Ethnobotanical study of plants from Pondoland used against diarrhoea

By

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In the

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DECLARATION BY SUPERVISORS

We hereby declare that we acted as Supervisors for this MSc student:

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Regular consultation took place between us and the student throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

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ABSTRACT

Diarrhoea and related diseases are the most common causes of death in children, especially from developing countries, killing about 1.5 million children under the age of five yearly. In South Africa, diarrhoea is the third leading cause of death. This condition results from food and water sources infected with *Campylobacter* spp., *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Giardia intestinalis* and *Cryptospondium parvum* amongst others. Diarrhoea spreads through faeces-contaminated water. Hence, infection is more common when there is a short supply of clean drinking and cooking water. Waterborne diseases are common in rural communities of Bizana because the majority of rural dwellers depend largely on water from unprotected sources.

Most of the pathogens that cause diarrhoea have developed resistance to several antibiotics. Therefore there is a need for new and safe antidiarrhoeal drugs. Most people in developing countries use traditional medicine to treat all kinds of diseases including diarrhoea and South Africa is no exception. Each cultural group in South Africa has different medical solutions for the prevention and curing of the same disease. The people from Pondoland (*AmaMpondo*), around Bizana have a strong tradition of using medicinal plants for the cure and prevention of several conditions including diarrhoea.

Although several researchers have conducted different types of studies in many parts of South Africa to evaluate the efficacy of traditional medicine used in the treatment of diarrhoea, there is, however, still a lot of undisclosed data that should be collected. The aims of this research were to record and collect medicinal plants that are used for treating diarrhoea in Bizana, Pondoland in the Eastern Cape and evaluate them for their pharmacological properties.

An ethnobotanical approach is one of several methods that have been useful in selecting plants for pharmacological research, yielding better results than other plant selection methods. Using questionnaires, this approach was used to record plants that are used for treating diarrhoea in Bizana for testing in pharmacological assays. From the completed questionnaires, nine plants were selected for bioassays based on their higher frequency index,

and the fact that the plants have never been evaluated against diarrhoea causing-microorganisms.

The study revealed 34 plant species belonging to 21 families as being used in treatment of diarrhoea in the study area. *Psidium guajava* was the most mentioned plant species. The dried, ground plant materials were each extracted non-sequentially using petroleum ether (PE), dichloromethane (DCM), 70% ethanol (EtOH) and water. Among all the extracts, 70% ethanol yielded the highest quantity of crude extract. The extracts were each evaluated for their antibacterial, anti-inflammatory and genotoxicity properties.

For the antibacterial activity, the following diarrhoea causing microorganisms were used: Gram-positive *Staphylococcus aureus* and Gram-negative *Campylobacter jejuni*, *E. coli* and *Shigella flexneri*. A microdilution assay (for *S. aureus*, *E. coli* and *S. flexneri*) and the disk diffusion technique (for *C. jejuni*) were used for antibacterial testing. The extracts were also evaluated for their ability to inhibit cyclooxygenase (COX-1 and -2) enzymes. Genotoxicity was evaluated using the *Salmonella* microsome assay without S9 metabolic activation. Three strains of *Salmonella typhimurium* TA98, TA1535 and TA1537 were used.

The evaluated plant extracts showed a broad spectrum of inhibitory activity with MIC values ranging from 0.098-12.5 mg/ml and mean zone inhibition percentage ranging from 0-73%. The best activity was exhibited by DCM extracts of *Rapanea melanophloeos*, EtOH extracts of *Ficus craterostoma* and *Maesa lanceolata* with MIC values of 0.098 mg/ml and EtOH extracts of *Searsia chirindensis* with 73% mean zone inhibition percentage.

The inhibitory activity against COX-1 enzyme was higher than COX-2, with 19 plant extracts for the former and 7 for the latter. The highest inhibition of COX-1 was shown by EtOH extracts of *F. craterostoma* and the DCM extract of *S. chirindensis* at 100%. Highest percentage COX-2 inhibition was shown by water extracts of *F. craterostoma* and DCM extracts of *Tecoma capensis* with 99.5% and 99.0% respectively. None of the tested plant extracts were mutagenic, at all concentrations tested against all tester strains of the bacteria.

The results of this study demonstrate that people still have a rich and diverse pool of knowledge concerning the uses of plants against diarrhoea. The data also show that plants form part of the cultural heritage of the communities in Pondoland. Therefore it is important

to urgently save the people's cultural heritage by recording the existing knowledge and confirming therapeutic uses of plants through scientific methods. This will prevent the information from vanishing together with the ageing knowledge holders. In light of the fact that the evaluated plants were selected based on their ethnobotanical use for treating diarrhoea, the activities reported here goes a long way in adding value to the plants used as part of traditional medicine.

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4NQO:	4-nitroquinoline-N-oxide
AA:	Arachidonic acid
AIDS:	Acquired immunodeficiency syndrome
ATCC:	American type culture collection
ATM:	African traditional medicine
CFU:	Colony forming units
COX:	Cyclooxygenase
DCM:	Dichloromethane
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DPM:	Disintegration per minute
DPMbackground:	Disintegration per minute background
DPMblank:	Disintegration per minute blank
DPMsample:	Disintegration per minute sample
DW:	Dry weight
EtOH:	Ethanol
FC:	Frequency of being
FI:	Frequency index
His^{+}:	Histidine
HCL:	Hydrochloric acid
HIV:	Human immune virus
I:	Inhibition
INT:	p-Iodonitrotetrazolium chloride
IPNI:	International plant names index
IUCN:	International union for conservation of nature
LPS:	Lipopolysaccharides
MH:	Mueller Hinton
MIC:	Minimum inhibitory concentration
MIZ:	Mean inhibition zone
N:	Number
No.1:	Number 1

NP:	Natural products
NSAID:	Nonsteroidal anti-inflammatory drugs
NRF:	National Research Foundation
NU:	Natal University
ORS:	Oral rehydration solution
PE:	Petroleum ether
PGG_2:	Prostaglandin endoperoxide
$PGEF_{2\alpha}$:	Prostaglandin EF_2
PGE_2:	Prostaglandin E_2
PGH_2:	Prostaglandin H_2
PGHS:	Prostaglandin HS
pH:	Potential hydrogen
SAAB:	South African Association of Botanists
SA's:	South Africa's
SANBI:	South African National Biodiversity Institute
TLR-4:	Toll-like receptor-4
TM:	Traditional medicines
TRIS:	Tris(hydromethyl)aminomethane
UK:	United Kingdom
UKZN:	University of KwaZulu-Natal
USA:	United States of America
US$:	United States Dollars
WHO:	World Health Organization

CHAPTER 1: General introduction

1.1.   Medicinal plants: Traditions of the past and drugs for the future

A "medicinal plant" can be defined as a plant that contains compounds with the potential to heal, and maintain health as well as prevent specific ailments and diseases (**RIOS and RECIO, 2005**). Worldwide, there is an increasing interest on the use of medicinal plants. The pharmaceutical industry and academic researchers are now paying more attention to medicinal plants. The interest is due to the growing public awareness of medicinal plants that are reported to contain pharmacologically active compounds. Researchers are beginning to fully appreciate the power and potential of medicinal plants that contain chemical entities for treating various health conditions (**HIRT and M'PIA, 1995**).

1.1.1. Ethnopharmacological approach to the discovery of novel medicinal drugs

An ethnopharmacological approach is a highly diversified approach for the discovery of drugs that involves observation, description, and experimental investigation of indigenous drugs and their biological activities. It is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines that contribute to the discovery of natural products that have biological activity (**FABRICANT and FARNSWORTH, 2001**).

The recorded documentation for African traditional medicine is very limited, because African traditional knowledge systems are usually oral, and are not written (**LIGHT et al., 2005**). Ethnopharmacological studies are divided into two informal standards of research, the anthropological survey, documenting plants that are used for certain illnesses by indigenous populations based on field work and also the biological or chemical laboratory analysis of the ethnopharmacological data that was documented previously (**CASE et al., 2006**).

The main goal of ethnopharmacology is to discover novel plant-derived compounds, based on the indigenous use of medicinal plants, which can be developed into new pharmaceuticals. Many drugs that are prescribed by physicians presently are either isolated directly from plants

or artificially modified versions of natural products (**WANG et al., 2007**). Historically, plants have proven to be a major source of drugs (**NEWMAN et al., 2003**). The promising candidates for new drug discovery are often natural products; which will play an important role in drug development programs (**GERTSCH, 2009**). While the pharmaceutical process makes use of bioassay screening platforms to find promising compounds for a particular disease, ethnopharmacology goes the opposite way; by putting claimed efficacy of medicinal plants to test in the laboratory. Ethnopharmacologists try to understand the pharmacological basis of plants that are important to different cultures (**GERTSCH, 2009**).

Although previously, the discovery of drugs was largely by chance and based on human practices, it provided a legacy and knowledge that benefits us even today. As understanding of the demands for the natural products increase and therapeutic costs cheapens, earlier unexpected discoveries that were made by coincidence evolve into active searches for new drugs (**WANG et al., 2007**).

Many useful drugs have been prepared from traditional medicines (TM) for the treatment of various diseases and ailments. It is estimated that 25% of drugs that are prescribed worldwide today come from plants and 60% of anti-tumour or anti-infection drugs already on the market or under clinical investigation are of natural origin (**WANG et al., 2007**). The use of plants and plant extracts to create therapeutic modality has stood the test of time. Pharmaceutical research conducted over decades has shown that natural products are a potential source of novel molecules for drug development (**WANG et al., 2007**).

The process of drug discovery is multi- and inter-disciplinary including different methods of analysis. It begins typically with informants, botanists, ethnobotanists, ethnopharmacologists, or plant ecologists who collect and identify the plants of interest. The collection may involve species with known biological activity for which active compound(s) have not been isolated (e.g. herbal remedies that are used traditionally) or taxa collected randomly for a large screening programme. It is important to respect the intellectual property rights of a given country where plant(s) of interest are collected (**BALUNAS and KINGHORN, 2005**).

Nowadays ethnobotany has become an integral part of drug discovery from plants. The plant based indigenous knowledge is passed from generation to generation in various parts of the

world and has significantly contributed to the development of different traditional systems of medicine (**JACHAK and SAKLANI, 2007**).

Drug discovery from medicinal plants has traditionally been lengthier and more complicated than other drug discovery methods (e.g. from microbes, synthetic chemistry, combinatorial chemistry and molecular modelling) and it is time-consuming. As a result natural product scientists and pharmaceutical industries will need to improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts. Innovative strategies to improve the process of plant collection are needed, especially with the legal and political issues surrounding benefit-sharing agreements (**BALUNAS and KINGHORN, 2005**). Improving the speed of active compound isolation is necessary. Even with all the challenges facing drug discovery from medicinal plants, natural products isolated from medicinal plants can be predicted to remain an essential component in the search for new medicines (**BALUNAS and KINGHORN, 2005**).

1.1.2. Traditional plant based medicine

All over the world, since the beginning of human civilization, medicinal plants have been used by all cultures for the treatment of various diseases and ailments, and they still continue to supply mankind with medicinal remedies (**BAKER et al., 1995; WHO, 1999; JACHAK and SAKLANI, 2007; HAWKINS, 2008**). They are the oldest known health care products. Irrespective of the ethnological, medical and historical background of each country the importance of medicinal plants is still growing worldwide. Plants are not only important in medical research when used directly as agents of therapy, but also as basic materials for the synthesis of drugs or as models for pharmacologically active compounds (**WHO, 1999**).

The World Health Organization (WHO) defines TM as the total sum of all the knowledge and practices that is used in the diagnosis, elimination, and prevention of imbalances that are physical, social or mental relying on practical experiences and observations that is handed down from one generation to another, whether in the form of writing or verbally (**HOAREAU and DASILVA, 1999**). Worldwide, there is the existence of traditional knowledge that is linked to health of human and animals which needs documentation and effective evaluation (**RUKANGIRA, 2001**). Africa is known generally as the cradle of

mankind with its rich cultural diversity and biodiversity that mark regional difference in healing practices (**GURIB-FAKIM, 2006; WATSON and PREEDY, 2008**). The African flora is estimated to have about 66000 higher plant species of which 35000 are endemic to the continent (**WATSON and PREEDY, 2008**). The main resource for managing diseases by man during human history have been plants and for thousands of years knowledge about medicinal plants and their properties has been handed down through generations. The majority of the countries worldwide, especially those in the African continent, still to a great extent rely on plant-derived medicine (**WATSON and PREEDY, 2008**). According to **AHMAD et al.** (**2006**), the discovery of the history of the curative properties of certain plants must have sprung from instinct. Ancient people first used plants as food. As a result of ingestion, a link with the properties of plants would have developed.

In South Africa there is a great cultural diversity, and several ethnic groups which have resulted in a massive use of medicinal plants throughout the provinces (**LIGHT et al., 2005**). Each culture in this country has medical solutions for the prevention and curing of diseases. Over 30000 species of higher plants are found in South Africa, of which most are endemic and approximately 3000 species of these plants are used in TM (**LIN et al., 1999; VAN WYK and GERICKE, 2000; FAWOLE et al., 2009a; VAN VUUREN, 2008**). This diversity of plants presents a very valuable resource (**VAN WYK, 2008**). Approximately 27 million people depend on traditional medicine for their primary health care needs (**STREET et al., 2008**). Almost 80% of black people use the services of traditional healers as a source of affordable and accessible health care. In South Africa an estimated 200000 of traditional healers are consulted by 60% of the population in preference or in addition to western doctors especially in rural areas (**ELGORASHI, et al., 2003; WHO, 2004a**).

The use of TM still forms an important part of the health care system of the South African population as is the case in most developing countries, because it is cheap and affordable. Traditional healers are consulted by numerous people for all, or some, of their health care needs. Traditional herbalists have long played an important role in the provision of primary health care by prescribing plant-based treatments especially to patients residing in areas where most of the population is poor and unable to afford modern drugs. TM practices remains strong in South Africa for the treatment and management of life threatening diseases, due to its availability, affordability and its role in belief systems (**VAN WYK, 2001**). Western medical care is provided by governmental health care services, as a result the

majority of communities in rural areas now have access to mobile clinics and hospitals, but still most people believe in herbal medicine, possibly because of lack of trust in the western health care system (**GRIERSON and AFOLAYAN, 1999**).

The unique situation of African traditional medicine (ATM) has been dealt with in South Africa by taking a number of initiatives. With respect to ATM the national department of health has shown fantastic commitment, by developing the national drug policy (1996), which recognises the benefits and potential role of available remedies (**NATIONAL REFERENCE CENTRE FOR AFRICAN TRADITIONAL MEDICINE, 2010**). The policy is aimed at: investigating TM use with the aim to integrate their use in the national health care delivery system; registration and control of marketed traditional medicine; provisions for the establishment of a national reference centre for the traditional medicines of Africa with the aim of gathering harnessing and synthezing information to promote, regulate and register the origin of African traditional medicines (**NATIONAL REFERENCE CENTRE FOR AFRICAN TRADITIONAL MEDICINE, 2010**).

The programmes that are used for *in vitro* screening of plant remedies are important for validating the use of herbal treatments traditionally and for providing leads in the discovery of new active principles. The products of nature play an important role in the discovery of drugs and development processes. Thus useful sources of highly active therapeutic compounds have been recognized as medicinal plants (**McGAW and ELOFF, 2008**). Reviews of traditional plants used are available in South Africa, although a lot of work needs to be done regarding the documentation of ethnobotanical knowledge that exists. South Africa is endowed with the richest temperate flora in the world, consequently there is much potential for the discovery of metabolites that are interesting and active. The country enjoys a strong cultural reliance of using plants in traditional medicine for healing a wide range of ailments (**LALL and MEYER, 1999; McGAW and ELOFF, 2008**). Due to the lack of interest from the younger generation to carry on traditions and the influence of the western lifestyle the knowledge of medicinal plants is decreasing rapidly (**OGIE-ODIA and OLUOWO, 2009**). Ethnobotanical studies are important in revealing locally important plant species, because knowledge on the uses of medicinal plants has provided many important drugs.

1.2. Diarrhoeal diseases

1.2.1. What is diarrhoea?

Diarrhoea is the passage of watery stools, usually at least three times in a 24 hour period, and its important indicators are changes in consistency of the stool (**APPIDI et al., 2008**). It is a symptom of infection either caused by viruses, bacteria, protozoa and helminths that are transmitted from the stool of one individual to the mouth of another, termed faecal-oral transmission (**WHO, 1995**). Infectious microorganisms multiply in the human gut, exit in excreta, and transit through the environment, causing diarrhoea in new hosts (**CURTIS and CAIRNCROSS, 2003**). The absorption and secretion of electrolytes and water in a normal intestinal tract are regulated to meet the body's physiological needs, but when there is an imbalance in the mechanism in the intestinal mucosa an increase in fluid and electrolyte loss into the gut lumen will result and that will lead into the production of unformed liquid faeces (**WHO, 1995**). This causes severe dehydration, which means the body lacks enough fluid to function properly. Diarrhoea can become life threatening if not treated especially when malnutrition is involved. Other symptoms of diarrhoea include abdominal cramps, abdominal pains, fever, bleeding and dizziness from dehydration (**PALOMBO, 2006**). Although changes in consistency of the stool are important indicators of diarrhoea, it can be classified into three categories, acute watery, persistent and bloody diarrhoea or dysentery. Acute diarrhoea has an abrupt onset, resolves within 14 days and is usually caused by an infectious agent (**WHO, 1995**). Drugs, poisons (including bacterial toxins) or acute inflammatory reactions can also contribute to diarrhoea. Persistent diarrhoea is manifested by loss of nutrients, abnormal absorption of nutrients from the digestive tract and lasts 14 days or longer. Dysentery or bloody diarrhoea is caused by damage of the intestinal mucosa due to invasive bacteria (**WHO, 1995**).

1.2.2. Worldwide effect of diarrhoea

Diarrhoea is one of the main causes of morbidity and mortality in children, especially those infected with HIV worldwide (**WHO, 2010**). It kills about 2.2 million people yearly and 1.5 million of those are children under the age of five especially in developing countries, 78% of these deaths occur in Africa and South-East Asia (**BARBOSA et al., 2006; WHO, 2009;**

WHO, 2010). It is the third biggest cause of death in South Africa, responsible for over 10000 deaths annually (**WHO, 2009**). Diarrhoea spreads through faeces-contaminated water, hence infection is more common when there is a short supply of clean water for drinking and cooking. As diarrhoea and related diseases are a major problem in developing countries, they are also a high risk to travellers who visit these countries. Dehydration is the main threat of death from diarrhoea, which is due to the loss of electrolytes in diarrhoea stools. Diarrhoea remains a leading global health problem which causes about 4% of all deaths (**CHEN et al., 2007; DAS et al., 2009**). An estimated 80% of deaths are due to acute watery diarrhoea, 10% for persistent diarrhoea and another 10% for dysentery (**MATHABE et al., 2006**). Inadequate sanitation and poor hygiene cause an estimated 88% of deaths related to diarrhoea (**DE WET et al., 2010**).

1.2.3. Microorganisms that cause diarrhoea

Diarrhoea is caused mostly by gastrointestinal infections caused by viruses, bacteria, protozoa and helminths. A significant number of deaths are due to a single genus of bacteria, *Shigella* Castelani and Chalmers, which causes dysentery or bloody diarrhoea (**MATHABE et al., 2006**). Diarrhoea also results from contaminated food caused by bacteria such as *Salmonella* spp. Lignieres, *Campylobacter* spp. Sebald and Veron, *Staphylococcus aureus* Rosenbach and *Escherichia coli* (Migula) Castelani, and contaminated water sources caused by protozoa such as *Giardia intestinals* (Lambl) Kofoid & Christiansen and *Cryptosporidium parvum* Tyzzer, viruses such as asrotavirus, astrovirus, adenovirus, calicivirust, rotavirus and helminths such as *Schistosoma mansoni* Sambon that are transmitted from the stool of one individual to the mouth of another (**MATHABE et al., 2006**). All these diarrhoea causative microorganisms differ in the number of organisms needed to cause infection and illness. The ability to survive stomach acid is an important determinant of the inoculum size needed to cause illness, as they require millions of organisms to cause infection (**WHO, 1995**).

1.3.   Incidence of diarrhoea in Eastern Cape

Diarrhoea is often associated with low standards of living, poor sanitation infrastructure and poor access to potable water sources. The Eastern Cape is known to have all these factors. Hence it is among the Provinces that are known to have many diarrhoea cases in South

Africa. Infections that are mostly treated with medicinal plants. During a rainy season diarrhoea cases are observed, more especially from rural areas, because the unprotected water sources on which most people depend for water supply are contaminated. The rural areas of the Eastern Cape Province are notorious for lack of proper sanitation and piped or clean water (**EASTERN CAPE DEPARTMENT OF HEALTH, 2009**). Hence waterborne diseases are common where the majority of rural dwellers depend largely on water from unprotected sources, shared with domestic animals.

1.4. Western medicine used for the treatment of diarrhoea

The use of oral rehydration therapy, breast feeding of children, zinc and other macronutrients for managing diarrhoea are recommended by the WHO (**MATHABE et al., 2006**). Oral rehydration solution (ORS) is formed by mixing glucose and several salts that are dissolved in water to form a mixture known as oral rehydration salts. ORS is absorbed in the small intestine even during copious diarrhoea, thus replacing the water and electrolytes lost in the faeces (**WHO, 1995**). However, oral rehydration therapy does not reduce the duration of diarrhoea nor the volume, therefore treatment with pharmacological agents that are able to suppress severe symptoms or are pathogen-specific would benefit patients suffering from prolonged diarrhoea (**PALOMBO, 2006**). When there is diagnostic doubt whether the infected individual has gastroenteritis or septicaemia, antibiotics have a limited part to play in treating specific infections. Vaccines have been considered as the most feasible approach to management of diarrhoea as a result various attempts for developing vaccines against organisms that cause diarrhoea have been made. However, in developing countries the response to such vaccines has not been encouraging probably due to their high cost (**CANDY, 1984**).

