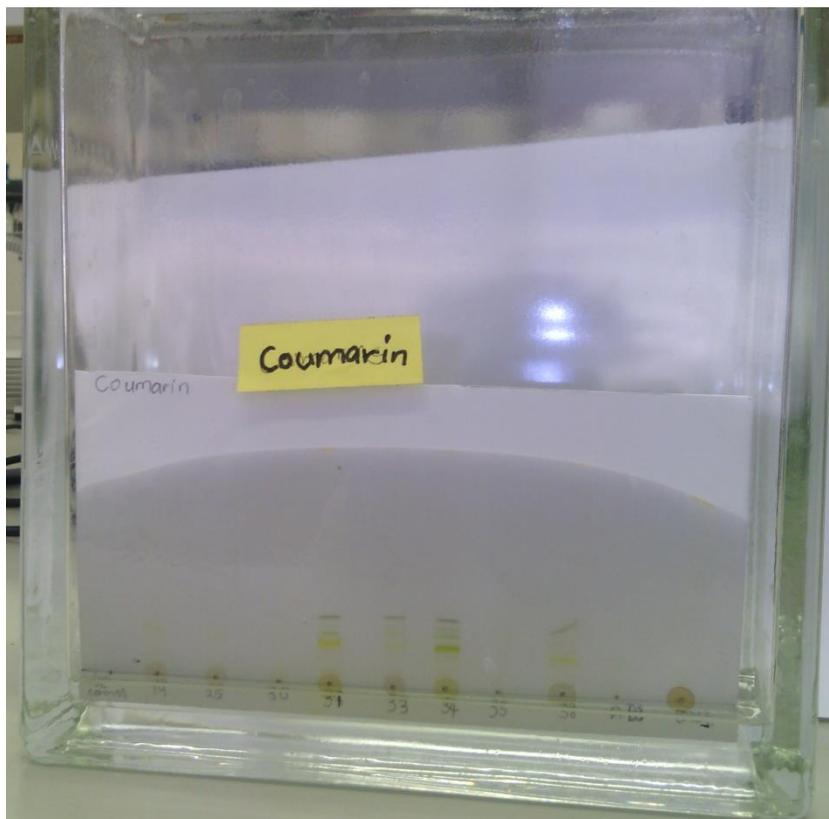


Figure 16: Coumarin detection by TLC using Chloroform as a mobile solvent.

3.4.7 Triterpenoids

Hexane: ethyl acetate in a ratio of 17:3 was used as the solvent system and Liebermann reagent (acetic acid, sulfuric acid and ethanol) was used as a chromogenic reagent. B- sitosterol was used as a positive control. A 1 cm line was drawn from the bottom of the TLC plate using a soft pencil. Approximately 10 μ l of each the plant extracts was applied on a TLC plate using capillary tubes. The plant extracts were applied in sequential application while allowing drying

of the spots. Spots that are 2 cm apart were drawn on a TLC plate and it was on these spots where the plant extracts were applied. The spotted TLC plates were placed in a tank containing not more than 1 cm volume of mobile solvent. The mobile phase was allowed to run up the TLC plate and the TLC plates were removed from the tank. The end solvent point was marked with a soft pencil before the TLC plate dried. After spraying the TLC plates with, the chromogenic reagent, the TLC plates were viewed under UV light at 360 nm and examined for the presence of blue, green and yellow fluorescence as depicted in figure 17. The R_f value for each spot was calculated.

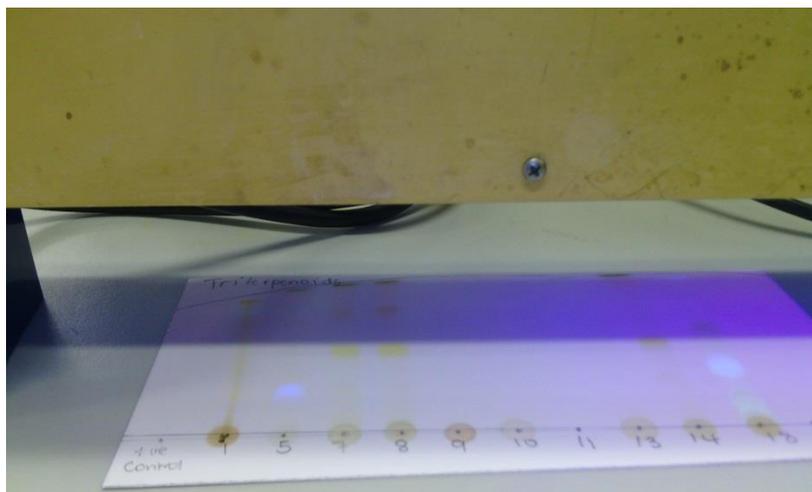


Figure 17: TLC plate viewed under Ultra-violet (UV) light at 366 nm.

3.5 Antibacterial assay on Bacterial laboratory pathogen strains

Two methods were used for antibacterial assay namely: Agar disk diffusion method and Broth dilution method. Agar diffusion method was used to determine the sizes of the inhibition zones and broth dilution to determine the minimum inhibitory concentrations.

3.5.1 Antibacterial assay by disc diffusion method

The Kirby-Bauer disc diffusion method was used for antibacterial testing as recommended by National Committee Clinical Laboratory Standards (NCCLS) (2008), with a few adjustments as described in section 3.5.2 to 3.5.7.

3.5.2 Preparation of Mueller-Hinton agar

Muller Hinton agar was prepared by adding 38 g of Muller Hinton agar powder to 1000 ml of distilled water in an autoclaving bottle. The mixture was autoclaved for 15 minutes at 121 degrees and cooled to 25°C. The media was poured into petri dishes and allowed to solidify. The plates were then stored at -4 degrees Celsius till use. The sterility of the media was determined by incubating an un-inoculated plate at 37 °C for 24 hours.

3.5.3 Preparation of plant extracts solution

Antibacterial activity of the extracts was tested at various concentrations ranging from 1000 – 250 µg/ml. The plant extracts for the five selected plants were weighted and dissolved in 90% ethanol and water for organic and aqueous extracts respectively; to prepare the stock solution of 1000 µg/ml concentrations. Stock solution was prepared using the formula: $C = M/V$, where M= Mass of the plant extract and V= Volume of the solvent. The same stock solution was used to get the desired concentrations of 500 and 250 µg/ml by serial dilutions method using the equation, $C_1V_1 = C_2V_2$, where C= concentration and V= volume.

3.5.4 Preparation of dried filter paper discs

Whatman no. 1 filter paper was used to prepare discs with a diameter of 6 mm. The discs were heated at 120 °C for 30 minutes for sterilization. The paper discs were stored at -4 °C in a sealed container till use.

3.5.5 Inoculum preparation (growth method)

At least three well isolated colonies of the same morphological type were selected from agar plates of *E. coli*, *Listeria monocytogenes*, *Shigella boydii* *Salmonella typhi* and *Staphylococcus aureus* respectively. The top of each colony was touched with a loop and transferred in to a conical flask containing 200 ml of nutrient broth. The broth culture was incubated at 37 °C for 9 hours. The turbidity of the actively growing broth culture was adjusted with sterile nutrient broth to obtain a turbidity of optimally comparable to that of 0.5 McFarland standard. This was done by determining the optical density of inoculum and comparing it to that of 0.5 McFarland standards.

3.5.6 Inoculation of test plate and application of discs to inoculated agar plates

Within 1 hour of adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. The dry surface of the Muller-Hinton agar plate was stroked using a swab over the entire agar surface. The striking procedure was repeated 3 times while rotating the plate 60 degrees each time to ensure even distribution of the inoculum.

The paper discs were taken out of the refrigerator 2 hours before performing the antibacterial assay. Three petri dishes were labeled with 250, 500 and 1000 µg/ml concentrations. Exact volumes of 2 ml of aqueous or organic extracts were poured into the petri-dishes and the paper discs were placed into respective extract concentrations for 3 minutes. The discs were then allowed to air dry for 3 minutes before they were placed onto the agar plates. For each plant extract, three impregnated paper discs were prepared per concentration in order to get the average. The plates were inverted and incubated at 37 °C for 24 hours. The experiment was done in duplicates.

Tetracycline was used as a positive control for *E. coli*, Vancomycin for *L. monocytogenes*, gentamycin for *Staphylococcus aureus* and ampicillin for *S. boydii* and *S. typhi*. Distilled water and 90% ethanol were used as negative controls. The concentrations of the positive controls were: Vancomycin (10µg/ml), Gentamycin (30µg/ml) and Tetracycline (35µg/ml). Three discs were prepared for each control and the plates were incubated for 24 hours at 37 °C. The experiment was done in duplicates.

3.5.7 Determination of Minimum Inhibitory Concentration by broth dilution method

(MIC)

Determination of Minimum Inhibitory Concentration (MIC) of the extracts on the test organisms. The MIC test was performed according to Sule & Agbabiaka (2008) with a few adjustments. The initial concentration of the plant extract (1000µg/ml) was diluted using double fold serial dilution by transferring 5ml of the sterile plant extract (stock solution) into 5ml of sterile Nutrient broth to obtain 500µg/ml concentration. The above process was repeated several times to obtain other dilutions: 250, 125, 62.5, 31.25, 15.625, 7.82 and 3.91µg/ml (Sule & Agbabiaka, 2008) as depicted in figure 18 from right to left. Having obtained the different concentrations of the extracts, each concentration was inoculated with 0.1 ml of the standardized bacterial cell suspension and incubated at 37°C for 24 hours. The growth of the inoculum in the broth was indicated by cloudiness of the broth and the lowest concentration of the extract which inhibited the growth of the test organism was taken as the (MIC). Controls were set up as follows: nutrient broth only; nutrient broth and plant extract; and nutrient broth, and a test organism. The negative controls were stored in the refrigerator after preparation until further use, while the positive control was incubated together with the sample tubes at °C for 24 hours. After incubation, a loop full from each tube was sub cultured on nutrient agar to see if bacterial growth was inhibited by

plant extracts (Minimum Bactericidal Activity). Growth of bacteria on solid media indicated that such particular concentration of the plant extract was unable to inhibit the bacteria growth. The MIC was defined as the lowest concentration of an antibacterial that inhibited the visible growth of a bacterium after overnight incubation. The experiment was repeated two times.

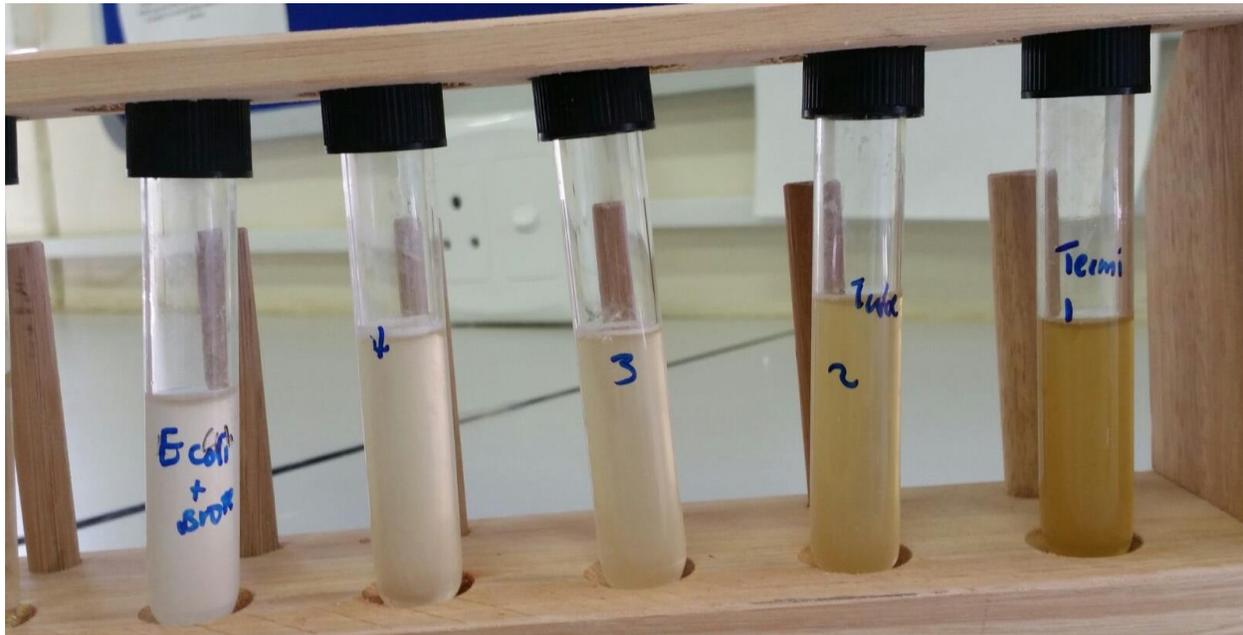


Figure 18: Determination of MICs by broth dilution method at different concentrations descending from right to left.

3.6 Clinical stool sample collection and bacteria isolation

After receiving a written consent and a sample collection form from Ministry of health and social services (MoHSS) (attached appendix A and B respectively), stool samples were collected from two different health centers in Windhoek (Katutura state hospital, and Okuryangava clinic). An ethical clearance from MoHSS was also obtained to allow the process of sample collection. Stool samples were collected by nurses from children under the age of 5. Only children who were admitted with acute diarrhea (diarrhea that is within 2 weeks) were included in the study. The selection of the participants was done by the nurses based on the report given by the doctors after

examining the patients. Before sample collection, parents of the patients involved in the study were informed and given time to decide on whether they would like their children to participate in the study or not. The content of the consent form was explained to the parents and parents who gave consent for their children to participate in the study were asked to sign the consent form. For the parents who were illiterate, a witness was requested to sign on their behalf. Only children whose parents gave consent were included in the study (appendix A) and a description of the patient's health condition was summarized in a form attached (appendix B). Stool samples were only collected from children who were admitted with acute diarrhea. From each child, a stool sample and a rectal swab (labeled with the patient code number) were collected. A trained nurse helped with the insertion of the rectal swab 2 to 3 cm (1 to 1.5 inches) into rectum and rotated 360 ° to collect fecal material, and carefully removed swab from rectum and with the collection of the stool samples. The collected stool samples and rectal swabs were kept on ice upon collection and taken to the laboratory within 4 hours of collection to ensure a high sample quality. In the laboratory, the samples were stored at – 80 degrees till further use. Figure 19 show the rectal swab and the stool sample after sample collection.

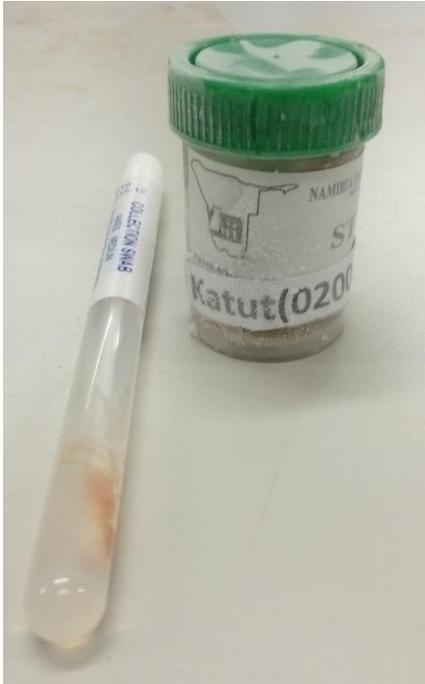


Figure 19. Rectal swab and stool container used in stool specimen collection.

3.7 Media preparation

XLD agar was prepared by weighing 56.68 g of XLD powder and adding to it distilled water in a 1000 ml conical flask. The agar was heated with continuous stirring until it boiled. Without cooling down (according to manufacturer's instructions), XLD agar was poured into agar plates and allowed to solidify. The plates were stored in the fridge at -4 °C till further use.

MAC was prepared by weighing out 52 g of the MAC agar powder on a weighing balance and adding it to a conical flask containing 1000 ml of distilled water. The mixture was heated to boil with continuous stirring until it completely dissolved. The MAC agar was sterilized in the autoclave at 121 °C for 15 minutes. After autoclaving, the media was cooled to 45 °C and poured in plates. The plates were stored at -4 degrees in the fridge till further use.

Selenite broth was prepared by adding 19 g of selenite broth base to a 1000 ml conical flask containing 4 g of bi-selenite and 1000 ml of distilled water was added to the conical flask with continuous shaking to completely dissolve the media in water. An exact volume of 5 ml of the

prepared selenite broth was added to the test tubes and the test tubes were placed in a boiling water bath to sterilize the broth for 10 minutes. After sterilization, the broth tubes were cooled down to room temperature (25°C) in tap water (WHO, 2010). The temperature of the broth was confirmed with a thermometer.

3.8 Inoculation of stool sample on XLD, MAC agar plates and Selenite broth

Stool specimens collected from Katutura State hospital and Okuryangava clinic were removed from – 80°C and placed on an ice bath to allow the stool specimen to thaw. The ice bath containing the stool specimen was taken to a level II biosafety cabinet. Using an applicator swab, a small amount of feces was collected from a container with stool specimen. A swab was rolled over the first quadrant of MAC and XLD agar plates. Using sterile 1 µl inoculating loop, the stool specimen was spread on the agar plate. The XLD and MAC agar plates were sealed with Para film. A new sterile swab inserted into the sample and the swab was dropped into the tube of selenite broth. The tubes were then loosely capped. The selenite tubes and XLD and MAC agar plates were incubated for 24 hours at 37 °C in an incubator. The experiment was repeated two times.

After incubation, the MAC agar plates were observed for the formation of *Salmonella* like and *Shigella* like colorless (lactose negative) colonies (2-4 mm) and the XLD agar plates were examined to identify any *Salmonella*-like or *Shigella*-like colonies (clear or pale-green colonies and colonies with black centers). The XLD and MAC agar plates were further examined for the formation of *E. coli* like colonies (yellow on XLD and red and pink on MAC). Most coliforms produce red colonies.

