# PHARMACOLOGY AND PHYTOCHEMISTRY OF SOUTH AFRICAN PLANTS USED AS ANTHELMINTICS

**By**

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Submitted in fulfilment of the requirements for the degree of

Master of Science

Research Centre for Plant Growth and Development

School of Biological and Conservation Sciences

University of KwaZulu-Natal

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# FACULTY OF SCIENCE AND AGRICULTURE

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## PHARMACOLOGY AND PHYTOCHEMISTRY OF SOUTH AFRICAN PLANTS USED AS ANTHELMINTICS

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# DECLARATION BY SUPERVISORS

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

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**Thesis Title:** PHARMACOLOGY AND PHYTOCHEMISTRY OF SOUTH AFRICAN PLANTS USED AS ANTHELMINTICS

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

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**SUPERVISOR:** PROFESSOR J. VAN STADEN

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**CO-SUPERVISOR:** DR J.F. FINNIE

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**CO-SUPERVISOR:** DR M.E. LIGHT

# ABSTRACT

Traditional medicine in South Africa is part of the culture of the people and has been in existence for a long-time. Although animal components form part of the ingredients used, plant material constitutes the major component. South Africa is endowed with vast resources of medicinal and aromatic plants which have been employed for treatment against various diseases for decades. A large number of South Africans still depend on traditional medicine for their healthcare needs due to its affordability, accessibility and cultural importance. Helminth infections are among the variety of diseases treated by traditional healers. These infections are regarded as neglected tropical diseases (NTDs) due to their high prevalence among the economically disadvantaged living in rural areas in different regions of the world.

For this study, eleven plants from nine families were selected based on their ethnopharmacological uses as anthelmintics. To validate the efficacy of the plants and for possible discovery of bioactive chemicals, it was deemed important to pharmacologically investigate the selected plants. Besides anthelmintic testing, antimicrobial activity and cyclooxygenase (COX) inhibitory activity screening of the plants were also carried out because microbial infections and inflammation are often associated with helminth infections. The plants investigated were also subjected to phytochemical analysis to test for the presence of different secondary metabolites that could be responsible for the biological activities observed.

The plant materials were collected in KwaZulu-Natal between February and April 2009. Plant parts used in traditional medicine were collected and for sustainable harvesting of medicinal plants in order to enhance conservation, alternative plant parts (leaves and twigs) of some of the plants were also investigated. Independently, the plant material was extracted using petroleum ether (PE), dichloromethane (DCM), ethanol (EtOH) and water. A total of 80 extracts were prepared from the selected plants which were screened for anthelmintic, antimicrobial and COX inhibitory activities. For the phytochemical analysis, 50% aqueous methanol (MeOH) extracts were used to determine the presence of phenolics and alkaloids, while water

extracts were used in the detection of saponins. Acetone extracts were used for the comparison of chemical ‘fingerprints’ on thin layer chromatography (TLC) plates.

For the *in vitro* anthelmintic screening, the free living nematode *Caenorhabditis elegans* var. Bristol (N2), which is morphological similar to parasitic nematodes, was used to evaluate the anthelmintic potential of the extracts. A modified microtitre plate technique, with the use of a viability indicator compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-formazan (MTT-formazan), was used to determine the minimum lethal concentration (MLC) values of the extracts against *C*. *elegans*. The DCM extract of *Hypoxis colchicifolia* leaves showed the best MLC value of 0.13 mg/ml. Besides *H*. *hemerocallidea* extracts, at least one of the extracts from other plant species investigated also exhibited high anthelmintic activity. Generally, ethanol extracts were the most active while water extracts displayed low or no anthelmintic activity in the bioassay.