1.5. Overview of medicinal plants used to treat diarrhoea

In many African countries, use of herbal drugs to combat the problem of diarrhoea is a common practice. In developing countries, most of the people use traditional medicine to treat all kinds of diseases including diarrhoea and South Africa is no exception (**LIN et al., 2002**). According to **PALOMBO** (**2006**) plants used for the treatment of diarrhoea have antispasmodic properties; they delay gastrointestinal transit, suppress the motility of the gut,

stimulate absorption of water and or reduce the secretion of electrolytes. Many medicinal plants have now become the focus of intensive studies to validate their traditional uses and provide scientific explanations for their pharmacological effects (**SPARG et al., 2002**). Numerous reports have been made on the use of traditional plants for treating diarrhoea and related diseases, as a result many plant-derived medicines that are used in African, American, Asian, European and other traditional medicinal systems have been recorded in pharmacopeias as agents used for treatment of diarrhoea (**PALOMBO, 2006**). Several studies have been conducted in many parts of South Africa to evaluate the effectiveness of traditional medicines used in the treatment of diarrhoea (**McGAW et al., 2000; LIN et al., 2002; MATHABE et al., 2006; APPIDI et al., 2008; FAWOLE et al., 2009a; BISI-JOHNSON et al., 2010**). For example, a study was conducted by **MATHABE et al.** (**2006**) to evaluate the effectiveness of *Indigofera daleoides* Benth. ex Harv. & Sond, *Punica granatum* L., *Syzygium cordatum* (Hochst.), *Gymnosporia senegalensis* (Lam.) Exell, *Ozoroa insignis* Delile, *Elephantorrhiza elephantine* (Burch.) Skeels, *Elephantorrhiza burkei* Benth., *Ximenia caffra* Sond., and *Schotia brachypetala* Sond. used in the treatment of diarrhoea. Another study was conducted by **LIN et al.** (**2002**) to evaluate the ability of *Psidium guajava* L. to inhibit the growth of bacteria that cause diarrhoea. All these studies demonstrated that there is a need to continue documenting medicinal plants and evaluate their effectiveness in treating diarrhoea, as plants play a major role in primary health care of rural people. There is still undiscovered ethnobotanical data that needs to be documented. WHO incorporates studies of traditional medicinal practice in its diarrhoeal disease control program. Plant-derived medicines that are used for the treatment of diarrhoea are available commercially, these include tormentil root, Seirogan and Kampo.

In poverty stricken areas, where there is ignorance in terms of hygiene, lack of clean drinking water, and sanitary ablutions are a problem, diarrhoea is a major concern. A study on traditional remedies around the Eastern Cape region of South Africa showed that diarrhoea is one of the most prominent conditions that is usually treated with medicinal plants (**DAMBISYA and TINDIMWEBWA, 2003**). The Pondoland people (*AmaMpondo*) in the eastern region of Eastern Cape have a strong tradition of using medicinal plants. People use several medicinal plants to cure diarrhoea (locally known as "*Utyatyazo*" in *isiXhosa*).

1.6.   Conservation of medicinal plants

Plant derived natural products have always stood for the treatment of diseases. The popularity of herbal medicines is increasing. Such an increase in demand leads to unsystematic harvesting of flora. This unsystematic harvesting of flora has generated conservation issues for plants. This has been increased by a lack of conservation knowledge among harvesters (**VERMA and SINGH, 2008**). In Africa collecting plants from wild populations is still a common practice as a result slow growing and slow reproducing popular species are the ones that are especially vulnerable to excessive collection. For example slow growing bulbous and tuberous plants like *Bowiea volubilis* Harv. Ex Hook.f., *Eucomis autumnalis* (Mill.) Chitt. and *Scilla natalensis* Planch. are threatened by over-exploitation. As a result many species are threatened and are in danger of extinction (**ZSCHOCKE et al., 2000**).

The African continent has the highest rates of endemism and is gifted with a huge wealth of plant resources that needs to be protected. According to **GURIB-FAKIM** (**2006**), Africa is reported to have one of the highest rates of deforestation in the world. There is a huge demand for bark, roots and whole plants which are causing a decline in population numbers of some species. This may lead to numerous extinctions (**GURIB-FAKIM, 2006**).

Conservation of medicinal plants is receiving attention in view of a surge of interest in herbal medicines for health care across the world (**DHAR et al., 2000**). Previously, harvesting of medicinal plants was the field of trained traditional medical practitioners, widely known for their skills as herbalists and diviners (**WILLIAMS et al., 2000**). As a result customary conservation practices which regulated times of plant collection and quantities to be collected were valued. However several factors such as affordability, accessibility, and acceptability of medicinal plants over western medicine, as well as high unemployment rates have resulted in overharvesting. The trend towards increased urbanization and commercialization of medicinal plants, created by a demand for plant derived medicines has resulted in overharvesting, and in some cases near-extinction of some valued indigenous plant species (**ZSCHOCKE et al., 2000**). As a result over harvesting of wild material is acknowledged as a serious threat to biodiversity as more than 700 plant species are known to be actively traded for medicinal purposes throughout the country. Growing demand of popular medicinal plants

is linked to increased harvesting pressures on traditional supply areas (**DOLD and COCKS, 2002**).

In Europe, China and India the growing demand for herbal medicine is often met by cultivating plants on a large scale. In southern Africa little attention was paid to issues relating to medicinal plant resources until the late 1980's. Responsible management of natural medicinal plant resources has become a matter of urgency, due to a rapid increase of informal trade in medicinal plants (**ZSCHOCKE et al., 2000**).

Barks and roots (54%), bulbs and whole plants (28%) represent the most important ingredients of Zulu herbal medicine that is sold on the street markets in KwaZulu-Natal (**ZSCHOCKE et al., 2000; MANDER et al., 2007**). In South Africa, high proportions (32%) of the most commonly used medicinal plants are trees. **CUNNINGHAM** (**1993**) reported that forest trees have been shown to be highly vulnerable to excessive exploitation, because mature bark is the most commonly used plant part in southern Africa. Therefore it seems that the problem of tree ring-barking and extinction of commonly used tree species is a particular problem in South Africa (**ZSCHOCKE et al., 2000**). However, a number of possible strategies to solve this problem were identified such as to establish conservation areas and enforce laws against bark collection, large scale cultivation and encouraging healers to collect and use alternative plant parts (**ZSCHOCKE et al., 2000**).

1.7. Trade of medicinal plants in South Africa

In South Africa, trade in medicinal plants is a large and growing industry with virtually no official trade statistics. Traditional medicine forms part of a multi-million rand trade in South Africa. The major stake holders in medicinal plant trade are the poor rural people. All the trade figures suggest that medicinal plants offer great motivation for those concerned in human healthcare and economy to increase worldwide supply of herbal drugs (**DHAR et al., 2000**). Women are more involved in the trade of medicinal plants as an alternative form of income generation. This could also be because women are generally responsible for the health care of their children and families (**ZOBOLO and MKABELA, 2006**). **MANDER** (**1998**) and **MANDER et al.** (**2007**) estimated that there are approximately 27 million consumers of traditional medicine in South Africa. The South African medicinal plant trade is

believed to be worth around US$39 million annually. When the value of that is added to these plants through prescription by traditional healers, the total annual value of the trade is considerably higher. This is 39% higher that the quantity of plants exported in Germany which is the third highest exporter of pharmaceuticals in the world (**MANDER, 1998**). The trade and use of traditional medicine is no longer confined to traditional healers but it has entered the formal and informal entrepreneurial sectors of the economy of South Africa resulting in a number of gatherers and traders. Crude traditional medicines are traded at local national markets in South Africa. TM is still part of everyday life in the Eastern Cape. Materials that are traded in Gauteng originate from Eastern Cape, thus revealing that trade is interprovincial (**DOLD and COCKS, 2002**).

1.8. Research questions

Many South African rural dwellers use untreated water domestically and are at risk to the devastating effects of diarrhoea. This is because diarrhoea-causing organisms may be transmitted as a result of poor quality of water, inadequate sanitation, and hygiene.

Information about medicinal plants and their medicinal value was usually passed on orally from one generation to another, resulting in few records being kept. Since oral information can never be as accurate as was told to the recipient, whole "libraries" of herbal information are gradually lost. The study of medicinal plants that are used for treating diarrhoea by local people can lead to valuable information that should be tested scientifically.

TM is still part of everyday life in Eastern Cape which is significantly poorer and less developed than other provinces and a home to approximately 6.3 million of SA's total population. Many people rely on natural resources for direct subsistence or for generating income thus having in a huge impact on natural resources of the province.

In rural areas of Bizana, hospitals are sparsely located and far away from the villages. This makes it very difficult for patients to regularly visit them for effective treatment. Even if the treatment is free, many rural dwellers find it very expensive to meet the travelling costs during their regular visits to clinics and hospitals. Hence, they resort to herbal remedies to alleviate diarrhoea. Concepts and terminologies for illnesses and the signs and symptoms

used by medically trained health workers do not necessarily mean the same to caregivers which may use local terms which do not translate simply into medical terms, and that may lead to misunderstandings during medical consultation. The people are reliant on traditional healers who usually reside among them, trusting their ingenuity on the use of herbs and other cultural and traditional beliefs. These facts thus provide a role for traditional healers among the rural dwellers' trust. This information needs to be documented and recorded in view of the rapid loss of natural habitats, traditional community life, cultural diversity and knowledge of medicinal plants. It also needs to be evaluated for pharmacological activities. This brings us to the research questions:

- Are there still medicinal plants in Bizana used for the treatment of diarrhoea that have not yet been recorded?
- Can the plants that are used for treating diarrhoea in Bizana inhibit the growth of diarrhoea causative bacteria?
- Are they safe to use?

1.9. Aims and objectives

African traditional medicine is the oldest and perhaps the most diverse system of all systems of medicine. Unfortunately, the systems of medicine are poorly recorded and remain so to date. Documentation of the uses of African plants is becoming increasingly urgent due to the rapid loss of the natural habitats of some of these plants because of anthropological activities, thus this study was conducted in Bizana, Pondoland of Eastern Cape. The aims of the study were:

- To record and document the plants used for treating diarrhoea in Bizana, Pondoland Eastern Cape through questionnaires.
- To investigate the activity of selected plant extracts against ATCC strains of bacteria that cause diarrhoea.
- To determine the anti-inflammatory activity of the selected plants using Cyclooxygenase-1 and-2 enzymes.
- To test the selected plant extracts for mutagenicity against *Salmonella typhimurium* tester strains in order to establish their safety.

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CHAPTER 2: Ethnobotanical study of plants from Bizana, Pondoland used against diarrhoea

2.1. Introduction

2.1.1. Ethnobotanical approach

Ethnobotany is the study concerned with the interactions between human beings and plants over time and space (**MARTIN, 1995; McCLATCHEY et al., 2009**). The definition of ethnobotany is varied, but the concept has some common elements. The prefix "ethno" refers to the way other people look at the world, while "botany" is the study of plants (**MINNIS, 2000**).

Ethnobotany began with direct observations about the ways in which people used plants and consisted mainly of compiling list of plants used (**MINNIS, 2000**). It was originally conceptualized as an art and skill practiced by outsiders who travelled to remote lands to document customs and beliefs (**MARTIN, 1995**). Hence, some of the early studies often recorded more than uses of plants, they also documented beliefs about plants and human ecology, and that made the research to be of a high quality. The study of Cahuilla ethnobotany in California done by Barrows (1900) is an example of early research that was of high quality which included more than plant uses, but also incorporated beliefs about plants and human ecological relationships. It was soon followed by scholars working in Africa, Asia, Australia and the Pacific (**MINNIS, 2000**). Ethnobotany has now grown in size and significance with scholars of many subdisciplines regenerating new beginnings in ethnobotanical research.

The concept of ethnobotany was first developed by Hershberger (1896), a botanist at the University of Pennsylvania who examined ancient plant remains from different dwellings in southwestern North America (**MINNIS, 2000**). It has now developed gradually into a much broader field that covers not only how people use plants to meet their needs, but also relationships that hold the emblematic, ecological and cognitive as well as the human–plant relationship in current settings (**SOEJARTO et al., 2005**). Ethnobotany is more about the cultural importance of the link between humans and plants. Indigenous knowledge of a

particular society is an important part of ethnobotany (**McCLATCHEY et al., 2009**). The discipline is based on knowledge by local people as understood by a particular cultural group since information about plant use varies from one cultural group to another. As a result, different cultures have their own understanding of plants and their uses. Indigenous knowledge is the means by which most communities survived for centuries by adapting themselves to their environment, using their inherent knowledge (**SOEJARTO et al., 2005**).

It is important to document indigenous knowledge through ethnobotanical studies for the purpose of preserving and utilizing it (**REVATHI and PARIMELAZHAGAN, 2010**). Since ancient times, plants have always played an important role in medicine and public health supporting human welfare, and always will. Writings from Babylon, China, Egypt, and India, are the earliest references of plants as medicines. The earliest records of medicinal plant therapy are written on clay tablets in cuneiform, from Mesapotamia and date from about 2600 BC (**GURIB-FAKIM, 2006**). Some of the materials include oils from cedar, cypress, licorice, myrrh and poppy juice. All of these plants are still in use today for the treatment of ailments ranging from coughs to parasitic infections and inflammation (**GURIB-FAKIM, 2006**). The prehistoric civilization of Egypt listed more than 850 therapeutic plant remedies in the Ebers Papyrus a medicinal scroll from 1500 BC. Medical and pharmaceutical knowledge of the Egyptians was documented in wall paintings of tombs dating from the old kingdom and on papyrus which is made from *Cyperus* L.. Of all these writings the most important is the Ebers Papyrus which originated from around 1500 BC and reported to contain ancient medicinal knowledge from before 3000 BC (**OUBRE et al., 1997; GURIB-FAKIM, 2006**). Over the years, in different parts of the world, traditional knowledge on medicinal plants and their use has proven to be an important guide towards present day screening of drugs. This has made the interest in ethnobotany increase dramatically especially in segments of the scientific community (**REVATHI and PARIMELAZHAGAN, 2010**).

With the introduction of modern medicine, traditional medical practices concerning use of medicinal plants declined to some extent (**HANIF et al., 2009**). However in recent years, traditional or herbal medicine has been receiving attention from both doctors and patients (**HANIF et al., 2009**). Various compounds that are active have been discovered on the basis of ethnobotanical information and used directly as patented drugs (**REVATHI and PARIMELAZHAGAN, 2010**). Medicinal plants form an important aspect of the daily lives of many people, and are an important part of the South African culture and heritage

(**RAMALIVHANA et al., 2010**). Hence, they are one of the reliable agents for drug discovery. An ethnobotanical study encourages the continuous search for natural products, and was recognized as one of the major approaches for selecting plants for pharmacological screening (**OGBOLE et al., 2010**). Ethnobotanical studies have been significant in revealing plant species used as medicine for crude drug development. According to **OGIE-ODIA and OLUOWO (2009)** documentation of traditional knowledge especially on the uses of medicinal plants has provided many important drugs of modern day.

In bioprospecting, an ethnobotanical approach involves selecting plants used in traditional medicine. Plant selection is based on the idea that, medicinal plants used to treat a particular disease may have an associated biological activity (**AHMAD et al., 2006**). The information obtained from traditional communities about the use of medicinal plants is combined with chemical/pharmacological studies done in laboratories (**OUBRE et al., 1997**). This approach is actually one of several methods that can be used in selecting plants for pharmacological studies and it has effectively identified novel bioactive molecules from various plants. Thus, it has been helpful in plant pharmacological research, yielding better results than other plant selection methods such as the random approach. However, this approach is challenging since it requires identification of people who possess knowledge of medicinal plants, and secure their cooperation.

Evaluating effectiveness of plants is difficult, as it requires keen understanding of culture, for example how and why people use plants for medicinal purposes. For this reason it is important to work directly with people who use medicinal plants (**OGIE-ODIA and OLUOWO, 2009**).

Several studies have been conducted on different types of methods including laboratory and survey based in many parts of South Africa to evaluate the efficacy of traditional medicine used in the treatment of diarrhoea (**LIN et al., 2002; MATHABE et al., 2006; APPIDI et al., 2008; FAWOLE et al., 2009a; BISI-JOHNSON et al., 2010**). There is, however, a lot of unrevealed ethnobotanical information that still needs to be collected from the local people. The data needs urgent documentation as most of the traditional knowledge about plants and their uses is diminishing as a result of socio-economic and land use changes.

2.2. The *AmaMpondo* people

The Mpondos are one of the Xhosa speaking tribes that reside in Pondoland which lies between uMtata River to the south, and uMtamvuna River to the north (**KEPE, 2005**). The citizens of the region speak a Xhosa dialect known as *isiMpondo* and thus are known as *AmaMpondo*, a group that successfully resisted colonial rule, showing the courage and tenacity to protect their livelihoods (**KEPE, 1997**). Historically, numerous natural resources have been an important part of *AmaMpodo* people's livelihoods, hence through oral information new generations are made aware of the importance of natural resources (**KEPE, 1997**).

2.3. Materials and methods

2.3.1. Study area

The study area is based in Bizana (formerly known as Mbizana), a rural district located in the north eastern part of Eastern Cape, in Pondoland, South Africa (**KEPE, 2005**). Mbizana is an *isiXhosa* word meaning "small pot". Previously, local peasant women used to make traditional clay pots in the river below Bizana village and displayed them in the sun to dry. According to **MADIKIZELA** (**2010**) (personal communication), one summer day, it is believed that an invisible force pushed all the pots that were displayed in the sun into the river and they disappeared, the women said, "Our small pots", and that is how the name originated. Bizana has a temperate climate, fertile soil, frost-free conditions and, annual rainfall of around 700 mm per year that peaks in summer, although there is substantial winter rainfall. The latitude of the area is -31.567 and the longitude is 29.400 and has an area of 2806 km^2. The area is dominated by grasslands. According to the 2001 census, the population of Bizana was approximately 244000 (**MBIZANA LOCAL MUNICIPALITY, 2005**). Each village is headed by a headman who reports to the chief. The settlements are loosely scattered throughout the entire area and are surrounded by arable grazing land. Proposed mining of titanium found in Bizana revealed a need for environment protection awareness, as the area has unique biodiversity value. New laws governing occupation of land, forests, grazing and

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movement of livestock have been seen by rural people as a threat to their livelihood and they resisted some of the rules, where possible. People make their livelihoods through farming, use of a range of natural resources and pensions (**KEPE, 2003**).

Most villages are further away from the town of Bizana and they do not access the fruits of modernization– such as piped clean water, infrastructure and easy access to modern health benefits. There are two hospitals, St Patricks and Greenville, and a few clinics. Most of the people in this area rely heavily on natural resources for their primary health care needs (**MBIZANA LOCAL MUNICIPALITY, 2005**).
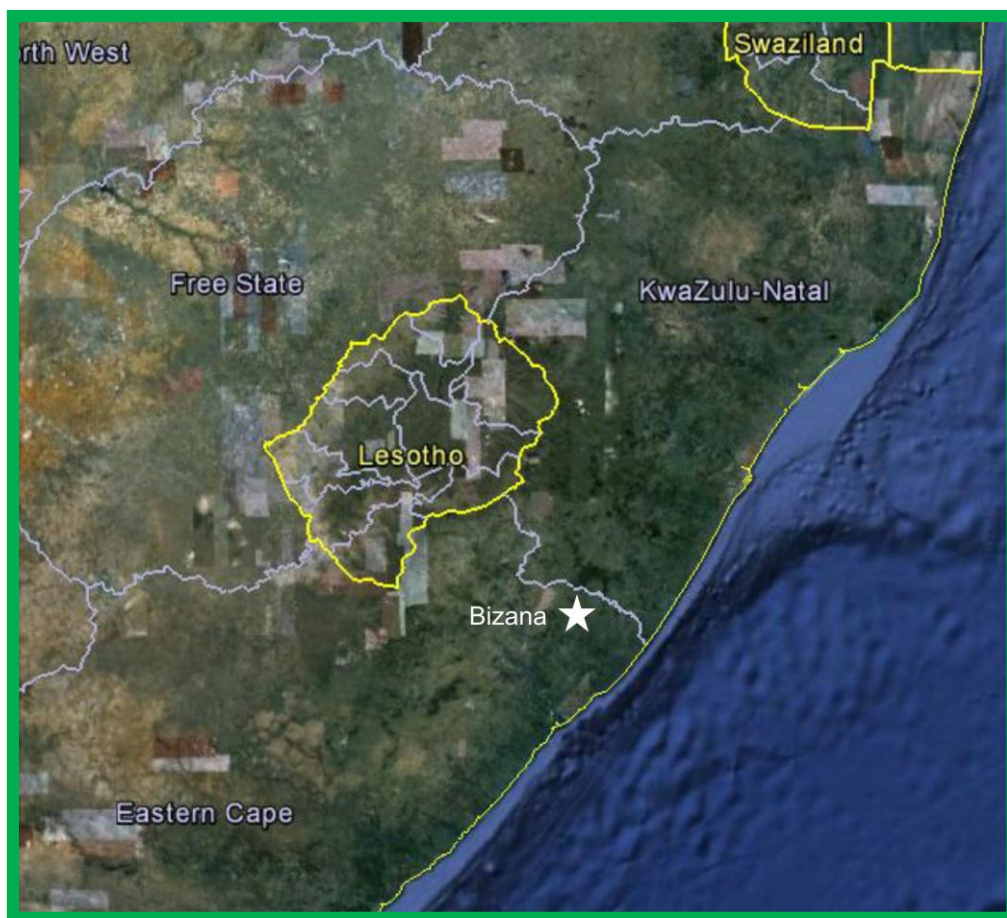
Figure 2.1: Map showing geographical position of Bizana (study area), in the Eastern Cape province, South Africa. Map obtained from AfriGIS (Pty) Ltd, Google Tele Atlas 2011(C).

2.3.2. An ethnobotanical approach to select plants for the study

An ethnobotanical study was done in six rural areas of Bizana (**Figure 2.1**), Pondoland in the Eastern Cape, South Africa to record plants that are used for treating diarrhoea. The six rural areas were Dutyini, Ludeke, Ndeyingana, Qanga, Redoubt and Sibomvini. The study areas were visited several times from January 2010 to October 2010. The ethnobotanical survey was conducted after seeking permission from the local authorities, and participation in this study was voluntary. The interviews and discussions were carried out using the local language (*isiMpondo*) for easy communication with the participants. The information on medicinal plants that are used for the treatment of diarrhoea in the study area was collected from traditional healers, herbalists, elderly people, young men and women using questionnaires (**Appendix 1 and 2**). The informants, except the healers which, were selected randomly. All the healers in the study area were interviewed. The questions asked included what plants are used for diarrhoea and how the remedies are prepared. Interviews were conducted in homesteads where rural people live and work. Prior to the administration of the questionnaires, conversations with the informants were held with the help of local representative to elaborate the objective of the study and to build trust with the common goal to document and preserve the knowledge on medicinal plants. The recorded information was expressed as a 'frequency index' (FI), i.e. a mathematical expression of the percentage of frequency of mentioning for a single plant species by informants and the information was calculated (**Table 2.1**), together with additional information such as vernacular names of the plants, parts used, preparation of remedies and a brief literature review. Plants were collected, pressed and dried in an oven. The following formula was used to calculate frequency index:

$$FI = \frac{FC}{N} \times 100$$

Where FC is the number of informants who mentioned the use of the species, and N is the total number of informants (100 in this study) (**TANGJANG et al., 2011**). Frequency index is high when there are many informants who have mentioned a particular plant and low when there are few reports.

The scientific names of plants were determined by using different textbooks (**WATT and BREYER-BRANDWIJK, 1962; POOLEY, 1993; HUTCHINGS et al., 1996; VAN WYK**

et al., 1997; VAN WYK et al., 2009) and **INTERNATIONAL PLANT NAMES INDEX (IPNI), 2011**. Herbarium specimens and monographs were used to identify the collected plants to species level. Additional usage of plants mentioned in the study was established by consultation with the literature. Plant species were grouped into their respective families along with local and common names. Voucher specimens were deposited at the Bews Herbarium, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg campus.