3.9 Morphological identification and Biochemical test for identification of clinical bacteria strains

3.9.1 Isolation using different media

Pure culture of different isolated colonies were cultured on MAC and XLD and incubated overnight at 37°C. After incubation, the MAC agar plates were observed for the formation of *Salmonella* and *Shigella* colorless (lactose negative) colonies (2-4 mm) and the XLD agar plates were examined to identify any *Salmonella* or *Shigella* colonies (clear or pale-green colonies and colonies with black centers). The XLD and MAC agar plates were examined for the formation of *E. coli* like colonies (yellow on XLD, red and pink on MAC).

3.9.2 Microscopic Appearance

Gram staining was performed in order to determine the microscopic appearance of the colonies. Bacterial culture of 10 microliters was spread on a glass slide and heat fixed. The fixed smear was flooded with crystal violet for 1 minute before it was rinsed off with tap water. The slide was further flooded with iodine solution and allowed to stand for 1 minute. The slide was further rinsed with tap water to remove iodine solution. The slide was then decolorized with ethanol for 30 seconds. Finally the slide was flooded with safranin and rinsed with distilled water. The slides were dried with paper and microscopically examined for bacterial organisms under a 100X objective and examined for gram-negative pink to red bacteria (Madigan, 2009).

Biochemical tests were done to further identify the bacteria isolated using method by Madigan, Martinko, Dunlap and Clark, (2009) as described in section 3.10.3 to 3.10.6.

3.9.3 Indole test

Indole test was done on Tryptophan media. Detection of Indole test was done on Tryptophan media. Detection of indole (a red color) on a culture media with dimethyl- amino benzaldehyde was considered a positive result. Expected results are: *E. coli* +, *Salmonella* – and *Shigella* -.

3.9.4 Lysine decarboxylase

Lysine decarboxylase broth was used as growth media. A bromcresol purple pH indicator was used to detect alkaline pH if there was an enzyme reaction. Decarboxylation of amino acid releases carbon dioxide and amine. Expected results are: *E. coli*+, *Salmonella* + and *Shigella*.

3.9.5 Carbohydrate fermentation

Tubes containing phenolred broth media with carbohydrates and phenol red as an indicator incubated at 35°C for 24 hours. Tubes were inverted to observe gas/ acid production. Expected results are *E. coli*+, *Salmonella* – and *Shigella* +.

3.9.6 Urease test

Urea broth and phenol red indicator was used and media plates incubated for 48 hours at 35 °C. Plates were examined for the formation of pink-red color (i.e. H_2NCONH_2) to 2NH_3 AND CO_2). Expected results are: *E. coli* -, *Salmonella* - and *Shigella* –

3.10 Antibacterial assay on clinical bacteria strains

Antibacterial assay on clinical bacteria strains was performed using the same method as described in section 3.5.1 to 3.5.7. The only exception was that, clinical antibacterial assay was only done against clinical *Shigella*, *Salmonella* and *E. coli*.

3.11 Data analysis

Microsoft Excel® was used to enter and capture data on zones of inhibitions at different concentrations. Tables for the phytochemical were developed using Microsoft Excel®. Averages, Standard deviation and standard error were calculated in Microsoft Excel and analysis and graphs showing antibacterial activity of different plants were extracted from this data. Data on inhibition zones was further analyzed using Gen Stat using a two-way Student's t-test, at 95% confidence level. P value < 0.05 to determine whether there is a significant difference in antibacterial activity of laboratory and clinical Diarrhea pathogens at different concentration.

3.12 Research ethics

An approval letter to conduct research using clinical samples was obtained from Biomedical Research Ethics Committee (Ministry of Health and Social Services) (APPENDIX A). A consent form and a sample collection form (APPENDIX B and APPENDIX C) to collect clinical specimens were drafted and sent for approval to the Biomedical Research Ethics Committee (Ministry of Health and Social Services), for approval of stool sample collection from patients in Katutura State hospital and Okuryangava clinic. In addition to the collection of plant material, voucher specimens of the plants were sent to the National Botanical Research Institute of Namibia (NBRI) for scientific identification. This type of identification was important for accurate documentation. A plant collection permit was obtained from the Ministry of Environment and Tourism of Namibia (MET) to license collection of plant materials used in this research.

CHAPTER 4: RESULTS

4.1 Effect of solvents on percentage yield from plant extracts

Aqueous and organic crude extracts were prepared from the various plant parts specified in table 3. The yields after preparation and drying are given in table 11 (Appendix D) and percentage yield is provided in table 3. The organic plant extracts for each plant showed the highest quantity of crude dry extract, except for *B. albitrunca* which showed higher percentage yield of 14% when extracted for aqueous extracts and the organic extract for *B. albitrunca* gave the percent yield of 7%. *Z. mucronata* and *T.sericea* showed higher percentage yield of 7% and 11% when extracted with organic solvents respectively. Moreover, *C. apiculatum* and *S. linnaeanum* showed the same % yield of 8 and 6 respectively in aqueous and organic extraction (table3).

Table 3. Effect of solvents on percentage yield from plant extracts

Plant name	Plant part used	% Yield	
		Organic extracts	Aqueous extracts
<i>B. albitrunca</i>	Roots	7	14
<i>Z. mucronata</i>	Bark	7	4
<i>C. apiculatum</i>	Bark	8	8
<i>S. linnaeanum</i>	Fruit	6	6
<i>T. sericea</i>	Twig	11	5

4.2 Screening for selected phytochemical classes

The TLC chemical profiles for the plant extracts investigated are portrayed in Table 4 and 5 below. The plants under investigation were tested for seven classes of phytochemicals with

known antibacterial properties which were alkaloids, coumarins, flavonoids, steroids, and triterpenoids, tannins and saponins. Only tannins and saponins were highly present in all aqueous extracts with an exception of *S. linnaeanum* aqueous extracts which did not have these classes of phytochemical. Coumarins and steroids were not present in any aqueous extract (table 4). Triterpenoids, saponins, coumarins and alkaloids were found in all organic plant extracts (table 5). Saponins were present in both aqueous and organic extracts. The organic extracts of *B. albitrunca* contained all the phytochemicals screened for whilst the aqueous extract of *B. albitrunca* only showed the presence of saponins and tannins.

Table 4: Phytochemical screening of aqueous plant extracts using TLC

Plant name	Triterpenoids	Saponins	Flavonoids	Steroids	Coumarins	Tannins	Alkaloids
<i>B. albitrunca</i>	-	+	-	-	-	++	-
<i>Z. mucronata</i>	-	++	+	-	-	+	+
<i>C. apiculatum</i>	-	+	-	-	-	++	-
<i>S. linnaeanum</i>	+	-	+	-	-	-	+
<i>T. sericea</i>	-	+++	-	-	-	+++	-

Key: +++ High presence, ++ moderate presence, + low presence, - absent

Table 5. Phytochemical screening of organic plant extracts using TLC

Plant name	Triterpernoids	Saponins	Flavonoids	Steroids	Coumarins	Tannins	Alkaloids
<i>B. albitrunca</i>	++	+	+	+	+++	+	++
<i>Z. mucronata</i>	++	++	++	+	+	-	+++
<i>C. apiculatum</i>	++	+	-	-	++	-	+
<i>S. linnaeanum</i>	+	+++	+++	-	+++	-	+++
<i>T. sericea</i>	+++	++	+++	+++	+++	-	++

Key: +++ High presence, ++ moderate presence, + low presence, - absent

4.3 Antibacterial activity of the plant extracts on laboratory strains of pathogenic bacteria

The 5 plants investigated *B. albitrunca*, *Z. mucronata*, *C. apiculatum*, *S. linnaeanum* and *T. sericea* were screened for antibacterial activity against laboratory strains of *E. coli*, *S. aureus*, *S. bodyii*, *L. monocytogenes* and *S. typhii* activity at concentrations of 250, 500 and 1000 µg/ ml.

The antibacterial activity in this study was measured as the formation of a clear zone of inhibition around the Whatman paper disc impregnated with plant extracts, the positive controls were antibiotic discs (vancomycin 10 µg, gentamycin 30 µg and tetracycline 35µg). The greater the zone of inhibition observed, the greater the antibacterial activity. Good antibacterial activity is the activity reported at lowest concentration at which antibacterial activity was determined. Figure 20 shows the formation of inhibition zones at different concentration and figure 21 showed inhibition against the positive controls. Only the inhibition zones of ≥ 7 mm were considered as positive results (NCCLS, 2008). The data for all zones of inhibition in each experiment is shown in Appendix D.

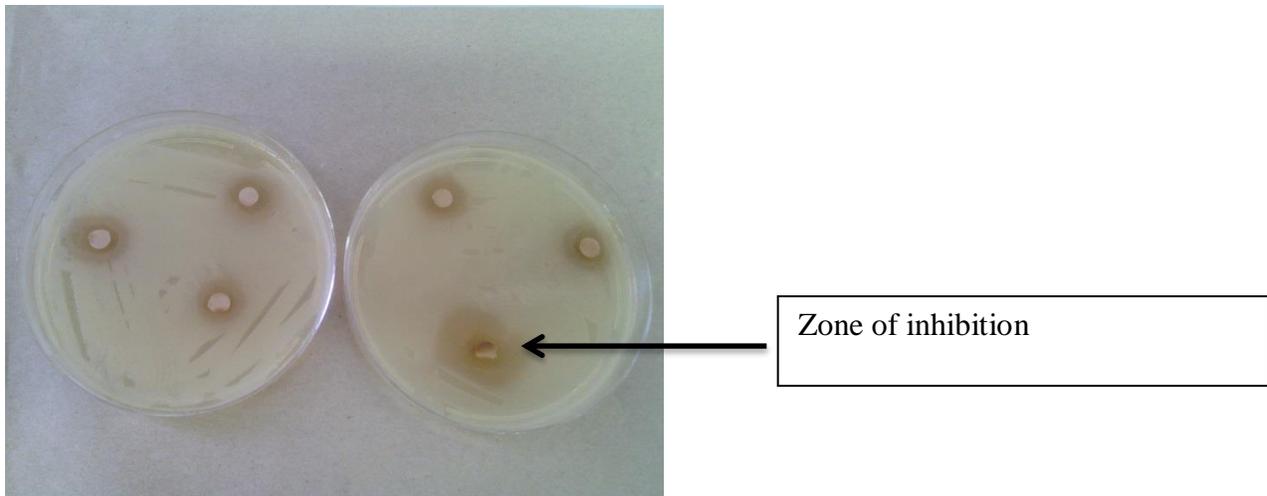


Figure 20. *S. boydii* growth inhibition by *T. sericea* plant extract on Muller Hinton agar.



Figure 21: *S. boydii* growth inhibition by Ampicillin (positive control) on XLD agar.

4.3. 1. Antibacterial activity of aqueous extracts against laboratory strains

Only *T. sericea* aqueous plant extracts showed antibacterial activity against all laboratory diarrheal strains of *E. coli*, *S. aureus*, *L. monocytogenes* and *S. boydii* with an exception of *S. typhi*. Growth inhibition of *E. coli*, *S. aureus*, *S. boydii* and *L. monocytogenes* by aqueous

extracts of *T. sericea* were observed at 500 and 1000 µg/ml. The good activity of antibacterial activity for aqueous extracts of *T. sericea* was recorded against *E. coli* and *S. boydii* (9 ± 0.57 , $n=3$) (i. e., average inhibition zone in mm, standard deviation and n = number of discs used per extract concentration) followed by growth inhibition against *S. aureus* (7.7 ± 0.58 , $n=3$) at 500 µg/ml as depicted in figure 22. There is a concentration dependent effect in antibacterial activity of aqueous extracts against different diarrheal pathogens.

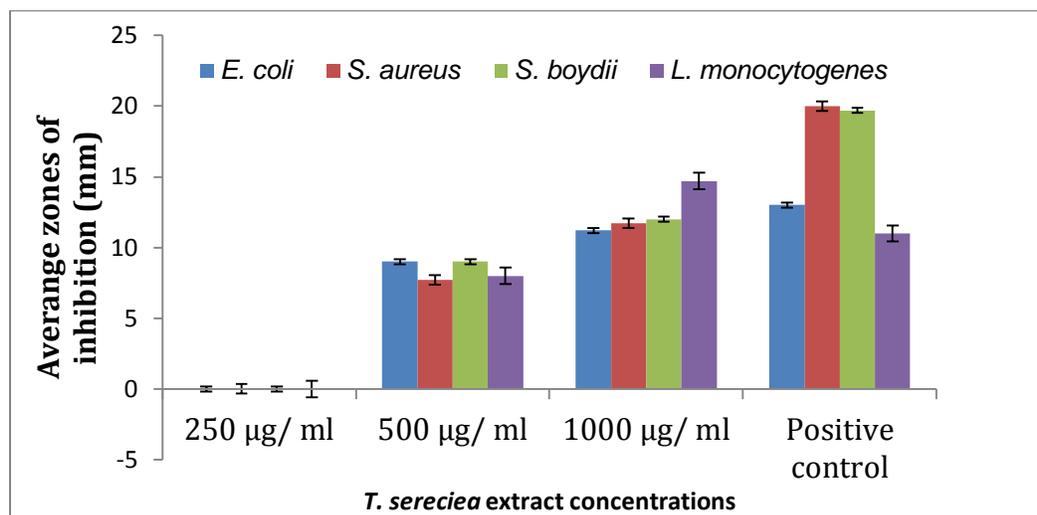


Figure 22: Antibacterial activity of aqueous extracts of *T. sericea* after 24 hours incubation against laboratory strains.

4.3.2 Antibacterial activities of organic extracts against Laboratory bacteria strains

T. sericea organic extracts showed antibacterial activity against *E. coli* at all three concentrations; with a concentration dependent effect. At 250 µg/ml average inhibition against *E. coli* was (7.7 ± 0.58 , $n=3$) (i. e., average inhibition zone in mm, standard deviation and n = number of replicates). *B. albitrunca* and *C. apiculatum* only showed antibacterial activity against *E. coli* (10.3 ± 0.58 , $n=3$ and 20.7 ± 0.57 respectively) at the highest concentration of 1000 µg/ml (figure 23). *S. linnaeanum* did not show antibacterial activity against *E. coli* at any concentration.

A positive control (tetracycline) showed antibacterial activity against *E. coli* with the average inhibition of 13.3 ± 0.57 (figure 23).

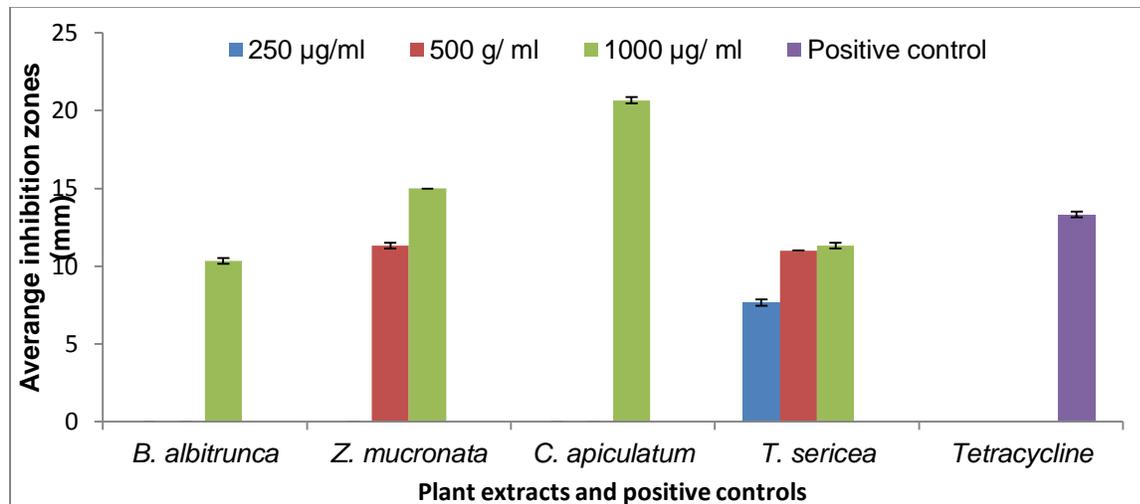


Figure 23: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum* and *T. sericea* against laboratory *E. coli* after 24 hours incubation.

For antibacterial activity against *S. aureus*, *T. sericea* showed antibacterial activity at all 3 concentrations with a concentration dependent effect. Average inhibitions of 7.7 ± 0.58 , $n=3$ at 250 µg/ml and at 500 µg/ml (11 ± 0.57 , $n=3$) followed by *Z. mucronata* (8 ± 0.29 , $n=3$). *T. sericea* also (11.3 ± 0.57 , $n=3$) at 1000 µg/ml and the lowest average zone of inhibition was for *B. albitrunca* against *S. aureus* (10.3 ± 0.58 , $n=3$) as depicted in figure 24. Positive control gentamycin showed higher antibacterial activity of 19.7 ± 0.58 , $n=3$.

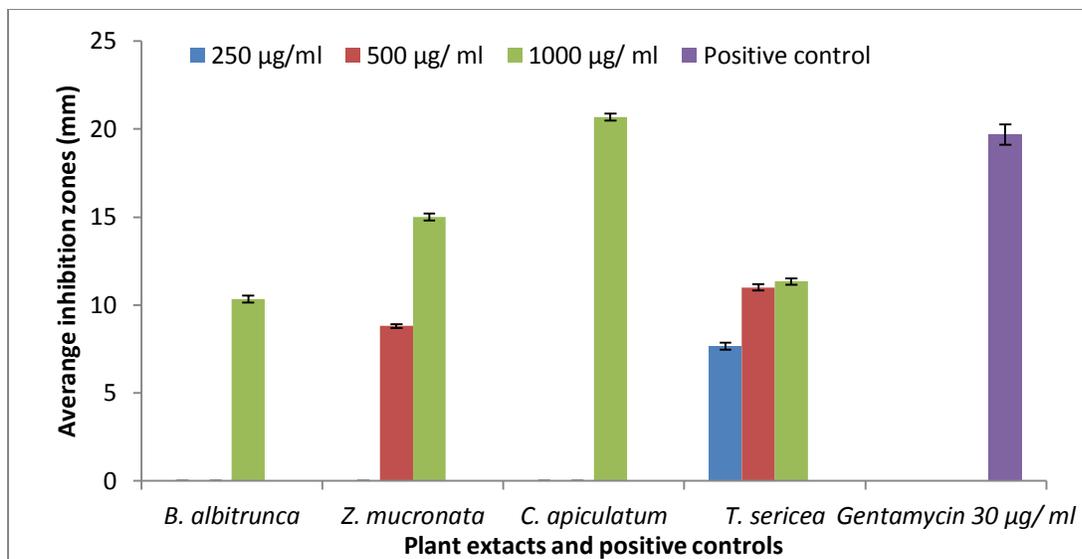


Figure 24: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum* and *T. sericea* against laboratory *S. aureus* after 24 hours incubation.

