PHARMACOLOGY AND PHYTOCHEMISTRY OF SOUTH AFRICAN TRADITIONAL MEDICINAL PLANTS USED AS ANTIMICROBIALS

By

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DECLARATION

I hereby declare that this thesis, unless otherwise acknowledged to the contrary in the text, is the result of my own investigation, under the supervision of Professor J. van Staden and Dr. J. F. Finnie, in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg.

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ABSTRACT

Among all the major infectious human diseases, gastro-intestinal infections caused by microbial pathogens are a major cause of morbidity and infant death in developing countries, largely due to inadequate sewage disposal and contaminated water. Traditional health practitioners in South Africa play a crucial role in providing health care to the majority of the population. Many plants are locally used by South African traditional healers to treat microbial infections related to gastro-intestinal tracts.

Ethnopharmacological and ethnobotanical studies using traditional knowledge as a selection strategy has given priority to certain plants for isolation and identification of plant novel bioactive compounds. Pharmacological and phytochemical studies of the investigated twelve medicinal plant species (from 10 families) extensively used as antimicrobials against gastro-intestinal infections was necessary to validate the use of the plants. Furthermore, to provide sufficient preliminary information for the isolation and identification of active compounds that are present in the investigated plants.

Plant parts were sequentially extracted using petroleum ether (PE), dichloromethane (DCM) and 70% ethanol (EtOH). Cold water and boiled (decoction) extracts of the plant materials were prepared non- sequentially. Among the extracts, EtOH yielded the highest amount of plant substances. A total number of 85 extracts were evaluated for antibacterial activity, 80 for antifungal activity, 64 for anti-inflammatory activity, and 27 biologically active extracts were tested for genotoxicity.

The microdilution method was used to determine the minimum inhibitory concentration values in the antibacterial assay against two Gram-negative bacteria (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) and two Gram-positive bacteria (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600). A modified microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration

(MFC) values in the antifungal assay against *Candida albicans*. Cyclooxygenase assay was used to evaluate the anti-inflammatory activity of the extracts against cyclooxygenase-1 and -2 (COX-1 and COX-2) enzymes. The plant extracts were screened first at a concentration of 250 µg/ml per test sample, and then further screened at concentrations of 125 and 62.5 µg/ml for extracts that inhibited the COX-2 enzyme. The Ames test was used to test for genotoxicity in extracts that showed interesting pharmacological activities using *Salmonella typhimurium* strain TA98.

Among the screened extracts, 25 extracts showed good antibacterial activity with MIC values ≤ 1.0 mg/ml. Dichloromethane extracts exhibited the greatest antibacterial activity, and Gram-positive bacteria were most susceptible. The best antibacterial activity was exhibited by *Becium obovatum* leaf EtOH extracts with an MIC value of 0.074 mg/ml. A broad spectrum antibacterial activity was observed by leaf extracts of *Cucumis hirsutus* (PE)*, Haworthia limifolia* (PE)*, Protea simplex* (PE and DCM) and *Dissotis princeps* (EtOH) against both Gram-negative and Gram-positive bacteria. No interesting antibacterial activity was exhibited by water extracts with the exception of *Dissotis princeps* water extract with a good antibacterial activity against Gram-positive and Gram-negative bacteria. In the antifungal assay, 6 extracts showed interesting antifungal activity. *Protea simplex* leaf PE extract showed the best fungicidal activity with an MFC value of 0.014 mg/ml. The best overall antifungal activity was observed in plant EtOH extracts. Some extracts from *Agapanthus campanulatus* (leaves and roots), *Dissotis princeps* (leaves), *Gladiolus dalenii* (corms) and *Protea simplex* (leaves) showed good activity against *Candida albicans*.

Twenty one extracts inhibited the COX-1 enzyme, while fifteen extracts inhibited the COX-2 enzyme at the lowest screening concentration of 62.5 µg/ml. The highest COX-1 inhibition at a concentration of 62.5 µg/ml was exhibited by *Diospyros lycioides* leaf PE extract (89.1%) while *Agapanthus campanulatus* root DCM extract showed the highest COX-2 inhibitory activity (83.7%) at the same concentration. In the Ames test, no genotoxicity was observed in any of the extracts, however more tests need to be done to confirm these results.

Thin layer chromatograms of the organic solvent plant extracts were developed. The fingerprints of the plant extracts showed colours of bands at different R_f values when viewed under UV_{254} and UV_{366} suggesting that the investigated plant species contained different compounds in the extracts.

In the quest to understand the source of the plants pharmacological activities, total phenolic compounds including condensed tannins, gallotannins and flavonoids were quantitatively investigated in terms of their amounts in the aqueous methanol extracts of the plants materials using spectrophotometric methods. Alkaloids and saponins were qualitatively determined. The amounts of total phenolics were determined by the Folin Ciocalteu assay, condensed tannins were determined by the butanol-HCl assay, while rhodanine and vanillin assays were used to determine the amounts of gallotannins and flavonoids respectively. Dragendorff reagent was used to detect alkaloids in the plant extracts on thin layer chromatographic plates, while the froth test was employed to detect saponins. Secondary metabolites varied with plant parts and species with *Cyperus textilis* (leaf) having the highest amounts of total phenolics, condensed tannins and flavonoids. The highest amount of gallotannins was detected in *Protea simplex* leaf extracts. All the investigated plant materials with the exception of *Haworthia limifolia* leaf*, Protea simplex* leaf, *Antidesma venosum* leaf and *Dissotis princeps* leaf tested positively to saponins. Alkaloids were detected in *Haworthia limifolia* leaf (PE and EtOH), *Cucumis hirsutus* leaf (EtOH), *Becium obovatum* root (DCM), *Protea simplex* root and bark (EtOH), *Agapanthus campanulatus* root (DCM) and leaf (EtOH), *Cyperus textilis* root (DCM), *Vernonia natalensis* leaf (PE), *Antidesma venosum* leaf (PE), *Diospyros lycioides* leaf (PE) and *Dissotis princeps* leaf (DCM) extracts.

The results obtained from the investigation of the pharmacology and phytochemistry of the plant species used to treat microbial infections related to gastro-intestinal tracts, provide sufficient preliminary information to validate the use of some of the plants in traditional medicine. The information provided might be considered sufficient for further studies aimed at isolating and identifying the active compounds in the plant species, and evaluating possible synergism amongst the isolated compounds.

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LIST OF ABBREVIATIONS

4-NQO..... 4-nitroquinoline-*N-oxide*
AA..... arachidonic acid
AIDS..... acquired immunodeficiency syndrome
ATCC..... American Type Culture Collection
CFU..... colony forming units
COX..... cyclooxygenase
CNS..... central nervous system
DCM..... dichloromethane
DMSO…….. dimethyl sulphoxide
DNA..... deoxyribonucleic acid
DPM..... disintegrations per minute
EtOH..... ethanol
GAE..... gallic acid equivalent
HPPD..... 4-hydroxyphenylpyruvate dehydrogenase
iCOX…… inducible cyclooxygenase
iNOS…… inducible nitric acid oxide synthase
INT..... *p* -iodonitrotetrazolium violet
IκB…… inhibitor κB kinase
LOX…… lipoxygenase enzyme
MFC..... minimum fungicidal concentration
MH..... Mueller-Hinton
MIC..... minimum inhibitory concentration
NFkB……. nuclear transcriptase factor kB
NSAIDs..... non-steroidal anti-inflammatory drugs
PE..... petroleum ether
PGE_2…… prostaglandin E_2
PGG_2…… prostaglandin G_2
PMN…….. polymorphonuclear neutrophil
R_f..... mobility relative to front
S.E..... standard error

TCM.....	traditional Chinese medicine
TLC.....	thin layer chromatography
TRIS.....	2-amino-2(hydroxymethyl)-1,3-propanediol buffer
USA.....	United States of America
USD.....	United States Dollars
UV.....	ultraviolet
WHO.....	World Health Organisation
YM.....	yeast malt

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Plants have been used as the basis of many traditional medicine systems throughout the world for thousands of years. They continue to provide mankind with new remedies. Fossil records date human use of plants as medicines at least to the middle Palaeolithic age some 60,000 years ago (**SANJAY and ARVIND, 2007**).

According to the World Health Organisation (WHO) the definition of traditional medicine is the sum total of all the knowledge and practical experience used in the diagnosis, prevention and elimination of physical, mental or social imbalance. This knowledge is handed down from generation to generation verbally or in writing (**BANNERMAN *et al.*, 1983; LEWIS and ELVIN-LEWIS, 2003**).

Every region has its own history of traditional medicine, for example traditional Chinese medicine (TCM), Arabic traditional medicine and African traditional medicine. Such practices are traditional because they are deeply rooted in a specific socio-cultural context, which varies from one community to another. Each community has its own particular approach to health and disease even at the level of ethno-pathogenic perceptions of diseases and therapeutic behaviour (**RUKANGIRA, 2001**).

In Africa, herbal medicine is gaining popularity as alternative and complementary therapies, largely as a result of cultural traditions and excessive cost of modern medicines. Traditional remedies made from plants play an important role in the health of millions of people. Low-income people such as subsistence farmers, people of small isolated villages and native communities use folk medicine for the treatment of common infections (**ROJAS *et al*., 2006**).

1.2 African Traditional Medicine

Infectious diseases account for approximately one-half of all deaths in tropical countries due to drug resistant micro-organisms and the emergence of unknown disease-causing microbes. In Africa, up to 80% of the population use traditional medicine for primary health care (**SOFOWORA, 1993**).

Medicinal plants are considered as wild plants though most can be cultivated (**COETZEE *et al.,* 1999; HAOREAU and DASILVA, 1999**). Plants have been used in African traditional medicine for centuries against a variety of diseases (**GILLS and AKINWUMI, 1986**). Plants of medicinal values include trees, shrubs, tubers, creepers and climbers that have healing properties. Medicinal plants are not only used by traditional practitioners to treat diseases attributed to both naturalistic and supernatural causes but also serve as ingredients in performing rituals or mystic practices (**OLIVER-BEVER and ZAHND, 1979**).

AMPOFO and JOHNSON-ROMAULD, (**1978**) defined a traditional healer as a person who is recognised by the community in which he or she lives as competent to provide health care using herbs, animals, mineral substances and some other methods based on social, cultural knowledge such as attitudes, as well as beliefs that are prevalent in the community regarding physical, mental and social well-being.

How and why people select particular plants for medicinal purpose is based on observations of what happened to animals or persons who had eaten certain plants accidentally. In other cases, it was noticed that plants produced some secretions which had relieving effects on itching skin or cleared up a sore on a person who touched the plant. Such observations suggest that the selection of specific plants is not solely a function of availability but the accumulated traditional medicinal knowledge of healing that had been passed on and developed from generation to generation (**BEP, 1986; ETKIN, 1986**).

Traditional medicine in Africa plays a significant role in human health with the use of medicinal plants which are active against a wide variety of human pathogens having shown great promise in the treatment of intractable infectious and life

threatening diseases including malaria, tuberculosis, and opportunistic diseases in HIV patients (**IWU *et al*., 1999**). Over the past two decades WHO has encouraged the use of traditional medicine, especially in developing countries by promoting the incorporation of its useful elements into national health care systems (**ISAAC *et al.,* 1995**).

In Zimbabwe, traditional healers are consulted on a large variety of health problems (**McIVOR, 1989**). **HEDBERG *et al.*** (**1982**) observed that the number of traditional medicinal practioners in Tanzania was estimated to be 30 to 40,000 in comparison with 600 medicinal doctors.

1.3 Traditional Medicine in South Africa

South Africa is blessed with a rich plant biodiversity of more than 24,000 indigenous plants, representing about 10% of all higher plants on Earth. The country has a long tradition of medicinal use of plants. It is estimated that at least 70% of all South Africans consult one of the more than 200,000 traditional healers in the country. Between 12 and 15 million South Africans still depend on traditional herbal medicines (**MEYER *et al.,* 1996; WHO, 2001**). Traditional medicine which is an integral part of South African cultural life is not likely to change to any significant degree in years to come (**BRANDT *et al.,* 1995**).

Traditional health practitioners in South Africa thus play a crucial role in providing health care to the majority of the population. In most cases, they are the first health care providers to be consulted, especially in rural areas where the practice is deeply interwoven into the fabric of cultural and spiritual lives of the people. It is for this reason that there is a wide recognition of traditional medicine practices in South Africa (**WHO, 2001**). Traditional healers in South Africa make use of several species of medicinal plants, differing with ethnic groups for the treatment of various ailments in both humans and domestic animals. With the ratio of medical doctors to patients in South Africa being 1:17,400, traditional medicine forms an important component in the nation's health care system (**PRETORIUS *et al.,* 1993**). The use of traditional medicine is not only confined to rural, low income

groups but is often used for treating conditions irrespective of education and income levels (**COCKS, 1997; MARSHALL, 1998; COCKS and DOLD, 2000**).

1.4 Trade of Medicinal Plants

Medicinal and aromatic plants play a significant role in meeting the demands of the traditional medicine markets which are found both domestically and in international markets (**TITZ, 2004**). They are usually traded as whole plant or plant parts (chopped) normally in dried form. In a few cases, they are traded fresh or preserved in alcohol (**LANGE, 1998**). In producer countries, plant materials are bought from collectors, cultivators, local dealers or district traders. They are then passed on to wholesalers, manufacturers or directly to retailers. The wide range of manufacturers involved can include those engaged in the production of pharmaceuticals, research laboratories, cosmetics, foods and colouring agents (**LANGE, 1998**). The largest global markets for medicinal plants exist in China, France, Germany, Italy, Japan, Spain, and the United Kingdom. Japan has the highest per capita consumption of botanical medicines in the world (**GLASER, 1999; LAIRD, 1999**).

In addition to the markets for medicinal plants, there is an expanding market in developed countries for botanical based products, such as health foods and supplements, herbal drinks and various health and personal care products. The market for herbal products throughout the world is currently worth an estimated US$60 billion per annum with a growth rate of 7 percent (**WHO, 2003; TITZ, 2004**).

The German phytomedical market grew at 30 percent between 1993 and 1995, from a value of US$2.5 billion to US$3.26 billion. The estimated growth rate in 1998 to 1999 was 5 to 10 percent (**LANGE, 1998**). Turkey is reported to export approximately 28,000 tonnes of medicinal and aromatic plants annually, generating nearly US$50 million (**OZHATAY *et al*., 1998**).

There has been substantial growth in herb and herbal product markets across the world in the last three decades. Rapidly rising exports of medicinal plants during

the past decade testifies to worldwide interest in these products as well as in traditional health systems (**SRIVASTAVA, 2000**).

In South Africa, the trade in traditional medicines forms part of a multi-million rand revenue due to high cultural value of traditional medicines. Demand generates a species specific trade network that extends across national boundaries (**CUNNINGHAM, 1997**). There are an estimated 27 million indigenous medicine consumers in South Africa with a large supporting industry (**MANDER, 1998**). More than 700 plant species are known to be actively traded for medicinal purposes throughout the country (**DOLD and COCKS, 2002**). To date, most documentation of the trade in medicinal plants has been undertaken in the Eastern Cape, Gauteng, KwaZulu-Natal and Mpumalanga provinces of South Africa. This evidently suggests that the trade in medicinal plants is not only local but also inter-provincial (**DAUSKARDT, 1990; WILLIAMS, 1996; MANDER, 1997; WILLIAMS *et al*., 1997; MANDER, 1998; WILLIAMS *et al.,* 2000**).

1.5 Ethnopharmacology Approach to Discovery of Novel Medicinal Drugs

Ethnopharmacology is an interdisciplinary area of research that deals with the identification, description, observation and investigation of ingredients used in various recipes of traditional medicine and their effect on animal models. It also studies the relevant forms of indigenous knowledge, practice and cultures implementing the ingredients (**JANARDHANAN and GEORGE, 2006**).

Nature is the best combinatorial chemist and possibly has answers to all diseases of mankind. Until now, natural compounds discovered from medicinal plants and their analogues have provided numerous clinically useful drugs. The fact that only about one-tenth of the flowering species occurring globally had been investigated for their pharmaceutical potential suggests that medicinal plants could be a major potential source of compounds for the treatment of human diseases, including HIV infection (**SANJAY and ARVIND, 2007**).

Screening of more medicinal plants for antimicrobial activities and phytochemicals is important for finding potential new compounds for therapeutic uses. The

research based on ethnopharmacological information is generally considered an effective approach in the discovery of new anti-infective agents from higher plants (**KLOUCEK *et al*., 2005**).

The use of plants in modern medicines involves the isolation of active compounds. This commenced with the isolation of morphine from opium in the early 19th century (**KINGHORN, 2001**). Subsequently this led to the isolation of early drugs such as cocaine, codeine, digitoxin and quinine, some of which are still in use today (**NEWMAN *et al.,* 2000; BUTLER, 2004**).

Drug discovery from plants has evolved to include numerous interdisciplinary fields and various methods of analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist, or plant ecologist who collects and identifies the plants of interest. Collection may involve species with known biological activity for which active compound(s) have not been isolated or may involve taxa collected randomly for a large screening programme (**SANJAY and ARVIND, 2007**).

As drug discovery from plants has traditionally been time-consuming, faster and better methodologies for plant collection, bioassay screening, compound isolation and compound development are now being employed (**KOEHN and CARTER, 2005**). The entire processes of new drug discovery from medicinal plants undergo a series of scientific stages before the drug can be commercially produced. These steps include, formulating an appropriate strategy, obtaining biologic extracts, screening those extracts, isolating active compounds, conducting preclinical tests and chemical modification, and commencing commercial production (**ARTUSO, 1997**). In spite of the various challenges encountered in medicinal plant-based drug discovery, natural products isolated from plants still remain an essential component in the search for new medicines.

1.6 Drugs Discovered from Medicinal Plants

Numerous methods have been utilized to acquire compounds for drug preparation, this include isolation from plants and other natural sources, synthetic chemistry,

combinatorial chemistry and molecular modelling (**GEYSEN *et al*., 2003; LOMBARDINO and LOWE, 2004**).

It is estimated that 61% of the 877 small-molecule new chemical entities introduced as drugs worldwide during 1981 to 2001 were inspired by natural products (**NEWMAN *et al*., 2003**). In addition to the biologically active plant-derived secondary metabolites which have found direct medicinal application as drug entities, many other bioactive plant compounds have proven useful as model compounds for drug syntheses or semi-syntheses.

Natural compounds of pharmaceutical importance that were once obtained from higher plant sources, but which are now produced commercially largely by synthesis, include caffeine, theophylline, theobromine, ephedrine, pseudoephedrine, papaverine, L-dopa, salicylic acid, and ∆-tetrahydrocannabinol (**KINGHORN and BALANDRIN, 1993**). A guanidine-type alkaloid, galegine, was found to be the active principle of *Galega officinalis* L., and is used clinically for the treatment of diabetes (**SNEADER, 1985**).

Recently, several new medicinal plant-derived drugs have been introduced to the U.S.A market. Arteether is a potent anti-malarial drug and is derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* L. (Asteraceae), a plant used in traditional Chinese medicine. Other derivatives of artemisinin are in various stages of use or clinical trials as anti-malarial drugs in Europe (**VAN AGTMAEL *et al*., 1999; GRAUL, 2001**).

Galantamine is a natural product discovered through an ethnobotanical lead and first isolated from *Galanthus woronowii* Losinsk (Amaryllidaceae) in Russia in the early 1950s. Galantamine is now approved for the treatment of Alzheimer's disease. It slows the process of neurological degeneration by inhibiting acetylcholinesterase as well as binding to and modulating the nicotinic acetylcholine receptor (**HEINRICH and TEOH, 2004; PIRTTILA *et al*., 2004**).

Nitisinone is a newly released medicinal plant-derived drug that works on the rare inherited disease, tyrosinaemia, demonstrating the usefulness of natural products as lead structures (**FRANTZ, 2005**). Nitisinone is a modification of mesotrione, an

herbicide based on the natural product leptospermone, a compound isolated from *Callistemon citrinus* Stapf. (Myrtaceae) (**MITCHELL *et al*., 2001**). Nitisinone inhibits enzyme 4-hydroxyphenylpyruvate dehydrogenase (HPPD), in humans and maize (**HALL *et al*., 2001; MITCHELL *et al*., 2001**). Inhibition of the HPPD enzyme in maize acts as an herbicide and results in reduction of plastoquinone and tocopherol biosynthesis, while in humans the HPPD enzyme inhibition prevents tyrosine catabolism and the accumulation of toxic bioproducts in the liver and kidneys (**HALL *et al*., 2001**).

Tiotropium has recently been released to the United States market for treatment of chronic obstructive pulmonary disease (**MUNDY and KIRKPATRICK, 2004; FRANTZ, 2005**). Tiotroprium is an inhaled anticholinergic bronchodilator, based on ipratropium, a derivative of atropine that has been isolated from *Atropa belladonna* L. (Solanaceae) and other members of the Solanaceae family (**BARNES *et al*., 1995**; **DEWICK, 2002; MUNDY and KIRKPATRICK, 2004**). Tiotropium has shown increased efficacy and longer lasting effects when compared with other available chronic obstructive pulmonary disease medications (**BARNES, 2002; MUNDY and KIRKPATRICK, 2004**). There are numerous other plant-derived drugs that are currently undergoing clinical tests.

1.7 Conservation of Medicinal Plants

The demand for medicinal plants in developing countries has been met by indiscriminate harvesting of flora including those in forests. This is due to the lack of conservation knowledge among traditional healers and other harvesters. As a result, many plant species have become extinct and some are endangered or threatened (**JOHN, 2006**).

The African continent is estimated to have about 2,163,400 hectares of closed forested areas. With a calculated annual loss of about 1% due to deforestation, some of the medicinal plants and other genetic materials may become extinct before they are even documented. Research has shown that the massive demand for bark, roots, and whole plants from wild populations is causing a critical decline in population numbers of some species, and may lead to numerous extinctions.

Africa has one of the highest rates of deforestation in the world with Cote d'Ivore and Nigeria having 6.5% and 5.0% deforestation rates per year respectively compared against a global rate of 0.6% (**RUKANGIRA, 2001**).

Increasing harvesting pressure on traditional supply areas are linked to a growing shortage in supply of popular medicinal plant species. At greatest risk of extinction are slow growing, popular and commonly used species that have a limited distribution. Demand for certain taxa exceeds supply, with traders reporting acute shortages and price increases. Several plant species have been greatly exploited by traditional healers, some of these plant species are therefore considered being vulnerable because they are only found in protected areas (**DOLD and COCKS, 2002**).