2.4.   Results and discussion

2.4.1. Plants reported to be used to treat diarrhoea in Bizana, Pondoland, in the Eastern Cape

Information on plants that are used for treating diarrhoea in Bizana, Pondoland, Eastern Cape recorded during the survey are listed in **Table 2.1**. The study revealed 34 plant species belonging to 21 families. For each species the following ethnobotanical information are provided: family, botanical and vernacular names, voucher specimen codes, plant parts used, types of plant, modes of preparation as well as the frequency index recorded during the study. Notes on reported antidiarrhoeal usage of the same plant elsewhere in the world were included, as the wide use of the plant for similar ailments may indicate rationale usage. The conservation status of each species was determined using the current **INTERNATIONAL UNION FOR CONSERVATION OF NATURE (IUCN)** (**2011**) data list, since all participants indicated that they were not practicing any method with regard to the conservation of these plants.

Table 2.1: Frequency index and plants used for the treatment of diarrhoea in Bizana, Eastern Cape

Family plant species (IPNI)	Voucher specimen number	Parts used	Vernacular name	Preparation	Type of plants	Reported antidiarrhoeal usage	Frequency index	IUCN Conservation status
Amaryllidaceae *Brunsvigia grandiflora* Lindl.	BALUNGI 31	Bulbs	Isichwe/ Isichu	Crushed bulbs are boiled in water and administered orally three times a day	Herb	No reported antidiarrhoeal usage	28	Least threatned
Anacardiaceae *Searsia chirindensis* (Baker f.) Moffett	BALUNGI 6	Leaves	Intlokoshiyane/ Intloboshane	Crushed leaves are boiled in water and taken orally three times a day	Shrub	Tubers are used for diarrhoea (**HUTCHINGS et al., 1996**)	25	Least threatened
Asteraceae *Senecio serratuloides* DC.	BALUNGI 19	Leaves, Stems	Untsukumbini	Leaves and stem are ground and boiled in water, decoction is taken orally three times a day	Herb	No reported antidiarrhoeal usage	29	Least threatened
Bignoniaceae *Tecoma capensis* (Thunb.) Lindl.	BALUNGI 1	Leaves	Umsilingi	Crushed leaves are soaked in water overnight and taken orally three times a day	Shrub	For diarrhoea and dysentery (**HUTCHINGS et al., 1996**)	37	Least threatened
Ulmaceae *Trema orientalis* (L.) Blume	BALUNGI 5	Leaves	Umbhangabhanga	Crushed leaves are boiled and administered orally three times a day	Tree	Dysentery (**HUTCHINGS et al., 1996**)	28	Least threatened

Family plant species (IPNI)	Voucher specimen number	Parts used	Vernacular name	Preparation	Type of plants	Reported antidiarrhoeal usage	Frequency index	IUCN Conservation status
Asteraceae *Aster bakerianus* Burtt Davy ex C.A.Sm.	BALUNGI 22	Roots	Unozixhekana	Roots are boiled and administered orally three times a day	Shrub	For stomach complaints (**VAN WYK et al., 2009**)	32	Least threatened
Cyperaceae *Cyperus dives* Delile	BALUNGI 25	Rhizomes	Intsikane	Rhizomes are ground, filtered through a clean cloth and administered orally three times a day	Herb	No reported antidiarrhoeal usage	24	Least threatened
Euphorbiaceae *Clutia pulchella* Spam. ex Sond.	BALUNGI 2	Leaves	Ungwalini	Leaves are crushed, boiled in water and taken orally three times daily	Shrub	Leaf infusions for diarrhoea (**HUTCHINGS et al., 1996**)	37	Least threatened
Mimosaceae *Acacia mearnsii* De Wild.	BALUNGI 13	Bark	Ubhulekwani	Mature bark, is ground and boiled in water. The decoctions is taken orally three times a day	Tree	Reported antidiarrhoeal usage (**BISI-JOHNSON et al., 2010**)	18	Least threatened
Fabaceae *Alysicarpus rugosu*s (Willd) DC.	BALUNGI 33	Leaves	Inkonazana	Crushed roots/leaves are boiled in water and administered orally three times a day	Herb	No reported antidiarrhoeal usage	13	Least threatened

Family plant species (IPNI)	Voucher specimen number	Parts used	Vernacular name	Preparation	Type of plants	Reported antidiarrhoeal usage	Frequency index	IUCN Conservation status
Mimosaceae *Elephantorrhiza elephantina* (Burch.) Skeels	BALUNGI 26	Roots	Intolwane	Crushed roots are boiled in water , and decoction is administered orally three times a day	Underground tree	Xhosa's use roots for diarrhoea and dysentery (**MATHABE et al., 2006; APPIDI et al., 2008; VAN WYK et al., 2009; BISI-JOHNSON et al., 2010**)	26	Least threatened
Geraniaceae *Pelargonium luridum* Sweet	BALUNGI 24	Bulbs	Ushwaqa	Crushed bulbs are boiled and taken orally three times a day	Herb	Tubers are administered orally for diarrhoea and dysentery (**VAN WYK et al., 2009; BISI-JOHNSON et al., 2010**)	21	Least threatened
Hydnoraceae *Hydnora africana* Thunb.	BALUNGI 9	Whole plant, Tubers	Umafumbuka	The whole plant is ground into powder and boiled, and the decoction is then administered orally three times a day	Succulent	Tubers are for diarrhoea (**HUTCHINGS et al., 1996**)	40	Least threatened
Asteraceae *Berkheya bipinnatifida* (Harv.) Roessler subsp. *bipinnatifida*	BALUNGI 34	Leaves	Ukhakhakha	The bulb is crushed and boiled in water. The decoction is taken orally three times a day	Herb	No reported antidiarrhoeal usage	23	Least threatened

Family plant species (IPNI)	Voucher specimen number	Parts used	Vernacular name	Preparation	Type of plants	Reported antidiarrhoeal usage	Frequency index	IUCN Conservation status
Hyacinthaceae *Eucomis autumnalis* (Mill.) Chitt.	BALUNGI 23	Leaves, Bulbs	Umathunga	Ground roots/leaves are boiled and administered orally three time a day	Bulb	Reported antidiarrhoeal usage (**BISI-JOHNSON et al., 2010**)	25	Declining
Hpoxidaceae *Hypoxis hemerocallidea* Fisch. C.A. Mey. and Ave`-Lall	BALUNGI 21	Corms	Ilabatheka	Crushed corms are boiled in water, and the decoction is administered orally three times a day	Tuber	Reported antidiarrhoeal usage (**BISI-JOHNSON et al., 2010**)	45	Declining
Lauraceae *Ocotea bullata* (Burch.) Baill.	BALUNGI 17	Leaves, Bulbs	Unukani	Leaves and bark are ground, soaked in water overnight, and administered orally three times a day	Tree	An infusion is said to be an effective remedy for infantile diarrhoea (**VAN WYK et al., 2009**)	34	Endangered
Hyacinthaceae *Ledebouria ovatifolia* (Baker) Jessop	BALUNGI 20	Bulbs	Unontangazibomvu	Crushed bulb is soaked in warm water overnight and administered orally three times a day	Herb	No reported antidiarrhoeal usage	24	Least threatened
Malvaceae *Malva parviflora* L.	BALUNGI 32	Leaves	Ujongilanga	Leaves are crushed, boiled in water and administered orally three times a day	Herb	Reported antidiarrhoeal usage (**APPIDI et al., 2008**)	34	Least threatened
Meliaceae *Turraea obtusifolia* Hochst.	BALUNGI 14	Roots, Leaves	Ikhambi lomsinga	Ground roots and leaves are boiled in water and taken orally three times a day	Shrub	Stomach and intestinal complaints (**HUTCHINGS et al., 1996**)	28	Least threatened

Family plant species (IPNI)	Voucher specimen number	Parts used	Vernacular name	Preparation	Type of plants	Reported antidiarrhoeal usage	Frequency index	IUCN Conservation status
Aloaceae *Aloe ferox* Mill.	BALUNGI 30	Leaves	Unomaweni	Leaves are crushed and soaked in water and the decoction is administered orally three times a day	Tree	No reported antidiarrhoeal usage	34	Least threatened
Moraceae *Ficus craterostoma* Warb. ex Mildbr. and Burret	BALUNGI 3	Leaves	Intendekwane/ Umthombe	Crushed leaves are soaked in warm water and administered orally three times a day	Tree	No reported antidiarrhoeal usage	48	Least threatened
Myrsinaceae *Maesa lanceolata* Forssk.	BALUNGI 4	Leaves	Umthendekwane/ Indendekwane	Leaves are crushed, soaked and administered orally three times a day	Shrub	No reported antidiarrhoeal usage	43	Least threatened
Myrsinaceae *Rapanea melanophloeos* Mez	BALUNGI 7	Leaves, Bark	Umaphipha	Bark/leaves are ground and boiled. The decoction is taken orally three times a day	Tree	No reported antidiarrhoeal usage	46	Declining
Myrtaceae *Eucalyptus camaldulensis* Dehnh.	BALUNGI 28	Leaves	Ugamthriya	Crushed and boiled leaves are sieved and taken orally three times a day	Tree	For dysentery (**HUTCHINGS et al., 1996**)	50	Least threatened

Family plant species (IPNI)	Voucher specimen number	Parts used	Vernacular name	Preparation	Type of plants	Reported antidiarrhoeal usage	Frequency index	IUCN Conservation status
Myrtaceae *Psidium guajava* L.	BALUNGI 27	Leaves	Igwava	Crushed leaves are boiled in water. Leaf decoctions are administered orally three times a day	Shrub	Used as a remedy for diarrhoea in various Africa, Asian and South American countries (**HUTCHINGS et al., 1996; VAN WYK et al., 2009; BISI-JOHNSON et al., 2010; DE WET et al., 2010**)	60	Least threatened
Rosaceae *Prunus persica* (L.) Stokes	BALUNGI 11	Leaves	Isiphitshi	Leaves are crushed and boiled in water, and the decoction is taken orally three times a day	Tree	Reported antidiarrhoeal usage (**APPIDI et al., 2008**)	34	Least threatened
Rubiaceae *Pentanisia prunelloides* (Klotzsch) Walp	BALUNGI 10	Leaves	Icimamlilo	Leaves are ground and soaked in warm water, boiled and aline added. The decoction is administered orally three times a day	Herb	Reported antidiarrhoeal usage (**BISI-JOHNSON et al., 2010**)	34	Least threatened

Family plant species (IPNI)	Voucher specimen number	Parts used	Vernacular name	Preparation	Type of plants	Reported antidiarrhoeal usage	Frequency index	IUCN Conservation status
Rhamnaceae *Ziziphus mucronata* Willd.	BALUNGI 16	Leaves	Umphafa	Ground bark is boiled in water and taken orally three times a day	Tree	Roots are popular for diarrhoea and dysentery. The leaves, fruit and stem are for diarrhoea (**HUTCHINGS et al., 1996; APPIDI et al., 2008; VAN WYK et al., 2009**)	36	Least threatened
Rutaceae *Zanthoxylum capense* (Thunb.) Harv.	BALUNGI 15	Leaves	Umlungumabele	The leaves are ground and boiled in water, and the decoction is administered orally three times a day	Tree	Leaves are ingredients for stomach complaints (**HUTCHINGS et al., 1996**)	36	Least threatened
Solanaceae *Physalis peruviana* L.	BALUNGI 12	Leaves	Igquzu	Leaves are crushed and boiled in water. The leaf decoction is taken orally three times a day	Herb	Leaves are used for diarrhoea (**HUTCHINGS et al., 1996; BISI-JOHNSON et al., 2010**)	45	Least threatened
Solanaceae *Solanum aculeastrum* Dunal	BALUNGI 18	Roots, Leaves, Fruit	Umthuma	Roots/leaves are ground into powder and boiled in water, and the decoction is taken orally three times a day	Shrub	Used for dysentery (**HUTCHINGS et al., 1996; BISI-JOHNSON et al., 2010**)	51	Least threatened

Family plant species (IPNI)	Voucher specimen number	Parts used	Vernacular name	Preparation	Type of plants	Reported antidiarrhoeal usage	Frequency index	IUCN Conservation status
Thymelaeaceae *Dais cotinifolia L.*	BALUNGI 8	Leaves	Intozane/ Isihlunge	Boiled leaves are taken orally as a decoction three times a day	Tree	No reported antidiarrhoeal usage	37	Least threatened
Vitaceae *Rhoicissus tridentata* (L.f.) Wild and R.B. Drumm.	BALUNGI 29	Roots	Uchithibhunga	Leaves are infused and the decoction is administered orally three times a day	Shrub	Stomach ailments (**HUTCHINGS et al., 1996; McGAW and ELOFF, 2008; VAN WYK et al., 2009**)	28	Least threatened

2.4.2. Botanical description, distribution, other medicinal uses, active components and pharmacological effects as well as conservation status of each plant that was recorded in the study to be used for treatment of diarrhoea, Bizana, Pondoland, Eastern Cape

This section discusses the nine selected plants for bioassays that were stated for the treatment of diarrhoea in Bizana (Pondoland), Eastern Cape. These plants were selected because of the following reasons; they had an higher frequency index (more mentioned by the informants), had never been investigated before (against diarrhoea causative microorganisms, for their anti-inflammatory properties, and for safety). Literature was used for the botanical description with details on the size, leaf, flower and fruit of each plant. Notes on usage of the same plant for treating other diseases were included, so as to ascertain if the plant is used for treating other ailments. Previous documentation on the distribution of each plant was checked to see where in South Africa each plant can be found. Reported pharmacological properties of each species were also included.

Figure 2.2: *Rapanea melanophloeos* Mez (Myrsinaceae)

Botanical description

Rapanea melanophloeos is a medium sized evergreen tree that is approximately 5-20 m in height. The leaves are oblong in shape narrowing towards the bases, and about 100 mm long. The leaf stocks and young leaves are purplish-red in colour (**VAN WYK et al., 1997**). The flowers of *R. melanophloeos* have small greenish or whitish fascicles. Fruit is spherical, thinly fleshy up to 5 mm, on short stalks (**PALGRAVE, 1977**). It is found in coastal, swamp, and mountain forest, on forest margins, and bush clumps, often in damp areas, from coast to mountains in KwaZulu-Natal, Transkei, and Orange Free State (**ARNOLD and DE WET, 1993; POOLEY, 1993**).

Other medicinal uses

This plant is used in steam baths to reduce fever, for the treatment of tuberculosis, snakebite, blood impurities, chest pain, toothache, haemorrhoids and to strengthen the heart (**VAN WYK et al., 1997; VAN WYK et al., 2009**). The Zulu people use the bark decoction as an expectorant and emetic. Leaves are used as an astringent and fruit is used as an anthelmintic in Tanzania and Kenya (**WATT and BREYER-BRANDWIJK, 1962**).

Active components and pharmacological effects

Sakurosaponins have been isolated from the leaves of *R. melanophloeos* (**OHTANI et al., 1993**). Twigs and bark contain tannins. So far there are no known pharmacological activities of any other parts of this plant (**WATT and BREYER-BRANDWIJK, 1962**).

Figure 2.3: *Tecoma capensis* (Thunb.) Lindl. (Bignoniaceae)

Botanical description

Tecoma capensis is an evergreen shrub which may grow up to approximately 5 m in height. The leaves are glossy green above and lighter below. This plant has dark green foliage and clusters of orange-red trumpet shaped flowers. *Tecoma capensis* flowers all year round. The fruit is a narrow, flat pod-like capsule up to 13 cm long (**VENTER and VENTER, 1996**). This shrub is widely distributed throughout the Northern Province, Mpumalanga, KwaZulu-Natal, and Western Cape (**VENTER and VENTER, 1996**).

Other medicinal uses

Powdered bark is used for the treatment of fever, pain, pneumonia, abdominal pains as well as chest ailments. The plant is also rubbed into bleeding gums to promote blood coagulation (**VENTER and VENTER, 1996; WATT and BREYER-BRANDWIJK, 1962**).

Active components and pharmacological effects

Leaf and flower parts have sterols. The leaves contain large quantities of benzoic and cinnamic esters, and the flowers have tecomoside and two phenylethanoid-derived glucosides (**GUISO et al., 1997**). Good antimicrobial activity was observed with the ethanol extracts of this plant against Gram-positive and Gram-negative bacteria as well as against some species of fungus (**SAINI et al., 2011**). The ethanol extracts of the flower exhibited antimicrobial activity (**AL-HUSSAINI and MAHASNEH, 2011**).
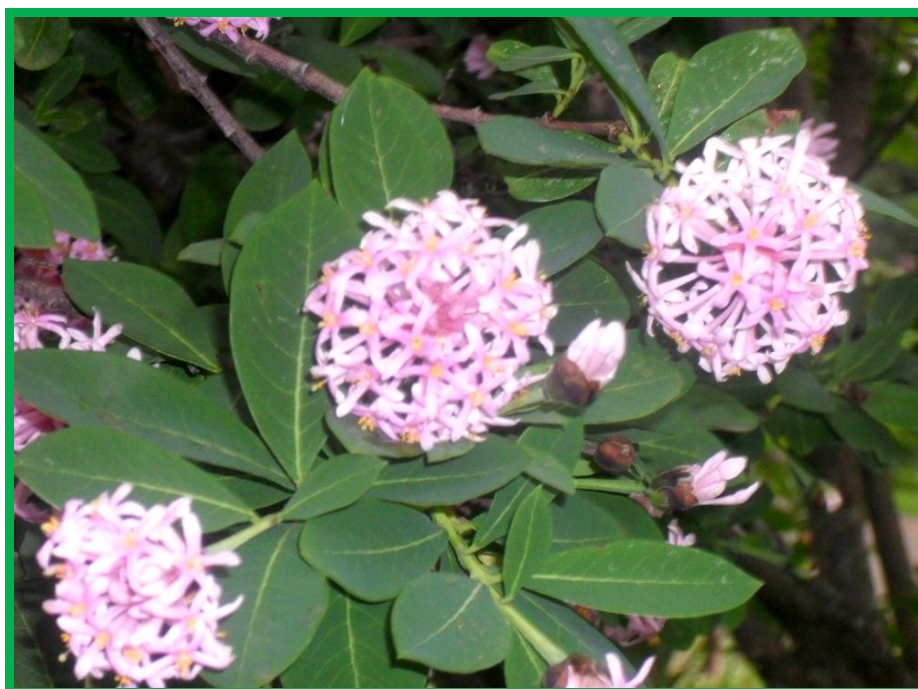
Figure 2.4: *Dais cotinifolia* L. (Thymelaeaceae)

Botanical description

Dais cotinifolia is a small to medium sized evergreen tree that grows to approximately 2-13 m in height. The leaves are bluish olive green above and lighter green beneath. The young leaves are pale green. The flowers are pale pink to pinkish mauve, and the fruit is a small brown nutlet enclosed in persistant calyx (**POOLEY, 1993**). It is found on forest margins, in thickets and riverine vegetation in rocky valley bushveld to the misbelt and montane forest in

KwaZulu-Natal and Transkei. It is also found in, Gauteng, North West, Mpumalanga, Limpopo and Eastern Cape (**POOLEY, 1993**).

Other medicinal uses

According to **NOMKHULEKO** (**2010**) (personal communication) boiled leaves are used for the treatment of coughs, fever and stomach ache.

Active components and pharmacological effects

There are no active compounds that have been isolated from this plant, and there are also no known pharmacological activities that have been observed.

Figure 2.5: *Searsia chirindensis* (Baker f.) Moffett (Anacardiaceae)

Botanical description

Searsia chirindensis is a deciduous shrub with a height of approximately 3-20 m with leaves that are glossy dark green and reddish when young. Flowers are in terminal branched sprays

and are up to 200 mm long. The fruit is pink to red-brown and fleshy when ripe (**POOLEY, 1993**). It is widespread in forest margins, riverine bush, coastal and scrub forest and on rocky hillsides in KwaZulu-Natal, Western Cape, Limpopo and Mpumalanga (**POOLEY, 1993**).

Other medicinal uses

Leaf decoctions are taken for biliousness and heart complaints. The bark is used to strengthen the body, for rheumatism, and for stimulating blood circulation (**VAN WYK et al., 2009**). The seed is taken as an anthelmintic agent in man and animal. The ripe fruit is used for sore throats and cramps of the abdomen. The root is taken for jaundice (**WATT and BREYER-BRANDWIJK, 1962**).

Active components and pharmacological effects

Anticonvulsant activity has been demonstrated by the extracts of *S. chirindensis* (**STAFFORD et al., 2008**). The tree has been reported to be toxic (**WATT and BREYER-BRANDWIJK, 1962**).

Figure 2.6: *Maesa lanceolata* Forssk. (Myrsinaceae)

Botanical description

Maesa lanceolata is a small shrub that grows to about 3-9 m in height with leaves that are thick, leathery, hairless and shiny green above, pale green below and finely hairy. It has very small creamy white flowers and fleshy small whitish pink flowers (**POOLEY, 1993**). *Maesa lanceolata* is found along the coast usually in drainage lines, forest edges, rocky mountain sides, bushveld thickets, and often in groves, widespread in KwaZulu-Natal and eastern parts of Eastern Cape. It is also found in Botswana, Lesotho and Swaziland (**POOLEY, 1993**).

Other medicinal uses

Leaves are used as a dressing for male initiation wounds and are also used as a vermifuge, as well as in the treatment of sore throats. Leaves and roots are also used for stinging rash and to alleviate rheumatic arthritis (**OKEMO et al., 2003**).

Active components and pharmacological effects

Extracts of *M. lanceolata* have shown activity against fungal pathogens (**OKEMO et al., 2003**). The compounds isolated from *M. lanceolata* have exhibited virucidal, haemolytic, molluscicidal and antiangiogenic activities (**THEUNIS et al., 2007**).

Figure 2.7: *Ficus craterostoma* Warb. ex Mildbr. and Burret (Moraceae)

Botanical description

Ficus craterostoma is a forest tree that grows to about 20 m in height with small blunt-tipped thin leathery leaves that are dark green. The figs are yellowish to red when ripe (**POOLEY, 1993**). It is found in KwaZulu-Natal**,** Eastern Cape to Gauteng, North West, Mpumalanga and Limpopo (**THIELTON-DYER, 1925; POOLEY, 1993**).

Other medicinal uses

According to **CANDLOVU** (**2010**) (personal communication) fresh leaves are taken for treating stomach ache problems.

Active components and pharmacological effects

Nothing is known about the pharmacological activities of this plant.

