PHARMACOLOGICAL ACTIVITIES OF SELECTED SOUTH AFRICAN MEDICINAL PLANTS

Ambrose Okem

Submitted in fulfilment of the academic requirements for the degree of Master of Science

In the
Research Centre for Plant Growth and Development
School of Biological and Conservation Sciences
University of KwaZulu-Natal
Pietermaritzburg

April 2011

STUDENT DECLARATION

Pharmacological activities of selected South African medicinal plants

I, **Ambrose Okem**, student number: **210551070**

i. The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg;

ii. This dissertation has not been submitted for any degrees or examination at any other University;

iii. This thesis does not contain data, Figures or writing, unless specifically acknowledged, copied from other researchers.

Signed at ……..……..on the…….…day of……2011.

SIGNATURE

DECLARATION BY SUPERVISORS

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

Student's Full Name: Ambrose Okem

Student Number: 210551070

Thesis Title: Pharmacological activities of selected South African medicinal plants

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.

SUPERVISOR: PROFESSOR J. VAN STADEN

CO-SUPERVISOR: DR J.F. FINNIE

FACULTY OF SCIENCE AND AGRICULTUR DECLARATION - PLAGIARISM

I, **Ambrose Okem**, Student number: **210551070**, declare that

1. The research reported in this thesis, except where otherwise indicated is my original research.

2. This thesis has not been submitted for any degree or examination at any other University.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
   b. Where their exact words have been used, then their writing has been placed inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed……..

ACKNOWLEDGEMENTS

I would to express my profound appreciation to:

- God almighty, the alpha and omega of my life for giving me the strength, courage and good health throughout my studies.
- My supervisor, Professor J. Van Staden for supervising my studies, and for his fatherly advice, enormous encouragement and understanding. I will always remain grateful to you.
- My co-supervisor, Dr. J.F. Finnie for his invaluable advice, encouragement and support throughout my research and the writing of the thesis.
- University of KwaZulu-Natal for awarding me a scholarship for my MSc programme.
- My family, Dad and Mum, Sylvester of the blessed memory, and my siblings for their amazing love, moral support and encouragement throughout my studies. You have all been the source of my strength.
- All my friends and colleagues at the Research Centre for Plant Growth and Development, for their friendly assistance, advice and encouragement. You have all been so supportive.
- Mrs A. Young (Horticulturalist UKZN Botanical Gardens) and Dr. C. Potgieter (NU Herbarium) for their assistance in plant identification and voucher specimen preparations.

ABSTRACT

The use of traditional medicine is a popular practice in South Africa especially among rural dwellers due to several reasons such as availability of natural products, cultural beliefs, preference of natural products to synthetically derived drugs and the high cost of modern drugs. Traditional healers in South Africa play key roles in administering treatment for all sorts of ailments using plants. The aim of this study was to evaluate the efficacy of seven selected medicinal plants that are used in traditional medicine to treat stomach-related ailments for their pharmacological and phytochemical properties.

Plant material was extracted sequentially with ethyl acetate (EtOAc), ethanol (EtOH) and water. The extracts were evaluated for their antimicrobial activities using the microdilution technique against two Gram-positive (*Enterococcus faecalis* ATCC 19433 and *Staphylococcus aureus* ATCC 12600) bacteria and a Gram-negative (*Escherichia coli* ATCC 11775) bacterium. A modified microdilution technique was used to screen for antifungal activity against a yeast-like fungus (*Candida albicans* ATCC 10231). Only the EtOAc extract of *Tetradenia riparia* demonstrated good antibacterial activity against the Gram-negative *E. coli,* all the other extracts that were active only showed good antibacterial activity against the two Gram-positive (*E. faecalis* and *S. aureus*) bacteria with MIC values <1 mg/ml. None of the extracts that exhibited good inhibitory activity showed corresponding bactericidal activity against the bacterial test strains, suggesting that the observed activity were all inhibitory. Good antifungal activity with an

MIC value <1 mg/ml was observed in only 5 extracts, and none of the extracts exhibited corresponding fungicidal activity.

The *in vitro* colorimetric assay for anthelmintic activity against *Caenorhabditis elegans* revealed that almost all the extracts possessed moderate to high anthelmintic properties. The EtOAc extract of *T. riparia* had the best activity at MLC value of 0.004 mg/ml.

The anti-inflammatory activity of the plant extracts was tested using the cyclooxygenase assays to determine their inhibitory potential against COX-1 and COX-2 enzymes. All the EtOAc extracts demonstrated both COX-1 and COX-2 inhibitory activity in the range of 50.7 ± 2.4 to 99.5 ± 0.5%. Apart from the EtOH extracts of *C. multicava* that showed high inhibitory activity against both COX-1 and COX-2, all the other EtOH extracts were COX-2 selective. Aqueous extracts exhibited poor inhibitory activity against both COX-1 and COX-2 enzymes with the exception of *T. riparia* and *Coddia rudis* that showed good inhibitory activity (69.1 ± 0.9 and 92.65 ± 0.7%) against COX-1 and COX-2 respectively.

The standard plate incorporation assay for the Ames test was carried out to determine the potential genotoxic effects of the plant extracts and this revealed that all the extracts were non-mutagenic towards *Salmonella typhimurium* tester strains TA98, TA100 and TA1537 without metabolic activation. However, further studies incorporating metabolizing enzymes are needed to confirm the safe use of the studied plants.

Phytochemical analysis revealed relatively high amounts of total phenolics, gallotannins and flavonoids in all the evaluated plants. Total and steroidal saponins were detected in only two

plant samples, *Canthium spinosum* and *Cassinopsis ilicifolia* (bark). These findings present useful information on the types of bioactive compounds that could be responsible for the pharmacological activities observed among some of the plant extracts.

The results obtained in this study showed different levels of pharmacological activities among all the evaluated medicinal plants which provide scientific validation for their use in traditional medicine as antimicrobial agents. Phytochemical analysis provides valuable information for further study that will be aimed at isolation and identification of the bioactive principles in the evaluated plant species.

CONFERENCE CONTRIBUTIONS

Oral presentation

1. Okem, A., Finnie, J.F. and Van Staden, J. Investigation of antibacterial and anthelmintic activity of South African medicinal plants. Faculty of Science and Agriculture Research Day, University of KwaZulu-Natal Piertermaritzburg. 23 September 2010.

2. Okem, A., Finnie, J.F. and Van Staden, J. Pharmacological effects of selected South African medicinal plants. 37th Annual Conference of the South African Association of Botanist (SAAB), Rhodes University, Grahamstown. South Africa. 17-19 January 2011.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

4-NQO…..	4-nitroquinoline-N-oxide	MBC….....	minimum bactericidal concentration
AA……	arachidonic acid	MFC……	minimum fungicidal concentration
AIDS…...	acquired immunodeficiency syndrome	MIC…….	minimum inhibitory concentration
AR……...	anthelmintic resistant	MLC…...	minimum lethal concentration
ATCC.....	American type culture collection	mRNA….	messenger ribonucleic acid
ATM….....	African traditional medicine	MRSA….	methicillin- resistant *Staphylococcus aureus*
CAM…...	complementary alternative medicine	NAM…...	N-acetylmuramic acid
CTE….....	catechin equivalent	NAG….....	N-acetylglucosamine
COX….....	cylooxygenase	NCEs…...	new chemical entities
CNS….....	central nervous system	NFkB…...	nuclear transcriptase factor kB
DE……...	diosgenin equivalent	NSAIDs...	non-steroidal anti-inflammatory drugs
DMSO.....	dimethyl sulphoxide	PBPs….....	penicillin-binding proteins
DNA…...	deoxyribonucleic acid	PGs….....	prostaglandins
EtOAc....	ethyl acetate	PGE_2…...	prostaglandin E_2
EtOH….	ethanol	PGG_2…...	prostaglandin G_2
GAE…...	gallic acid equivalent	PGH_2…...	prostaglandin H_2
iCOX….	inducible cyclooxygenase	PGI_2….....	prostaglandin I_2
IBDs.....	inflammatory bowel diseases	ROS…...	reactive oxygen species
IkB…...	inhibitor kB kinase	TNF-α….	tumor necrosis factor
IL-1……	interleukin-1	TXA_2…..	thromboxane A_2
iNOS.....	inducible nitric acid oxide synthesis		
INT…...	*p*-iodonitrotetrazolium violet		
LOX…..	lipoxygenase enzyme		

Chapter 1

Literature review

1.1 Introduction

The use of plants as herbal medicines since ancient times is evident in man's quest for knowledge to cure ailments. Throughout the history of mankind, different kinds of infectious diseases have been treated with plant extracts (**BUWA and VAN STADEN, 2006**), through traditional preparations like decoctions, concoctions, teas and infusions (**VAN WYK and WINK, 2004**). Traditional health care preparations made from plants continue to provide mankind with new remedies. Some of these practices have been described in ancient texts such as the Vedas and the Bible (**HOAREAU and EDGAR, 1999**).

Dioscorides inscription 'Great Pharmacology' was the first book published on the use of plants as complementary alternative medicine (CAM) which detailed about 500 healing plants used in traditional medicine (**DÖRFLER and ROSELT, 1989**). The ancient Egyptian literature, which dates back to about 3000 BCE, mentions the use of *Ricinus communis* seeds, *Citrilus colocynthes*, *Senna alexandrina* and *Prunica granatum* roots in large quantities for therapeutic purposes. Hence, it is believed that modern medicine had its origin in Egypt along the Nile Valley. However, one of the most important pharmaceutical records was the Ebers Papyrus (1500 BCE) which give precise diagnostic, symbolic and traditional plant-based treatments for all sorts of illness (**HEINRICH *et al.*, 2004**).

1.1.1 Traditional medicine

Traditional medicine (TM) is the sum total of knowledge, skills and practices which are based on beliefs and experiences indigenous to different cultures. The information on TM is usually passed orally from one generation to the next, and this information has been utilized in maintaining health, preventing, diagnosing and treating illnesses (**WHO 2003**). In some cultures around the world the use of TM is often associated with witchcraft and superstition, due to lack of scientific proof to explain the healing power of plants. One example of such a concept is the doctrine of signatures. This is based on the assumption that the morphology of plants may give clues to their medicinal properties. For instance red juice and sap are associated with blood and menstrual ailments (**VAN WYK *et al.*, 1997**).

The use of indigenous medicine especially at the primary health care level is becoming a top priority among developed and developing countries (**McGAW *et al.*, 2000**). The revival of interest in the use of phytomedicine is at a global level, as demonstrated in the annual sales of herbal products worth over US$ 100 billion in the world. For instance in 2002 more than 62% of Americans used CAM contributing over US$ 20 billion to the industry. The Eastern world is already well known for its adherence to herbal medicine. China and India are two leading countries in this regard. Even in the Western world, popularity of phytomedicine is increasing rapidly. Germany is the leading country in Europe followed by France in the use of botanical supplements (**BARNES *et al.*, 2004; GILANI and RAHMAN, 2005**). The increasing interest in the use of medicinal plants has prompted scientific studies into natural products to determine whether their traditional uses are supported with pharmacological effects or if their use is merely based on folklore (**SPARG *et al.*, 2002**).

1.1.2 Traditional medicine in South Africa

African Traditional Medicine (ATM), as a major socio-cultural heritage, has been in existence for thousands of years. This is the oldest and perhaps the most diverse of all medical systems that was once believed to be primitive and strongly opposed during colonial days and subsequently by medical practitioners (**ELUJOBA *et al.*, 2005; GURIB-FAKIM, 2006**).

Comprehensive documentation on the use of medicinal plants in ATM is lacking and with the rapid urbanization; oral transmission of traditional knowledge is dwindling very fast. This implies that the reservoir of traditional knowledge will be completely lost if not retrieved from the herbal healers, because the death of an old traditional healer will result in the loss of a valuable mental library (**RAMAWAT, 2009**).

South Africa is renowned for its plant biodiversity. More plant species occur here than in any other region of comparable size, making South Africa the richest country on earth in terms of floral wealth (**VAN JAARSVELD, 2005**). In South Africa, more than 60-80% of the population relies mainly on TM for their primary health care needs (**DAUSKARDT, 1990**). In most cases traditional healers are the first health care providers to be consulted, especially in rural areas where the practice is deeply interwoven into the cultural and spiritual life of the people (**WHO, 2001**). Traditional healers in South Africa make use of a wide variety of plant species to treat different kinds of infectious diseases which are prevalent in rural areas (**McGAW *et al.*, 2000**). For instance, in KwaZulu-Natal, more than 1032 plant species from over 147 families are used in TM (**LIGHT *et al.*, 2005**). Most of the commonly utilized medicines in South Africa are still derived from plants, many of which are sold in both the

informal and commercial sectors of the economy (**VERSCHAEVE *et al*., 2004**). Due to the increasing interest in the use of TM, the need to meet some of the pressing challenges such as: the general lack of research, the demand for patenting rights, evidence of safety, efficacy and good quality of TM products and, the need to integrate and maximize natural products as potential sources of primary health care remedies, must all be quantified for the acceptable use of TM in modern therapeutics (**GAMANIEL and JSSELMUIDEN, 2004; MUHAMMAD and AWAISU, 2008**). The prescription and use of TM in South Africa is currently not regulated, which implies that there is a danger of misadministration. The prolonged use of some of the most popular herbal products might have some potential genotoxic effects (**FENNELL *et al*., 2004**).

1.2 Prospects in drug discovery and development from plants

Drug discovery is generally based on three principal approaches: screening of natural products based on ethnobotanical uses or by random screening; the modification of structures of a known drug; and the synthesis of substances based on the knowledge of the biological process with which the drug is to interact (**LAURENCE and BENNETT, 1980**). Biologically active compounds from plant species used in TM have now become the major focus for developing new antimicrobial agents. This is as a result of the side effects and the resistance that pathogenic organisms develop against the antibiotics currently used (**ESSAWI and SROUR, 2000**). It is estimated that more than 50% of drugs in clinical use today are derived from plant products and their derivatives (**GURIB-FAKIM, 2006**).

In spite of the great advancement in instrumentation and achievement in the pharmaceutical industries in the search for drug lead compounds from plants, much of the plant biodiversity still remains unstudied as sources of novel principles (**CRAGG and NEWMAN, 2007**). The process of drug discovery and development from plants is faced with several challenges some of which include: most of the natural products from plants are present in very small quantities which are insufficient for drug lead development and optimization; the problem of innovative deficits that are adversely affecting the pharmaceutical industry (such as the application of ethnopharmacological research findings in pharmaceutical development, advancing the research findings to the level of biosciences, and making the research work meaningful for the local population); and it was estimated that only one out of 5000 lead compounds isolated from a plant will successfully advance through clinical trials and subsequently be approved for use (**ETKIN, 2001; BALUNAS and KINGHORN, 2005**). Hence, the pharmacognosists, phytochemists and other natural scientists need to drastically improve the quality and quantity of lead compounds that will enter the drug development phase in order to keep up with the pace of other drug discovery fields such as the genomics and proteomics (**BALUNAS and KINGHORN, 2005**).

1.3 Ethnobotanical approach in drug development from plants

The advantage of choosing plants as a starting point for the elaboration of new chemical entities (NCEs) through ethnobotanical surveys is that, the active constituents of plants which have undergone long-term usage by human are likely to be safer than the active compounds isolated from plants with no history of ethnomedical use (**FABRICANT and**

FARNSWORTH, 2001). Ethnopharmacology has played a significant role in the progress of conventional medicine and is likely to become increasingly important in years to come. A cooperative approach by ethnobotanists, ethnopharmacologists, physicians and phytochemists is therefore essential to spur the progress of medicinal plant research (**GILANI and RAHMAN, 2005**).

Plants have been the source of natural chemical structures which provide almost unimaginable chemical diversity with many functional groups. Some of the natural products from plants have been identified as ideal molecules that can interact specifically with biological target units, which can be utilized in the treatment of human and animal diseases **(McCHESNEY *et al*., 2007; AKINPELU *et al*., 2008**). Bioactive natural products from plants have enormous economic importance. They can be used as novel; feedstock products, dietary supplements, flavours, fragrances, dyes, cosmetics and insecticides (**GURIB-FAKIM, 2006**).

Drug discovery from plants requires the combined efforts of botanists, pharmacognosists, phytochemists and other natural scientists to screen plant products through the use of improved isolation techniques to meet the demand of pharmaceutical companies (**RAMAWAT, 2009**). This is done by using a large number of samples from correctly identified plant species for high throughput screening, elaborate arrangements of novel compounds and preclinical screening which include pharmacological, toxicological and pharmacokinetic studies (**BORRIS, 1996**). The use of separation techniques, identification and structural determination of biologically active compounds from natural products has been facilitated by continual development of chromatographic and spectroscopic methods of

analysis (**PHILLIPSON, 2007**). An important development in the drug discovery process in recent years is the use of biological assay systems. These modern assays can test many samples within a short period of time, thus providing sufficient data for biostatistical analysis (**YEH *et al.*, 2007**).

The process of drug discovery from medicinal plants has led to the isolation of well known drugs such as aspirin; atropine, resveratrol, calanolide, digoxin, ephedrine, morphine, silvestrol, pilocarpine, quinine, quinidine, reserpine, taxanes, vincristine, and vinblastine. Most of these plant-derived drugs were originally discovered through the study of traditional cures and folk knowledge of indigenous peoples and some of these drugs cannot be substituted despite the enormous advancement in synthetic chemistry (**BALUNAS and KINGHORN, 2005; GILANI and RAHMAN, 2005**). Some of the known compounds from medicinal plants have exhibited promising and possibly selective activity. Several known compounds isolated from traditionally used medicinal plants have already been shown to act on newly validated molecular targets, as illustrated by indirubin (a constituent of Chinese antileukaemia medicine) which selectively inhibits cyclin-dependent kinases (that regulate the cell cycle and transcription of mRNA) (**HOESSEL *et al.*, 1999; EISENBRAND *et al.*, 2004**).

1.4 Toxicity of natural products

There is a growing interest in the use of medicinal plants as therapeutic agents especially in developing countries, largely because of the high cost in the synthesis of modern drugs, the availability of natural products and the cultural beliefs of the people (**POPAT *et al.*, 2001;**

REID *et al.*, 2006). It has recently been demonstrated that several side effects such as allergic reactions, cramp, diarrhoea, fever, gastrointestinal disorders, renal damage and vomiting are some of the common effects associated with poisoning from traditional medicine (**VERSCHAEVE *et al.*, 2004**). Studies on South African medicinal plants have indicated that some plant species used in traditional medicine have potential genotoxic effects. For instance, *Callilepis laureola* has been used as a multi-purpose remedy in traditional Zulu medicine but has been found to have adverse toxicity effects with generalized symptoms of poisoning such as renal failure leading to hypoglycemia, metabolic acidosis and a high fatality rate. The incidence of poisoning from *C. laureola* is very high in KwaZulu-Natal province where large volumes of the plant have been exploited for medicinal purposes (**POPAT *et al.*, 2001**).

The mechanism for herbal toxicity remains elusive, but there are accumulating data suggesting that the role of reactive metabolites/intermediates through the bioactivation of major herbal constituents might be responsible for herbal toxicity and carcinogenicity. It has also been hypothesized that the resultant reactive metabolites following herbal bioactivation covalently bind to cellular proteins and DNA, leading to toxicity via multiple mechanisms such as direct cytotoxicity, oncogene activation, and hypersensitivity reactions (**ZHOU *et al.*, 2004**). The adverse effects such as mutagenicity and lipid peroxidation demonstrated by some plant flavonoids are a major threat facing the use of natural products as therapeutic agents (**HODEK *et al.*, 2002**). Hence, thorough screening of natural products for toxicity and mutagenicity before applying them as therapeutic agents is becoming increasingly important.

1.5 Stomach-related ailments

Most diseases of the digestive tract are caused by several factors such as diet, infection, immune processes and other environmental factors. They may all play different roles in causing stomach disorders (**KANTSEVOY, 2006**). Stomach disorders encompass a spectrum of diseases involving the oesophagus, and duodenum. Symptoms range from the histological evidence of inflammation such as gastritis to the endoscopic finding of erosions and mucosal ulceration (**DOHIL, 2005**). Gastroenteritis is the inflammation of the digestive tract, involving both the stomach and the small intestines which are characterized by symptoms such as stomach pain, diarrhoea, dysentery, vomiting, fever, inflammatory infections of the colon and abdominal cramp (**WHO, 2003**). The incidence of dysentery or rectal bleeding associated with diarrhoea strongly suggests an inflammatory process in the colon. The infections may result from infective colitis or by non-specific inflammatory bowel diseases (IBDs) e.g. ulcerative colitis (**FARTHING, 2007**).

Diarrhoea is an increase in the fluidity and frequency of stools and is one of the most common disorders of man. Diarrhoea and intestinal parasitic diseases are usually referred to as 'tropical diseases' because of the environmental conditions in the region which favour recurrent cases after treatment. The prevalence of diarrhoea in tropical and subtropical regions is extremely high affecting nearly all inhabitants at some point in their lives. The infections may persist for long periods posing challenges to clinicians. However, in many cases, the disorder is short-lived or responds well to a number of well-tried remedies (**LEWIS and ELVIN-LEWIS, 1977; BLACK *et al.*, 1982; MILLER *et al.*, 2003**). In the case of severe diarrhoea, there is the potential for very rapid dehydration with the risk of prerenal impairment and hypokalemia

(electrolytes imbalance) especially among predisposed individuals (**CHERNY, 2008**). The fact that the burden of stomach-related ailments retards growth and intellectual development in millions of children is largely ignored by researchers (**HOTEZ and BROWN, 2009**).

1.5.1 Disease causing bacteria

Intestinal infection is the most common cause of diarrhoea worldwide and is estimated to be responsible for the death of 3-4 million individuals each year, particularly infants and children below the age of 5 years (**WHO, 1996**). Bacterial food-borne agents have, to date, been the most well investigated and monitored causes of intestinal infectious diseases. The burden of food-borne diseases caused by methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli,* and *Enterococcus faecalis* are on the increase globally due to intake of food contaminated with faecal materials (**NEWELL *et al.*, 2010**). Pathogenic bacteria have been implicated in gastroenteritis by attaching to the intestinal epithelium and producing enterotoxins, or they may directly invade and destroy the mucosal cells. This invasion may result in intense inflammation and bloody diarrhoea, with patients having severe abdominal pains and cramps (**CALAM, 1995**).

1.5.1.1 *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped bacterium, which belongs to the genus Enterobacteria and they inhabit the intestine of humans and animals (**HUGO, 1992**). *E. coli* is mostly associated with food poisoning, producing enterotoxins that induce watery diarrhoea and abdominal tissue damage through plasmid-encoded invasion factors. These may cause

acute or chronic abdominal pains and cramps leading to the destruction of the epithelia cells of the intestine which may result in haemorrhagic colitis (**NAIK and SKETH, 1976**; **SLEISENGER and FORDTRAND, 1973**). Cases of haemorrhagic colitis caused by non-motile *E. coli* have been identified in some of the South African provinces such as Mpumalanga, KwaZulu-Natal and the adjacent Swaziland. *E. coli* have been implicated in water-borne diseases and the outbreak of infection is common in rural areas where people drink contaminated water (**MÜLLER *et al.*, 2001**).

1.5.1.2 *Enterococcus faecalis*

Enterococcus faecalis is a ubiquitous Gram-positive bacterium of the genus Enterococcus which comprises more than 20 different species (**KAYSER, 2003**). They are part of the normal flora of the gut of both humans and animals but are also important pathogens responsible for serious infectious diseases of the gastrointestinal tract, genitourinary tract, and tissue infections which are common among immunocompromised individuals. *E. faecalis* has both intrinsic and acquired resistance to many antibiotics including β-Lactams, aminoglycosides and lincosamides which makes them an important nosocomial bloodstream pathogen (**MALATHUM and MURRAY, 1999; KAYSER, 2003**). The incidence of vancomycin-resistant enterococcal infections is usually seen among the most debilitated individuals who require prolong hospitalization with the use of broad spectrum antibiotics. High mortality rates have been observed in patients with vancomycin-resistance bacteremia (**NOSKIN, 1997**).

1.5.1.3 *Staphylococcus aureus*

Stapylococcus aureus is a Gram-positive bacterium which has the ability to ferment glucose anaerobically. They are mostly found on human skin and have been tagged as the most common etiologic organisms of infectious diseases such as gastroenteritis, impetigo and genitourinary tract infections. They have several virulent characteristics and resistance mechanisms. An example is the methicillin-resistant *Stapylococcus aureus* (MRSA) (**KLOOS and SCHLEIFER, 1981**). Cases of *S. aureus* infections have been reported to cause life-threatening deep sited infections such as bacteremia, endocarditis and pneumonia. Experience with some strains indicates that aggressive and costly measures are necessary to prevent cross-infections and morbidity rates (**COX *et al.*, 1995**). **KLOOS and SCHLEIFER** (**1981**) reported that the enterotoxins of *S. aureus* are responsible for common food poisoning.