Concern about this problem has brought conservationists and resource users together to investigate possible solutions (**COLLINS, 2001**). This can be done through the establishment of medicinal plant gardens and farms. For sustainable harvesting, plant part substitution is a means of alleviating the pressure placed on highly popular medicinal plants. For example, because of the destructive harvesting nature of underground or bark plant parts, the use of leaves should be encouraged if they contain and or provide the same active compounds. Conservation and cultivation of medicinal plants primarily in community gardens must be given priority along with other conservation options and market incentives (**RUKANGIRA, 2001**).

1.8 Major Infectious Diseases of Humans

Only a small proportion of the micro-organisms that abound in nature are pathogenic to man. Most are free-living in soil, water and similar habitats, and are unable to invade the living body (**GREENWOOD *et al*., 1992**). According to **SLEIGH and TIMBURY** (**1998**), major microbial infections in humans are infections of the upper respiratory organs, gastro-intestinal tract, skin, urinary tract, and sexually transmitted infections.

Among all these major infectious diseases, gastro-intestinal microbial infections are a major cause of morbidity and infant death in developing countries, largely due to inadequate sewage disposal and contaminated water. Well over 500 million episodes of diarrhoea as a result of gastro-intestinal infections in children under five are estimated to occur annually in Asia, Africa, and Latin America. At least five million children die due to such disorders (**WHO, 1996; BARBARA, 1998**). Some of the causes of gastro-intestinal microbial infections include viruses, bacteria, bacterial toxins, fungi and protozoa. Gastro-intestinal microbial infection (gastroenteritis) is an illness triggered by the infection and inflammation of the digestive system particularly the stomach and intestine. It is frequently referred to as stomach or intestinal flu. Gastroenteritis is characterized by loss of appetite, bloating, nausea, vomiting, abdominal cramps and pains, bloody stools, pus in the stools, and is accompanied by fever and overall weakness (**BARBARA, 1998**).

Infectious gastroenteritis typically lasts about three days if well treated. Adults usually recover without difficulty, but children, the elderly, and or HIV patients with an underlying disease are more vulnerable to complications such as dehydration and even death if proper medical attention is not employed. Clinically, the secretion and absorption processes in the small and large intestines are disrupted resulting in dehydration (**GERALDINE and KELLY, 2007**). Dysentery caused as a result of intestinal infections is described as the configuration of fever, pain, and bloody diarrhoea. Diarrhoea accounts for approximately 80% of microbial gastro-intestinal infections in humans (**BARBARA, 1998; BHAN, 2000**).

Infectious gastroenteritis is caused by many bacterial species such as *Shigella* species*, Vibro cholerae, Escherichia coli, Staphylococcus aureus, Bacillus* species, *Salmonella* species, *Klebsiella* species, and *Campylobacter* species. Other pathogens that invade the mucosa and produce cytotoxins or primarily affect the colon in patients suffering from gastro-intestinal infections include *Entamoeba histolytica*, *Candida albicans*, *Giardia lamblia*, *Cryptosporidium* and *Cyclospora* (**ROBERT *et al*., 1990; SLEISENGER and FORDTRAND, 1993; BURTON and ENGELKIRK, 1995; SLEIGH and TIMBURY, 1998; NIYOGI *et al*., 2000; ADEGUNLOYE, 2006**). Patients with inflammatory gastroenteritis are typically more ill, with higher fever and more severe abdominal pain or cramp. Non-

inflammatory gastroenteritis causes watery diarrhoea of larger volume, resulting from the attack of the intestinal walls by viruses such as Rotavirus, Adenovirus and Astrovirus. Severe loose stools usually result in loss of about 10% of total body weight or 250 millilitres per kilogram per day or more (**KEUSCH *et al*., 1992; BARBARA, 1998; SLEIGH and TIMBURY, 1998**). Dehydration as a result of diarrhoea may also cause decreased skin elasticity, absent tears, dry mucous membranes, sunken eyes, decreased urine output and abnormal pains (**GERALDINE and KELLY, 2007**).

The organisms or toxins responsible for gastro-intestinal infections are readily transmitted via food, water, environmental contacts, pets, and from person to person, with morbidity rates in developing countries three-fold higher than in the developed countries as a result of poor sanitation, malnutrition, drinking of unclean water, poor living conditions and lack of access to vaccines that protect against diarrhoeal pathogens in poor and remote communities (**RYAN and CALDERWOOD, 2000; GUERRANT *et al.*, 2001**).

According to **KRAMER and CHALMERS** (**2001**), there are a few steps that can be taken to prevent microbial gastro-intestinal infections. Exclusive breast feeding can reduce gastroenteritis in infants by 40% as breast milk build strong immunity in infants against diarrhoeal pathogens. Furthermore, household treatment of water and consumption of uncontaminated water are associated with a 39% reduction in diarrhoea incidence. Food poison can be prevented by ensuring that foods are well-cooked and uncontaminated before they are consumed. Children are at particular risk where faeces are disposed at ground level. It is reported that sanitation interventions produce a 32% reduction in diarrhoea incidence. Hygienic living environments are also paramount in the prevention of gastro-intestinal microbial infections.

1.9 Aims and Objectives

Gastro-intestinal microbial infections account for 40% of deaths among children under the ages of five in rural areas, and it is also one of the opportunistic diseases in HIV patients with a direct cause of death in many children. There are

many plants that are used by South African traditional healers in the treatment of many microbial infections and the associated symptoms. The aim of the study was to pharmacologically evaluate some traditional medicinal plants locally used for the treatment of gastro-intestinal microbial infections for antibacterial and antifungal activities against some gastro-intestinal pathogens using *in vitro* microdilution bioassays. The anti-inflammatory activity was also determined to know how effectively the plants alleviate stomach and intestinal pains as well as cramps that are often associated with gastroenteritis in humans. Plant extracts showing good antimicrobial activities were subjected to genotoxicity testing to establish how safe it is to use the plant material. Furthermore, in order to understand the source of the plant biological activities, total phenolic compounds including condensed tannins, gallotannins and flavonoids were quantitatively investigated in terms of their amounts in the aqueous methanol of the plant materials using spectrophotometric methods. Alkaloids and saponins were qualitatively determined.

The output of the research work will later assist in the isolation and identification of active compounds that are present in the medicinal plants for the development of cheap, novel, safe and effective preparations to combat gastro-intestinal microbial infections.

CHAPTER 2

COLLECTION AND EXTRACTION OF PLANT MATERIAL

2.1 Materials and Methods

2.1.1 Collection of Plant Material

The selection of medicinal plants was based on the indigenous ethno-pharmacological records compiled by different researchers on the use of the medicinal plants by various South African tribes. Twelve traditional medicinal plants that have not been investigated but extensively used for the treatment of gastro-intestinal microbial infections and other related problems (Table 2.1) were collected from Mt. Gilboa (29º 16.766' S, 30º 17.627' E), Midmar (29º 29.703' S, 30º 12.417' E), the University of KwaZulu-Natal botanical garden and Pietermaritzburg National Botanical Garden in KwaZulu-Natal Province, South Africa. Due to availability and consideration of potential sustainable harvesting of medicinal plants, the leaves of some plant species were substituted for their roots. Voucher specimens were identified and lodged in the University of KwaZulu-Natal, Pietermaritzburg Herbarium. Different parts of the collected medicinal plants were oven-dried at 50 ºC, ground into powder and stored in airtight containers at room temperature in the dark until they were required for extraction.

2.1.2 Extraction of Plant Material

Ground plant material (5 g of each plant sample) was sequentially extracted with 100 ml of solvents; petroleum ether (PE), dichloromethane (DCM) and 70% ethanol (EtOH) in a sonication bath (Julabo GMBH sonicator) for 1 h at room temperature. Ice was added to water in the sonicator to cool the plant material. Water extracts were prepared non-sequentially. All the extracts were filtered under vacuum through Whatman No.1 filter paper in Büchner funnels. Water extracts

were freeze-dried while organic extracts were concentrated *in vacuo* with a Büchner rotary evaporator at 30 ºC. The resultant extracts were transferred to weighed pill vials and placed under a stream of air for complete dryness. Dried extracts were kept in the dark at 10 ºC until required.

2.1.3 Preparation of Extracts from Boiled Medicinal Plants

Ground plant material (5 g of each plant sample) was added into 20 ml of distilled water (pH 7), and then placed on a hot stove regulated at 100 ºC. The plant material was allowed to boil in water for 15 min before being removed from the stove and allowed to cool to room temperature. The resultant extract was filtered *in vacuo* through Whatman No.1 filter paper and freeze-dried. Dried extract was weighed and stored in the dark at 10 ºC until required.

2.1.4 Percentage Yield of Dried Extracts

In order to establish the amount of dried crude extract obtained from the extraction of dried plant sample in each solvent, the percentage yield of the dried extracts was calculated (Table 2.2). The percentage yield was represented as the percentage ratio of the mass of the dried extract to the mass of the dried plant sample extracted.

2.2 Results and Discussion

Table 2.1: Traditional medicinal plants used for the treatment of gastro-intestinal microbial ailments in South Africa

Species, (Family) Voucher number[1] , Plant part evaluated[2]	Ethnopharmacological information
Agapanthus campanulatus Leighton (Agapanthaceae) FAW 4 NU, LF & RT	Root decoctions are taken orally or as enemas to ensure that a child is free from stomach trouble (**WATT and BREYER-BRANDWIJK, 1962**).
Antidesma venosum E. Mey.ex Tul. (Euphorbiaceae) FAW 7 NU, LF	Leaf decoctions are used for abdominal disorders and dysentery (**HUTCHINGS *et al*., 1996**).
Becium obovatum E. Mey. Ex Benth. (Lamiaceae) FAW 8 NU, LF & RT	Warm water infusions of pounded roots and leaves are administered as enemas to treat children with stomach ailments and abdominal pain (**POOLEY, 1998**).
Cucumis hirsutus Sond (Cucurbitaceae) FAW 2 NU, LF	Leaf and root decoctions are used for abdominal pain and diarrhoea (**HUTCHINGS *et al*., 1996**).
Cyperus textiles Thunb. (Cyperaceae) FAW 9 NU, LF & RT	Root infusions are used as enemas to children with various stomach ailments (**HUTCHINGS *et al*., 1996**).
Diospyros lycioides Desf. (Ebenaceae) FAW 10 NU, LF & ST	Bark and root decoctions are taken for bloody faeces and dysentery (**HUTCHINGS *et al*., 1996**).

Dissotis princeps (Kunth) Triana (Melastomataceae) FAW 11 NU, LF	Leaf infusions are administered as enemas for dysentery and diarrhoea (**HUTCHINGS *et al*., 1996**).
Gladiolus dalenii van Geel (Iridaceae) FAW 12 NU, CM	Corm ground down to a fine meal and taken with warm water in small quantities to treat dysentery, diarrhoea and stomach upsets (**ROBERTS, 1990**).
Haworthia limifolia Marloth (Asphodelaceae) FAW 3 NU, LF	Decoction made from the leaves is used for stomach trouble (**HUTCHINGS *et al*., 1996**).
Protea simplex E. Phillips (Proteaceae) FAW 1 NU, LF & BK	Decorticated root infusions are used for dysentery and diarrhoea in humans and calves (**HUTCHINGS *et al*., 1996**).
Vernonia natalensis Sch.Bip. e x Walp. (Asteraceae) FAW 6 NU, LF	Decoctions from leaves and stems are used for stomach cramps, nervous spasms of the stomach and other stomach ailments (**HUTCHINGS *et al*., 1996**).
Watsonia tabularis Bak (Iridaceae) FAW 5 NU, CM	Corms are used for diarrhoea in humans and calves (**HUTCHINGS *et al*., 1996**).

[1]Voucher number: NU = Natal University, Pietermaritzburg campus Herbarium.

[2]Plant part evaluated: BK= Bark, CM= Corm, LF = Leaf, RT= Root, ST= Stem.

2.2.1 Extraction of Plant Material

Extraction of bioactive compounds from medicinal plants is to a great extent, dependent on the surface area of the material to be extracted and the extracting solvents used in the extraction. The surface area of plant materials is increased by grinding the dried plant materials to powders in order to enhance the penetration of the extracting solvents into the cell components of the plant materials, thereby increasing the yield of biological compounds present in them. According to **ALBU *et al.*** (**2004**), sonication has been proven to increase the yield of compounds from plant material. It also reduces extraction time. Ice was added to cool the temperature of the plant material during sonication so as to prevent breakdown of thermolabile biological compounds in the plant material (**ELOFF, 1998**).

Polar and non-polar biological compounds readily dissolve in polar and non-polar solvents respectively. The extracting solvents (PE, DCM, EtOH and water) were used because of their wide polarity range. This ensured that majority of the plant compounds were extracted in at least one of the extracting solvents. A sequential extraction method was employed in order to possibly have different biological compounds or different amounts in each of the solvent extracts. The water extracts were prepared non-sequentially in order to imitate the extraction or preparation techniques in traditional medicine. The extracts were kept in the dark at 10 ºC to prevent or reduce growth of any phototropic micro-organism on the extracts and also to reduce the decomposition of active compounds in the plant extracts.

2.2.2 Preparation of Extract from Boiled Medicinal Plants

In traditional medicine systems, herbal remedies are prepared in several but rather standardized ways which usually vary based on the plant utilized, and sometimes, the kind of condition or disease to be treated. Some of these methods include infusions (hot teas), decoctions (boiled teas), tinctures (alcohol and water extracts), and macerations (cold-soaking) (**JEAN, 1990**). In South Africa, most of the medicinal plants are prepared by infusion and decoction (**HUTCHINGS *et al*.,**

1996). Boiling of medicinal plants in this study is a close imitation of plant extract preparation in traditional medicine. It also helped to determine the effects heat had on the biological activity of the extracts prepared by boiling the plant materials.

Table 2.2: Dried residues yielded from the extraction of dried plant materials

Species	Plant part	Percentage yield per extract				
		PE	DCM	ETOH	Water	Boiled[a]
Agapanthus campanulatus	Leaves	0.01	1.5	9.2	17.3	21.2
Agapanthus campanulatus	Roots	3.4	0.9	18.1	3.9	7.3
Antidesma venosum	Leaves	0.7	3.1	4.0	4.6	8.3
Becium obovatum	Leaves	1.4	1.1	16.3	5.3	5.8
Becium obovatum	Roots	3.4	4.2	9.1	7.2	11.7
Cucumis hirsutus	*Leaves*	1.3	0.7	11.8	9.8	14.2
Cyperus textiles	Roots	2.9	6.2	10.4	3.5	8.8
Cyperus textiles	Leaves	3.8	5.1	18.1	9.8	10.1
Diospyros lycioides	Leaves	0.03	3.4	17.6	6.8	7.2
Diospyros lycioides	Stems	0.2	0.08	4.7	1.9	2.1
Dissotis princeps	Leaves	1.5	1.1	9.6	4.4	5.4
Gladiolus dalenii	Corms	1.6	3.1	25.1	12.8	13.3
Haworthia limifolia	Leaves	2.7	0.4	2.8	15.5	19.8
Protea simplex	Leaves	0.4	0.2	24.8	12.6	17.3
Protea simplex	Bark	0.8	2.9	5.3	3.9	5.5
Vernonia natalensis	Leaves	9.2	5.5	14.2	2.0	7.8
Watsonia tabularis	Corms	0.3	2.3	7.4	7.5	9.4

[a]Residues obtained from boiled plant materials.

2.2.3 Percentage Yield of Dried Extracts

For most plant species, EtOH extracted the largest quantity of plant substances, while boiled plant materials better yielded substances compared with water extracts (Table 2.2). Relatively, small yields were obtained with DCM and PE. According to **EVANS** (**1996**), alcohol is a general solvent and tends to provide a more complete extraction of compounds with a variety of polarities. Larger amount of yields obtained with EtOH could be as a result of its affinity to extract many plant substances particular polar compounds present in the plant materials. Plant substances obtained in decocted (boiled) plant materials could probably be due to the solubility effect of heat that better breakdown cellular membranes and components of the plant materials.

2.3 Conclusions

The extraction of all possible active principles from medicinal plants for pharmacological evaluation is to some extent dependent on the solvents used in the extraction method. In extracts where good biological activity is observed, it will help to know possible suitable solvent combinations to use for the isolation of active compounds that may be responsible for the biological activity. The use of scientific methods in the preparation of traditional medicinal plants is effective in the discovery of active novel medicinal principles.

CHAPTER 3

EVALUATION OF ANTIBACTERIAL ACTIVITY IN INVESTIGATED PLANT EXTRACTS

3.1 Introduction

Bacterial diseases are still widespread and common, but its spectrum is changing because diseases which were once familiar are now rare and new infections are being recognized (**SLEIGH and TIMBURY, 1998**). Food poisoning as a result of bacterial contamination is one of the major microbial infections which are now being reported in many parts of the world, particularly in Australia, Canada, Japan, South Africa, USA, European countries, and in most African countries (**BAUMLER *et al*., 2000; ADAK *et al*., 2002**). A major concern with regards to gastro-intestinal bacterial infections is the alarming increase of antimicrobial resistance of key causative species (**BAR-MEIR *et al*., 2005**).

3.1.1 Gastro-intestinal Bacterial Infections

Diarrhoea which is a major symptom of gastro-intestinal infection is one of the leading causes of death in most developing countries; its greatest impact is seen in infants and children. In the United States, there is an average of two episodes of diarrhoea per year in children under five years of age with 500 deaths per year. In developing countries, the rate of diarrhoea is two to three times higher. Medical costs and loss of productivity due to infectious diarrhoea amounts to US$23 billion per year in the United States (**SLEISENGER and FORDTRAND, 1993**).

Acute bacterial diarrhoea can be classified into two types. Toxigenic type, which occur when enterotoxins are the major pathogenic mechanism, while the invasive type occurs when the organism penetrates the mucosal surface, however, enterotoxins may be produced as well (**SLEISENGER and FORDTRAND, 1993**).

Bacterial gastro-intestinal infections are characterized by loss of appetite, bloating, nausea, vomiting, abdominal cramps and pains, bloody stools, pus in the stools, and accompanied by fever as well as overall weakness (**JULIA, 2006**).

Common types of intestinal bacterial infections can be linked to *Salmonella enteritadis* and *Campylobacter* species. However, *Escherichia coli*, *Listeria monocytogenes, Staphylococcus aureus* and *Bacillus* species are creating increased concern in developed nations while cholera and shigellosis remain two diseases of great concern in developing countries. *E. coli* is a major cause of traveller's diarrhoea, infantile gastroenteritis and haemorrhagic diarrhoea. The bacterium attaches to the intestinal epithelial cells and produces enterotoxins that cause acute watery diarrhoea in adults by mechanisms similar to cholera toxin (**SLEIGH and TIMBURY, 1998**). *Klebsiella pneumoniae* has also been reported to cause food poison which results in diarrhoea (**ROBERT *et al*., 1990**). *Staphylococcus aureus* is an important pyogenic organism causing toxic food poisoning and superficial infections such as abscesses. *Bacillus* species (*B. subtilis, B. pumilus* and *B. licheniformis*) cause food poisoning which results in food poisoning, abdominal pains and diarrhoeal diseases. Other gastro-intestinal bacteria include *Yersinia enterocolitica, Clostridium perfringens, Clostridium difficile* and *Vibrio cholerea*. They are associated with symptoms ranging from illness, headache, anorexia, lower abdominal pains and lassitude. With the increasing rate of mortality among children, the immuno-compromised and old people, including HIV patients, as a result of gastro-intestinal bacterial pathogens other than commonly identified pathogens should not be ignored (**BARBARA, 1998; AYYAGARI *et al*., 2003; ADEGUNLOYE, 2006; MICHAEL and PAUL, 2007**).

3.1.2 Treatment of Gastro-intestinal Bacterial Infections

Bacterial infections are among the few diseases in medicine for which specific therapies are available. However, despite this, infections are still common and treatment may fail. Bacteria are generally classified as either Gram-positive or Gram-negative by testing them with the Gram stain. The stain attaches to part of

the bacterial cell wall called peptidoglycan and causes a purple colouration. The cell wall of Gram-positive bacteria is a relatively simple structure which forms no appreciable barrier to the entry of antibiotics. It contains many layers of peptidoglycan which is closely associated with the cytoplasmic membrane. On the other hand, the cell wall of Gram-negative bacteria contains an outer membrane which is made up of lipopolysaccharides, and the peptidoglycan layer is separated from the cytoplasmic membrane by the periplasm. It also contains specific proteins, which include pore-forming proteins through which hydrophilic molecules are transported. Teichoic acids which help in the maintenance of the level of divalent cations outside the cytoplasmic membrane are also found in the cell wall of Gram-negative bacteria (**SLEIGH and TIMBURY, 1998**).

There are various treatments for bacterial infections in the public health system in all countries. Patients suffering from diarrhoea as a result of gastro-intestinal infections are subjected to fluid and electrolyte replacement to restore lost body fluids. Commercially available rehydration fluids which contain glucose and electrolytes (0.9% saline) are prescribed to facilitate water absorption in patients with watery stools except when losses are very severe or it is associated with profound vomiting (**BARBARA, 1998; SLEIGH and TIMBURY, 1998**). Antimicrobial therapy is also used with antibiotics against pathogens for the treatment of infectious gastro-intestinal bacterial ailments (**MICHAEL and PAUL, 2007**). Antimicrobial drugs are often classified as bactericidal when they kill the infecting bacteria or as bacteriostatic when they prevent multiplication but do not kill the bacteria. Some of the common antimicrobial drugs that are used against gastro-intestinal pathogens include trimethoprim-sulfamethoxazole, quinolone, doxycycline and chloramphenicol. Unfortunately, resistance increasingly limits their usefulness. A rapid increase in fluoroquinolone resistance in *Salmonella* in some regions has been extremely worrisome (**SLEIGH and TIMBURY, 1998; STEPHEN *et al*., 2003**).

South African traditional medicine makes use of a wide variety of plant species to treat stomach-related diseases which are prevalent in the rural areas (**McGAW *et al.*, 2000**). Herbal medications often are high in tannins, which act as astringents and thereby act to control diarrhoea, and also reduce or prevent the colonization of

the infectious agent. According to **LEWIS and ELVIN-LEWIS** (**2003**), the therapeutic value of a known South African diarrhoea mixture is attributed to specific tannins from *Areca catechu* contained in the mixture.

3.1.3 Ethnopharmacological Search for Antimicrobial Plants

Studies based on the ethnobotanical use of plants have often proved to be a more efficient method of drug discovery than random plant screening (**SLISH *et al*., 1999**; **KHAFAGI and DEWEDAR, 2000**). In the past decades, several ethno-pharmacological studies aimed at evaluating the antimicrobial potentials of traditional medicinal plants used as antimicrobials in many countries such as South Africa, China and Nigeria have been reported (**MENEZES and RAO, 1998; MUKHERJEE *et al*., 1998; TONA *et al*., 1999; RAHMAN *et al*., 2003; TANGPU and YADAV, 2004**).