The test organisms used for the *in vitro* antimicrobial investigation were Gram-positive bacteria (*Bacillus subtilis* American type culture collection (ATCC) 6051 and *Staphylococcus aureus* ATCC 12600), Gram-negative bacteria (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) and the fungus *Candida albicans* ATCC 10231. The microplate dilution technique was used to evaluate the minimum inhibitory concentration (MIC) of the plant extracts against the test organisms. In addition, the minimum fungicidal concentration (MFC) of the extracts was evaluated against the *C*. *albicans* culture. A total of 25 (31.3%) plant extracts exhibited noteworthy antibacterial activity and *S*. *aureus* was the most susceptible bacterium. PE and DCM extracts from the leaves of *Leucosidea sericea* had the best antibacterial activity against *B*. *subtilis* and *S*. *aureus* with an MIC value of 0.025 mg/ml. Although poor antifungal activity was displayed by most plant extracts, the PE extract of *H*. *colchicifolia* leaves exhibited both fungistatic (MIC: 0.78 mg/ml) and fungicidal (MFC: 0.78 mg/ml) activity. Also, the water extract of *Ocimum basilicum* leaves had an MIC value of 0.78 mg/ml.

COX-1 and -2 enzymes were used to determine the anti-inflammatory potentials of the plant extracts. Initially, the organic solvent and water extracts were tested at a final concentration of 250 µg/ml and 2 mg/ml, respectively. Many of the plant extracts

had higher inhibitory activity against COX-1 than the COX-2 enzyme. The DCM extracts of *Acokanthera oppositifolia* leaves and *Felicia erigeroides* stems exhibited the best COX-1 and COX-2 inhibition, respectively. As a result of the fewer side effects associated with COX-2 inhibition, it is preferred to COX-1 inhibition. Of the extracts tested, 32 showed COX-2 inhibition above 70% and were further evaluated at two lower concentrations (organic solvent extracts: 125 and 62.5 µg/ml; water extracts: 1 and 0.5 mg/ml). Both COX-1 and -2 inhibition of these extracts were determined. Apart from the DCM extract of *Senna petersiana* leaves and the EtOH extract of *O*. *basilicum* leaves, that exhibited approximately the same level of inhibition at the three different concentrations tested, the other 30 extracts displayed a dose-dependent COX inhibition.

Phytochemical analysis using spectrophotometric methods revealed the presence of phenolics including flavonoids, condensed and hydrolysable tannins at varying amounts in the plant extracts. Condensed tannins were not detected in the extracts of *F*. *erigeroides* stems and *Cotyledon orbiculata* var. *dactylopsis* leaves. A qualitative test for saponins was positive for the extracts of *A*. *oppositifolia* twigs, *Clerodendrum myricoides* stems, *Cyathea dregei* leaves and roots, *F*. *erigeroides* leaves and stems, *Hypoxis* species corms and *L*. *sericea* stems while alkaloids were only detected in leaf extracts of *A*. *oppositifolia*, *C*. *orbiculata* var*. orbiculata*, *Hypoxis* species and *L*. *sericea*. TLC fingerprinting of acetone extracts allowed for the visualization of the compounds present in the plant extracts. The leaf extracts were shown to contain more compounds than the other plant part extracts, as would be expected.

The screening of the plants for pharmacological activity and phytochemical compositions provided valuable preliminary information validating the use of some of the plants in the traditional medicine. In addition, this study has further confirmed the need to screen medicinal plants for other pharmacological activities related to the diseases for which the plant is traditionally used. Many of the plants investigated have potential to be used as source of new treatments against common infections of humans.

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## PUBLICATIONS FROM THIS THESIS

**AREMU, A.O., NDHLALA, A.R., LIGHT, M.E., FINNIE, J.F. and VAN STADEN, J.** *In vitro* pharmacological evaluation and phenolic composition of ten South African medicinal plants used as anthelmintics (Manuscript in preparation)

**AREMU, A.O., FAWOLE, O.A., CHUKWUJEKWU, J.C., LIGHT, M.E., FINNIE, J.F. and VAN STADEN, J.** *In vitro* pharmacology and phytochemistry of *Leucosidea sericea* (Manuscript in preparation)