1.5.2 Opportunistic fungal infections

Fungi are ubiquitous in the environment and are able to metabolize a wide range of substrates for their metabolic activities (**QUIROGA *et al.*, 2001**). Fungal infections of the skin and mucosal membranes constitute major health problems particularly in tropical and subtropical countries (**PORTILLO *et al.*, 2001**). Recent increases in the number of immunosuppressed and/or debilitated patients in modern hospitals have resulted in an increase in the number of invasive infections such as persistence diarrhoea, and candidemia caused by *Candida spp* (**HOBSON, 2003**).

Candida albicans is a yeast-like microorganism which has fungal characteristics (**SALTARELLI, 1989**). *C. albicans* is a normal commensal of man which can become pathogenic and cause infections such as oral thrush, vaginal thrush and as a superinfection of the gastrointestinal tract (**MOSS, 1987; PRASAD, 1991**). This opportunistic pathogen is the major cause of morbidity and mortality among predisposed persons, especially HIV/AIDS patients (**GARBINO *et al.*, 2001**). The infestation occurs when the yeast-like form of *C. albicans* is altered to the mycelium fungal stage which is invasive and can penetrate the intestinal mucosal membrane, to release mycotoxins into the blood stream (**PRASAD, 1991; CHAITOW, 1996**). This has been reported to be the cause of pulmonary infections leading to fatal meningoencephalitis and nosocomial bloodstream infections in the USA (**MOSS, 1987; EGGIMANN *et al.*, 2003**). The toxic effects observed with *Candida* infections result from its ability to produce acetaldehyde under appropriate conditions. This compound has carcinogenic potential (**CHAITOW, 1996**).

1.6 Morphological characteristics of selected medicinal plants

The selection of the medicinal plants for study was based on their ethnobotanical history and uses in South African traditional medicine for the treatment of stomach-related ailments (Tables 1.1 and 1.2). The information about the selected medicinal plants was sourced from available literature including: **BOON** (**2010**), **HUTCHINGS *et al.*** (**1996**), **POOLEY** (**1998**), and **VAN WYK and WINK** (**2004**). Of the seven selected medicinal plants, *Canthium spinosum, Cassinopsis ilicifolia, Lagynia lasaintha* and *Tetradenia riparia* are shrubs; *Crassula multicava* is a perennial plant while *Conostomium natalensis* and *Coddia rudis* are

shrublet plants. Apart from *Conostomium natalensis, Crassula multicava* and *T. riparia* that have terminal inflorescences, the rest have small flowers with varying shapes and colours. Scientific study of these selected medicinal plants might lead to the development of new and novel compounds that will help in combating the problems of microbial resistance.

Table 1.1: Morphological characteristics of selected South African medicinal plants used in treating stomach-related ailments

Plant family	Scientific name (Voucher specimen number)	Morphological characteristics	Distribution	Reference
Crassulaceae	*Crassula multicava* (Lem) A OKEM 3 NU	Perennial plant, the leaves are somewhat succulent, yellowish grey-green. The flowers are rounded inflorescences with colours varying from cream, white to tinged red	Found mostly on rock outcrops and in shade, common in KwaZulu-Natal	**POOLEY, 1998**
Icacinacea	*Cassinopsis ilicifolia* (Hochst.) Kuntze A OKEM 5 NU	Shrubs, canopy climber about 6 meters tall. It has narrow leaves shiny dark green with tapered apex. Flowers are small, creamy-white and clustered on short stalks opposite each spine	Common in high altitude forest especially in the Western Cape	**BOON, 2010**
Lamiaceae	*Tetradenia riparia* (Hochst.) Codd A OKEM 7 NU	Shrub with several branches, about 3-5 meters tall. The leaves and stem are somewhat succulent and have grandular hairs. It has terminal inflorescences, flower spikes with colours varying from white to mauve	Found mostly along stream banks and rocky slope in the Eastern part of south Africa. The distribution also extend into Namibia and Angola	**VAN WYK and WINK, 2004**

Table 1.1: Continued

Plant family	Scientific name (Voucher specimen number)	Morphological characteristics	Distribution	Reference
Rubiaceae	*Canthium spinosum* (Klotzsch) Kuntze A OKEM 4 NU	Usually small about 4 meters tall with multi-stemmed and clustered leaves on dwarf side shoots below each spine. Flowers are small and greenish-white.	Found in forest, bushveld and rock outcrops in coastal areas of the Eastern Cape and KwaZulu-Natal	**BOON, 2010**
Rubiaceae	*Coddia rudis* (E. Mey. Ex Harv.) Verdc. A OKEM 2 NU	Shrublet plant with clustered leaves on dwarf side of twigs. The flowers are small, white in colour and funnel-shaped	Found in bushveld, forest and on rock outcrops of the Eastern Cape, Mozambique and Zimbabwe	**BOON, 2010**
Rubiaceae	*Conostomium natalensis* (Stapf) Cufod A OKEM 1 NU	Woody shrublet plant, about 1 meter tall. The leaves are narrow with tapered apex and have terminal inflorescences with colours ranging from pink, lilac to white	Mostly found on forest margins, they are common in the Eastern Cape and the distribution extend to the Northern Province	**POOLEY, 1998**
Rubiaceae	*Lagynia lasiantha* (Sond.) bullock A OKEM 6 NU	Scrumbling shrubs with narrow leaves taper to rounded apex. Flowers are clustered small, greenish-yellow	Common in bushveld, sand and along the coastal forest of KwaZulu-Natal and Limpopo	**BOON, 2010**

Table 1.2: Selected South African medicinal plants used in traditional medicine in treating stomach-related ailments

Scientific name	Traditional preparation	Reference
Canthium spinosum	Leaf infusion is used in treating diarrhoea	**HUTCHINGS *et al.*, 1996**
Cassinopsis ilicifolia	Leaf and bark infusion is used to treat diarrhoea and inflammation of ear	**WATT and BREYER-BRANDWIJK, 1962**
Coddia rudis	Leaf infusion is used to treat diarrhoea and unspecified parts are used to treat malaria and fever. Pounded root decoction is used in treating impotency and infertility	**HUTCHINGS *et al.*, 1996**
Conostomium natalensis	Whole plant infusion is used to treat diarrhoea, root infusions are used as emetics in the Transkei	**HUTCHINGS *et al.*, 1996**
Crassula multicava	Decoction of whole plant is used as strong emetic and as love charm	**HUTCHINGS *et al.*, 1996**
Lagynia lasiantha	Powdered leaf decoction is used to treat diarrhoea	**HUTCHINGS *et al.*, 1996**
Tetradenia riparia	Leaf infusion is used to treat gastroenteritis. The leaf decoctions and infusions are widely taken for cough and sore throats and as antimalarial. This plant is also used in treating livestock diseases	**VAN WYK and WINK, 2004**

1.7 Aims and objectives

The incidence of stomach-related ailments caused by microorganisms is responsible for high morbidity and mortality rate among rural dwellers whose diet is marginal in quality, together with poor hygiene and no access to a good water source. Children and infants below the age of 5 years are the most vulnerable, especially among predisposed individuals such as HIV/AIDS infected persons. Traditional healers in South Africa make use of a wide variety of medicinal plants as therapeutic agents in combating microbial infections. This study was aimed at validating the efficacy of the selected medicinal plants as antimicrobial agents used in the treatment of stomach-related ailments.

- The *in vitro* microdilution bioassay was used to determine the antibacterial and anti-fungal activities of various plant extracts;
- The colorimetric assay for anthelmintic activity was used to determine the efficacy of plant extracts on pathogenic helminths;
- The cyclooxygenase assay for anti-inflammatory activity was used to establish the efficacy of the plants in alleviating stomach pain, cramps and various inflammatory responses associated with stomach-related ailments;
- The Ames test was used to determined the potential mutagenic effects of plant extracts that demonstrated good antimicrobial activity to establish if it is safe to use the plants as therapeutic agents; and
- The colorimetric assay was used to evaluate the total phenolics content e.g. gallotannins and total flavonoids; and saponin of the plant extracts.

Chapter 2

Antimicrobial activity of the selected medicinal plant extracts

2.1 Introduction

Bacteria, viruses and parasitic organisms were identified long ago as the major etiologic agents of infectious diseases that have plagued man for centuries. The menace of diarrhoea and cholera in tropical and subtropical countries has been reported as one of the worst scenarios of disease outbreaks, as these have claimed lives of millions of people especially children and infants (**COHEN and TARTASKY, 1997; SARKAR *et al*., 2007**). Infectious diseases erupt with the release of enterotoxins. These may have direct microbial damage on the host tissues or cells, leading to the release of inflammatory mediators (**MIMS, 1987**). Besides being pathogenic, microorganisms also have enormous economic importance. Some of them have great applications in the pharmaceutical and food industries, whilst others are found as normal commensals of the guts and skin of mammals.

2.1.2 Antimicrobial agents

The discovery that penicillium fungi can inhibit the growth of bacterial cultures, by Sir Alexander Fleming in 1928, was a turning point in the history of health sciences. These findings have greatly influenced the development of chemotherapeutic agents in the twentieth century (**LAURENCE and BENNETT, 1980**). Antibiotics were seen initially as truly miraculous drugs, but the availability of the first synthetic antibiotics (penicillin and sulfonamides) was not made immediately to the general public. In fact, these drugs were

scarce and very expensive and were reserved for military use during World War II (**ALANIS, 2005**). In the last few years several antibiotic agents have been discovered with simplified manufacturing processes, and are readily available for use by the general public. The use of antibiotics has become widespread as the panacea of medicine which are been utilized in the treatment of various kinds of infectious diseases (**ALANIS, 2005**).

Antibiotics are substances produced by microorganisms or they are semisynthetic substances, which at low concentrations kill or inhibit the growth of microorganisms but with little or no host damage (**QUESNEL and RUSSELL, 1983**). Recently different groups of antibiotic drugs have been developed which are aimed at inhibiting or antagonizing the mechanisms of microbial physiology and metabolism. Examples of some of the common antibiotic drugs in clinical use today are presented in Table 2.1.

Table 2.1: Classes of antibiotic families and their mechanisms of action. Modified Table from LEVY and MARSHALL (2004)

Antibiotic families	Common examples	Mechanism of action
Beta-lactams, Daptomycin	Penicillins, Cephalosporins, Glycopeptides, Cyclic lipopeptides	Inhibition of cell wall synthesis
Tetracyclines, Streptogramins, Lincosamides	Aminoglycosides, Oxazolidonones (linezolid), (quinupristin-dalfopristin), Ketolides, macrolides	Inhibition of protein synthesis
Fluoroquinolones, Rifampin	Cyprofloxacin	Inhibition of DNA synthesis
Sulfonamides	Trimethoprim	Competitive inhibition of folic acid synthesis
Polymyxins	Polymyxin-B, Colistin	membrane disorganizing agents
Metronidazole	Flagyl	Other mechanisms

2.2 Actions of antimicrobial agents

2.2.1 Actions of antibacterial agents

Bacteria are prokaryotes, and mostly single-celled, except when they exist in colonies. These ancestral cells reproduce by means of binary fission, duplicating their genetic material and splitting to form two daughter cells identical to the parent (**STARR, 1981**). Bacteria are generally classified as either Gram-positive or Gram-negative, based on their staining properties (**CHARTRAND et al., 1996**). Bacteria have a compact cell wall which is made of

peptidoglycan consisting of alternating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). The polysaccharide chains are cross-linked by peptide bridges which function as an envelope, protecting the protoplast that is surrounded by its delicate cytoplasmic membrane, and this confer the characteristic rigidity and shape of the cell (**GALE, 1981**).

Gram-positive bacteria have cell walls that are simple in structure but lack an outer membrane and form little or no barrier to the entry of antibiotics. The cell wall contains layers of peptidoglycans, in the range of 15-50 nm which is about 90% of the thick compact cell wall and can easily be separated from the plasma membrane (**RANG and DALE, 1987; PAGE *et al*., 1997**).

Gram-negative bacteria have a cell wall that is thermodynamically complex in structure, thinner and less compact than Gram-positive bacteria. From the plasma membrane outwards, there is a periplasmic space containing enzymes and other components; a 2 nm peptidoglycan layer which is about 5-20% of the cell wall, a lipid bilayer, and protein molecules with complex lipopolysaccharides. Each bacterial strain has distinct polysaccharides which form the endotoxins that determine the antigenicity of the organism. Polysaccharides are known to activate the inflammatory response in bacterial infections (**CHARTRAND et al., 1996**).

The use of antibiotics as therapeutic agents has gained worldwide recognition, and understanding their mechanism of actions is very important. Some antibiotics act by interfering with mRNA thus inhibiting bacterial protein synthesis. For instance,

aminoglycoside antibiotics demonstrate selective toxicity by inhibiting bacterial protein synthesis more than the host protein synthesis. They bind specifically to the bacterial ribosome at its decoding site, thereby reducing the potency of its protein biosynthesis. Classes of antibiotics with this mode of actions include: aminoglycosides (e.g. gentamicin, streptomycin), amphenicols (e.g. chloramphenicol), lincosamides, macrolides (e.g. erythromycin), and tetracyclines (**OGLE *et al.*, 2003; PAGE *et al.*, 1997**).

Two classes of antibiotics that inhibit bacterial cell wall synthesis are: β-lactams and glycopeptides. β-lactam antibiotics prevent the transpeptidation or the cross-linkage of the peptide chains of the bacteria cell wall (**QUESNEL and RUSSELL, 1983**). This antibiotic drug has structural similarity with the D-alanyl-D-alanine units of the peptidoglycan layer of the bacteria cell wall. These facilitate their binding to the active site of the penicillin-binding proteins (PBPs). The β-lactam units of the drug molecules bind irreversibly to (acylates) the Ser_{403} residue of the PBPs active site. This irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis. Drugs with this mode of action include: penicillins, cephalosporins, carbapenems and monobactams (**PAGE *et al.*, 1997; HANCOCK, 2005**).

Glycopeptides are high molecular weight drugs consisting of sugars and amino acids. These drugs bind specifically to the D-ala-D-ala group on the peptide chain membrane-bound intermediate units, thereby inhibiting the synthesis of peptidoglycan. Drugs with this mode of action are vancomycin and bleomycin (**QUESNEL and RUSSELL, 1983**).

The discovery of sulfonamides has been considered as a hallmark in the development of new antimicrobial agents. This drug has a unique mechanism of action by inhibiting the biosynthesis and metabolism of folate. This compound is needed in the production and maintenance of the new cell wall, and the synthesis of microbial DNA and RNA. This type of action will help in combating the problem of antibiotic resistance (**MONAGHAN and BARRET, 2006**). In most cases, many antibiotics are less active against Gram-negative than Gram-positive bacteria. Perhaps this could be because of the complex outer layer of Gram-negative bacteria which makes it difficult for antibiotics to penetrate (**RANG and DALE, 1987**).

2.2.2 Action of antifungal agents

The fungal cell wall is made of crystalline polysaccharides, β-glucans, lipids and chitin. Chitin is an important structural component in the fungal cell wall which is not found outside fungi and invertebrates; hence a specific inhibitor of chitin synthesis would be potentially useful in the treatment of fungal pathogens (**GALE, 1981**).

In the last few years, different classes of antifungal agents have been developed which are less toxic and have been used in the treatment of several fungal infections ranging from superficial to systemic mycosis. Amphotericin B is a broad spectrum antimycotic drug that has been used extensively for the past 30 years in the treatment of invasive fungal infection. This drug acts by disrupting fungal membrane synthesis. However, amphotericin B has recently been reported to have some severe side effects such as renal damage (**SIONOV *et al.*, 2006**). Polyenes have similar modes of action as amphotericin B and they exert their fungicidal

effects by binding to the ergosterol in the fungal cell membrane, causing osmotic instability and loss of membrane integrity (**ESPINEL-INGROFF and PFALLER, 1995**).

Azoles exhibit their inhibitory effects by blocking the functions of cytochrome P450 (Erg11p) involved in the demethylation of the lanosterol molecules. This process inhibits the biosynthesis of ergosterol; an essential component in fungal cell walls that maintain membrane functions. This inhibition may lead to ergosterol depletion and consequently retard fungal growth (**SANGLARD, 2002**).

Echinocandins are cyclic hexapeptides which exhibit their fungicidal effects by disrupting the glucan formation through the inhibition of β-(1-3)-glucansynthase. This polysaccharide provides structural integrity and osmotic stability to the fungal cell wall (**MARCO *et al.*, 1998**). Sordarins, act by selectively inhibiting fungal protein synthesis via specific interaction with fungal elongation factor 2 (EF2), stabilizing the EF2-ribosomal complex. This action made sordarins attractive fungicidal agents particularly against *Candida spp* (**JUSTICE *et al.*, 1998**). *5*-Flucytosine is another antifungal agent which acts by converting its structures to *5*-fluorouracil within the target cell, which is then incorporated into the RNA, causing premature chain termination, and inhibition of fungal DNA synthesis (**ODDS *et al.*, 2003**). One of the major challenges in the development of new antifungal agents is selectivity, since fungi and humans are eukaryotes. Hence, understanding the mode of action of each antifungal agent to distinguished toxic effects by chemical derivatization of compounds is very important (**ZIEGELBAUER *et al.*, 1998**).

2.3 Mechanism of antimicrobial resistance

2.3.1 Bacterial antibiotic resistance

There has been an increase in antibiotic resistance over the past four decades in the bacteria's response to the selective pressure imposed by the medical and the veterinary use of antibiotics. Studies on the emergence and dissemination of antibacterial resistance genes have clearly illustrated the genetic flexibility of bacteria (**CHARTRAND *et al*., 1996; QUESNEL and RUSSELL, 1983**). In 1945, Sir Alexander Fleming warned that the inappropriate use of penicillin could lead to the development of resistant forms of *S. aureus* that could cause more serious infections, and that the resistant microbes may proliferate and pass on the resistant genes (**LEVY, 2002**). The rates of infections caused by bacteria that have become resistant to commonly used antibiotics are today one of the major global healthcare problems. They are not only more severe and require longer and more complex treatments, but they are also very expensive to diagnose and treat (**ALANIS, 2005**).

The mechanism of antibiotic resistance starts with the bacteria producing enzymes which can degrade or modify the antimicrobial agents, making them less effective or inactive against the bacterium. This mechanism of resistance is a common type that has affected several antibiotics more especially β-lactam antibiotics, in which the bacteria produces β-lactamases (**JACOBY and MUNOZ-PRICE, 2005**). There are four major biochemical mechanisms of bacterial resistance to antibiotic agents and these include: inactivation or modification of penicillin by β-lactamase, resulting in the opening of the β-lactam ring or inactivation of cephalosporins to cephalosporic acids; reducing the permeability of antibiotics into the cell

wall by Gram-negative bacteria; alteration of target sites by changing relevant ribosomal binding sites; and the tolerance of the resistant mutant to grow in the presence of β-lactam antibiotics (**QUESNEL and RUSSELL, 1983; HANCOCK, 2005**).

The mechanisms of antibacterial resistance to aminoglycoside antibiotics are divided into two major categories: intrinsic or nonenzymatic, in which the mutation occurs in the chromosomal genes encoding ribosomal proteins; and acquired or enzyme-mediated resistance. Acquired or enzyme-mediated resistance is the most extensively studied mechanism of antimicrobial resistance, due to its prevalence among various pathogenic bacteria. The genes coding for aminoglycoside-modifying enzymes are usually disseminated by mobile genetic elements such as plasmids or transposons, and some of these are chromosomal in origin (**MAJUMDER, 2007**). The fact is that many strains of bacteria have become resistant, and in some cases multi-resistant to antibiotic agents, thus rendering antibiotic drugs ineffective as treatments of choice for severe infections caused by pathogenic microorganisms (**LEVY and MARSHALL, 2004**).

2.3.2 Fungal antibiotic resistance

Resistance of fungal pathogens evolved due to indiscriminate use of antifungal agents in which the fungi try to overcome effects of inhibitory agents through specific mechanisms of resistance (**DIXON *et al.*, 1996; SANGLARD, 2002**). Resistance or failure in antifungal therapy to treat fungal infections has been classified into primary or intrinsic (present before exposure to antifungal agents), secondary or acquired (which develops after exposure to antifungal agents owing to stable or transient genotypic alteration) and clinical resistance,

these includes progression or relapse of fungal infection. This particular type is very common among HIV/AIDS patients (**KONTOYIANNIS and LEWIS, 2002**).

Resistance of fungi to azoles has developed very fast in which the fungi alter the structure of the drug targets so that it cannot bind preferentially to the binding units in the fungal cell. Resistant fungal can also stimulate the overproduction of target enzymes through gene amplification or upregulation to avoid complete inhibition of biochemical processes. Sometimes, resistant mutants prevent drug entry into the cell using efflux systems at the membrane level (**GHANNOUM and RICE, 1999; SANGLARD, 2002**). Recent studies have shown another interesting mechanism of azole resistance in which the pathogenic fungus build a biofilm on a synthetic or natural surface, and these constitute a physical barrier to the penetration of antifungal agents, thereby making the cells embedded in the structure, to become recalcitrant to actions of antifungal agents (**SANGLARD, 2003**). Among the fungal pathogens that have become drug resistance, the most extensively studied is *C. albicans* due to its clinical relevance (**COWEN *et al*., 2002**).

Certain strains of *Candida* species and some isolates of *C. albicans* exhibit low susceptibility to amphotericin B. The rate at which this resistance has developed is yet to be quantified, and the precise mechanism of resistance to amphotericin B has not yet been completely elucidated (**SANGLARD and ODDS, 2002**). Literally no fungus has yet shown the ability to biochemically degrade an antifungal agent, compared to the way and manner in which bacteria degrade β-lactam antibiotics (**ODDS, 1996**). However, because of the increase in the emergence of antibiotic resistant pathogenic fungi, it has become very important to develop

new antifungal agents of plant origin, that will be less expensive, highly effective and non toxic, which will help in combating the problem of anti-fungal resistance (**MASOKO *et al.*, 2007**).

2.4 Testing for antimicrobial activity

There are several methods that have been developed for measuring the preliminary pharmacological activity of natural products. The simplest way is the use of sensitivity discs and the relationship between the diameters of zones of inhibition to agar diffusion methods, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (**LINTON, 1983**). The choice of technique to be used depends greatly on the extracts or compounds of study and understanding the limitations and the advantages of each method is very important. Sometimes the results from the antimicrobial assays might vary significantly due to several factors such as assay technique, culture medium (bacterial strain) and the amount of the resuspended extract tested (**JANSSEN *et al*., 1987; THOMAS, 1989; COS *et al*., 2006**).

2.4.1 Microdilution methods

This is the most widely used technique for the quantitative study of an organism's sensitivity to antibiotics. This technique involves the dilution of the antibiotic solution in the growth medium and the determination of the minimum inhibitory concentration (MIC) values. The protocol involves two-fold serial dilutions of the test extracts in microtitre plates and the inoculation of the bacterial strain in each well of the plate. The microtitre plate is incubated at

37 °C for 24 h and the result is taken as that concentration of the sample in which there is no visible colour change after adding an indicator. Control plates containing culture-free broth and water are incubated alongside to serve as negative and solvent controls respectively (**LINTON, 1983; GREENWOOD, 1995, COS *et al*., 2006**).

This technique has certain advantages over the agar diffusion method. In this assay the antibiotic is incorporated in the test medium and does not require diffusion, hence antibiotics of large molecular size can be tested by this method. Also this technique uses a wide range of organisms, including anaerobes. Results from this assay are a direct measure of the inhibitory concentration on the test organism. However, this technique has a number of disadvantages which include: the dilution steps may render the end point difficult to read; contamination may occur during the dilution process; diluted solution of antibiotics, either in broth or in agar plates, may lose their potency during incubation (**LINTON, 1983**).

Popularity of herbal remedies among developing countries is on the increase due to the availability of natural products, preference of natural products to modern drugs and the high cost of synthetically derived drugs. In South Africa, TM plant-based remedy is used by the larger part of the population especially among rural dwellers in treating various kinds of infectious diseases. The evaluated medicinal plants were chosen based on their ethnobotanical history in the treatment of stomach-related ailments and were investigated for their antimicrobial activity using microdilution technique against two Gram-positive (*E. faecalis* and *S. aureus*) bacteria, Gram-negative (*E. coli)* bacteria and a fungus *C. albicans.*

2.5 Materials and methods

2.5.1 Preparation of plant materials

Plant material from the selected medicinal plants as used in traditional medicine for the treatment of stomach-related ailments were oven dried at 50 °C and stored in brown paper bags at room temperature. The dried plant material was milled into powders using a Retsch® ZM 200 ultra centrifugal mill. Thereafter samples of 1 g each were extracted sequentially with 10 ml of ethyl acetate (EtOAc), ethanol (EtOH) and water in order to extract both polar and non-polar compounds (**ELGORASHI and VAN STADEN, 2004**). The extraction was done in a sonication bath (Julabo GmbH sonicator) for 1 h. The plant extracts were then filtered using Whatman No 1 filter paper in Büchner funnels under vacuum. The filtrates were concentrated using a rotary evaporator and were then transferred into pre-weighed pill vials. The concentrates were air-dried under a stream of cold air. The aqueous extracts were freeze-dried. The dried extracts were kept in the dark at 10 °C until ready for use.