Figure 2.8: *Clutia pulchella* L. (Euphorbiaceae)

Botanical description

Clutia pulchella is a deciduous shrub that grows to approximately 2-4 m in height with leaves that are light green above and bluish green beneath. The flowers are very small and cream coloured. The fruits are small round, warty capsules that split when ripe (**POOLEY, 1993**). This shrub is found in KwaZulu-Natal eastern part of Eastern Cape, as well as Free State (**ARNOLD and DE WET, 1993; POOLEY, 1993**).

Other medicinal uses

Milk infusion of leaf, stem and root is used for griping pains in children and calves (**WATT and BREYER-BRANDWIJK, 1962**). Roots are mixed with urine of a bull and applied to

scarification near the point of fracture to aid union during fracture (**WATT and BREYER-BRANDWIJK, 1962**).

Active components and pharmacological effects

Nothing is known about the pharmacological activities of *Clutia pulchella*.

Figure 2.9: *Hydnora africana* Thunb. (Hydnoraceae). The picture on the right was obtained from http://www.plantzafrica.com/planthij/hydnorafr ic2.jpg, 2011.

Botanical description

Hydnora africana is a parasitic plant that looks astonishingly similar to fungi and is only distinguishable from fungi when the flower has opened. This is a leafless plant with a fleshy and warty stem. It contains a single flower that rises from the thick rhizome-like vegetative body. As it ages, the plant turns dark grey to black (**SKENE, 2006**). Fruit subglobose, is filled with gelatinous pulp (**RETIEF and HERMAN, 1997**). The plant is found in the dry and semi-arid parts of the Succulent Karoo, Little Karoo, Eastern Cape Karoo, and the dry coastal thickets between the Eastern Cape and KwaZulu-Natal (**BURGER et al., 1988**).

Other medicinal uses

Hydnora africana is used as an astringent for inflamed throats and swollen tonsils (**WATT and BREYER-BRANDWIJK, 1962**).

Active components and pharmacological effects

The whole plant has been reported to have tannins (**WATT and BREYER-BRANDWIJK, 1962**).

Figure 2.10: *Trema orientalis* (L.) Blume (Ulmaceae). The picture on the right was obtained from www.google.co.za/imgres? Trema+orientalis, 2011.

Botanical description

Trema orientalis is an evergreen shrub that grows to approximately 10 m with grey or brown bark, finely toothed leaves and small flowers that are greenish-white or green (**PANOFF, 1970**). It occurs in Gauteng, Limpopo, Mpumalanga, Northern Province, KwaZulu-Natal, as well as tropical Africa, Arabia, Madagascar, Namibia and Swaziland (**ARNOLD and DE WET, 1993**).

Other medicinal uses

An infusion of this plant is used as a liniment and decoction for backache, earache, throat and mouth infections. Infusions of leaves and fruit are taken as tea for bronchitis, pneumonia and pleurisy. It is also used to treat wounds, fever, cough, black tongue, jaundice, malaria and convulsion (**PANOFF, 1970; PARSONS, 1995**)

Active components and pharmacological effects

Bark water extracts of *T. orientalis* exhibited a glucose lowering effect in diabetic rats (**DIMO et al., 2006**).

2.4.3. Ethnobotanical information

The rural areas of Bizana are located in Pondoland which is one of the most fertile areas in South Africa (**BISI-JOHNSON et al., 2009**). The elderly people and traditional healers of Bizana are well versed in traditional medicinal practices, thus most people depend on them for their primary health care needs. An ethnobotanical study on plants used by the people in six rural areas of Bizana for treating diarrhoea was carried out. A total of 100 informants were interviewed for their knowledge on plants used for the treatment of diarrhoea. All the informants used or knew plants to treat diarrhoea. Approximately 60% of the informants had no formal education, with the rest having primary or secondary education. Fifty five of the informants were females with their ages ranging from 20-75 and 45 were males with their ages ranging from 23-80 years. Women have vast knowledge of medicinal plants because they are generally responsible for the upkeep of their children and families (**ZOBOLO and MKABELA, 2006**). Out of all the people who were interviewed 30 were traditional healers. They believe that the information about the plants was given to them by their ancestors in their dreams. The knowledge about the plants was passed onto them by their parents or experienced mentors. The informants knew that there are other diseases that are associated with diarrhoea, such as AIDS and cancer.

For the first questionnaire (**Appendix 1**), all the 30 interviewed informants who were trained traditional medicine practitioners reported to know diarrhoea and its symptoms and also to

treat the condition using medicinal plants. Twenty three informants knew other diseases that are linked to diarrhoea and only two mentioned that they can treat one of those diseases (cancer). All the informants mentioned that the medicinal plants that they use are very effective in treating diarrhoea, as treatment takes less than 24 h and sometimes two to three days. Most patients (16) tried self treatment before consulting traditional healers. Out of the 30 informants, 10 mentioned that their herbal remedies can be kept for 3 months, 9 for 6 months and 11 for a year. Though it was surprising when the informants mentioned that they are not doing anything to conserve the plants they are using, it is worth mentioning that all these medicinal plants are least threatened according to the current **INTERNATIONAL UNION FOR CONSERVATION OF NATURE** (**2011**) data list. However, according to the 2009 South African red data list, three plants that were recorded in this study are declining, those are *Eucomis autumnalis*, *Hypoxis hemerocallidea*, and *Rapanea melanophloeos*, *Ocotea bullata* is endangered (**RAIMONDO et al., 2009**). This is of concern as *Ocotea bullata* is slow growing, slow producing and highly vulnerable to excessive collection. Therefore, action must be taken to conserve these plants before they are lost in the wild, such as responsible collection and finding a suitable propagation technique.

Out of the 70 informants that were interviewed using the second questionnaire (**Appendix 2**), all knew diarrhoea and how to treat it. They also all mentioned that they use medicinal plants and that the herbal remedies that they prepare from these plants are very effective (within less than 24 h after taking the medication the patient will be having less or no diarrhoea). Twenty seven of the informants do not often use and 43 very often use medicinal plants. Only seven mentioned that they do not visit hospitals for further treatment, the rest (63) visit health care centres (clinics and hospitals) for more treatment. All the informants mentioned that the plants they are harvesting are in greatest demand, but only 10 of them are practising conservation of the plants (by either harvesting less, using leaves and growing some of the plants).

Thirty-four plant species belonging to 21 families were cited by the informants as being traditionally used in the treatment of diarrhoea in the area. Of the 34 plants that were mentioned in the study, 12 species were trees, 10 were herbs and nine were shrubs, while bulbs, succulents, and tubers consisted of one species each. **Table 2.1** represents the frequency index of the plants mentioned by the informants. *Psidium guajava* was the most used plant species, having a frequency index of 60% but was not selected for bioassays,

because it has already been evaluated as an antidiarrhoeal plant (**GHOSH et al., 1993; LIN et al., 2002; SALGADO et al., 2006; OJEWOLE et al., 2008**). On the other hand, *Alysicarpus rugosu*s was the least utilized plant with a frequency index of 13%.

Asteraceae was the most represented family comprising of nine percent, followed by Mimosaceae, Hyacinthaceae, Myrsinaceae, Myrtaceae and Solanaceae comprising of six percent each. All the other families had three percent each from the total plants cited. Leaves, constituting 65% of the preparations, were the most used part of the plants mentioned. Roots constituted 18%, barks 12% while corms, rhizomes and fruits constituted nine percent each. In surveys done by **MATHABE et al.** (**2006**) and **BISI-JOHNSON et al.** (**2010**), roots and bark were cited as the most preferred plant parts for making remedies for the treatment of diarrhoea. **DE WET et al.** (**2010**) recorded the leaf as the most used plant part in a survey conducted in Northern Maputaland, KwaZulu-Natal. The use of leaves to treat diarrhoea promotes conservation, as the use of bark and roots cause destructive harvesting of trees (**MATHABE et al., 2006**).

In a survey done by **APPIDI et al.** (**2008**) in the Eastern Cape, four plant species out of 17 species that were mentioned in their study were also cited in our study, and those were *E. elephantina*, *M. parviflora*, *P. persica* and *Z. mucronata*. In yet another survey done by **BISI-JOHNSON et al.** (**2010**) in other parts of the Eastern Cape, 32 plant species were recorded for the treatment of diarrhoea of which 10 were also mentioned in our study. They were *A. mearnsii*, *E. elephantina*, *E. automnales*, *P. luridum*, *H. africana*, *H. hemerocallidea*, *P. guajava*, *P. pruneloides*, *P. peruviana* and *S. aculeastrum*. In **MATHABE et al.** (**2006**) research survey in Limpopo, 21 species were cited for the treatment of diarrhoea; only one plant species (*E. elephantina*) corresponds with the plants mentioned in our study. In a survey done by **DE WET et al.** (**2010**), out of 23 plant species recorded only *P. guajava* corresponds with plants mentioned in our survey.

The variation in the plants mentioned in all the studies may be attributed to the cultural differences in different provinces of South Africa. The climatic diversities also influence the types of plants and their secondary metabolites, thus affecting the uses of these plants in different regions. Distribution also plays a role, since people make use of what is available.

The informants reported the use of both combinations of several species as well as the use of single plant species for preparing the remedies. For instance, *E. elephantina*, *P. luridum*, *P. persica*, *T. capensis*, *Z. mucronata* and *T. orientalis* were either prepared as mixtures or used as single entities. In traditional healing, it is a common practice to combine plants when making healing remedies to obtain a more potent mixture and also to mask or reduce toxicity (**NDHLALA et al., 2011**).

R. melanophloeos, *F. craterostoma*, *A. ferox*, *L. ovatifolia*, *D. cotinifolia*, *B. bipinnatifida* subsp. *bipinnatifida, A. rugosus*, *C. dives*, *S. seratulloides*, *M. lanceolata* and *B. grandiflora* have never been recorded in South Africa for the treatment of diarrhoea. Species like *P. guajava*, *Z. mucronata*, *O. bullata*, *P. luridum*, *T. capensis*, *C. pulchella*, *T. orientalis*, *E. elephantina*, *E. camaldulensi*, *H. africana*, *P. peruviana*, and *S. aculeastrum* have been recorded by **HUTCHINGS et al.** (**1996**), **VAN WYK et al.** (**2009**) and **BISI-JOHNSON et al.** (**2010**) for the treatment of diarrhoea.

The informants pointed out the use of these plant remedies by all ages and gender. They also pointed out that the remedies are safe for circumcised boys and pregnant women to use. What differed were the doses given to patients which depended on age; ranging from three teaspoons of decoction per day for children to one cup, three times a day for adults. The informants also highlighted that decoctions were the most common method of preparing the remedies. Oral administration was preferred.

Only 10% of the informants knew that plants can sometimes have adverse effects, the majority were ignorant about this fact. The absence of side effects was reported in most cases. This implies that the plants used for treating diarrhoea have no side-effects. It is also possible that the informants never took notice of the side-effects.

Out of all the informants 90% claimed to never have used cultivated plants but have always depended on harvesting from the wild. The majority of the respondents confirmed regular supply of their plants from the forest, and only a few people sourced some of their plants from their gardens. All the informants noticed these plants growth in spring, and the traditional healers believe that it is the right season for them to start growing so that they can use them in summer when there is a high rate of diarrhoea. Since the Eastern Cape is one of the provinces that have summer rainfall, water is highly contaminated by various infections,

hence this season has a high percentage of diarrhoeal cases. The plants are preferably collected in the morning. The maximum time for the majority of the respondents to keep their medication is for the whole year, and the minimum time is six months.

Nine plant species that had higher frequency indices compared to the others were selected and collected for laboratory based *in vitro* pharmacological bioassays. The selected plants were *C. pulchella*, *D. cotinifolia*, *F. craterostoma*, *H. africana*, *M. lanceolata*, *T. capensis*, *R. melanophloeos*, *S. chirindensis* and *T. orientalis*.

2.5. Conclusions

The study presents new plants used in the community of Bizana that have not been previously reported to be remedies for treating diarrhoea. Out of the 34 plant species recorded in this study 11 plant species have not been previously reported as remedies to treat diarrhoea. These plant species are *R. melanophloeos*, *F. craterostoma*, *A. ferox*, *L. ovatifolia*, *D. cotinifolia*, *B. bipinnatifida* subsp. *bipinnatifida*, *A. rugosus*, *C. dives*, *S. seratulloides, M. lanceolata* and *B. grandiflora*. The results obtained in this study show that people still possess a rich and diverse knowledge regarding traditional uses of plants against diarrhoea, and that plants form part of the cultural heritage of the communities. However, these knowledge holders are ageing, and the information is likely to vanish fairly soon. It is therefore urgent to save the cultural heritage of the people by confirming therapeutic uses of plants with scientific criteria. Thus the results presented in this study will go a long way in documenting indigenous knowledge on the use of traditional plants in Bizana, Pondoland.

CHAPTER 3: Plant material collection and preparation

3.1. Introduction

Plant collection, preservation, extraction and storage are vital steps of a pharmacological study. These vital steps may result in the loss of access to the active compounds if done inappropriately (**BENTHIN et al., 1999**). Additionally, inappropriate collection and extraction methods may result in the degradation of the natural products (**GURIB-FAKIM, 2006**). As biotic and abiotic factors affect chemical composition of plant material, fresh plants that have been dried are preferred for pharmacological studies (**MAKKAR, 2000**). Based on the biological concepts of a plant cells permeability, plant material can be extracted with different solvents. Different solvents with different polarities extract different or similar compounds. Polarity is the sum of all the molecular properties responsible for all the interaction forces between solvent and soluble molecules (**LIN and GIUST, 2005**). It is vital to compare extraction efficacy of different solvents based on their polarity. Dried plant material is ground to smaller particles and extracted using solvent systems, such as sequentially (using a series of solvents) or non-sequentially (using a single solvent). However, in the process of validation, the utilisation by the traditional healers or lay person must be mimicked as far as possible so that the same natural bioactive products are extracted (**GURIB-FAKIM, 2006**).

3.2. Materials and methods

Below are the methods and techniques used for collection, extract preparation and storage of plant materials from different species studied.

3.2.1. Sample collection

After data collecting using questionnaires, plants were ranked based on the number of times they were mentioned by the informants. Plants with an higher frequency index (mentioned more by the participants), as shown in **Table 3.1**, that have never been investigated before against diarrhoea causative microorganisms were selected for bioassay. The selected nine

plants were: *C. pulchella*, *D. cotinifolia*, *F. craterostoma*, *H. africana*, *M. lanceolata*, *R. melaphloeos*, *S. chirindensis*, *T. capensis*, and *T. orientalis*.

3.2.2. Preparation of plant extracts

The leaves of *C. pulchella*, *D. cotinifolia*, *F. craterostoma, M. lanceolata*, *R. melaphloeos*, *S. chirindensis*, *T. capensis*, and *T. orientalis* and tubers of *H. africana* were oven-dried at 50 °C, ground into powders and stored in airtight containers in the dark at room temperature until used. The ground plant material (10 g) was each extracted non-sequentially with 200 ml of dichloromethane (DCM), petroleum ether (PE), 70% ethanol (EtOH) and water by sonication for 1 h at 15 °C, temperature being maintained by addition of ice to the water baths. After sonication the extracts were filtered through Whatman No.1 filter paper then transferred into Buchner flasks and the filtrates concentrated to dryness under reduced pressure using a rotary evaporator. Aqueous filtrates were freeze-dried and kept in airtight containers. The organic extracts were dried under a steam of cold air. Percentage yield for each dried plant extract was determined per solvent. This was achieved as a percentage ratio of the mass of the dried plant extracts to mass of the starting plant material. Dried plant extracts were kept in the dark at 10 °C until required for bioassays.

3.3.   Results and discussion

The percentage yield of the extracts are presented in **Table 3.1**. Plant material was ground into powders in order to increase the surface area for maximum diffusion of the extractable compounds. Sonication was used as a technique to increase the yield of bioactive compounds from plants while reducing extraction time. The temperature was lowered by adding ice during sonication to prevent decomposition of plant compounds that are sensitive to high temperatures (**ELOFF, 1998a**).

Plants produce a wide range of secondary metabolites with different functional groups and polarities. The extraction process of bio-active substances from raw plant material is an important stage in the techniques used to make natural medicinal preparations. Numerous improvements have been put forward in order to perfect the extraction process (**IVANOV et al., 2004**). Several approaches can be employed to extract plant natural products. Although

water is used as an extractant in many traditional protocols, organic solvents of varying polarities are generally selected in modern methods of extraction to exploit various solubilities of plant constituents (**SARKER et al., 2005**). Four solvents (PE, DCM, 70% EtOH and water) were used in this study because of their wide polarity range. The highest yield was obtained with the 70% EtOH extracts of *D. cotinifolia* (26.07%), and the lowest yield was obtained from the PE extract of *D. cotinifolia* (2.18%). The highest quantity of crude extracts was obtained from 70% EtOH. Lowest yields were recorded with PE. The general trend of yields was 70% EtOH > water > DCM > PE. Higher yields obtained when 70% EtOH was used could be due to the wide range of phytochemicals extracted with polar and non-polar solvent.

The plant extracts were stored at 10 °C in the dark to reduce photodecomposition of active compounds by radiant energy.

Table 3.1: Percentage yields of nine plant extracts from material collected from Bizana, Eastern Cape province

Species	Plant part	Yield (% DW)			
		PE	DCM	70% EtOH	Water
Clutia pulchella	Leaf	7.22	5.86	20.11	25.70
Dais cotinifolia	Leaf	2.18	5.54	26.07	22.80
Ficus craterostoma	Leaf	5.66	7.22	8.94	14.20
Hydnora africana	Tubers	5.38	10.17	24.87	17.10
Maesa lanceolata	Leaf	6.11	7.19	21.40	11.80
Rapanea melanophloeos	Leaf	3.84	4.56	24.85	7.20
Searsia chiridensis	Leaf	2.92	4.69	23.69	15.80
Tecoma capensis	Leaf	3.65	3.99	16.81	21.00
Trema orientalis	Leaf	3.88	4.55	15.45	11.30

DCM = dichloromethane, PE = petroleum ether, EtOH = ethanol, % DW=percentage dry weight

3.4.   Conclusions

Plants with higher frequency indices were collected and extracted non-sequentially with different solvents. The extraction of active plant extracts from the medicinal plants for bioassays was to some extent dependent on the polarity of the solvents used in the extraction. Ethanol yielded the highest quantity of crude extract.

CHAPTER 4: Antibacterial screening of plants used in Bizana, Pondoland for the treatment of diarrhoea

4.1. Introduction

Infectious diseases caused by bacteria, viruses, fungi and other parasites are among the main causes of death in humans despite successful developments in modern medicine. Throughout human civilization, people have been victim to cholera, plague, influenza, typhoid, tuberculosis and other infectious maladies (**WHO, 2000**). Infectious diseases accounts for approximately one half of all deaths and are the leading cause of death in tropical countries (**PINNER et al., 1996; AGUNU et al., 2005**). Increased mortality rates, due to infectious diseases such as diarrhoea and dysentery that are mainly caused by intestinal infections, have been witnessed in developing countries (**LONGANGA-OTSHUDI et al., 2000**). In financially poor countries, infectious diseases still contribute to the premature death and ongoing miseries amongst the disadvantaged component of the population. This fact is disturbing as it was assumed that infectious diseases would have been eliminated by the end of the millennium (**WHO, 1996**). This rise in infectious diseases mortality rate is credited to the increase in diarrhoea, respiratory tract infections and HIV/AIDS. Another contributing factor is an increase in antibiotic resistance in nosicomial and community acquired infections (**PINNER et al., 1996**).

More than half of infectious diseases that affect immune-compromised individuals involve bacterial species. Bacterial infections are widespread and common in developing countries due to inadequate sanitation, poor hygiene, and food poisoning (**RASOANAIVO and RATSIMAMANGA-URVEG, 1993**). The main concern with regard to bacterial infections is the emergence of resistance to known drugs (**BAR-MEIR et al., 2005**).

These negative health trends call for new strategies on prevention and cures and have enhanced the interest in infectious disease in the medical and public health communities. One of the proposed solutions to the problems was to develop new antimicrobials (**FAUCI, 1998**). Therefore, there is an urgent need to find new antimicrobial compounds with different chemical structures and novel mechanisms of action (**FAUCI, 1998**).

4.1.1. Microbes and human health

Humans are exposed to microorganisms from birth, some of which become resident to the skin, mucous membranes and gastrointestinal tract. Bacteria are both beneficial and harmful to human beings. They play an important part in maintaining human health by exerting protective effects in the gut (**VAUGHAN and JUDD, 2003**). Some bacteria play a major role in certain chronic diseases not formerly associated with bacterial infections, for instance *Helicobacter pylori* Marshall et al. and Goodwin et al. cause ulcers and also contribute to stomach cancer (**VAUGHAN and JUDD, 2003**). Bacteria coexist with the commensal organisms stimulating host immunity and providing some essential nutrients and co-factors to humans (**MOXON and TANG, 2000**). Most microbes are beneficial, playing an essential function in the production of antibiotics against human diseases. Colonic microflora ferment non-digestible dietary residues and endogenous mucus produced by the epithelia (**MOXON and TANG, 2000**).

4.1.2. Bacterial infections

Bacteria are heterogeneous unicellular organisms that have prokaryotic cellular organization with a rigid cell wall. The cell wall determines the shape of the bacterium, protects the cell from mechanical disruption and being burst by turgor pressure. It also provides a barrier against toxic, chemical and biological agents (**SALTON and KIM, 1996**). Gram-negative and Gram-positive are the two main classes of bacteria that are determined by using a staining technique. Gram-positive bacteria have thick walls that are composed of peptidoglycan polymers that are cross-linked by polypeptides to form a rigid protective coat. Whereas, Gram-negative bacteria have a complex cell-wall plasma membrane structure. The cell wall of Gram-negative bacteria is thinner than that of Gram-positive bacteria and is interposed between the outer and inner membrane. Both the inner and outer membranes have a characteristic lipid bilayer, but the outer membrane has a lipopolysaccharide component that permits or excludes certain macromolecules. When bacterial cells are stained with crystal violet, after being discolourized with alcohol, treated with safronine and washed in water, those that retain the crystal violet are Gram-positive and those that do not retain the dye are Gram-negative (**SALTON and KIM, 1996; JAMPALA, 2007; O'LEARY and CAPOTE,**

2008). Regardless of their small size bacteria are, the most abundant group of unicellular organisms on earth, able to adapt to almost any living condition. They have diverse metabolic capabilities. They are the smallest living cells, are nearly colourless and transparent with a refractive index that is almost close to that of the surrounding liquid. Major forms of bacteria can be recognized as spheres, rods (bent or curved rods) and spirals (**RYAN and RAY, 2004**).

Bacterial infections can be caused by a wide range of bacteria, resulting in mild to life-threatening illnesses that need abrupt interference. Tuberculosis caused by *Mycobacterium tuberculosis* Zopf, ranks among the world's leading cause of death. *Streptococci* Rosenbach continue to be a common cause of life-threatening infections during the first two months of human life. Diarrhoeal diseases are caused by food and water borne bacteria such as *Salmonella* spp. and *Campylobacter* spp. (**GEORGIEV and FAUCI, 2009**). During the past decade, many new strains of familiar bacteria such as *E. coli* have been discovered by scientists. These organisms can injure or kill their host or can cause diseases that represent challenges to human health. Worldwide approximately 17 million deaths per annum are reported to be caused by bacterial infections especially in children (**WATSON and PREEDY, 2008; SLEIGH and TIMBURY, 1998**).