In South Africa, similar studies on the antimicrobial activities of medicinal plants that are used against gastro-intestinal pathogens have been reported. **McGAW *et al*.** (**2000**) reported on the antimicrobial activities of some medicinal plants. Among the plants evaluated *Cassine transvaalensis, Catha edulis, Combretum apiculatum, Harpephyllum caffrum, Heteromorpha trifoliate, Schotia brachypetala* and *Sclerocarya birrea* showed interesting antibacterial activities with MIC value less than 200 µg/ml against some diarrhoea-causing bacteria.

MATHABE *et al*. (**2006**) also evaluated the antibacterial activities of medicinal plants that are used for the treatment of diarrhoea in Limpopo Province using both agar-well diffusion and microdilution methods. *Punica granatum* and *Ozoroa insignis* were active against all bacterial strains used in the assay.

3.2 Materials and Methods

3.2.1 Extract Preparation for Antibacterial Assay

The extraction of different plant materials were carried out as described in Sections 2.1.2 and 2.1.3. Dried plant extracts were resuspended to a concentration of 50 mg/ml in water for aqueous extracts or 70% ethanol for organic solvent extracts on the day of the experiment.

3.2.2 Storage and Maintenance of Bacterial Strains

Two Gram–positive (*Bacillus subtilis*, ATCC 6051 and *Staphylococcus aureus*, ATCC 12600) and two Gram–negative (*Escherichia coli*, ATCC 11775 and *Klebsiella pneumoniae*, ATCC 13883) bacteria were used. From the bacteria stocks kept at -70 ºC, suspension cultures were prepared in 5 ml Mueller-Hilton (MH) broth (Oxoid) at 37 ºC overnight in an orbital shaker. After 18 h the suspension cultures were streaked on MH sterile media rich agar and then incubated overnight at 37 ºC in an incubator. The solid cultures were removed from the incubator and kept at 4 ºC in the fridge. This process was repeated every month in order to maintain the strength of the bacteria (**WISTREICH, 1997; THIEL, 1999**).

3.2.3 The Antibacterial Microdilution Assay

Plant extracts were evaluated for their antibacterial activity using the microdilution antibacterial assay by determining minimum inhibitory concentration (MIC) as described by **ELOFF** (**1998**). Overnight cultures of two Gram-negative bacteria ((*Escherichia coli* ATCC 11775 (7.0×10^{10} CFU/ml) and *Klebsiella pneumoniae* ATCC 13883 (2.5×10^{9} CFU/ml)) and two Gram-positive bacteria (*Bacillus subtilis* ATCC 6051 (4.8×10^{9} CFU/ml) and *Staphylococcus aureus* ATCC 12600 (2.7×10^{9} cfu/ml)) were diluted with sterile Mueller-Hinton (MH) broth (Oxoid). One hundred microlitres of each of the suspended extract were two-fold serially diluted

with sterile water in a sterile 96-well micro-plate (Greiner Labortechnik) (one microplate per test bacterium) with each extract concentrations ranging from 12.5 mg/ml to 0.098 mg/ml in the micro-plate wells. A similar two-fold serial dilution of neomycin was used as the positive control while 70% ethanol and bacteria-free broth were used as the negative controls. The bacterial saturated suspension cultures were diluted (1:100) with sterile MH broth, with 100 µl being added to each of the wells containing the test and control solutions. The micro-plates were covered with parafilm and incubated at 37 ºC for 24 h. The MIC values were obtained by adding 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma) and incubated further at 37 ºC for 2 h. Since the colourless tetrazolium salt is biologically reduced to a red product due to the presence of an active organism, the MIC values were recorded as the concentrations in the last wells in which no colour change was observed after adding the INT indicator. The screening was repeated three times in duplicate for each extract to determine an average MIC value.

3.3 Results and Discussion

3.3.1 Antibacterial Activity of Plant Extracts

The results of antibacterial minimum inhibitory concentration (MIC) of evaluated plant extracts are presented in Table 3.1. A range of antibacterial activity was exhibited by the screened extracts. According to **VAN VUUREN** (**2008**), natural products with MIC values below 1.0 mg/ml are considered noteworthy. In this study, plant crude extracts showing antibacterial activity with MIC values less than 1.0 mg/ml are considered to have good activity. Some degree of antibacterial activity was exhibited by most of the plant extracts with 25 extracts showing good antibacterial activity against at least one of the test bacteria.

Table 3.1: Antibacterial minimum inhibitory concentration of screened extracts

Plant name	Plant part	Extract	Antibacterial MIC (mg/ml) E. c	K. p	B. s	S.a
A. campanulatus	L	PE	6.25	1.56	3.125	3.125
		DCM	3.125	3.125	**0.39***	1.56
		EtOH	6.25	3.125	4.69	9.38
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled[a]	>12.5	>12.5	>12.5	>12.5
	R	PE	3.125	3.125	3.125	3.125
		DCM	3.125	3.125	1.56	3.125
		EtOH	3.125	3.125	3.125	3.125
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
A. venosum	L	PE	4.2	5.2	5.2	8.3
		DCM	3.125	4.69	3.52	9.4
		EtOH	3.125	3.125	5.21	**0.650**
		Water	12.5	12.5	12.5	7.8
		Boiled	>12.5	>12.5	>12.5	>12.5
B. obovatum	L	PE	**0.195**	**0.195**	**0.195**	**0.098**
		DCM	1.95	1.95	**0.52**	2.3
		EtOH	3.125	3.125	**0.074**	**0.195**
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
	R	PE	3.91	3.65	3.65	3.91
		DCM	3.125	3.125	1.56	3.125
		EtOH	3.125	3.125	2.343	1.56
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5

Plant name	Plant part	Extract	Antibacterial MIC (mg/ml) E. c	K. p	B. s	S.a
C. hirsutus	L	PE	**0.098**	**0.26**	**0.26**	**0.13**
		DCM	**0.59**	2.08	**0.78**	1.04
		EtOH	4.69	3.125	1.56	1.56
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
C. textilis	R	PE	4.69	4.69	**0.975**	4.69
		DCM	3.125	4.69	**0.78**	1.56
		EtOH	3.125	1.56	**0.29**	**0.195**
		Water	12.5	12.5	12.5	12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
C. textilis	L	PE	4.69	4.69	3.125	3.125
		DCM	3.125	6.25	3.125	3.125
		EtOH	4.69	2.34	**0.59**	**0.15**
		Water	12.5	9.38	9.38	12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
D. lycioides	L	PE	3.125	4.69	2.34	3.65
		DCM	4.69	4.69	3.125	3.125
		EtOH	3.125	3.125	1.56	1.3
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
D. lycioides	S	PE	6.25	4.69	1.56	3.65
		DCM	6.25	6.25	**0.59**	**0.59**
		EtOH	4.69	3.125	2.3	1.3
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5

Plant name	Plant part	Extract	Antibacterial MIC (mg/ml)			
			E. c	K. p	B. s	S.a
D. princeps	L	PE	9.38	6.25	4.63	3.125
		DCM	3.125	6.25	3.125	6.25
		EtOH	1.56	2.08	**0.78**	**0.46**
		Water	1.56	**0.78**	**0.78**	**0.78**
		Boiled	>12.5	12.5	6.25	6.25
G. dalenii	C	PE	3.125	3.125	1.56	1.56
		DCM	1.95	4.69	**0.39**	**0.39**
		EtOH	6.25	6.25	6.25	4.29
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
H. limifolia	L	PE	**0.52**	**0.91**	**0.163**	**0.098**
		DCM	2.6	2.6	1.3	1.56
		EtOH	3.125	3.125	3.125	3.125
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
P. simplex	L	PE	**0.098**	**0.33**	**0.26**	**0.195**
		DCM	**0.32**	**0.49**	**0.44**	**0.59**
		EtOH	3.125	1.56	1.56	**0.78**
		Water	>12.5	>12.5	>12.5	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
P. simplex	B	PE	1.56	1.56	1.56	**0.19**
		DCM	3.125	3.125	1.56	**0.78**
		EtOH	2.34	3.125	**0.29**	**0.147**
		Water	6.25	12.5	3.125	>12.5
		Boiled	>12.5	>12.5	>12.5	>12.5

Plant name	Plant part	Extract	Antibacterial MIC (mg/ml)			
			E. c	K. p	B. s	S.a
V. natalensis	L	PE	3.125	4.69	1.17	3.125
		DCM	3.125	4.69	**0.59**	**0.78**
		EtOH	4.69	3.125	3.125	1.56
		Water	12.5	12.5	12.5	12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
W. tabularis	C	PE	3.125	3.125	**0.15**	**0.39**
		DCM	3.125	3.125	**0.59**	1.56
		EtOH	4.69	3.125	2.3	1.56
		Water	12.5	12.5	12.5	12.5
		Boiled	>12.5	>12.5	>12.5	>12.5
Neomycin			1.58×10^{-3}	7.8×10^{-4}	1.47×10^{-4}	3.9×10^{-4}

E.c; *Escherichia coli,* K.p; *Klebsiella pneumoniae,* B.s; *Bacillus subtilis,*
S.a; *Staphylococcus aureus,* B- bark, C-corm, L-leaf, R-root, S-stem.
[a] Extracts from boiled plant parts; *Extracts with values in bold are considered active

Gram-positive *B. subtilis* was the most susceptible bacterium (Figure 3.1). Twenty one extracts showed good activity against the bacterium. Nineteen extracts showed good activity against *S. aureus,* six extracts showed activity against both Gram-negative bacteria (*K. pneumoniae* and *E. coli*). The observed results agree with **MARTIN, 1995; SLEIGH and TIMBURY, 1998** that Gram-negative bacteria are more resistant than Gram-positive ones as a result of the protective cell membrane composition. The same observation has also been reported by other authors (**VLIETINCK *et al*., 1995; VALSARA *et al*., 1997; McGAW *et al.*, 2000; SAMY and IGNACIMUTHU, 2000; SRINIVASAN *et al*., 2001; BUWA and VAN STADEN, 2005**).

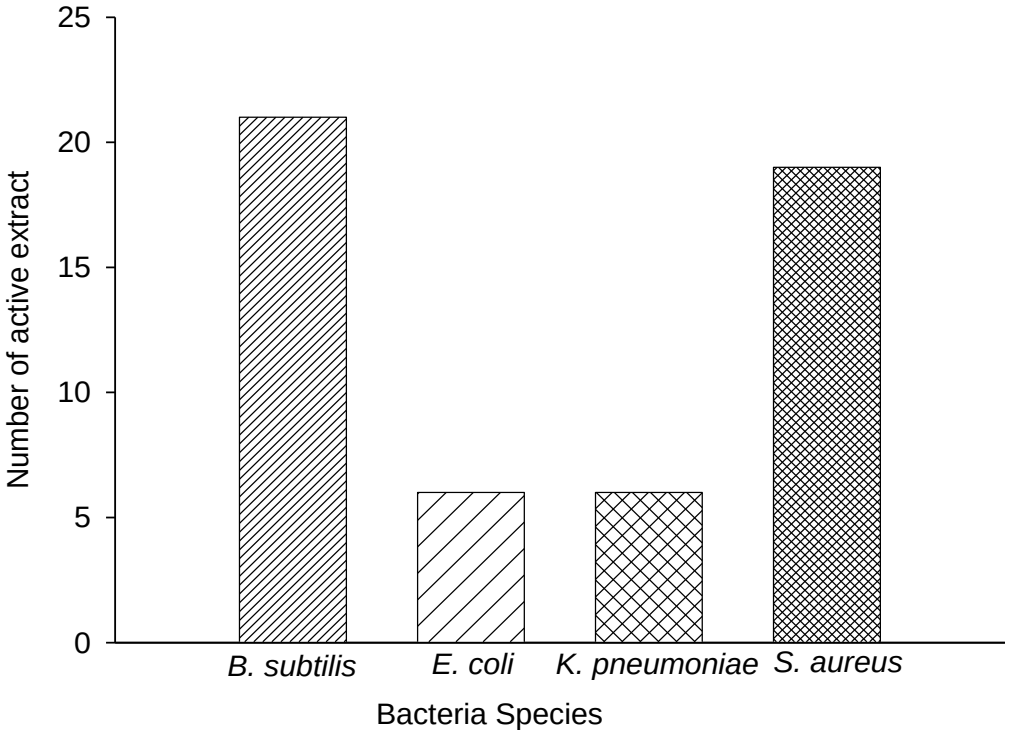


Figure 3.1 Susceptibility of test bacteria to plant extracts (MIC ≤ 1.0 mg/ml).

The EtOH extract of *Becium obovatum* (leaf) showed the best antibacterial activity with an MIC value of 0.074 mg/ml against *B. subtilis.* Petroleum ether leaf extracts of *Becium obovatum*, *Cucumis hirsutus*, *Haworthia limifolia* and *Protea simplex* showed antibacterial activity with MIC values ranging between 0.098 and 0.910 mg/ml against all the test bacteria. All the organic extracts of *Becium obovatum* (leaf)*, Protea simplex* (leaf and bark), and *Cyperus textiles* (root) showed antibacterial activity with MIC values ranging between 0.098 to 0.975 mg/ml against at least one of the test bacteria. Good antibacterial activity was exhibited by the some extracts of *Becium obovatum* leaves but a relatively weak antibacterial activity was observed in all the root extracts. Interestingly, the traditional healers make use of decoctions prepared from the roots to treat diarrhoea and dysentery (**HUTCHINGS *et al*., 1996**). The observed antibacterial activity showed by leaf extracts of *Becium obovatum* could be as a result of larger amount of active compound present in the plant part. According to **STEENKAMP *et al*.** (**2006**), it is equally possible that different compounds could be responsible for the activity observed in the plant part. Similarly, the antibacterial activity

exhibited by the organic leaf extracts of *Protea simplex* (between 0.098 and 3.125 mg/ml) was better than the antibacterial activity showed by the bark extracts. It is noteworthy that the bark is used traditionally for the treatment of diarrhoea and dysentery (**WATT and BREYER-BRANDWIJK, 1962**). Moreover, better antibacterial activity showed by the leaves of *Becium obovatum* and *Protea simplex* compared to the activity showed by the root and bark supports the idea of plant part substitution for sustainable use (**ZSCHOCKE and VAN STADEN, 2000**). Cineole, hydrocyanic acid and myristic acid are present in *Cyperus* species. *Haworthia* species are said to contain anthraquinones, and a medicinal compound called cucumin was reported in *Cucumis hirsutus* (**HUTCHINGS *et al*., 1996**). It can be hypothesized that some of these compounds may be contributing to the antibacterial activity exhibited by the plant extracts.

Agapanthus campanulatus (leaf), *Cyperus textiles* (leaf and root), *Vernonia natalensis* (leaf), *Watsonia tabularis* (corm), *Antidesma venosum* (leaf), *Diospyros lycioides* (stem), and *Gladiolus dalenii* (corm) exhibited good but selective antibacterial activity against at least one of the Gram-positive test bacteria. Generally, aqueous extracts showed poor antibacterial activity in most of the plant species however the aqueous extract of *Dissotis princeps* showed exceptional activity with an MIC value of 0.78 mg/ml against Gram-positive and Gram-negative bacteria. Surprisingly, no bacterial growth inhibition was observed in any of the boiled plant extracts at the highest concentration assayed except in *Dissotis princeps* extract with a poor antibacterial activity against the test bacteria. According to **IEVEN *et al*.** (**1979**), polyphenols have antimicrobial activities and are soluble in water. This could explain the exceptional antibacterial activity exhibited by *Dissotis princeps* aqueous extract. In comparison, there was a significant decrease in activity with boiled plant extracts compared with water extracts. This evidently suggests that heat could have some destructive effects on the active compounds present in the medicinal plants. The positive control (neomycin) inhibited all bacterial growth with MIC values ranging between 0.147-1.580 µg/ml. Ethanol (70%) used as a negative control showed no bacterial growth inhibition at the highest concentration equivalent to the screened plant extracts, this ruled out false positive results in the antibacterial activity observed in all the active plant extracts.

Of all the plants evaluated, leaf extracts of *Cucumis hirsutus, Becium obovatum*, *Dissotis princeps*, *Haworthia limifolia* and *Protea simplex* showed a broad spectrum and non-selective antibacterial activity against the test bacteria. These plant extracts could be equally active against other resistant bacteria especially Gram–negative ones such as *Salmonella typhi, Shigella dysenteriae* and *Vibrio cholerae* that are responsible for acute gastro-intestinal infections. Further investigation aimed at isolating and identifying the compounds responsible for the activity against Gram–negative bacteria is necessary.

3.4 Conclusions

At least one extract from all the investigated plant species showed antibacterial activity with varying MIC values. This suggests the presence of antibacterial principles in them. Bacteria account for more than 60% of acute gastro-intestinal microbial infections and other related ailments which are prevalent in rural areas (**BAUMLER *et al*., 2000; ADAK *et al*., 2002**). Most of the test bacteria in this study are reported to be involved in microbial infections such as acute diarrhoea, dysentery, abdominal pains and other microbial infections (**ROBERT *et al*., 1990; SLEISENGER and FORDTRAND, 1993; BURTON and ENGELKIRK, 1995; SLEIGH and TIMBURY, 1998; BRIAN, 1996; NIYOGI *et al*., 2000**; **MERCY, 2005; ADEGUNLOYE, 2006**). The results presented in this study validate the use of most of these plants as antimicrobials in traditional medicine. Although PE, DCM and EtOH extracts were evaluated in this study, PE and DCM are not accessible to traditional healers; water is the most commonly used solvent by many traditional healers or water-ethanol mixtures in some cases (**JEAN, 1990; ZSCHOCKE and VAN STADEN, 2000**). The activity of aqueous extracts from medicinal plants could be said to be dose dependent. Weak antibacterial activity observed in aqueous extracts could suggest that most of the active compounds present in the evaluated medicinal plants are less polar therefore they are extracted in small quantities because of their weak affinity to water. However, good antibacterial activity could be achieved at higher concentrations. Practically, more cups of tea or decoction prepared from the medicinal plants would be prescribed for effective treatment to be achieved in traditional medicine.

CHAPTER 4

EVALUATION OF ANTIFUNGAL ACTIVITY IN INVESTIGATED PLANT EXTRACTS

4.1 Fungal Gastro-intestinal Infections

Infections due to fungal pathogens have become more frequent with the rise of HIV infection (**WALSH and GROLL, 1999; FLEMING *et al*., 2002**). Opportunistic fungal pathogens have become a common cause of morbidity and mortality (**GARBINO *et al.*, 2001**). In adults, fungal gastroenteritis is encountered in the tropics. It is associated with persistent diarrhoea, anorexia, abdominal bloating, substantial weight loss and overt steatorrhoea (**MICHAEL and PAUL, 2007**).

Major intracellular gastro-intestinal fungal pathogens are *Microsporidia, Candida albicans*, *Aspergillus*, and *Cryptococcus*. They are the common types that cause persistent and severe diarrhoea particularly in the immuno-compromised such as HIV-positive patients. People most likely get fungal infections by ingesting contaminated foods. These organisms may also contaminate fresh water streams, making villagers and campers part of the risk groups of the infection. Pathogenic fungi invade the intestinal mucosa in patients while the opportunistic ones become pathogenic and virulent when the immune system is compromised (**BARBARA, 1998; LEWIS and ELVIN-LEWIS, 2003**).

Candida albicans is an opportunistic pathogen that causes severe inflammatory diarrhoea. Mortality associated with systemic *C. albicans* infections is approximately 35 to 50%. In South Africa where many patients are immunocompromised as a result of the AIDS pandemic, infections caused by *C. albicans* are common (**SHAI *et al*., 2008**). *Isospora belli* is a common pathogen that causes gastro-intestinal infections in immunocompetent hosts (**BARBARA, 1998**).

4.2 Treatment of Fungal Infections

With the limited availability of antifungal drugs and the increasing incidence of opportunistic fungal infections, the emergence of drug resistant fungal pathogens poses a serious public health concern. Antifungal drug resistance has been studied most extensively with the yeast *Candida albicans* because it is an opportunistic pathogen (**COWEN *et al*., 2002**).

There has been extensive research into the development of antifungal drugs. Some of the widely used antifungal drugs for the treatment of fungal infections include Amphotericin B, azoles, imiazoles, miconazole and ketoconazole, triazole and itraconazole as well as pyrimidine inhibitors (**PFALLER *et al.,* 1996**). In traditional medicine, a lot of medicinal plants are used to treat *Candida* infections. Many researchers have investigated numerous medicinal plants for anti-candidal potentials (**CLARK and HUFFORD, 1993**). A few antifungal compounds have been isolated from medicinal plants species. Medicinal plant antifungal activity has been associated with plant secondary metabolites such as phenols, glycosides, lactones and saponins (**AGARWAL *et al*., 2000**).

4.3 Materials and Methods

4.3.1 Extract Preparation for Antifungal Assay

Plant extracts were prepared as described in Sections 2.1.2 and 2.13. Dried plant extracts were resuspended to a concentration of 50 mg/ml in water for aqueous extracts or in ethanol (70%) for organic solvent extracts for antifungal evaluation on the day of the assay.

4.3.2 Storage and Maintenance of Fungal Strain

Candida albicans (ATCC 10231) obtained from the South African Bureau of Standards was used in the assay. From the *Candida albicans* stock kept at -70 ºC, suspension culture was incubated overnight in 5 ml Yeast Malt (YM) broth at 37 °C on an orbital shaker. The overnight culture was streaked on Yeast Malt (YM) broth rich sterile media agar in a Petri-dish. The agar culture was incubated at 37 °C in an incubator. After 18 h the agar culture was removed and kept at 4 °C in the fridge. This process was repeated every 30 days in order to maintain the potency of the fungus (**WISTREICH, 1997; THIEL, 1999**).

4.3.3 The Antifungal Microdilution Assay

A microdilution method as described by **MASOKO *et al*.** (**2007**) was used to determine the antifungal activity. An overnight fungal culture was prepared in 5 ml Yeast Malt (YM) broth. Sterile saline (0.85% NaCl) was added to the fungal culture such that the absorbance of the fungal culture at 530 nm was in the range of 1 ml of a 0.5 M McFarland standard solution (which is between 0.2500 and 0.2800). The standardised fungal culture was further diluted with sterile YM broth (1:1000) (approximate 5 x $l0^5$ CFU/ml). One hundred microlitres of each resuspended extracts were two-fold serially diluted with sterile water in a 96-well micro-plate (Greiner Labortechnik) with each extract concentrations ranging from 12.5 mg/ml to 0.0123 mg/ml in the wells. A similar two-fold dilution of solution of Amphotericin B was prepared as the positive control while ethanol (70%) and fungal-free wells were used as the negative controls. One hundred microlitres of the dilute *Candida* culture were added to each well. The plates were covered with parafilm and incubated at 30 °C for 48 h. The MIC values were obtained by adding 50 µl of (0.2 mg/ml) *p*-iodonitrotetrazolium (INT) (Sigma, Steinham, Germany) and then incubating for another 2 h. Fungicidal activity of the plant extracts was determined by adding 50 µl YM broth to all the wells with no colour change and further incubated at 37°C for 24 h. *Candida* growth in the wells was indicated by a reddish-pink colour, whereas clear wells indicated growth inhibition by plant extracts. The concentrations of extracts in the last clear wells were taken as the

minimum fungicidal concentration (MFC). The screening was done in triplicate and repeated twice for each extract, the average MIC and MFC values are reported.