2.5.2 Antibacterial microdilution assay

The evaluation for the antibacterial activity of the plant extracts was achieved using the minimum inhibitory concentration technique as described by **ELOFF** (**1998**). Overnight culture of a Gram-negative bacterium (*E. coli* American type culture (ATCC) 11775) and two Gram-positive bacteria (*S. aureus* ATCC 12600 and *E. faecalis* ATCC 19433) were diluted with sterile Mueller-Hinton (MH) broth (Oxoid). The plant extracts were resuspended to a concentration of 50 mg/ml with sterile distilled water for the aqueous extracts and 70% ethanol for the organic extracts. A two-fold serial dilution of 100 µl of the resuspended

extracts was prepared with sterile water in a 96-well microtitre plate (Greiner Labortechnik), for each extract resulting in concentrations ranging from 12.5 mg/ml to 0.098 mg/ml. A similar two-fold serial dilution of neomycin was used as the positive control while 70% ethanol, water and bacteria-free broth were used as the solvent and negative controls. The bacteria stock cultures were diluted (1:100) with sterile MH broth and 100 µl was added to each of the wells containing the samples and control solutions. The microtitre plates were then covered with parafilm and incubated at 37 °C for 24 h. The MIC values were obtained by adding 50 µl of 0.2 mg/ml *ρ*-iodonitrotetrazolium chloride (INT) (Sigma-aldrich, Steinham, Germany) and incubated for another 1 h at 37 °C. The bacteria growth was indicated by a pink-red colour, while clear wells indicated growth inhibition by the plant extracts. The MIC values were recorded as the concentrations in the last wells in which no colour change was observed after adding the INT. The minimum bactericidal concentration (MBC) was determined by adding 50 µl of sterile MH broth to each of the clear wells in which there was no bacterial growth and further incubated at 37 °C for 24 h. The MBC values were recorded as the concentration in the last well in which no bacterial growth was observed after adding sterile MH broth. The assay was repeated two times in duplicate for each extract.

2.5.3 Antifungal microdilution assay

Investigation of antifungal activity of the plant extracts was determined using a microdilution technique as described by **ELOFF** (**1998**) and modified for fungi by **MASOKO *et al.*** (**2007**). An overnight culture of the test fungus *C. albicans* (ATCC 10231) was prepared by adding 4 ml of sterile saline (0.85% NaCl) to 400 µl of the overnight *C. albicans* culture. The optical density was read at 530 nm and was adjusted with sterile saline to match that of a 0.5 M

McFarland standard solution (which is between 0.2500 and 0.2800). From the prepared stock fungal culture a 1:1000 dilution with Yeast Malt (YM) broth was prepared to give approximately 10^6 CFU/ml. Using 96-well microtitre plates (Greiner Labortechnik), 100 µl of the resuspended extracts were two-fold serially diluted with sterile water for each extract resulting in concentrations ranging from 12.5 mg/ml to 0.098 mg/ml in the wells. A similar two-fold serial dilution of amphotericin B (sigma) (0.25 mg/ml) was used as the positive control while ethanol (70%), water and fungal-free broth were used as the solvent and negative controls. To each of the wells 100 µl of the dilute *C. albicans* cultures were added and the plates were then covered with parafilm. Thereafter the microtitre plates were incubated at 37 °C for 24 h. The MIC values were obtained by adding 50 µl of (0.2 mg/ml) INT and then incubated for a further 24 h at 37 °C. Fungal growth in the wells was indicated by a reddish-pink colour, and clear wells indicated growth inhibition by the plant extracts. The concentration in the clear well was taken as the minimum inhibitory concentration (MIC) in which there was no fungal growth. The minimum fungicidal concentration (MFC) was then determined by adding 50 µl of YM broth to each of the clear wells in which there was no fungal growth and again incubated at 37 °C for 24 h. The MFC value was taken as the concentration in the clear well in which there was no fungal growth after adding sterile YM broth. The screening was done in duplicate and repeated twice for each of the extracts and the MIC and MFC values were recorded.

2.6 Results and discussion

2.6.1 Antibacterial activity of plant extracts

Antimicrobial activities of the investigated medicinal plant extracts are presented in Table 2.2. In the present study plant extracts that demonstrated MIC value <1 mg/ml, were considered as having good antimicrobial activities (**GIBBONS, 2005**). They are highlighted in bold in Table 2.2. Only 12 extracts exhibited good antibacterial activity; and 5 extracts exhibited good antifungal activity. Gram-positive (*E. faecalis* and *S. aureus)* bacteria were more susceptible than the Gram-negative *E. coli*. This is an indication that Gram-negative bacteria are generally more resistant to antibiotic agents than the Gram-positive bacteria (**QUESNEL and RUSSELL, 1983**).

The EtOAc extract of *Tetradenia riparia* was the only extract that exhibited promising antibacterial activity (0.19 mg/ml) against Gram-negative (*E. coli*) bacteria. The leaf extract of *T. riparia* has been reported to contain diterpenediol, which possesses significant antimicrobial activity against an array of bacterial and fungal strains, as well as protozoans (**VAN PUYVELDE *et al.*, 1986**). The presence of this compound in *T. riparia* might be responsible for the good antibacterial activity in the present study. The leaf infusion of *T. riparia* has been used extensively by traditional healers to treat gastroenteritis and other kinds of infectious diseases such as fever, malaria, haemorrhoids, skin diseases and sores which are prevalent in rural areas. This plant is also used in the treatment of livestock diseases (**HUTCHINGS *et al.*, 1996; VAN WYK *et al.*, 1997**). **HUTCHINGS *et al.*** (**1996**) reported that a strong decoction of *T. riparia* is believed to be poisonous. Hence, it should not be taken

continuously for more than four days. Most importantly it should not be administered to children.

Coddia rudis was the only plant that did not exhibit good antibacterial activity against any of the test bacteria strains in the present study. Although, this plant has been reported in traditional medicine to be used in treating different kinds of infectious diseases such as colds, fever, malaria and it has been used as an emetic. Pounded root decoction of this plant has also been reported to be used in TM in the treatment of impotence and infertility (**HUTCHINGS *et al.*, 1996**). The lack of good antibacterial activity observed in the present study does not mean the complete absence of bioactive compounds (**TAYLOR *et al.*, 2001**). It could be that the bioactive compounds are present in small amounts, or it could be that their actions were antagonized by the presence of other compounds. Rigorous isolation and purification of such compounds might yield potent antimicrobial agents.

The EtOH extracts of *C. multicava, C. spinosum, C. ilicifolia* (leaf) and *T. riparia* demonstrated promising antibacterial activity against the Gram-positive (*E. faecalis* and *S. aureus*) bacteria. All the other EtOH extracts demonstrated relatively low antimicrobial activity against the bacterial test strains. The EtOAc extracts generally show more antibacterial activity. It could possibly be that ethyl acetate, extracts mainly lipophilic (fatty acids) compounds which have widespread occurrence in plants and they are known for their potent antimicrobial activity (**HEINRICH *et al.*, 2004**). The antibacterial activity of long-chain unsaturated fatty acids has been well known for many years. Fatty acids function mainly by inhibiting the growth of microorganisms through a unique pattern of mechanisms, either by

interacting with the bacterial cell wall, cell membrane or other biosynthetic activities (**ZHENG *et al.*, 2005**). It could possibly be that, some of the extracts that demonstrated promising activity might have similar patterns of antimicrobial activity on the test organisms.

There has never been any report on the antibacterial activity or phytochemical studies for *C. rudis, C. natalensis, C. ilicifolia, C. multicava* and *L. lasiantha*. Considering the observed antibacterial activity in some of these plant extracts, isolation and identification of the bioactive compounds from plants in which specific activity has been reported for the first time might serve as leads in the development of new chemotherapeutic agents. Phytochemical studies on *Canthium* species have led to the isolation of calmatin; a compound known to have potent antibacterial activity (**WATT and BREYER-BRANDWIJK, 1962**), and this might be responsible for the observed antibacterial activity in *C. spinosum*. In determining the minimum bactericidal concentration (MBC) values, none of the plant extracts tested in the present study that exhibited noteworthy MIC (<1 mg/ml) values demonstrated corresponding good MBC effects on any of the bacterial test strains. This implies that the observed antibacterial activities were all bacteriostatic.

Generally, aqueous extracts are known to exhibit low antimicrobial activities (**RABE and VAN STADEN, 1997; LUSEBA *et al.*, 2007**). Water is the most frequently used solvent as an extractant in traditional plant-based remedies. This could possibly explain the reasons why traditional healers prescribe considerable volume of aqueous extracts to be consumed per day to ensure good antimicrobial activity **(HOFFMANN, 1989**).

Table 2.2: Antimicrobial activity of the investigated medicinal plant extracts as determined by microdilution techniques

Plant species	Plant Part	Extracts	Antibacterial activity MIC (mg/ml)						Antifungal activity (mg/ml)	
			E. c.		E. f.		S. a.		C. a.	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
Canthium spinosum	Leaf	EtOH	3.13	12.5	1.56	12.5	**0.39**	6.25	1.56	3.13
		EtOAc	3.13	12.5	**0.78**	6.26	1.56	6.25	**0.19**	12.5
		Water	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Cassinopsis ilicifolia	Leaf	EtOH	3.13	6.25	3.13	3.13	**0.39**	3.13	1.56	12.5
		EtOAc	3.13	3.13	**0.19**	12.5	1.56	12.5	3.13	6.25
		Water	3.13	6.25	1.56	12.5	3.13	3.13	12.5	12.5
	Bark	EtOH	3.13	6.25	3.13	12.5	1.56	12.5	1.56	6.25
		EtOAc	3.13	12.5	**0.78**	12.5	1.56	12.5	3.13	6.25
		Water	6.25	6.25	3.13	6.25	3.13	6.25	12.5	12.5

Table 2.2: Continued

Plant species	Plant part	Extracts	Antibacterial activity MIC (mg/ml)						Antifungal activity (mg/ml)	
			E. c.		E. f.		S. a.		C.a.	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
Coddia rudis	Leaf	EtOH	6.25	3.13	6.25	6.25	6.25	12.5	3.13	3.13
		EtOAc	12.5	6.25	1.56	6.25	3.13	3.13	**0.39**	12.5
		Water	3.13	6.25	1.56	12.5	3.13	6.25	6.25	6.25
Conostomium natalensis	Leaf	EtOH	6.25	12.5	3.13	3.13	3.13	3.13	**0.39**	6.25
		EtOAc	6.25	6.25	**0.09**	1.56	3.13	3.13	1.56	6.25
		Water	6.25	12.5	6.25	6.25	6.25	6.25	3.13	12.5
Crassula multicava	Whole plant	EtOH	3.13	12.5	**0.78**	6.25	1.56	6.25	**0.19**	1.56
		EtOAc	1.56	3.13	**0.09**	1.56	1.56	3.13	3.13	12.5
		Water	6.25	6.25	3.13	3.13	3.13	6.13	6.25	6.25

Table 2.2: Continued

Plant species	Plant part	Extracts	Antibacterial activity (mg/ml)						Antifungal activity (mg/ml)	
			E. c.		E. f.		S. a.		C. a.	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
Lagynia lasiantha	Leaf	EtOH	3.13	6.25	1.56	3.13	1.56	3.13	1.56	3.13
		EtOAc	1.56	6.25	**0.78**	3.13	1.56	6.25	6.26	12.5
		Water	12.5	12.5	6.25	12.5	6.25	6.25	6.25	12.5
Tetradenia riparia	Leaf	EtOH	1.56	6.25	1.56	6.25	**0.39**	3.13	1.56	1.56
		EtOAc	**0.19**	6.25	**0.04**	3.13	1.56	1.56	**0.39**	1.56
		Water	6.25	3.13	6.25	3.13	6.25	6.25	12.5	6.25

MIC = Minimum inhibitory concentration, MBC = Minimum bactericidal concentration, MFC = Minimum fungicidal concentration, E. c. = *Escherichia coli*, E. f. = *Enterococcus faecalis*, C. a. = *Candida albicans*, S. a. = *Staphylococcus aureus*.

Neomycin at 2 µg/ml E. c= 0.39 µg/ml, E. f. = 6.25 µmg/ml, S. a. = 0.19 µg/ml, amphotericin B at 0.25 mg/ml, C. a. = 0.97 µg/ml

2.6.2 Antifungal activity of plant extracts

The antifungal (MIC) values of the investigated medicinal plant extracts are presented in Table 2.2. Plants that demonstrated good antifungal activities against *C. albicans* were the EtOAc extracts of *C. spinosum, C. rudis* and *T. riparia,* and the EtOH extracts of *C. natalensis* and *C. multicava*. The inhibitory activities among these plant extracts are very interesting considering the low concentration at which some of the extracts inhibited the growth of *C. albicans*. Fungi are known to develop more resistance to commonly used antibiotics than pathogenic bacteria (**BUWA and VAN STADEN, 2006**). Plants that did not exhibit noteworthy antifungal (1< mg/ml) activity against *C. abicans* were the extracts of *C. ilicifolia* (bark and leaf) and *L. lasiantha*, but these plants showed some good antibacterial activity. Aqueous extracts exhibited poor antifungal activity. This could possibly be the reasons for the relatively low antifungal activity observed amongst most of the plant extracts. In determining the minimum fungicidal concentration (MFC) none of the extracts that exhibited good MIC (<1 mg/ml) values showed corresponding good MFC values. This suggests that such extracts possessed only inhibitory activity and not direct lethal effects on the test organism *C. albicans*.

2.7 Conclusions

The results of the present study validate some of the selected medicinal plants based on their ethnobotanical history and use in traditional medicine as antimicrobial agents. At least some of the extracts investigated exhibited good antibacterial and antifungal activities, although there was no noteworthy (<1 mg/ml) bactericidal or fungicidal effects. The EtOAc extracts demonstrated the most antimicrobial activity, followed by EtOH extracts and aqueous extracts. The observed antimicrobial activity in some of the plant extracts indicates the presence of bioactive compounds

such as alkaloids, phenolics, flavonoids, and saponins which have widespread occurrence in plants (**POLYA, 2003**). The antimicrobial activity observed in some of the plant extracts suggests that they could equally have similar activity against other microbial strains that cause life threatening infectious diseases. It is interesting to note that the antibacterial activity of *T. riparia* against the Gram-negative *E. coli* is very promising because Gram-negative enteropathogenic bacteria are known to cause severe cases of acute gastro-intestinal disorders and other related stomach ailments which are prevalent in rural areas (**MÜLLER *et al.*, 2001**). Plant extract that showed good fungistatic activity at MIC value 1< mg/ml are very promising because *C. abicans* are known to change the morphological state easily due to the presence of mobile transposon. Hence, it will be interesting to investigate the bioactive compounds that are responsible for the observed activity in the present study as they could be active against other fungal pathogens that cause stomach-related ailments. However, further investigation is needed to isolate and identify the bioactive compounds present in the medicinal plants that demonstrated good antimicrobial activity especially for those plants species for which relatively high activity is reported for the first time and these might serve as a blue print in developing novel compounds.

Chapter 3

Anthelmintic activity of the selected medicinal plant extracts

3.1 Introduction

3.1.1 Helminthiasis

Helminthiasis is a parasitic disease involving helminth parasites living inside or on their host for nourishment and life processes. This biological interaction might have detrimental effects on their host, causing either symptomatic or asymptomatic infections. Helminths are multicellular organisms with a life cycle involving an intermediate host for the development of larva stages and a definitive host for the adult stage. Adults may be dioecious or hermaphroditic (**PETERS and GILLES, 1995; VAN RIET *et al.*, 2007**). Helminth infections may have a life threatening effect on the health of both human and animals, especially in tropical and sub-tropical Africa, Asia and South America. The infection rate is extremely high in these regions due to environmental contamination with eggs and infective larvae of parasitic nematodes. Globally, millions of people are infected with soil-transmitted helminths (STHs) and schistosomes (**CROMPTON, 1999; ASAOLU and OFOEZIE, 2003**). Helminth infection has for a long time been known as one of the 'neglected tropical diseases' which has high prevalence among rural dwellers, particularly people of low socio-economic status that have a problem of poor hygiene **(BUNDY *et al.*, 1995; HOTEZ and BROWN, 2009). EVANS *et al.* (1987**) reported that the prevalence of nematode infection in KwaZulu-Natal children is about 64.5% for *Ascaris lumbricoides* and 61.1% for *Trichuris trichuria*.

Helminth infection occurs when the parasitic nematodes interfere with the nutrition of their hosts. For instance, ascaris, hookworms, and strongyloides spend most of their life in the small intestine where digestion and absorption of nutrients take place. Trichuris and schistosomes inhabit the large intestine and the nearby mesenteric veins. They affect the host by decreasing its ability for nutrient intake; increase nutrient excretion or loss and decrease nutrient utilization within the body (**STEPHENSON, 1980**). Helminthiasis has been linked with an increased risk of nutritional anaemiasis, protein-energy malnutrition and growth deficits. These can impair behavioral, cognitive and motor development especially among children, and in some cases the impairment is irreversible (**RODRGUEZ-MORALES *et al.*, 2006; HOTEZ and BROWN, 2009**).

Schistosomes are digenetic trematodes that were first described by Bilharz (1852) (**WOOLHOUSE, 1994**). The adult parasite is about 30 mm long and occurs as male-female pairs in the abdominal blood vessels of their mammalian host. Two variants (i.e. *mansoni* and *japonicum*) infect humans, causing schistosomiasis. Current estimates suggest that up to 200 million people in the world are infected with schistosomiasis (**WOOLHOUSE, 1994**). Schistosomiasis and STHs were probably the most widespread serious worm infections at the end of the 20th century (**EDDLESTON and PIERINI, 2001**).

Hookworm infections remain one of the most prevalent forms of chronic human parasitic infections in developing countries; the most common species infecting humans are *Ancylostoma duodenale* and *Necator americanus* (**BROOKER *et al.*, 2007**). The half-inch-long worms attach to the small intestine and suck blood, like an intestinal leech, persisting over a period of several months or years, resulting in severe iron-deficiency anaemia and protein malnutrition. In most

cases, children with chronic hookworm anaemiasis take on a sickly and sallow complexion and have trouble learning at school (**HOTEZ, 2010**). It is probable that hookworm infection is a significant contributor to the burden of disease among women of reproductive age (**BUNDY *et al.*, 1995**).

3.1.2 Actions of anthelmintic agents

Several therapeutic agents have been developed in the treatment of STHs but for the past 30 years only four drugs have been approved for use by the World Health Organization (WHO), namely: albendazole, levamisole, mebendazole and pyrantel pamoate (**WHO, 2002**). Albendazole, like other benzimidazoles (BZ), is absorbed by the host and most of its anthelminthic action operates directly in the gastrointestinal tract. After it has been metabolised by the liver, the active metabolite (albendazole sulphoxide) is found at high concentration in the plasma fluid (**JASMER *et al.*, 2000**). These compounds bind selectively to nematode tubulin, inhibiting tubulin polymerase, which prevents the formation of microtubules and thereby inhibiting cell division. The drug also impairs the uptake of glucose, which may increase glycogen depletion, and hampering the formation of ATP which is an essential energy source for the nematodes (**LACEY, 1990; ROBINSON *et al.*, 2004**).

Levamisole and pyrantel pamoate act by disrupting neuromuscular transmission in nematodes. These anthelmintic drugs penetrate the nematode cuticle and intercept or antagonize the cholinergic receptors at the neuromuscular junctions, which gradually paralyzes the nematodes and render the worms immobile, allowing for easy excretion from the bowel of an infected person (**FRAYHA *et al.*, 1997**). The avermectins and milbemycins have a similar mode of action

in that, they are able to stimulate the conductance of chloride ions across muscular membranes thereby antagonizing the axial muscles of nematodes (**HABER *et al.*, 1991**).

Modern anthelmintic drugs are known to be highly effective against the mature and larvae stages of different kinds of gastrointestinal nematodes as well as many extra-intestinal helminth species. Many of these drugs are well tolerated by the host and in most cases treatment requires only a single oral dose **(KÖHLER, 2001**). The introduction of praziquantel as a new schistosomicidal and cestocidal drug was a major breakthrough in antiparasitic chemotherapeutic development. This drug exhibits selective toxicity on nematodes causing muscle contraction and vacuolisation of the tegument thereby increasing calcium flux across the tegumental membranes which may lead to death of immature worms and immobilization of the adult worms (**FRAYHA *et al.*, 1997; KÖHLER, 2001**).

3.1.3 Anthelmintic resistance

There has been a decline in the efficacy of anthelmintic agents against parasitic nematodes that were once susceptible to all the broad spectrum anthelmintic drugs currently used, due to improper administration of drugs (**WALLER, 1997; SANGSTER, 1999**). Despite remarkable progress in the discovery and development of new anthelmintic drugs, nematode parasitic diseases remain one of the greatest limiting factors to successful, and sustainable ruminant livestock production in the world (**PERRY and RANDOLPH, 1999**). Anthelmintic resistance (AR) among sheep and horses were the first incidences reported, but AR in many animal industries and among humans are now major causes of alarm (**SANGSTER and GILL, 1999**). Although human parasitic infections naturally are more complex to study than veterinary

parasites, because field statistics are hard to obtain and it is also very difficult to detect resistant parasites in human populations. However, in the past few years it was observed that hookworms are most likely to develop resistance faster than other pathogenic human parasites (**SANGSTER and GILL, 1999**). The rate at which AR develop can be determined by two factors; selection pressure and the extent to which the worm surviving treatment pass their genes onto the next generation. These processes have led to high frequency of resistant genes in the population and are on exponential increase (**GEARY *et al*., 1999**).

Resistance to benzimidazole occurs when there is mutation in the β-tubulin gene. This process alters or degrades microtubulin to a level where it may gradually disappear leading to resistant mutants, thereby reducing the fidelity of BZ-binding compounds to the target molecules (**VON SAMSON-HIMMELSTJERNA, 2006**). Anthelmintic resistance to levamisole has also been identified in which the resistant helminth changes the levamisole-activated receptor channel, thus reducing the potency of the drug. Recently there was a report on the decline in the efficacy of praziquantel, but the exact mechanisms of resistance and the extent to which the resistance has developed has not been elucidated (**KÖHLER, 2001**).

3.1.4 Control of helminth infections

Control of helminth infections involve different practices or programs that will help in reducing the incidence of helminth infections, prevalence, morbidity and mortality rates to an acceptable level within the endemic regions (**HOTEZ *et al*., 2004**). The most effective control measure is to avoid the use of contaminated water, and to reduce the level of environmental contamination of parasitic helminths through indiscriminate sewage disposal. Mass treatment with broad spectrum

anthelmintic drugs is considered to be the key strategy for controlling morbidity among impoverished populations, since it is difficult to eliminate factors such as lack of sanitation, poor hygiene and overcrowding that make individuals vulnerable to helminth infections (**URBANI and ALBONICO, 2003**). In order to realize an effective mass-treatment strategy, it is necessary to know how often and in which season helminth infections are common and more specifically which chemotherapeutic agents are used to reduce the morbidity rate (**JORDAN and WEBBE, 1982**). The use of nematophagous fungi (nematode trapping fungi) has been considered as one of the best control strategies in reducing the incidence of helminth infections. Nematophagous fungi prey on the infective larvae of helminths in faeces and in the process releases anthelmintic agents. This method can effectively control the level of environmental contamination by pathogenic helminths (**BARNES *et al*., 1995**).