Gastrointestinal infections caused by bacteria are categorized by abdominal cramps and pains, bloody stool, pus in the stool, loss of appetite, bloating, nausea, vomiting as well as fever (**BARBARA et al., 2006**). The major burden of infection is due to food-borne infections caused by *Salmonella* spp., *Campylobacter* spp., *Shigella* spp., *S. aureus* and *E. coli*. The bacterium attaches to the intestinal epithelial cells producing enterotoxins that cause acute watery diarrhoea in adults (**SLEIGH and TIMBURY, 1998**). While shigellosis and cholera remain two diseases of great concern in developing countries, *E. coli*, *Listeria monocytogenes* (Murray et al.) Pirie and *S. aureus* are causing increasing concern in developing countries because of their resistance to main line antibiotics (**SLEIGH and TIMBURY, 1998**).

The levels of infectious diseases are increasing in South Africa, due to increasing drug resistance and high incidences of HIV/AIDS infection amongst the population (**ELDEEN et al., 2005**). A lot of microorganisms have been isolated in drinking water supplies in the Eastern Cape and Limpopo provinces. These include *E. coli*, *Shigella* spp., non-typhoidal

Salmonella strains, *Aeromonas* spp. Stanier, *Campylobacter jejuni* (Jones et al.) Veron and Chatelain, *Campylobacter coli* (Doyle) Veron and Chatelain, *Vibro cholerae* Pacini, *Vibro parahaemolyticus* (Fujino et al.) Sakazaki et al. and *Plesiomonas shigellosis* corrig (Bader) Habs and Schubert (**MOMBA et al., 2010**). Diarrhoea and gastrointestinal symptoms are frequent in immune-compromised patients. More or less all AIDS patients report diarrhoea at some time during the course of their disease (**MADDOX et al., 1992**). Since HIV/AIDS patients use water to take medication (antiretroviral), diarrhoea causative microorganism have been frequently isolated from the stools. Hence, there is a connection between drinking water, diarrhoeal diseases and HIV/AIDS in South Africa (**MADDOX et al., 1992**).

4.1.3. Drug resistance

Increasing and persistent resistance of pathogens to main-line antibiotics and the fast emergence of multi-drug resistant Gram-negative bacteria are of concern (**WATSON and PREEDY, 2008**). Pathogen resistance has increased although pharmaceutical industries have produced a number of new antibiotics in the last three decades (**AKINSULIRE et al., 2007**). This resistance of drugs to human pathogens has been reported worldwide and the situation has been surprising in both developing and developed countries (**AKINSULIRE et al., 2007**).

Multi-drug resistant strains of bacteria like methicillin-resistant *S. aureus* and multi-drug resistant *Salmonella typhi* (Schroeter) Warren and Scott, vancomycin-resistant enterococci have necessitated a need for the development of new antimicrobial substances (**GEORGIEV and FAUCI, 2009**). Generally, bacteria have the genetic ability to transmit and acquire resistance to drugs. Transfer of genes among different strains and between different species of bacteria is understood to be a common means by which they acquire resistance (**GEORGIEV and FAUCI, 2009**). The problem of the resistance of microorganisms is still growing and there is still uncertainty about the outlook for the use of antimicrobial drugs in the future. New drugs should be able to either inhibit pathogen growth or destroy them, yet not have the slightest toxicity to host cells in order for them to be considered as candidates (**AHMAD and BEG, 2001**).

Action must be taken to reduce the problem of drug resistance, for instance finding ways of understanding the genetic mechanisms of resistance and continue with the studies to develop new drugs either synthetically or naturally. Plants have been a valuable source of natural products (NP) for the maintenance of human health for a long period of time (**NASCIMENTO et al., 2000**). They may provide a natural source of antimicrobial drugs that will provide novel or lead compounds that may be employed in controlling some infectious diseases (**IBRAHIM et al., 2009**). A pivotal role has been played by NP in the development of antibacterial drugs as most of the currently used drugs have been derived from NP leads (**BUTLER, 2004**).

4.1.4. Diarrhoea causing bacteria used in this study

4.1.4.1. *Escherichia coli*

Escherichia coli is a rod-shaped, Gram-negative, non-spore forming microorganism that can be classified as a facultative anaerobe and coliform enteric bacterium that ferments lactose to acid end products. This bacterium is transmitted through faecal contamination and is the largest colonizer of human and animal intestines (**DUGGAN et al., 2008**). This bacterium can survive with minimum nutrients and grows optimally at a temperature of 37 °C. Characteristic symptoms of *E. coli* infection include intense abdominal pain and non-bloody diarrhoea which progresses to bloody diarrhoea within one to two days (**DUGGAN et al., 2008**).

4.1.4.2. *Shigella flexneri* Castellani and Chalmers

Shigella flexneri is a Gram-negative, nonmotile, non-spore forming, rod-shaped bacterium that is very closely related to *E. coli*. It is a facultative anaerobic bacillus. This bacterium is highly infectious with ingestion of as few 100 microorganisms resulting in disease development (**HECHT, 2003**). Infection causes shigellosis or bacillary dysentery in humans, which is an acute inflammatory disease characterized by fever, intestinal cramps and discharge of mucoporulant and bloody faeces. People infected with *Shigella* develop diarrhoea that is often bloody, and characterized by fever and stomach cramps starting a day or two after they are exposed to the bacterium. Shigellosis usually resolves in five to seven

days and can be so severe that the patient needs to be hospitalized. A severe infection with high fever may be associated with seizures in children less than two years old. Some persons who are infected may have no symptoms at all, but may still transmit the bacteria to others (**HECHT, 2003**).

4.1.4.3. *Campylobacter jejuni*

Campylobacter jejuni is a slender, curved, spiral-shaped, catalase positive and Gram-negative flagellated motile rod bacterium that is microaerophillic (requires reduced levels of oxygen), relatively fragile and sensitive to environmental stress (**REDMAN, 2007**). It requires 5% oxygen, 10% carbon dioxide and 85% nitrogen to grow and a temperature range between 37 and 42 °C. The bacterium was believed to be primarily an animal pathogen causing abortion and enteritis in sheep (**BARCELOUX, 2008**). However, it is now recognized as an important enteric pathogen. This pathogen is the leading cause of bacterial illness and causes more disease than *Shigella* spp. and *Salmonella* spp. combined in both the developing and developed worlds, where it is acquired by ingesting contaminated food or water (**REDMAN, 2007**). It is not easy to differentiate pathogenic from non pathogenic strains, because the mechanism of pathogenesis of these bacteria is still being studied. The bacterium causes campylobacteriosis often known as gastroenteritis, with diarrhoea, fever, abdominal pain, nausea, headache and muscle pain as symptoms of infection (**REDMAN, 2007**). The bacterium possesses lipopolysaccharide and lipo-oligosaccharide endotoxins which develop adherence, increase invasiveness and improve survival in human serum during bacteremia (**BARCELOUX, 2008**).

4.1.4.4. *Staphylococcus aureus*

Staphylococcus aureus is a spherical Gram-positive non-motile, catalase and coagulase-positive, non-spore forming microorganism that is arranged in grape-like clusters. It is a facultative anaerobe that grows by aerobic respiration or by fermentation to yield principally lactic acid. This bacterium can grow at a temperature range of 15 to 45 °C and at NaCl concentrations as high as 15%. The staphylococci grow in clusters, because the cells divide successively in three perpendicular planes with the sister cells remaining attached to each other following a successive division. Since the exact point of attachment of sister cells may

not be within the divisional plane and the cells may change position slightly while remaining attached, the result is formation of an irregular cluster of cells (**WHO, 2004b**). This bacterium causes a variety of infections in humans. It causes superficial skin lesions such as boils, styes and furuncules, infections such as pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, deep-seated infections, such as osteomyelitis and endocarditis. It is the main cause of nosocomial infection of surgical wounds and infections associated with indwelling medical devices. It causes food poisoning by releasing enterotoxins into food (**WHO, 2004b**).

4.2. Materials and methods

4.2.1. Antibacterial assay

All the bacterial strains used in this study were obtained from the American type culture collection (ATCC). The following protocols were designed for storage and maintenance of bacterial cultures.

4.2.2. Storage of *Campylobacter jejuni* (ATCC 29428)

4.2.2.1. Preparation of agar plates

Brucella agar (Sigma-Aldrich, Switzerland) (20.5 g) was dissolved in 500 ml of distilled water and autoclaved at 120 °C with a stirring rod for 15 min and cooled to 50 °C. The campylobacter growth supplement (liquid, 500 ml) SR 0232E (Oxoid, England) was then added to the media and mixed thoroughly. Brucella agar with the supplement (30 ml) was poured into Petri dishes and allowed to cool before they were closed and turned upside down.

4.2.2.2. Procedure for micro organism preparation

The Kwik-stik^{TM} unit was removed from 2-8 °C storage and allowed to equilibrate to room temperature. The hydrating fluid was released by breaking the ampoule by pinching in the middle of the ampoule cap of the unit. The hydrating fluid was allowed to flow through the swab shaft and into the bottom portion of the unit containing the gelatine pellet. The bottom

portion of the pellet was pinched repeatedly to crush it and the pellet in the fluid so as to mix the pellet to uniform size forming a homogeneous suspension. Immediately, the swab with the hydrated material was saturated and transferred to Petri dishes with brucella agar supplemented with campylobacter growth supplement. The same swab was used to streak repeatedly the inoculated area. The inoculated agar plates were quickly placed in an anaerobic jar AG 0025 (Oxoid, England). A sachet (Campylobacter system CN 0025A, Oxoid, England) was opened and placed in an anaerobic jar which was then closed within less than 20 min. An indicator BR 0055B (Oxoid, England) was opened and placed inside an anaerobic jar containing a sachet, this was done within less than a minute. The sachet was for absorbing oxygen and providing CO_2, producing a proper microaerobic environment and an anaerobic indicator for CO_2 monitoring. The inoculated agar plates in an anaerobic jar were incubated at 42 °C for 48 h.

4.2.2.3. Long term storage of bacterial cells

Brucella broth BD 211088 (Becton, Dickinson, MD 21152 USA) (5.6 g) was dissolved in 200 ml of distilled water in a 250 ml Schotts bottle and autoclaved at 120 °C for 15 min. Sterilized glycerol (3 ml) were added to 17 ml of brucella broth in a 100 ml Schott bottle to obtain a 15% glycerol solution. The glycerol solution was dispensed into McCartney bottles (10 ml per bottle). Flame sterilized loops were used to inoculate the solution (10 ml) with the 48 h incubated bacteria from the agar plates. Aliquots of the inoculated broth (100 µl) were transferred into sterilized Eppendorf tubes which were then stored in the freezer set at -70 °C.

4.2.3. Storage of *Shigella flexneri* ATCC 12022

4.2.3.1. Procedure for microorganism preparation

The same procedure as described in **Section 4.2.2.2** was followed, however nutrient agar 213000 (Becton, Dickinson, MD 21152 USA) was used for *S. flexneri*. The Kwik-stik™ unit was removed from 2-8 °C storage and allowed to equilibrate to room temperature (about 22 °C). The hydrating fluid was released by breaking the ampoule using a pinching action in the middle of the ampoule in the cap of the device. The hydrating fluid was allowed to flow through the swab shaft and into the bottom portion of the unit containing the gelatine pellet.

The bottom portion of the pellet was pinched to crush and mixed until the pellet particles were uniform in size and the suspension was homogeneous. Immediately, the swab with the hydrated material was saturated and was transferred to nutrient agar. The same swab was used to streak pure cultures of *S. flexneri* through the inoculated area repeatedly and the remainder of the agar surface was streaked for isolation and incubated for 24 h at 37 °C. The cells were harvested from the slant and a suspension in sterile skim milk was made. The suspension was dispensed into cryovials and frozen rapidly by placing sealed vials in an alcohol dry ice bath until frozen. The frozen vials were then transferred to a freezer set at -70 °C.

4.2.4. Storage of *E. coli* and *S. aureus*

The same procedure as described in **Section 4.2.3.1** was followed however, MH broth 23 4000 (Oxoid, England) was used for *E.coli* and *S. aureus*. Each of the microorganisms was stored in sterile cryovials containing glycerol in a freezer set at -70 °C until needed for bioassays. Mueller-Hilton (MH) agar (Merck, South Africa) was prepared according to the manufactures instructions, and sterilized at 121 °C for 20 min. Sterilized MH broth (30 ml) were poured into sterile plastic Petri dishes and allowed to gel. These MH agar plates were stored upside down at 4 °C for 24 h before the stock bacteria were sub-cultured on the MH agar plates. The inoculated plates were incubated upside down at 37 °C overnight. After incubation, the cultured bacterial growth was transferred to a freezer set at 4 °C until required for bioassays.

4.2.5. Preparation of plant extracts

For the microdilution assays, dried plant extracts were resuspended in sterile distilled water for aqueous extracts or 70% ethanol for organic solvent extracts to a concentration of 50 mg/ml on the day of the experiment. For the disc diffusion method against *C. jejuni*, the extracts were dissolved in 10% dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml. Each extract with a concentration of 50 mg/ml was sterilized by filtration through 0.45 µm millipore filters and diluted ten times with 10% DMSO to make a concentration of 5 mg/ml. The 5 mg/ml extract was further diluted to a concentration of 0.5 mg/ml (**MURRAY et al., 2007**). The aqueous extracts were dissolved in sterile distilled water.

4.2.6. Preparation of bacteria for bioassay

For the preparation of *C. jejuni* the following protocol was followed: an Eppendorf containing *C. jejuni* was removed from -70 °C storage and placed on alcohol dry ice. Sterilized cotton swabs were deepped in an Eppendorf containing *C. jejuni* and streaked on brucella agar plates with campylobacter growth supplement. The plates were then incubated in a microaerophillic environment at 42 °C for 48-72 h.

For *S. flexneri*, the following procedure was followed: nutrient agar was prepared and dispensed as 4 ml aliquots in test tubes and sterilized at 121 °C for 15 min. The tubes were placed in a slanted position to provide a short slant and deep butt while they were still hot. A sterilized inoculating needle was stabbed into the butt of the medium once or twice, and then the slant was streaked. The tubes were sealed with cork stoppers soaked in hot paraffin. Incubation was overnight at 37 °C. The cultures were then stored at room temperature (about 22 ± 3 °C) in the dark. Sterile mineral oil was used to prevent drying of the slants. Growth from the slant was scraped when the bacterium was needed for bioassay.

For *E. coli* and *S. aureus* the following protocol was followed: Petri dishes containing *E. coli* and *S. aureus* were removed from 4 °C storage. The test organisms were prepared by transferring healthy colonies of each bacterium into a tube containing 10 ml Mueller-Hinton (MH) broth which were then incubated overnight at 37 °C.

4.2.7. Microdilution technique

The microdilution technique using 96-well microtitre plates (**ELOFF, 1998b**) was used to obtain minimum inhibitory concentration (MIC) values of crude extracts against Gram-negative *E. coli* (ATCC 11775), *S. flexneri* (ATCC 12022) and Gram-positive *S. aureus* (ATCC 12600) bacteria. For *S. flexneri* the turbidity of the culture was adjusted with sterile nutrient broth to match 0.5 McFarland standard, equivalent to 10^8 colon forming units/ml (CFU/ml). For *E. coli* and *S. aureus* the same procedure for adjusting turbidity of cultures of *S. flexneri* was followed except that MH broth was used for adjusting. Dried PE, DCM and 70% EtOH plant extracts were dissolved in 70% aqueous EtOH, aqueous extracts were

dissolved in sterile water to make a 50 mg/ml concentration. Sterile water (100 µl) was used to serially dilute (two fold) each plant extract in a 96-well microtitre plate for each test bacterium. A similar two-fold microdilution was made for neomycin (100 µl) (positive control). In the other free wells, 70% EtOH and water were used as solvent and negative controls respectively. Bacterial cultures (100 µl) were added to each well. The microplates were covered with parafilm and incubated for 24 h at 37 °C. *p*-Iodonitrotetrazolium chloride (INT) (Sigma 0.2 mg/l) was added (50 µl) to each well and incubated for 1 h at 37 °C to obtain MIC values. The MIC values were recorded as the concentrations in the last wells in which no colour change was observed after adding the INT indicator. The INT indicator is biologically reduced to a red product in the presence of active microorganisms. For each plant extract, the screening was done in duplicate and repeated three times.

4.2.8. Antibacterial testing against *Campylobacter jejuni* (ATCC 29428)

4.2.8.1. Disk diffusion method

A disc diffusion method as described by **NATIONAL ANTIMICROBIAL RESISTANCE MONITORING SYSTEM** (**2002**) and **KLANČNIK et al.** (**2010**) was used for the bioassay. Brucella agar was prepared according to the manufacturer's instructions and autoclaved at 120 °C for 15 min. The sterilized brucella agar (1000 ml) was allowed to cool to 50 °C before 500 µl of campylobacter growth supplement was added. Brucella agar with campylobacter growth supplement (30 ml) was then poured into Petri dishes (**ASLIM and YUCEL, 2007**). Four colonies from an overnight culture were suspended in 5 ml of brucella broth. The turbidity of the cell suspension at 450 nm was adjusted by adding brucella broth or organism as required, until the turbidity of the suspension was equivalent to that of a 0.5 McFarland BaSO$_4$ standard, equivalent to approximately 10^8 CFU/ml (**KLANČNIK et al., 2010**). Bacterial suspension (1 ml) was spread uniformly on a solid growth medium using a bacteria spreader. Sterile paper disks (6 mm in diameter) were impregnated with 5 µl of diluted plant extract and dried under a stream of sterile air before being placed on the surface of each agar plate. Each concentration was applied in triplicate (**SUNILSON et al., 2009; KLANČNIK et al., 2010**). The disks impregnated with sterile distilled water, DMSO, 70 % EtOH, DCM, and PE served as negative controls (**KLANČNIK et al., 2010**). Disks impregnated with streptomycin (an antibiotic) served as positive control. Plates were incubated at 42 °C for 48

h under a microearophillic environment (**SILVA et al., 1995**). The clear zone of inhibition (mm) around the disk of *C. jejuni* growth was measured to determine antimicrobial activity of the plant extracts and the findings were tabulated (**SUNILSON et al., 2009**). Inhibition percentage was calculated by comparing the distance (place inhibited around the disk) of the sample in mm to the distance of the positive control.

4.3. Results

A total of 36 extracts from nine plants were screened for antibacterial activity. The extracts exhibited broad-spectrum activity against the bacterial strains used. Most of the investigated plant species exhibited some activity at the maximum screening concentration of 12.5 mg/ml when using a microdilution technique and at a zone of inhibition of 22 mm for disk diffusion.

4.3.1. Antibacterial activity of plant extracts (microdilution assay)

The results of the antibacterial assay expressed as MIC values are represented in **Table 4.1**. **FABRY et al.** (**1998**) defined potentially useful activity for crude solvent extracts of plants to be considered as having good activity if they have MIC values < 8 mg/ml, whilst **GIBBONS (2005)** suggested that isolated phytochemicals should have MIC < 1.0 mg/ml. However, in this study, antibacterial MIC values equal to or less than 1.0 mg/ml for crude extracts were considered very active against tested bacteria (**NDHLALA et al., 2009**). Some degree of antibacterial activity was exhibited by most of the plant extracts with at least 21 extracts showing good antibacterial activity against at least one of the test bacteria.

The DCM extract of *R. melanophloeos* and 70% EtOH extracts of *F. craterostoma* and *M. lanceolata* showed the best antibacterial activity with MIC values of 0.098 mg/ml each against the Gram-negative bacterium, *S. flexneri*. The water extracts of *R. melanophloeos* and *S. chirindensis* exhibited good antibacterial activity against *E. coli*, *S. aureus* and *S. flexneri* with MIC values ranging from 0.39 to 0.78 mg/ml. This is in contrast to work done by other researchers and its encouraging as water extract is reported to lack activity in many studies (**MULAUDZI et al., 2009**). The observed antibacterial activity exhibited by water extracts of *R. melanophloeos* and *S. chirindensis* could be of interest since traditional healers use water as a way of preparing remedies. However, the water extracts of *T. capensis*, *F.craterostoma*,

and *M. lanceolata* showed poor activity against *E. coli*, *S. aureus* and *S. flexneri*. This implies that most of the active compounds in these plants are non-polar, in view of the fact that water extracts mainly polar compounds. Generally, PE extracts showed poor antibacterial activity as exhibited by most of the plants tested. However, the PE extract of *F. craterostoma* and *S. chirindensis,* showed good activity against *S. flexneri* with MIC values of 0.195 and 0.78 mg/ml respectively.

All the PE, DCM and EtOH extracts of *T. capensis*, *C. pulchella*, *D. cotinifolia*, *T. orientalis*, and *H. africana* had poor activity (MIC >1 mg/ml) against *E. coli* and *S. aureus*. The DCM, EtOH and water extracts of *T. orientalis*, *S. chirindensis*, *R. melanophloeos*, *D. cotinifolia* and *H. africana* showed good activity against *S. flexneri*. Only six extracts showed good activity against *S. aureus* and four extracts against *E. coli* (**Table 4.1**).

The bacterium most susceptible to inhibition by the plant extracts was *S. flexneri,* while, *E. coli* was the most resistant microorganism. Gram-negative bacteria are usually impermeable to most antibacterial compounds due to the presence of lipopolysaccharides present on their outer membrane (**FENNELL et al., 2004; VORAVUTHIKUNCHAI et al., 2004**). This can explain the low number of active extracts against *E. coli*. The 70% EtOH extracts were the most active with 14 of the extracts having MIC values of less than 1 mg/ml. The positive controls (neomycin and streptomycin) inhibited all bacterial growth with MIC values ranging between 0.003 and 0.006 μg/ml. The 70% EtOH used as a negative control showed no bacterial growth inhibition at the highest concentration equal to that of the screened plant extracts, ruling out possibilities of false positive results in the antibacterial activity observed for the active plant extracts.