4.4 Results and Discussion

4.4.1 Antifungal Activity of Plant Extracts

According to **SLEIGH and TIMBURY** (**1998**) *C. albicans* is resistant to all antibacterial antibiotics. The results obtained evidently showed that *C. albicans* is resistant to most of the plant extracts. Only six plant extracts showed interesting antifungal activity with MIC values less than 1.0 mg/ml. **BUWA and VAN STADEN** (**2005**) also reported that pathogenic fungi are more resistant than pathogenic bacteria.

Table 4.1: Antifungal activity (MIC and MFC) of investigated plant extracts used traditionally against *C. albicans*

Plant species	Plant part	Extracts	Antifungal concentration (mg/ml) MIC	MFC
A. campanulatus	L	PE	9.38	12.5
		DCM	3.125	6.25
		EtOH	**0.29***	**0.29**
		Water	> 12.5	> 12.5
		Boiled[a]	> 12.5	>12.5
	R	PE	6.25	6.25
		DCM	4.69	6.25
		EtOH	**0.39**	**0.39**
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
A. venosum	L	PE	3.125	4.69
		DCM	3.125	4.69
		EtOH	3.125	6.25
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
B. obovatum	R	PE	4.69	6.25
		DCM	3.125	3.125
		EtOH	4.17	6.25
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
C. hirsutus	L	PE	6.25	6.25
		DCM	4.69	4.69
		EtOH	3.125	4.17
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
C. textilis	R	PE	6.25	6.25
		DCM	4.69	6.25
		EtOH	3.125	3.125
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
	L	PE	6.25	9.38
		DCM	1.17	1.56
		EtOH	3.125	5.21
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5

Plant species	Plant part	Extract	Antifungal concentration (mg/ml) MIC	MFC
D. lycioides	L	PE	3.125	3.125
		DCM	3.125	4.69
		EtOH	4.17	8.33
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
	S	PE	6.25	6.25
		DCM	3.125	3.125
		EtOH	4.17	8.33
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
D. princeps	L	PE	1.56	1.56
		DCM	6.25	6.25
		EtOH	**0.014**	**0.037**
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
G. dalenii	C	PE	6.25	6.25
		DCM	3.125	3.125
		EtOH	**0.39**	**0.39**
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
H. limifolia	L	PE	6.25	6.25
		DCM	4.69	6.25
		EtOH	6.25	6.25
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
P. simplex	L	PE	**0.014**	**0.014**
		DCM	**0.39**	**0.39**
		EtOH	3.125	3.125
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
	B	PE	12.5	12.5
		DCM	9.38	12.5
		EtOH	3.125	12.5
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
V. natalensis	L	PE	5.21	6.25
		DCM	3.125	4.69
		EtOH	3.125	5.21
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5

W. tabularis	C	PE	4.69	6.25
		DCM	4.69	7.81
		EtOH	3.125	6.25
		Water	> 12.5	> 12.5
		Boiled	> 12.5	>12.5
Amphotericin B			6.9×10^{-3}	1.3×10^{-2}

[a] Extracts from boiled plants; * Extracts with values in bold are considered active MIC - minimum inhibitory concentration; MFC - minimum fungicidal concentration B- bark, C-corm, L-leaf, R-root, S-stem.

The best fungicidal activity was shown by the petroleum ether extract of *Protea simplex* leaf with MIC and MFC value of 0.014 mg/ml, the same fungistatic activity was also exhibited by the EtOH extract of *Dissotis princeps* leaf. The EtOH leaf and root extracts of *Agapanthus campanulatus* exhibited good fungicidal activity with MFC values of 0.29 and 0.39 mg/ml respectively. Interesting MFC value was also exhibited by EtOH extracts of *Gladiolus dalenii*. Similarly, good fungicidal activity was shown by the DCM leaf extracts of *Protea simplex* (0.39 mg/ml) and the EtOH leaf extract of *Dissotis princeps* (0.037 mg/ml). Amphotericin B exhibited antifungal activity with MIC and MFC values of 6.9 and 13 µg/ml respectively.

The antifungal activity exhibited by *Protea simplex* leaf PE extracts is impressive considering the low concentration at which the *Candida* growth was inhibited. This suggests that the activity may reside in the lipophilic leaf compounds extracted in PE. A comparable difference in the antifungal activity exhibited by *Protea simplex* leaves and bark could be attributed to the types of active compounds in the plant parts. Several saponins and sapogenins of the furistane and spirostane types that have a large number of biological activities and biochemical effects have been isolated from the roots of *Agapanthus* species (**VAN WYK *et al*., 1997**). The antifungal activity exhibited by *Agapanthus campanulatus* leaf and root EtOH extracts could in part be attributed to the reported compounds. No visible antifungal activity was observed in all the water and boiled extracts at the highest concentration assayed. However, negative results do not mean that the plant extracts are inactive or absence of bioactive compounds. Active compounds may

be present in insufficient quantities in the crude aqueous extracts for them to show activity with the concentrations assayed, therefore lack of activity can be proven by using large doses in extracts that showed no anticandidal activity.

4.5 Conclusions

Plant species that showed good fungistatic and or fungicidal activity at a concentration less than 1.0 mg/ml are *Protea simplex, Dissotis princeps, Agapanthus campanulatus* and *Gladiolus dalenii*. *C. albicans* is a morphologically changing organism; therefore it has the ability to develop resistance against many antifungal compounds. It will be interesting to investigate the compound or group of compounds that are responsible for this activity as they could be more active against other fungal pathogenic species that cause gastro-intestinal infections.

CHAPTER 5

BIOACTIVITY-GUIDED GENOTOXICITY TEST OF ACTIVE PLANT EXTRACTS

5.1 Introduction

Mutagenesis is a health problem because the mechanism may lead to different types of cancer which are increasing at an alarming rate in humans. Mutagenic mechanisms involve anything that damages the DNA or interferes with the structure of the DNA, or with cell division. A given mutagen interacting with the cell may induce different types of genetic lesions, ranging from a single-based modification, which might completely be restored in its initial form, or results in the loss of a chromosome. These different types of mutations occur in somatic cells and might lead to cancer or alterations on seminal cells giving rise to congenital diseases, degenerative disorders, aging, and genetic defects in the offspring. However, often, human cells are able to protect themselves against defective replications through DNA repair processes (**KIRSCH-VOLDERS, 1984; CUZZOCREA *et al*., 2001; MIGLIORE and COPPEDÈ, 2002**).

Plants in general, synthesize toxic substances, which in nature act as a defence against infections, insects and herbivores; however, they also can affect man (**CUZZOLIN *et al*., 2006**). Based on the long-term use of medicinal plants by humans, an assessment of their mutagenic and cytotoxic potential is necessary to ensure a relatively safe use of plant-derived medicines. The genotoxic effects of widely used plants are not well documented in the literature. However, recent investigations have revealed that some plants used as food or in traditional medicine have mutagenic effects in *in vitro* assays (**HIGASHIMOTO *et al*., 1993; SCHIMMER *et al*., 1994; KASSIE *et al*., 1996; ELGORASHI *et al*., 2003**). Extracts of *Crinum macowanni*, *Ziziphus mucronata*, *Chaetacme aristata*, *Plumbago auriculata*, *Catharanthus roseus*, *Diospyros whyteana* and *Combretum mkhzense* have been reported to be mutagenic for strain TA98 of *Salmonella*

typhimurium (**ELGORASHI *et al*., 2003**). Mutagenic plant compounds can themselves be intrinsically mutagenic, or can become mutagenic after activation and transformation by enzymatic and metabolic processes. This raises concern about the potential mutagenic hazards resulting from the long-term use of such plants in humans.

5.2 Tests for Genotoxicity

Many *in vivo* and *in vitro* tests have been developed to give accurate results of genotoxic effects of natural products. The two principal systems used in bacteria are the Rec-assay in *Bacillus substilus* and the reversion assay in *Salmonella typhimurium.* Positive results in the Rec-assay usually indicate a covalent binding to DNA or a chemical breakage of DNA while the reversion assay detects frame-shift mutations and base-pair substitutions and often parallels the carcinogenic potential of a compound (**MARON and AMES, 1983; KIRSCH-VOLDERS, 1984**).

A few tests in yeast and mammalian cells also aim to detect gene mutation, but the great majority of tests in eukaryotes assess the capability to induce structural or numerical chromosome aberrations with no proof of an induction of gene mutations and carcinogenic properties of a compound (**KIRSCH-VOLDERS, 1984**). The alkaline comet and micronucleus assays for the screening of potential genotoxic compounds utilize a variety of cell types. The alkaline comet assay detects primary single- and double-strand breaks and alkali-labile sites before and after DNA repair process, while the micronucleus test detects DNA lesions after their fixation as chromosome mutations (**SINGH *et al*., 1988**).

5.3 The Ames Test

The Ames test procedure is described in a series of papers from early 1970s by Bruce Ames and his group at the University of California. The Ames test is a biological assay widely used for detecting carcinogens and mutagens with *Salmonella*. As cancer is often linked to DNA damage, the test also serves as a quick assay to estimate the carcinogenic potential of a compound since the

standard tests for carcinogenicity done on rodents take years to complete. The test has been used to determine the mutagenicity of complex environmental and biological mixtures. The Ames test using *Salmonella* was first validated in a study of 300 chemicals most of which were known carcinogens (**McCANN *et al*., 1975**). A considerable number of mutagens first detected by the Ames test have been shown subsequently to be carcinogenic in other *in vivo* tests. A set of strains that require histidine for growth is used for mutagenicity testing, with each tester strain containing a different type of mutation in the histidine operon (**MARON and AMES, 1983**). The standard tester strains used in Ames test include TA 97, TA98, TA100, and TA 102. These strains contain the R-factor plasmid (pKM101) which carries the hisG428 mutation and a tetracycline resistance gene. In *Salmonella*, pKM101 increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present in the organism. The strain TA98 detects various frame-shift mutagens; the mutagens can stabilize the shifted pairing that often occurs in repetitive sequences of the DNA resulting in a frame-shift mutation which restores the correct reading frame for histidine synthesis. In some cases, rat liver homogenates are added to stimulate the effect of metabolism, as some compounds, like benzopyrene are not mutagenic themselves but their metabolic products are found to be mutagenic (**AMES *et al*., 1973; MARON and AMES, 1983**).

5.4 Materials and Methods

5.4.1 Preparation of Plant Extracts

Plant materials were extracted as described in Sections 2.1.2 and 2.1.3. Biologically active extracts were dissolved in 10% DMSO to make a concentration of 5 mg/ml and filtered through 0.22 µm filter tips to remove impurities. Three concentrations of 50 µg/ml, 500 µg/ml, and 5000 µg/ml were made from each of the filtered extracts.

5.4.2 Genotoxicity Testing of Active Plant Extracts

Bioactivity-guided genotoxicity study was employed to test for genotoxicity in crude extracts that showed good antimicrobial activities with a minimum inhibitory concentration of ≤ 1.0 mg/ml in the antibacterial and antifungal assays reported in Chapters 3 and 4. Genotoxic compounds are known to be dose dependent and active in living cells. Considering the antimicrobial activities the extracts exhibited against living microbial cells, one can suggest that the activity could probably be due to the presence (or larger amounts) of biologically active compounds in the various active extracts. The Ames assay was performed using the plate incorporation procedure with *Salmonella typhimurium* strain TA98 as described by **MARON and AMES** (**1983**). Ten millilitres of Oxoid nutrient broth No.2 were inoculated with 100 µl stock bacterium (kept at -70 ºC) at 37 ºC for 16 h on an orbital shaker. Hundred microlitres of each extract concentration were dispensed in culture tubes, followed by adding 500 µl of phosphate buffer. The bacterial culture (100 µl) was then added to all the test tubes containing the mixture of plant extract and phosphate buffer, after which 2 ml of agar containing traces of biotin and histidine were added. Sterile water (100 µl) was used as a negative control while 2 µg/ml of 4-Nitroquinoline-*N-oxide* (4-NQO) which is a known mutagen served as a positive control. The mixture was vigorously mixed using a vortex mixer (Chiltern), poured over minimal agar plates and allowed to set before being inverted and incubated at 37 ºC for 48 h. The positive control and plant extracts were tested in triplicate for each concentration, while the negative control was tested in five replicas. According to **MARON and AMES** (**1983**), for a substance to be considered mutagenic in the Ames test, the number of revertant colonies (His^+) on the plates containing the test compounds must be more than twice the number of colonies produced on the negative control plates. After incubation, the number of revertant colonies was counted using a colony counter. Cytotoxicity effects of the plant extracts on *S. typhimurium* was determined by comparing the background of all the plates with the negative control.

5.5 Results and Discussion

5.5.1 Genotoxicity in Plant Extracts

The Ames test using *S. typhimurium* strain (TA98) detects frame-shift mutations based on the spontaneous reversion of *S. typhimurium* strain from His^- to His^+ caused by crude plant extracts. The bacterium (His^+) can then be able to synthesis histidine for proper growth. Although no positive results were detected in any of the plant extracts, the mean numbers of revertant colonies produced are reported in Table 5.1 for reference purposes.

Quercetin, furoquinoline alkaloids and isothiocyanates are among the possible mutagens of plant origin (**SCHIMMER *et al*., 1994; KASSIE *et al*., 1996**). There are no reports on mutagenic compounds from any of the investigated plant species. None of the number of revertant colonies counted was twice the colonies in the negative control. This suggests that the plant extracts have no mutagenic potential in the Ames test. Most mutagens are also toxic and are dose dependent to some extent (**MARON and AMES, 1983**). Dense background of the agar plates after 48 h as compared with the negative control showed that all the plant extracts are not toxic to *S. typhimurium* strain. The absence of genotoxicity in any of the screened plant extracts against *S. typhimurium* bacterial strain is a positive step forward in determining the safety of long-term consumption of the plants in traditional medicine. Inversely, some genotoxicity is exhibited in the presence of metabolic and enzymatic activation. The plant species are administered orally for the treatment of gastro-intestinal ailments and may therefore be subjected to metabolic and enzymatic activation within the human body. However, further investigation of the plant extracts in other genotoxicity tests that make use of human white blood cells need to be carried out to confirm the results observed in the Ames test.

Table 5.1: Number of His+ colonies in *S. typhimurium* strain TA98 produced by antimicrobial crude plant extracts used in traditional medicine (colony numbers are reported as mean ± S.E. Where n=3)

Plant species	Plant part	Bio-active extract	Extract concentration (µg/ml)		
			5000	500	50
A. campanulatus	L	DCM	29.5 ± 7.5	29.0 ± 6.0	45.5 ± 0.5
		EtOH	37.0 ± 2.5	25.3 ± 3.7	23.0 ± 4.6
	R	DCM	31.0 ± 1.2	28.5 ± 1.5	41.0 ± 1.0
		EtOH	24.3 ± 1.2	27.3 ± 3.8	43.5 ± 3.5
A. venosum	L	EtOH	19.7 ± 2.9	22.7 ± 4.3	15.7 ± 1.3
B. obovatum	L	PE	27.3 ± 4.1	25.1 ± 3.0	23.0 ± 4.3
		DCM	25.3 ± 2.3	22.3 ± 1.2	24.3 ± 2.2
		EtOH	26.3 ± 3.3	24.3 ± 1.5	29.3 ± 2.0
C. hirsutus	L	PE	13.0 ± 1.5	13.3 ± 0.9	15.7 ± 1.5
		DCM	14.0 ± 1.7	14.0 ± 0.6	17.3 ± 2.6
C. textilis	R	PE	25.7 ± 3.8	30.0 ± 0.6	27.7 ± 3.3
		DCM	16.3 ± 2.2	31.3 ± 1.9	26.7 ± 1.5
		EtOH	17.3 ± 0.7	19.0 ± 3.2	26.7 ± 8.2
D. lycioides	S	DCM	16.3 ± 0.9	27.3 ± 4.2	25.3 ± 2.4
D. princeps	L	EtOH	22.3 ± 3.8	26.3 ± 2.6	32.3 ± 1.2
G. dalenii	C	DCM	23.0 ± 1.0	22.0 ± 1.0	25.3 ± 4.4
		EtOH	24.0 ± 2.7	38.0 ± 8.4	27.7 ± 2.9
H. limifolia	L	PE	14.3 ± 1.7	14.7 ± 0.9	18.7 ± 2.2
P. simplex	L	PE	18.7 ± 3.2	18.7 ± 2.6	16.3 ± 1.5
		DCM	20.7 ± 0.9	19.0 ± 1.0	17.3 ± 2.3
		EtOH	21.3 ± 2.4	25.5 ± 5.5	17.0 ± 1.0
	B	PE	20.0 ± 2.0	15.5 ± 0.5	24.0 ± 5.0
		DCM	17.7 ± 0.7	23.7 ± 2.3	30.5 ± 1.5
		EtOH	18.0 ± 6.0	18.3 ± 4.7	22.0 ± 3.5
V. natalensis	L	DCM	21.0 ± 2.0	23.5 ± 0.5	21.3 ± 4.9
W. tabularis	C	PE	20.3 ± 2.9	33.0 ± 1.5	26.0 ± 2.6
		DCM	20.0 ± 4.0	22.3 ± 0.7	19.3 ± 1.7

Negative control revertant colony number =**26.0 ± 0.6**

Positive control (4-NQO) (2 µg/ml) revertant colony number = **130.3 ± 17.0**

B- bark; C-corm; L-leaf; R-root; S-stem.

5.6 Conclusions

It is interesting that screened plant extracts that exhibited good antimicrobial activities showed negative genotoxicity results in the Ames test. The negative result observed suggests that isolated compounds from the plant species could be potentially non-genotoxic, hence preferable in the preparation of safe antimicrobial drugs. Therefore, further pharmacological investigation in the isolation and identification of active principles that are responsible for the observed antimicrobial activities is relevant and worthy. However, it will be important to subject any isolated compounds from the plant species to human based genotoxicity tests to ensure their safety in humans.

CHAPTER 6

EVALUATION OF ANTI-INFLAMMATORY ACTIVITY IN INVESTIGATED PLANT EXTRACTS

6.1 Introduction

NAIK and SHETH (**1976**) defined inflammation as a complex, vascular lymphatic and local tissue reaction elicited in mammals by the presence of viable and non-viable irritants. Gastro-intestinal infections involve the inflammation of the gastro-intestinal tracts and result in intense abdominal pains and cramps of varying degrees (**BARBARA, 1998**). The intestine is vulnerable to muscle spasm in patients suffering from gastro-intestinal infections, and most patients suffering from such conditions often complain of abdominal cramps and pains. Some types of chronic inflammations are caused by self replicating parasite like bacteria, viruses or other pathogens. *Escherichia coli* and other gastro-intestinal pathogens that are associated with food poison produce enterotoxins that induce watery diarrhoea and abdominal tissue damage through plasmid-encoded invasion factors that permit invasion of the mucosa thereby resulting to abdominal pains and chronic cramps (**NAIK and SHETH, 1976; SLEISENGER and FORDTRAND, 1993**).

Inflammation is broadly classified into two categories; acute and chronic inflammations. Acute inflammation occurs when a tissue injury is caused by a single event such as mechanical trauma, a thermal or chemical burn. It is characterized by the exudation of plasma proteins, and the migration of leukocytes into the injured area. The chronic inflammation is of a more prolonged duration and it is manifested by the presence of lymphocytes and macrophages, resulting in fibrosis and tissue necrosis. Persistent chronic inflammation increases the development of the degenerative diseases such as rheumatoid arthritis, heart diseases, asthma, cancer, acquired immunodeficiency disorder, infections (bacteria, fungi, viruses and parasites) and inflammatory bowel disease (**IWALEWA *et al*., 2007**). There is growing interest in anti-inflammatory activity of

plant extracts by pharmaceutical companies as well as the herbal industry. In the last few years, a number of anti-inflammatory compounds have been introduced in order to find potent and safe anti-inflammatory drugs which would be as effective as steroids but without any deleterious effects. Therefore, it is worth evaluating medicinal plants that are used traditionally in the treatment of pain related infections in search for potent anti-inflammatory agents with reduced or no side effects.

6.1.1 Inflammatory Process

The inflammatory response is a complex process triggered by several factors ranging from bacterial infection and chemical injury to environmental pollution that result in cell injury or death. The inflammatory process is characterised by the activation of monocytes, granulocytes and lymphocytes, as well as the release and activation of inflammatory mediators, and humoral mediators (**SHEK and SHEPHARD, 1998**). These substances bind to specific target receptors on the cells and may increase vascular permeability, promote neutrophil-chemotaxis, stimulate smooth muscle contraction, increase direct enzymatic activity, induce pain and mediate oxidative damage (**COLEMAN, 2002**). The inflammatory process mediators are termed pro-inflammatory fundamental factors, and they result in inflammations of different severity depending on the duration of the injury. The overall inflammatory process involves kinins and kinin-generating protease, chemoattractant chemokines, pro- and anti-inflammatory cytokines, cell surface proteins involved in cell-cell interactions, small bioactive molecules (such as platelet activating factor, eicosanoids and histamine), cell surface receptors for the foregoing and downstream signal transduction components (**POLYA, 2003**).

Cyclooxygenase (COX) is a bi-functional enzyme that first catalyses the addition of two molecules of oxygen to arachidonic acid to form the hydroperoxide prostaglandin G_2 (PGG_2), then reduces the hydroperoxide by a peroxidase activity (**SMITH and SONG, 2002**). Cyclooxygenase enzymes exist in two isoforms (COX-1 and COX-2), coded by distinct genes on different chromosomes. The two enzymes show about 50% homology and have similar catalytic activity, but are

physiologically distinct. COX-1 is generally constitutive; it has “housekeeping” functions (regulation of normal cell activity) including gastric cytoprotection and platelet aggregation. In contrast, COX-2 can also be constitutive in some tissues but its expression and activity is largely responsive to adverse stimuli (**PASINETTI, 2001; CRONSTEIN, 2002**).