The use of plants in TM in treating stomach-related ailments caused by parasitic helminths is mostly for their purgative effects, by expelling the parasitic helminths from the bowel of an infected person. The aim of this study was to investigate the anthelmintic activity of the selected medicinal plants against a free-living nematode *Caenorhabditis elegans.* This nematode has been used extensively as a model organism to address fundamental questions in biology, due to its simple nature, ease of cultivation and maintenance in the laboratory, reproducibility, and supplies an ideal broad spectrum anthelmintic activity to test compounds within a short time interval (**MEGALOU and TAVERNARAKIS, 2009; HELMCKE*, et al*., 2010).**

3.2 Materials and methods

3.2.1 Anthelmintic colorimetric assay

An *in vitro* determination of free-living nematode larvae viability assay, as described by **JAMES and DAVEY** (**2007**) with slight modifications (**AREMU *et al.*, 2010**), was used to evaluate the minimum lethal concentration (MLC) values of the plant extracts. A 3-day-old *C. elegans* var. Bristol (N2) culture was prepared by subculturing a stock culture and seeded with autoclaved *E. coli*. The subcultured *C. elegans* was washed with 5 ml of M9 buffer and the optical density (OD) at 530 nm was measured using a UV–visible spectrophotometer (Varian Cary 50, Australia). Thereafter, 5 ml of M9 buffer (sufficient for inoculating one microtitre plate) were adjusted with appropriate volumes of the prepared stock culture of *C. elegans* to obtain a culture mixture in the OD_{530} range of 0.04–0.06 (approximately 100 worms per 50 µl). Plant extracts were redissolved to a concentration of 25 mg/ml with sterile distilled water for the aqueous extracts and 70% ethanol for the organic extracts. From each of the redissolved extracts 100 µl was two-fold serially diluted with sterile distilled (100 µl) water in a 96-well microtitre plate. A similar two-fold serial dilution of levamisole (100 µl, 1 mg/ml) (Sigma-Aldrich, Germany) was used as a reference drug. Fifty microlitres of the prepared *C. elegans* culture was added to each well of the microtitre plate. Control plates containing 70% ethanol and water were included as solvent and negative controls. The microtitre plates were covered with parafilm and incubated at 20 °C for 48 h in the dark. Thereafter, 50 µl of 1.25 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) was added to the wells of the microtitre plate and further incubated at 20 °C for 24 h. Presence of an active organism was indicated by a pink colour. The concentration of the lowest clear well was recorded as the MLC value of the extract. The assay was done in duplicate and repeated twice for each extract.

3.3 Results and discussion

The MLC values of the investigated plant extracts are presented in Table 3.1. In this study, plant extracts that exhibited MLC values lower than 1 mg/ml, 1 to 4 mg/ml and above 4 mg/ml were considered as having high, moderate and low anthelmintic activity, respectively (**AREMU *et al.*, 2010**). Plant extracts with high anthelmintic activity are highlighted in bold. Most of the plant extracts demonstrated promising anthelmintic activity against *C. elegans.* All the extracts of EtOAc exhibited high anthelmintic activity and most interestingly *T. riparia* showed the best anthelmintic activity with an MLC value of 0.004 mg/ml. Terpene (1,8-cineole) and ishelenine (eudesmanolide sesquiterpene lactone) have been isolated from leaf extract of *T. riparia* which are known to have diverse pharmacological properties (**VAN PUYVELDE *et al.*, 1986; POLYA, 2003**). This might be responsible for the exceptional anthelmintic activity exhibited by *T. riparia* in the present study. Terpenes are essential oil fractions, synthesized from the acetate unit, highly enriched in compounds based on an isoprene structure and have been recognized for their potent pharmacological properties (**POLYA, 2003**).

Table 3.1: Anthelmintic activity of medicinal plants used in treating stomach-related ailments in South African traditional medicine

Plant species	Minimum lethal concentration (mg/ml)			
	Plant part	EtOAc	EtOH	water
Canthium spinosum	Leaf	**0.260**	**0.016**	**0.033**
Cassinopsis illicifolia	Leaf	**0.130**	**0.033**	**0.065**
	Bark	**0.270**	**0.016**	**0.016**
Coddia rudis	Leaf	**0.520**	**0.008**	2.083
Conostomium natalensis	Leaf	**0.270**	1.042	2.083
Crassula multicava	Whole plant	**0.008**	**0.521**	8.330
Lagynia lasiantha	Leaf	**0.065**	2.083	8.330
Tetradenia riparia	Leaf	**0.004**	2.083	2.083

Levamisole at 1 mg/ml = 1.04 µg/ml

The EtOH extracts of *C. natalensis, L. lasiantha and T. riparia* exhibited moderate anthelmintic activity against *C. elegans.* Interestingly, aqueous extracts of *C. spinosum* and *C. ilicifolia* (leaves and bark) were exceptionally active. The anthelmintic activity of the aqueous and organic extracts validated the use of the studied medicinal plants in traditional medicine as agents for the treatment of gastrointestinal ailments caused by parasitic helminths. The aqueous extracts of *C. rudis, C. natalensis, T. riparia, C. multicava* and *L. lasiantha* exhibited moderate to low activity. The observed activities in the present study were unevenly distributed between the aqueous and the organic extracts. For example the organic extracts of *C. rudis* and *C. natalensis* showed good

activity whereas the aqueous extract of these plants exhibited moderate activity. In other cases, the organic extracts of *C. multicava* exhibited promising anthelmintic activity but the aqueous extract did not show any significant activity. EtOAc extract of *L. lasiantha* showed good activity whereas the EtOH and aqueous extracts did not show significant activity. Most interestingly the EtOAc extract of *T. riparia* that exhibited the best anthelmintic activity demonstrated low anthelmintic activity in the EtOH and aqueous fractions respectively.

The different levels in anthelmintic activity across the test extracts clearly indicates the importance of testing both the organic and aqueous extracts, which can lead to the isolation and identification of diverse range of bioactive compounds in plants (**WATERMAN *et al.*, 2010**). In the present study plant materials were extracted sequentially using organic solvents first, and then followed by water extraction. The detection of anthelmintic activity in both the organic and aqueous extracts indicates the presence of more than one type of anthelmintic agent in the same plant (**WATERMAN *et al.*, 2010**).

3.4 Conclusions

Anthelmintic activity exhibited by the organic and aqueous extracts in this investigation support the use of the selected medicinal plants in traditional medicine as anthelmintic agents. The different levels in anthelmintic activity among the aqueous and the organic extracts confirmed the need to use more than one solvent in the extraction of bioactive compounds from the plants. These might lead to the extraction of useful pharmacologically active compounds that will serve as a blue-print in developing novel anthelmintic agents. Generally plants are used in traditional medicine for their purgative effects which can expel parasitic helminths from the body of an

infected person, but the results of the present study showed direct lethal effects on *C. elegans*. Hence, the moderate or low activity of some of the extracts does not mean complete absence of bioactive compounds as good anthelmintic activity could be achieved by high doses of the extracts. The low anthelmintic activity could also suggest that some of the extracts might have purgative properties. This investigation indicates the presence of potential anthelmintic compounds in all the studied plant species. Isolation and identification of these compounds could be used as a supplement to current clinical treatment in the hope of preventing anthelmintic resistance in human populations.

Combating the rate of morbidity and impairment caused by parasitic nematodes among disadvantaged communities remain a great challenge. Basically because of several cases of reinfection after treatment, hence, the need to improve the standard of living, with access to a good water supply should be considered as the key strategy for controlling helminth infections.

Chapter 4

Anti-inflammatory activity of the selected medicinal plant extracts

4.1 Introduction

Inflammation is a complex protective mechanism initiated by the cells/tissues of the body to different kinds of hostile agents such as parasites, allergens, chemical irritations, as well as injury or infections. The symptoms are characterized by pains and swellings which may result from the dilation of blood vessels (**IWALEWA *et al.*, 2007**). Any damage to tissues in the form of inflammatory stimulation may give rise to activation of blood clotting factor XII (a protease), this in turn may lead to the activation of a further specific protease (kallikrein) and the formation of kinins. Kinins generally cause vasodilation, which increase vascular permeability to proteins and access to the damaged tissues by introducing neutrophiles from capillaries into the extravascular spaces of the tissues (**POLYA, 2003**).

Acute inflammation occurs with rapid onset and short duration which quickly degrades in the tissues once the stimulus is removed. It is characterized by exudation of fluids and plasma proteins, and the migration of leukocytes into the injured area. While chronic inflammation is of prolonged duration, the symptoms are characterized by the presence of lymphocytes and macrophages, resulting in fibrosis and tissue necrosis. Persistent chronic inflammation may lead to the development of degenerative diseases such as rheumatoid arthritis, cardiovascular diseases, cancer, asthma, acquired immunodeficiency disorders, infections and inflammatory bowel diseases (IBDs) (**BENGMARK, 2004; McKAY, 2008**).

Tissue injury induced by the trauma of inflammatory processes may result in the activation of monocytes, granulocytes and lymphocytes. It may also involve the release of inflammatory mediators such as cytokines and tumor necrosis factor (TNF-α), and interleukin-1 (IL-1) from leukocytes, and macrophages (**IWALEWA *et al.*, 2007**). Cytokines has been reported to activate the up-regulation of other pro-inflammatory cytokines, chemokines and immunoglobulins, and can also increase the expression of many cellular adhesion molecules (**HOPKINS, 2003**). These substances bind to specific target receptors of the cells and may increase vascular permeability, promote neutrophil-chemotaxis and stimulate smooth muscle contraction. It can also increase direct enzymatic activity, induce pain and mediate oxidative damage (**COLEMAN, 2002**).

Neutrophils are usually the first cells deployed to the sites of tissue injury to deal with the causes of inflammation. They destroy invading pathogens and compounds by phagocytosis or opsonisation. These processes may involve the production of reactive oxygen species (ROS) that could react with nucleic acids, lipids and proteins, causing extensive oxidative damages. The process can also lead to the release of tissue-damaging enzymes such as proteases, myeloperoxidase (MPO) and arachidonic acid (**SCOTT *et al.*, 1999; LABIENIEC and GABRYELAK, 2005**).

Arachidonic acid is a 20-carbon, polyunsaturated fatty acid containing four double bonds. It is abundant in cellular membranes of mammalian cells, where it can be esterified into glycerophospholipids. The phospholipase enzyme is activated by hormonal and various forms of inflammatory stimuli, to release arachidonate from the bimolecular nucleophilic substitution (sn-$_2$) position of the membrane-bound phospholipids through hydrolysis of the ester linkage.

Once released, arachidonate is oxygenated by either cyclooxygenase (COX) or lipoxygenase (LOX) to form prostaglandins (PGs) and leukotrienes (**BELLEY and CHADEE, 1995**).

4.1.1 Functions of prostaglandin

Prostaglandins, a group of ubiquitous long chain fatty acids also known as eicosanoids were first discovered in 1935 and have been used in the control of a wide range of physiological processes. There are 14 naturally occurring PGs which are named in a complex fashion using their chemical structures and physiological properties. They are among the most important mediators and modulators of inflammatory reactions (**LAURENCE and BENNETT, 1980**). All cells with the exception of red blood cells have the capacity to synthesize PGs. Once the conversion of prostaglandin H_2 (PGH_2) has occurred, the specific PGs is rapidly catabolized to an inactive product, because PGs are not stored intracellularly, but are rather synthesized and released immediately. They are very labile and only small quantities of the active compounds are measurable in the systemic circulation during normal physiologic states (**MILLER, 2006**).

These inflammatory mediators have physiological roles which involve inflammation, pain and fever, the regulation of blood pressure and the induction of blood clotting (**MANTRI and WITIAK, 1994**). Prostaglandins have several other functions which may differ according to how they are formed. They may activate the enzymatic pathway and the physiological state of the target organ. They can also cause smooth muscle (vascular, uterine, and bronchial) to contract or relax (**LAURENCE and BENNETT, 1980**). For instance thromboxanes are found in blood platelets which function as vasoconstrictors and facilitate platelet aggregations. Leukotrienes have biological effects such as respiratory and intestinal regulations (**VANE, 1971; ROBERT**

and NEWTON, 1982). Prostaglandins have also been implicated in the defence processes of gastric mucosa, which is in the front line of attack against many substances, including its own secretions of acids, pepsin and the refluxed bile (**WHITTLE, 1980**).

4.1.2 Cyclooxygenase isoenzyme

There are two major isoforms of the COX enzyme (COX-1 and COX-2) which is coded by distinct genes on different chromosomes and they show 50% homology. They have similar catalytic properties, but are physiologically distinct. COX-1 is a constitutive enzyme. It performs normal physiological functions of the body including gastric cytoprotection and platelet aggregation. However, COX-1 has recently been shown to up regulate various carcinomas (**PASINETTI, 2001; BOURS *et al*., 2006**). In contrast COX-2 is induced by growth factors, carcinogens and tumor-promoting phorbol esters. It is known to play an important role in rheumatoid diseases, inflammatory responses and tumorigenesis (**MORITA, 2002**). COX-2 has been identified as inducible isoform in pathological conditions due to its involvement in various forms of inflammatory stimulation, and this has made COX-2 a drug target in the development of new anti-inflammatory agents (**LI *et al*., 2006**).

Generally, COX enzymes are bound to the endoplasmic reticulum and catalyse the formation of cyclic endoperoxides, prostaglandin G_2 (PGG_2) and PGH_2. The process incorporates both COX and peroxidase activity, and bisdioxygenates arachidonic acid to the hydroperoxyl endoperoxyl PGG_2, which is then reduced by the peroxidase to form the hydroxyl endoperoxide PGH_2 (**DeWITT and SMITH, 1988; BELLEY and CHADEE, 1995; VANE and BOTTING, 1998**). This dual reaction occurs in almost every cell type in the body but the subsequent steps in the

COX metabolic pathway differ depending on the cell type. This tissue-specificity is as a result of the selective presence of the enzymes which convert PGH_2 to prostaglandins F_2 ($PGF2_\alpha$) (**MANTRI and WITAK, 1994**). In blood platelets the pathway leads to the synthesis of thromboxane A_2 (TXA_2), and prostacyclin I_2 (PGI_2) in vascular endothelium and prostaglandin E_2 (PGE_2) in macrophages (**RANG and DALE, 1987**).

4.1.3 Actions of anti-inflammatory agents

Anti-inflammatory drugs are used to control pain and inflammatory disorders. It relieves inflammation and the associated pain by blocking certain enzymes such as COX which is needed in the synthesis of PGs. Drugs which are used as anti-inflammatory agents are categorized into two groups, namely the non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoid drugs. Most anti-inflammatory agents such as aspirin, ibuprofen, and indomethacin are NSAIDs that inhibit PGs synthesis (**HOWARD and DELAFONTAINE, 2004**).

Conventional NSAIDs are the most widely prescribed therapeutic agents used for the treatment of various kinds of inflammatory conditions and pains. Some of these drugs such as aspirin and paracetamol are readily available for the treatment of headaches, toothaches and other minor complaints. However, toxic manifestation associated with most of these synthetic agents is a matter of great concern (**RANG and DALE, 1987; LI *et al*., 2006**). Most NSAIDs have similar effects, these include the modification of the inflammatory reaction (anti-inflammatory effects), the reduction of certain types of pain (analgesic effect), and the lowering of a raised body temperature (antipyretic effect). In general, all these effects are related to the primary action of

the NSAIDs which is the inhibition of the COX enzyme that will lead to the production of PGs and TXA_2 (**RANG and DALE, 1987; DESMUELES, 2002**).

Plant extracts have been evaluated for their anti-inflammatory effects by using different kinds of enzymatic inhibition assays such as the cyclooxygenase inhibition test and other similar *in vitro* tests (**WAGNER and JURCIC, 1991**). Important aspects of inflammatory responses that have been exploited for screening for anti-inflammatory activities were the various functions of neutrophils, the metabolic products of arachidonic acid and the role played by ROS in the activation of inducible enzymes (**LAUPATTARAKASEM *et al.*, 2003**). Various methods involving the use of mammalian enzymes contained within peritoneal leukocytes have been exploited extensively to evaluate the potential anti-inflammatory activity of a wide range of plant extracts and derived natural products (**LAUPATTARAKASEM *et al.*, 2003**).

Anti-inflammatory properties of several phytomedicines have proven to be good inhibitors of various pro-inflammatory mediators. Some of these compounds include phytoestrogens, flavonoids and its derivatives; phytosterol, tocopherol, ascorbic acid, curcumin, genistein, and other compounds. The presence of alkaloids, tannins, saponins, anthraquinones, triterpenoids and other constituents in plants have been reported to possess a diverse range of bioactivities including anticancer and anti-rheumatoid properties (**IWALEWA *et al.*, 2007**). They inhibit the formation of pro-inflammatory signalling molecules such as prostaglandin or leukotrienes. Some plant substances have been reported to inhibit the nuclear transcriptase factor (NFKB)-mediated signalling pathway in immune cells that leads to the production of inducible nitric acid oxide

synthesis (iNOS), pro-inflammatory cytokines and inducible cyclooxygenase (iCOX) (**POLYA, 2003**).

The use of plants in traditional medicine for treating cases of inflammatory responses associated with stomach-related ailments such as cramp, gastritis and IBDs are not uncommon. Selected medicinal plants were evaluated for their anti-inflammatory properties using COX-1 and COX-2 bioassays.

4.2 Materials and methods

4.2.1 Anti-inflammatory assay

Both COX-1 and COX-2 assays was conducted as described by **JÄGER *et al.*** (**1996**) and modified as outline by **ELDEEN and VAN STADEN** (**2008**). The basic protocol is the same for both assays allowing a comparison of the inhibitory effects of the plant extracts on the enzymes. The stock solution of COX-1 enzyme (60 µl) (Sigma-Aldrich) stored at -70 ºC was activated with 1250 µl of a co-factor solution (0.3 mg/ml L-epinephrine, 0.3 mg/ml reduced glutathione and 100 µl hematin in 10 ml Tris buffer, at pH of 8); COX-2 enzyme was also activated with 1250 µl of a co-factor solution (0.6 mg/ml L-epinephrine, 0.3 mg/ml reduced glutathione and 100 µl hematin in 10 ml of Tris buffer, at pH of 8) and pre-incubated on ice for 5 min. The enzyme/co-factor solution (60 µl) was added to sample solutions (20 µl of aqueous plant extracts or 2.5 µl of organic solvent plant extracts + 17.5 µl of distilled water; bringing the final concentration to 250 µg/ml for organic solvent plant extracts and 2 mg/ml for aqueous plant extracts) and then pre-incubated at room temperature for 5 min. In each assay three controls were run (2.5 µl ethanol +

17.5 µl distilled water). Two were the negative controls: the background in which the enzyme was inactivated with 2N HCl before the addition of ^{14}C-arachidonic acid, and a solvent blank. Indomethacin® was used as a positive control at a concentration of 5 µM for COX-1 and 200 µM for COX-2 to determine the efficiency of the assay-system. The reaction was started by adding 20 µl of ^{14}C-arachidonic acid (Amersham) to each of the test solution, with the enzymes in the background being inactivated by adding 10 µl of 2N hydrochloric acid (HCl) before incubating the test solution at 37 °C for 10 min. Thereafter the reaction was terminated by adding 10 µl of 2N HCl to each test solution, followed by 4 µl of 0.2 mg/ml unlabeled prostaglandins (Sigma-Aldrich) (a carrier solution). The samples were loaded onto individual Pasteur pipettes packed with silica gel with 1 ml of hexane:1,4 dioxan:acetic acid (70:30:0.2 v/v/v), to separate the PGs from the unmetabolized arachidonic acid. Column chromatography was used to elute the unmetabolized arachidonic acid with 4 ml of hexane: 1,4-dioxan: acetic acid (1 ml at a time) and collection of PGs products was done with 3 ml of ethyl acetate: methanol (85:15 v/v) into scintillation vials and the radioactivity was measured using a Beckman L S 6000LL scintillation counter. Percentage inhibition for the test extracts was calculated using:

$$\text{COX inhibition (\%)} = \left(1 - \frac{\text{DMPsample} - \text{DMPbackground}}{\text{DMPblank} - \text{DMPbackground}}\right) \times 100$$

4.3 Results and discussion

The percentage inhibitory activity against COX-1 and COX-2 by all the plant extracts at 250 µg/ml was reported as percentage inhibition of prostaglandin biosythensis in Figure 4.1. Minimum inhibitory activity of <50% was considered as good inhibition of the COX enzymes

(**ELDEEN and VAN STADEN, 2008**). All the EtOAc extracts in this investigation showed percentage inhibition in the range of 50.7 to 94.7% against both COX-1 and COX-2 respectively except for *L. lasiantha* that was not tested due to lack of plant material. The percentage inhibitory activity of EtOAc extracts against COX-2 enzyme were generally higher compared to that of COX-1 at the same concentration. This finding is very important because COX-2 specific inhibitors have been suggested to potentiate the development of non-steroidal anti-inflammatory agents due to their low side-effects and non-negligible risk of platelet aggregation, gastric haemorrhage colitis and gastro-intestinal toxicity (**MacAULAY and BLACKBURN, 2002; NURTJAHJA-TJENDRAPUTRA *et al.*, 2003; BERTIN, 2004; WARNER and MITCHELL, 2008**). It should be noted that anti-inflammatory effects of selective COX-2 inhibitors can only be possible if the dose is not increased above the levels which can also inhibit COX-1 activity (**LI *et al.*, 2006**). This suggest that plant extracts with selective COX-2 inhibtory activity posses anti-inflammatory agent that can only bind specifically to COX-2 and these can be exploited for design and development of superior anti-inflammatory agents.

The EtOH extract of *C. multicava* demonstrated the highest percentage inhibitory activity at 99.5 ± 0.5% and 98.8 ± 0.4% against COX-1 and COX-2 respectively. The EtOAc extract of this plant species demonstrated inhibitory activity against COX-2 to a greater extent than COX-1. However aqueous extract of the same plant showed relatively low inhibitory activity against both COX enzymes. The EtOH extract of *L. lasiantha* exhibited good inhibitory activity (63.2 ± 0.2%) against COX-1 enzyme but showed poor inhibitory activity (38 ± 2.0%) against COX-2 enzyme. The EtOH extracts of *C. spinosum* and *T. riparia* demonstrated high inhibitory activity against COX-2 enzyme but exhibited relatively low inhibitory activity against COX-1 enzyme. The low

inhibitory activity against COX-1 isoenzyme in the present study is noteworthy due to the fact that COX-1 enzyme is constitutively expressed in most tissues of the body, hence, their inhibition can lead to serious adverse effects, therefore it is undesirable to have remedy that has high COX-1 activity (**LI *et al.*, 2006**). It has been shown that prolonged use of NSAIDs with high COX-1 inhibitory activity can induce gastro-intestinal toxicity (**WHITTLE, 2004**).

Except for the aqueous extract of *T. riparia* and *C. rudis* that showed exceptional inhibitory activities against both COX-1 and COX-2 enzymes respectively, all other aqueous extracts in this investigation demonstrated poor inhibitory activity against both COX enzymes. Contrary to the previously study (**NDHLALA *et al.*, 2011**), aqueous extracts of *T. riparia* exhibited COX-1 inhibitory activity to a greater extended than COX-2 enzyme. In traditional plant-based remedies, water is the most frequently used solvent for extraction and traditional healer's use predominantly boiled or hot water extracts. The presence of saponins or other compounds that have effect on surface tension, in the course of boiling, can bring into the aqueous phase water-insoluble compounds. To some extend this could be the reason for the efficacy of aqueous preparation using hot water (**TAYLOR and VAN STADEN, 2001**).

It has been demonstrated that, enzyme inhibition assays are highly specific and a mechanism-based process, hence any bioactive compound acting by a mechanism that is not related to the assay will automatically be bypassed (**TAYLOR and VAN STADEN, 2001**). This implies that, extracts exhibiting poor inhibitory activity in the present study cannot be eliminated as lacking anti-inflammatory properties as they may contain compounds with different mechanisms of action in reducing pain and inflammatory responses (**McGAW *et al.*, 1997**).