Table 4.1: The antibacterial (MIC values) effects of some plants used as remedies for the treatment of diarrhoea in Bizana, Eastern Cape

Plant species	Plant part	Extract	Antibacterial activity MIC (mg/ml)		
			E. coli	*S. aureus*	*S. flexneri*
Tecoma capensis	Leaves	PE	6.25	6.25	3.13
		DCM	3.13	6.25	1.56
		EtOH	6.25	1.56	**0.78**
		Water	6.25	3.13	1.56
Clutia pulchella	Leaves	PE	6.25	6.25	3.13
		DCM	3.13	12.50	1.56
		EtOH	6.25	6.25	3.13
		Water	6.25	6.25	**0.78**
Ficus craterostoma	Leaves	PE	6.25	3.13	**0.195**
		DCM	6.25	6.25	1.56
		EtOH	6.25	**0.39**	**0.098**
		Water	6.25	3.13	3.13
Maesa lanceolata	Leaves	PE	6.25	3.13	3.13
		DCM	6.25	6.25	1.56
		EtOH	6.25	**0.78**	**0.098**
		Water	3.13	1.56	1.56
Trema orientalis	Leaves	PE	6.25	12.5	3.125
		DCM	6.25	6.25	**0.78**
		EtOH	1.56	1.56	**0.78**
		Water	1.56	6.25	**0.78**
Searsia chirindensis	Leaves	PE	6.25	1.56	**0.78**
		DCM	1.56	1.56	**0.195**
		EtOH	**0.78**	**0.78**	**0.78**
		Water	**0.39**	**0.39**	**0.78**
Rapanea melanophloeos	Leaves	PE	6.25	6.25	1.56
		DCM	6.25	3.13	**0.098**
		EtOH	**0.78**	**0.39**	**0.78**
		Water	**0.39**	**0.39**	**0.78**

Plant species	Plant part	Extract	Antibacterial activity MIC (mg/ml)		
			E. coli	*S. aureus*	*S. flexneri*
Dais cotinifolia	Leaves	PE	6.25	12.50	1.56
		DCM	3.13	3.13	**0.78**
		EtOH	1.56	1.56	**0.78**
		Water	6.25	6.25	**0.78**
Hydnora africana	Tubers	PE	6.25	1.56	1.56
		DCM	3.13	3.13	**0.78**
		EtOH	3.13	1.56	**0.78**
		Water	3.13	3.13	**0.78**
Neomycin			3.063×10^{-3}	3.063×10^{-3}	
Streptomycin					6.125×10^{-3}

MIC = minimum inhibitory concentration, DCM = dichloromethane, PE = petroleum ether,

EtOH = ethanol, Values in bold are considered very active (MIC < 1.0 mg/ml)

4.3.2. Disc diffusion assay

Campylobacter jejuni is the leading human cause of gastroenteritis in many developing countries (**McDERMOTT et al., 2005**). Infection caused by this bacterium is characterized by watery and/ or bloody diarrhoea. With increasing human infections caused by *C. jejuni*, this bacterium has become resistant to antimicrobial therapy (**McDERMOTT et al., 2005**). As shown in Table 4.2, the extracts from different plant species exhibited broad-spectrum antibacterial activity against *C. jejuni*, with the diameters of zones of inhibition ranging between 0 and 22 mm at all concentrations. Three levels of percentage activity were used: 0–44% not active; 45–70% moderate; 70–100% good activity (**MULAUDZI et al., 2011**). The EtOH extract of *S. chirindensis* showed good activity at a concentration of 50 mg/ml (percentage inhibition zone of 73%). At a concentration of 50 mg/ml the DCM extracts of *C. pulchella*, *T. capensis*, *S. chirindensis*, *R. melanophloeos*, *D. cotinifolia* and *H. africana* and the 70% EtOH extracts of *T. capensis*, *F. craterostoma*, *C. pulchella*, *S. chirindensis*, *R. melanophloeos*, *D. cotinifolia* and *H. africana* exhibited moderate activity against *C. jejuni* with inhibition percentages ranging from 47-67. The PE extracts of *T. orientalis*, *S. chirindensis*, *R. melanophloeos*, *D. cotinifolia* and *H. africana* exhibited moderate activity with inhibition percentages ranging from 47-57. As for water extracts, only *T. orientalis* inhibited *C. jejuni* at an inhibition percentage of 47 at a concentration of 50 mg/ml. All the other extracts at concentrations of 50 mg/ml, 5 mg/ml and 0.5 mg/ml showed poor activity against *C. jejuni* since their percentage inhibitions were lower than 44%. At a concentration of 50 mg/ml, the EtOH extracts showed the highest activity compared to the other extracts. The positive control (streptomycin) inhibited bacterial growth with inhibition percentages of values between 14.5 and 30 mm.

Table 4.2: The antibacterial (MIZ) effects against *Campylobacter jejuni* of some selected plants used as remedies for the treatment of diarrhoea in Bizana, Eastern Cape

Plant species	Plant Part	Extract	Concentration (mg/ml)					
			50mg/ml		5 mg/ml		0.5mg/ml	
			MIZ (mm)	I (%)	MIZ (mm)	I (%)	MIZ (mm)	I (%)
Tecoma capensis	Leaves	PE	6	20	5	23	4	28
		DCM	20	**67**	6	30	5	34
		EtOH	18	**60**	7	33	6	41
		Water	13	43	5	23	0	0
Clutia pulchella	Leaves	PE	7	23	7	33	0	0
		DCM	16	**53**	7	33	0	0
		EtOH	14	**47**	5	23	0	0
		Water	12	40	5	23	0	0
Ficus craterostoma	Leaves	PE	8	27	0	0	0	0
		DCM	7	23	4	23	0	0
		EtOH	17	**57**	6	30	0	0
		Water	12	40	5	23	0	0
Maesa lanceolata	Leaves	PE	14	**47**	5	23	5	34
		DCM	6	20	5	23	0	0
		EtOH	10	33	5	23	3	21
		Water	11	37	5	23	0	0
Trema orientalis	Leaves	PE	15	**50**	5	23	4	28
		DCM	4	13	0	0	0	0
		EtOH	13	43	6	30	4	28
		Water	14	**47**	6	30	5	34

Plant species	Plant Part	Extract	Concentration (mg/ml)					
			50mg/ml		5 mg/ml		0.5mg/ml	
			MIZ (mm)	I (%)	MIZ (mm)	I (%)	MIZ (mm)	I (%)
Searsia chirindensis	Leaves	PE	16	**53**	9	42	7	**48**
		DCM	14	**47**	0	0	0	0
		EtOH	22	**73**	10	**47**	5	34
		Water	13	43	5	23	0	0
Rapanea melanophloeos	Leaves	PE	17	**57**	9	42	0	0
		DCM	19	**63**	5	23	0	0
		EtOH	17	**57**	8	37	0	0
		Water	12	40	6	30	4	28
Dais cotinifolia	Leaves	PE	15	**50**	6	30	0	0
		DCM	14	**47**	5	23	0	0
		EtOH	17	**57**	9	42	0	0
		Water	9	30	4	23	0	0
Hydnora africana	Tubers	PE	14	**47**	6	30	0	0
		DCM	18	**60**	6	30	0	0
		EtOH	15	**50**	6	30	0	0
		Water	13	43	5	23	0	0
Streptomycin			30	100	21.5	100	14.5	100

DCM = dichloromethane, PE = petroleum ether, EtOH = ethanol; Values in bold are considered active. Microorganism: *Campylobacter jejuni*

Standard drug: Streptomycin positive control. Grading of results: 0-44%, not active; 45-70%, moderate; 70-100%, good activity

MIZ = Mean inhibition zone, I = inhibition

Values in bold are considered active

4.3.3. Discussion

As it is ideal to test plant extracts against specific target microorganisms, plant extracts were tested against specific bacteria that cause diarrhoea. It was expected that medicinal plants that demonstrated a broad-spectra of activity might help in the discovery of new antibacterial antibiotics that could serve as selective agents for human health maintenance.

A decoction of *M. lanceolata* is used in traditional medicine in Rwanda against various diseases including infectious hepatitis, bacillary dysentery and some types of dermatoses and neuropathies. It is also used in East African folk medicine to prevent cholera (**FOUBERT et al., 2010**). The antimicrobial activity observed in the extracts of *M. lanceolata* could be from the saponins, reportedly isolated from its leaves that have been shown to have virucidal, haemolytic and anthelmitic activities (**APERS et al., 1998**). The extracts of *R. melanophloeos* also exhibited good antibacterial activity, probably because of potent sakuraso-saponins which have been reported to be present in the leaves. Saponins are widely distributed in the plant kingdom and are known to possess a broad spectrum of biological and pharmacological activities. Several reports are available in support of antimicrobial activity of plant extracts that is due to the presence of saponins (**LACAILLE-DUBOIS and WAGNER, 2000; KHANNA and KANNABIRAN, 2008**).

The antibacterial activities of *S. chirindensis* observed in this study confirm for the first time its antibacterial properties. It was noteworthy that the EtOH and water extracts of *S. chirindensis* showed good antibacterial activity against *E. coli*, *S. aureus* and *S. flexneri*. It was also interesting to observe that the DCM, PE and EtOH extract of the same plant showed moderate to good antibacterial activity against *C. jejuni*. This suggests that this plant contains important bioactive molecules that have antibacterial properties.

A decoction of *T. capensis* is known for the treatment of fever, pain, pneumonia, abdominal pains as well as chest ailments (**VENTER and VENTER, 1996**). The results

obtained in this study on antibacterial activity in EtOH extracts of *T. capensis* seem to agree with those observed by **SAINI et al.** (**2011**) who worked on *E. coli* and *S. aureus*. It was also interesting to observe good antibacterial activity on the EtOH extract of this plant against *S. flexneri* (0.78 mg/ml).

The extracts of most of the plant species had good antibacterial activity, and the findings were interesting as they confirm antibacterial activities of these plants. To our knowledge results found in this study confirm for the first time antibacterial activities of *C. pulchella*, *F. craterostoma*, *S. chirindensis*, *M. lanceolata*, *R. melanophloeos*, *D. cotinifolia* and *H. africana* and that was noteworthy. Bioactivity cannot be completely ruled out in some of the extracts that did not show positive results as there is a possibility that the plant extracts may have activity against other pathogenic bacteria other than those tested or may act by a different mechanism that was not tested in the reported study. These plant extracts could be equally active against other resistant bacteria especially Gram–negative such as *S. typhi, Shigella dysenteriae* (Shiga) Castelani and Chalmers and *V. cholerae* that are responsible for acute gastro-intestinal infections. Further investigation aimed at isolating and identifying the compounds responsible for the activity observed against Gram–negative bacteria is necessary.

4.4. Conclusions

In view of the fact that the plants were selected based on their ethnobotanical usage for treating diarrhoea, the antibacterial activity of the plant extracts was noteworthy. From all the investigated plant extracts at least two plants exhibited antibacterial activity against all test bacteria. It is noteworthy that the 70% EtOH and water extracts of *S. chirindensis* and *R. melanophloeos* exhibited the highest activity against both Gram-negative and Gram-positive bacterial strains tested. Findings found in this study confirm for the first time presence of active constituents of most of the evaluated plant species except for *T. capensis*. This confirms the presence of important bioactive principles that are active against bacteria in the studied plants. These plants could be active against other resistant bacteria that cause diarrhoea such as *S. typhi*, S*higella* spp. and *V. cholera*. Therefore

further investigation using *in vivo* and/ or *in vitro* bioassays is required in order to reach sound conclusions on the potential of these plants as sources of novel antimicrobial agents.

Chapter 5: Inhibition of cyclooxygenase enzymes as an evaluation for anti-inflammation properties of plants used in Bizana, Pondoland against diarrhoea

5.1. Introduction

Pathologically, the term inflammation means a succession of changes which occurs in living tissue in response to physical trauma, chemical irritation and infections caused by pathogens and parasites (**BALARAM, 2000**). Biochemically, it is defined as a disorder involving localized increases in the number of leukocytes and a variety of complex mediator molecules (**MANTRI and WITIAK, 1994; BALARAM, 2000**). Although inflammation progresses to pain, it is one of the most important processes involved in the defence mechanisms of an organism (**CELLOTI and LAUFER, 2001**). Inflammation is an essential function of the innate immune system that is required to protect the host against pathogens and to commence specific resistance (**SHIN et al., 2010**). Gastro-intestinal ailments are associated with inflammation of the digestive tract, resulting in abdominal pains and cramps of varying degrees (**BARBARA, 1998**). In patients suffering from gastro-intestinal infections, the intestines are susceptible to muscle contraction leading to abdominal cramps and pains. Enterotoxins produced by *E. coli* and other gastro-intestinal pathogens associated with water and food poisoning induce watery diarrhoea and abdominal tissue damage through plasmid-encoded invasion factors resulting in pain (**BARBARA, 1998**).

When blood cells dilate, blood supply increase resulting in movement of leukocytes, proteins and fluids into inflamed regions and that cause pain, heat, redness, swelling and loss of function (**IWALEWA et al., 2007**). Inflammatory responses are major contributors to diseases and disorders as its symptoms appear when the body recognizes the injury and repairs the damage (**IWALEWA et al., 2007**). Inflammation can be classified as either acute or chronic and can be beneficial or harmful. Acute inflammation is the initial response of the body to harmful stimuli that is caused when there is an

increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. Under normal circumstances it is helpful and results in the process of healing (**VASSILEVA AND PIQUETTE-MILLER, 2010**). Chronic inflammation leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue during the inflammatory process. Cells of the immune system liberate reactive oxygen species, chemokines, cytokines, and other small molecules that maintain and intensify the inflammatory response. Simultaneous degradation and regeneration of the affected tissue occurs as precursor of a wide variety of pathologies. Thus, a persistent stimulus converts a normal and essential host defence mechanism into an injurious response (**VASSILEVA AND PIQUETTE-MILLER, 2010**).

5.2. Overview of the inflammation process

Upon invasion by a pathogen, mammalian monocytes or macrophages identify lipopolysaccharides (LPS) of bacteria through the transmembrane signaling Toll-like receptor-4 (TLR-4), thus signalling normal host defense mechanism commencement (**SHIN et al., 2010**). Inflammation has two effects, to demolish/eliminate invading pathogens, or to repair damaged tissues (**BALARAM, 2000**). The process of inflammation involves a range of enzyme reactions, release of chemical mediators, as well as repair and breakdown of tissues. Cyclooxygenase (COX) enzymes catalyze the arachidonic acid (AA) conversion to prostaglandins, prostacyclins, and thromboxanes, which are involved in an inflammatory response (**DUBOIS et al., 1998**). Inflammation is an essential function of the innate immune system and it is necessary to protect the host against microorganisms and commence definite immunity (**SHIN et al., 2010**). During the process of inflammation, arachidonic acid (AA) is released from the cell membrane by phospholipase A_2. After cleavage, AA is converted by COX enzymes to form an unstable prostaglandin endoperoxide (PGG_2). PGG_2 is converted into PGH_2 by PGHS peroxide function and finally isomerized to prostaglandins (which play a pivotal role in health and gastrointestinal tract diseases) by cell-specific isomerases (**CURTIS-PRIOR, 2004; BLOBAUM and MARNETT, 2007**).

5.2.1. Cyclooxygenase enzymes

COX enzymes are the main enzymes involved in the production of prostaglandins from the substrate arachidonic acid (**JÄGER and VAN STADEN, 2005**). These enzymes exist in two isoforms (COX-1 and COX-2) that are coded by distinct genes on different chromosomes and have unique patterns of expression (**MAHLER et al., 1996**). The two COX enzymes show about 50% homology and have similar catalytic activity, but are physiologically distinct (**ELDEEN and VAN STADEN, 2008**). COX-1 is believed to produce prostaglandin that is involved in the maintenance of normal physiological functions, thus known as a house keeping enzyme (**DAVIES et al., 1997; DEY et al., 2003**). COX-2 is an inducible enzyme, and its expression is activated by tissue damage and inflammation. COX-1 is generally constitutive (**DEY et al., 2003; ELDEEN et al., 2005**). Its housekeeping functions include gastric cytoprotection, platelet aggregation and controlling of renal blood flow in afferent vessels of the kidneys. In contrast, COX-2 is inducible in inflammatory cells (**DEY et al., 2003; ELDEEN and VAN STADEN, 2008**). The expression and activity of COX-2 is responsive to adverse stimuli, such as inflammation although it can be constitutive in some tissues (**ELDEEN and VAN STADEN, 2008**). COX-2 is involved in many processes of inflammation and plays a key role. The identification of COX-2 inhibitors is therefore considered to be a desirable approach to combat inflammation.

5.2.2. Anti-inflammatory agents

Anti-inflammatory refers to the property of a substance or treatment to reduce inflammation. Anti-inflammatory drugs reduce inflammation by blocking prostaglandin inducing enzymes. There are two groups of anti-inflammatory agents: non-steroidal and steroidal or glucocorticoid drugs. Nonsteroidal anti-inflammatory drugs (NSAID) have analgesic, antipyretic and anti-inflammatory activities (**AWOFISAYO et al., 2008**). NSAID inhibit COX activity thus blocking the synthesis of prostaglandins and thromboxane (**UMARU et al., 2009**). Glucocorticoids inhibit COX-2, down regulating its

transcription (**AWOFISAYO et al., 2008**). Anti-inflammatory action of NSAIDs is associated with their COX-2 inhibition. However, several side effects of NSAIDs have been reported to be due to their COX-1 enzymatic pathway inhibition.

Non-steroidal and steroidal treatment used to treat inflammation has a number of limitations. For example, COX-2 inhibitors have harmful actions on the upper gastrointestinal tract (**WHITTLE, 2004**). NSAIDs are associated with the enhancement of bacterial infection progression by their actions, such as granulocyte function impairment (adherence, phagocytosis, cidal activity), augmentation of inflammatory cytokine release and inhibition of renal prostaglandin synthesis. Steroids, such as prednisone, prednisolone, and hydrocortisone are associated with side effects such as immunosuppression, muscle breakdown, glaucoma and increased blood pressure (**WILSON, 1991; BOTTING, 2006**).

The deleterious side effects associated with the use of NSAIDs demands continual exploration for effective agents of anti-inflammation with reduced or no side-effects. A range of chemical compounds having diverse pharmacological activities are present in plants. Hence, greater interest on anti-inflammatory activity of medicinal plants has been shown by pharmaceutical industries and scientific research as they search for novel drugs that can be used in the treatment of pain-related ailments (**FAWOLE et al., 2010**). A number of studies to evaluate anti-inflammatory activity of medicinal plants have been conducted in South Africa (**AREMU et al., 2010; FAWOLE et al., 2009b; FAWOLE et al., 2010**).

It is vital to screen medicinal plant extracts in different *in vitro* assays, because there is a possibility of losing other potentially useful bioactive compounds when there is evaluation of only a single biological activity. Gastro-intestinal ailments are associated with inflammation of the digestive tract. Herbal remedies have long been used in traditional medicine to treat human ailments such as gastritis, ulcers, and other inflammation-related diseases (**FAWOLE et al., 2010**). Thus, medicinal plants that are

used in traditional remedies for treating diarrhoea were investigated for inhibition of COX enzymes.

5.3.   Materials and methods

Preparation of plant extracts and COX-1 and -2 bioassays were performed as detailed below.

5.3.1. Preparation of plant extracts

Dried plant materials that were prepared as describe in **Section 3.2.2** were dissolved in 70% EtOH for organic solvent extracts and in water for aqueous extracts to make a final concentration of 10 mg/ml.

5.3.2. Substrate and enzyme preparation

The substrate ^{14}C-arachidonic acid (radio labeled AA) was obtained from Amersham (GE Healthcare, UK). Radio-labeled AA (100 µl) were diluted with unlabelled AA (6.75 µl) (Sigma-Aldrich, Germany) to obtain the final concentration (17 Ci/mol, 30 µM) required for the bioassay.

The COX-1 and -2 enzymes were obtained from Sigma-Aldrich (USA). Stock solutions of COX-1 and -2 enzymes were kept at -70 ℃ until use. The COX enzymes were diluted with Tris(hydromethyl)aminomethane (TRIS) storage buffer (pH 8.0) to obtain 50 µl of 75 units enzyme concentration per aliquot. The prepared COX enzymes (50 µl, 75 units) were kept in an ultra-freezer at -70 ℃ until required.

5.3.3. Cyclooxygenase-1 inhibition bioassays

The method described by **JÄGER et al.** (**1996**) as modified by **ELDEEN and VAN STADEN** (**2008**) was used to evaluate the ability of the plant extracts to inhibit COX-1 enzyme. One thousand two hundred and fifty microlitres of co-factor solution (3 mg/ml *L*-epinephrine and 3 mg/ml reduced glutathione in 0.1 M Tris buffer, at pH 8.0) were used to activate the enzyme. To each sample solution (2.5 µl of the plant extract and 17 µl of water) and 60 µl of enzyme solution were added and incubated at room temperature for 5 min. The extracts were tested at a concentration of 10 µg/ml (resuspended in 70% EtOH) giving a final concentration of 250 µg/ml per test solution for organic extracts and 200 µg/ml for aqueous extracts. As background, the enzyme was inactivated by adding 10 µl 2N HCL before the addition of ^{14}C-arachinodic acid, a solvent blank and indomethicin at 5 µM were used as controls. Twenty microlitres of ^{14}C-arachidonic acid (17 Ci/ mol, 30 µM) were added to each sample to initiate the reaction. A water bath was used to incubate the samples for 10 min after which 10 µl 2N HCL were added to terminate the reaction. Four microlitres of unlabelled prostaglandins (PGE$_2$: PGEF$_{2\alpha}$ 1:1) from Sigma-Aldrich were added to each Eppendorf as a carrier solution. Silica gel (silica 60, 0.63-0.200, Merck) was used to pack silica columns to a height of 3 cm using Pasteur pipettes. To separate the prostaglandins and unmetabolized arachinodic acid, the test solution was applied to the column with 1 ml of eluent 1 [hexane: 1, 4-dioxan: glacial acetic acid (70:30:0.20)]. The arachidonic acid was eluted first with 4 ml of eluent 1 and the products of prostaglandin were eluted with 3 ml of eluent 2 [ethyl acetate: methanol (85:15)] and collected in scintillation vials. Four millilitres of scintillation fluid were added to each vial and a scintillation counter (Beckman LS 6000LL scintillation counter) was used to count the disintegration per minute (DPM) of radioactive material. All the samples were tested in duplicate. The percentage of inhibition was calculated using the following equation:

$$COX\ inhibition\ (\%) = \left[1 - \left(\frac{DPMSample - DPMbackground}{DPMblank - DPMbackground}\right)\right] \times 100$$

Where DPMsample, DPMbackground and DPMblank represent the disintegration per minute for sample, background and solvent blank respectively.

5.3.4. Cyclooxygenase inhibition bioassays (COX-2)

For COX-2 bioassay the procedure used was the same as the one for COX-1 (**Section 5.2.3**) with slight modifications (**ZSCHOCKE and VAN STADEN, 2000**). The co-factor solution was prepared by adding 6 mg *L*-epinephrine and 3 mg reduced glutathione into 10 ml of TRIS buffer and 100 µl of haematin. A concentration of 200 µM of reference anti-inflammatory drug indomethacin was used.