In summary, the inflammatory process begins with a stimulus that causes the release of prostaglandins from cells. Stimuli such as lipopolysaccharides can induce an enzyme responsible for the production of nitric oxide (inducible nitric oxide synthase) and the COX enzymes. COX-2 acts by producing prostaglandins, particularly prostaglandin E_2 (PGE_2). In turn, PGE_2 acts inside the cell to produce various types and quantities of cytokines, which are pro-inflammatory agents that complete the process by bringing active leukocytes to the injury site (**PASS *et al*., 1995**).

6.1.2 Anti-Inflammatory Agents

Anti-inflammatory medications are used to manage pain and inflammatory disorders. Anti-inflammatory drugs relieve inflammation and the associated pain by blocking specific enzymes that the body needs in order to make prostaglandins as inflammation cannot develop without prostaglandins. Drugs which are used as anti-inflammatory agents are categorised into two groups namely the non-steroidal and the glucocorticoid drugs. Most anti-inflammatory agents such as aspirin, ibuprofen, indomethacin, and COX-2 specific drugs are non-steroidal drugs that inhibit prostaglandin synthesis (**RANG and DALE, 1987**).

Non-steroidal corticosteroid such as cortisol causes inhibition of the arachidonic acid, cascade inhibits the synthesis of pro-inflammatory proteins such as inducible nitric acid oxide synthase (iNOS) and of pro-inflammatory cytokines as well as the immune responses. As a result of the inhibition of COX-1, adverse effects on the gastro-intestinal mucosa may arise resulting in gastric ulceration and increase cardiovascular problems. A newer class of non-steroidal anti-inflammatory drug (NSAID) which includes the brand name Celebrex works by stopping the chemical reaction that leads to inflammation in the body, but unlike other NSAIDs, it does

not harm the production of chemicals that protect the stomach lining (**CRONSTEIN, 2002**).

A variety of plant-derived compounds are used as anti-inflammatories by inhibiting the formation of pro-inflammatory signalling molecules such as prostaglandin or leukotrienes. Some plant substances are reported to inhibit the nuclear transcriptase factor (NFkB)-mediated signalling pathway in immune cells that leads to the production of iNOS, pro-inflammatory cytokines and inducible cyclooxygenase (**POLYA, 2003**). In some medicinal plants, bioactive flavones namely, apigenin, luteolin, and matricine have been identified to be responsible for the anti-inflammatory activities by inhibiting cytokine-induced gene expression (**HERTOG *et al*., 1993; MERFORT *et al*.,1994; SAFAYHI *et al*., 1994**). *Zingiber officinale* is a common anti-inflammatory in traditional medicine. The main ingredients (6-gingerol, 6- paradol, 8-paradol and 8-shogaol) identified in the plant are reported to have an inhibitory effect on the COX-2 enzyme system as well as antitumor and anti-proliferative properties against tumour cells (**TJENDRAPUTRA *et al*., 2001**). Numerous herbs possess anti-inflammatory effects by inhibiting COX-2 enzyme or the activation of transcription factors. Other plant species reported to have anti-inflammatory properties include *Hamamelis virginiana, Echinacea* species, *Ananas comosus*, *Arnica montana*, *Salix alba*, *Glycyrrhiza glabra* and *Cucuma longa* (**KROES *et al*., 1997; RININGER *et al*., 2000; SURH *et al*., 2001; HUGHES-FORMELLA *et al*., 2002; SPERONI *et al*., 2002; BALTINA, 2003; MERFORT, 2003**).

6.2 Materials and Methods

6.2.1 Extract Preparation

The extraction of different plant parts was carried out as described in Sections 2.1.2 and 2.1.3. For each plant sample, dried crude extracts were dissolved in ethanol (80%) for organic extracts (PE, DCM and EtOH) or in water for water extracts.

6.2.2 Testing COX-1 Inhibition

The COX-1 assay was conducted using the method as described by **ELDEEN and VAN STADEN** (**2008**). A stock solution of COX-1 enzyme (60 µl) (Sigma-Aldrich) stored at -70°C was activated with 1250 µl of co-factor solution (0.3 mg/ml Epinephrine, 0.3 mg/ml reduced glutathione in 10 ml of Tris buffer pH 8.0, and 100 µl hematin solution) (Sigma) and pre-incubated on ice for 5 min. Plant extracts were first screened at 10 mg/ml of crude extracts. In duplicate, plant extract (2.5 µl) were added to 17.5 µl distilled filtered water in Eppendorf tubes (for water extracts only 20 µl of the extract was added) to give a final concentration of 250 µg/ml of organic extract per test solution (2 mg/ml for water extracts per test solution). A similar preparation of 5 µM indomethacin (Sigma) was used as a positive control. Solvent blank and background (2.5 µl ethanol + 17.5 µl of water) were used as negative controls. The enzyme solution (60 µl) was added to the test solution and pre-incubated at room temperature for 5 min. The reaction was started by adding 20 µl of ^{14}C-arachidonic acid (Amersham) to the test solutions, with the enzymes in the background being inactivated by adding 10 µl 2N hydrochloric acid (HCl) before incubating the test solution at 37 ºC for 10 min. The incubation was stopped and the reaction terminated by adding 10 µl 2N HCl to each test solution. Unlabeled prostaglandins (4 µl of 0.2 mg/ml) (Sigma) which is a carrier solution were then added to each test solution. The samples were loaded onto individual Pasteur pipettes packed with silica gel (particle size 0.063-0.200 mm, Merck) with 1 ml of hexane:1,4-dioxan:acetic acid (70:30:0.2 v/v/v) to separate prostaglandins 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) while the elution and collection of prostaglandin products was done with 3 ml of ethyl acetate:methanol (85:15 v/v) into scintillation vials. Four ml of scintillation fluid was added to the eluate in the scintillation vials and the radioactivity was measured using a Beckman L S 6000LL scintillation counter. Percentage inhibition of the screened extracts was calculated using:

COX Inhibition (%) = $$\left[1-\left(\frac{DPM_{sample}-DPM_{background}}{DPM_{blank}-DPM_{background}}\right)\right]\times 100$$

DPM =Disintegrations per minute.

6.2.3 Testing COX-2 Inhibition

The same experimental protocol as for COX-1 was followed but with a different type of COX enzyme and the amount of cofactor used in activating the enzyme (**ELDEEN and VAN STADEN, 2008**). Prepared stock solution of COX-2 enzyme (60 µl) (Cayman Chemicals) at -70 ºC was activated with 1250 µl of co-factor solution (0.3 mg/ml Epinephrine, 0.6 mg/ml reduced glutathione in 10 ml Tris buffer pH 8.0, and 100 µl hematin solution) (Sigma-Aldrich) and pre-incubated on ice for 5 min. Plant extracts were first screened at 10 mg/ml of crude extracts. In duplicate, plant extract (2.5 µl) were added to 17.5 µl distilled filtered water in Eppendorf tubes (for water extracts only 20 µl of the extract was added) to give a final concentration of 250 µg/ml of each organic extract per test solution (2 mg/ml for water extracts per test solution). A similar preparation of 200 µM indomethacin (Sigma) was used as a positive control. Solvent blank and background (2.5 µl ethanol + 17.5 µl of water) were used as negative controls. The enzyme solution (60 µl) was added to the test solution and pre-incubated at room temperature for 5 min. The reaction was started by adding 20 µl of ^{14}C-arachidonic acid (Amersham) to the test solutions. The enzyme in the background was inactivated by adding 10 µl 2N hydrochloric acid (HCl) before incubating the test solution at 37 ºC for 10 min. The incubation was stopped and the reaction terminated by adding 10 µl 2N HCl to each test solution. Unlabeled prostaglandins (4 µl of 0.2 mg/ml) (Sigma) which is a carrier solution were added to each test solution. The samples were loaded onto individual Pasteur pipettes packed with silica gel (particle size 0.063-0.200 mm, Merck) with 1 ml of hexane:1,4-dioxan:acetic acid (70:30:0.2 v/v/v) to separate prostaglandins 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) while the elution and collection of prostaglandin products was done with 3 ml of ethyl acetate:methanol (85:15 v/v) into scintillation vials. Four ml of scintillation fluid was added to the eluate in the scintillation vials and the radioactivity was measured using a Beckman L S 6000LL scintillation counter. COX-2 inhibition was determined as described for the COX-1 assay. Plant extracts showing COX-2 inhibition greater than 50% were tested at lower concentrations (125 and 62.5 µg/ml) for COX-1 and -2 to determine if they are dose dependent. Plant extracts were tested in duplicate and repeated twice.

6.3 Results and Discussion

6.3.1 COX Inhibition by Investigated Plant Extracts

Inhibitory activities of COX-1 and COX-2 enzymes by plant extracts were reported as percentage inhibition of prostaglandin biosynthesis. The results expressed as means ± standard errors are presented in Table 6.1. In this report, minimum inhibitory activity of ≥ 50% was considered as good inhibition of COX enzymes (**ELDEEN and VAN STADEN, 2008**). The inhibition of COX-1 enzyme causes significant damage to the gastro-intestinal tract such as bleeding and stomach ulcers (**KIM *et al*., 2005**). Therefore it is desirable to obtain plant products that are COX-2 selective. Plant extracts were first screened at a maximum concentration of 250 µg/ml. Good percentage inhibition of COX-2 enzyme was thereafter used for the selection of plant extracts that were further evaluated at concentrations of 125 and 62.5 µg/ml for both enzymes. At a concentration of 250 µg/ml (Table 6.1), virtually all the plant extracts showed stronger inhibition of COX-1 than COX-2. Plant extracts were considered to be COX selective because they inhibited either COX-1 or COX-2 enzyme at a percentage inhibition of prostaglandin of ≥ 50%. Inhibition of COX enzymes were observed mostly in the PE and DCM extracts of the evaluated plants. PE and DCM extracts from all the plant material (except *Antidesma venosum* leaves and *Protea simplex* bark) showed good COX-1 and COX-2 inhibition ranging from 60.6 to 103%. Generally, all the ethanol extracts (except *Diospyros lycioides* leaves and *Watsonia tabularis* corms extracts) showed weak or no activity against COX-2. However, COX-1 inhibition was exhibited by some EtOH extracts of the investigated plants with the exception of the EtOH extracts of *Agapanthus campanulatus* (leaves and roots), *Becium obovatum* (leaves), *Cucumis hirsutus* (leaves), *Haworthia limifolia* (leaves) and *Protea simplex* (leaves). All the plant extracts of *Cyperus textilis* (leaves) showed selective COX-1 inhibitory activity ranging from 61.3 to 91.7%. At a concentration of 250 µg/ml, water extracts showed poor or no inhibitory activity against both enzymes. Interestingly water extracts from *Agapanthus campanulatus* (leaves), *Becium obovatum* (leaves), *Cyperus textilis* (roots), *Protea simplex* (leaves and bark) and *Watsonia tabularis* (corms) showed moderate to high activity against COX-1 enzyme with percentage inhibitory activity ranging from 50.3 to 90.5%.

Furthermore, most of the plant extracts evaluated at other lower concentrations (125 and 62.5 µg/ml) showed dose dependent activity against COX-1 and COX-2 enzymes. However, *Diospyros lycioides* leaf PE extract (COX-1) (Figure 6.1a), *Gladiolus dalenii* corm PE, *Diospyros lycioides* stem PE, *Vernonia natalensis* leaf DCM, *Agapanthus campanulatus* root DCM, *Becium obovatum* root DCM as well as *Watsonia tabularis* corm EtOH extracts (COX-2) (Figure 6.1b) did not show dose response activities. At the lowest screening concentration (62.5 µg/ml), 46.7% of the evaluated plant extracts screened showed non-selective inhibitory activity, 23% were COX-1 selective, while 6.7% were COX-2 selective with percentage inhibition of prostaglandins ≥ 50%. The highest COX-1 inhibition at a concentration of 62.5 µg/ml was exhibited by *Diospyros lycioides* leaf PE extract (89.1%) while *Agapanthus campanulatus* root DCM extract showed the highest COX-2 inhibitory activity (83.7%) at the same concentration.

Table 6.1: Anti-inflammatory activity (COX-1 and COX-2) of plant extracts evaluated at concentration of 250 µg/ml (% inhibition was reported as mean ± S.E. Where n=2)

		Percentage inhibition							
	Plant	COX-1				COX-2			
Plant species	part	PE	DCM	EtOH	Water	PE	DCM	EtOH	Water
Agapanthus campanulatus	L	92.6 ± 1.1	78.4 ± 7.2	12.8 ± 0.8	74.2 ± 5.9	72.3 ± 9.1	68.1 ± 3.8	16.9 ± 9.5	47.5 ± 3.7
	R	97.7 ± 1.4	98.4 ± 1.0	48.1 ± 9.3	33.4 ± 3.3	78.0 ± 4.4	97.0 ± 1.2	9.1 ± 21.0	28.8 ± 4.0
Antidesma venosum	L	103.0 ± 0.8	72.8 ± 4.3	84.3 ± 7.0	36.2 ± 6.1	46.6± 12.3	40.9 ± 9.9	40.9 ± 10.5	0
Becium obovatum	R	78.5 ± 4.0	86.4 ± 2.6	4.1 ± 3.0	85.3 ± 1.9	76.6 ± 0.8	62.6 ± 9.3	0	1.2 ± 17.0
Cucumis hirsutus	L	91.7 ± 2.1	101.8 ± 1.1	29.1 ± 5.0	26.0 ± 9.4	80.3 ± 3.5	81.5 ± 4.1	0	0
Cyperus textilis	R	91.7 ± 5.0	88.5 ± 4.9	81.4 ± 8.9	61.3 ± 0.69	75.6 ± 9.8	73.5 ± 2.4	47.9 ± 24.3	0
	L	86.3 ± 0.7	88.4 ± 0.5	79.8 ± 8.9	0	75.0 ± 1.8	83.0 ± 0.1	32.8 ± 1.0	0
Diospyros lycioides	L	92.8 ± 0.9	94.0 ± 5.7	90.4 ± 4.3	37.1 ± 0.2	91.6 ± 1.9	84.8 ± 1.3	72.0 ± 2.3	0
	S	73.8 ± 3.6	81.0 ± 2.1	70.6 ± 1.2	13.1 ± 5.7	67.7 ± 0.3	65.9 ± 1.7	37.9 ± 10.1	0
Dissotis princeps	L	58.8 ± 1.2	82.7 ± 2.3	87.1 ± 4.0	4.8 ± 2.8	60.6 ± 7.7	67.2 ± 9.5	22.1 ± 1.1	0
Gladiolus dalenii	C	88.3 ± 1.5	101.8 ± 0.3	53.1 ± 4.4	34.4 ± 0.1	68.4 ± 5.7	100.6 ±3.5	35.6 ± 5.7	0
Haworthia limifolia	L	88.3 ± 3.8	83.5 ± 1.9	1.3 ± 4.1	30.7 ± 10.5	82.4 ± 2.3	72.3 ± 2.8	0	0
Protea simplex	L	100.1 ± 0.8	80.6 ± 8.7	23.7 ± 6.1	57.8 ± 14.4	72.4 ± 1.1	68.4 ± 3.7	0	20.9 ± 6.9
	B	94.2 ± 3.7	86.1 ± 7.1	89.2 ± 7.8	90.5 ± 1.2	41.0 ± 7.4	35.8 ± 2.4	20.0 ± 2.1	16.7 ± 0.3
Vernonia natalensis	L	88.5 ± 3.2	77.5 ± 3.4	51.2 ± 12.4	38.7 ± 3.3	86.7 ± 0.9	63.4 ± 0.5	0	0
Watsonia tabularis	C	97.6 ± 5.3	73.9 ± 5.1	90.3 ± 3.4	50.3 ± 1.8	91.5 ± 0.9	80.5 ± 8.7	51.1 ± 8.8	0

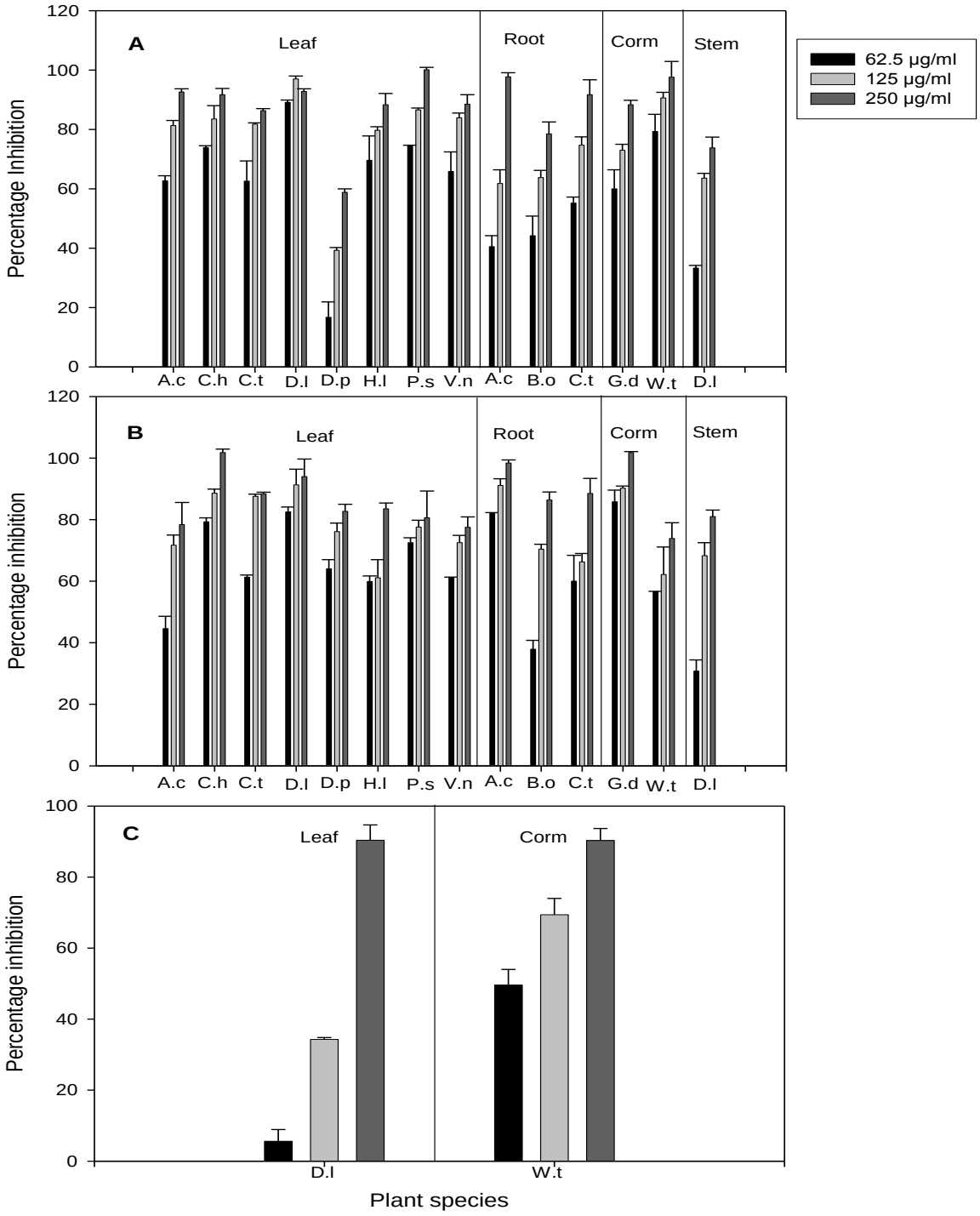
Percentage inhibition of prostaglandin synthesis by indomethacin was **70 ± 3.3** for COX-1 and **68.9 ± 2.5** for COX-2.

B- bark, C-corm, L-leaf, R-root, S-stem.

Figure 6.1a: Dose-dependent COX-1 percentage inhibition by different plant extracts.

(A) Petroleum ether extracts, (B) Dichloromethane extracts and (C) Ethanol extracts.

A.c = *Agapanthus campanulatus*; B.o = *Becium obovatum*; C.h = *Cucumis hirsutus*; C.t = *Cyperus textilis*; D.l = *Diospyros lycioides*; D.p = *Dissotis princeps*; G.d = *Gladiolus dalenii*; H.l = *Haworthia limifolia*; P.s = *Protea simplex*; V.n = *Vernonia natalensis*; W.t = *Watsonia tabularis*.

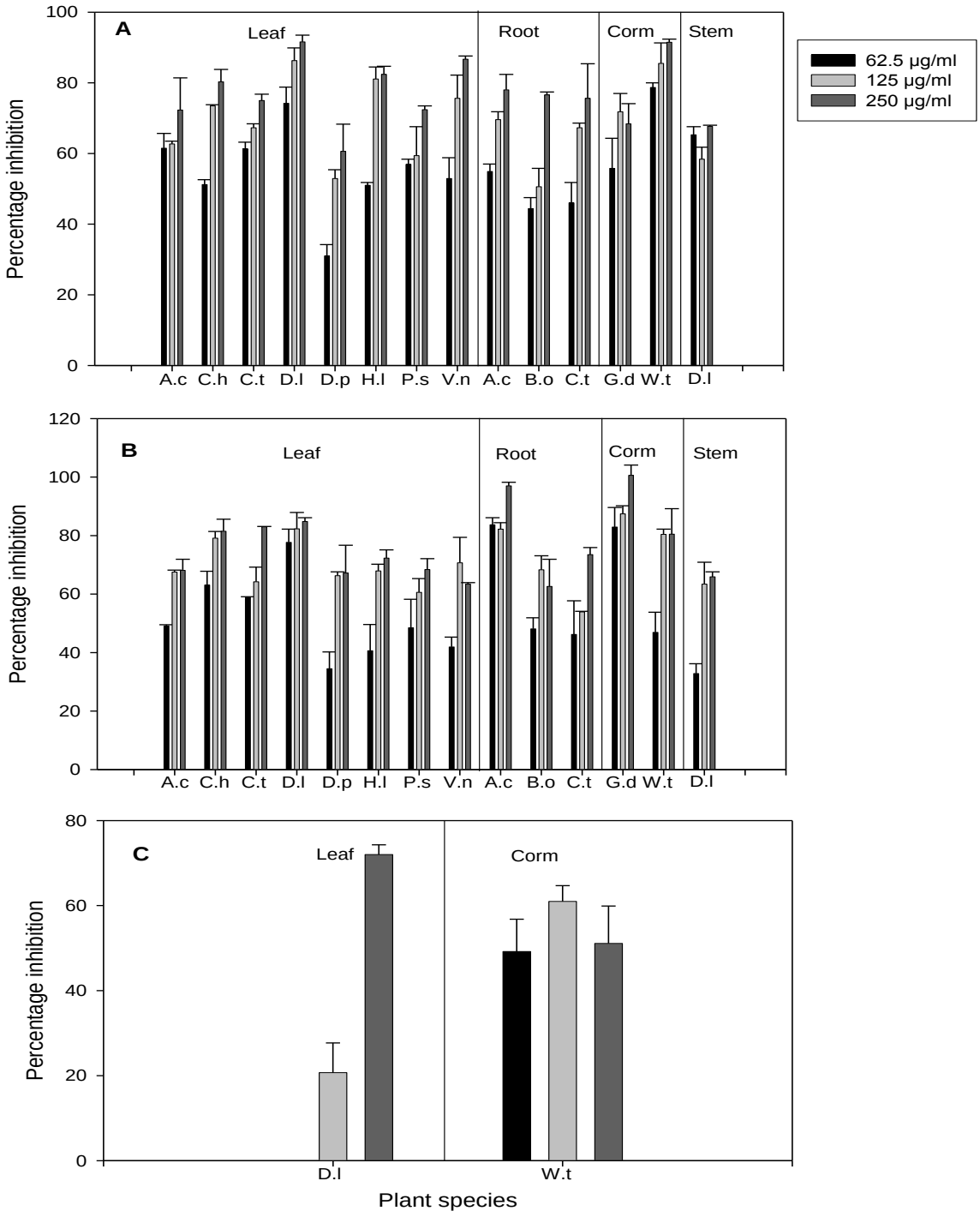


Percentage inhibition of prostaglandin synthesis by indomethacin was **70 ± 3.3**.