**AREMU, A.O., CHUKWUJEKWU, J.C., LIGHT, M.E., FINNIE, J.F. and VAN STADEN, J.** Isolation of anthelmintic and antibacterial bioactive compounds from the leaf extracts of *Leucosidea sericea* (Manuscript in preparation)

## CONFERENCE CONTRIBUTION FROM THIS THESIS

**AREMU, A.O., CHUKWUJEKWU, J.C., LIGHT, M.E., FINNIE, J.F. and VAN STADEN, J.** Preliminary studies on the *in vitro* pharmacology of *Leucosidea sericea*

Oral presentation:

36th Annual Conference of the South African Association of Botanists (SAAB)

North-West University, Potchefstroom (11-14 January, 2010)

# FACULTY OF SCIENCE AND AGRICULTURE
# DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

**Publication 1**

**AREMU, A.O., NDHLALA[a], A.R., FAWOLE[b], O.A., LIGHT, M.E., FINNIE, J.F. and VAN STADEN, J.** *In vitro* pharmacological evaluation and phenolic composition of ten South African medicinal plants used as anthelmintics (Manuscript in preparation)

**Publication 2**

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**Publication 3**

**AREMU, A.O., CHUKWUJEKWU[c], J.C., LIGHT, M.E., FINNIE, J.F. and VAN STADEN. J.** Isolation of anthelmintic and antibacterial bioactive compounds from the leaf extracts of *Leucosidea sericea* (Manuscript in preparation)

[a]Assisted with experiments on the phenolics investigation of the plant extracts

[b]Assisted in cyclooxygenase-1 and -2 inhibition bioassays

[c]Assisted in Thin layer chromatography (TLC) chemical profiling of the plant extracts and isolation techniques

Signed:_____

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# LIST OF ABBREVIATIONS

ADT..... Adult development test
AIDS..... Acquired immunodeficiency syndrome
ATCC..... American type culture collection
CFU..... Colony forming units
COX..... Cyclooxygenase
CTE..... Catechin equivalents
DALYs..... Disability-adjusted life years
DCM..... Dichloromethane
DMSO..... Dimethyl sulphoxide
DPM..... Disintegrations per minute
EHT..... Egg hatch test
EtOH..... Ethanol
GAE..... Gallic acid equivalent
GR..... Glucocorticoid receptor
HIV..... Human acquired immune deficiency
IDA..... Iron-deficiency anaemia
IL..... Interleukin
INT..... *p*-iodonitrotetrazolium chloride
L3..... Larval (third) stage
LAMA..... Larval arrested morphology assay
LCE..... Leucocyanidin equivalents
LK..... Leukotriene
LMA..... Larval motility assay
LOX..... Lipoxygenase
MeOH..... Methanol
MFC..... Minimum fungicidal concentration
MH..... Mueller-Hinton
MIC..... Minimum inhibitory concentration
MLC..... Minimum lethal concentration
MTT..... 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NG..... Nematode growth (NG agar)

NO..... Nitric oxide
NSAIDs..... Non-steroidal anti-inflammatory drugs
NTDs..... Neglected tropical diseases
OD..... Optical density
PE..... Petroleum ether
PG..... Prostaglandin
$PGE_2$..... Prostaglandin $E_2$
$PGF_{2\alpha}$…….. Prostaglandin $F_{2\alpha}$
$PGG_2$..... Prostaglandin $G_2$
$PGH_2$..... Prostaglandin $H_2$
PGHS..... Prostaglandin endoperoxide synthase
$PLA_2$..... Phospholipase $A_2$
STH..... Soil-transmitted helminth
TLC..... Thin layer chromatography
TNF..... Tumor necrosis factor
TRIS..... 2-amino-2(hydroxymethyl)-1,3-propanediol (buffer)
UK..... United Kingdom
UKZN..... University of KwaZulu-Natal
US$..... United States Dollars
USA..... United States of America
UV..... Ultraviolet
WHO..... World Health Organisation
YM..... Yeast malt