5.4.   Results and discussion

5.4.1. Inhibition of COX-1 and COX-2

A total of 36 plant extracts were screened for their ability to inhibit COX-1 and COX-2 enzymes. The results as percentage inhibition of prostaglandin synthesis by the extracts in COX-1 and-2 assays are shown in **Figure 5.1**. Inhibition of COX-1 enzyme has been reported to cause damage to the gastrointestinal tract, such as stomach ulcers and bleeding. COX-1 is also involved in the homeostasis of various physiological functions and thus high activity is undesirable (**KIM et al., 2005; NDHLALA et al., 2009**). COX-2 has inflammatory effects. Therefore it is desirable to find extracts that selectively inhibit COX-2, since COX-2 inhibition provides therapeutic effects (**BLOBAUM and MARNETT, 2007**). In this study, minimum inhibitory activity below 20% was considered insignificant, 20-40% low, 40-70% moderate, and 70-100% high at the extract concentration tested (**TAYLOR and VAN STADEN, 2001**). Moderate to high levels were considered to be good activity. Nearly all the investigated plant extracts showed stronger inhibition against COX-1 than the COX-2 enzyme.

High COX-1 inhibition was exhibited by 17 extracts, 11 exhibited moderate, and four exhibited low, while insignificant activity was shown by four plant extracts. Only DCM

and PE extracts of *D. cotinifolia* inhibited COX-1 more than the EtOH and PE extracts. The highest inhibition of COX-1 was exhibited by DCM extract of *S. chirindensis* and EtOH extract of *F. craterostoma* each with 100% inhibitory activity. With the exception of *M. lanceolata*, all the DCM extracts showed good COX-1 inhibitory activity. The DCM extract of *T. capensis*, *F. craterostoma*, *S. chirindensis*, *D. cotinifolia* and *H. africana* showed high COX-1 inhibition with inhibitory percentage ranging from 90.65 to 100%. Most of the PE extracts showed good COX-1 activity with the exception of *C. pulchella* and *F. craterostoma*. Most EtOH extracts showed good COX-1 activity except *T. capensis* and *D. cotinifolia*. For water extracts, only *D. cotinifolia*, *H. africana* and *F. craterostoma* showed low activity, whereas the rest of the extracts exhibited good COX-1 inhibitory properties. Of all the plant extracts evaluated, the DCM extract of *M. lanceolata*, PE extract of *C. pulchella* and *F. craterostoma*, EtOH extracts of *T. capensis* and *D. cotinifolia* and water extracts of *F. craterostoma*, *D. cotinifolia* and *H. africana* exhibited insignificant to low COX-1 inhibitory activities. This is worth mentioning as drugs with low COX-1 enzyme are required.

High inhibition of COX-2 was shown by seven extracts, 11 showed moderate inhibition and five showed low activity while 13 showed insignificant activity. DCM and PE extracts of *T. capensis*, *M. lanceolata* and *T. orientalis* inhibited COX-2 more than EtOH and water extracts. The highest percentage inhibition of COX-2 was exhibited by DCM extracts of *T. capensis* and water extracts of *F. craterostoma* with 99.5 and 99.0% inhibition respectively. The EtOH extracts of *D. cotinifolia* also exhibited high activity against COX-2 (98%). Most DCM extracts showed moderate to high inhibitory activity against COX-2 with the exception of *C. pulchella* and *H. africana* that exhibited low inhibition of the enzyme. The PE extracts of *T. capensis*, *F. craterostoma*, *M. lanceolata*, *T. orientalis*, and *S. chirindensis* showed moderate to high COX-2 activity. For EtOH extracts, only *T. capensis*, *D. cotinifolia* and *H. africana* exhibited moderate to high COX-2 inhibitory activity, the rest of the extracts exhibited low to insignificant activities. Most of the water extracts showed no inhibition (0% inhibition) against COX-2 enzyme except for *F. craterostoma*, *S. chirindensis* and *R. melanophloeos* that exhibited insignificant to high activities.

(A)
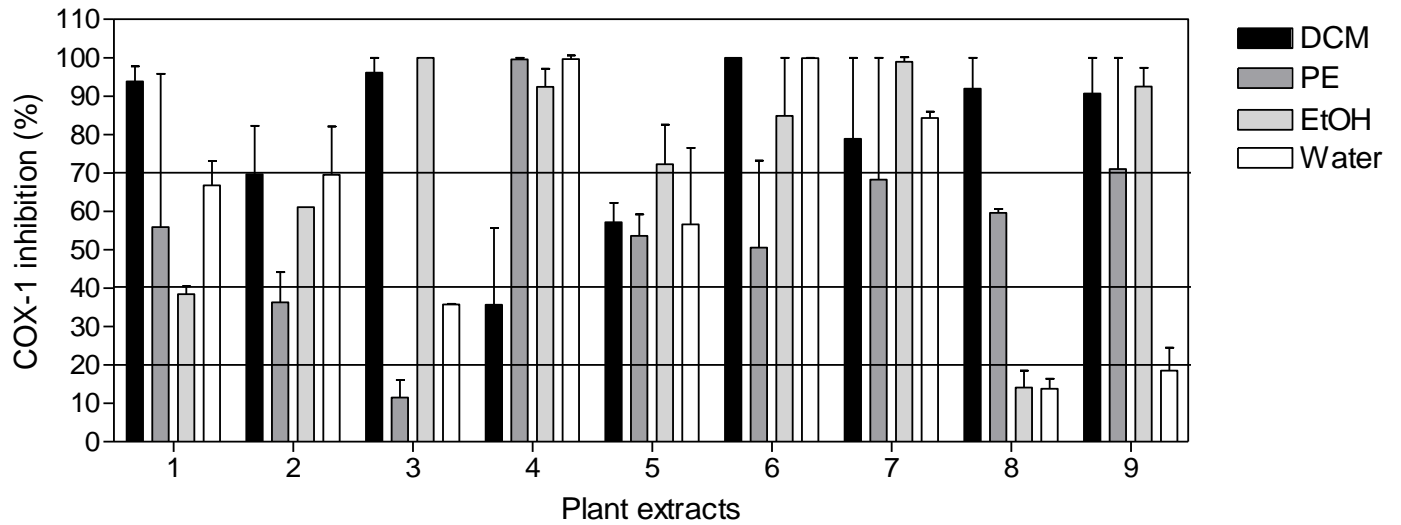
(B)
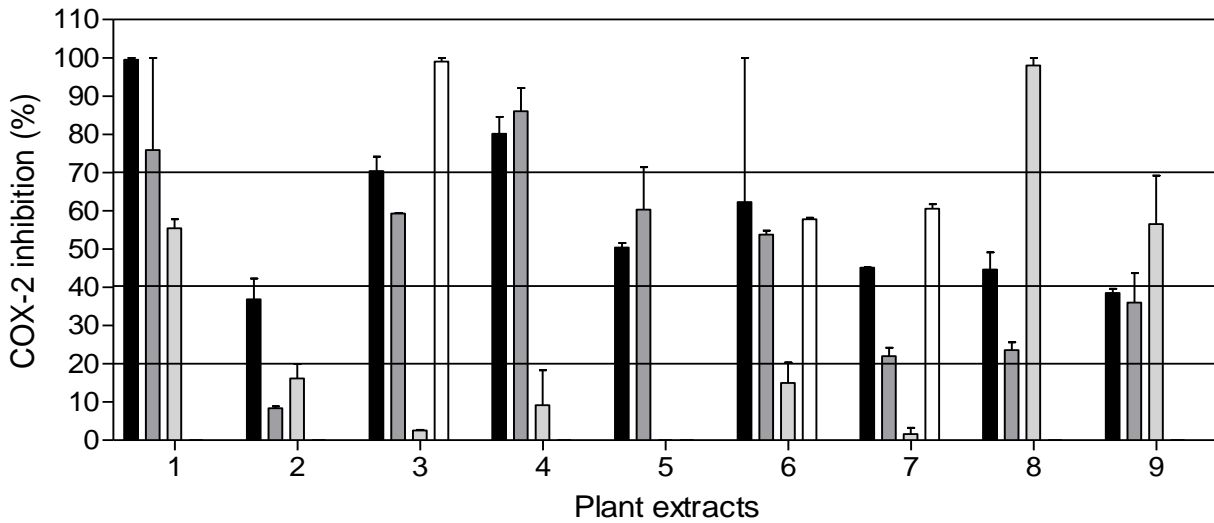
Figure 5.1: Percentage inhibition of cyclooxygenase -1 (A) and -2 (B) enzymes with leaf extracts of (1) *T. capensis*, (2) *C. pulchella*, (3) *F. craterostoma*, (4) *M. lanceolata*, (5) *T. orientalis*, (6) *S. chirindensis*, (7) *R. melaphloeos*, (8) *D. cotinifolia*, and tubers of (9) *H. africana*. Water extracts were tested at 2 mg/ml. Organic extracts were tested at 250 µg/ml. Extracts with inhibition above 70% were considered to be highly active. Concentrations and inhibitions for indomethacin® were 5 µM; 64.18 ± 3.10% and 200 µM; 68.50 ± 2.57% for COX-1 and COX-2 enzymes respectively

It is interesting to note that DCM extracts of *T. capensis* and *M. lanceolata*, PE extracts of *T. capensis*, *F. craterostoma*, *T. orientalis* and *S. chirindensis*, EtOH extracts of *T. capensis* and *D. cotinifolia* and water extracts of *F. craterostoma* showed stronger inhibition of COX-2 than COX-1.

A number of extracts in this study inhibited both COX-1 and -2 enzymes at a moderate to high percentage, however the inhibitory activity against COX-1 enzyme was higher than COX-2. This could be due to the differences in the active sites between these two isoenzymes. COX-2 has a slightly larger active site than COX-1 (**VANE and BOTTING, 1998**). Hence, the smaller active site is easily inhibited more than a larger active site (**BOTTING, 2006**). Most of the plant extracts exhibited better activity against COX-1 enzyme, with 53% of the plant extracts exhibiting high activity. Only 19 % of the plant extracts exhibited high activity against the COX-2 enzyme. That is noteworthy as inhibition of COX-2 enzyme is the ideal goal of therapy (**IWALEWA et al., 2007**). For all the plant species evaluated, water extracts showed the least inhibitory activity against the COX-2. In the case of extracts showing weak or no activity in these assays, it is worth mentioning that high dosages are frequently used in traditional medicine (**McGAW et al., 1997**). The extracts that exhibited weak or no activity might be active when concentrations higher than those evaluated are used. Since lipophilic compounds are more extractable by polar solvents and have far better resorption through the cell membrane, activity exhibited by non-polar solvents specifically at low concentrations is noteworthy (**ZSCHOCKE and VAN STADEN, 2000**). Significant is that the water extracts of *S. chirindensis, R. melanophloes* and *F. craterostoma* exhibited moderate to high COX-2 inhibition with inhibitory percentage of 57%, 60% and 99% respectively. This is of importance since plant decoctions are prepared using water in traditional medicine and this therefore supports the therapeutic uses of these plants traditionally. However, more *in vivo* tests still need to be done. The high to moderate inhibitory activity exhibited by the leaf extracts of these plants against COX-2 and COX-1 inhibition is important for medicinal plant species conservation, as the leaves can be sustainable harvested while using the plants for medicine without threat to their survival. The results show the potential of the plant extracts to reduce inflammation. According to

our knowledge, the results of this study also confirm for the first time anti-inflammatory evaluation of these plants.

All the plant species that were evaluated in this study for inhibition of COX-1 and 2 are used for the treatment of diarrhoea. Diarrhoea is associated with pain and cramps, as is the case with dysentry. Moderate to high inhibition of COX enzymes was exhibited by at least one plant extract from most of the evaluated plant species, except for the extracts of *C. pulchella* against COX-2 enzyme. The inhibition of COX enzymes shown by some of the evaluated plants supports their uses in South African traditional medicine for the treatment of pain-related ailments. The high COX-1 and -2 inhibition percentage observed for the extracts of *M. lanceolata* could be from the saponins, reportedly isolated from its leaves (**APERS et al., 1998**). Inhibition of COX-1 and -2 shown by *R. melanophloeos* could probably be because of potent sakuraso-saponins which have been reported to be present in the leaves of this plant as saponins are known to possess a broad spectrum of biological and pharmacological activities (**LACAILLE-DUBOIS and WAGNER, 2000**).

5.5. Conclusions

COX-1 was inhibited by most plant extracts. More so than COX-2. For both enzymes the non-polar solvent extracts were more active than polar solvent extracts. The highest inhibition of COX-1 was shown by EtOH extracts of *F. craterostoma* and DCM extract of *S. chirindensis* at 100% and highest COX-2 inhibition was shown by water extracts of *F. craterostoma* and DCM extracts of *T. capensis* at 99.5% and 99.0% respectively. The water extracts of *F. craterostoma* showed high inhibition of COX-2 at 99%. Further studies are required to elucidate the observed activities. Isolation and identification of COX inhibiting compounds in extracts of *T. capensis*, *F. craterostoma*, *M. lanceolata* and *D. cotinifolia* that showed high inhibitory activity of COX-2 enzymes could potentially be of great importance in the search for novel anti-inflammatory drugs from natural sources.

Chapter 6: Genotoxicity evaluation of nine selected plants used in Bizana, Pondoland for treating diarrhoea

6.1. Introduction

Medicinal plants have been used since ancient times for treatment of various diseases and ailments. They are still used by a large proportion of the population in developing countries. The increase in the use of medicinal plants is probably due to the belief that they are harmless, merely because they are natural (**VERSCHAEVE et al., 2004; HONG and LYU, 2011**). Just because medicinal plants are natural and have been used by humans for long does not mean they are safe. They might have detrimental side effects from overuse or misuse and some may cause deoxyribonucleic acid (DNA) mutations (**PHILOMENA, 2011**). Therefore, safety evaluation of plants that are used in herbal remedies are required, especially before they can be developed into herbal medicine products (**CHICHIOCO-HERNANDEZ et al., 2011**).

Mutagenesis is a process by which an organism's genetic information is changed in a stable, heritable manner, either in nature by the use of chemicals or radiation. Chemicals that are capable of inducing mutation can potentially damage germ lines leading to fertility problems and mutations in future generations. Due to the cancer causing capability of mutagens, the analysis for substances that can induce mutations has become an important procedure in safety assessment (**MORTELMANS and ZEIGER, 2000**). Mutation events may result in several degenerative diseases, such as cancer and arteriosclerosis.

Generally, plants produce toxic substances which act as defence against infections, herbivores and insects, which may also affect man. Based on long-term use of medicinal plants in traditional medicine, assessment of their mutagenic potential is necessary to ensure their safe use. Furthermore, one might expect medicinal plants to have low toxicity based on their long-term use. However many plants used in traditional medicine

or as food have been shown in recent studies to be potentially toxic, mutagenic and/or carcinogenic (**ELGORASHI et al., 2003; VERSCHAEVE et al., 2004**). This raises concern about potential mutagenic hazards caused by long-term use of plants and there is still limited scientific confirmation concerning safety of plant sources (**ELGORASHI et al., 2003; VERSCHAEVE et al., 2004**). Investigation of medicinal plants used in TM is thus valuable at two levels: firstly as a possible chemotherapeutic drug source and secondly as a measure of safety for continued use of medicinal plants (**VERSCHAEVE et al., 2004**). Medicinal plants showing mutagenicity should be considered as unsafe for human consumption and should be evaluated further before recommendation of their continued use, and those showing antimutagenicity should be considered interesting for therapeutic use and tested further (**VERSCHAEVE and VAN STADEN, 2008**).

Numerous studies have led to the validation of traditional remedies. However, some of these studies have shown that some plants used in traditional medicine have mutagenic effects in *in vitro* assays (**ELGORASHI et al., 2003**). The potential genotoxicity effects that follow long term use of some of the more popular herbal remedies, are a cause for alarm (**VERSCHAEVE et al., 2004**). Therefore it would be dangerous to assume that all plant extracts are safe to use.

6.2. Genotoxicity testing methods

Chemicals that exert adverse effects through interaction with genetic material (DNA) of cells are called genotoxics (**JENA et al., 2002**). Genotoxicity is the alteration of structure and expression of the genetic material of cells (**STELLMAN, 1998**). These alterations are responsible for the incidence of heritable mutations in man and impose risks to future generations (**JENA et al., 2002**). Many *in vivo* and *in vitro* tests have been developed to give indications of genotoxicity effects of natural products. These tests enable hazard identification with respect to DNA damage and fixation in the form of gene mutations, large scale chromosomal damage, recombination and numerical chromosome changes (**JENA et al., 2002**). They have been used to detect carcinogenicity and genotoxicity.

Extracts of medicinal plants which are positive in these tests, have a potential to be human carcinogens or mutagens (**JENA et al., 2002**).

Different techniques for genotoxicity testing have been designed and guidelines to conducting the assays have been developed. These include microbial and mammalian studies. Suspension and plate (spot) tests are used for genotoxicity evaluation of plants using *E. coli* (**GAD, 2008**). In suspension assays, cells are treated in suspension whereas in the spot assay, a test chemical is applied directly to the center of a selective agar medium in small amounts. The area/spot where the chemical was applied will be surrounded by a ring of revertent colonies that would have resulted from a concentration gradient (**GAD, 2008**). This test is not well known due to relative insensitivity of *E. coli* mutation in reverting from streptomycin dependence to streptomycin independence and lack of knowledge of metabolic activation of mutagens, (**GAD, 2008**). Ames test, a plate incorporation procedure is a widely accepted short-term bacterial test used to assess genotoxicity (**MORTELMANS and ZEIGER, 2000**). It detects point mutations and is more sensitive than the spot test. It detects chemicals that induce reverse mutations in *Salmonella typhimurium* (Loeffler) Castelani and Chalmers strains and restores the functional competence of a bacterial strain that is defective in synthesizing important amino acids (**MORTELMANS and ZEIGER, 2000; GAD, 2008**). Bacterial strains used in the Ames test carry a faulty (mutant) gene that prevents them from synthesizing a vital amino acid, histidine, from the ingredients in standard bacterial culture medium (**MORTELMANS and ZEIGER, 2000**). Thus, the tester strains can only survive and grow on excess histidine containing medium. nonetheless, the defective histidine gene may be mutated back to the functional state if a mutagenic chemical is present , allowing the bacterium to grow on standard medium that does not contain supplemental histidine (**MORTELMANS and ZEIGER, 2000; GAD, 2008**). Numerous strains of the *S. typhimurium* bacterium may be used for testing genotoxicity. Each strain is genetically different, so using several strains in a test increases the likelihood of detecting a mutagenic chemical. The tester strains used in the Ames test are TA97, TA98, TA100, TA102, TA104, TA1535, TA1537 and TA1538 (**MORTELMANS and ZEIGER,**

2000). Three different strains were used in this study, these were TA98, TA1535 and TA1537. A positive response in strains TA98 and TA1537 indicate frameshifts and in TA1535 base-pair substitution (**MORTELMANS and ZEIGER, 2000**).

6.3. Materials and methods

6.3.1. Preparation of plant extracts

Plant extracts were dissolved in 10% DMSO to make a concentration of 5000 µg/ml. This was then filtered through 0.22 µm Millipore filtertips to remove biological contaminants. After being filtered, the 5000 µg/ml concentration of the sample extracts was diluted with sterile 10% DMSO to make concentrations of 500 and 50 µg/ml.

6.3.2. *In vitro* mutagenic evaluation (Ames test)

The genotoxicity testing of the plant extract was done using the *Salmonella* microsome assay in histidine poor growth medium. In this medium histidine (His$^+$) mutants were able to form observable colonies. The *Salmonella* microsome assay was used to test mutagenicity based on the plate-incorporation procedure. The mutagenecity assay was performed by incorporating test compounds with *S*. *typhimurium* tester strains TA98, TA1535 and TA1537. The procedure was done according to **MARON and AMES** (**1983**). A stock (100 µl) bacterial culture in 10 ml Oxoid No. 2 nutrient broth was incubated for 16 h at 37 °C. Bacterial cultures (100 µl) were added to 100 µl of plant extract in 500 µl of phosphate buffer and 2 ml of agar containing biotin and histidine (0.5 mM). The mixture was mixed by vortexing and then gently poured on minimal agar plates, allowed to solidify (2 min) and then incubated in an inverted position at 37 °C for 48 h. The samples were tested in triplicate. The positive control, 4-nitroquinoline-N-oxide (4NQO) at a concentration of 2 µg/ml, was used. After 48 h of incubation, the number of bacterial colonies was counted using a colony counter. In the Ames test, for a substance to be considered mutagenic, the number of revertant colonies (His$^+$) must be

more than twice the number of colonies produced on the negative control plates and have an increase in colonies with an increase in concentration (**MARON and AMES, 1983**). Absence of toxicity was examined by observing background bacterial growth, which should be normally present.

6.4. Results and discussion

6.4.1. Mutagenicity results using the Ames test

The Ames test is normally used as a first genotoxicicity screening tool, specifically for point-inducing mutation. **Table 6.1** presents results of the mutagenic effects of extracts of selected antidiarrhoeal plants in the Ames test performed in the absence of S9 metabolizing enzymes. Positive results from strains TA98 and TA1537 gives an indication of frame-shift mutations while that of strain TA1535 indicates base-pair substitutions (**MORTELMANS and ZEIGER, 2000**). The standard plate incorporation method for Ames test using *S. typhimurium* tester strain TA98, TA1535 and TA1537 exposed to three dilutions of the DCM, PE, 70% EtOH and water extracts of nine plant species used as remedies for treatment of diarrhoea was performed to investigate their mutagenicity. None of the tested extracts were mutagenic, as the numbers of revertant colonies at all the concentrations did not satisfy the criteria for mutagenicity. This means that the extracts were not able to increase the number of His^- to His^+ revertants by a factor of two or more above the spontaneous background level. The background level as well as the positive control values were in all cases within the normal limits found in the laboratory and are in accordance with the literature (**ELGORASHI et al., 2003**). In addition no dose–response was observed for the various concentrations of the mutagen tested as this may indicate toxicity (**NDHLALA et al., 2011**).

The negative genoxicity results in the bacterial reverse mutation test of *S. chirindensis* obtained in this study seem to agree with those observed in a study done by **REID et al.** (**2006**). In a study done by **HONG and LYU** (**2011**) *T. orientalis* also showed negative results. The methanol extracts of maesasaponin (a compound extracted from the leaves of

M. lanceolata) showed negative mutagenic results in a study done by **SINDAMBIWE et al.** (**1998**), and this is in agreement with the results obtained in this study for *M. lanceolata*. This is a step forward in validating the potential safety in the usage of these plants in traditional medicine.

Medicinal plants with antimutagenic properties should be considered interesting for potential medicinal use and further investigation for their pharmacological properties are required. If a substance screened does not indicate a mutagenic response it does not necessary confirm that it is not mutagenic nor carcinogenic. It confirms that the substance is not mutagenic to the particular bacterial strain used and for the genetic endpoint tested. Hence the absence of mutagenicity on the tested plant extracts is a positive step forward in determining their safe use as a diarrhoea treatment. However, they should be further investigated for therapeutic properties. Furthermore, medicinal plants used for treating diarrhoea are administered orally and may therefore be subjected to metabolic and enzymatic activation within the human body. Thus, further genotoxicity testing of these plants is required using other screening methods, as well as performing the Ames test in the presence of S9 metabolizing enzymes.