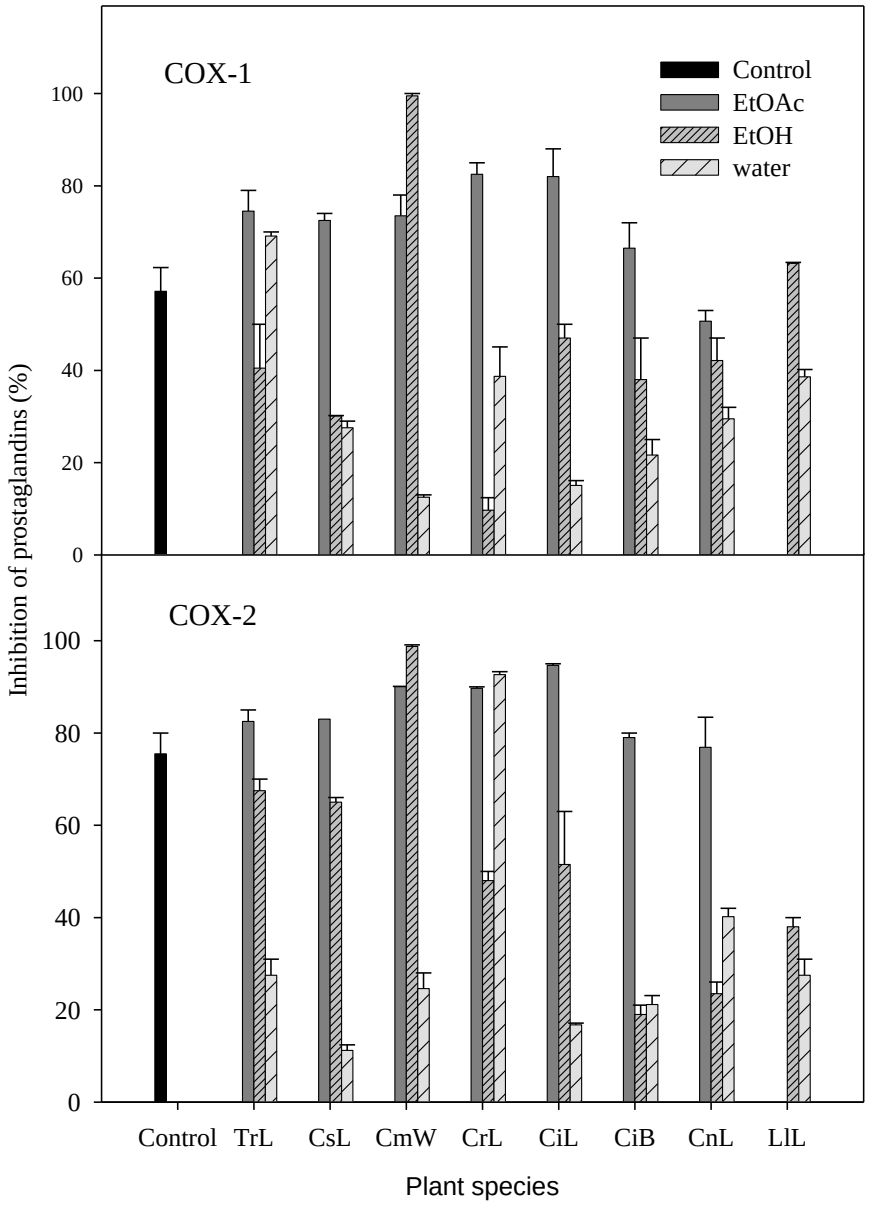

Fig. 4.1 Percentage inhibitory activity against COX-1 and COX-2 by the evaluated plant extracts. TrL = *Tetradenia riparia* leaf, CsL = *Canthium spinosum* leaf, CmW = *Crassula multicava* whole plant, CrL = *Coddia rudis* Leaf, CiL = *Cassinopsis ilicifolia* leaf, CiB = *Cassinopsis ilicifolia* bark, CnL = *Conostomium natalensis* leaf, LlL = *Lagynia lasiantha* leaf. Plant extracts with inhibitory activity above 50% were considered to be active. Aqueous extracts were evaluated at 2 mg/ml, organic extracts at 250 µg/ml. Percentage inhibition by indomethacin® in COX-1 was 57.15 ± 5.2% and COX-2 was 75.5 ± 4.5% respectively.

Plant remedies are becoming popular and often preferred to synthetically derived drugs due to their diverse range of anti-inflammatory potential and relatively low side effects. Traditionally, the evaluated plant species are used for the treatment of inflammatory responses in the gastro-intestinal tract which is characterized by stomach pains, cramps, diarrhoea, dysentery and other stomach-related ailments. Inhibition of the biosynthesis of inflammatory mediators by blocking the activities of enzymes would be an important treatment for many causes of inflammatory disorders (**ZSCHOCKE and VAN STADEN, 2000**).

4.4 Conclusions

Despite the widespread use of NSAIDs for the treatment of various kinds of inflammatory diseases over the last century, their mechanism of action was not fully appreciated until 1971 when Vane elucidated the precise molecular target for the COX enzyme (**LI *et al.*, 2006**). The anti-inflammatory activity exhibited by the evaluated medicinal plants support their uses in traditional medicine as anti-inflammatory agents in treating stomach pains and cramps associated with gastro-intestinal ailments. Plant extracts that exhibited good anti-inflammatory activity especially against COX-2 enzyme needed further studies to determine their inhibitory potential against other pro-inflammatory mediators such as the NF_kB mediated signalling pathway in the immune cells that leads to the production of iNOS, pro-inflammatory cytokines and iCOX (**POLYA, 2003**). A number of bioactive compounds from plants have been identified to have significant anti-inflammatory effects especially those that are COX-2 selective have been sought by pharmaceutical companies in the search for novel anti-inflammatory agents and can be used as blue-prints in the development of superior anti-inflammatory drugs.

In traditional medicine, plant decoction are prepared by using water, hence, low inhibitory activity of aqueous extracts against COX enzymes does not mean complete absence of bioactive compounds as they may have inhibitory potential against other pro-inflammatory mediators.

Further studies are needed to isolate and identify the anti-inflammatory compounds in the plant species for which specific activity has been reported for the first time and possibly subject them to *in vitro* and *in vivo* tests. This could be a lead in developing new anti-inflammatory drugs that will be capable of inhibiting other pro-inflammatory mediators found in inflammatory responses.

Chapter 5

Genotoxic properties of bioactive plant extracts

5.1 Introduction

Mutation occurs when an alteration in the genomic sequence of a cell takes place. It can occur as a gene (point) mutation involving the modification of a single base in the DNA; or as an insertion/deletion of one or more bases which may result in a gain or loss of a whole chromosome. Mutagenic substances are capable of damaging the germ line of a cell which can lead to fertility problems or mutation/degenerative diseases in future generations (**MORTELMANS and ZEIGER, 2000**). The mechanism of mutation is a complex process which may involve direct mutagenesis, metabolic activation or detoxification of mutagenic substances. This process may lead to induction of DNA molecules causing structural damage and loss of its ability to transcribe the gene for which the affected DNA encodes (**FAVOR, 1999; NIWA, 2006**). The integrity of DNA is vital to cell division and any oxidative alteration can disrupt its transcription, translation and replication. This may subsequently give rise to mutations and the death of a cell (**LABIENIEC and GABRYELAK, 2005**). Oxidative modification can also damage proteins, causing impairment of their structural or enzymatic functions. This process may proceed along several pathways such as direct oxidation of side chains, by indirectly reacting with the products of lipid and carbohydrate oxidation or by modifying the sugar moieties of the glycoproteins (**LABIENIEC and GABRYELAK, 2005**).

Tandem repeat sequences have been used extensively as mutation markers in studying the frequency of mutation in germ lines and at the somatic level where most repeats in gene sequences occur. Repeat sequences are generally unstable and due to their unusual biophysical properties, it can lead to cellular senescence and cancer (**NIWA, 2006**). The use of 2,4-dinitrophenylhydrazine (DNPH) as the marker of oxidative damage is a recent development which determines the level of carbonyl groups (CO). This technique is used in studying the progression of oxidative damages in DNA sequence (**LABIENIEC and GABRYELAK, 2005**).

Understanding of the human genome is a great advancement in recent years, which enables biologists to establish genetic links to many known diseases. This has greatly influenced the development of therapeutic agents that are used in treating several diseases particularly cancers that emerge from cell mutagenicity (**NATARAJAN *et al*., 2003**).

Plant extracts are believed to be harmless and safe because they are "natural" and have been utilized in the treatment of infectious diseases for centuries. However, scientific studies have shown that some of the plants used in traditional medicine contain substances that are potentially toxic and/or carcinogenic (**POPAT *et al*., 2001; VERSCHAEVE *et al*., 2004**). Due to the rate at which plants are being exploited for medicinal purposes the need to determine the toxic or genotoxic potential of natural products is becoming very important. Studies on the mutagenic and antimutagenic activities of various plant compounds, including some derivatives of flavonoids, known to have toxic effects, will help in reducing the risk of using substances that could interact and cause severe cellular and molecular disorders in both humans and animals (**HORN and VARGAS, 2008**).

Studies have shown that more than 26% of the annual death rate from acute poisoning in South Africa is due to poisoning from the use of traditional medicine (**POPAT *et al.*, 2001**). Hence, evaluating South African medicinal plants for their genotoxicity potential to determine the safety of some of the commonly sold herbal products has become the top research priority (**POPAT *et al.*, 2001**).

It is not sufficient merely to identify substances which may pose a genetic hazard to the human population; some of these compounds have been identified to have significant beneficial activity in the treatment of cancer and other degenerative diseases. This implies that some of these compounds cannot reasonably be eliminated from use. However, there is need to determine the safety and the right dose of such compounds before they can be recommended for human use (**THYBAUD *et al.*, 2007**).

5.1.1 Ames test

The Ames test is a simple and rapid technique that is widely used to detect the presence of mutagenic compounds. This technique is used to predict the possible carcinogenicity of compounds, because most of the mutagenic compounds can remain inactive until bio-activated before binding to DNA and causing mutation leading to cancer and cell death (**HAKURA *et al.*, 1999**). Although recent studies have shown that some of the current *in vitro* test systems such as the Ames test can generate both false negative and false positive results in relation to predicting carcinogenicity. These potential discrepancies could be attributed to metabolic activities, such as the limited capability of *in vitro* exogenous activation systems, substrate preference for specific

enzyme, the artificial metabolic activity of the liver S9- mix, and the different biotransformation of chemicals in cells of different tissues from different species (**KU *et al.*, 2007**).

Mutations in gene sequences are measured in bacteria and other cell systems especially when the mutagens cause a change in the growth requirements of the cell (**MORTELMANS and ZEIGER, 2000**). A range of bacterial tester strains have been engineered for investigating various mutagens. The introduction of the plasmid *pKM101* into the *Salmonella typhimurium* tester strains TA1535 and TA1538 resulted in the corresponding isogenic strains of TA98 and TA100, and in TA97 it resulted in TA102 and TA104. The Plasmid *pKM101* generally enhances chemical and UV-induced mutagenesis via an increase in the error-prone recombinational DNA repair pathway (**CARROLL *et al.*, 2002**). All the bacterial tester strains have different mutations in various genes in the histidine operon which are designed to act through different mechanisms with high sensitivity to mutagens. Strain TA98 detects various frame-shift mutagens. The *hisD3052* mutation in strains TA98 and TA1538 carries a -1 frame-shift mutation which affects the reading frame of a nearby repetitive sequence of cytosine/guanine and this can be reverted to the wild-type state by various frame-shift mutagens in the test compounds (**MORTELMANS and ZEIGER, 2000**). The *hisD6610* mutation in strain 97 carries a +1 frame-shift mutation (cystosine) resulting in a run of 6 cystosines which is believed to be more sensitive than strain TA1537 to frame-shift mutagens The *hisG46* mutation in strains TA1535 and TA100 results from the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC) and these can be reverted to the wild-type state by mutagens that cause base-pair substitution at one of the GC pairs. Strain TA102 has the mutation *hisG428* inserted on the multi-copy plasmid *pAQ1* with the aim of amplifying the number of target sites which will enhance the strain to detect DNA cross-

linking agents and mutagens that cause oxidative damages (**MORTELMANS and ZEIGER, 2000**).

This study was aimed at evaluating the mutagenic effects of the bioactive plant extracts on three histidine dependent strains of *Salmonella typhimurium* (TA98, TA100 and TA1537). A positive response in any single bacterial strain either with or without metabolic activation is sufficient to designate a substance as a mutagen (**ZEIGER, 2001**).

5.2 Materials and methods

5.2.1 Plant extract preparations

The biologically active plant extracts were redissolved in 10% DMSO to make a concentration of 5 mg/ml which were then filtered through 0.22 µm filter to remove impurities. From the stock solution two other dilutions were made resulting in three different concentrations (50, 500 and 5000 µg/ml) each with sterile 10% DMSO for the organic solvent extracts and distilled water for the aqueous extracts.

5.2.2 Plate incorporation assay

The genotoxicity potential, of crude plant extracts that showed good antimicrobial and anthelminthic activities with MIC and MLC values <1 mg/ml as reported in Chapters 2 and 3 respectively were determined. The *Salmonella* microsome assay was used based on the standard plate-incorporation procedure with *Salmonella typhimurium* tester strains TA98, TA100 and TA1537 without metabolic activation (**MARON and AMES, 1983; MORTELMANS and**

ZEIGER, 2000). The bacterial tester strains were grown overnight in 10 ml Oxoid nutrient broth No. 2 for 16 h at 37 °C with agitation (100 rpm) to obtain a density of 1×10^9 (CFU/ml). Top agar was melted at the beginning of the assay and supplemented with 10 ml of histidine/biotin (0.5mM) and kept in a water bath at 50 °C. In triplicate, 100 µl of each of the three dilutions (50, 500 and 5000 µg/ml) per sample was added to each sterile glass tube, followed by 500 µl of phosphate buffer (0.1 mM, pH 7.4). Thereafter, 100 µl of the overnight bacterial culture was added to each tube. From the supplemented top agar, 2 ml was added to the mixture in the sterile tubes. The contents of the tubes were then mixed with a vortex mixer and poured onto the labeled minimal agar plates. After 2-3 min as the top agar hardened, the plates were inverted and incubated at 37 °C for 48 h. The revertant colonies were counted using a colony counter. The assay was performed for each bacterial strain and the results were expressed as the mean (± standard error) number of the revertant colonies per plate. 4-Nitroquinoline-N-oxide (4NQO) (2 µg/plate) was used as the positive control and 10% DMSO as the negative control.

5.3 Results and discussion

The spontaneous reversion response of the *Salmonella typhimurium* tester strains to different dilutions of plant extracts are presented in Table 5.1. The Ames test without metabolic activation is designed to detect only direct mutagens. The results indicated that all the evaluated extracts were non-mutagenic towards *Salmonella typhimurium* strains TA98, TA100 and TA1537. None of the evaluated plant extracts exhibited a dose dependent increase in the number of revertants and the numbers of revertants were not equal to, or greater than, two times that of the negative control (**MARON and AMES, 1983**). This implies that the evaluated plant extracts were devoid of any direct mutagenic compounds. The dense background of the plates as compared to the

negative control after 48 h showed that all the extracts were not toxic to the *Salmonella typhimurium* tester strains.

Several compounds isolated from plants have been identified as carcinogens, some of these compounds such as the aromatic amines, furoquinoline alkaloids, isothiocyanates and several polycyclic aromatic hydrocarbons like benzo-[a]-pyrene can remain biologically inactive until they are metabolized to active diol epoxide products as the ultimate carcinogen (**RETHER *et al.*, 1990; POLYA, 2003**). Metabolic activation or detoxification of xenobiotic compounds in humans mostly take place in the liver, lungs and kidneys and sometimes the process may result in bioactivation of metabolites capable of damaging DNA (**MORTELMANS and ZEIGER, 2000**). Hence, the evaluated medicinal plants which are administered orally in the treatment of stomach-related ailments may result in many potential carcinogenic compounds to be metabolized after enzymatic activation within the body which could lead to adverse effects.

The absence of mutagenic activity shown by the extracts does not in any way indicate absolute safety of these extracts as their metabolites could be mutagenic. At this stage, the consumption of these plants in traditional medicine could be said to be reasonably safe dependent on further studies; incorporating metabolizing enzymes that will enhance bioactivation of mutagenic metabolites that might be present in the extracts.

Table 5.1: Number of His^+ revertants observed in *Salmonella typhimurium* strains TA98, TA100 and TA1537 induced by the bioactive extracts

Plant species	Plant part	Plant extracts	Number of His^+ revertants (µg/ ml)								
			TA98			TA100			TA1537		
			5000	500	50	5000	500	50	5000	500	50
T. riparia	Leaf	EtOAc	14.7±2.2	15.7±2.9	22.0±2.0	165.0±1.7	176.0±5.1	170.3±5.6	30.3±5.5	30.7±3.3	22.0±2.8
C. spinosium	Leaf	EtOAc	15.7±1.2	19.7±0.7	18.3±4.4	184.0±11	174.0±10	167.7±6.4	39.0±1.7	32.1±3.4	18.3±4.4
		EtOH	16.0±3.2	15.0±2.5	19.7±1.5	162.7±6.4	149.6±2.6	158.0±2.5	55.7±3.4	62.3±6.1	19.7±1.5
		Water	17.0±2.5	15.3±0.0	17.7±1.2	166.7±8.8	197±18.2	169.0±4.9	49.3±2.6	57.7±5.0	17.7±1.2
C. multicava	Whole plant	EtOAc	16.7±0.9	16.0±1.7	19.3±1.2	174.7±6.9	163.3±4.4	153.0±4.9	30.5±2.3	33.7±2.8	19.3±1.2
		EtOH	17.7±0.9	14.3±1.3	15.3±1.7	160.0±7.2	170.0±10	168.0±9.0	58.3±5.4	57.0±6.8	15.3±1.7
C. rudis	Leaf	EtOAc	15.7±1.9	16.7±1.2	16.0±2.5	196.3±6.6	187.3±8.7	174.0±15	22.7±5.8	29.0±4.1	16.0±2.5
		EtOH	18.3±2.4	18.7±2.0	20.0±1.5	164.3±0.7	158.0±11	174.0±2.1	56.3±2.3	49.7±2.9	20.0±1.5
C. ilicifolia	Leaf	EtOAc	21.7±1.2	20.7±0.9	14.0±0.6	158.0±9.3	145.7±9.4	134.7±7.3	29.7±1.8	29.7±2.7	14.3±0.6
		EtOH	18.3±1.8	15.3±1.3	16.3±1.5	148.3±1.2	152.0±7.2	173.7±7.8	51.7±3.9	47.3±1.8	16.3±1.5

Table 5.1: ***continued***

Plant species	part	Plant extracts	Number of His $^{+}$ revertant (µg/ml)								
			TA98			TA100			TA1537		
			5000	500	50	5000	500	50	5000	500	50
		EtOH	18.3±1.8	15.3±1.3	16.3±1.5	148.3±1.2	152.0±7.2	173.7±7.8	51.7±3.9	47.3±1.8	16.3±1.5
		Water	19.7±2.3	17.3±3.5	18.3±1.2	185.3±7.4	189.7±9.3	182.7±2.9	59.7±4.9	62.0±4.5	18.3±1.2
C. spinosum	Bark	EtOAc	19.7±1.8	11.3±2.5	14.7±2.6	179.3±8.7	171.3±9.8	161.3±6.3	25.7±2.4	29.0±3.7	14.7±2.6
		EtOH	17.0±3.8	21.5±4.5	14.3±0.7	159.3±8.1	163.0±8.6	142.7±8.4	51.0±3.8	48.3±2.3	14.3±0.7
		Water	12.3±0.3	11.7±1.2	32.0±6.0	124.3±8.1	181.0±9.5	197.7±9.9	58.7±14	57.7±1.2	32.0±6.0
C. natalensis	Leaf	EtOAc	18.7±2.6	19.7±0.9	15.3±1.2	171.0±2.5	179.7±7.4	187.3±4.7	28.0±4.4	30.3±2.7	15.3±1.2
L. lasiantha	Leaf	EtOAc	16.7±2.3	23.0±3.7	19.0±1.7	160.0±6.1	189.3±4.4	172.0±10	40.0±2.3	32.3±3.2	19.0±1.7
4-QNO			271.7±10			898.7±9.8			84.6±4.8		
10% DMSO			25.0±2.3			165.7±5.5			58.7±1.5		

Number of His^{+} revertants/plate: mean values of three triplicates per sample. 4-NQO; 4-nitroquinoline-oxide at 5 µg/ml was used as the positive control for the assay, DMSO = dimethyl sulfoxide, EtOAc = ethyl acetate, EtOH = ethanol.

5.4 Conclusions

The determination of toxicity and mutagenicity of South African medicinal plants is becoming increasingly important, due to several cases of poisoning resulting from the use of traditional medicine leading to high morbidity and mortality (**POPAT *et al.*, 2001**). The evaluated medicinal plant extracts exhibited non-mutagenic activity against the *Salmonella typhimurium* tester strains TA98, TA100 and TA1537 at different dilutions when metabolic activation did not occur. The absence of mutagenic response in the Ames test among all the evaluated extracts provides evidence to support the safe consumption of the medicinal plants in traditional medicine. This finding is a step toward determining the safety of these plant extracts. At this stage the bioactive compounds from plant extracts with good antimicrobial activities could be used in the preparation of safe antimicrobial agents. To ensure the safe use of the plant extracts on long-term consumption, it will be important to subject the selected medicinal plants to further studies that will incorporate metabolizing enzymes (e.g. induced rat liver S9) capable of detecting the presence of promutagens.

Chapter 6

Plant secondary metabolites

6.1 Introduction

Plant secondary metabolites are compounds with a variety of unique carbon skeletons and functional group modifications that are not required for the basic metabolic activities of plants, but play vital roles as mediators in the interactions of the plant with its biotic and abiotic environment; such as plant-insect, plant-microorganism, and plant-plant interactions (**VERPOORTE and MEMELINK, 2002; KUTCHAN and DIXON, 2005**). Secondary metabolites in plants comprise numerous compounds that are produced at various stages during development which may have distinct functions. For instance; alkaloids play important roles as defensive agents by deterring herbivores due to their bitter taste, and volatile oils act as pheromones (for attracting pollinating insects) and protecting the plant against ultraviolet radiation, microbial infections and pest attack (**HODEK *et al.*, 2002; DANIEL, 2006; CROZIER *et al.*, 2006; MAKKAR *et al.*, 2007**). The diverse functions of plant secondary metabolites show that they are not aberrations in the biosynthesis of plants but are prepared through specially designed mechanistic pathways (**DANIEL, 2006**). Plant secondary metabolites demonstrate several biological activities and have been used for centuries. Some of them have important applications in the pharmaceutical, cosmetic, and nutraceutical industries (**BOURGAUD *et al.*, 2001**). Plant secondary metabolites are classified into three main groups based on their biosynthetic pathways, namely phenolics, terpenes and alkaloids.

Phenolics are the most widespread metabolites and are made of several compounds such as condensed tannins, gallatonnins and flavonoids (**BOURGAUD *et al*., 2001**).

6.1.1 Properties of plant secondary metabolites

6.1.1.1 Phenolics

The term phenolic acid includes all compounds of secondary nature that possess an aromatic ring and bear a hydroxyl group or its substitute. Phenolic compounds include metabolites derived from the condensation of acetate units (e.g. terpenoids), and those produced by the modification of aromatic amino acids (e.g. phenylpropanoids, cinnamic acid, lignin precursors and catechols) (**RAMAWAT, 2009**). Plant phenolics, represent a large group of defensive compounds that have a phenol (hydroxybenzene) moiety and are mainly produced by the phenylpropanoid pathway (**IGNAT *et al*., 2011; GRUZ *et al*., 2011**). Phenolics are one of the most widely occurring phytochemicals with considerable morphological importance in the plant kingdom. These compounds range in complexity from simple phenolics and quinones, through chalcones and stilbenes to a range of phenolics with three rings namely anthocyanins, anthochlors, benzofurans, chromones, coumarins, flavonoids, isoflavonoids, neoflavonoids, stilbenoids and xanthones. There are some other complex polycyclic phenolics that exist, notably the hydrolysable tannins and the condensed tannins (**BRUNETON, 1995; POLYA, 2003; DANIEL, 2006**).

6.1.1.2 Flavonoids

Flavonoids are secondary metabolites of low molecular weight, which are widely distributed in the plant kingdom. This group of phytochemicals are produced by various plants in high quantities at different stages of development. They occur naturally in fruits, vegetables, nuts, seeds, flowers and bark, and have been reported to have numerous biological activities including antibacterial, antiviral, anti-inflammatory and vasodilatory activities (**COOK and SAMMAN, 1996; WINK, 1999; ISHIGE *et al*., 2001**). In several instances, they act as analogues of cellular signal compounds or substrates which inhibit mechanisms such as prostaglandin and leukotriene formation, enzyme inhibition, oestrogenic properties (e.g. coumarins, isoflavone and stilbenes) and DNA alkylation (e.g. furocoumarins) (**HERNÄNDEZ *et al*., 2000; HEIM *et al*., 2002; IGNAT *et al*., 2011**). In addition to several biochemical and pharmacological activities of flavonoids, they also have effective chemopreventive activity which can be used in reducing the incidence of cancer in humans (**ERLUND, 2004**).

6.1.1.3 Saponins

Saponins are glycosides of triterpenes or steroids which include the group of cardiac glycosides and steroidal alkaloids. Saponins are amphipathic (hydrophilic and hydrophobic) compounds that have water-soluble sugar residues linked (via glycosidic links formed between the sugar hemiacetal and terpenoid hydroxyls) (**WINK, 1999; POLYA, 2003; VINCKEN *et al*., 2007**). Saponins are non-volatile surface-active compounds which have widespread occurrence in higher plants, and have been detected in nearly 100 plant families

(**OLESZEK, 2002**). Steroidal saponins are common in monocotyledonous families such as the Liliaceae, Amaryllidaceae, and Dioscoreaceae, whereas triterpene saponins are predominant in dicotyledonous angiosperms (**DANIEL, 2006**).