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1 Plant medicinal value

The relationship existing between plants and humans is as old as mankind, dating back to the origin of human civilization **(RATES, 2001)**. Humans have relied on plants for food, clothing, shelter, fuel and medicine **(NEWMAN *et al.*, 2000)**. Plants continue to be an integral part of human existence, making ethnobotany an interesting and important research field **(SUMNER, 2000; OKUJAGU *et al.*, 2006)**. **GURIB-FAKIM (2006)** has identified plants as forming the basis of a sophisticated traditional medicine from antiquity to date. Most medicinal plants were used against ailments such as coughs, colds, parasitic infections and inflammation; probably based on trial and error. This ancient Knowledge was verbally transferred from one generation to another **(GURIB-FAKIM, 2006; OKIGBO *et al.*, 2008)**. Therapeutic plant use can be a herbal tea, a crude extract, a phytopharmaceutical or herbal mixture or isolated compounds **(RATES, 2001)**.

Globally, many pharmacological and economically noteworthy medicinal plant species have had multiple uses **(AJIBESIN *et al.*, 2008)**. The variation in African traditional medicine is due to rich biological and cultural diversity on the continent **(GURIB-FAKIM, 2006)**. Medicinal plants are found and frequently used in China, India, Japan, Pakistan, Thailand and particularly to this study, in South Africa **(MUKHTAR *et al.*, 2008)**. Globally, the Indian Ayurvedic and Chinese traditional medicine are recognized as the oldest and most developed respectively **(GURIB-FAKIM, 2006)**. Traditional medicine also exists in southern America and Australia, but these are not as developed as in Asia or Africa. Distinct traditional medicinal systems are found universally in each geographical region, so that medicinal plant use is diverse globally **(AJIBESIN *et al.*, 2008)**.

## 1.2 Traditional medicine in South Africa

The World Health Organization (WHO) defined traditional medicine as "*the health practices, approaches, knowledge and beliefs incorporating plant, animal and*

*mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well being*" **(WHO, 2008)**. Plant and animal-based, traditional medicine provide holistic treatments as opposed to the chemical-based, scientific western medicinal treatment providing cause and effect units. In a holistic approach, factors such as individual mental, social, spiritual, physical, and ecological states are considered in addressing the symptoms, causes and cures for an ailment **(CUNNINGHAM, 1993; BODEKER, 2004)**. **CUNNINGHAM (1997)** studied different African ethnobotanical systems and established that plants are the main ingredients used in most traditional medicines.

Traditional medicine has been in existence for a long-time and forms an integral part of the culture of many South Africans **(VAN STADEN, 2008)**. **VAN WYK *et al.* (1997)** highlighted approximately 3000 plant species that are used for medicinal purpose by an estimated 200 000 indigenous traditional South African healers. The country is regarded as having one of the most diverse temperate floras in the world due to its abundant and rich floral biodiversity, with an estimated 24 000 plant taxa **(LOW and REBELO, 1996; VAN STADEN, 2008)**. South Africa, like most developing countries, has traditional medicine as an informal alternative to the rural healthcare system due to shortage of Western-trained medical doctors, inadequate healthcare facilities and medical equipment in most rural areas **(VAN WYK *et al.*, 1997; LIGHT *et al.*, 2005)**. In the urban areas, a large proportion of the black population patronise traditional medicinal healers in addition to the western-trained doctors **(MANDER *et al.*, 2007)**.

An estimated 27 million South Africans depend on traditional medicine because of its presumed affordability, availability and cultural importance **(MANDER, 1998)**. Research findings by **MANDER (1998)** revealed a diverse range in users of medicinal plants, indicating that educational level, occupation, age, and religion were not determining factors for medicinal plants use. This observed diversity in medicinal plant users was also found in other African countries such as Ethiopia **(MANDER *et al.*, 2007)**. The increase in patronage of traditional medicine by South Africans has been linked to the escalating HIV/AIDS epidemic and other common infectious diseases **(MANDER, 1997)**. Unfortunately, South Africa, like most African countries,

is gradually losing this rich traditional knowledge due to lack of proper documentation of medicinal knowledge **(LIGHT *et al.*, 2005)**.