For antibacterial activity of organic extracts against *L. monocytogenes*, *S. linnaeanum*, *T. sericea* and *Z. mucronata* showed antibacterial activity at all three concentrations. *S. linnaeanum* showed the highest antibacterial activity at 250 µg/ml (15 ± 0.57 , n=3) followed by *T. sericea* (7.7 ± 0.57 , n=3). The least potent antibacterial activity was for *Z. mucronata* (7.3 ± 0.58 , n=3) at 250 µg/ml as depicted in figure 25. Positive control vancomycin showed antibacterial activity with the average inhibition of 11 ± 0.57 , n=3.

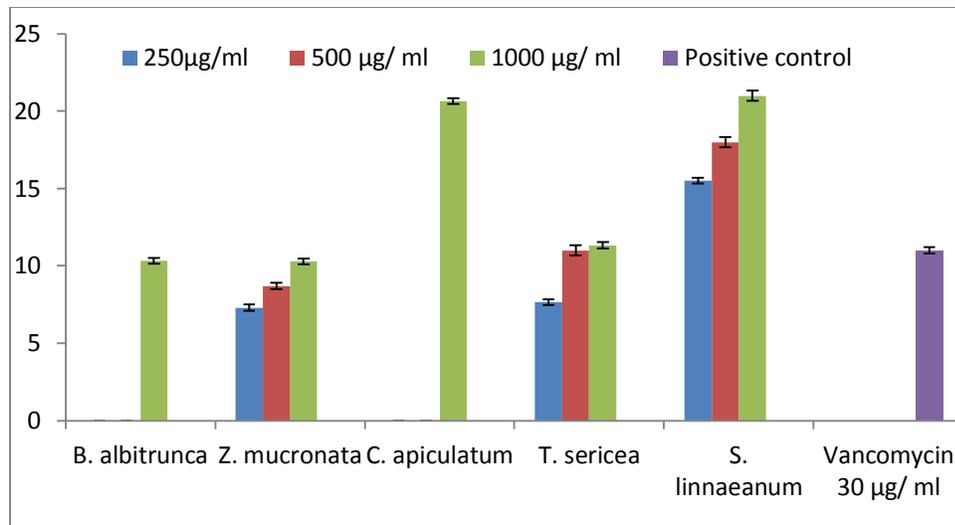


Figure 25: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum*, *T. sericea* and *S. linnaeanum* against laboratory *L. monocytogenes* after 24 hours incubation.

For antibacterial activity against *S. boydii*, only *T. sericea* and *Z. mucronata* showed antibacterial activity. *T. sericea* at all three concentration with at 250 µg/ml (8.7 ± 0.58 , n=3) and at 500 µg/ml (15.3 ± 0.57 , n=3) and at 1000 µg/ml (17.7 ± 2.08 , n=3) with a concentration dependent effect. *Z. mucronata* showed antibacterial activity (11.3 ± 0.58 , n=3) at 1000 µg/ml as depicted in figure 26. Positive control ampicillin showed antibacterial activity against *S. boydii* 19.7 ± 0.58 , n=3.

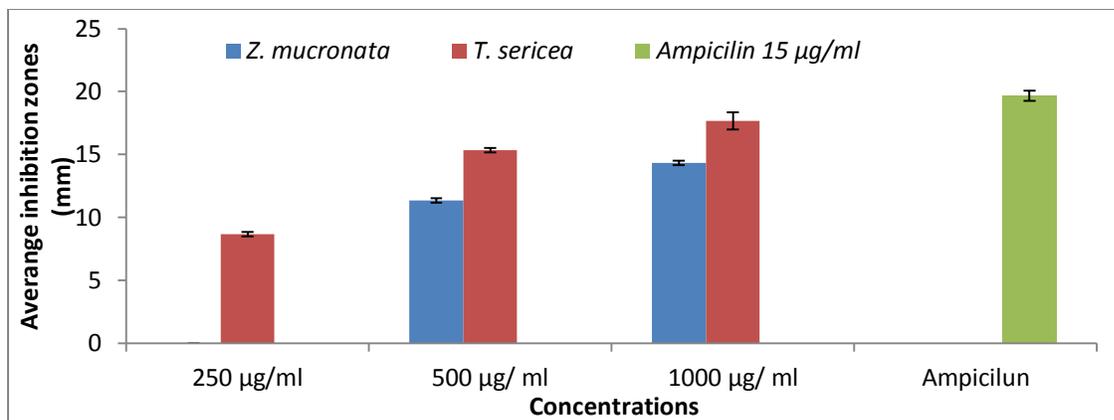


Figure 26: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum*, *T. sericea* and *S. linnaeanum* against laboratory *S. boydii* after 24 hours incubation.

Only *S. linnaeanum* organic extracts showed antibacterial activity at 1000 µg/ml (18 ± 1.15 , $n=3$) and there was no antibacterial activity at 500 and 250 µg/ml as depicted in figure 27. Positive control ampicillin showed antibacterial activity of 19.7 ± 0.56 , $n=3$ against *S. boydii*.

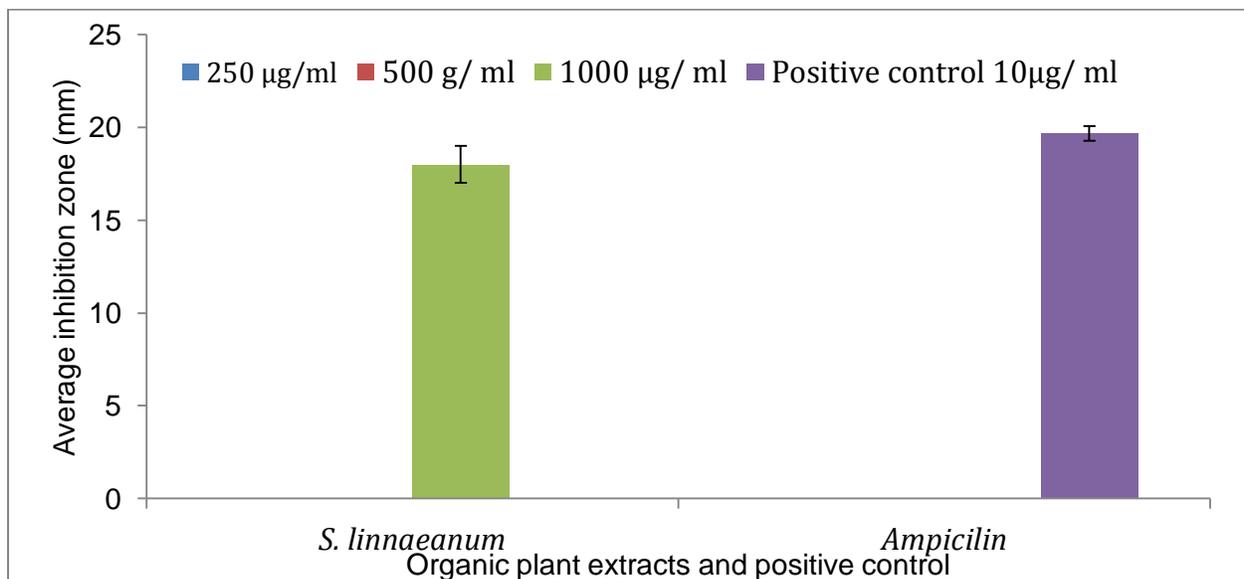


Figure 27: Antibacterial activity of organic extracts of *S. linnaeanum* against laboratory *S. typhi* after 24 hours incubation and ampicillin was used as a positive control.

Overall, irrespective of the concentrations, *T. sericea* aqueous and organic extracts showed the highest antibacterial activity against *E. coli*, *S. aureus*, *S. boydii* and *L. monocytogenes*. Only *S. typhi* was not inhibited by *T. sericea* aqueous extracts. Furthermore, the antibacterial activities of each plant extract (aqueous and organic) showed activity with a concentration dependent effect (i.e. the antibacterial activity increase with the increase in extract concentration). In addition, *S. linnaeanum* showed activity against the least number of pathogens in comparison to other plant extracts (i.e. *S. linnaeanum* only showed good antibacterial activity against *L. monocytogenes* at all 3 concentrations and against *S. typhi*).

The organic extracts showed a broad spectrum in MICs ranging from 62.5 to 1000 µg/ml. The organic plant extracts of *C. apiculatum* and *T. sericea* showing the lowest MIC of 62.5 µg/ml against clinical *E. coli* and *S. aureus* respectively. The highest MIC of 1000 µg/ml was recorded for *S. linnaeanum* and *B.albitrunca* against *S. typhi* and *L. monocytogenes* respectively. The MIC for *T. sericea* aqueous extract was 500µg/ml against *E. coli*, *S. aureus*, *L. monocytogenes* and *S. boydii* (table 6). Generally, the organic extracts exhibited stronger activity and a much broader spectrum of action than the aqueous extracts. Furthermore, only *T. sericea* showed the same MIC of 500µg/ml against laboratory strain of *E. coli* in both aqueous and organic plant extracts (table 6 and 7).

Table 6. Minimum inhibitory concentrations of *T. sericea* aqueous extracts against different selected diarrhea causing bacteria

Plant names	Test organisms			
	<i>E. coli</i> ATCC25922 (µg/ml)	<i>S. aureus</i> ATCC25923 (µg/ml)	<i>L. monocytogenes</i> ATCC13932 (µg/ml)	<i>S. boydii</i> ATCC9207 (µg/ml)
<i>T. sericea</i>	500	500	500	500

Table 7. Minimum inhibitory concentrations for organic plant extracts against selected diarrhea causing bacteria

Plant names	Test organisms				
	<i>E. coli</i> ATCC2592 2 (µg/ml)	<i>S. aureus</i> ATCC2592 3 (µg/ml)	<i>L. monocytogene</i> ATCC13932 (µg/ml)	<i>S. typhi</i> ATCC (µg/ml)	<i>S. boydii</i> ATCC920 7 (µg/ml)
<i>B. albitrunca</i>	125	125	1000	>100 0	
<i>Z. mucronata</i>	250	250	500	>1000	500
<i>C. apiculatum</i>	62.5	250	500	>1000	
<i>T. sericea</i>	500	62.5	250	>1000	125
<i>S. liannaeanum</i>	>1000	>1000	250	1000	>1000

4.4 Clinical description of collected stool samples

A total of 12 clinical stool samples were collected. Six stool samples were collected from Katutura state hospital and 4 stool samples were collected from Okuryangava Clinic. The samples were collected from children under 5 years with acute diarrhea. The pathogens identified in each sample after biochemical tests and selective media identifications are listed in table 8.

4.5 Biochemical test confirmation

All the bacteria isolates obtained from stool samples listed in table 8 were confirmed using biochemical tests. The obtained results were consistent with the characteristics of *Salmonella*, *E. coli* and *Shigella* as described by Madigan (2009). The results obtained for *Salmonella*, *Shigella* and *E. coli* in table 8 were acquired after comparing the obtained results to diagnostic results for biochemical tests in table 11 (APPENDIX D).

E. coli colonies appeared yellow on XLD agar while pink on MAC agar. *Salmonella* colonies appeared red with black centers on XLD and colorless on MAC. *Shigella* appeared red or clear on XLD while colorless on MAC. Figures 28 to 29 shows some of the plate cultures of *Salmonella*, *Shigella* and *E. coli* isolated from stool specimen collected.

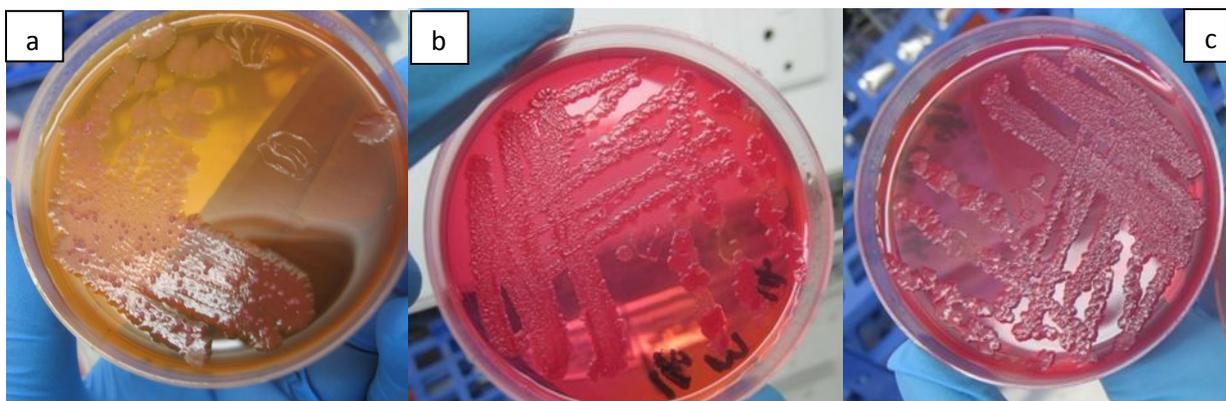


Figure: 28 MAC agar with a) *Salmonella* lactose negative colonies, b) *E. coli* lactose fermenting positive colonies, c) *Shigella* lactose positive colonies.

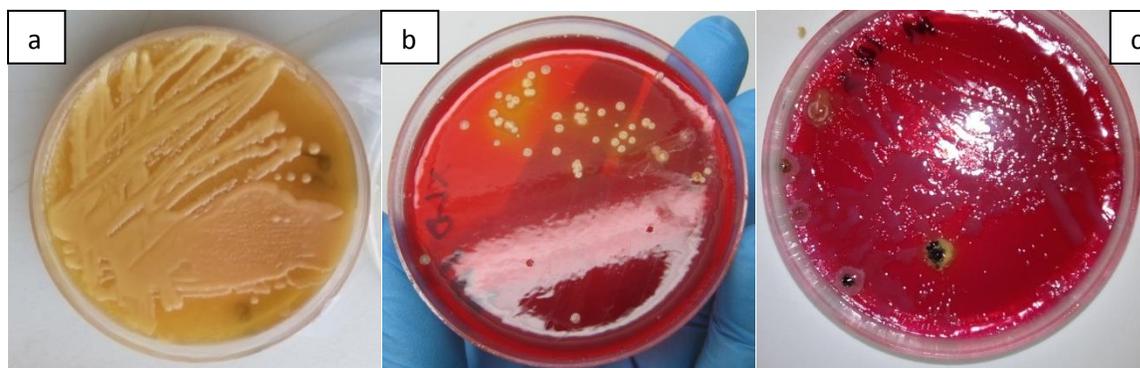


Figure: 29. XLD agar with a) clinical *Shigella* clear colonies, b) clinical *E. coli* yellow colonies c) clinical *Salmonella* pale-red colonies with black centers.

Table 8. Characterization and isolation of Bacteria on XLD and MAC

	XLD	MAC
Sample code	Isolated bacteria	Isolated bacteria
Okury (0200- 29)	<i>Shigella</i>	<i>Shigella</i>
Okury (0200-14)	<i>Salmonella</i>	<i>Salmonella</i>
Katut(0200-141)	<i>E. coli</i>	<i>E. coli</i>
Katu(0200-159)	<i>E. coli</i>	<i>E. coli</i>
Katu(0200-158)	<i>E. coli</i>	<i>E. coli</i>
Katu(0200-154)	<i>E. coli</i>	<i>E. coli</i>
Katu(0200-144)	<i>E. coli</i>	<i>E. coli</i>
Katu(0200-155)	<i>E. coli</i>	<i>E. coli</i>
Katu(0200-123)	<i>E. coli</i>	<i>E. coli</i>

4.6 Antibacterial assay on isolated clinical strains

The 5 plants investigated *B. albitrunca*, *Z. mucronata*, *C. apiculatum*, *S. linnaeanum* and *T. sericea* were screened for antibacterial activity against the isolated clinical bacterial strains of *E. coli*, *Salmonella* and *Shigella* at different concentrations of 250, 500 and 1000 µg/ml (as done on laboratory bacterial strains).

4.6.1 Antibacterial activity of aqueous plant extracts against clinical strains

Only *T. sericea* aqueous plant extracts showed antibacterial activity. Antibacterial activity was only observed against *Shigella* clinical strains at 1000 µg/ml with an average zone of inhibition of 7.3 ± 0.47 , n=3 (figure 30). Positive control ampicillin 10 µg/ml did not show antibacterial activity against *Shigella* (figure 30).