Some saponins are stored as bidesmosidic compounds in the plant vacuole and are broken down into monodesmosidic compounds by β-glucosidase upon wounding. Monodesmosidic saponins are amphiphilic compounds, which form complexes with cholesterol in biomembranes with their lipophilic terpenoid moiety and bind to surface glycoproteins and glycolipids with their sugar side chain. These haemolytic properties of saponins can leads to severe tension of the biomembrane and leakage thereby solubilizing the cell membrane of red blood cells (**WINK, 1999; POLYA, 2003**).

A number of analytical techniques have been developed for analysis of plant secondary metabolites. The colorimetric method is the most frequently used technique due to its accuracy, rapid results and ease of set up (**XU and DIOSADY, 1997; SCHOFIELD *et al.*, 2001**). The selected medicinal plants were evaluated for their phenolic constituents including the total phenolic compounds, total flavonoids, hydrolysable tannins, and saponins using colorimetric methods.

6.2 Materials and methods

6.2.1 Preparation of plant extracts

Phenolic compounds were extracted from plant materials as described by **MAKKAR** (**1999**). Dried plant samples (2 g) were extracted with 10 ml of 50% aqueous methanol by sonication on ice for 20 min. The extracts were then filtered under vacuum through Whatman No. 1 filter paper. The extracts were transferred into pill vials for analysis.

6.2.2 Determination of total phenolic compounds

The amounts of total phenolic compounds in plant samples were determined using the Folin Ciocalteu (Folin C) assay as described by **MAKKAR** (**1999**) with slight modifications. In triplicate, 50 µl of each plant extract were transferred into test tubes and was made up to 1 ml by adding 950 µl of distilled water followed by 500 µl of 1 N Folin C phenol reagent and 2.5 ml of 2% sodium carbonate. A blank that contained aqueous methanol instead of plant extracts was also prepared. The test mixtures were incubated for 40 min at room temperature, thereafter the absorbance was read at 725 nm using a UV–vis spectrophotometer (Varian Cary 50, Australia). Total phenolic concentration was expressed as gallic acid equivalents (GAE).

6.2.3 Determination of hydrolysable tannin

The determination of hydrolysable tannins as gallotannin concentration was done using rhodanine assay for gallotannin as described by **MAKKAR** (**1999**) with modifications (**NDHLALA *et al.*, 2007**). In triplicate, 50 µl of plant extracts were transferred into test tubes and were made up to 1 ml with distilled water. One hundred ml of 0.4 N sulphuric acid and

600 µl of rhodanine were added to the diluted extracts. After incubating for 5 min at room temperature, 200 µl of 0.5 N potassium hydroxide was added followed by 4 ml distilled water after a further 2.5 min incubation. The mixtures were incubated for an additional 15 min at room temperature, after which the absorbance at 520 nm was read using a UV–vis spectrophotometer against a blank that contained 50 µl of methanol instead of the plant extract. Gallotannin concentrations were expressed as GAE, derived from a standard curve.

6.2.4 Determination of total flavonoid

The aluminium chloride colorimetric assay as described by **ZHISHEN *et al.*** (**1999**) and **MARINOVA *et al.*** (**2005**) was used to determine the total flavonoid content. In triplicate, 500 µl of plant extracts were pipetted into test tubes and 2 ml of distilled water were added to all the test tubes, followed by 150 µl of 5% $NaNO_2$. After incubating for 5 min, 150 µl of 10% $AlCl_3$ was added to all the test tubes. At the 6^{th} min of incubation, 1 ml of 1M NaOH was transferred to all the test tubes and the volume was made up to 5 ml by adding 1.2 ml of distilled water. Thereafter, the mixture was vortexed and the absorbance was then read at 510 nm using using a UV–vis spectrophotometer against reagent blank containing 50% methanol instead of the plant extracts. Total flavonoid content was expressed as catechin equivalents (CTE).

6.3 Saponin content

6.3.1 Qualitative determination of saponins

The saponin content was determined as described by **TADHANI and SUBHASH** (**2006**). Ten ml of distilled water was added to 0.1 g of plant samples in test tubes. The test tubes were corked and vigorously shaken for 2 min. The appearance of stable and persistent foam on the liquid surface for 15 min indicated the presence of saponins. Presence of saponins was confirmed by the formation of an emulsion upon addition of ten drops of olive oil to the 2 ml aqueous extract.

6.3.2 Saponin extraction

Saponins were extracted from the plant material as described by **MAKKAR *et al.*** (**2007**). The powdered plant samples were defatted with hexane in a Soxhlet apparatus for 3 h. After air-drying, saponins were extracted twice from the defatted samples (10 g of sample in 100 ml of 50% aqueous methanol by incubating at room temperature overnight with continuous stirring). The extracts were then centrifuged at 3000 rpm for 10 min and the supernatant was collected. The procedure was repeated with the original residue to obtain a second supernatant. The two supernatants were combined and filtered under vacuum through Whatman No. 1 filter paper. Methanol from the filtrate was evaporated from the solution under vacuum at 40 °C to yield the saponin sample in the aqueous phase. The aqueous phase was then centrifuged at 3000 rpm for 10 min to remove water insoluble materials, and transferred to a separating funnel and extracted three times with an equal volume of chloroform to remove pigments. The concentrated saponins in the aqueous solution were then extracted twice with an equal volume

of *n*-butanol. The *n*-butanol was evaporated under vacuum at 45 °C. The dried fractions containing saponins were dissolved in 10 ml of distilled water and air-dried under a stream of cold air.

6.3.3 Quantitative determination of total saponin

Total saponin content in plant samples that tested positive in the Froth test was determined using a spectrophotometric method as described by **HIAI *et al.*** (**1976**) with modifications. The crude saponin extracts were dissolved in 50% aqueous methanol to a concentration of 10 mg/ml. In triplicate, 250 µl of each sample were transferred to test tubes into which an equal volume of vanillin reagent (8 g/100 ml ethanol) was added followed by 2.5 ml of 72% (v/v) sulphuric acid. The mixture was vortexed and placed in a water bath at 60 °C for 10 min. The tubes were cooled on ice for 3 to 4 min and absorbance was measured at 544 nm using a UV–vis spectrophotometer against a blank that contained 50% aqueous methanol instead of plant extract. The saponin concentrations were expressed as diosgenin equivalents (DE), derived from a standard curve.

6.2.4 Quantitative determination of total steroidal saponin

The amounts of total steroidal saponin in plant samples that tested positive in the Froth test was determined using the method described by **BACCOU *et al.*** (**1977**). Crude saponin extracts were dissolved in 50% aqueous methanol (0.1 mg/ml) from which 300 µl aliquots (corresponding to a sapogenin content of between 1 and 40 µg) were transferred into test tubes and placed in a boiling water bath at 100 °C to remove the methanol by evaporation. After

cooling, 2 ml of ethyl acetate was added followed by 1 ml of anisaldehyde–ethyl acetate reagent (0.5:95.5, v/v) and 1 ml sulphuric acid–ethyl acetate reagent (50:50, v/v). The reaction mixtures were vortexed and incubated in a water bath at 60 °C for 20 min. After cooling for 10 min in a water bath at room temperature, absorbance was read at 430 nm using a UV–vis spectrophotometer against a blank that contained ethyl acetate instead of plant extract. Each extract was evaluated in triplicate and steroidal saponin concentrations were expressed as diosgenin equivalents (DE), derived from a standard curve.

6.3 Results and discussion

6.3.1 Total phenolic composition of the plant extracts

The concentration of total phenolic compounds in each of the evaluated plant extracts are presented in Figure 6.1. Phytochemical analysis revealed varying concentrations of total phenolic compounds in all the evaluated plant extracts which could be attributed to the observed pharmacological activities. The leaf extract of *C. natalensis* had the highest amount of total phenolics (1.7 mg GAE/g DW). It is important to note that phytochemical compounds at lower concentrations have significant beneficial effects such as antimicrobial, antioxidant and anti-inflammatory, antiviral, antimutagenic and chemopreventic effects. Higher concentrations of phytochemical compounds on the other hand have been reported to have negative physiological effects such as neurological disfunction, gastrointestinal toxicity, and reproductive failure (**POLYA, 2003; MAKKAR *et al.*, 2007**). The extract of *T. riparia* had the lowest amount of total phenolic compounds (0.02 mg GAE/g DW) but exhibited the best pharmacological activity among all the evaluated extracts. This result suggests that *T. riparia*

might contain more bioactive compounds than the other extracts evaluated. The observed pharmacological activities by *T. riparia* could also be due to other types of phytochemicals that were not screened for, or synergism of the bioactive compounds (**SADHU *et al.*, 2006**).

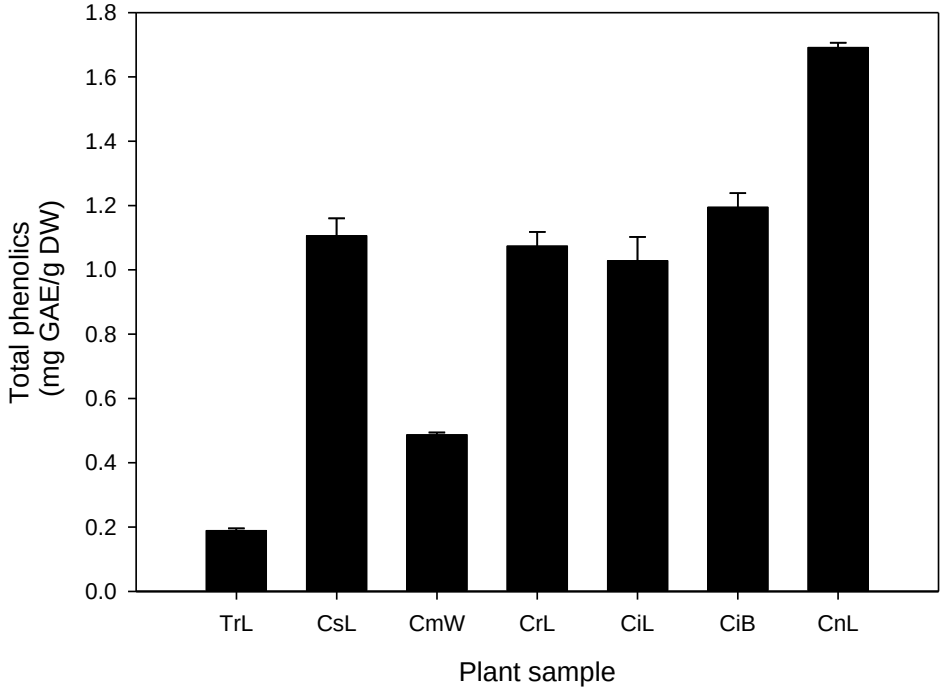

Figure 6.1: Total phenolic compositions expressed as gallic acid equivalents detected in the evaluated medicinal plants. DW = dry weight, GAE = gallic acid equivalents, TrL = *Tetradenia riparia* leaf, CsL = *Canthium spinosum* leaf, CmW = *Crassula multicava* whole plant, CrL = *Coddia rudis* leaf, CiL = *Cassinopsis ilicifolia* leaf, CiB = *Cassinopsis ilicifolia* bark, CnL = *Conostomium natalensis* leaf.

6.3.2 Gallotannin content in the evaluated medicinal plants

The amount of gallotannins detected in the evaluated plant extracts, expressed as gallic acid equivalents, is presented in Figure 6.2. Gallotannins were detected in all the evaluated

medicinal plants. The highest amount of gallotannin was detected in the leaf extract of *C. rudis* and *C. natanlensis* (0.12 and 0.11 mg GAE/g DW) respectively. Extract of *T. riparia* had the lowest (0.01 mg GAE/g DW) amount. Gallotannins have been reported to exhibit significant biological activities including antimicrobial, anti-inflammatory and anticancer effects (**BRUNETON, 1995**). The mechanism of anti-inflammatory activity of gallotannin is based on its ability to scavenge free radicals that can initiate inflammatory responses and the inhibition of various pro-inflammatory mediators, such as the COX-2 enzyme, iNOS and PGs (**POLYA, 2003**). The presence of gallotannins in all the evaluated medicinal plants may explain the observed antimicrobial activities as well as good anti-inflammatory effects especially against COX-2 by most of the plant extracts. This provides evidence indicating why the selected medicinal plants are used in traditional medicine in treating stomach pains and cramp associated with gastrointestinal ailments.

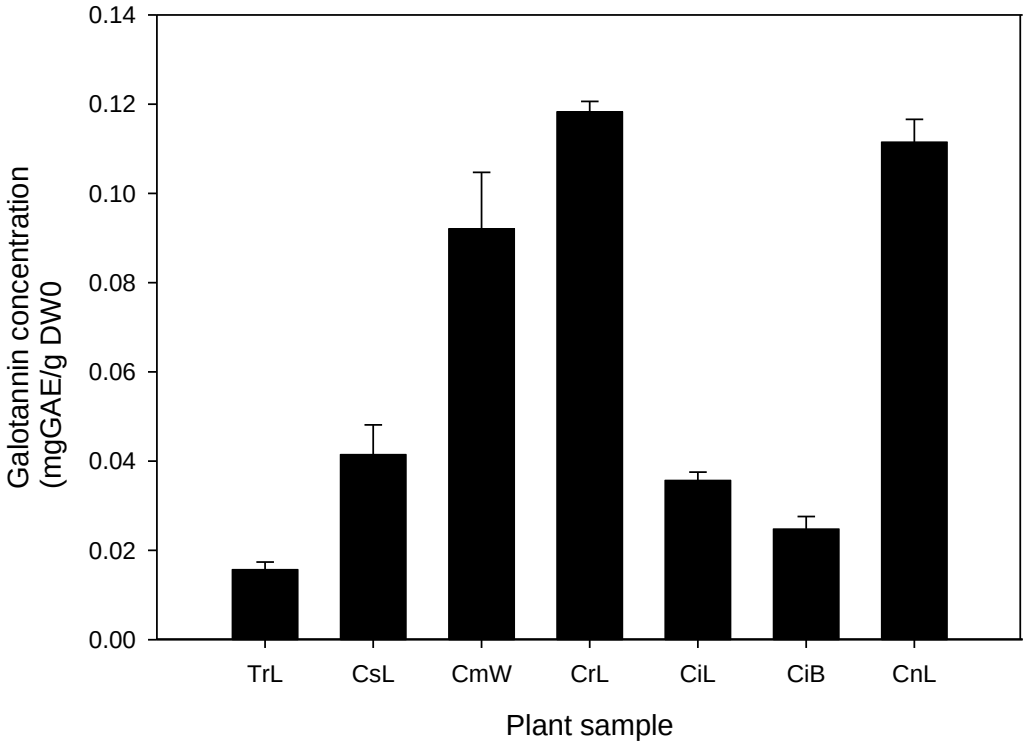

Figure 6.2: Gallotannin concentrations expressed as gallic acid equivalents, detected in the investigated medicinal plants. DW = dry weight, GAE = gallic acid equivalents, TrL = *Tetradenia riparia* leaf, CsL = *Canthium spinosum* leaf, CmW = *Crassula multicava* whole plant, CrL = *Coddia rudis* leaf, CiL = *Cassinopsis ilicifolia* leaf, CiB = *Cassinopsis ilicifolia* bark, CnL = *Conostomium natalensis* leaf.

6.3.3 Flavonoid concentration in evaluated plant extracts

The concentrations of flavonoids in the evaluated plant extracts, measured as catechin equivalents, are presented in Figure 6.3. Relatively high levels of flavonoids were detected in all the evaluated plant extracts. The highest concentrations of flavonoids were detected in the leaf extracts of *C. spinosum* and *C. rudis* (0.26 and 0.27 mg CTE/g DW) respectively. The anti-inflammatory and antimicrobial activity of several flavonoids such as flavonol monomers

and biflavonoids are well known. They inhibit various pro-inflammatory mediators such as PGs, COX and LOX by modulating essential biosynthetic and signal transduction pathways in organisms and this activity may be directly related to their radical scavenging capability (**PELZER *et al*., 1998; GUARDIA *et al*., 2001; HODEK *et al*., 2002**). Flavonoids are known to inhibit phosphodiesterases involved in specific cell activation responsible for mediating leukocyte adhesion to the sites of injury and this explains their anti-platelet aggregation activity (**LIN *et al*., 1993; WENG *et al*., 2006**). The presence of flavonoids at considerably high levels in all the evaluated plant extracts could partly be responsible for the observed pharmacological activities among some of the plant extracts. Flavonoids are known to exhibit numerous beneficial effects, but control should be exercised in the application of these compounds as therapeutic agents, because some metabolites of these compounds could be genotoxic and induce adverse effects.

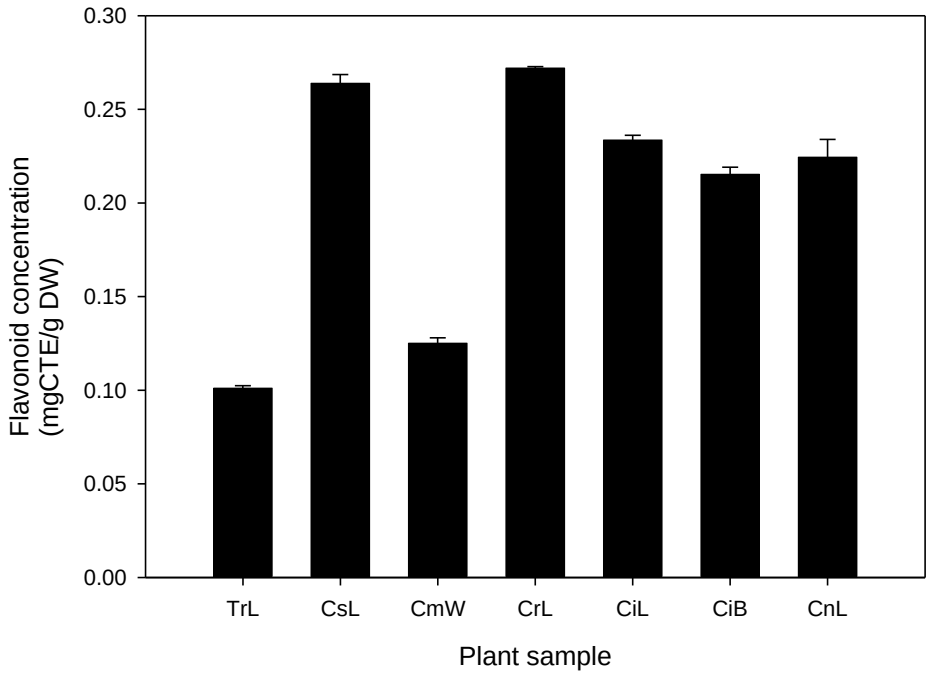

Figure 6.3: Flavonoid concentration, as catechin equivalents, detected in the evaluated medicinal plants. DW = dry weight, CTE = catechin equivalent, TrL = *Tetradenia riparia* leaf, CsL = *Canthium spinosum* leaf, CmW = *Crassula multicava* whole plant, CrL = *Coddia rudis* leaf, CiL = *Cassinopsis ilicifolia* leaf, CiB = *Cassinopsis ilicifolia* bark, CnL = *Conostomium natalensis* leaf.

6.3.5 Total saponin and steroidal saponin in the evaluated plant extracts

The qualitative and colorimetric results for the presence of saponins in the evaluated plant extracts are presented in Table 6.1. Based on the "Froth" test, saponins were detected in only two plant extracts; *C. spinosum* (leaf) and *C. ilicifolia* (bark). *Canthium spinosum* had the highest amount of both total and steroidal saponins compared to *C. ilicifolia* (bark). There could be synergism between the saponins and other bioactive molecules responsible for the good antimicrobial, anthelmintic and anti-inflammatory activities observed in the extracts of

C. spinosum and *C. ilicifolia* (bark). Saponins are structurally diverse bioactive compounds of plant origin. They consist of non-polar aglycones coupled with one or more monosaccharide moieties and are well known for their numerous properties which include sweetness and bitterness, foaming and emulsifying, and pharmacological activities (**SPARG *et al.*, 2004; VINCKEN *et al.*, 2007**). Saponins have also been shown to lower the levels of serum cholesterol in animals and humans which might reduce the risk of cardiovascular diseases and have the abilities to scavenge free radicals and counteract oxidative stress (**FRANCIS *et al.*, 2002; QIN *et al.*, 2009**). Generally, saponins are found in plant tissues that are most vulnerable to fungal or bacterial attack or insect predation (**WINA *et al.*, 2005**). Therefore, it is believed that one of their roles in plants is to act as a chemical barrier against potential pathogens, which may explain their potent pharmacological activities (**SPARG *et al.*, 2004; VINCKEN *et al.*, 2007**).

Table 6.1: Saponin composition of the evaluated plant extracts

Plant species	Plant part	Froth test	Total saponin (mg DE/g)	Steroidal saponin (mg DE/g)
Canthium spinosum	leaf	+	14.2 ± 6.2	13.9 ± 4.8
Cassinopsis ilicifolia	leaf	-	ND	ND
	bark	+	11.7 ± 12.3	4.5 ± 9.1
Coddia rudis	leaf	-	ND	ND
Conostomium natalensis	leaf	-	ND	ND
Crassula multicava	whole plant	-	ND	ND
Lagynia lasiantha	leaf	-	ND	ND
Tetradenia riparia	leaf	-	ND	ND

- = absence of saponins, + = present of saponins, DE = diosgenin equivalence, ND = not determined.

6.4 Conclusions

The results of the present study revealed the diverse range of phytochemical composition in the selected plant species. This indicates the type of bioactive compounds that could be responsible for the antimicrobial, anthelmintic and anti-inflammatory activities. These activities could result from the synergism of the bioactive compounds present in the evaluated extracts or other phytochemical compounds that were not screened for. This study also revealed the presence of saponins in two plant samples and this provides useful information for further scientific studies. Further studies are needed to isolate and identify the bioactive

compounds present in the evaluated plant species and this might lead to developing superior drugs with potent pharmacological activities.

Chapter 7

General conclusions

South Africa is renowned for its high plant biodiversity, and because of the long history of traditional healing and the cultural belief of the people, the majority of the rural population still make use of traditional plant-based medicine for their primary health care needs. Traditional healers in this region play a key role in administering medications for various kinds of infectious diseases that are prevalent in rural areas. The rate at which plants are being exploit for therapeutic purposes in recent years warrant scientific validation to establish the efficacy and the safety of their uses in traditional medicine.

The results presented in this thesis dealt with the general pharmacological studies of the investigated plant species used in treating stomach-related ailments. These involved broad evaluation for antibacterial, antifungal, anthelmintic, anti-inflammatory and mutagenic activities. The study also evaluated the phytochemical compositions of the investigated plant species which include total phenolics, gallotannins, flavonoids, and total saponins.

The results of the general pharmacological screening of medicinal plants as highlighted in Chapters 2, 3, and 4 showed that some of the plants used in traditional medicine in treating infectious diseases exhibited good antimicrobial, anthelmintic and anti-inflammatory activities. Of all the evaluated plant species only the extracts of *C. rudis* exhibited poor antibacterial activity. The EtOAc extract of *T. riparia* was the only extract that demonstrated

promising antibacterial activity against the Gram-negative (*E. coli*) bacterium. Good antifungal activity with an MIC value <1 mg/ml was observed in only 5 extracts, and none of the extracts exhibited corresponding fungicidal activity. The results of this study provide scientific validation of the evaluated medicinal plants used in traditional medicine as antimicrobial agents.

The *in vitro* colorimetric assay for anthelmintic activity indicates the presence of potent anthelmintic compounds in all the evaluated plant species and the different levels of activities among the evaluated extracts revealed the need to use more than one solvent to extract bioactive compounds from plants in the search for novel anthelmintic drugs. The EtOAc extract of *T. riparia* exhibited the best anthelmintic activity (0.004 mg/ml) against *C. elegans*, but the EtOH and aqueous extracts of the same plant demonstrated relatively low activity. The aqueous extracts of *C. spinosum* and *C. ilicifolia* (bark and leaf) exhibited promising anthelmintic activity, all the other aqueous extracts showed relatively low activity. The observed anthelmintic activity provides evidence to support the use of the evaluated plants in traditional medicine in treating stomach-related ailments caused by parasitic helminths.

The percentage inhibitory activity against COX-1 and COX-2 enzymes by the evaluated plant species revealed that the EtOH extracts of *C. multicava* exhibited relatively high inhibitory activity (99.5 ± 0.50) against both enzymes; all the other EtOH extracts demonstrated high COX-2 selective inhibitory activity. The EtOAc extracts of all the evaluated plant extracts demonstrated high inhibitory activity against both COX enzymes except for *L. lasiantha* that was not evaluated due to lack of plant material. All the aqueous extracts showed low

inhibitory activity against both COX enzymes with the exception of *T. riparia* and *C. rudis* that exhibited exceptional activity (69.1 ± 0.9 and 92.7 ± 0.6%) against COX-1 and COX-2 respectively, Plant extracts that exhibited good anti-inflammatory activity especially against COX-2 enzyme need further studies to determine their inhibitory potential against other pro-inflammatory mediators such as the NF_kB mediated signalling pathway in the immune cells that leads to the production of iNOS, pro-inflammatory cytokines and iCOX (**POLYA, 2003**).