Table 6.1: Number of revertant colonies of *Salmonella typhimurium* strains TA1537, TA1535 and TA98 induced by extracts of some plants used as remedies for the treatment of diarrhoea in Bizana, Eastern Cape

Plant species	Extract	Bacterial strain								
		TA1537			TA1535			TA98		
		Extract concentration (µg/ml)								
		5000	500	50	5000	500	50	5000	500	50
Tecoma capensis	DCM	14.0 ±0.6	12.0 ± 1.5	11.7 ± 1.8	14.33 ± 0.8	13.7 ± 0.8	13.7 ± 1.8	60.3 ± 3.4	47.7 ± 0.7	39.0 ± 0.6
	PE	11.0 ± 1.0	8.7 ± 0.3	9.3 ± 0.3	8.0 ± 1.0	8.3 ± 1.5	5.0 ± 0.0	31.3 ± 2.7	35.0 ± 2.5	36.0 ± 1.2
	EtOH	13.0 ±1.5	11.0 ± 1.5	14.0 ± 1.0	10.3 ± 1.8	8.3 ± 0.9	6.7 ± 1.3	43.0 ± 4.5	36 ± 1.5	36.7 ± 2.3
	Water	9.0 ±0.6	10.0 ± 1.2	9.7 ± 1.2	8.0 ± 1.0	7.7 ± 1.3	7.7 ± 1.5	63.7 ± 5.4	59.0 ± 0.6	45.0 ± 1.2
Clutia pulchella	DCM	10.0 ± 1.0	8.7 ± 2.0	10.0 ± 1.0	14.1 ± 1.2	8.7 ± 2.0	14.0 ±0.6	63.3 ± 0.9	60.0 ± 3.2	53.0 ± 2.5
	PE	11.3 ± 2.0	9.7 ± 0.3	9.3 ± 0.3	7.7 ± 0.9	9.3 ± 0.9	6.3 ± 0.3	56.3 ± 0.7	33.3 ± 0.9	46.3 ± 2.9
	EtOH	13.3 ± 2.0	13.3 ± 1.7	12.0 ± 1.5	13.0 ± 2.1	9.0 ± 2.1	8.0 ± 0.6	64.3 ± 2.2	52.7 ± 0.9	29.7± 0.9
	Water	9.3 ± 0.7	11.7 ± 2.4	12.3 ± 1.9	7.0 ± 0.0	8.7 ± 1.7	12.0 ± 1.5	63.7 ± 1.9	35.3 ± 1.3	33.3 ± 1.8
Ficus craterostoma	DCM	13.0 ± 2.6	10.0 ± 0.0	11.7 ± 1.2	12.0 ± 1.5	9.0 ± 0.6	8.0 ± 1.0	63.7 ± 1.9	35.3 ± 1.3	33.3 ± 1.8
	PE	9.0 ± 1.2	12.3 ± 1.2	12.0 ± 0.0	6.0 ± 1.0	6.3 ± 0.3	6.3 ± 1.2	34.7 ± 1.5	42.0 ± 1.5	45.7 ± 2.3
	EtOH	14.0 ± 1.5	13.3 ± 1.8	15.3 ± 0.8	8.3 ± 1.7	7.7 ± 1.2	7.7 ± 1.2	45.7 ± 2.4	35.7 ± 0.7	38.0 ± 0.6

Plant species	Extract	Bacterial strain								
		TA1537			TA1535			TA98		
		Extract concentration (µg/ml)								
		5000	500	50	5000	500	50	5000	500	50
	Water	11.7 ± 0.9	11.3 ± 1.9	11.0 ± 1.5	7.3 ± 1.2	11.0 ± 1.5	10.0 ± 0.6	64.3 ± 2.3	60.3 ± 0.3	50.3 ± 5.8
Maesa lanceolata	DCM	11.0 ±1.5	9.7 ± 0.7	12.7 ± 1.5	6.7 ± 1.2	10.3 ± 0.7	9.3 ± 0.3	39.7 ± 4.3	46.3 ± 4.1	34.7 ± 0.3
	PE	13.3 ± 1.7	14.0 ± 0.6	14.3 ± 0.9	10.0± 0.6	10.0 ± 0.6	14.3 ± 0.3	58.3 ± 0.9	51.7 ± 0.9	51.3 ± 0.3
	EtOH	9.3 ± 0.3	13.0 ± 1.5	11.3 ± 2.4	14.6 ± 0.9	14.6 ± 0.8	13.6 ± 1.2	42.7 ± 1.8	44.7 ± 0.7	42.0 ± 1.5
	Water	9.7 ± 1.7	10.3 ± 0.3	9.3 ± 0.3	11.0 ± 0.6	8.7 ± 0.9	9.7 ± 1.7	39.7 ± 2.0	33.7 ± 1.3	38 ± 1.7
Trema orientalis	DCM	12.3 ± 1.9	10.7 ± 1.8	9.3 ± 1.8	11.7 ± 0.9	7.3 ± 0.3	9.3 ± 0.3	63.7 ± 2.6	51.3 ± 0.9	51.0 ± 3.6
	PE	12.7 ± 1.3	8.7 ± 0.3	14.0 ± 1.0	12.7 ± 1.2	14.3 ± 1.5	11.3 ± 0.9	62.7 ± 1.5	67.3 ± 3.8	65.3 ± 2.0
	EtOH	10.3 ± 0.9	14.7 ± 0.7	14.7 ± 0.7	10.7 ± 0.3	12.0 ± 0.6	10.3 ± 0.3	34.3 ± 2.6	34.7 ± 2.0	38.7 ± 3.3
	Water	8.7 ± 0.9	13.7 ± 0.3	10.7 ± 0.7	8.3 ± 0.8	7.3 ± 1.3	8.0 ± 1.0	57.7 ± 3.4	46.3 ± 3.2	36.0 ± 1.5
Searsia chirindensis	DCM	9.3 ± 2.4	10.0 ± 1.0	14.6 ± 0.3	7.3 ± 2.6	12.3 ± 1.7	6.3 ± 0.3	61.3 ± 0.9	59.7 ± 3.8	66.3 ± 7.8
	PE	14.7 ± 0.7	11.3 ± 1.9	15.0 ± 0.6	14.3 ± 0.7	9.3 ± 0.7	13.6 ± 0.3	37.0 ± 0.5	36.3 ± 1.2	36.0 ± 3.0
	EtOH	13.0 ± 1.5	9.3 ± 2.4	12.0 ± 2.0	9.3 ± 1.2	11.3 ± 0.7	10.0 ± 0.0	33.0 ± 2.5	37.0 ± 1.5	37.3± 1.2
	Water	8.7 ± 0.3	9.0 ± 0.6	10.3 ± 0.3	6.3 ± 0.7	6.3 ± 0.3	6.0 ± 0.0	56.3 ± 4.7	52.3 ± 1.5	44.7 ± 1.5

Plant species	Extract	Bacterial strain								
		TA1537			TA1535			TA98		
		Extract concentration (µg/ml)								
		5000	500	50	5000	500	50	5000	500	50
Rapanea melanophloeos	DCM	10.3 ± 0.9	12.7 ± 1.9	8.7 ± 0.9	7.7 ± 0.3	7.7 ± 1.2	8.0 ± 1.5	40.0 ± 1.7	46.0 ± 0.3	42.3 ± 1.9
	PE	11.7 ± 1.7	13.6 ± 0.9	12.3 ± 0.9	14.3 ± 1.2	13.7 ± 0.3	14.0 ± 1.5	44.3 ± 2.3	46.3 ± 0.9	43.0 ± 2.5
	EtOH	9.7 ± 0.3	9.3 ± 1.3	10.3 ± 0.9	10.7 ±1.2	13.7 ± 0.3	12.0 ± 0.6	37.3 ± 0.9	34.3 ± 1.9	35.7 ± 1.2
	Water	9.3 ± 0.3	9.0 ± 0.6	9.7 ± 0.3	5.7 ± 0.3	6.3 ± 0.9	5.7 ± 0.9	49.3 ± 5.4	52.0 ± 3.5	43.0 ± 2.6
Dais cotinifolia	DCM	11.3 ± 1.5	10.7 ± 0.3	14.0 ± 0.6	6.7 ± 1.9	9.0 ± 1.5	8.7 ± 2.0	42.7 ± 3.8	45.7 ± 1.9	40.0 ± 4.7
	PE	14.3 ± 0.3	9.3 ± 0.7	12.3 ± 1.5	13.0 ± 1.0	14.0 ± 0.6	14.0 ± 0.6	61.3 ± 0.9	68.3 ± 7.4	60.3 ± 1.2
	EtOH	14.3 ± 0.3	12.7 ± 1.7	10.3 ± 0.9	8.3 ± 1.2	10.7 ± 2.2	13.3 ± 0.9	34.3 ± 1.2	36.3 ± 1.2	34.7 ± 1.7
	Water	14.0 ± 2.3	11.7 ± 1.2	12.3 ± 0.9	8.3 ± 0.9	10.0 ± 1.0	7.3 ± 0.7	56.7 ± 3.3	43.7 ± 1.9	32.3 ± 1.2
Hydnora africana	DCM	15.0 ± 0.0	12.7 ± 1.4	12.3 ± 1.2	7.7 ± 0.9	9.3 ± 2.3	10.7 ± 1.2	46.3 ± 0.9	40.0 ± 1.2	35.3 ± 1.2
	PE	8.7 ± 0.7	9.0 ± 0.6	10.3 ± 0.8	6.0 ± 0.0	6.7 ± 0.7	6.3 ± 1.2	51.3 ± 1.9	40.7 ± 0.7	32.0 ± 0.6
	EtOH	13.0 ± 1.5	11.0 ± 0.6	11.0 ± 0.6	7.3 ± 0.3	7.7 ± 1.7	8.0 ± 0.6	46.0 ± 1.7	48.0 ± 0.5	47.0 ± 0.6
	Water	13.3 ± 0.3	14.0 ± 0.0	12.3 ± 1.2	12.7 ± 1.7	11.0 ± 0.6	7.7 ± 0.3	44.3 ± 0.7	38.0 ± 1.2	35.0 ± 1.2
4NQO (+ve control)		133.0 ± 2.8			109.3 ± 3			144.7 ± 4.4		
Water (-ve control)		8.0 ± 0.6			9.0 ± 1.5			36.7 ± 1.5		

4NQO = 4-nitroquinoline-N-oxide

6.5. Conclusions

The extracts of selected plants that showed good antibacterial activities showed negative genotoxicity results in the Ames test. These results offer supporting evidence of a prolonged safe use of the tested plant extracts. Compounds from these plants need to be individually isolated as is the case with maesasaponin isolated from *M. lanceolata* by **SINDAMBIWE et al.** (**1998**), as they could be none genotoxic thus preferable in the preparation of antibacterial drugs. However more assays *in vitro* and *in vivo* are urgently required to confirm the lack of mutagenicity.

Chapter 7: General conclusions

Worldwide, medicinal plants have been used for managing diseases since the beginning of human civilization, and they still continue to supply mankind with medicinal remedies, with their benefits being handed down through generations. They form an important aspect of the daily lives of many people, and are an important part of the South African cultural heritage (**RAMALIVHANA et al., 2010**). When evaluating the effectiveness of medicinal plants it is important to work directly with people who use them to have an understanding of how and why they are used (**OGIE-ODIA and OLUOWO, 2009**). Ethnobotanical studies are important in revealing locally important plant species and are one of the major methods for selecting plants for pharmacological evaluation (**OGBOLE et al., 2010**). Several studies have been conducted in South Africa using different techniques, including laboratory and survey based approaches, to evaluate the effectiveness of traditional medicine used in the treatment of diarrhoea (**LIN et al., 2002; MATHABE et al., 2006; APPIDI et al., 2008; FAWOLE et al., 2009a; BISI-JOHNSON et al., 2010**). There is, however, a lot of unrecorded ethnobotanical information that still needs to be collected from the local people for pharmacological evaluation (**REVATHI and PARIMELAZHAGAN, 2010**). For these reasons, an ethnobotanical study was done in six rural areas of Bizana, Pondoland in the Eastern Cape, South Africa to record plants that are currently used in the treatment of diarrhoea for pharmacological evaluation.

The study revealed 34 plant species belonging to 21 families that are used in Bizana. These are: *Brunsvigia grandiflora*, *Searsia chirindensis*, *Senecio serratuloides*, *Tecoma capensis*, *Trema orientalis*, *Aster bakerianus*, *Cyperus dives*, *Clutia pulchella*, *Acacia mearnsii*, *Alysicarpus rugosu*s, *Elephantorrhiza elephantine, Pelargonium luridum*, *Hydnora africana, Berkheya bipinnatifida* (Harv.) Roessler subsp. *Bipinnatifida*, *Eucomis autumnalis*, *Hypoxis hemerocallidea*, *Ocotea bullata*, *Ledebouria ovatifolia*, *Malva parviflora*, *Turraea obtusifolia*, *Aloe ferox*, *Ficus craterostoma*, *Maesa lanceolata*, *Rapanea melanophloeos*, *Eucalyptus camaldulensis*, *Psidium guajava*, *Prunus persica*, *Pentanisia prunelloides*, *Ziziphus mucronata, Zanthoxylum capense*, *Physalis peruviana*,

Solanum aculeastrum, Dais cotinifolia and *Rhoicissus tridentate*. It was disconcerting to hear from most of the informants that nothing was being done to conserve the plants they are using. It is worth mentioning that the mentioned medicinal plants are least threatened according to the current **INTERNATIONAL UNION FOR CONSERVATION OF NATURE** (**2011**) data list. However, three (*E. autumnalis*, *H. hemerocallidea*, and *R. melanophloeos*) are reported to be declining according to the 2009 South African red data list and one (*O. bullata*) is endangered (**RAIMONDO et al., 2009**). This raises concern as *O. bullata* is a slow growing, slow reproducing species highly vulnerable to excessive collection (**ZSCHOCKE et al., 2000**). Therefore sustainable utilization and suitable propagation techniques of these plants are needed as a matter of urgency before these plants become seriously threatened or extinct.

Eleven plant species recorded in this study have not been previously reported as remedies for diarrhoea. These plant species are *R. melanophloeos*, *F. craterostoma*, *A. ferox*, *L. ovatifolia*, *D. cotinifolia*, *B. bipinnatifida* subsp. *bipinnatifida*, *A. rugosus*, *C. dives*, *S. seratulloides, M. lanceolata* and *B. grandiflora*. Of the 34 plants mentioned, nine were selected for bioassays because; they had higher frequency indices (mentioned more by the informants) and had never been investigated before (against diarrhoeal pathogens, anti-inflammatory properties, and for safety). These were *C. pulchella*, *D. cotinifolia*, *F. craterostoma*, *H. africana*, *M. lanceolata*, *R. melaphloeos*, *S. chirindensis*, *T. capensis*, and *T. orientalis*.

Four solvents (PE, DCM, 70% EtOH and water) were used for extraction in this study because of their wide polarity range. The highest yield was obtained from the 70% EtOH extracts of *D. cotinifolia* (26.07%), and the lowest yield for the PE extract of the same plant (2.18%).

The extracts of most of the plant species evaluated had good antibacterial activity at least against one of the test bacteria. At least two species of all the investigated plants exhibited good antibacterial activity against all test bacteria. It is noteworthy that the 70% EtOH and water extracts of *S. chirindensis* and *R. melanophloeos* exhibited high activity

against both Gram-negative and Gram-positive bacterial strains tested. This suggests that these plants have important antibacterial properties and their compounds need to be selectively isolated and evaluated further. Findings from this study were interesting as they confirm for the first time the antibacterial activities of all the evaluated plant species except for *T. capensis*. This confirms the presence of important bioactive principles active against bacteria in the studied plants. In view of the fact that these plants were selected based on their ethnobotanical usage for treating diarrhoea, the good antibacterial activity of the plant extracts is of paramount importance to the users. Bioactivity cannot be completely ruled out in some of the extracts that did not show positive results as there is a possibility that the plant extracts may have activity against other pathogenic bacteria than those tested or may act through a different mechanism than the one tested in this study.

In light of the fact that gastro-intestinal ailments are associated with inflammation of the digestive tract, resulting in abdominal pains and cramps of varying degrees (**BARBARA, 1998**), the nine selected medicinal plants were investigated for their inhibition of COX enzymes. COX-1 was inhibited by more plant extracts (estimate in %) than COX-2. For both enzymes, the non-polar solvent extracts were more active than polar extracts. The highest inhibition of COX-1 was shown by EtOH extracts of *F. craterostoma* and DCM extract of *S. chirindensis* at 100%. The highest COX-2 inhibition was shown by water extracts of *F. craterostoma* and DCM extracts of *T. capensis* at 99.5% and 99.0% respectively. It is interesting to note that the extracts of some plants showed higher inhibition of COX-2 than COX-1 as inhibition of this enzyme is vital for the treatment of inflammation.

A plate incorporation procedure for the Ames test using *S. typhimurium* strains TA98, TA1535 and TA1537 without S9 metabolic activation was used to evaluate genotoxicity of plant extracts. The results revealed that extracts of the selected plants were non-mutagenic in the Ames test. These results offer encouraging evidence of prolonged safe use of the tested plant extracts. Compounds need to be selectively isolated from these

plants to see if they are not genotoxic, as is the case with maesasaponin isolated from *M. lanceolata* (**SINDAMBIWE et al., 1998**).

The results obtained in this study show that people still possess a rich and diverse knowledge regarding the traditional uses of plants against diarrhoea, and that plants form part of the cultural heritage of the communities. However, these knowledge holders are ageing, and the information is likely to disappear fairly soon. It is therefore urgent to save the cultural heritage of the people by confirming therapeutic uses of plants through scientific methods. Thus the results presented in this study go a long way in documenting indigenous knowledge on the use of traditional plants in Bizana, Pondoland. Measures need to be taken to protect declining *E. autumnalis*, *H. hemerocallidea*, and *R. melanophloeos* and threatened *O. bullata* populations. People still need to be educated about the importance of sustainable use of plants to prevent or avoid overexploitation. The plants evaluated in this study could be active against other resistant bacteria that cause diarrhoea such as *S. typhi*, S*higella* spp. and *V. cholera*. Thus, further testing is required to investigate inhibition of some bacterial strains not tested in this study. Additionally, it is important to test for other parameters which could involve bioavailability of those extracts that were found to be active. Isolation and identification of COX inhibiting compounds in extracts of *T. capensis*, *F. craterostoma*, *M. lanceolata* and *D. cotinifolia* that showed high inhibitory activity of COX-2 enzyme will be of great importance in the search for novel anti-inflammatory drugs from natural sources. Therefore, further studies using both *in vitro* and *in vivo* models are urgently required in order to reach a solid conclusion on the potential of these plants as sources of novel antimicrobial agents and to confirm the results observed.

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Cover page of questionnaires

The study	Plants used for treating diarrhoea in Bizana, Pondoland

Name	

Title	

Age group	

Sex	

Occupation	

Trained or untrained	

Appendix 1

Questionnaire 1: Traditional healer questionnaire

1.   Do you know diarrhoea?

Yes	No

2.   Do you know diarrhoea symptoms?

Yes	No

3.   Do you know any diseases that are linked to diarrhoea?

Yes	No

4.   What are some of the disease linked to diarrhoea that you treat?

5.   Do you treat diarrhoea?

Yes	No

6.   IF yes, what do you use to treat diarrhoea?

7.   How effective is your treatment of diarrhoea?

8.   Do you experience cases where patients show adverse reactions to your treatment?

Yes	No

9.   Do patients try self-treatment of diarrhoea before consulting you?

Yes	No

10.   How do you feel about patients going to hospitals or clinics for further treatment?

11.   Do you use medicinal plants to treat diarrhoea?

Yes	No

12.   Which plants do you use for the treatment of diarrhoea?

13.   Which medicinal plants have you used the most for treating diarrhoea?

14.   Which part do you use for treating diarrhoea?

15.   Where are those plants usually found (distribution)?

16.   When do those plants grow (season)?

17.	Which time of the year do people consult you the most for treatment of diarrhoea?

Summer	Autumn
Winter	Spring

18.	Are those plants that you use for treating diarrhoea available in your area?

19.	Do you use cultivated plants for the treatment of diarrhoea?

Yes	No

20.	If no, why?

21.	Do you move to new areas to buy medicinal plants?

Yes	No

22.	How do you prepare your medication?

23.	Do you mix or use your plant singly?

Mix	Don't mix

24.	If you do, do you mix them with other plants or western mixtures? (Salt, vinegar)

25.	Are there any other diseases that you treat using the same plants that you use to treat diarrhoea?

26.	How long can you keep medicinal plants before they loose their healing properties?

27.	Do you treat both males and females?

28.	Do you treat pregnant women?

29.	Do you treat young man during circumcision school?

30.	Where do most of the people you treat come from?

31.	Are the people you treat (tick only in the appropriate box):

Well dressed	[ ]	Poorly dressed	[ ]
Literate	[ ]	Illiterate	[ ]
Speak Xhosa	[ ]	Speak other languages	[ ]
Wealthy	[ ]	Poor	[ ]

32.	What do you do to ensure that there are plants for you to harvest in the future?

33.	Are the medicinal plants that you use in the greatest demand?

34.	Which of the age categories do you belong to?

15-25	26-35
36-45	46-55
56-65	66+

35.   Do you treat diarrhoea at any age group (children, young adults and   adults)?

36.   What are the age categories of your patients?

0-7	8-14
15-25	26-35
36-45	46-55
56-65	66+

37.   What is the gender of the patients who visits you the most?

Male   [ ]   Female   [ ]

38.   How many people do you treat per day?

39.   What unit of measure do you use for medication? (Cup/ spoon)

40.   What is the average cost of   treatment per patient?

41.   Where do you buy or collect these medicinal plants? (name of place)

42.   Do you have to move to new areas to collect your medicinal plants?

Appendix 2

Questionnaire 2: A questionnaire for ordinary villagers/domestic users

1.   Do you know diarrhoea?
2.   Do you treat diarrhoea?
3.   What do you use to treat diarrhoea?
4.   How effective is your treatment of diarrhoea?
5.   How often do you use medicinal plants?
6.   Do you know plants that are used for treating diarrhoea?
7.   Which plants do you use for treating diarrhoea?
8.   Which parts do you use for the treatment of diarrhoea?
9.   How is the medication prepared?
10. What unit of measure do you use for medication? (Cup/ spoon)
11. Is the treatment for males or females or both?
12. Do pregnant women use it for treating diarrhoea?
13. Do young men use it for treating diarrhoea during circumcision school?
14. If no, why?
15. Do you go to hospitals or clinics for further treatment?
16. Where are those plants usually found? (distribution)
17. When do those plants grow (season)?
18. Would you mind showing me where they grow?
19. Are those plants that you use for treating diarrhoea available in your area?
20. Are the medicinal plants that you use in greatest demand?
21. What do you do to ensure that there are plants for you to harvest in the future?