Figure 6.1b: Dose-dependent COX-2 percentage inhibition by extracts plant species used for the treatment of gastro-intestinal infections.

(A) Petroleum ether extracts, (B) Dichloromethane extracts and (C) Ethanol extracts.

A.c = *Agapanthus campanulatus*; B.o = *Becium obovatum*; C.h = *Cucumis hirsutus*; C.t = *Cyperus textilis*; D.l = *Diospyros lycioides*; D.p = *Dissotis princeps*; G.d = *Gladiolus dalenii*; H.l = *Haworthia limifolia*; P.s = *Protea simplex*; V.n = *Vernonia natalensis*; W.t = *Watsonia tabularis*.



Percentage inhibition of prostaglandin synthesis by indomethacin was **68.9 ± 2.5**.

The evaluated plant species are used traditionally for the treatment of inflammation of the gastro-intestinal tract characterised by stomach pains, cramps, diarrhoea, dysentry and general stomach troubles. Inhibition of the biosynthesis of inflammatory mediators by blocking the activities of enzymes would be an important treatment of many inflammatory disorders (**ZSCHOCKE and VAN STADEN, 2000**). Some of these symptoms are treated clinically with pain relievers such as codeine phosphate (**SLEIGH and TIMBURY, 1998**). At least one extract from most the evaluated plant species showed good COX-1 and or COX-2 inhibitory activity with the percentage inhibition ≥ 50% at the maximum screening concentration with exception of *Antidesma venosum* and *Protea simplex* bark that are COX-1 selective. Plant medicinal compounds such as flavonoids and its derivatives, sterols, tocopherol, ascorbic acid, alkaloids, tannins, saponins, anthraquinones, triterpenoids, curcumin, genistein, and others have inhibitory properties on the molecular targets of pro-inflammatory mediators in inflammatory responses (**IWALEWA *et al*., 2007**).

No anti-inflammatory study has been reported on the evaluated medicinal plants although some medicinal bioactive compounds with anti-inflammatory properties have been isolated from some of them. *Haworthia* species are reported to contain anthraquinones (**HUTCHINGS *et al*., 1996**). A range of anthraquinones are cathartic, antimicrobial and have anti-inflammatory properties (**IWALEWA *et al*., 2007**). The inhibition of COX-1 and COX-2 enzymes with percentage inhibition of prostaglandins (> 50%) by some of *H. limifolia* leaf extracts could be attributed to the presence of anthraquinones in the plant species. The roots of *Agapanthus* species are reported to contain saponins and sapogenins of the furistane and spirostane types that have many biological activities and biochemical targets such as anti-inflammatory properties (**VAN WYK *et al*., 1997; POLYA, 2003; IWALEWA *et al*., 2007**). *A. campanulatus* root PE extract inhibited the COX-2 enzyme with activity of 54.9%. The root DCM extract of the same plant inhibited COX-1 and COX-2 enzymes with activity of 82 and 83.7% respectively. *Cyperus* species are reported to contain cineole, hydrocyanic acid and myristic acid (**POLYA, 2003**). These compounds could be attributed to the activity shown by the *C. textilis* leaf PE and DCM extracts against COX-1 and COX-2 at a concentration of 62.5 µg/ml. Bioactive medicinal compounds such as cucumin and γ-linolenic

acid, are reported to be present in Curcubitaceae (**VAN WYK *et al*., 1997; IWALEWA *et al*., 2007**). Naphthalene glycosides, diospyrosides (A, B, C, and D) and two known bioactive naphthoquinones, juglone and 7-methyljuglone are reported in *D. lycioides* (**LINING *et al*., 2000**). The activity of *D. lycioides* leaf PE and DCM extracts against COX-1 (ranging from 73.9 to 79.3%) and COX-2 (ranging from 51.2 to 63.1%) at a concentration of 62.5 µg/ml could be attributed to the bioactivity of some of the compounds as they are reported to have biological activities such as anti-inflammatory properties (**POLYA, 2003**). Interestingly, the stem PE extract of the same plant showed COX-2 selective activity of 65.3% at a concentration of 62.5 µg/ml.

The anti-inflammatory activity showed by some of the evaluated plants supports their traditional uses in South African traditional medicine. In the human inflammatory process, anti-inflammatory activity of medicinal plants could be manifested in the inhibition of nuclear transcriptase factor (NFκB) mediated signalling pathway in immune cells that lead to the production of inducible nitric oxide synthase (iNOS), pro-inflammatory cytokines and inducible cyclooxygenase (iCOX). Inhibitor κB kinase (IκB) leads to NFκB de-inhibition, nuclear translocation and expression of pro-inflammatory proteins such as iCOX. Various plant compounds are responsible for the inhibition of inhibitor κB kinase (IκB), thus preventing NFκB activation and expression of pro-inflammatory proteins such as cytokines, COX and iNOS (**POLYA, 2003**).

Plant decoctions are prepared with water in traditional medicine. In case of extracts showing weak or no activity in these assays, it will be noteworthy to mention that high dosage is often used in traditional medicine. Moreover, some of these extracts might be active at other sites in the inflammatory pathways and or contain compounds showing better activity *in vivo* as they undergo metabolic transformation (**McGAW *et al.,* 1997; GARCIA *et al*., 2003**).

6.4 Conclusions

Anti-inflammatory activity observed with some of the investigated plant extracts in this study is interesting. Non-steroidal anti-inflammatory drugs NSAIDs that are COX-2 selective human are highly sought after by pharmaceutical companies. For the purpose of novel drug discovery, further studies aimed isolating and identifying active compounds are necessary. Isolated compounds from the evaluated traditional plants could be effective against other enzyme such as lipoxygenase enzyme (LOX) found in lipoxygenase pathway that result in inflammation.

CHAPTER 7

THIN LAYER CHROMATOGRAPHIC FINGERPRINTING AND DETERMINATION OF PLANT SECONDARY METABOLITES

7.1 Introduction

Plant secondary metabolites are compounds that have no recognised role in the maintenance of fundamental life processes in the plants that synthesize them, but they have important roles in the interaction of the plant with its environment (**NEMDEO, 2007**). Secondary metabolites in plants comprise a long list of compounds produced at various steps in the metabolic pathway that are not directly related to regulating photosynthesis or other primary metabolic pathways, such as respiration (**COLEY *et al*.,1985**). The production of these compounds is often low and dependent on the physiological stages of plants (**DIXON, 2001**). A diversity of chemical compounds serves to defend plants. In some plants, the defensive chemical also routinely serves a number of functions, while in other cases a plant uses different chemicals under different stress conditions (**JONES and COLEMAN, 1991**). Although plant secondary products are very common, not all plants can produce every product. Some compounds are restricted to single species, others to related genus. But they are nearly always found only in certain specific plant organs, often in just one type of cell or cell organelle. Furthermore, they are often generated only during a specific developmental period of the plant. Some of the secondary metabolites (chemical substances) are biologically active and have phyto-pharmaceutical uses. Common secondary metabolites include alkaloids, phenols, tannins, glycosides, terpenoids, saponins, steroids and essential oils. During the last 20 to 30 years, the analysis of secondary plant products has advanced a lot with the use of modern analytical techniques like chromatography, electrophoresis, isotope techniques used in the isolation of bioactive compounds.

7.1.1 Properties of Some Plant Secondary Metabolites

7.1.1.1 Alkaloids

Alkaloids are a group of nitrogen-containing bases. The large majority are produced from amino acids, while only a few are derived from purines or pyrimidines. In a given plant, the concentration of alkaloids can vary widely from part to part, and some parts may contain none. Generally, alkaloids as bases are not soluble or sparingly soluble in water but are soluble in acidic solutions. Different alkaloids may also be found in different parts of the same plant. Alkaloids are widespread in plants and include some very well-known poisons, notably (coniine and strychnine), hallucinogens (morphine, cocaine and muscimol) and other potentially lethal compounds that are nevertheless used in medical practice (atropine, codeine, colchicine and morphine) (**BRUNETON, 1995; POLYA, 2003**). Atropine is a CNS stimulant and is used as a treatment for nerve gas poisoning. Scopolamine, another member of this class is used as a treatment for motion sickness. Alkaloid from *Erythroxylum coca* (cocaine) is also a central nervous system (CNS) stimulant, and has been used as a topical anaesthetic in ophthalmology. A similar compound is also contained in *Datura stramonium* (**POLYA, 2003**). The morphine alkaloids derived from the opium poppy, *Papaver somniferum* are powerful pain relievers and narcotics. Other useful alkaloids include agents to treat fibrillation (quinidine), antibacterial (berberine), and amebicide (emetine) (**BRUNETON, 1995**).

7.1.1.2 Phenols

The term phenolic acid applies to all compounds with at least one carboxyl group and one phenolic hydroxyl group. Plant phenolics represent a very large group of defensive compounds defined as having a phenol moiety. The phenolics range in complexity from simple phenolics and quinines, through chalcones and stilbenes to a range of phenolics with three rings namely anthocyanins, anthochlors, benzofurans, chromones, chromenes, coumarins, flavonoids, isoflavonoids, neoflavonoids, stilbenoids and xanthones. More complex polycyclic phenolics

exist, notably the hydrolysable tannins and condensed tannins. Notable simple phenol-related odorants include 4-methoxybensaldelhyde, guaiacol, 4-hydroxybenzaldehyde, phenethyl alcohol, piperonal and vanilla. Some simple phenolics inhibit cyclooxygenate (COX) enzymes and 5-lipoxygenase (5-LOX), and have antibacterial and antifungal properties, particularly against phytopathogenic organisms (**BRUNETON, 1995; POLYA, 2003**). Some known phenolic-containing plants include *Cynara scolymus*, *Rosmarinus officinalis*, *Orthosiphon stamineus*, *Filipendula ulmaria*, *Salix alba*, *Solidago virga-aurea*, *Myroxylon balsamum* and *Lithospermum officinale* (**BRUNETON, 1995**).

7.1.1.3 Tannins

Historically, the importance of tannin-containing drugs is linked to their tanning properties, which is their ability to transform fresh hides into imperishable leathers. In higher plants, hydrolysable and condensed tannins are the two groups of tannins that are generally distinguished based on their structure, as well as their biogenetic origin. Hydrolysable tannins are esters of a sugar and of a variable number of phenolic acid molecules, while condensed tannins or proanthocyanidins are polymeric flavans. Tannins dissolve in water to form colloidal solutions, but their solubility varies with their degree of polymerization. Most of the biological properties of tannins are linked to their ability to form complexes with macromolecules, particularly with protein. Externally, they waterproof the external layers of the skin and the mucosas, thus protecting the underlying layers. They are also known to have anti-diarrhoeal properties. They also have vasoconstrictor effects on small superficial vessels, and enhance tissue regeneration in case of superficial wounds or burns by limiting fluid losses and preventing external aggressions. Other tannin-containing plants include *Quercus infectoria*, *Krameria lappacea*, *Lythrum salicaria*, *Acacia catechu*, *Crataegus monogyna* and *Cupressus sempervirens* (**BRUNETON, 1995**).

7.1.1.4 Saponins

Saponins are a large form of glycosides present in plants. Saponins can be classified into two groups based on the nature of their aglycone. The first group consists of the steroidal saponins, which are almost exclusively present in the monocotyledonous angiosperms. Triterpenoid saponins consist of the second group which are the most common and occur mainly in the dicotyledonous angiosperms. Some authors distinguish a third category of saponins, namely the steroidal amines which are treated by others as steroidal alkaloids (**BRUNETON, 1995**). Saponins are water soluble, have detergent properties, and can be haemolytic through solubilising the cell membrane of red blood cells. Many saponin-containing drugs are traditionally used for their antitussive and expectorant properties. Several drugs owe their anti-inflammatory and anti-edema activities to saponins (**BRUNETON, 1995; LI *et al.*, 2002; IWALEWA *et al*., 2007**). Triterpenoid saponins from *Chenopodium quinoa* have been reported to have antifungal activity (**WOLDEMICHAEL and WINK, 2001**). Saponins from Ivy wood have also been reported to have antibacterial and anti-amoebic properties. They also have toxic effect on molluscs (**BRUNETON, 1995; KILLEEN, *et al*., 1998; KINJO *et al.*, 2000**). Some known saponin-containing plants include *Dioscorea* species, *Agave sisalana*, *Glycyrrhiza glabra*, *Ruscus aculeatus* and *Ranunculus ficarial* (**BRUNETON, 1995**).

7.2 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a rapid method of drug analysis that can be used to efficiently demonstrate the characteristic constituents of a drug or raw plant extract. The technique clearly illustrates differences in chemical composition of plant extracts. TLC also enables the quantitative and qualitative detection of known natural products, and the associated metabolites or breakdown products. The use of photographic plates with a suitable solvent combination can be used to develop catalogue spectra of different compounds. The use of stain reagents can facilitate secondary metabolites identification (**WAGNER and BLADT, 1996; VAN MIDDLESWORTH and CANNELL, 1998**).

7.3 Materials and Methods

7.3.1 Sample Preparation

Evaluated medicinal plants were oven-dried at 50 ºC, ground into powders and stored in airtight containers at room temperature in the dark until they were required.

7.3.2 Extraction of Plant Material

Ground plant materials (5 g) were sequentially extracted with 100 ml of petroleum ether (PE), dichloromethane (DCM) and 70% ethanol (EtOH) in a sonication bath for 1 h (Julabo GMBH, West Germany) at room temperature with the temperature being cooled by adding ice. All the extracts were filtered under vacuum through Whatman No.1 filter papers in Büchner funnels and concentrated using Büchner rotary evaporator *in vacuo* at 30 ºC. The resultant extracts from the rotary evaporator were transferred to weighed pill vials and completely dried under a stream of air. Dried extracts were kept in the dark at 10 ºC for 72 h.

7.3.3 TLC Fingerprinting

Ten µl of PE, DCM and EtOH plant extracts (50 mg/ml) were loaded on thin layer chromatographic (TLC) plates (5 x 20 cm Silica gel, 60 F_{254}) (Merck, Germany). The plates were developed using hexane:benzene:ethyl acetate (5:2:3 v/v/v) for PE and DCM extracts, while ethyl acetate:methanol:water (100:16.5:13.5 v/v/v) was used for EtOH extracts. After developing the fingerprints over a distance of 7.5 cm, the plates were dried and viewed under ultraviolet (254 and 366 nm) and the photographs of the fluorescent bands were taken to compare the extracts fingerprints. Presence of phenolic compounds in the plant extracts was detected by spraying the plates with 1 N Folin-Ciocalteu reagent (**MUCHUWETI *et al*., 2006**). After 60 min, the spots were visible and the plates were scanned using a high resolution Hp scan jet 7400 scanner.

7.3.4 Phytochemical Analysis

7.3.4.1 Extraction of Phenolic Compounds

Extraction of plant materials was done as described by **MAKKAR** (**1999**). Dried plant samples (2 g) were extracted with 10 ml of 50% aqueous methanol by sonication at room temperature for 20 min. All the extracts were filtered *in vacuo* through Whatman No.1 filter papers in Büchner funnels and transferred into pill vials for analysis.

7.3.4.2 Folin Ciocalteu Assay for Total Phenolics

The Folin Ciocalteu (Folin C.) assay was done as described by **MAKKAR** (**1999**) with slight modification. In triplicate, each extract (50 µl) was transferred into test tube and made up to 1 ml with distilled water (950 µl), followed by the addition of 1 N Folin C. reagent (500 µl) and 2% sodium carbonate (2.5 ml). A similar preparation of a blank control containing aqueous methanol instead of plant extracts, and concentrations of gallic acid (**Appendix**) were made. The test mixtures were incubated for 40 min at room temperature then absorbance read at 725 nm using a UV-spectrophotometer. Total phenolics were expressed as gallic acid (GAE) equivalents. Biostatistical analysis was done using Graphpad prism (Graphpad software incorporation).

7.3.4.3 The Butanol-HCl Assay for Condensed Tannins

In triplicate, 3 ml of butanol-HCl (95:5 v/v) reagent was added to plant extracts (500 µl), followed by 100 µl ferric reagent (**Appendix**). The test solutions were vigorously mixed using a vortex mixer (Chiltern) and heated in a water bath at 100 ºC. After 60 min, absorbance at 550 nm was measured using a UV-spectrophotometer against a blank prepared by mixing tannin-containing extract (500 µl) with butanol reagent (3 ml) and ferric reagent (100 µl), but without heating. Condensed tannins (% per dry matter) as leucocyanidin equivalents were

calculated using the formula: ($A_{550\ nm}$ X 78.26 X dilution factor)／(% dry matter), as described by **PORTER *et al.*** (**1986**). Biostatistical analysis was done using Graphpad prism (Graphpad software incorporation).

7.3.4.4 Rhodanine Assay for Gallotannins

Plant extracts (50 µl) were made up to 1 ml with distilled water in triplicate. To the diluted extracts, 100 µl of 0.4 N sulphuric acid and rhodanine (600 µl) were added. After 5 min, 200 µl potassium hydroxide (0.5 N) (**Appendix**) was added, and distilled water (4 ml) after 2.5 min. Concentrations of gallic acid (**Appendix**) were made. After a further 15 min at room temperature, absorbance at 520 nm was read using a UV-visible spectrophotometer (Varian) against a blank test that contained methanol instead of sample. Gallotannins were expressed as gallic acid (GAE) equivalents (**MAKKAR, 1999**).

7.3.4.5 Vanillin Assay for Flavonoids

Plant extracts (50 µl) were made up to 1 ml with distilled water in test tubes before adding 2.5 ml of methanol-HCl (95:5 v/v) and finally 2.5 ml vanillin reagent (1 g/100 ml distilled water). Similar preparations of blank controls that contained methanol instead of plant extracts, and concentrations of catechin (4 mg/ml) (sigma) were made. The test tubes were left for 20 min at room temperature before absorbance at 500 nm was read using a UV- visible spectrophotometer against the blank. Plant extracts and controls were assayed in triplicate. The flavonoids in the plant extracts were expressed as catechin equivalents (**MAKKAR, 1999**). Biostatistical analysis was done using Graphpad prism (Graphpad software incorporation).

7.3.4.6 Thin Layer Chromatography for Alkaloid Detection

Ten microlitres each of PE, DCM and EtOH plant extracts (50 mg/ml) (prepared as described in Section 7.2.2) were loaded on thin layer chromatographic (TLC) plates (5 x 20 cm Silica gel, 60 F_{254}) (Merck, Germany). The plates were developed using hexane:benzene:ethyl acetate (5:2:3 v/v/v) for PE and DCM extracts, while ethyl acetate:methanol:water (100:16.5:13.5 v/v/v) was used for EtOH extracts. After developing the fingerprints over a distance of 7.5 cm, the plates were dried and viewed under UV light (UV_{254} and UV_{366} nm). The photographs of the fluorescence were taken before being sprayed with modified Dragendorff reagent (**Appendix**) to detect the presence of alkaloids (**ROBERT, 1962; WILFRED and RALPH, 2006**). After 10 min, the spots were visible and the plates were scanned using a high resolution Hp scan jet 7400 scanner.

7.3.4.7 Froth Test for Saponins

Ten ml of distilled water were added to 0.1 g of ground plant sample in test tubes in duplicate. The test tubes were corked and vigorously shaken for 2 min. The appearance of stable foam or froth (3 cm high from the surface of water) for 45 min showed the presence of saponins. Presence of saponins was further tested by taking aqueous extract (2 ml) into a test tube, followed by adding olive oil (10 drops). The test tube was corked and vigorously shaken. The forming of an emulsion confirmed the presence of saponins (**TADHANI and SUBHASH, 2006**).

7.4 Results and Discussion

7.4.1 TLC Fingerprinting

Comparing the distance moved by the plant extract components and different colours on the TLC chromatogram (Figure 7.1), it can be suggested that some extracts contained different compounds. Under UV_{254nm}, most of the bands appeared blue-green inflorescence at different R_f values. However, under UV_{366nm},

colours ranging from deep red to blue fluorescence were observed (Figure 7.2). Phenolic compounds appeared blue-black on the plates after spraying with Folin Ciocalteu reagent (**Appendix**). Phenolic compounds were detected at differing colour intensity on the TLC chromatogram in all of the plant extracts (Figure 7.3). Variation in the intensity of the colour (blue-black) of the phenolic compositions after spraying with Folin C. reagent suggests that the amounts present in some plant extracts varied with plant parts, extracts and species. More phenolic compounds are contained in DCM extracts of most of the plant materials. The pharmacological activities exhibited by some of the plant extracts could be attributed to the presence of phenolic compounds probably in synergism with other compounds.