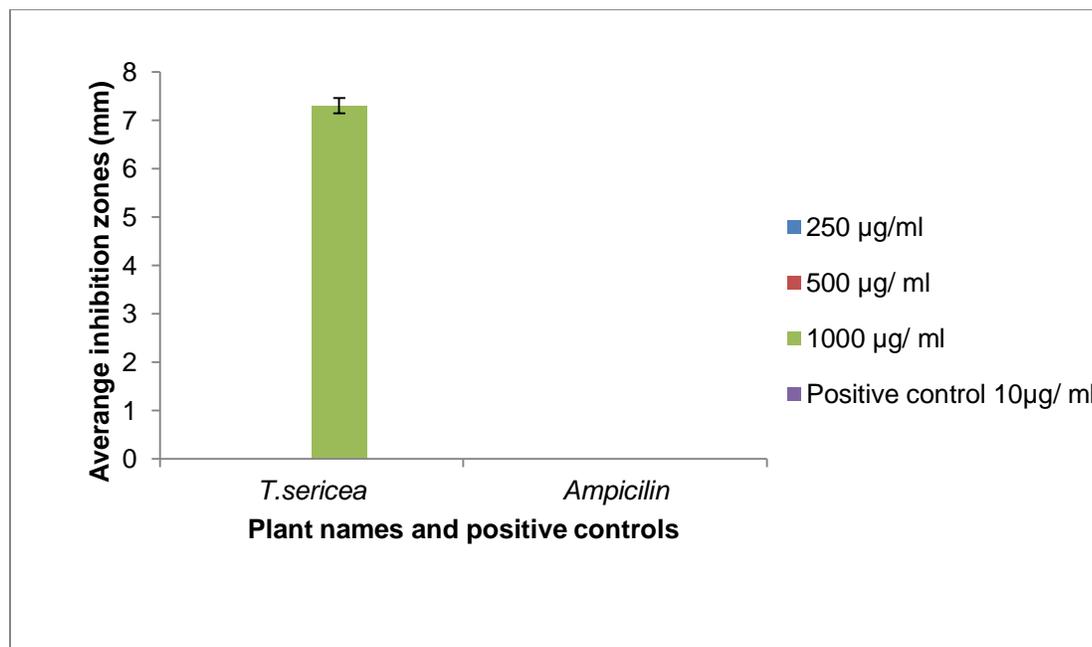


Figure 30: Antibacterial activity of aqueous extract of *T. sericea* against clinical *Shigella* after 24 hours incubation.

4.6.2 Antibacterial activity of organic extracts against different clinical strains

For antibacterial activity of organic extracts against *Shigella*, no antibacterial activity was observed at 250 and 500 $\mu\text{g/ml}$ in all organic plant extracts. However, *C. apiculatum* organic extract showed good antibacterial activity at 1000 $\mu\text{g/ml}$ (16 ± 0.57 , $n=3$) and the lowest antibacterial activity was for *Z. mucronata* against *Shigella* (9.3 ± 0.57 , $n=3$). The rest of the plants showed no antibacterial activity against *Shigella* (Figure 31). Positive control ampicillins 10 $\mu\text{g/ml}$ did not show antibacterial activity against clinical *Shigella* (although it was able to inhibit laboratory strain of *Shigella*) could not inhibit the growth of *Shigella* (figure 31 and 32).

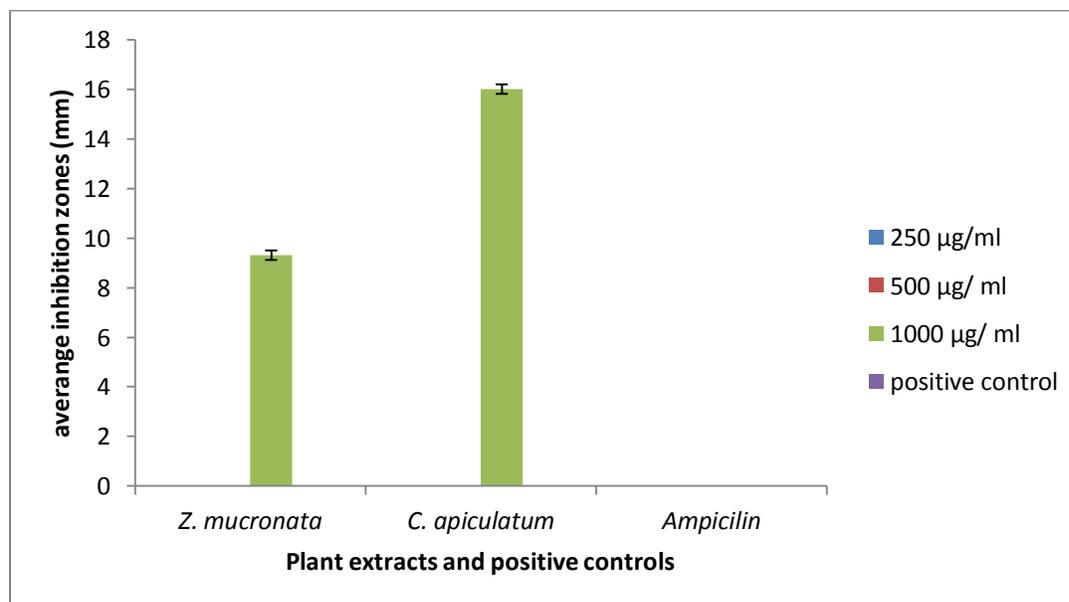


Figure 31: Antibacterial activity of organic extracts of *Z. mucronata* and *C. apiculatum* against clinical *Shigella* after 24 hours incubation

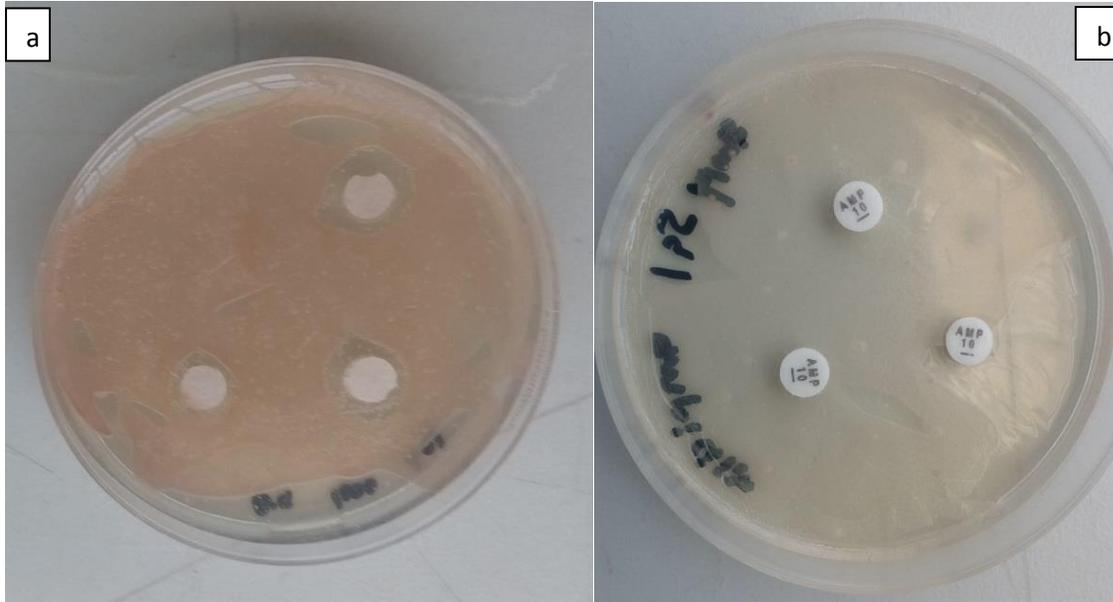


Figure 32: *Shigella* growth inhibition by a) *C. apiculatum* organic plant extracts 1000 µg/ml b) Ampicillin (positive control) on Muller hinton agar.

For antibacterial activity of organic extracts against *Salmonella*, *C. apiculatum* showed antibacterial activity at all 3 concentrations with a concentration dependent effect. *C. apiculatum* showed antibacterial activity at 250 µg/ml (12 ± 2.51 , n=3). *Z. mucronata* only showed antibacterial activity at 1000 µg/ml (9.7 ± 0.57 , n=3) as depicted in figure 33. Ampicillin which was used as a positive control could not inhibit the growth of *Salmonella* as depicted in figure 33 and 34.

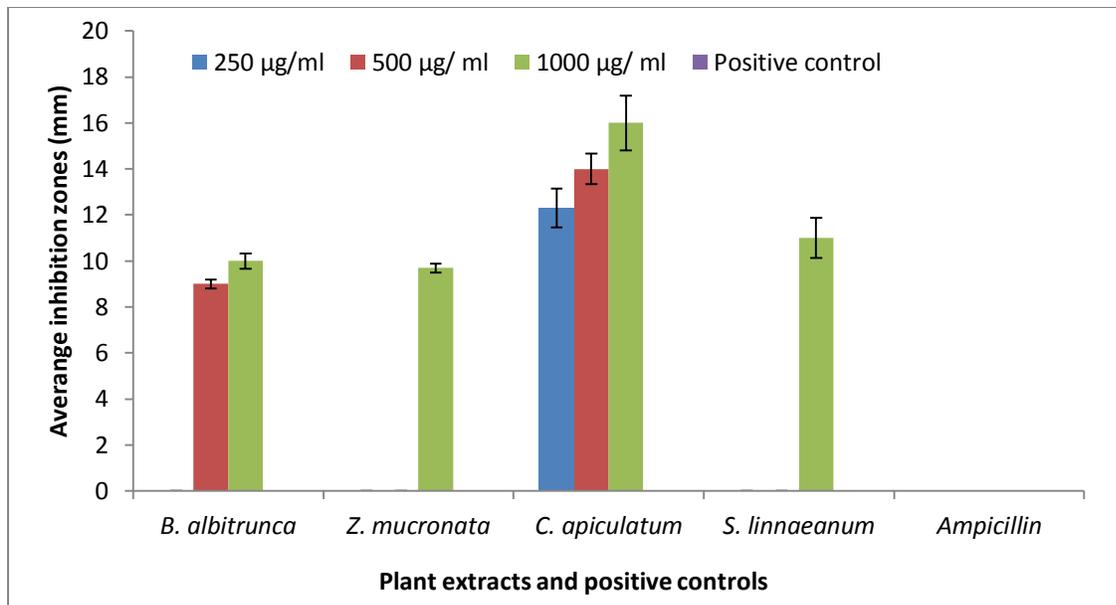


Figure 33: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum* and *S. linnaeanum* against clinical *Salmonella* after 24 hours incubation.

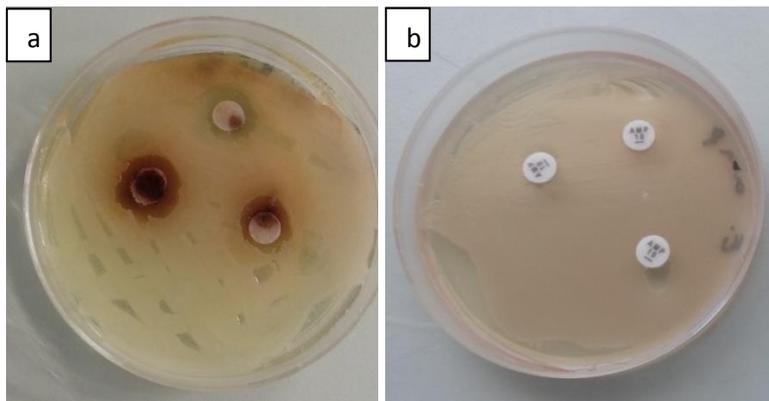


Figure 34: *Salmonella* growth inhibition by a) *C. apiculatum* organic plant extracts 250 µg/ml b) ampicillin (positive control) on Muller hinton agar.

For antibacterial activity of organic extracts against *E. coli*, *T. sericea* showed highest antibacterial activity at all 3 concentrations. *T. sericea* showed antibacterial activity of 7.3 ± 0.57 , $n=3$ at 250 µg/ml. *C. apiculatum* only showed antibacterial activity at 1000 µg/ml (8 ± 1.0 , $n=3$) as depicted in figure 35. Positive control tetracycline showed good antibacterial activity of 20 ± 0.58 .

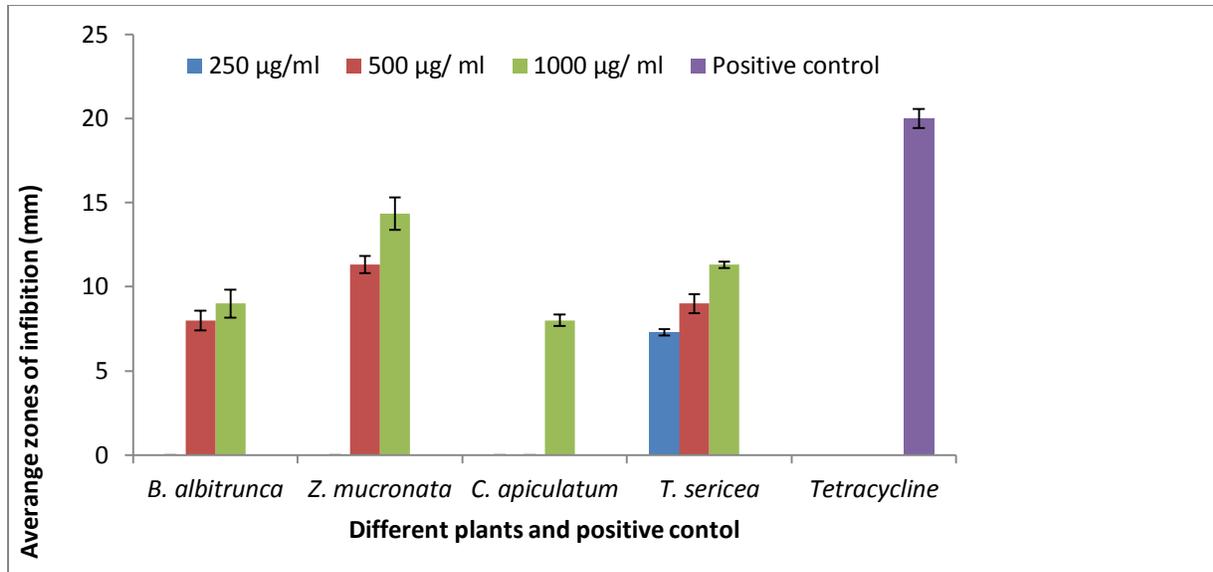


Figure 35: Antibacterial activity of organic extracts of *B. albitrunca*, *Z. mucronata*, *C. apiculatum* and *T. sericea* against clinical *E. coli* after 24 hours incubation.

Figure 36 a) shows the formation of inhibition zones at 1000 µg/ml concentration and figure 36 b) showed inhibitions against the positive controls. Only the inhibition zones of ≥ 7 mm were considered as positive results.

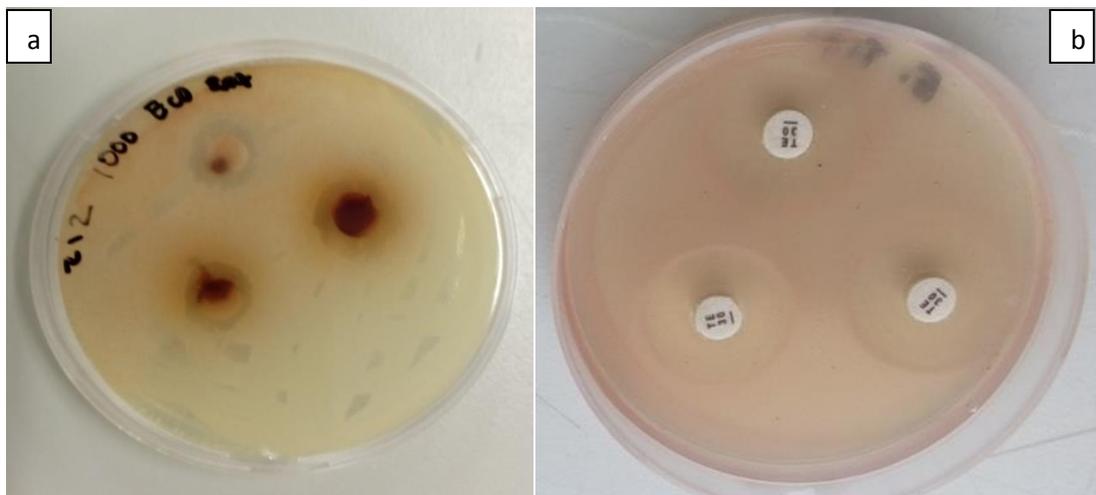


Figure: 36. *E. coli* growth inhibition by a) *Z. mucronata* organic plant extracts 1000µg/ml b) Tetracycline (positive control) on Muller Hinton agar.

Based on the statistical analysis results of two-way Student's t-test, at 95% confidence level on the zones of inhibition of *T. sericea* (plant with highest antibacterial activity for both aqueous and organic extracts), by comparing the level of significance of antibacterial activity of organic extracts of *T. sericea* against laboratory *Shigella species*, a P- value of 0.008 and F- value of 1.68 were calculated $P < 0.05$. Hence we reject the null hypothesis and conclude that there is significant variation in the mean of inhibition zones of *T. sericea* organic and aqueous extracts against laboratory *S. boydii*.

By comparing the level of significance of antibacterial activity of aqueous and organic extracts of *T. sericea* against clinical *Shigella*, a P- value of 0.787 and F- value of 1.45 were calculated $P > 0.05$. Hence we accept the null hypothesis and conclude that there is no significant variation in the mean of inhibition zones of *T. sericea* aqueous and organic extracts against clinical *Shigella*.

By comparing the level of significance of antibacterial activity of organic extracts of *T. sericea* against laboratory *Shigella boydii* and clinical *Shigella species*, a P- value of 0.001 and F- value of 1.24 were obtained $P < 0.05$. Hence we reject the null hypothesis and conclude that there is significant variation in the mean of inhibition zones of *T. sericea* organic extract against laboratory *S. boydii* and clinical *Shigella species*. Interestingly, aqueous extracts of *T. sericea* inhibited both clinical and laboratory *Shigella species* with a difference in antibacterial activity. Hence, when the level of significance of antibacterial activity of aqueous extracts of *T. sericea* was compared between laboratory *Shigella boydii* and clinical *Shigella species*, a P- value of 0.063 and F- value of 1.96 were calculated $P > 0.05$. Hence we do not reject the null hypothesis and conclude that there is no significant variation in the mean of inhibition zones of *T. sericea* aqueous extracts against laboratory and clinical *Shigella species*.