South Africa has experienced an increase in ethnopharmacology research due to increased government financial support and researchers' recognition of the economic value of available botanicals **(LIGHT *et al.*, 2005; VAN VUUREN, 2008)**. Many workers have reported ethnobotanical usage of plants as medicines in various provinces such as KwaZulu-Natal **(HUTCHINGS *et al.*, 1996)**, Western Cape **(THRING and WEITZ, 2006)**, and Eastern Cape **(BHAT and JACOBS, 1995)** Provinces. This has resulted in the investigation of many South African plants for bioactivities such as antibacterial **(KELMANSON *et al.*, 2000)**, antifungal **(MASOKO *et al.*, 2007)**, anti-inflammatory **(IWALEWA *et al.*, 2007)**, and anthelmintic **(McGAW *et al.*, 2000)** activities. These works were aimed at validating and isolating of bioactive compounds from the investigated plants. Interestingly, most of the screened plants were found to be effective, validating traditional usage as medicines by many South Africans. Also, a number of bioactive compounds have been isolated from South African plants **(VAN VUUREN, 2008)**.

Some popular and widely used South African medicinal plant species include *Agathosma betulina* (Buchu), *Aloe ferox* (Cape aloe), *Harpagophytum procumbens* (Devil's claw), *Siphonochilus aethiopicus* (African wild ginger) and *Hypoxis hemerocallidea* (African potato) **(VAN WYK *et al.*, 1997)**. Presently, most herbal preparations containing some of these species are well packaged and available in most traditional health shops in the country.

The South African government has proposed the integration of traditional medicine into the national healthcare system but a number of problems that might hinder the plan have been identified **(GQALENI *et al.*, 2007 )**. For instance, **JÄGER (2005)** raised concerns on the low number of research projects dealing with diseases affecting the majority of the population who are the main users of traditional medicine. Some of these diseases include diarrhoea, malaria, schistosomiasis, leishmaniasis and helminthiasis. Furthermore, the lack of regulation in the prescription, usages of traditional medicines and the need for proper toxicological tests to determine its safety has been recommended **(FENNELL *et al.*, 2004)**.

In view of this**, BODEKER (2004)** suggested the need for national governments to encourage more research into traditional medicinal plants and emphasized the importance of developing good policies, regulations and trade standards due to the increase in number of people that currently depend on traditional medicine as the sole source of their primary healthcare.

### 1.3 Drug discovery from medicinal plants

**RATES (2001)** defined a drug as "*a pharmacologically active compound, which is a component of a medicine, irrespective of its natural, biotechnological or synthetic origin*". It is used in the treatment, cure, prevention or diagnosis of diseases. According to **WHO (1998)**, "*a medicine is a product prepared according to legal and technical procedures, which is used for the diagnosis, prevention and treatment of disease and has been scientifically characterized in terms of its efficacy, safety and quality*". Many methods have been used in obtaining compounds for drug production, these include isolation from plants and other natural sources like microbes, synthetic chemistry, combinatorial chemistry and molecular modelling **(BALUNAS and KINGHORN, 2005)**. There are challenges associated with each method, necessitating the need for one method to complement the other **(SHU, 1998)**.

Natural product sources such as plants and animals have been utilized as drugs for centuries, with a deep attachment to the socio-cultural life of early humans **(RATES, 2001)**. The isolation of morphine from opium (*Papaver somniferum*) by the German pharmacist Friedrich Sertürner in 1805 marked the beginning of isolation of pure compounds from plants **(KINGHORN, 2001)**. This development led to the isolation of drugs such as cocaine (*Erythroxylum coca)*, codeine (*Papaver somniferum*), digitoxin (*Digitalis* species) and quinine (*Cinochona* species), with the majority still in use **(NEWMAN *et al.*, 2000; BUTLER, 2004)**. The importance of plants as one of the natural sources of drugs cannot be over-emphasized as about 25% of the drugs prescribed worldwide come from plants **(RATES, 2001)**. **NEWMAN *et al*. (2003)** researched 55 categorized human diseases (such as cancer, microbial and parasitic infections) and found that 87% of the medications in use were derived from natural products from plants.