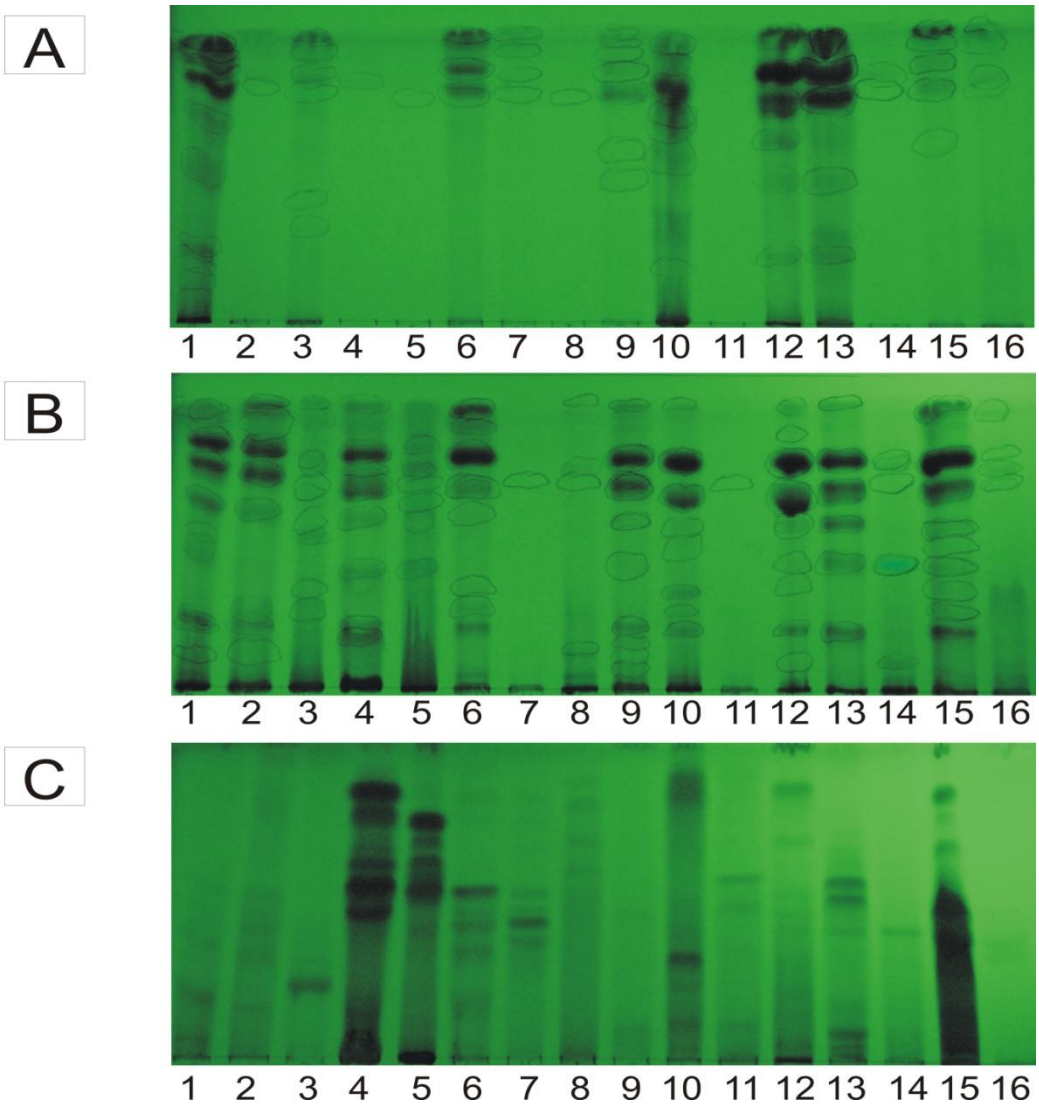


Figure 7.1: TLC Fingerprinting profiles of (A) PE extracts, (B) DCM extracts and (C) EtOH extracts of investigated plant species viewed under UV $_{254\ nm}$.

PE and DCM extracts were developed with hexane:benzene:ethyl acetate (5:2:3 v:v:v), EtOH extracts were developed with ethyl acetate:methanol:water (100:16.5:13.5 v:v:v).

1- *Haworthia limifolia* (leaf), 2- *Cucumis hirsutus* (leaf), 3- *Becium obovatum* (root), 4- *Protea simplex* (leaf), 5- *P. simplex* (bark), 6- *Agapanthus campanulatus* (leaf) 7- *A. campanulatus* (root), 8- *Cyperus textilis* (root), 9- *C. textilis* (leaf), 10- *Vernonia natalensis* (leaf), 11- *Watsonia tabularis* (corm), 12- *Antidesma venosum* (leaf), 13- *Diospyros lycioides* (leaf), 14- *D. lycioides* (stem), 15- *Dissotis princeps* (leaf), 16- *Gladiolus dalenii* (corm).

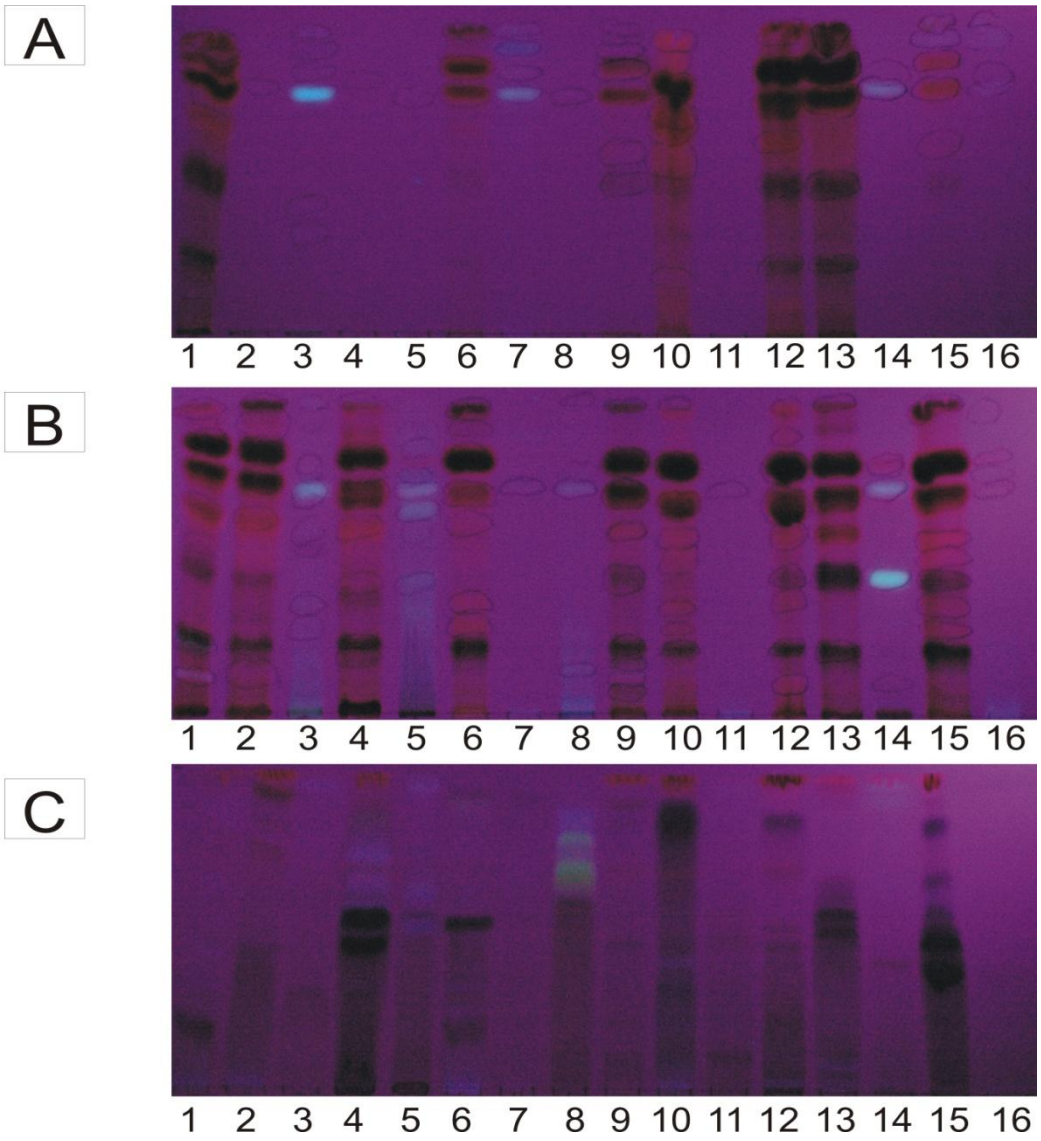


Figure 7.2: TLC Fingerprinting profiles of (A) PE extracts, (B) DCM extracts and (C) EtOH extracts of investigated plant species viewed under UV $_{366\ nm}$.

PE and DCM extracts were developed with hexane:benzene:ethyl acetate (5:2:3 v:v:v), EtOH extracts were developed with ethyl acetate:methanol:water (100:16.5:13.5 v:v:v).

1- *Haworthia limifolia* (leaf), 2- *Cucumis hirsutus* (leaf), 3- *Becium obovatum* (root), 4- *Protea simplex* (leaf), 5- *P. simplex* (bark), 6- *Agapanthus campanulatus* (leaf) 7- *A. campanulatus* (root), 8- *Cyperus textilis* (root), 9- *C. textilis* (leaf), 10- *Vernonia natalensis* (leaf), 11- *Watsonia tabularis* (corm), 12- *Antidesma venosum* (leaf), 13- *Diospyros lycioides* (leaf), 14- *D. lycioides* (stem), 15- *Dissotis princeps* (leaf), 16- *Gladiolus dalenii* (corm).

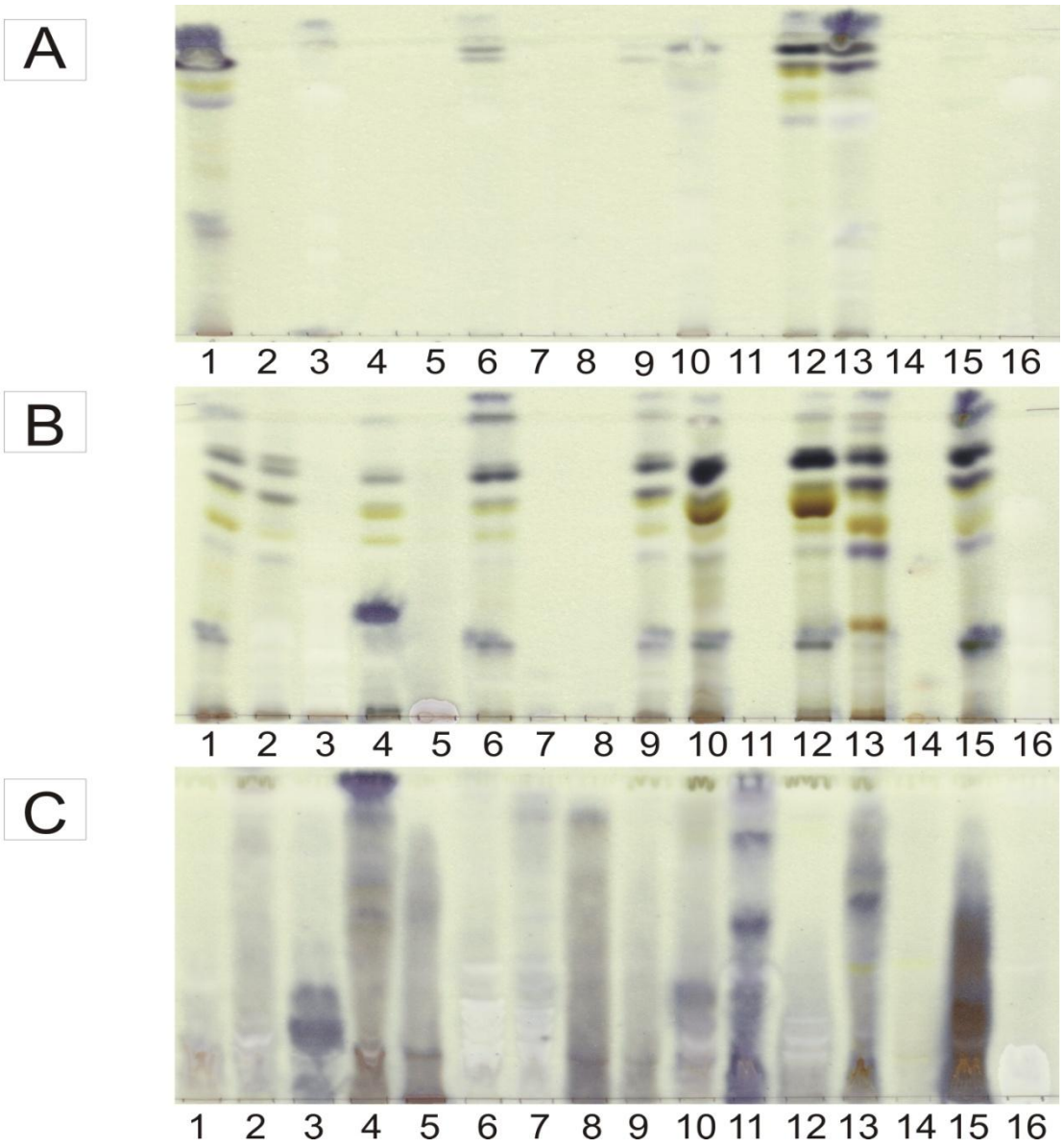


Figure 7.3: TLC Fingerprinting profiles of (A) PE extracts, (B) DCM extracts and (C) EtOH extracts of investigated plant species.

Presence of phenolic compounds (blue-black) was detected by spraying with Folin C reagent.

1- *Haworthia limifolia* (leaf), 2- *Cucumis hirsutus* (leaf), 3- *Becium obovatum* (root), 4- *Protea simplex* (leaf), 5- *P. simplex* (bark), 6- *Agapanthus campanulatus* (leaf) 7- *A. campanulatus* (root), 8- *Cyperus textilis* (root), 9- *C. textilis* (leaf), 10- *Vernonia natalensis* (leaf), 11- *Watsonia tabularis* (corm), 12- *Antidesma venosum* (leaf), 13- *Diospyros lycioides* (leaf), 14- *D. lycioides* (stem), 15- *Dissotis princeps* (leaf), 16- *Gladiolus dalenii* (corm).

7.4.2 Total Phenolic Compositions

The results of the total phenolic contents of the investigated plant species are presented in Figure 7.4. The largest amount of total phenolics is contained in *C. textilis* (leaves) (84.5 mgGAE/g). *B. obovatum* (root), *P. simplex* (leaves and bark), and *C. textilis* (roots) contained total phenolics ≥ 50 mgGAE/g. Phenolic compounds are of important pharmacological values which include antibacterial, antifungal and anti-inflammatory activities. All the plant materials evaluated showed considerable amounts of phenolic compounds that could in one way or another be attributed to the pharmacological activities observed (**FRANKEL *et al*., 1993; HARBORNE, 1994; SHARMA *et al*., 1994; POLYA, 2003**). It is however noted that the plant extracts with high phenolic contents showed moderate to high antibacterial, antifungal and anti-inflammatory activities. This suggests that the quality of the phenolic compounds also plays a major role. Therefore investigation of specific phenolic compounds such as flavonoids, gallotannins and condensed tannins which have been reported to have a range of biological activities was made.

7.4.3 Condensed Tannin Contents

Figure 7.5 showed the amounts of condensed tannins expressed as percentage dry matter. Condensed tannins are measured as leucocyanidins (flavan-3, 4-diol) equivalents which are the products of the breakdown of condensed tannins in the assay (**SHARON *et al*., 2002; CROZIER *et al*., 2006**). Highest amounts (4.13%) of condensed tannins were detected in *C. textilis* (leaves), while low levels were detected in *G. dalenii* (corms). No condensed tannins were detected in *B. obovatum* (roots), *A. campanulatus* (roots) and *V. natalensis* (leaves). Condensed tannins have been reported to accumulate in root and leaf tissues of many plants, probably as a defence mechanism against microbes (**BRUNETON, 1995**). The root and leaf extracts of *C. textilis* showed why the observed pharmacological activity could be attributed to the condensed tannins detected here. Condensed tannins (proanthocyanidin) essentially contained (+) gallocatechin, (–) epicatechin, (+) catechin and epigallocatechin, and their derivatives via carbon-carbon links.

These compounds are primarily responsible for inhibiting the generation of chemiluminescence by activated human polymorphonuclear neutrophil (PMN), indicating that these compounds inhibit the oxidative burst of PMN during inflammation (**POLYA, 2003**).

7.4.4 Gallotannins Contents

The assay is based on the hydrolysis of gallotannins to gallic acid. Gallic acid reacts with the rhodanine to give an intense colour that can be measured using a spectrophotometer. Figure 7.6 presents the amounts of gallotannins detected in the investigated 12 medicinal plants. Gallotannins were detected in all of the investigated plant species with the exception of *A. campanulatus* (roots) and *G. dalenii* (corms). The largest amounts of gallotannins were detected in *P. simplex* (leaves) (13.5 µg/g of dry matter). Relatively high amounts of gallotannins were also detected in *D. lycioides* and *D. princeps* leaves. Gallotannins have been reported to exert various biological effects ranging from antimicrobial to anti-inflammatory and anticancer (**ERDE`LYI *et al*., 2005**). The mechanisms underlying the anti-inflammatory effect of gallotannins include the scavenging of radicals and inhibition of the expression of inflammatory mediators, such as some cytokines, inducible nitric-oxide synthase, and cyclooxygenase-2 (**POLYA, 2003; ERDE`LYI *et al*., 2005**). This could possibly explain the best antibacterial and antifungal, as well as good anti-inflammatory activities exhibited by *P. simplex*. Pharmacological activities of other plant materials could in part be influenced by high amounts of gallotannins detected.

7.4.5 Flavonoids Contents

As shown in Figure 7.7, flavonoids were detected in all the evaluated plant species. The flavonoid content is represented as catechin equivalents. The highest amount (7.4 mg/g) of flavonoids was detected in *C. textilis* (leaves), while the lowest amount was detected in *H. limifolia* (0.24 mg/g). Several flavonoids and biflavonoids are potent inhibitors of lipoxygenase, or cyclooxygenase

(**BRUNETON, 1995; POLYA, 2003**). According to **TALHOUK *et al*.** (**2007**), flavonoids are known to act on the inflammatory response via many routes and block molecules like COX, iNOS, cytokines, nuclear factor-κB and matrix metalloproteinases. Some flavonoids have been reported to be effective against acute inflammation *in vivo* using a carrageenin-induced mouse paw oedema model (**PELZER *et al*., 1998**).

Flavonoid could be major bioactive compounds responsible for the anti-inflammatory properties exhibited by some extracts of *C. textilis* as well as other active extracts. Furthermore, flavonoids derivatives such as epicatechin, quercetin and luteolin are reported to inhibit the development of fluids that result in diarrhoea and abdominal pains. This could explain why these plant species are used in traditional medicine to treat diarrhoeal related ailments (**SCHUIER *et al*., 2005**).

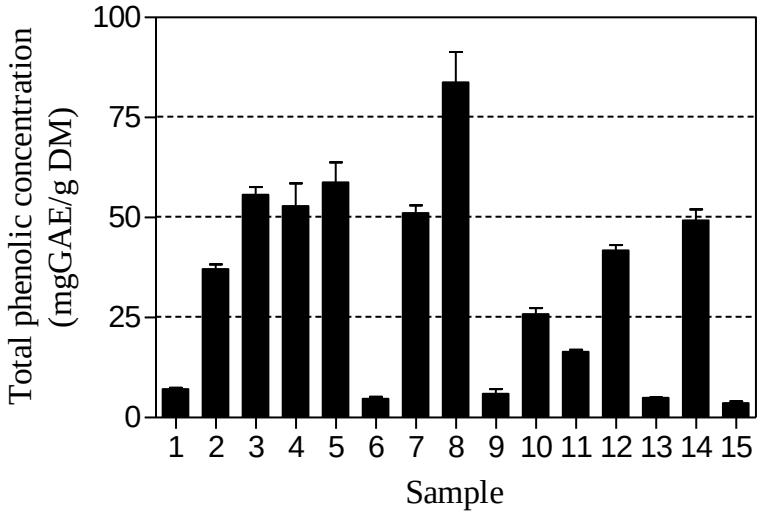


Figure 7.4: Total phenolics content as gallic acid equivalents detected in medicinal plants used for treating gastro-intestinal ailments in traditional medicine.

DM – dry matter; GEA- gallic acid equivalents

1- *Haworthia limifolia* (leaf), 2- *Cucumis hirsutus* (leaf), 3- *Becium obovatum* (root), 4- *Protea simplex* (leaf), 5- *Protea simplex* (bark), 6- *Agapanthus campanulatus* (root), 7- *Cyperus textilis* (root), 8- *Cyperus textilis* (leaf), 9- *Vernonia natalensis* (leaf), 10- *Watsonia tabularis* (corm), 11- *Antidesma venosum* (leaf), 12- *Diospyros lycioides* (leaf), 13- *Diospyros lycioides* (stem), 14- *Dissotis princeps* (leaf), 15- *Gladiolus dalenii* (corm).

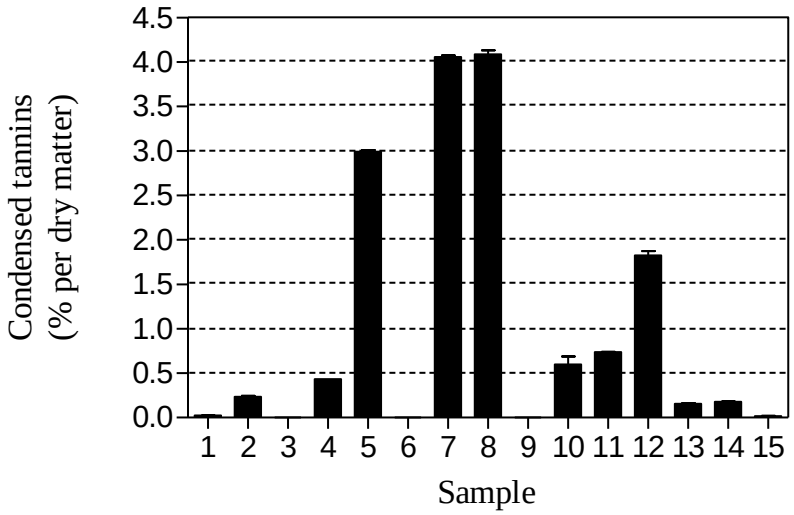


Figure 7.5: Percentage of condensed tannins as leucocyanidins equivalents detected in medicinal plants used for treating gastro-intestinal ailments in traditional medicine.

1- *Haworthia limifolia* (leaf), 2- *Cucumis hirsutus* (leaf), 3- *Becium obovatum* (root), 4- *Protea simplex* (leaf), 5- *Protea simplex* (bark), 6- *Agapanthus campanulatus* (root), 7- *Cyperus textilis* (root), 8- *Cyperus textilis* (leaf), 9- *Vernonia natalensis* (leaf), 10- *Watsonia tabularis* (corm), 11- *Antidesma venosum* (leaf), 12- *Diospyros lycioides* (leaf), 13- *Diospyros lycioides* (stem), 14- *Dissotis princeps* (leaf), 15- *Gladiolus dalenii* (corm).