By comparing the level of significance of antibacterial activity of organic extracts of *T. sericea* against clinical and laboratory *E. coli* species, a P- value of 0.093 and F- value of 7.92 were calculated $P > 0.05$; *S. aureus* strain a P- value of 0.197 and F- value of 28.06 were calculated $P > 0.05$. Hence we do not reject the null hypothesis and conclude that there is no significant variation in the mean of inhibition zones of *T. sericea* organic and aqueous extracts against laboratory and clinical strains of *E. coli* and laboratory strains of *S. aureus*.

By comparing the level of significance of antibacterial activity of organic and aqueous extracts of *T. sericea* against laboratory *L. monocytogenes* a P- value of 0.006 and F- value of 1.63 were calculated $P < 0.05$. Hence we reject the null hypothesis and conclude that there is significant variation in the mean of inhibition zones of *T. sericea*

Similarly, all the plant extracts that showed antibacterial activity against laboratory *E. coli* and laboratory *Shigella* also showed activity against clinical *E. coli* and *Shigella* respectively. Although the laboratory strain of *Salmonella typhi* was the least sensitive to the plant extracts (i.e. its growth was only inhibited by *S. linnaeanum*), Clinical *Salmonella* was highly sensitive to the plant extracts (i.e. it was inhibited by more than one plant extract).

The MIC concentrations for organic extracts ranged from 250 to 1000 $\mu\text{g/ml}$. The organic plant extracts of *C. apiculatum* and *T. sericea* showed the lowest MIC of 250 $\mu\text{g/ml}$ against *Salmonella* and *E. coli* respectively as depicted in table 9. Aqueous extracts of *T. sericea* showed MIC of 1000 $\mu\text{g/ml}$ against clinical *Shigella* isolate

Table 9. MIC of organic and aqueous plant extracts against clinical bacterial strains

Organic plant extracts	Test organisms' MIC in µg/ml		
	<i>E. coli</i>	<i>Salmonella</i>	<i>Shigella</i>
<i>B. albitrunca</i>	500	500	>1000
<i>Z. muronata</i>	500	1000	1000
<i>C. apiculatum</i>	>1000	250	1000
<i>T. sericea</i>	250	>1000	>1000
<i>S. linnaenum</i>	1000	1000	>1000
Aqueous plant extracts			
<i>T. sericea</i>	>1000	>1000	1000

Over all, organic extracts of *C. apiculatum* and *T. sericea* showed the lowest MICs against clinical *Salmonella* and *E. coli* respectively (250 µg/ml) as well as laboratory *E. coli* (62.5µg/ml).

CHAPTER 5: DISCUSSION

Diarrheal diseases are major causes of morbidity and mortality especially among children in developing countries (Gunasegaran, Kasi, Sathasivam, Sreenivasan and Subramaniam, 2011). Statistics have shown that 5.9 million children around the world die before reaching their fifth birthday. Of these 5.9 million reported 16% deaths were due to pneumonia and 9% due to diarrhea; making them two of the leading killers of children worldwide. Together, these diseases claimed the lives of nearly 1.5 million children under the age of 5 (International Vaccine Access Center (IVAC), 2015).

Various medicines have been developed to combat diarrhea such as rotavirus immunization, cholera immunization, measles immunization, Oral rehydration solution, zinc supplements, tetracycline, ampicillin and fixed ratio combination treatment of antibiotics (WHO, 2010; IVAC, 2015). However, despite the available medicine and vaccines, diarrhea remained to be among the top causes of preventable morbidity and mortality especially in developing countries (WHO, 2010). This is because most people in developing countries are exposed to poor sanitation and lack of clean drinking water due to poverty (Water Aid, 2011; United Nation, 2014). In addition, many people in rural areas are far from hospitals hence, it takes several hours or days to reach the health facilities and they have no access to the antibacterial pharmaceuticals (Nguyen, Van, Hey, Gia & Weintraub, 2006). Another factor for the continuing morbidity and mortality is that some pathogens such as *Shigella*, *Campylobacter*, *Nontyphoidal Salmonella*, and *enterotoxigenic Escherichia coli* have become resistant to most of the antibiotics (Pendota, Aderogba and Van Staden, 2015; Miljković, Selimović, Babić, Stojanović, 2010). As a result, incidents and death cases due to diarrhea continue to intensify. The present study aimed at investigating the antibacterial activity of selected plants used traditionally to treat diarrheal diseases since

according to Payyappallimana, (2009) there is a lack of sufficient scientific evidence on the efficacy of medicinal plants that 80 % of people in developing countries rely on for treatment.

T. sericea had the highest antibacterial activity against most diarrheal bacteria and it also showed broad spectrum activity inhibiting growth of not only *S. boydii*, *E. coli*, *S. aureus* and *L. monocytogenes* laboratory strains but also clinical strains of *Shigella*. *T. sericea* is used traditionally to treat diarrhea, coughs and infected wounds (Masupa, 2012); Vuuren *et al* (2015) also reported higher antibacterial activity in *T. sericia* organic and aqueous extracts against *S. aureus*, *E. coli*, *S. typhi* and *S. flexneri* in which organic extracts showed higher antimicrobial activities than aqueous extracts at the concentrations ≤ 1.0 mg/ ml. In this study *T. sericia* extracts also showed higher activity for organic and lower activity for aqueous extracts which is similar to the findings of Vuuren *et al* (2015). It has been reported that organic extract had more phytochemicals compounds than aqueous extracts (Tiwari *et al.*, 2011; Kumar *et al.*, 2010; Satar *et al.*, 2010). Saponins were found in both aqueous and organic extracts and may have contributed to the activity as the *T. sericia*. Kannabiran *et al.*, (2009) isolated saponin fraction that exerted antimicrobial activity higher than the aqueous, organic extracts and standard antibiotics. The presence of alkaloids, tannins, coumarins, steroids, flavonoids and other phytochemical compounds may have contributed to this activity. This is because secondary metabolites inhibit microbial activities through different mechanisms such as by forming complexes with bacteria cell walls, membrane disruption, and protein lysis and by enhancing membrane absorption of Sodium and water (Tiwari, 2011).

Clinical bacterial strains confirmed to belong to *E. coli*, *Salmonella* and *Shigella* species were isolated in stool samples of the children under the age of five from Katutura state hospital and Okuryangava clinic in Windhoek. These diarrheal pathogens are responsible for 1.3 million

global deaths per year among the children under five years, making diarrhea the second most common cause of death worldwide (UNICEF, 2012). Clinical bacterial strains of *Salmonella* and *Shigella* species isolated in this study did not respond to low doses of ampicillin (standard) at 10ug/ml, which is equivalent to the clinic dose (Gunasegaran *et al.* , 2011). This could mean that the clinical *Salmonella* and *Shigella* have developed resistance to the used antibiotics or that the concentration used to prepare the used antibiotic discs is lower. These findings were similar to those of Gunasegaran *et al* (2011) and Sosa *et al* (2009) that showed a 100% resistance of the isolated *Salmonella* and *Shigella species* to ampicillin and tetracycline in Africa. This may also be due to the presence of more than one serovar of *Salmonella* and *Shigella* in the stool samples which usually results in decrease antibacterial activity (Zhang *et al.*, 2013; Gunasegaran *et al.*, 2011). In this study, although ampicillin was ineffective against clinical isolates of *Salmonella* and *Shigella*, it was effective in inhibiting laboratory strains of *S. typhi* and *S. boydii* (appendix D). This could be due to the difference in virulence between clinical and laboratory strains of *Salmonella* and *Shingella*.

An increase in microbial resistance calls for new antibacterial drugs with a new mode of action (Cheruyot and Kateregga, 2009), one of which could be traditional medicinal plants. In this study, organic plant extracts of *B. albitrunca*, *T. sericea*, *Z. mucronata*, *B. albitrunca* and *C. apiculatum* showed a broad spectrum of antibacterial activity against clinical strains of *Salmonella*, *Shigella* and *E. coli*. Only *T. sericea* and *C. apiculatum* showed antibacterial activities against *Salmonella* and *E. coli* at 250 µg/ml. Organic plant extracts of *B. albitrunca*, *T. sericea*, *Z. mucronata*, *B. albitrunca* and *C. apiculatum* showed the higher presence of phytochemical compounds such as triterpenoids, saponins, coumarins and alkaloids. The bioactive constituents present could be responsible for the antibacterial activity of these plant

extracts (Murugan, Wins and Murugana, 2013). Tannins have been found to form irreversible complexes with proline-rich proteins resulting in the inhibition of the cell protein synthesis. This phytochemical compound was present in *B. albitrunca* hence it could have attributed to its antibacterial activity against test organisms. A study by Cheruiyot, Olila and Kateregga, (2009) also showed that secondary plant metabolites constitute an important source of microbicides, pesticides and many pharmaceutical drugs.

Although clinical *Salmonella* was resistant to the ampicillin antibiotic used, Plant extracts showed good antibacterial activity against clinical *Salmonella*. These findings are on the contrary with the antibacterial activity of plant extracts and ampicillin against laboratory *S. typhi*, since only *S. linnaeanum* showed antibacterial activity against laboratory strain of *S. typhi*. Moreover, the 10 µg/ml of ampicillin used inhibited the growth of laboratory *S. typhi*.

Among the laboratory bacterial strain, *L. monocytogenes* was most sensitive to the plant extracts since all five plant extracts under study showed activity against *L. monocytogenes* at least at one extract concentration used. These findings are significant since to date, there are no commercially available vaccines to protect against infection by *Listeria* which is acquired by consuming raw (unpasteurized) milk or food that contain unpasteurized milk (Davis, Nettleman and Stoppeler, 2015). This makes these medicinal plants potential sources of anti- listeria agents that can be used in treatment against listeriotic diarrhea.

Sample preparation is the crucial first step in analysis of herbs, because it is necessary to extract the desired chemical components from the herbal material for further separation and characterization (Gupta, Naraniwal & Kothari, 2012). According to Dupreez, (2012) the moisture content of fresh plant material changes the chemical composition and properties of the plant over

time and grinding of the plant material into fine particles helps to create a larger surface area to allow higher extraction of bioactive compounds. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate (Tiwari, Kumar, Kaur, Kaur, Kaur, 2011). In traditional settings, water is used as a solvent for preparing plant extracts, however, literature recommend the use of organic solvents such as ethanol, methanol, chloroform and hexane etc. Hydro-alcoholic solvent mixture (i.e., mixture of alcohol and water in varying proportions) is generally considered to give high extraction yields which is owing to their expanded polarity range (Gupta, Naraniwal & Kothari, 2012). Hence in this study, apart from distilled water that was used to prepare aqueous extracts, a hydro-alcoholic solvent containing 90 % ethanol and 10% distilled water was used to prepare organic extracts. Organic and aqueous extracts were prepared to enable extraction of polar bioactive compounds. The results of this study complied with Gupta, Naraniwal & Kothari, (2012), since most bioactive compounds such as coumarins, alkaloids, saponins, steroids, triterpenoids, and flavonoids were highly present in hydro- organic extracts of 90% ethanol and only tannins and saponins were highly present in aqueous extracts. Apart from other phytochemical compounds present in *T. sericea* extracts, higher presence of saponins was detected in both organic and aqueous extracts of *T. sericea*. This is because this phytochemical compound can be extracted using both water and organic solvents (Tiwari *et al* 2011).

Although aqueous extracts of *B. alibitrunca* provided high percentage yield of extract than its organic extract, the organic extract showed the presence of all phytochemical compounds and aqueous extracts only showed the presence of tannins and saponinns. The ability of aqueous extracts of *B. alibitrunca* to yield higher extract than its organic extract is on the contrary to other

findings since according to Parekh *et al* (2005), extraction with organic solvent lead to high yield of dry weight of the plant extracts than aqueous extracts, although this could mean that the aqueous extracts of *B. albitrunca* contained more polar compounds that are easily soluble in water mainly, carbohydrates, glycosides (sugar containing compounds) (Parekh *et al.*, 2005). Although studies such as Tiwari *et al.*, 2011; Rawani, Pal and Chandra (2011) and Murugan *et al.*, (2013) have linked the antibacterial activity of plant extracts to the phytochemical compounds in the plant extracts studied, there have be no studies that linked the yield of the plant extracts, phytochemical compounds and antibacterial activities of the plant extracts. Based on the findings of this study, the yield of the plant extracts cannot be linked directly proportional to their phytochemical compounds and antibacterial activities because, plant extracts such as aqueous extract of *B. albitrunca* that showed higher % yield did not show higher presence of phytochemical compounds neither did it show potent antibacterial activities. On the contrary organic extracts of *B. albitrunca* that showed lower yield showed a broad spectrum of antibacterial activity and better antibacterial activity than aqueous extracts against both clinical and laboratory strains.

In this study all organic extracts showed the presence of more phytochemical compounds than aqueous extracts. This is because more bioactive compounds such as flavonoids, steroids and alkaloids are more soluble in organic solvents such as ethanol, methanol, chloroform, ether and acetone (Du Preez and Mumbengegwi, 2012), while water can only extract sugar, tannins and saponins (Tiwari *et al.*, 2011). This could mean that the effectiveness of traditional medicine could be due to the presence of compounds such as tannins and saponins, since traditional medicine is prepared using water. In the present study, apart from tannins and saponins, flavonoids and alkaloids were also present in aqueous extracts of *S. linnaeanum* and *Z.*

muronata, while triterpenoids were only present in aqueous extracts of *S. linnaeanum*. This finding contradicts Tiwari *et al* (2011), since according to Tiwari, water can only extract sugars, tanins and saponin and no other phytochemical compounds.

Although Kirsoy, (2006); Gürbüz *et al.*, (2015) reported on the toxicity of *S. linnaeanum* and the presence of solamargine and solasonine glycoalkaloids with medicinal properties in *S. linnaeanum*. There is no other study to date that reported on the presence of other phytochemical compounds in *S. linnaeanum*. Hence, the findings of the presence of phytochemical compounds such as saponins, triterpenoids, coumarins and flavonoids with antibacterial activity in *S. linnaeanum* obtained in this study are new and have not been reported in other studies. Furthermore, the antibacterial activity of *S. linnaeanum* has also not been documented.

Moreover, it is interesting to note that although water is universal solvent and it is used by many traditional healers in preparing herbal medicine, organic solvents have been found to give more consistent antimicrobial activity compared to water extracts. This could be because bioactive compounds such as water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics only important as antioxidant compound (Tiwari *et al.*, 2011). This could explain the lower antibacterial activities reported for aqueous extracts in comparison to organic extracts.

Qualitative phytochemical screening of plant extracts revealed that different plant parts used in the study exhibited different kinds of secondary metabolites. However, in organic extracts, root extracts were found to contain all phytochemical compounds. This findings support Srivastava and Srivastava, (2007), who explained that the hairy root cultures offer promise for high production and productivity of valuable secondary metabolites (used as pharmaceuticals, pigments and flavors) in many plants. Although this could mean that to get more phytochemical

compounds, roots of the plants have to be used. However, the findings of this study showed that the number of phytochemical compounds present in an organic extract is not directly proportional to its antibacterial activity, other parts of the plants extracts such as leaves, twig and bark can be used since they also showed the presence of phytochemical compounds (although not all) with potent antibacterial activity than the root extracts. This will help to maintain the vegetation and avoid extinction of medicinal plants.

Each plant included in this study inhibited the growth of at least one diarrheal pathogen, with a concentration dependent effect. The findings of Abu-Gharbia *et al* (2014) and Bhalodia and Shukla (2011) also showed antibacterial activities of *Cassia fistula*, *C. cassia*, *S. aromaticum* and *H. sabdarffa* with a concentration dependent effect against different oral pathogens.

The findings of this study also showed a difference in MIC of plant extracts against clinical and laboratory diarrheal strains. It is important to note that a higher concentration of 1000 µg/ml was the MIC for the plant extracts against clinical strains while for plants such as *C. apiculatum* and *T. sericea* an MIC of 62.5 µg/ml was found to be the MIC. The findings of this study are the first to date in comparing antibacterial activity of plant extracts and antibiotics against both laboratory and clinical strains.

Based on the findings from statistical analysis done on organic and aqueous extracts of *T. sericea* there was a significant difference in antibacterial activity of plant extracts at different concentrations against different bacterial strains as well as in antibacterial activities of aqueous and organic extract of *T. sericea* at different concentrations with an exception in antibacterial activity of organic and aqueous extracts against *L. monocytogenes* that showed no significant difference in antibacterial activities between aqueous and organic extracts of *T. sericea*.

Moreover, there was significant difference in the antibacterial activities of organic extracts of *T. sericea* against both clinical and laboratory *Shigella* species and in the antibacterial activity of aqueous extracts of *T. sericea* at different concentrations against different pathogens. In addition, there was also a significant difference in antibacterial activity of *T. sericea* against clinical and laboratory strains of *E. coli*.

Although traditional uses for selected plants in this study is documented, scientific validations and antibacterial efficacy of these plants comparing activity of the extracts against clinical and laboratory strains does not exist. However, the information provided by traditional healers and other knowledge holders during ethnobotanical surveys serves an important goal toward documentation of the uses, of many medicinal plants. All these validates that traditional plant knowledge is of significant value, not just for local uses, but also to researchers for the development of novel antidiarrheal medicines. The findings of this study served as justification to validate the use of *T. sericea*, *Z. mucronata*, *S. linnaeanum*, *B. albitrunca* and *C. apiculatum* in fighting diarrheal causing pathogens in Namibia. It also verified the presence of phytochemical compounds associated with antibacterial activities.