The plate incorporation assay for the Ames test using *Salmonella typhimurium* tester strains TA98, TA100 and TA1537 exposed to three dilutions without S9 metabolic activation of the plant extracts was performed to detect the presence of direct mutagens. The results showed that all the evaluated plant extracts were non-mutagenic towards the bacterial tester strains used in this investigation. The absence of mutagenic response in the Ames test among all the evaluated extracts provides evidence to support the safe consumption of the medicinal plants in traditional medicine. At this stage, the consumption of these plants in traditional medicine could be considered reasonably safe until further studies; incorporating metabolizing enzymes are done to determine the safety of the extracts metabolites.

Phytochemical analysis revealed the presence of various secondary metabolites at varying concentrations. Total phenolic compounds, gallotannins and flavonoids were detected in all the investigated plant species except for *L. lasiantha* that was not evaluated due to lack of plant materials. Saponins were detected in *C. spinosum and C. ilicifolia* (bark). The various

phytochemicals detected in the evaluated plant species could be responsible for the observed antimicrobial, anthelmintic and COX activities in some of the plant extracts.

In vitro screening of plant materials is an important step to validate the traditional uses of medicinal plants. The various levels of pharmacological activities in this study provide basic understanding of the efficacy of the investigated medicinal plants as potential source of novel and useful drugs, but do not in any way confirm that they are effective medicine. There has never been any scientific report of the evaluated plant species except for *T. riparia* that has been studied extensively. Plant species for which relatively high activity has been reported for the first time provide fresh ground for further scientific studies to isolate and identify the bioactive compounds responsible for the pharmacological activities, and possibly subject any bioactive compounds that will be isolated from these plant species to metabolizing enzymes using *in vivo* screening. The application of these findings in pharmaceutical development and making this research work meaningful to the local population will be considered as great steps to combating cases of stomach-related ailments.

References

ALANIS, A.J. 2005. Resistance to antibiotics: are we in the post-antibiotic era? *Achieve of Medicinal Research* 36: 697-705

ALIGIANNIS, N., KALPOUTZAKIS, E., MITAKU, S. and CHINOU, I.B. 2001. Composition and antimicrobial activity of the essential oils of two *Origanum* species. *Journal of Agriculture and Food Chemistry* 49: 4168-4170

AREMU, A.O., FAWOLE, O.A., CHUKWUJEKWU, J.C., LIGHT, M.E., FINNIE, J.F. and VAN STADEN, J. 2010. *In vitro* antimicrobial, anthelmintic and cyclooxygenase-inhibitory activities and phytochemical analysis of *Leucosidea sericea*. *Journal of Ethnopharmacology* 131: 22-27

ASAOLU, S.O. and OFOEZIE, I.E. 2003. The role of health education and sanitation in the control of helminth infections. *Acta Tropica* 86: 283-294

BACCOU, J.C., LAMBERT, F. and SAUVAIRE, Y. 1977. Spectrophotometric method for the determination of total steroidal sapogenin. *Analyst* 102: 458-465

BALUNAS, M.J. and KINGHORN, A.D. 2005. Drug discovery from medicinal plants. *Life Sciences* 78: 431-441

BARNES, E.H., DOBSON, R.J. and BARGER, A.I. 1995. Worm control and anthelmintic resistance: adventures with a model. *Parasitology Today* 11: 56-63

BARNES, P.M., POWELL-GRINER, E., McFANN, K. and NAHIN, R.L. 2004. Complementary and alternative medicine use among adults: United States, 2002. *Seminars in Integrative Medicine* 2: 54-71

BELLEY, A. and CHADEE, K. 1995. Eicosanoid production by Parasites: from pathogenesis to immunomodulation? *Parasitology Today* 11: 327-334

BENGMARK, S. 2004. Acute and ‘‘chronic’’ phase reaction-a mother of disease. *Clinical Nutrition* 23: 1256-1266

BERTIN, P. 2004. Should gastroprotective agents be given with COX-2 inhibitors? A question worthy of scrutiny. *Joint Bone Spine* 71: 454-456

BLACK, R.E., BROWN, K.H., BECKER, S. and YUNUS, M. 1982. Longitudinal studies of infectious diseases and physical growth of children in rural area of Bangladesh. *American Journal of Epidemiology* 115: 305-314

BOON, R. 2010. Pooley's Trees of Eastern South Africa a Complete Guide. Flora and Fauna Publication Trust, South Africa

BORRIS, R.P. 1996. Natural product research: perspective from a major pharmaceutical company. *Journal of Ethnopharmacology* 51: 29-38

BOURGAUD, F., GRAVOT, A., MILESI, S. and GONTIER, E. 2001. Production of plant secondary metabolites: a historical perspective. *Plant Sciences* 161: 839-851

BOURS, M.J.L., SWENNEN, E.L.R., DI VIRGILIO, F., CRONSTEIN, B.N. and DAGNELIE, P.C. 2006. Adenosine 5′-triphosphate and adenosine as endogenous signalling molecules in immunity and inflammation. *Pharmacology and Therapeutics* 112: 358-404

BROOKER, S., JARDIM-BOTELHO, A., QUINNELL, R.J., GEIGER, S.M., CALDAS, I.R., FLEMING, F., HOTEZ, P.J., CORREA-OLIVEIRA, R., RODRIGUES, L.C., and BETHONY, J.M. 2007. Age-related changes in hookworm infection, anaemia and iron deficiency in an area of high *Necator americanus* hookworm transmission in South-Eastern Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 101: 146-154

BRUNETON, J. 1995. Pharmacognosy Phytochemistry and Medicinal Plants. Lavoisier, France

BUNDY, D.A.P. CHAN, M.S. and SAVIOLI. L. 1995. Hookworm infection in pregnancy. Short Report. *Transaction of the Royal Society of Tropical Medicine and Hygiene* 89: 521-522

BUWA, L.V. and VAN STADEN, J. 2006. Antibacterial and antifungal activity of traditional medicinal plants used against venereal diseases in South Africa. *Journal of Ethnopharmacology* 103: 139-142

CALAM, J. 1995. 4 pathogenic mechanisms. *Baillière's Clinical Gastroenterology* 9: 487-506

CARROLL, C.C., WARNAKULASURIYARACHCHI, D., NOKHBEH, M.R. and LAMBERT, I.B. 2002. *Salmonella typhimurium* mutagenicity tester strains that overexpress oxygen-insensitive nitroreductases *nfsA* and *nfsB. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 501: 79-98

CHAITOW, L. 1996. *Candida albicans*: the non-drug approach to the treatment of candida infection: the proliferation of a parasite yeast that lives inside all of us. Thorsons, London

CHARTRAND, S.A., THOMPSON, K.J. and SANDERS, C.C. 1996. Antibiotic-resistant, Gram-negative bacillary infections. *Seminars in Pediatric Infectious Diseases* 7: 187-203

CHERNY, N.I. 2008. Evaluation and management of treatment-related diarrhoea in patients with advanced cancer. *Journal of Pain and Symptom Management* 36: 413-423

COHEN, F.L. and TARTASKY, D. 1997. Microbial resistant to drug therapy. *American Journal of Infection Control* 25: 51-64

COLEMAN, J.W. 2002. Nitric oxide: a regulator of mast cell activation and mast cell-mediated inflammation. *Clinical and Experimental Immunology* 129: 4-10

COOK, N.C. and SAMMAN, S. 1996. Flavonoids-chemistry, metabolism. cardioprotective effect, and dietary sources. *Journal of Nutritional Biochemistry* 7: 66-76

COS, P., VLIETINCK, A.J., BERGHE, D.V. and MAES, L. 2006. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. *Journal of Ethnopharmacology* 106: 290-302

COWEN, L.E., ANDERSON, J.B. and KOHN, L.M. 2002. Evolution of drug resistance in *Candida albicans. Annual Review of Microbiology* 56: 139-165

COX, R.A., CONQUEST, C., MALLAGHAN, C. and MARPLES, R.R. 1995. A major outbreak of methicillin-resistant *Staphylococcus aureus* caused by a new phage-type (EMRSA-16). *Journal of Hospital Infection* 29: 87-106

CRAGG, G.M. and NEWMAN, D.J. 2007. Drug from nature: past achievement, future prospect. *Advances in Phytomedicines* 1: 23-37

CROMPTON, D.W.T. 1999. How much human helminthiasis is there in the world? *Journal of Parasitology* 85: 397-403

CROZIER, A., CLIFFORD, M.N. and ASHIHARA, H. 2006. Plant Secondary Metabolites: Occurance, Structure and Role in the Human Diet. Blackwell Publishing, Tokyo

DANIEL, M. 2006. Medicinal Plants Chemistry and Properties. Science Publishers, Enfield, New Hampshire

DAUSKARDT, R.P.A. 1990. The changing geography of traditional medicine: urban herbalism of the Witwatersrand, South Africa. *Geographical Journal* 22: 275-283

DESMUELES, J.A., DOCENT, P., CEDRASCHI, C., PIGUET, V., ALLAZ, A-F. and DAYER, P. 2002. Advances with analgesics and NSAIDs for the treatment of spinal disorders. *Best Practice and Research Clinical Rheumatology* 16: 105-121

DeWITT, D.L. and SMITH, W.L. 1988. Primary structure of prostaglandin G/H synthesis from sheep vesicular gland determined from the complementary DNA sequence. *Proceedings of the National Academy of Sciences of the United States of America* 85: 1412-1416

DIXON, D.M., McNEIL, M.M., COHEN, M.L., GELLIN, B.G. and La MONTAGNE, J.R. 1996. Fungal infections: a growth threat. *Public Health Reports* 111: 223-235

DOHIL, R. 2005. Disorders of the stomach. *Journal of Pediatric* 3: 665-669

DÖRFLER, H-P. and ROSELT, G. 1989. The Dictionary of Healing Plants. Blandford Press, London

EDDLESTON, J. and PIERINI, S. 2001. Schistosomiasis. In: Oxford Handbook of Tropical Medicine. Oxford University Press, London

EGGIMANN, P., GARBINO, J. and PITTET, D. 2003. Management of Candidiasis, Management of *Candida* species infections in critically ill patients. *Lancet Infectious Diseases* 3: 772-785

EISENBRAND, G., HIPPE, F., JAKOBS, S. and MUEHLBEYER, S. 2004. Molecular mechanisms of indirubin and its derivatives: novel anticancer molecules with their origin in traditional Chinese phytomedicine. *Journal of Cancer Research and Clinical Oncology* 130: 627-635

ELDEEN, I.M.S. and VAN STADEN, J. 2008. Cyclooxygenase inhibition and antimycobacterial effects of extracts from Sudanese medicinal plants. *South African Journal of Botany* 74: 225-229

ELGORASHI, E.E. and VAN STADEN, J. 2004. Pharmacological screening of six Amaryllidaceae species. *Journal of Ethnopharmacology* 90: 27-32

ELOFF, J.N. 1998. A sensitive and quick microplate method to determine the minimum inhibitory concentration of plant extract for bacteria. *Planta Medica* 64: 711-714

ELUJOBA, A.A., ODELEYE, O.M. and OGUNYEMI, C.M. 2005. Traditional medicine development for medical and dental primary health care delivery system in Africa. *African Journal of Traditional Complementary and Alternative Medicines* 2: 46-61

ERLUND, I. 2004. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutrition Research* 24: 851-874

ETKIN, N.L. 2001. Perspectives in ethnopharmacology: forging a closer link between bioscience and traditional empirical knowledge. *Journal of Ethnopharmacology* 76: 177-182

ESPINEL-INGROFF, A. and PFALLER, M.A. 1995. Antifungal Agents and Susceptibility Testing. In: Manual of Clinical Microbiology. MURRAY, P.R., BARON, E.J., PFALLER, M.A., TENOVER, F.C. and YOLKEN, R.H. (Eds.). ASM Press, Washington D.C

ESSAWI, T. and SROUR, M. 2000. Screening of some Palestinian medicinal plants for antibacterial activity. *Journal of Ethnopharmacology* 70: 343-349

EVANS, A.C., DU PREEZ, L., MAZIYA, S.P., VAN DERMERWE, C.A. and SCHKUTTE, C.H.J. 1987. Observations on the helminth infections in black pupils of the Eastern Transvaal lowveld of South Africa. *South African Journal of Epidermiological Infection* 2: 7-14

FABRICANT, D.S. and FARNSWORTH, N.R. 2001. The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives Supplement* 109: 69-75

FARTHING, M.J.G. 2007. The patient with refractory diarrhoea. *Best practice and research*. *Clinical Gastroenterology* 21: 485-501

FAVOR, J. 1999. Mechanisms of mutation induction in germ cells of the mouse as assessed by the specific locus test. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 428: 227-236

FENNELL, C.W., LINDSEY, K.L., McGAW, L.J., SPARG, S.G., STAFFORD, G.I., ELGORASHI, E.E., GRACE, O.M. and VAN STADEN, J. 2004. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *Journal of Ethnopharmacology* 94: 205-217

FRANCIS, G., MAKKAR, H.P.S. and BECKER, K. 2002. Dietary supplementation with a *Quillaja* saponin mixture improves growth performance and metabolic efficiency in common carp (*Cyprinus carpio* L.). *Aquaculture* 203: 311-320

FRAYHA, G.J., SMYTH, J.D., GOBERT, J.G. and SAVEL, J. 1997. The mechanism of action of antiprotozoal and anthelmintic drugs in man. *General Pharmacology: The Vascular System* 28: 273-299

GALE, E.F. 1981. Inhibitors of Bacterial and Fungal Cell Wall Synthesis. In: The Molecular Basis of Antibiotic Action. 2nd Ed. GALE, E.F., CUNDLIFFE, E., REYNOLDS, P.E. and RICHMOND, F.R.S. (Eds.). John Wiley & Sons Ltd, Great Britain

GAMANIEL, K.S. and JSSELMUIDEN, C.I. 2004. Ethical challenges posed by herbal traditional medicines research. Global forum for health research, forum 8, Mexico City

GARBINO, J., KOLAROVA, L., LEW, D., HIRSCHEL, B. and ROHNER, P. 2001. Fungemia in HIV-infected patients: a 12-year study in a tertiary care hospital. *AIDS Patients Care and STDs* 15: 407-410

GEARY, T.G., SANGSTER, N.C. and THOMPSON, D.P. 1999. Frontiers in anthelmintic pharmacology. *Veterinary Parasitology* 84: 275-295

GHANNOUM, M.A. and RICE, L.B. 1999. Antifungal agents: mode of Action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clinical Microbiology* 12: 501-517

GIBBONS, S. 2005. Plants as a source of bacterial resistance modulators and anti-infective agents. *Phytochemistry Review* 4: 63-78

GILANI, A.H. and RAHMAN, A.U. 2005. Trends in ethnopharmacology. *Journal of Ethnopharmacology* 100: 43-49

GREENWOOD, D. 1995. Inhibitors of Bacterial Protein Synthesis. In: Antimicrobial Chemotherapy. 3rd Ed. Oxford University Press Inc. New York

GRUZ, J., AYAZ, F.A., TORUN, H. and STRNAD, M. 2011. Phenolic acid content and radical scavenging activity of extracts from medlar (*Mespilus germanica* L.) fruit at different stages of ripening. *Food Chemistry* 124: 271-277

GUARDIA, T., ROTELLI, A.E., JUAREZ, A.O. and PERZER, L.E. 2001. Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *IL FARMACO* 56: 683-687

GURIB-FAKIM, A. 2006. Medicinal plants: Tradition of yesterday and drugs of tomorrow. *Molecular Aspect of Biology* 27: 1-93

HABER, C.L., HECKAMAN, C.L., LI, G.P., THOMPSON, D.P., WHALEY, H.A and WILEY, V.H. 1991. Development of a mechanism of action-based screen for anthelmintic microbial metabolites with avermectin-like activity and isolation of milbemycin-producing S*treptomyces* strains. *Antimicrobial Agents and Chemotherapy* 35: 1811-1817

HAKURA, A., SUZUKI, T. and SATOH, T. 1999. Advantage of the use of human liver S9 in the Ames test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 438: 29-36

HANCOCK, R.E.W. 2005. Mechanisms of action of newer antibiotics for Gram-positive pathogens. *Lancet Infectious Diseases* 5: 209-218

HEIM, K.E., TAGLIAFERRO, A.R. and BOBILYA, D.J. 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry* 13: 572-584

HEINRICH, M., BARNES, J., GIBBON, S. and WILLIAMSON, E.M. 2004. Fundamentals of Pharmacognosy and Phytotherapy. Churchill Livingstone, Edinburgh

HELMCKE, K., AVILA, D.S. and ASCHNER, M. 2010. Utility of *Caenorhabditis elegans* in high throughput neurotoxicological research. *Neurotoxicology and Teratology* 32: 62-67

HERNÄNDEZ, N.E., TERESCHUK, M.L. and ABDALA, L.R. 2000. Antimicrobial activity of flavonoids in medicinal plants from Tafi´ del Valle (Tucuma´n, Argentina). *Journal of Ethnopharmacology* 73: 317-322

HIAI, S., OURA, H. and NAKAJIMA, T. 1976. Color reaction of some sapogenins and saponins with vanillin and sulfuric acid. *Planta Medica* 29: 116-122

HOAREAU, L. and EDGAR, D.J. 1999. Medicinal plants: are-emerging health aid. *Plant Biotechnology* 2: 57-70

HOBSON, R.P. 2003. The global epidemiology of invasive *Candida* infections is the tide turning? *Journal of Hospital Infection* 55: 159-168

HODEK, P., TREFIL, P. and STIBOROVA, M. 2002. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. *Chemico-Biological Interactions* 139: 1-21

HOESSEL, R., LECLERC, S., ENDICOTT, J.A., NOBEL, M.E., LAWRIE, A., TUNNAH, P., LEOST, M., DAMIENS, E., MARIE, D., MARKO, D., NIEDERBERGER, E., TANG, W., EISENBRAND, G. and MEIJER, L. 1999. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. *Nature Cell Biology* 1: 60-67

HOFFMANN, D. 1989. The Holistic Herbal: A Herbal Celebrating the Wholeness of Life. Element Book Ltd, Dorset

HOPKINS, S.J. 2003. The pathophysiological role of cytokines. *Legal Medicine* 5: S45-S57

HORN, R.C. and VARGAS, V.M.F. 2008. Mutagenicity and antimutagenicity of teas used in popular medicine in the *salmonella*/microsome assay. *Toxicology in Vitro* 22: 1043-1049

HOTEZ, P.J. 2010. A plan to defeat neglected tropical diseases. *Scientific American* 302: 90-99

HOTEZ, P.J.H. and BROWN, A.S. 2009. Neglected tropical disease vaccines. *Biological* 37: 160-164

HOTEZ, P.J., REMME, J.H.F., BUSS, P., ALLEYNE, G., GEORGE, G., MOREL, C. and BREMAN, J.G. 2004. Combating tropical infectious diseases: Report of the disease control priorities in developing countries project. *Clinical Infectious Diseases* 38: 871-878

HOWARD, P.A. and DELAFONTAINE, P. 2004. Nonsteroidal anti-inflammatory drugs and cardiovascular risk. *Journal of the American College of Cardiology* 43: 519-525

HUGO, W.B. 1992. Biology of Microorganisms. In: Pharmaceutical Microbiology. HUGO, W.B. and RUSSELLE, A.D. (Eds.). Blackwell Scientific Publications, Oxford

HUTCHINGS, A., SCOTT, A.H., LEWIS, G. and CUNNINGHAM, A.B. 1996. Zulu Medicinal Plants: An Inventory. Natal University Press, Pietermaritzburg

IGNAT, I., VOLF, I. and POPA, V.I. 2011. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry* 126: 1821-1835

ISHIGE, K., SCHUBERT, D. and SAGARA, Y. 2001. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biology and Medicine* 30: 433-446

IWALEWA, E.O., McGAW, L.J., NAIDOO, V. and ELOFF, J.N. 2007. Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. *African Journal of Biotechnology* 6: 2868-2885

JACOBY, G.A. and MUNOZ-PRICE, L.S. 2005. The new *β*-lactamases. *New England Journal of Medicine* 352: 380-391

JÄGER, A.K., HUTCHINGS, A. and VAN STADEN, J. 1996. Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. *Journal of Ethnopharmacology* 52: 95-100

JAMES, C.E. and DAVEY, M.W. 2007. A rapid colorimetric assay for the quantitation of the viability of free-living larvae of nematodes *in vitro*. *Parasitology Research* 101: 975-980

JANSSEN, A.M., SCHEFFER, J.J.C. and SVENDSEN, A.B. 1987. Antimicrobial activity of essential oils: A 1976-1986 literature review. Aspects of the test methods. *Planta Medica* 53: 395-398

JASMER, D. P., YAO, C., REHMAN, A. and JOHNSON, S. 2000. Multiple lethal effects induced by a benzimidazole anthelmintic in the anterior intestine of the nematode *Haemonchus contortus. Molecular and Biochemical Parasitology* 105:81-90

JORDAN, P. and WEBBE, G. 1982. Control of Schistosomes. In: Schistosomiasis, Epidermiology, Treatment and Control. JORDAN, P. and WEBBE, G. (Eds.). William Heinemann Medical Books Ltd, London

JUSTICE, M.C., HSU, M-J., TSE, B., KU, T., BALKOVEC, J., SCHAMTZ, D. and NIELSEN, J. 1998. Elongation factor 2 as a novel target for selective inhibition of fungal protein synthesis. *Journal of Biological Chemistry* 273: 3148-3151

KANTSEVOY, S.V. 2006. Digestive Disorders. In: Digestive Disorder. Health Report. Johns Hopkins White Papers, New York

KAYSER, F.H. 2003. Safety aspects of enterococci from the medical point of view. *International Journal of Food Microbiology* 88: 255-262

KLOOS, W.E. and SCHLEIFER, K-H. 1981. The Genus *Staphylococcus*. In: The Prokaryotes a Handbook on Habitats, Isolation, and Identification of Bacteria. STARR, M.P., STOLP, H., TRŰPER, H.G., BALOWS, A. and SCHLEGEL, H.G. (Eds.). Springer-Verlag Berlin Heidelberg, New York

KÖHLER, P. 2001. The biochemical bases of anthelmintic action and resistance. *International Journal for Parasitology* 31: 336-345

KONTOYIANNIS, D.P. and LEWIS, R.E. 2002. Antifungal drug resistance of pathogenic fungal. *The Lancet* 359: 1135-1144

KU, W.W., BIGGER, A., BRAMBILLA, G., GLATT, H., GOCKE, E., GUZZIE, P.J., HAKURA, A., HONMA, M., MARTUS, H-J., OBACH, R.S. and ROBERTS, S. 2007. Strategy for genotoxicity testing-metabolic considerations. *Mutation Research* 627: 59-77

KUTCHAN, T. and DIXON, R.A. 2005. Physiology and metabolism: secondary metabolism: nature's chemical reservoir under deconvolution. *Current Opinion in Plant Biology* 8: 227-229

LABIENIEC, M. and GABRYELAK, T. 2005. Measurement of DNA damage and protein oxidation after the incubation of B14 Chinese hamster cells with chosen polyphenols. *Toxicity Letters* 155: 15-25

LACEY, E. 1990. Mode of action of benzimidazoles. *Parasitology Today* 6: 112-115

LAUPATTARAKASEM, P., HOUGHTON, P.J., HOULT, J.R.S. and ITHARAT, A. 2003. An evaluation of the activity related to inflammation of four plants used in Thailand to treat arthritis. *Journal of Ethnopharmacology* 85: 207-215

LAURENCE, D.R. and BENNETT, P.N. 1980. Clinical Pharmacology. 5th Ed. Churchhill Livingstone, Edinburg

LEVY, S.B. 2002. From Tragedy the Antibiotic Age is Born. In: The Antibiotic Paradox: How the Misuse of Antibiotics Destroys Their Curative Powers. 2nd Ed. LEVY. S.B. (Ed.). Perseus Publishing, Cambridge

LEVY, S.B. and MARSHALL, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine* 10: S122-S129

LEWIS, W.H. and ELVIN-LEWIS, M.P.F. 1977. Medical Botany: Plants Affecting Man's Health. John Wiley and Son, Inc. Canada

LI, R.W., LIN, G.D., LEACH, D.N., WATERMAN, P.G. and MYERS, S.P. 2006. Inhibition of COXs and 5-LOX and activation of PPARs by Australian *Clematis* species (Ranunculaceae). *Journal of Ethnopharmacology* 104: 138-143