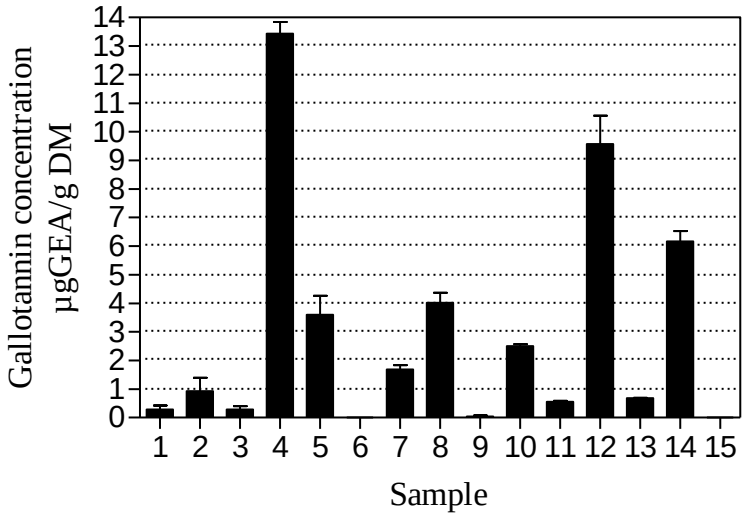


Figure 7.6: Gallotannins concentration as gallic acid equivalents detected in medicinal plants used for treating gastro-intestinal ailments in traditional medicine. DM – dry matter; GEA- gallic acid equivalents

1- *Haworthia limifolia* (leaf), 2- *Cucumis hirsutus* (leaf), 3- *Becium obovatum* (root), 4- *Protea simplex* (leaf), 5- *Protea simplex* (bark), 6- *Agapanthus campanulatus* (root), 7- *Cyperus textilis* (root), 8- *Cyperus textilis* (leaf), 9- *Vernonia natalensis* (leaf), 10- *Watsonia tabularis* (corm), 11- *Antidesma venosum* (leaf), 12- *Diospyros lycioides* (leaf), 13- *Diospyros lycioides* (stem), 14- *Dissotis princeps* (leaf), 15- *Gladiolus dalenii* (corm).

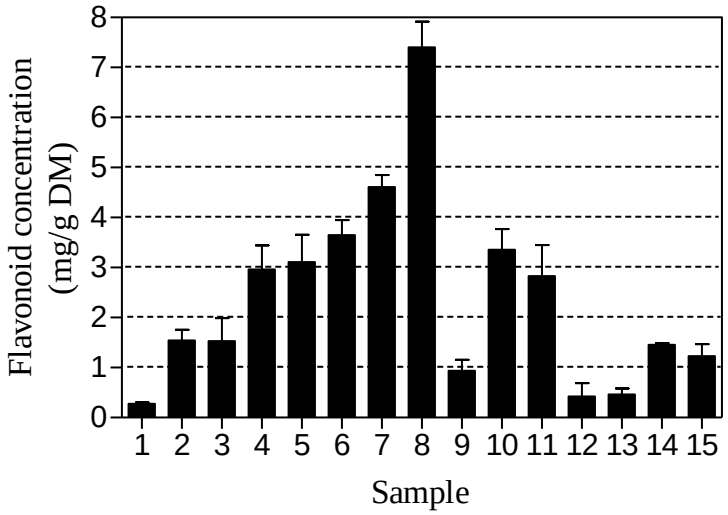


Figure 7.7: Flavonoid concentration as catechin equivalents detected in medicinal plants used for treating gastro-intestinal ailments in traditional medicine. DM – dry matter.

1- *Haworthia limifolia* (leaf), 2- *Cucumis hirsutus* (leaf), 3- *Becium obovatum* (root), 4- *Protea simplex* (leaf), 5- *Protea simplex* (bark), 6- *Agapanthus campanulatus* (root), 7- *Cyperus textilis* (root), 8- *Cyperus textilis* (leaf), 9- *Vernonia natalensis* (leaf), 10- *Watsonia tabularis* (corm), 11- *Antidesma venosum* (leaf), 12- *Diospyros lycioides* (leaf), 13- *Diospyros lycioides* (stem), 14- *Dissotis princeps* (leaf), 15- *Gladiolus dalenii* (corm).

7.4.6 Alkaloid Detection

Alkaloids produce orange-red colouration with Dragendorff reagent on TLC plates (**ROBERT, 1962; WILFRED and RALPH, 2006**). Alkaloids were detected in *H. limifolia* leaf (PE and EtOH), *C. hirsutus* leaf (EtOH), *B. obovatum* root (DCM), *P. simplex* root and bark (EtOH), *A. campanulatus* root (DCM) and leaf (EtOH), *C. textilis* root (DCM), *V. natalensis* leaf (PE), *A. venosum* leaf (PE), *D. lycioides* leaf (PE) and *D. princeps* leaf (DCM) (Figure 7.8). Alkaloids have been reported for *A. venosum* leaves (**WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS *et al*., 1996**). **HUTCHINGS *et al*.** (**1996**) and **NDUKWE *et al*.** (**2004**) have also reported alkaloids for *Vernonia* species and *D. lycioides* in preliminary tests. Alkaloids are

interesting substances because of their multiple pharmacological activities ranging from depressants or stimulants, sympathomimetics, sympatholtics and ganglioplegics. In addition, alkaloids include curare, local anaesthetics (cocaine), agents to treat fibrillation (quinidine), antitumor agents (vinblastine), antimalarials (quinine), antibacterial substances (berberine), and amebicides (emetine) (**BRUNETON, 1995**). Some alkaloids such as Isoquinoline, indole and diterpene alkaloids are known to have good anti-inflammatory activity (**BARBOSA-FILHO *et al*., 2006**). The activity showed by extracts of plant species where alkaloids were detected could well be attributed partly to the alkaloid present.

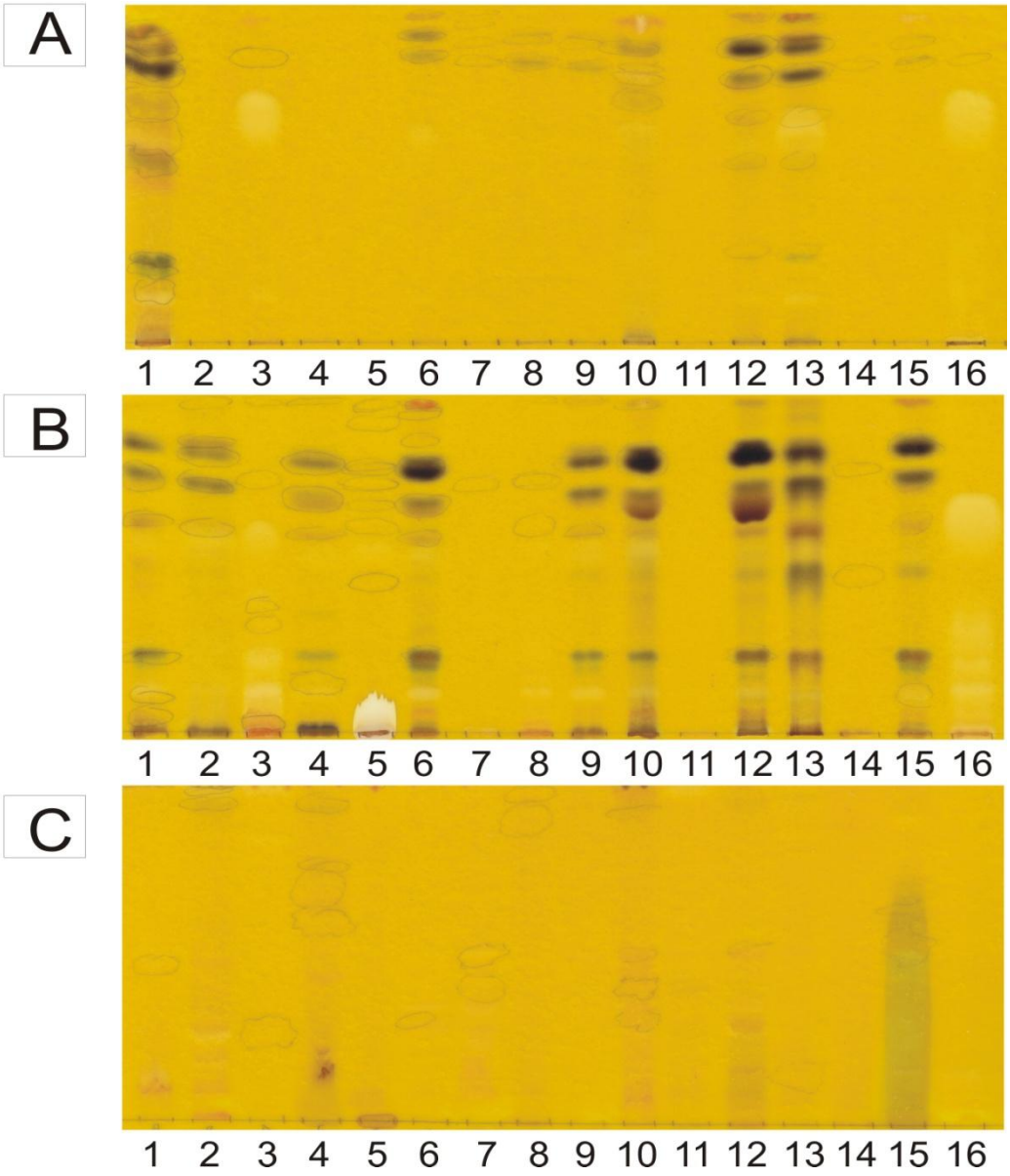


Figure 7.8: TLC Fingerprinting profiles of (A) PE extracts (B) DCM extracts and (C) EtOH extracts of investigated plant species.

Presence of alkaloids (red-orange) was detected by spraying with Dragendorff reagent.

1- *Haworthia limifolia* (leaf), 2- *Cucumis hirsutus* (leaf), 3- *Becium obovatum* (root), 4- *Protea simplex* (leaf), 5- *P. simplex* (bark), 6- *Agapanthus campanulatus* (leaf) 7- *A. campanulatus* (root), 8- *Cyperus textilis* (root), 9- *C. textilis* (leaf), 10- *Vernonia natalensis* (leaf), 11- *Watsonia tabularis* (corm), 12- *Antidesma venosum* (leaf), 13- *Diospyros lycioides* (leaf), 14- *D. lycioides* (stem), 15-*Dissotis princeps* (leaf), 16- *Gladiolus dalenii* (corm).

7.4.7 Saponin Detection

Saponins display a creamy, even foamy texture that distinguishes them from other plant metabolites (**POLYA, 2003**). All of the evaluated plant samples (Table 7.1) with the exception of *H. limifolia, P. simplex*, *A. venosum* and *D. princeps* leaves tested positively for saponins. According to **SPARG *et al*.** (**2004**), many saponins extracted from plant sources produce an inhibition of inflammation in the mouse carrageenan-induced oedema assay and are also known to have antimicrobial, antifungal or antiyeast activities. The anti-inflammatory activities of some saponins derivatives such as triterpenoids saponins have been reported (**SAHU and MAHATO, 1994**). There could be a possible synergism among saponins and other active metabolites in the anti-inflammatory activity exhibited by some investigated plant extracts such as *D. lycioides* leaf and *A. campanulatus* root extracts where interesting anti-inflammatory activities were exhibited.

Table 7.1: Detection of saponins in the investigated medicinal plants used for treating gastro-intestinal ailments in traditional medicine

Plant name	Plant part	Saponins
Agapanthus campanulatus	Roots	+
Antidesma venosum	Leaves	-
Becium obovatum	Roots	+
Cucumis hirsutus	Leaves	+
Cyperus textiles	Roots	+
Cyperus textiles	Leaves	+
Diospyros lycioides	Leaves	+
Diospyros lycioides	Stems	+
Dissotis princeps	Leaves	-
Gladiolus dalenii	Corms	+
Haworthia limifolia	Leaves	-
Protea simplex	Leaves	-
Protea simplex	Bark	+
Vernonia natalensis	Leaves	+
Watsonia tabularis	Corms	+

- = Absence of saponins

+ = Presence of saponins

7.5 Conclusions

The results obtained on the phytochemistry of the plant species provide preliminary information on some important groups of plant principles that are present in the investigated plant species, and also suggest the types of plant bioactive compounds that may be responsible for the biological activities exhibited by some of the plant extracts. The investigated plants can be seen as potential sources of novel and useful drugs. Further studies to isolate and identify the plant principles from the medicinal plants will be considered in the future. It may also be necessary to investigate possible synergism among the isolated bioactive compounds.

CHAPTER 8

GENERAL SUMMARY

Thousands of plant species growing throughout the world have medicinal value, containing active constituents that have a direct action on the human body. In recent years, research to discover promising potential medicinal plants used in various traditional, complementary and alternative systems has attracted a lot of attention globally (**BRUNETON, 1995**). Several South African medicinal plants have been studied for pharmacological and phytochemical activities using modern scientific methodologies and tools in recent years. Research on twelve South African traditional medicinal plants used as antimicrobials presented in this thesis encompassed different areas of study that provide scientific information on the possible reasons why some of the investigated medicinal plants are commonly used in traditional medicine.

Three areas of study were focused on in this thesis. The pharmacology of the medicinal plants, which involved the extraction of dried medicinal plant materials with solvents ranging in polarity, and a broad evaluation of the extracts for antibacterial, antifungal, anti-inflammatory and mutagenic properties. Thin layer chromatographic (TLC) fingerprints of the evaluated plant extracts were developed to compare the compositions of the evaluated plant species. Finally, in the quest to understand the source of the plant pharmacological activities, total phenolic compounds including condensed tannins, gallotannins and flavonoids were quantitatively investigated in terms of their amounts in the aqueous methanol extracts of the medicinal plants.

Agapanthus campanulatus leaves and roots exhibited a range of pharmacological and phytochemical properties. The leaf dichloromethane (DCM) extract exhibited interesting antibacterial activity (0.39 mg/ml) against *Bacillus subtilis*. However, 70% ethanol extracts (EtOH) of both leaves and roots showed good antifungal activity with minimum concentration values of 0.29 and 0.39 mg/ml respectively. At

a concentration of 62.5 µg/ml, the leaf petroleum (PE) extract and the root DCM extract inhibited both cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2) enzymes with a percentage inhibition value greater than 50%. Genotoxicity test showed no positive results in active extracts of both plant parts. Phytochemistry screening revealed that *A. campanulatus* leaves and roots contain phenolic compounds, saponins, alkaloids and flavonoids.

No interesting antifungal and anti-inflammatory activities were observed in *Antidesma venosum*. However, the leaf EtOH extract showed good antibacterial activity (0.65 mg/ml) against *Staphylococcus aureus*. The active extract showed no positive result in the Ames test. Positive tests for condensed tannins, phenolics, alkaloids, flavonoids and gallotannins were detected in the plant species.

A broad spectrum antibacterial activity was exhibited by *Becium obovatum* leaf PE extract against both Gram-negative and Gram-positive bacteria. However, no interesting antibacterial and antifungal activities were observed in the root. Anti-inflammatory screening revealed some degree of COX-1 and -2 enzyme inhibition by the root non-polar extracts at a concentration of 250 µg/ml. No genotoxic properties were observed in the active extracts. The leaves were not evaluated in the phytochemical analysis due to lack of plant material. However, phenolics, saponins, alkaloids, flavonoids and gallotannins were detected in the root.

Cucumis hirsutus leaf PE and DCM extracts showed a broad spectrum antibacterial activity however no antifungal activity was exhibited by any of the plant extracts. Leaf PE and DCM extracts indicated good anti-inflammatory activity against COX-1 and -2 enzymes with inhibitory concentration values greater than 60% at a concentration of 62.5 µg/ml. Bioactive leaf extracts showed no genotoxicity in the Ames test. Phytochemical screening showed the presence of condensed tannins, phenolics, alkaloids, saponins, flavonoids and gallotannins in the leaves.

Pharmacological evaluation of *Cyperus textiles* showed that root PE and DCM as well as leaf and root EtOH extracts inhibited Gram-positive bacteria at minimum inhibitory concentration values less than 1.0 mg/ml. No antifungal and genotoxic

activity was revealed in both the leaf and root extracts. COX-1 selective inhibition was exhibited by the root PE extracts at a concentration of 62.5 µg/ml. The phytochemistry of *C. textiles* showed the presence of alkaloids in the leaves. Saponins and large amounts of condensed tannins, phenolic compounds, flavonoids and gallotannins are present in both plant parts.

Diospyros lycioides stem DCM extract showed good antibacterial activity. Non-polar leaf extracts showed good anti-inflammatory activity against COX-1 and -2 enzymes at a concentration of 62.5 µg/ml. Interestingly, the stem PE extract showed a COX-2 selective inhibitory activity at the same concentration. No genotoxic properties were observed in the active extracts. Phytochemical studies revealed the presence of alkaloids only in the plant leaves. However, condensed tannins, phenolic compounds, flavonoids, gallotannins and saponins are present in both plant parts.

Dissotis princeps leaf EtOH and water extracts exhibited interesting antibacterial activity against Gram-negative and Gram-positive bacteria with a minimum inhibitory concentration of 0.78 mg/ml. The EtOH extracts also showed good antifungal activity with a MIC value of 0.014 mg/ml. COX-1 selective anti-inflammatory activity was exhibited by the DCM extracts. Genotoxicity tests showed no positive result with all the plant extracts. *D. princeps* leaves tested positive to condensed tannins, phenolic compounds, saponins, flavonoids, gallotannins and alkaloids.

The pharmacological analysis of *Gladiolus dalenii* corms showed good antibacterial activity of DCM extract with a MIC value of 0.39 mg/ml. The EtOH extract exhibited antifungal activity with the same MIC value. The highest COX-1 and -2 percentage inhibition was observed with the non-polar extracts of the corms. No genotoxic properties were observed with the active extracts. Phytochemical screening of *G. dalenii* corms revealed the presence of phenolic compounds, flavonoids and saponins.

Haworthia limifolia leaf PE extracts showed broad spectrum antibacterial activity with a minimum inhibitory concentration value less than 1.0 mg/ml. However, no interesting antifungal activity was exhibited by any of the plant extracts. Good

COX-1 and -2 inhibition was shown by leaf non-polar extracts with percentage inhibition greater than 50% at a concentration of 62.5 µg/ml. Genotoxicity screening revealed no cytotoxic effects in the active extracts. The pharmacological activities exhibited by *H. limifolia* could be as a result of condensed tannins, phenolics, flavonoids, gallotannins and alkaloids contained in the leaves.

Protea simplex bark and leaf extracts showed interesting pharmacological activities. The leaf non-polar extracts showed a broad spectrum (and the best) antibacterial activity against Gram-negative and Gram-positive bacteria while the bark non-polar extracts, including the EtOH extract, showed activity against Gram-positive bacteria. Good antifungal activity was exhibited by the leaf PE and DCM extracts with a minimum fungicidal concentration of 0.014 and 0.39 mg/ml respectively. Non-polar leaf extracts showed interesting COX-1 and -2 inhibition at a concentration of 62.5 µg/ml. No anti-inflammatory activity was exhibited by the bark extracts at the same concentration. Active extracts showed no positive tests in the Ames test. Phytochemical analysis revealed that the leaves tested negatively to saponins while the bark tested positively for saponins. Condensed tannins, phenolic compounds, alkaloids, flavonoids and gallotannins are apparently present in both plant parts.

Vernonia natalensis leaf DCM extract inhibited Gram-positive bacteria. No interesting antifungal activity was observed by any of the extracts. Anti-inflammatory screening revealed that non-polar leaf extracts showed good inhibition of COX-1 and -2 enzymes at a concentration of 62.5 µg/ml. No genotoxic properties were observed in the active extracts. Phytochemical screening indicated that alkaloids, flavonoids and gallotannins and phenolic compounds are contained in *V. natalensis* leaves.

Watsonia tabularis corm non-polar extracts inhibited Gram-positive bacterial growth. No interesting antifungal activity was exhibited. Anti-inflammatory activity was observed by non-polar extracts against COX-1 and -2 enzymes. No genotoxic properties were observed in the active extracts. *W. tabularis* corms tested positively to condensed tannins, phenolics, flavonoids, gallotannins and saponins.

Plant extracts were loaded onto thin layer chromatographic (TLC) plates. The plates were developed using hexane:benzene:ethyl acetate (5:2:3 v/v/v) for PE and DCM extracts, while ethyl acetate:methanol:water (100:16.5:13.5 v/v/v) was used for EtOH extracts. After development, the plates were dried and viewed under UV (254,366 nm) and the photographs of the fluorescence were taken. Different coloured bands with varying R_f values were observed in some of the extract fingerprints suggesting that some of the extracts contain different plant compounds.

The results presented in this thesis provide results of the investigation of medicinal plants which can lead to the isolation of pure biologically active components from the evaluated medicinal plants. With regards to the pharmacological and phytochemical studies of 10 plant families, isolation of active and effective antimicrobial plant ingredients from plant species belonging to the family Agapanthaceae, Melastomataceae, Iridaceae and Proteaceae are necessary. This may potentially lead to the preparation of cheap and effective antimicrobial drugs.

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APPENDIX

Ferric Reagent Recipe

Ferric reagent is 2% ferric ammonium sulphate in 2 N HCl

Solution A

Make 16.6 ml of concentrated HCl up to 100 ml with distilled water to make 2 N HCl.

Dissolve 2.0 g ferric ammonium sulphate in Solution A. The reagent should be stored in a dark bottle (**MAKKAR, 1999**).

Gallic Acid Standard Solution

Solution A

100 mg gallic acid in 80 ml of 0.4 N sulphuric acid.

Make Solution A up to 100 ml with 0.4 N sulphuric acids (can be stored for at least a month).

Note: 95 to 98% commercially available sulphuric acid is approximately 36 N (**MAKKAR, 1999**).

Modified Dragendorff Reagent Recipe

Solution A

0.85 g of bismuth subnitrate in glacial acetic acid: distilled water (10 ml: 40 ml).

Solution B

8 g of potassium iodide in 20 ml of distilled water.

Solution C

20 ml of glacial acetic acid and 100 ml of distilled water.

Mix Solution A with Solution B and store in a bottle and keep away from light (wrap with aluminium foil).

Dilute 1 ml of the mixture before use with 2.3 ml of a mixture of Solution C. The reagent is stable for several weeks when protected from light.
Add 1 ml of Solution A with 1 ml of Solution B, mix well and then add 2.3 ml of 20 ml of glacial acetic acid in 100 ml of distilled water (**ROBERT, 1962**).

Preparation of 0.5 N Potassium hydroxide Solution

Dissolve 2.8 g potassium hydroxide in 100 ml distilled water (**MAKKAR, 1999**).

Preparation of 1N Folin Ciocalteu Reagent

Dilute commercially available Folin Ciocalteu (Folin C.) reagent (2 N). Add 5 ml of Folin C. reagent with 5 ml of distilled water. Store away from light in a refrigerator.
Note: The reagent should be golden in colour (Do not use it if it turns olive green) (**MAKKAR, 1999**).