The findings of this study showed interesting results, since the plants under study inhibited the growth of at least one diarrheal pathogen. This showed the value of this plants and their potential in being considered for further studies in antidiarrheal drug development. Plants such as *T. sericea*, *Z. mucronata*, *B. albitrunca* and *S. linnaeanum* that showed activity against ampicillin resistant *Salmonella* and *Shigella* showed significance of medicinal plants in inhibiting the growth of diarrheal pathogens. The difference in the antibacterial activity possessed by different plants against clinical and laboratory diarrheal strains have proven the significance of using both clinical strains and laboratory standards during the development of antimicrobial drugs; this is

due to the difference in virulence between the laboratory strains and clinical strains and the difference in efficacy possessed by different plant extracts against the two classes of diarrheal bacteria. These could help to determine the degree of effectiveness for the medicine to be developed.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

Namibian indigenous plants, *T. sereciea*, *B. albitrunca*, *C. apiculatum*, *S. linnaenum* and *Z. muronata* were observed to possess phytochemical compounds such as alkaloids, flavonoids, coumarins, triterpenes, steroids and tannins, which are known to have antibacterial activity against different clinical and laboratory diarrheal bacteria. At least each plant showed antibacterial activity against one diarrheal pathogen used in the study. Organic and aqueous extracts of *T. sereciea* were found to possess higher antibacterial activity against clinical and laboratory diarrheal bacteria strains.

The findings of this study provide significant support for the use of, *T. sereciea*, *B. albitrunca*, *C. apiculatum*, *S. linnaenum* and *Z. muronata* for treatment of diarrheal diseases in resource poor settings to meet the healthcare needs of patients from distant rural areas who cannot easily access Western treatment or have to endure the long referral process. In conclusion this study demonstrated that traditional knowledge serves as guidance in the fight against diarrheal diseases. Furthermore the findings of this study also showed that, *T. sereciea*, *B. albitrunca*, *C. apiculatum*, *S. linnaenum* and *Z. muronata* can serve as alternative treatment for diarrheal diseases. This is because based on the results obtained from this study, plant extracts especially for *T. sereciea* and *C. apiculatum* showed good antibacterial activity against the main clinical diarrheal pathogens such *Salmonella*, *E. coli* and *Shigella* species in children under the age of five. Hence, these plants can serve great use in fighting diarrheal pathogens especially when the etiological agents are *Salmonella*, *E. coli* and *Shigella*.

Further studies should focus on optimization of the extraction process using different solvent systems to achieve a greater yield of bioactive compounds. The extracts can also be fractionated to identify the compounds in the extracts. The toxicity in the plant extracts also have to be

determined. In addition, the panel of diarrheal pathogens can be expanded to yield a wide range of activity profiles of the five plant extracts. Furthermore, plant extracts should be evaluated for antidiarrheal activity and toxicity in a mammalian such as mice. Moreover, antibiotic resistance profile of isolated clinical strains should be determined in order to detect the presence of resistance genes in these clinical isolates. Finally, future research should differentiate which phytochemical compounds are originating from endophytes and those originating from the five plants investigated in this study.

CHAPTER 7: REFERENCES

Aderogba, M. A., Kgatle, D. T., McGaw, J. N., and Eloff, J. N. (2011). Isolation of antioxidant constituents from *Combretum apiculatum* subsp. *apiculatum*. *South African Journal of Botany*, 79, 125-131.

Ahmed (2006). Assessment of mother's knowledge toward management of child with diarrheal disease in Khartoum state. Thesis. The national Ribat University college of Postgraduate studies. Retrieved from: http://repository.ribat.edu.sd/public/uploads/upload/repository/%20%D8%AE%D8%AF%D9%8A%D8%AC%D8%A9_2522024549.pdf.

Allen, S. J., Okoko, B., Martinez, E., Gregorio, G., & Dans, L.F. (2010). Probiotics for treating infectious diarrhea. *Infectious disease group*. Retrieved from: <http://www.thecochranelibrary.com>.

American Academy of Pediatrics (2006). The Use of Systemic Fluoroquinolones. 118 (3) 1287.

Retrieved from: <http://pediatrics.aappublications.org/content/118/3/1287>.

Andino, A., and Hanning, I. (2015). Salmonella enterica: Survival, colonization and virulence differences among serovars. *The science world journal*, 2015, 1-16.

Argudín, M. A., Mendoza, M. C., and María Rosario Rodicio, M. R. (2010). Food Poisoning and *Staphylococcus aureus* Enterotoxins. *Toxins*, 2, 1751-1773; doi:10.3390/toxins2071751.

Auala, J. R. N., Mumbengegwi, D. R., Du Preez, I., & Bock, R. (2012). Evaluation of

- Tarchonanthus camphoratus* plant extracts for antimicrobial activity against food-borne pathogens. *Journal of Research in Microbes*, 1 (2), 096-104.
- Asrat D. (2008). Shigella and Salmonella serogroups and their antibiotic susceptibility patterns in Ethiopia. *East Mediterr Health J*, 14: 760-767.
- Andrade, J.A., & Fagundes-Neto, U. (2011). Persistent diarrhea: still an important challenge for the pediatrician. *J Pediatr (Rio J)*. 8;87(3):199-205.doi:10.2223/JPED.2087.
- Babić, T., Stojanović, P. (2010). Bacterial Etiology of Diarrheal Syndrome. *Scientific Journal of the Faculty of Medicine in Niš* 27(2):55-62.
- Bad, B., Slippers, B., Wingfield, M. J., and Roux, J. (2009). *Botryosphaericeous* fungi as endophytes on Terminalia species in Cameroon. *Forest Pathology*.106-141.
- Balemba, O. B., Bhattarai, Y., Stenkamp-Strahm, C., Lesakit, M. S. B., & Mawe, G. M. (2010). The traditional anti-diarrheal remedy, *Garcinia buchananii* stem bark extract, inhibits propulsive motility and fast synaptic potentials in the guinea pig distal colon. *Neurogastroenterology and Motility: The Official Journal of the European Gastrointestinal Motility Society*, 22(12), 1332–1339. <http://doi.org/10.1111/j.1365-2982.2010.01583.x>.
- Bell, C., & Kyriakides, A. (2002). *Salmonella*: A practical approach to the organism and its control in foods. Blackwell Science, Oxford.
- Bille, P. G. (2013). Effect of *Boscia albitrunca* (omukunzi) root on the bacteriology and

- viscosity of omashikwa, traditional fermented buttermilk from Namibia. *African journal of food, agriculture, nutrition and development*. 12 (4) 1-17.
- Bhalodia, N. R., & Shukla, V. J., (2011). Antibacterial and antifungal activities from leaf extracts of *Cassia fistula* L.: An ethnomedicinal plant. *Journal of Advanced Pharmaceutical Technology & Research*. 2(2):104-109. doi:10.4103/2231-4040.82956.
- Bhavani, P. K., Teoh, T. W., and Roufogalis, B. D. (2012). Pharmacology of Traditional Herbal Medicines and Their Active Principles Used in the Treatment of Peptic Ulcer, Diarrhea and Inflammatory Bowel Disease, *New Advances in the Basic and Clinical Gastroenterology*, 51, 978-953.
- Bussmann, R.W., Malca-García, G., & Glenn, A. (2010). Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterial remedies. *Journal of ethnopharmacology*. 132(1):101-108. doi:10.1016/j.jep.2010.07.048.
- Bryan, C. (2015). Infectious disease: Gastrointestinal and Intra-abdominal Infections. <http://www.microbiologybook.org/Infectious%20Disease/Gastrointestinal%20Infections.htm>.
- Casburn-Jones, A. C., & Farthing, M. J. G. (2004). Management of infectious diarrhoea. *Gut*, 53(2), 296–305. <http://doi.org/10.1136/gut.2003.022103>.
- Cheruiyot, K. R., Olila, D., & Kateregga, J. (2009). In-vitro antibacterial activity of selected medicinal plants from Longisa region of Bomet district, Kenya. *African Health Sciences*, 9, 42–46.
- Centers for Disease Control and Prevention (CDC). (2012). Centers for Disease Control and Prevention in Namibia. Retrieved from: www.cdc.gov/global.

Center for Disease control and Prevention. (2015). Diarrhea: common illness, global killer.

Retrieved from: <http://www.cdc.gov/healthywater/global/diarrhea-burden.html>.

Centers for Disease Control and Prevention (CDC). (2016). Listeria (Listeriosis). Retrieved

from: www.cdc.gov.

Center of Food Security and Public health. (2005). Iowa state university.

www.c.fsp.h.iastate.edu.

Cheikhoussef, A., Mapaure, I., & Shapi, M. (2011). The use of some indigenous plants for medicinal and other purposes by local communities in Namibia with emphasis on Oshikoto region. *Research Journal of Medicinal Plant*, 5, 406-419.

Chinenye, J., Ugwah, O., Yusuf, I., Alkali, O., Ugwah, M., & Kabiru, A. (2013). Anti-diarrheal potential of the aqueous root extract of *Ziziphus abyssinica* . *Scholars Academic Journal of Pharmacy*, 2, 419-423.

Chinsebu, K. C. (2009). Model and experiences of initiating collaboration with traditional healers in validation of ethnomedicine for HIV/AIDS in Namibia. *J. Ethnobiology and Ethnomedicine*, 5: 30: doi 10.1186/1746-4269-5-30.

Chinsebu, K. C., & Hedimbi, H. (2012). An ethnobotanical survey of plants used to manage HIV/AIDS opportunistic infections in Katima Mulilo, Caprivi region, Namibia. *Journal of Ethnobiology and Ethnomedicine*, 6, 25. 1-9.

Chinsebu, K., C., & Hedimbi, M. (2010). An ethnobotanical survey of plants used to manage

- HIV/AIDS opportunistic infections in Katima Mulilo, Caprivi region, Namibia. *Journal of Ethnobiology and Ethnomedicine*, 6(25), 1-9.
- Davis, C. P., Nettleman, M. D., and Stoppler, M. C. (2015). Listeriosis (*Listeria monocytogenes* infection). MedicineNet.com.
- De Wet, H. (2008). An ethnobotanical and chemotaxonomic study of South African Menispermaceae. <http://hdl.handle.net/10210/233>.
- Djeussi, D.E., Noumedem, J. A., Seukep, J. A. (2013). Antibacterial activities of selected edible plants extracts against multidrug-resistant Gram-negative bacteria. *BMC Complementary and Alternative Medicine*.13:164. doi:10.1186/1472-6882-13-164.
- Diniz-Santos, D. R., Silva, L. R., & Silva, N. (2006). Antibiotics for the Empirical Treatment of Acute Infectious Diarrhea in Children. *The Brazilian Journal of Infectious Diseases*, 10(3): 217-227.
- Division of Public Health Services, Communicable Disease Control Section (2009). Diarrhea (Infectious Diarrhea). Disease Handbook for Childcare Providers.
- Dorn, S. (2012). What are the common treatments for diarrhea? *UNC Center for functional GI & Mortality Disorders*. 11-22.
- Doughari, J. H., Human, I. S., Bennade, S., & Ndakidemi, P. A. (2009). Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria. *J. Med. Plant. Res*, 3(11), 839-848.

Doughari (2012). Phytochemicals: Extraction Methods, Basic Structures and Mode of Action as Potential Chemotherapeutic Agents, Phytochemicals - A Global Perspective of Their Role in Nutrition and Health, Dr Venketeshwer Rao (Ed.), ISBN: 978-953-51-0296-0, InTech, Available from: <http://www.intechopen.com/books/phytochemicals-a-global-perspective-of-their-role-in-nutrition-andhealth/> phytochemicals-extraction-methods-basic-structures-and-mode-of-action-as-potentialchemotherapeutic.

Dushimemaria, F., Mumbengegwi, D. R., & Du Preez, I. (2012). Qualitative phytochemical screening and *in vitro* antimicrobial effects of *Searsia tenuinervis*. *Jornal of Research in Microbes*, 1, 088-095.

Dushimemaria, F. (2014). *An investigation into the antineoplastic properties of Schinziophyton rautanenii and Colophospermum mopane*. Unpublished PhD thesis, University of Namibia.

Du Preez, I. (2012). *Evaluation of antimalarial properties of indigenous plants used by traditional healers in Namibia*. Thesis. University of Namibia.

Du Preez, I; Mumbengegwi, D. (2012). Phytochemical investigation on Namibian plants for anti-malaria compounds. *Journal for studies in humanities and social sciences* 2026-7215.

Elumalai, E. K., Ramachandran, M., Thirumalai, T., & Vinothkumar, P. (2011). Antibacterial activity of various leaf extracts of *Merremia emarginata*. *Asian Pac J Trop Biomed*, 1, 406-408.

Feng, L., Tao, J., Guo, H., Xu, J., Li, Y., Rezwani, F., Reeves, P. & Wang, L. (2004). Structure of

the *Shigella dysenteriae* 7 O antigen gene cluster and identification of its antigen specific genes. *Microb pathog.* 36, 109–115.

Fletcher, S. M., McLaws, M. J., Ellis, J. T. (2013). Prevalence of gastrointestinal pathogens in developed and developing countries: Systematic review and Meta-analysis. *Journal of public health research* 2 (1), 42-53.

Food Standards Australia, (2013). *Listeria monocytogenes*. Retrieved from:

<http://www.foodstandards.gov.au>.

Fouche, G., Cragg, G. M., Pillay, P., Kolesnikova, N., Maharaj, V. J., & Senabe, J. (2008). *In vitro* anticancer screening of South African plants. *J. Ethnopharmacol*, 119, 455-461.

Fyhrquist, P. (2007), Traditional medicinal uses and biological activities of some plant extracts of African *Combretum* L., *Terminalia* L. and *Pteleopsis* Engl. species (Combretaceae). Published Thesis. Faculty of Biosciences of the University of Helsinki, Retrieved from <http://ethesis.helsinki.fi>.

Healthline Networks. (2016). *Staphylococcus aureus* food poisoning. Retrieved from

<http://www.healthline.com/health/staph-aureus-food-poisoning#Overview1>

Hedimbi, M., & Chinsebu, K. C. (2012). Ethnomedicinal study of plants used to manage

HIV/AIDS-related disease conditions in the Ohangwena region, Namibia. *International Journal of Medicinal Plant Research* 1, 004-011.

Helle, W., Line, K., Mahiuddin, A., Anne, B., Samuelsen, C., & Karl, E. M. (2013).

Can Scientific Evidence Support Using Bangladeshi Traditional Medicinal Plants in the Treatment of diarrhoea? A review on seven plants. *Nutrients*, 5, 1757-1800.

- Hong, K. S., & Kim, J. S. (2011). Rifaximin for the treatment of acute infectious diarrhea. *Therapeutic Advances in Gastroenterology*, 4(4), 227–235. <http://doi.org/10.1177/1756283X11398734>.
- Ibtisam, M. A. (2010). Antimicrobial activity of ethanolic extracts from some medicinal plant. *Australian Journal of Basic and Applied Sciences*, 5, 678-683.
- International Vaccine Access Center (IVAC), John Hopkins Bloomberg School of Public Health. (2015). Pneumonia and Diarrhea progress report: Sustainable progress in the post- 2015 Era.
- Ganiyat, K. O., Patricia, A. O., Julius, S., Oluwaseyi, O., & Emmanuel, T. (2010). Phytochemical screening, antimicrobial and antioxidant activities of four Nigerian medicinal plants. *Annals of Biological Research*, 1, 114- 120.
- Gilani, A.H., & Rahman, A.U. (2005). Trends in ethnopharmacology. *J Ethnopharmacol.* 100, 43-49.
- Graz, B., Kitua, A. Y., & Malebo, H. M. (2011). To what extent can traditional medicine contribute a complementary or alternative solution to malaria control programmes? *Malaria Journal*, 10; 1. DOI: 10.1186/1475-2875-10-S1-S6.
- Gupta, A., Naraniwal, M., and Kothari, V. (2012). Modern extraction methods for preparation of bioactive plant extracts. *International Journal of Applied and Natural Sciences*, 1, 8- 26.
- Gunasegaran, T., Kasi, R., Sathasivam, Sreenivasan, K., and Subramaniam, S. (2011). Isolation and identification of Salmonella from curry samples and its sensitivity to commercial antibiotics and aqueous extracts of *Camelia sinensis* (L) and *Trachyspermum ammi* (L). *Asian pac J Trop Biomed*, 1 (4),1-56.