LIGHT, M.E., PSARG, S.G., STAFFORD, G.I. and VAN STADEN, J. 2005. Riding the wave: South Africa's contribution to ethnopharmacological research over the last 25 years. *Journal of Ethnopharmacology* 100: 127-130

LIN, C.N., SHIEH, W.L., KO, F.N. and TENG, C.M. 1993. Antiplatelet activity of some prenylflavonoids. *Biochemistry and Pharmacology* 45: 509-512

LINTON, A.H. 1983. Theory of Antibiotic Inhibition Zone Formation, Disc Sensitivity Methods and MIC Determinations. In: Antibiotics: Assessment of Antimicrobial Activity and Resistance. RUSSELL, A.D. and QUESNEL, L.B. (Eds.). Academic Press, London

LUSEBA, D., ELGORASHI, E.E., NTLOEDIBE, D.T. VAN STADEN, J. 2007. Antibacterial, anti-inflammatory and mutagenic effects of some medicinal plants used in South Africa for the treatment of wounds and retained placenta in livestock. *South African Journal of Botany* 73: 378-383

MacAULAY, S. and BLACKBURN, D. 2002. Selective COX-2 inhibitors for patients with arthritis: are they safer than traditional nonsteroidal anti-inflammatory drugs? *Canadian Pharmacists Journal* 135: 30-34

MAJUMDER, K. 2007. Aminoglycoside Antibiotics. In: Enzyme-Mediated Resistance to Antibiotics, Mechanisms, Dissemination, and prospects for Inhibition. WEI, L., ANNEDI, S.C., KOTRA, L.P., BONOMO, R.A. and TOLMASKY, M. (Eds.). ASM Press, Washington DC

MAKKAR, H.P.S., 1999. Quantification of tannins in tree foliage: a laboratory manual for the FAO/IAEA Co-ordinated Research Project on "Use nuclear and Related Techniques to Develop Simple tannin Assays for Predicting and Improving the Safety and Efficiency of Feeding Ruminants on the Tanniferous Tree Foliage". In: Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria

MAKKAR, H.P.S., SIDHURAJU, P., BECKER, K., 2007. Plant Secondary metabolites. Humana Press Inc., New Jersey

MALATHUM, K. and MURRAY, B.E. 1999. Vancomycin-resistant enterococci: recent advances in genetics, epidemiology and therapeutic options. *Drug Resistance Update* 2: 224-243

MANTRI, P. and WITIAK, D.T. 1994. Inhibitors of Cyclooxygenase and 5-Lipoxygenase.In: Current Medicinal Chemistry. RAHMAN, A. (Ed.). Bentham Science Publishers, Schipol

MARCO, F., PFALLER, M.A., MESSER, S.A. and JONES, R.N. 1998. Activity of MK-0991 (L-743,872), a new echinocandi, compared with those of LY303366 and four other antifungal agents tested against blood stream isolates of *Candida Spp*. *Diagnostic Microbiology and Infectious Diseases* 32: 33-37

MARON, D.M. and AMES, B.N. 1983. Revised methods for *Salmonella* mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects* 113: 173-215

MARINOVA, D., RIBAROVA, F. and ATANASSOVA, M. 2005. Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *Journal of the University of Chemical Technology and Metallurgy* 40: 255-260

MASOKO, P., PICARD, J. and ELOFF, J.N. 2007. The antifungal activity of twenty-four Southern African *Combretum* species (Combretaceae). *South African Journal of Botany* 73: 173-183

McCHESNEY, J.D., VENKATARAMAN, S.K. and HENRI, J.T. 2007. Plant natural products: Back to the future or into extinction? *Phytochemistry* 68: 2015-2022

McGAW, L.J., JÄGER, A.K. and VAN STADEN, J. 2000. Antibacterial, anthelmintic and anti-amoebic activity in South Africa medicinal plants. *Journal of Ethnopharmacology* 72: 247-263

McGAW, L.J., JÄGER, A.K. and VAN STADEN, J. 1997. Prostaglandin synthesis inhibitory activity in Zulu, Xhosa and Sotho medicinal plants. *Phytotherapy Research* 11: 113-117

McKAY, C.J. 2008. Chronic inflammation and pancreatic cancer. *Best Practice and Research Clinical Gastroenterology* 22; 65-73

MEGALOU, E.V. and TAVERNARAKIS, N. 2009. Autophagy in *Caenorhabditis elegans. Biochemica et Biophysica Acta-Molecular Cell Research* 1793: 1444-1451

MILLER, S.B. 2006. Prostaglandins in health and diseases: An overview. *Seminars in Arthritis and Rheumatism* 36: 37-49

MILLER, S.A., ROSARIO, C.L., ROJAS, E. and SCORZA, J.V. 2003. Intestinal parasitic infection and associated symptoms in children attending day care centres in Trujillo, Venezuela. *Tropical Medicinal and International Health* 8: 342-347.

MIMS, C.A. 1987. Mechanisms of Cell and Tissue Damage. In: The Pathogenesis of Infectious Disease. 3rd Ed. Academic Press, London

MONAGHAN, R.L. and BARRET, J.F. 2006. Antibacterial drug discovery-then, now and the genomics future. *Biochemical Pharmacology* 71: 901-909

MORITA, I. 2002. Distinct functions of COX-1 and COX-2. *Prostaglandins and other Lipid Mediators* 68-69: 165-175

MORTELMANS, K. and ZEIGER, E. 2000. The Ames *Salmonella*/microsome mutangenicity assay. *Mutation Research*/*Fundamental and Molecular Mechanism of Mutangenesis* 455: 29-60

MOSS, M.O. 1987. Food Mycology. In: Mycotoxins in Food. KROGH, P. (Ed.) Academic Press, San Diego

MUHAMMAD, B.Y. and AWAISU, A. 2008. The need for enhancement of research, development, and commercialization of natural medicinal products in Nigeria: Lessons from the Malaysian experience. *African Journal of Complementary and Alternative Medicines* 5: 120-130

MÜLLER, E.E., EHLERS, M.M. and GRABOW, W.O.K. 2001. The occurrence of *E. coli* 0157: H7 in South African water sources intended for direct and indirect human consumption. *Water Research* 35: 3085-3088

NAIK, S.R. and SHETH, U.K. 1976. Inflammatory process and screening methods for anti-inflammatory agents. *Journal of Postgraduate Medicine* 22: 5-21

NATARAJAN, L., BERRY, C.C. and GASCHE, C. 2003. Estimation of spontaneous mutation rates. *Biometrics* 59: 555-561

NEWELL, D.G., KOOPMANS, M., VERHOEF, L., DUIZER, E., AIDARA-KANE, A., SPRONG, H., OPSTEEGH, M., LANGELAAR, M., THREFALL, J., SCHEUTZ, F., VAN Der GIESSEN, J. and KRUSE, H. 2010. Food-borne diseases-the challenges of 20 years ago still persist while new ones continue to emerge. *International Journal of Food Microbiology* 139: S3-S15

NDHLALA, A.R., FINNIE, J. and VAN STADEN, J. 2011. Plant composition, pharmacological properties and mutagenic evaluation of a commercial Zulu herbal mixture: Imbiza ephuzwato. *Journal of Ethnopharmacology* 133: 663-674

NDHLALA, A.R., KASIYAMHURU, A., MUPURE, C., CHITINDINGU, K., BENHURA, M.A. and MUCHUWETI, M. 2007. Phenolic composition of *Flacourtia indica*, *Opuntia megacantha* and *Sclerocarya birrea*. *Food Chemistry* 103: 82-87

NIWA, O. 2006. Indirect mechanisms of genomic instability and the biological significance of mutations at tandem repeat loci. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 598: 61-72

NOSKIN, G.A. 1997. Vancomycin-resistant enterococci: Clinical, microbiologic, and epidemiologic features. *Journal of Laboratory and Clinical Medicine* 130: 14-20

NURTJAHJA-TJENDRAPUTRA, E., AMMIT, A.J., ROUFOGALIS, B.D., TRAN, V.H. and DUKE, C.C. 2003. Effective anti-platelet and COX-1 enzyme inhibitors from pungent constituents of ginger. *Thrombosis Research* 111: 259-265

ODDS, F.C. 1996. Resistance of clinically important yeasts to antifungal agents. *International Journal of Antimicrobial Agents.* 6: 145-147

ODDS, F.C., BROWN, A.J.P. and GOW, N.A.R. 2003. Antifungal agents: mechanisms of action. *Trends in Microbiology* 11: 272-279

OGLE, J.M., CARTER, A.P. and RAMAKRISHNAN, V. 2003. Insights into the decoding mechanism from recent ribosome structures. *Trends in Biochemical Sciences* 28: 259-266

OLESZEK, W.A. 2002. Chromatographic determination of plant saponins. *Journal of Chromatography A* 967: 147-162

PAGE, C.P., CURTIS, M.H., SUTTER, M.C., WALKER, M.H.A. and HOFFMAN, B.B. 1997. Integrated Pharmacology. Mosby-Wolfe, New York

PASINETTI, G.M. 2001. Cyclooxygenase and Alzheimer's disease: implication for preventive initiatives to slow the progression of dementia. *Archives of Gerontology and Geriatrics* 33: 13-28

PELZER, L.E., GUARDIA, T., JUAREZ, A.O. and GUERREIRO, E. 1998. Acute and chronic anti-inflammatory effects of plant flavonoids. *IL FARMACO A* 53: 421-424

PERRY, B.D. and RANDOLPH, T.F. 1999. Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. *Veterinary Parasitology* 84: 145-168

PETERS, J., GILLES, H.M. 1995. Reviews and Notes: Tropical medicine: Color Atlas of Tropical Medicine and Parasitology. Mosby-Wolfe, New York

PHILLIPSON, J.D. 2007. Phytochemistry and pharmacognosy. *Phytochemistry* 68: 2960-2972

POLYA, G.M. 2003. Biochemical Targets of Plant Bioactive Compounds. A Pharmacological Reference Guide to Sites of Action and Biological Effects. CRC Press, Florida

POOLEY, E. 1998. A Guide to Wild Flowers of KwaZulu-Natal and the Eastern Region. Williams, R. (Ed.). Natal Floral Publications Trust, Durban

POPAT, A., SHEAR, N.H., MALKIEWICZ, I., STEWART, M.J., STEENKAMP, V., THOMSON, S. and NEUMAN, M.G. 2001. The toxicity of *Callilepis laureola,* a South African traditional herbal medicine. *Clinical Biochemistry* 34: 229-236

PORTILLO, A., VILA, R., FREIXA, B., ADZET, T. and CAÑIGUERAL, S. 2001. Antifungal activity of Paraguayan plants used in traditional medicine. *Journal of Ethnopharmacology* 76: 93-98

PRASAD, R. 1991. *Candida abicans*; Cellular and Molecular Biology. Springer-Verlag, Berlin

QUESNEL, L.B. and RUSSELL, A.D 1983. Antibiotics In: Assessment of Antimicrobial Activity and Resistance. RUSSELL, A.D. and QUESNEL, L.B. (Eds.). Academic Press, London

QIN, Y., WU, X., HUANG, W., GONG, G., LI, D., HE, Y. and ZHAO, Y. 2009. Acute toxicity and sub-chronic toxicity of steroidal saponins from *Dioscorea zingiberensis* C.H.Wright in rodents. *Journal of Ethnopharmacology* 126: 543-550

QUIROGA, E.N., SAMPIETRO, A.R. and VATTUONE, M.A. 2001. Screening antifungal activity of selected medicinal plants. *Journal of Ethnopharmacology* 74: 89-96

RABE, T. and VAN STADEN, J. 1997. Antibacterial activity of South African plants used for medicinal purposes. *Journal of Ethnopharmacology* 56: 81-87

RAMAWAT, K.G. 2009. The Chemical Diversity of Bioactive Molecules and Therapeutic Potential of Medicinal Plants. In: Herbal Drugs: Ethnomedicine to Modern Medicine. DASS, S. and MATHUR, M. (Eds.). Springer-Verlag, Berlin Heidelberg

RANG, H.P. and DALE, M.M. 1987. Pharmacology. Churchhill Livingstone, Edinburgh

REID, K.A., MAES, J., MAES, A., VAN STADEN, J., De KIMPE., MULHOLLAND, D.A. and VERSCHAEVE, L. 2006. Evaluation of the mutagenic and antimutagenic effects of South African plants. *Journal of Ethnopharmacology* 106: 44-50

RETHER, B., PFOHL-LESZOKWICZ, A., GUILLEMAUT, P. and KEITH, G. 1990. Benzo(a)pyrene induces nuclear-DNA adducts in plant cell suspension culture detection by [^{32}P] postlabelling. *Federation of European Biochemical Societies Letters* 263: 172-174

ROBERTS, S. M. and NEWTON, R.F. 1982. History, Nomenclature and Potential Uses of Prostaglandins and Thromboxanes in the Clinic. In: Butterworths Monograph in Chemistry: Prostaglandins and Thromboxanes. ROBERS, S.M. and NEWTON, R.F. (Eds.). Butterworth Scientific, London

ROBINSON, M.W., McFERRAN, N., TRUDGETT, A., HOEY, L. and FAIRWEATHER, I. 2004. A possible model of benzimidazole binding to *β*-tubulin disclosed by invoking an inter-domain movement. *Journal of Molecular Graphics and Modelling* 23: 275-284

RODRIGUEZ-MORALES, A.J., BARBELLA, R.A., CASE, C., ARRIA, M., RAVELO, M., PEREZ, H., URDANETA, O., GERVASIO, G., RUBIO, N., MALDONADO, A., AGUILERA, Y., ViILORIA, A., BLANCO, J.J., COLINA, M., HARNANDEZ, E., ARAUJO, E., CABANIEL, G., BEITEZ, J. and RIFAKIS, P. 2006. Intestinal parasitic infections among pregnant women in Venezuela. *Infectious Diseases in Obstetrics and Gynecology* 14: 1-5

SADHU, S.K., OKUYAMA, E., FUJIMOTO, H., ISHIBASHI, M. and YESILADA, E. 2006. Prostaglandin inhibitory and antioxidant components of *Cistus laurifolius*, a Turkish medicinal plant. *Journal of Ethnopharmacology* 108: 371-378

SALTARELLI, C.G. 1989. *Candida albicans*: The Pathogenic Fungus. Hemisphere Publishing Corporation, New York

SANGLARD, D. 2002. Resistance of human fungal pathogens to antifungal drugs. *Current Opinion in Microbiology* 5: 379-385

SANGLARD, D. 2003. Resistance and tolerance mechanisms to antifungal drugs in fungal pathogens. *Mycologist* 17: 74-78

SANGLARD, D. and ODDS, F.C. 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infectious Diseases* 2: 73-85

SANGSTER, N.C. 1999. Pharmacology of anthelmintic resistances in cyathostomes: will it occur with the avermectin/milbemycins? *Veterinary Parasitology* 85: 189-204

SANGSTER, N.C. and GILL, J. 1999. Pharmacology of anthelmintic resistance. *Parasitology Today* 15: 141-146

SARKAR, R., PRABHAKAR, A.T., MANICKAM, S., SELVAPADIAN, D., RAGHAVA, M.V., KANG, G. and BALRAJ, V. 2007. Epidemiological investigation of an outbreak of acute diarrhoeal disease using geographic information systems. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 101: 587-593

SCHOFIELD, P., MBUGUA, D.M. and PELL, A.N. 2001. Analysis of condensed tannins. *Animal Feed Science and Technology* 91: 21-40

SCOTT, K.F., BRYANT, K.J. and BIDGOOD, M.J. 1999. Functional coupling and differential regulation of the phosholipase A_2-cycloxygenase pathways in inflammation. *Journal of Leukocyte Biology* 66: 535-541

SLEISENGER, M.H. and FORDTRAND, J.S. 1973. Gastrointestinal Disease: pathophysiology, Diagnosis, management. Saunders, Philadelphia

SIONOV, E., MENDLOVIC, S. and SEGAL, E. 2006. Efficacy of amphotericin B or amphotericin B–intralipid in combination with caspofungin against experimental aspergillosis. *Journal of infection* 53: 131-139

SPARG, S.G., VAN STADEN, J. and JÄGER, A.K. 2002. Pharmacological and phytochemical screening of two Hyacinthaceae species: *Scilla natalensis* and *Ledebouria ovatifolia*. *Journal of Ethnopharmacology* 80: 95-101

SPARG, S.G., LIGHT, M.E. and VAN STADEN, J. 2004. Biological activities and distribution of plant saponins. *Journal of Ethnopharmacology* 94: 219-243

STARR, M.P. 1981. Prokaryote Diversity. In: The Prokaryote, A Handbook on Habitats, Isolation and Identification of Bacteria. Volume 1. STARR, M.P., STOLP, H., TRÜPER, H.G., BALOWS, A. and SCHLEGEL, H.G. (Eds.). Springer-Verlag, New York

STEPHENSON, L.S. 1980. The contribution of *Ascaris lumbricoides* to malnutrition in children. *Parasitology* 81: 221-233

TADHANI, M. and SUBHASH, R. 2006. Preliminary studies on *Stevia rebaudiana* leaves: proximal composition, mineral analysis and phytochemical screening. *Journal of Medical Science* 6: 321-326

TAYLOR, J.L.S., RABE, T., McGAW, L.J., JÄGER, A.K. and VAN STADEN, J. 2001. Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation* 34: 23-37

TAYLOR, J.L.S. and VAN STADEN, J. 2001. COX-1 inhibitory activity in extracts from *Eucomis* L'Herit species. *Journal of Ethnopharmacology* 75: 257-265

THOMAS, O.O. 1989. Re-examination of the antimicrobial activities of *Xylopia aethiopica, Carica papaya, Ocimum gratissimum* and *Jathropha curcas*. *Fitoterapia* 60: 147-155

THYBAUD, V., AARDEMA, M., CLEMENTS, J., DEARFIELD, K., GALLOWAY, S., HAYASHI, M., JACOBSON-KRAM, D., KIRKLAND, D., MAcGREGOR, J.T., MARZIN, D., OHYAMA, W., SCHULER, M., SUZUKI, H. and ZEIGER, E. 2007. Strategy for genotoxicity testing: Hazard identification and risk assessment in relation to *in vitro* testing. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 627: 41-58

URBANI, C. and ALBONICO, M. 2003. Anthelminthic drug safety and drug administration in the control of soil-transmitted helminthias in community campaigns. *Acta Tropica* 86: 215-221

VANE, J.R. and BOTTING, R.M. 1998. Ant-inflammatory drugs and their mechanism of action. *Inflammation Research* 47: 578-587

VAN JAARSVELD, E.J. 2005. Succulent of South Africa: A Guide to Regional Diversity. Sunbird Publishing (Pty) Ltd, Cape Town

VAN PUYVELDE, L., NYIRANKULIZA, S., PANEBIANCO, R., BOILY, Y., GEIZER, I., SEBIKALI, B., De KIMPE, N. and SCHAMP, N. 1986. Active principles of *Tetradenia riparia* .I. Antimcrobial activity of 8(14), 15-Sandaracopimaradiene-7α, 18-diol. *Journal of Ethnopharmcology* 17: 269-275

VAN RIET, E., HARTGERS, F.C. and YAZDANBAKHSH, M. 2007. Chronic helminth infections induce immunomodulation: Consequences and mechanisms. *Immunobiology* 212: 475-490

VAN WYK, B-E. and WINK, M. 2004. Medicinal Plants of the World. Timber Press Inc, Portland

VAN WYK, B-E., VAN OUTSHOORN, B. and GERICKE, N. 1997. Medicinal Plants of South Africa. Briza Publications, Pretoria

VANE, J.R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biology* 231: 232-235

VERPOORTE, R. and MEMELINK, J. 2002. Engineering secondary metabolite production in plants. *Current Opinion in Biotechnology* 13: 181-187

VERSCHAEVE, L., KESTENS, V., TAYLOR, J.LS., ELGORASHI, E.E., MAES, A., VAN PUYVELDE, L., De KIMPE, N. and VAN STADEN, J. 2004. Investigation of the antimutagenic effects of selected South African medicinal plants extracts. *Toxicology in Vitro* 18: 29-35

VINCKEN, J-P., HENG, L., De GROOT, A. and GRUPPEN, H. 2007. Saponins, classification and occurrence in the plant kingdom. *Phytochemistry* 68: 275-297

VON SAMSON-HIMMELSTJERNA, G. 2006. Molecular diagnosis of anthelmintic resistance. *Veterinary Parasitology* 136: 99-107

WAGNER, H. and JURCIC, K. 1991. Assays for Immunomodulation and Effects on Mediators of Inflammation. In: Methods in Plant Biochemistry. volume. 6. Assays for Bioactivity. 8th Ed. DEY, P.M., HARBORNE, J.B. and HOSTETTMANN. P. (Eds.). Academic Press, London

WALLER. P.J. 1997. Anthelmintic resistance. *Veterinary Parasitology* 72: 391-412

WARNER, T.D. and MITCHELL, J.A. 2008. COX-2 selectivity alone does not define the cardiovascular risks associated with non-steroidal anti-inflammatory drugs. *The Lancet* 371: 270-273

WATT, J.M. and BREYER-BRANDWIJK, M.G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa: Being an Account of Their Medicinal and Other Uses, Chemical Composition, Pharmacological Effect and Toxicology in Man and Animal. 2nd Ed. Livingstone, London

WATERMAN, C., SMITH, R.A., PONTIGGIA, L. and DERMARDEROSIAN, A. 2010. Anthelmintic screening of Sub-Saharan African plants used in traditional medicine. *Journal of Ethnopharmacology* 127: 755-759

WENG, J-R., CHAN, S-C., LU, Y-H., LIN, H-C., KO, H-H. and LIN, C-N. 2006. Antiplatelet prenylflavonoids from *Artocarpus communis. Phytochemistry* 67: 824-829

WHITTLE, B.J.R. 1980. Role of Prostaglandins in the Defense of the Gastric Mucosa. *Brain Research Bulletin* 5: 7-14

WHITTLE, B.J.R. 2004. Mechanisms underlying intestinal injury induced by anti-inflammatory COX inhibitors. *European Journal of Pharmacology* 500: 427-439

WINA, E., MUETZEL, S. and BECKER, K. 2005. The impact of saponins or saponin containing materials on ruminant production. *Journal of Agricultural and Food Chemistry* 53: 8093-8105

WINK, M. 1999. Functions of Plant Secondary Metabolites and their Exploitation in Biotechnology. Annual Plant Reviews, Volume 3. WINK, M. (Ed.). Sheffield Academic Press, England

WOOLHOUSE, M.E. J. 1994. Epidermiology of Human Schistisomiasis.In: Parasitic and Infectious Diseases, Epidermiology and Ecology. SCOTT, M.E. and SMITH,G. (Eds.). Academic Press San Diego, California

WORLD HEALTH ORGANIZATION. 1996. Water and Sanitation: Fact sheet, vol. 112. Geneva

WORLD HELATH ORGANIZATION. 2001. Legal Status of Traditional Medicine and Complementary/Alternative Medicine: Worldwide Review

WORLD HEALTH ORGANIZATION. 2002. First Report of the Joint WHO expert Committee on the Prevention and Control of Schistosomiasis and Soil Transmitted Helminths. No 268 Geneva

WORLD HEALTH ORGANIZATION. 2003. Traditional Medicine Fact Sheet No 134 Geneva

XU, L. and DIOSADY, L.L. 1997. Rapid method for total phenolic acid determination in rape seed/canola meals. *Food Research and International* 30: 571-574

YEH, C-H., WANG, H-E. and CHENG, J-T. 2007. Application of bioassay in the safety and/or quality control of herbal products. *Journal of Food and Drug Analysis* 15: 372-376

ZEIGER, E. 2001. Mutagens that are not carcinogenic; faulty theory or faulty tests? *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 492: 29-38

ZHENG, C.J., YOO, J-S., LEE, T-G., CHO, H-Y., KIM., Y-H. and KIM W-G. 2005. Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *Federation of European Biochemical Societies* 579: 5157-5162

ZHISHEN, J., MENGCHENG, T. and JIANMING, W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry* 64: 555-559

ZHOU, S., KOH, H-L., GAO, Y., GONG, Y-Z. and LEE, E.J.D. 2004. Herbal bioactivation: The good, the bad and the ugly. *Life Sciences* 74:935-968

ZIEGELBAUER, K., BABCZINSKI, P. and SCHÖNFELD, W. 1998. Molecular mode of action of antifungal *β*-Amino acid BAY10-8888. *Antimicrobial Agents and Chemotherapy* 42: 2197-2205

ZSCHOCKE, S. and VAN STADEN, J. 2000. *Cryptocarya* species-substitute plants for *Ocotea bullata*? A pharmacological investigation in terms of cyclooxygenase-1 and -2 inhibition. *Journal of Ethnopharmacology* 71: 473-478