- Gürbüz, N., Karabey, F., Kurtulu, T., Öztürk, S., Kiliñç, A., Frary, A and Doganlar, S. (2006). Etiology and epidemiology of diarrhea in children in Hanoi, Vietnam. *Int J Infect Dis.* 10 (4):298-308.
- Hashempour. A., Eslamieh, M., Jamshidi, S., Taei. R., Adeh, M. A. A., Mavaneh, E. J. (2015). Comparable Anti-bacterial Activity of Three Herbal Plants with two Antibiotic Drugs against Pathogenic Bacteria. *Bull. Env. Pharmacol. Life Sci*, 4, 3, 05-09.
- Indu, M.N., Hatha, C A.A.M., Abirosh; U., & Vivekanandan ,G. (2006). Antimicrobial activity of some of the south-Indian spices against serotypes of Escherichia coli, Salmonella, Listeria monocytogenes and Aeromonas hydrophila. *Braz. J. Microbiol.* 37. 2 . <http://dx.doi.org/10.1590/S1517-83822006000200011>.
- Isenbarger, D. W., Hoge, C. W., Srijan, A., Pitarangsi, C., Vithayasai, N., Bodhidatta, L., Hickey., K., and Cam, P. D. (2002). Comparative Antibiotic Resistance of Diarrheal Pathogens from Vietnam and Thailand, 1996-1999. *Emerging Infectious Diseases.* 8(2):175-180.
- Jamoson, T., Breman, J. G., Measharm, A. R., Alleyne, G., Claeson, M., Evans, D.B., Jha, P., Mills, A., & Musgrove, P. (2006). Disease control priorities in Developing countries, 2nd edition. World Bank, Washington (DC). ISBN-10:0-8213-6179-1.
- Joy, P. P., Thomas, J., Mathew, S., & Skaria, B. P. (1998). Medicinal plants (pp. 1-211). Kerala, India: Kerala Agricultural University Aromatic and Medicinal Plants Research Station.
- Kazembe, L. N., and Neema, I. (2013). Today, tomorrow, forever: A Bayesian ordered categories

- model for treatment seeking in febrile children. *Int. Sci. Technol. J. Namibia*. 1, 1-14.
- Kannabiran, K., Mohankumar, T., & Gunaseker, V. (2009). Evaluation of Antimicrobial Activity of Saponin isolated from *Solanum xanthocarpum* and *Centella asiatica*. *International Journal of Natural and Engineering Sciences 3 (1)*: 25-28.
- Kariuki, A. C., & Njoroge, G. N. (2011). Ethnobotanical and Antimicrobial Studies of Some Plants used in Kibwezi (Kenya) for Management of Lower Respiratory Tract Infections. *Afr J Tradit Complement Altern Med*. 8, 144–149.
- Keusch, G. T., Fontaine, O., Bhargava, A., Boschi-Pinto, C., Zlfiqar, Z. A. Bhutta, A. (2006). Disease Control Priorities in Developing Countries. 2nd edition. Washington (DC).
- Kirsoy, O. (2006). Characterization of glycoalkaloids content and molecular mapping in Eggplant. Thesis. School of Engineering and Sciences, Izmir institute of technology. 1-150.
- Kisangau DP, Lyaruu HV, Hosea KM, Joseph CC.(2007). Use of traditional medicines in the management of HIV/AIDS opportunistic infections in Tanzania: a case in the Bukoba rural district. *J Ethnobiol Ethnomed*. 3:29.
- Kubmarawa, D., Khan, M. E., & Shuaibu, A. (2012). Comparative phytochemical screening and biological evaluation of n-hexane and water extracts of *Acacia tortilis*. *Research I Pharmaceutical Biotechnology*, 4, 18-23.
- Kumar, R., Sharma, R. J., Bairwa, K., Roy, R. K., & Kumar, A. (2010). Pharmacological review on natural antidiarrheal agents. *Der Pharma Chemica 2(2)*: 66-93.
- Lakhanpa, P, Kumar, G. (2007). Quercetin: a versatile flavonoid. *Internet journal of medical*

2(2):22-37.

Li, S.-T. T., Grossman, D. C., & Cummings, P. (2007). Loperamide Therapy for Acute Diarrhea in Children: Systematic Review and Meta-Analysis. *PLoS Medicine*, 4(3), e98. <http://doi.org/10.1371/journal.pmed.0040098>.

Lima, G. C., Loiko, M. R., Casarin, L.S., and Tondo, E. C. (2013). Assessing the epidemiological data of *Staphylococcus aureus* food poisoning occurred in the state of Rio Grande do Sul, Southern Brazil. *Brazilian journal of Microbiology*, 44 9 (3), 759- 763. <http://doi.org/10.1590/S151783822013005000063>.

Mahady, G. B. (2005). Medicinal Plants for the Prevention and Treatment of Bacterial Infections. *Current Pharmaceutical Design*, 11, 2405-2427.

Manuel, F., Balandrin, M. F., Kinghorn, A. Dand ., & Farnsworth, N. R. (1993). Plant-Derived Natural Products in Drug Discovery and Development. DOI: 10.1021/bk-1993-0534.ch001.

Masupa, T. (2012). *Terminaliasericca*.

<http://www.plantzafrica.com/planttuv/terminaliasericca.htm>.

Mendez-Vila, S, A. (2013). Worldwide research efforts in the fighting against microbial pathogens. *Universal –Publishers. com*

Ministry of Health and Social Services (MoHSS). (2008). Strengthening public health through

- leadership development and health systems innovation. Retrieved from: www.africanhealthleadership.org/wp-content/.../ChildMortality finding.
- Ministry of Health and Social Services (MoHSS). (2014). Cholera outbreaks in Katutura and Opuwo. Retrieved from <http://www.rosa.humanitarianresponse.info>.
- Morgan, E., Campbell, J. D., Rowe, S. C., Bispham, J., Stevens, M. P., Bowen, A. J., Barrow, P. A., Maskell, D. J., & Wallis, T. S. (2004). Identification of host-specific colonization factors of *Salmonella enterica* serovar Typhimurium. *Molecular Microbiology*, 54 (4), 994 – 1010.
- Miljković-Selimović, B., Moshi, M. J., Mbwambo, Z. H. (2006). Some pharmacological properties of extracts of *Terminalia sericea* roots. *J Ethnopharmacol.* 10, 97(1):43-7.
- Murugan, T., Wins, J. A., & Muruganants, M. (2013). Antimicrobial activity and phytochemical constituents of leaf extracts of *Cassia auriculata*. *Indian J Pharm Sci*, 75, 122-125.
- Myra, N., Widjoatmodjo, C., & Verhoef, J. (1994). Rapid Identification of Bacteria by PCR-Single-Strand Conformation Polymorphism. *Journal of Clinical Microbiology*, 3002-3007.
- Namibia Demographic Profile. (2014). CIA World fact book. Retrieved from: www.indexmundi.com.
- National Digestive Diseases. (2011). Diarrhea. Retrieved from: www.digestive.niddk.nih.gov.
- National Institution of Allergy and Infectious Diseases. (2009). NIAID's Antibacterial Resistance Program: Current Status and Future Directions.

- National Institutes of Health. (2016). Zinc: Strengthening knowledge and understanding of Dietary supplements. Retrieved from: <https://ods.od.nih.gov/factsheets/Zinc-HealthProfessional/>.
- Ndamane, Y., Kambizi, L., Songca, S. P., & Oluwafemi, O. S., (2013). Antibacterial effectiveness of *Tetradenia riparia* extract, a plant traditionally used in the Eastern Cape province to treat diseases of the respiratory system. *Journal of Medicinal Plants Research*, 7, 2755-2760.
- Nguyen, T. V., Le Van, P., Huy, L. C., Gia, K. N., Weintraub, A. (2006). Etiology and epidemiology of diarrhea in children in Hanoi, Vietnam. *Int J Infect Dis*. 10, 298-308.
- Nyota, S., & Mapara, J. (2008). Shona Traditional Children's Games and Play: Songs as Indigenous Ways of Knowing. *The Journal of Pan African Studies*, 2, 4.
- Ochoa, B., and Surawicz, C. M. (2012). Diarrheal Diseases – Acute and Chronic. University of Washington School of Medicine.
- Olajuyigbe, O. O., Afolayan, A. J. (2013). Evaluation of combination effect of Ethanolic extracts of *Ziziphus mucronata* wild. Subsp. *mucronata* wild and antibiotics against clinically important bacteria. *The Scientific world journal*, 2013, 1-9.
- Oloyede, G. K., Onocha, P.A., Soyinka, J., Oguntokun, O. W., and THONDA, E. (2010). Phytochemical screening, antimicrobial and antioxidant activities of four Nigerian medicinal plants. *Annals of Biological Research*, 1:114-120.
- Pan, S., Litscher, G., Gao,S., Zhou, S., Yu, Z., Chen, H., Zhang,S., Tang,M., Sun,J and Ko, K.

- (2014). Historical Perspective of Traditional Indigenous Medical Practices: The Current Renaissance and Conservation of Herbal Resources. *Evidence-Based Complementary and Alternative Medicine*. <http://dx.doi.org/10.1155/2014/525340>.
- Panda, S.K., Patra, N., Sahoo, G., Bastia, A. K., Dutta, S. K. (2012). Anti-diarrheal activities of medicinal plants of Similipal Biosphere Re-serve, Odisha, India. *Int. J. Med. Arom. Plants*. 2123-134.
- Payyappa;;imana, U. (2009). Role of traditional medicine in primary health care: An overview of perspectives and Challenges. *Yokohama Journal of Social Sciences*, 14. 723-743.
- Pendota, S. C., Aderogba, A. M., and Van Staden, J. (2015). In vitro antimicrobial activity of extracts and an isolated compound from *Boscia albitrunca* leaves. *South African journal of Bortany*, 96, 91-93.
- Pendota, S. C., Aderogba, A. M., and Van Staden, J. (2016). Germination, seedling growth requirements and antimicrobial properties of *Boscia albitrunca*- A keystone species in arid Southern Africa. *South African journal of Bortany*, 104, 105-111.
- Pichler, J., Much, P., Kasper, S., Fretz, R., Auer, B., Kathan, J *et al.*, (2009). An outbreak of febrile gastroenteritis associated with jellied pork contaminated with *Listeria monocytogenes*. *The Central European Journal of Medicine*.121, 149- 156.
- Population action international. (2011). Why Population Matters to Infectious diseases and HIV/AIDS.
- Population act. (2011). Why population matters to infectious diseases and HIV/AIDS.
- Washington, DC 20036 USA www.populationaction.org.

Protected Trees. (2013). Department of water affairs and forestry, Republic of South Africa.

Retrieved from: <https://en.m.wikipedia.org>.

Protiva, R. D., Shakila, A., Tabibul, I, Mohammad, H, Kabir., Megbahul, H., Zubaida, K.,

Rownak, J., Mohammed, R., & Zehedina, K. (2012). A selection of medicinal plants used for treatment of diarrhea by folk medicinal practitioners Of Bangladesh. *American-Eurasian Journal of Sustainable Agriculture*, 6, 153-161.

Raveendra, K., Vijayasankar, G. R., Prema, R., Jeevanandham, S., Lakshmana, G., & Sekar

, M. M. (2011). Prelude studies of antidiarrheal activity of ethyl acetate extract of aerial part of *Indigofera purpurea* on isolated rabbit ileum. *International Journal of Biological*, 209-218.

Ryan, Kenneth James; Ray, C. & George, E. D. (2004). Sherris medical microbiology: an

introduction to infectious diseases (4th ed.). McGraw-Hill Professional Med/Tech. ISBN 978-0-8385-8529-0.

Reda A, Seyoum B, Yimam J, (2011). Antibiotic susceptibility patterns of *Salmonella* and

Shigella isolates in Harare. *Eastern Ethiopia. J Infect Dis Immun*, 3, 134-139.

Roma, B., Worku, S., Mariam, S., & Langeland, N. (2000). Antimicrobial susceptibility

pattern of *Shigella* isolates in Hawassa. *Ethiop J Health Dev*, 14: 149-154.

Roberts, M. F., and M. Wink. (1998). Alkaloids: Biochemistry, ecology and medicinal

applications; Plenum Press, New York and London.

- Robertson, S. (2015). What are flavonoids? Retrieved from: <http://www.news-medical.net/health/What-are-Flavonoids.aspx>
- Salam, M., Farthing, M., Lindberg, G., Dite, P., Khalif, I., Salazar-Lindo, E., Ramakrishna, B. S., Goh, K., Thomson, K. J., Krabshuis, A. G., & Mair, A. (2012). Acute diarrhea in adults and children: a global perspective. *World Gastroenterology Organisation Global Guidelines*. 1-24.
- 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. *African Journal of Biotechnology* 4 (12), 1443-1451.
- Stanley, S. (2003). Amoebiasis. *The lancet*, 361, (9362)1025–1034, [http://dx.doi.org/10.1016/S0140-6736\(03\)12830-9](http://dx.doi.org/10.1016/S0140-6736(03)12830-9).
- Stephanie, M. F., MCLaws, M. L., and Ellis, J. T. (2013). Prevalence of Gastrointestinal Pathogens In Developed and Developing Countries: Systematic Review and Meta-Analysis. *J Public Health Res.* 2 (1): 42–53.
- Saxena, M., Saxena, J., Nema, R., Singh, D., and Gupta, A. (2013). Phytochemistry of medicinal plants. *J. Pharm. Phytochem.* 1(6): 168 -82.
- Sevda, K., Fikriye, Z., & Murat, K. (2009). Antimicrobial activities of extracts of some plants. *Pak. J. Bot.*, 41, 2067-2070.
- Shalini, S., & Prema S. (2012). Phytochemical screening and antimicrobial activity of plant extracts for disease management. *Int J Curr Sci*, 209-218.
- Sharma, R., & Lall, N. (2014). Antibacterial, antioxidant activities and cytotoxicity of plants

- against *Propionibacterium acnes*. *S Afr J Sci*. 110, 11-12. Retrieved from: <http://dx.doi.org/10.1590/sajs.2014/20130293>.
- Shiv, G., Navneet, S., & Kumar, P. (2012). Screening of antibacterial activity of *Nepeta ciliaris benth* against respiratory tract pathogens. *Journal of Science, Engineering and Technology*, 8,100-103.
- Srivastava, S., & Srivastava, A. K. (2007). Hairy root culture for mass-production of high-value secondary metabolites. *Crit Rev Biotechnol*, 27(1):29-43.
- Soliman, S. S. M., Trobacher, C. P., Tsao, R., Greenwood, J. S. & Raizada, M. N. (2013). A fungal endophyte induces transcription of genes encoding a redundant fungicide pathway in its host plant. *BMC Plant Biol*, 13, 93.
- Sonia, S., Preeti, D., Mahesh, P., & Saksham, G. (2013). Antimicrobial activity of medicinal plants on respiratory tract pathogens. *International Journal of Biological & Pharmaceutical Research*, 4, 148-152.
- Sosa, A. J., Byarugaba, D. K., and Amabile, C. (2009). Antimicrobial resistance in Developing countries). Retrieved from: <http://books.gogle.com.na>
- Sule, O. I., & Agbabiaka, T. O. (2008). Antibacterial Effect of some Plant Extracts on Selected Enterobacteriaceae. *Ethnobotanical Leaflets*, 12: 1035-42.
- Sutar, N., Garai, R., Sharma, U. S., Sharma, U. K. (2010). Anthelmintic activity of *Platycladus orientalis* leaves extract. *International Journal of Parasitology Research*, 2(2): 1-3.
- Symington, V., (2011). Cholera: Death by Diarrhoea. *Society for General Microbiology*. www

.microbiologyonline.org.uk.

Tambe, R., Kulkarni, M., and Bhise, K. (2013). Preliminary Phytochemical Screening and HPTLC Fingerprinting of Bark Extracts of *Symplocos racemosa*. *Journal of Pharmacognosy and Phytochemistry*, 2 (3): 45-49.

Tilak, K. V. G. K. (2013). Infectious diseases. [HTTP://WWW.apiindia.org/medicine-updated2013/chap01.pdf](http://www.apiindia.org/medicine-updated2013/chap01.pdf).

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., and Kaur, H. (2011). Phytochemical screening and Extraction: A Review. *Internationale Pharmaceutica Scientia*. 1, 1-9.

The United Nations Children's Fund (UNICEF)/World Health Organization (WHO). (2009).

Diarrhea: Why children are still dying and what can be done. United Nations Plaza, New York 10017, USA.

The United Nations Children's Fund (UNICEF). Retrieved from: UNICEF> health> index_43834.

Traditional Knowledge Sector Paper. (1999). Namibia National Biodiversity Programme (NNBP). Retrieved from <http://www.the-eis.com/data/RDPs/RDP66.pdf>.

United Nation (UN). (2014). Access to sanitation.

<http://www.un.org/waterforlifedecade/index.shtml>.

Vince, M., and Zoltán, Ö. (2011). Plant Physiology: Secondary metabolites in plant defenses.

Retrieved:http://www.tankonyvtar.hu/hu/tartalom/tamop425/0010_1A_Book_angol01_nov_enelettan/ch03s05.html

- Virgilio, B. G., María, R. C. C., Mónica, R. C., Gildardo, R., & Elvira, G. G. (2009). The bioactivity of plant extracts against representative bacterial pathogens of the lower respiratory tract. *BMC Research Notes*, 2, 1-95.
- Vuuren, S. F., Nkwanyana, M. N., and Wet, H. (2015). Antimicrobial evaluation of plants used for the treatment of diarrhea in a rural community in northern Maputaland, Kwaxulu-Natal, South Africa. *BMC Complementary and alternative medicine*. 15, 53.
- Wardlaw, T., Salama, P., Brocklehurst, C., Chopra, M., and , Mason, E. (2009). Diarrhoea: why children are still dying and what can be done. DOI:10.1016/S0140-6736(09)61798-0.
- WaterAid. (2011). The sanitation problem: What can and should the health sector do? www.wateraid.org/publications.
- World Health Organization (WHO). (2001). Infections and infectious diseases. Regional office, Europe.
- WHO (2007). The top ten causes of death. Fact sheet
- WHO (2013). "Diarrhoeal disease Fact sheet N°330".
- WHO (2014). The 10 leading causes of death in the world, 2000 and 2012.
- WHO and UNICEF. (2014). Progress on Drinking water and Sanitation. Geneva, Swaziland.
- Yanling, J., Xin, L., & Zhiyuan, L. (2013). The antibacterial drug discovery. DOI: 10.5772/52510.
- Yismaw G, Negeri C, Kassu A. A. (2006). Five-Year Antimicrobial Resistance Pattern

Observed In Shigella Species Isolated From Stool Samples In Gondar University Hospital, Northwest Ethiopia .*J. Health Dev.* 2006; 20(3):194-198.

Zhang, J., Qian,L., Wu, Y., Cai, X., Li, X., Cheng, X., and Qu, D. (2013). Deletion of *pic* results in decreased virulence for a clinical isolate of *Shigella flexneri* from China. *BMC Microbiology*, 13:31.

APPENDIX A

9 - 0/0001



REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198
Windhoek
Namibia

Ministerial Building
Harvey Street
Windhoek

Tel: 061 - 203 2034
Fax: 061 - 222558
E-mail: nforster@mhss.gov.na

OFFICE OF THE PERMANENT SECRETARY

Ref: 17/3/3

Enquiries: Dr Norbert Forster

Date: 20 June 2014

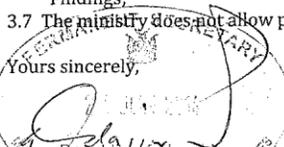
Prof Isack Quaye
University of Namibia
Private Bag 13301
Windhoek

Dear Prof Quaye

Re: Toward vaccine and probiotic solution to diarrheal disease in children living in Namibia: Determination of gut microbes in children with and without acute diarrhoea.

1. Reference is made to your application to conduct the above-mentioned study.
2. The proposal has been evaluated and found to have merit.
3. **Kindly be informed that permission to conduct the study has been granted under the following conditions:**
 - 3.1 The data to be collected must only be used for the completion of the above state project;
 - 3.2 No other data should be collected other than the data stated in the proposal;
 - 3.3 A quarterly report to be submitted to the Ministry's Research Unit;
 - 3.4 Preliminary findings to be submitted upon completion of the study;
 - 3.5 Final report to be submitted upon completion of the study;
 - 3.6 Separate permission should be sought from the Ministry for the publication of the Findings;
 - 3.7 ~~The ministry does not~~ allow participants to be photographed during the execution of this project.

Yours sincerely,


Andrew Ndishishi (Mr)
Permanent Secretary

"Health for All"

APPENDIX B

PARENTAL/GUARDIAN CONSENT FORM

Project Title: *Pre/post vaccination surveillance for rotavirus associated gastroenteritis in Namibia.*

Your child is being invited to take part in a research study being conducted by The University of Namibia School of Medicine. This form is to explain to you what we are going to do and why we need to ask your consent to enroll your child.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to find out how well the rotavirus vaccine is working and to better understand what germs cause diarrhea in children in Namibia.

WHY IS MY CHILD BEING ASKED TO BE IN THIS STUDY?

Because your child is admitted to hospital with diarrhea.

HOW MANY PEOPLE WILL BE ASKED TO BE IN THIS STUDY?

A total of about 1000 subjects will be enrolled at multiple study centers in Namibia.

WHAT ARE THE ALTERNATIVES TO BEING IN THIS STUDY?

You do not have to participate in this study.

WHAT WILL YOUR CHILD BE ASKED TO DO IN THIS STUDY?

We will obtain either a stool sample and/or a rectal swab sample. The samples will be labelled with a number, stored, and tested for germs. In future the sample may be sent to partner laboratories for further testing. We will also be asking you questions about your child's health.

ARE THERE ANY RISKS TO MY CHILD?

There should be no increased risk to your child. The sample collection is done routinely at the hospital.

ARE THERE ANY BENEFITS TO MY CHILD?

There may be no direct benefit to your child by being in this study. The information from this study will help inform the government to make important decisions about diarrhea in Namibian.

WILL THERE BE ANY COSTS TO MY CHILD OR ME?

No.

WILL MY CHILD BE PAID TO BE IN THIS STUDY?

No.

WILL INFORMATION FROM THIS STUDY BE KEPT CONFIDENTIAL?

Version 2.0 December 14, 2014

Yes.

WHOM CAN MY CHILD OR I CONTACT FOR MORE INFORMATION?

You or your child can call the Principal Investigator to tell him/her about a concern or complaint about this research study. The Principal Investigator can be called at (Prof Isaac Quaye) +264818536057).

MAY MY CHILD OR I CHANGE MY MIND ABOUT PARTICIPATING?

You or your child may change your mind at any time without any effect on your care or that of your child.

STATEMENT OF CONSENT

The procedures, risks, and benefits of this study have been told to me and I agree to allow my child to be in this study and sign this form. My questions have been answered. I may ask more questions whenever I want. I do not give up any of my child's or my legal rights by signing this form. A copy of this signed consent form will be given to me.

Subject's Name

Parent/Guardian Signature

Date

Parent/Guardian Name

Signature of Interviewer

Date

Signature of Witness (if illiterate)

Date

APPENDIX C

CASE INFORMATION									
1 Date of interview and abstraction: ____/____/____ D D M M Y Y Y Y					2 Medical Record #: ____/____/____ D D M M Y Y Y Y				
3 Hospital name:					4 Region of Residence:				
5 Treated at:		1 A&E room (with IV fluids)			2 Admitted to ward				
6 Source of information from : Mother Y/N Father Y/N Other Y/N					7 Contact #				
8 Date of admission or A&E visit: ____/____/____ D D M M Y Y Y Y					9 Approximate time of admission or A&E visit: ____:____ H H M M				
10 Child's age in months: _____			11 Child's date of birth: ____/____/____ D D M M Y Y Y Y						
12 Child's sex:		1 Male		2 Female					
RECORDED VACCINATION HISTORY, MEASUREMENTS, AND HIV STATUS FROM CHILD WELFARE CLINIC CARD/Passport card (Take a picture of the card where the vaccines are documented that includes the Medical record number but not the name.									
13 Birth weight			_____ kg		Birth Length		_____ cm		
14 Has the child ever been vaccinated?		1 Yes		2 No		9 Don't know			
15 What is the source of the vaccination information?			1 Vaccination card		2 Vaccine card not available (cannot complete vaccine history)				
16 Has the child received the following vaccines?									
a. Rotavirus (Rotarix) Dose 1?		1 Yes	2 No	9 Unknown		Date received: ____/____/____ D D M M Y Y Y Y			
b. Rotateq		1 Yes	2 No	9 Unknown		Date received: ____/____/____ D D M M Y Y Y Y			
CLINICAL HISTORY									
17 Date of symptom onset: ____/____/____ D D M M Y Y Y Y									
18 How many days before admission did the diarrhea begin?					_____ days (enter 99 if don't know)				
19 During this illness, what was the maximum number of diarrhea episodes in 24 hours?					_____ episodes (enter 99 if don't know)				
20 Did the child vomit? (If NO or DON'T KNOW go to Q17)			1 Yes		2 No		9 Don't know		
21 How many days before admission did the vomiting begin?					_____ days (enter 99 if don't know)				
22 What was the maximum number of vomiting episodes in 24 hours?					_____ episodes (enter 99 if don't know)				
23 Did the child receive any oral rehydration solution (ORS) before presenting to the hospital?			1 Yes		2 No		9 Don't know		
24 Did the child have a history of fever?			1 Yes		2 No		9 Don't know		

2

25 On arrival or admission (i.e. before rehydration), what was the child's general condition?	1	Well, alert	2	Restless, irritable	3	Lethargic or unconscious	9	Don't know		
26 On arrival, admission, or during the hospital visit, did the child have sunken eyes?			1	Yes	2	No	9	Don't know		
27 On arrival or admission (i.e. before rehydration), what was the child's thirst status?	1	Drank normally, not thirsty	2	Thirsty, drank eagerly	3	Drank poorly or not able to drink	9	Don't know		
28 On arrival or admission (i.e. before rehydration), describe the child's skin turgor when pinching the skin of the abdomen?	1	Goes back quickly (i.e. immediately)	2	Goes back slowly (i.e. 1-2 seconds)	3	Goes back very slowly (i.e. more than 2 seconds)	9	Don't know		
29 Level of dehydration	1	None	2	Some	3	Severe	4	Shock		
30 Did the child receive IV fluids during the hospital stay?	1	Yes	2	No	9	Don't know				
31 Did the child receive ORT during the hospital stay?	1	Yes	2	No	9	Don't know				
32 What was the child's highest temperature?	_____ ° Centigrade (enter 99 if don't know)									
33 Was the child transferred to ICU during this visit?	1	Yes	2	No						
DISCHARGE AND OUTCOME										
34 Date of discharge: _____ / _____ / _____	35 Approximate time of discharge: _____ : _____									
	D	D	M	M	Y	Y	Y	Y		
			H	H	M	M				
36 Outcome:	1	Recovered	2	Died	3	Transferred to another ward	4	Left against medical advice	9	Don't know
37 What is the child's HIV status?	1	Infected	2	Uninfected	99	Unknown				

PARENT INTERVIEW	
38 What is the age (in years) of the mother at the time of the child's hospital visit? (enter 99 if don't know)	

STOOL TESTING RESULTS AND MEASUREMENTS				Completed by:			
39 Child's current weight:	_____ kilograms			40 Child's current height:	_____ centimeters		
41 Was a stool specimen collected?	1	Yes	2	No	42 Date of collection: _____ / _____ / _____		
					_____	D D M M Y Y Y	
					_____	D D M M Y Y Y Y	
43 Was a rectal swab specimen collected?	1	Yes	2	No	44 Date of collection: _____ / _____ / _____		
					_____	D D M M Y Y Y Y	
45 Result of rapid rotavirus test	1	Rotavirus positive	2	Adenovirus positive	3	Negative	
46 Date stool specimen received in the lab?	_____ / _____ / _____ (Enter 9s if don't know)						
	D	D	M	M	Y	Y	Y
47 Stool specimen adequate? (covered base of cup)	1	Yes	2	No	9	Don't know	

APPENDIX D

Table 10: Yield of the organic and aqueous plant extracts

Plant name	Plant part used	Plant material weight (g)	Extract weight (g)	
			Ethanol extracts	Aqueous extracts
<i>B. albitrunca</i>	leaves	10	0.7	1.3
<i>Z. mucronata</i>	Bark	10	0.7	0.4
<i>C. apiculatum</i>	Bark	10	0.8	0.1
<i>S. linnaeanum</i>	Fruit	10	0.6	0.4
<i>T. serecia</i>	Twig	10	1.1	0.5

Table 11. Important diagnostic tests for biochemical identification of bacteria

Biochemical tests	<i>E. coli</i>	<i>Shigella</i>	<i>Salmonella</i>
Growth on XLD	+	+	+
Growth on MAC	+	+	+
Growth in selenite broth	-	+	+
Carbohydrates fermentation	+	+	-
Urease test	-	-	-
Indole test	+	+	-
Lysin decarboxylase	+	-	+
Motility	+	-	-

(+) growth/ positive (-) no growth/ negative

Antibacterial activity of organic and aqueous plant extracts against Clinical diarrheal pathogens



Figure 37: Antibacterial activity of a) *S. linnaeanum* against b) *C. apiculatum* against clinical *Salmonella* at 1000 µg/ml.



Figure 38: Antibacterial activity of a) *T. sericea* b) *B. albitrunca* c) *Z. mucronata* against clinical *E. coli* at 500 µg/ml.

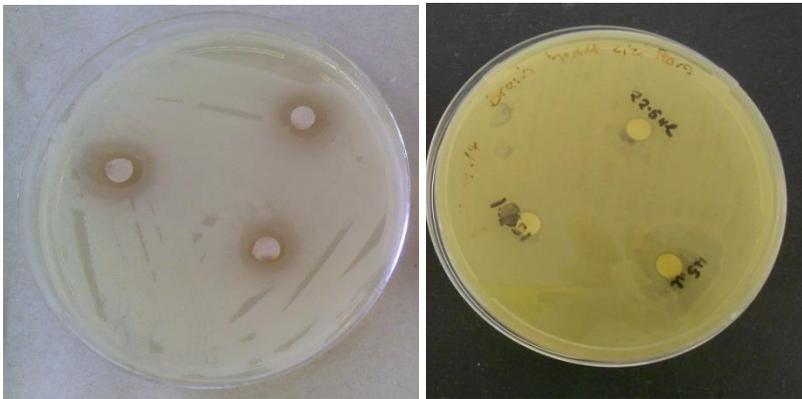


Figure 39: Antibacterial activity of a) organic extract of *Z. mucronata* b) aqueous extract of *T. serisea* against clinical *Shigella* at 1000 μ g/ml.

Antibacterial activities of positive controls against clinical and laboratory bacterial strains

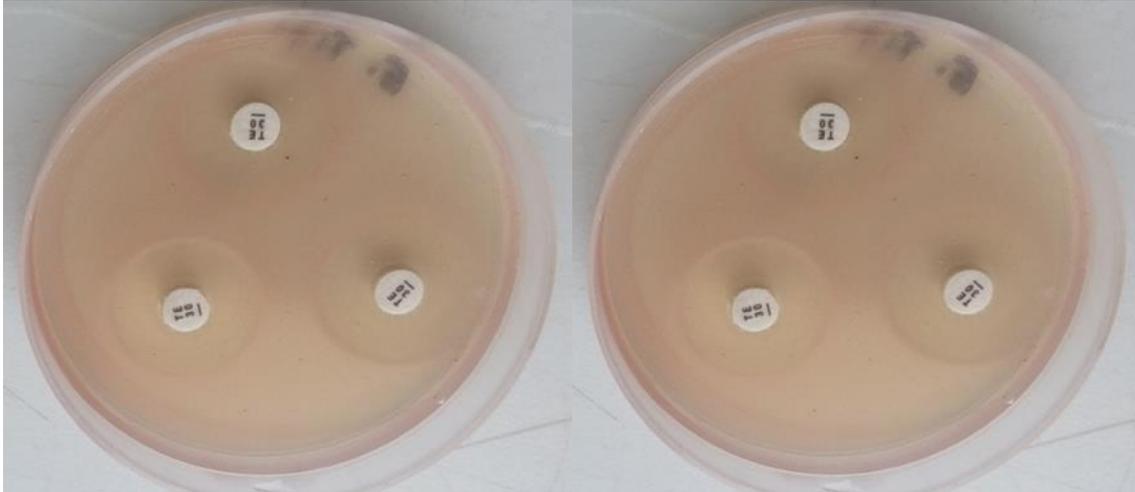


Figure 40: Antibacterial activity of tetracycline 30 μ g/ ml against a) Clinical b) laboratory strain of *E. coli*.

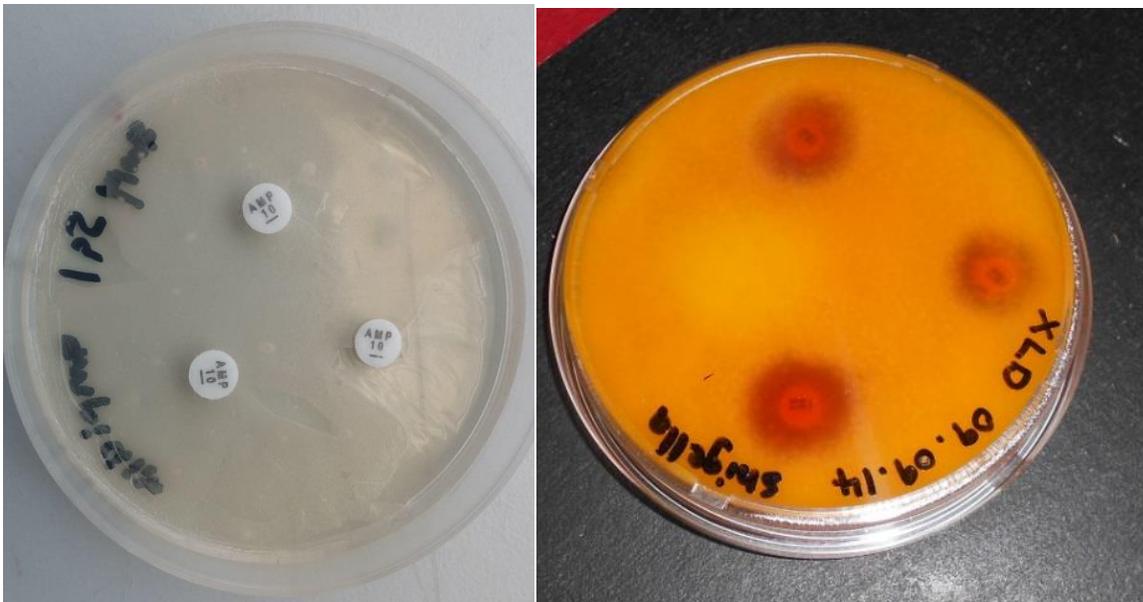


Figure 41: Antibacterial activity of Tetracycline 10 μ g/ ml against a) Clinical (no bacterial inhibition observed) b) laboratory strain of *E. coli* (Bacterial inhibition observed).

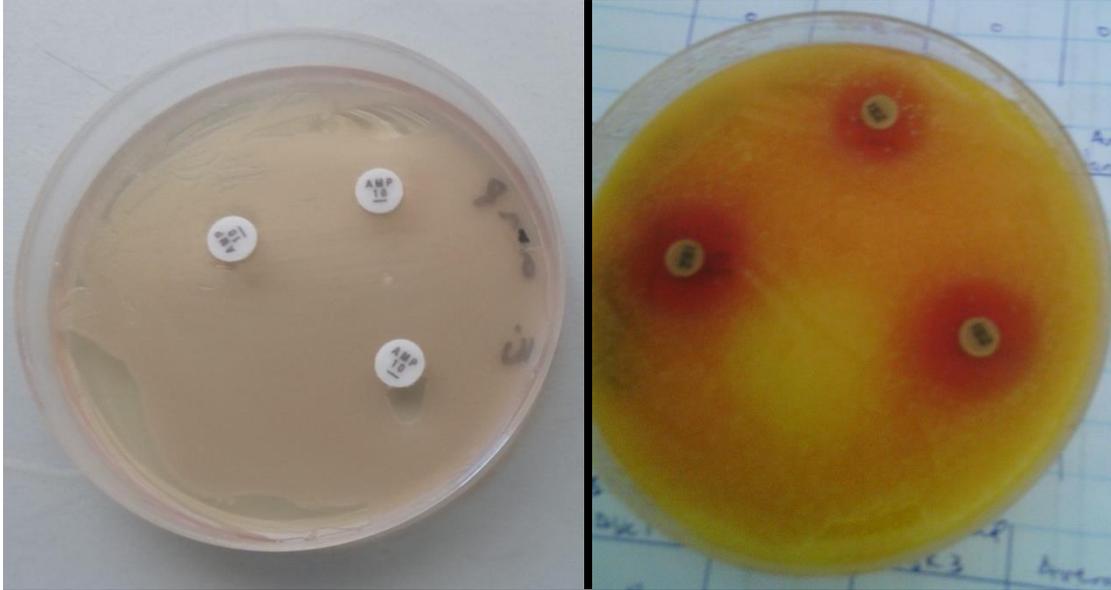


Figure 42: Antibacterial activity of tetracycline 10 µg/ ml against a) Clinical (no bacterial inhibition observed) b) laboratory strain of *Salmonella* (Bacterial inhibition observed).



Figure 43: Antibacterial activity of vancomycin 30µg/ ml against Laboratory strain of *L. monocytogenes*.