There are many challenges encountered in the drug discovery process, some of these include the high cost involved, which has been estimated to be over US$ 800 million, the long period of time required for the process (minimum of 10 years), and the low average yield of isolated compounds from natural products which are mostly insufficient for lead optimization, development and clinical trials **(REICHERT, 2003; DICKSON and GAGNON, 2004; BALUNAS and KINGHORN, 2005)**. This process of drug discovery is also characterized by a very low chance of success as it has been estimated that only one in 5000 lead compounds will successfully advance through clinical trials for final approval as a drug **(BALUNAS and KINGHORN, 2005)**.

Despite the challenges and obstacles encountered in drug discovery from plants, a number of clinically useful isolated bioactive compounds from natural products are currently in use and many are also being investigated **(NEWMAN *et al.*, 2003; BALUNAS and KINGHORN, 2005)**. For this reason, natural products isolated from plants have been predicted to continue to remain an essential part of the search for novel medicines against human diseases **(NEWMAN *et al.*, 2000; BALUNAS and KINGHORN, 2005)**. To achieve significant success, **BUTLER (2004)** emphasized the need for all concerned scientists to develop faster and better techniques for plant collection, crude extract preparation, bioassay screening, compound(s) isolation and development to keep pace with other drug discovery efforts. There is a bright future for the discovery and development of more drugs from plants due to the large number of plant species that have not been pharmacologically and phytochemically investigated **(SUMNER, 2000; GURIB-FAKIM, 2006)**. The use of medicinal plants will continue to play an important role as an effective health aid, especially for the large number of poor people in the third world and developing countries.

### 1.4 Economic benefits of medicinal plants

According to **WHO (2008)**, 80% of the population in some Asian and African countries depend on traditional medicine for primary healthcare. In the last two decades, the developed and developing countries have witnessed a significant rise in the demand for herbal medicines with global sales estimated at US$ 60 billion **(HOAREAU and DaSILVA, 1999; WHO, 2003)**. Herbal treatment has become

lucrative in the international market generating US$ 14 billion for China in 2005 and US$ 160 million for Brazil in 2007. In addition, countries like Germany, Japan and Turkey have contributed significantly to the economic boom in the phytomedical market **(LANGE, 1998; LAIRD *et al.*, 2003; COSKUN and GENÇLER ÖZKAN, 2005)**.

In southern Africa, an estimated 700 000 tonnes of plant material valued at US$ 150 million is consumed annually **(WIERSUM *et al.*, 2006)**. In South Africa, an estimated 20 000 tonnes of plant material derived from around 700 different plant species, and valued at US$ 60 million (R 270 million) is traded annually **(MANDER, 1998)**. **MANDER *et al*. (2007)** reported an estimated 133 000 income earning opportunities generated by the traditional medicinal plant trade sector which was comparable to 5.6% of South Africa National Health budget. Rural people, especially women, are the major players involved in the harvesting, trading and serving as the traditional healers in this informal sector of the economy of South Africa **(MANDER, 1998; WIERSUM *et al.*, 2006)**.

In KwaZulu-Natal, which is well known for its rich indigenous traditional knowledge system, the trade in ethnomedicinal plants was estimated to be worth US$ 10 million (R 60 million) and between 20 000 to 30 000 people derive an income from the trade of medicinal plant materials annually **(MANDER, 1998)**. The actual economic value of the medicinal plant trade may be higher than estimated by the different researchers because the market is informal and the volume of sales are not easily quantified due to lack of records and the often illicit nature of the trade **(BOTHA *et al.*, 2004)**.

Apart from KwaZulu-Natal, with probably the highest volume of trade, the trade of medicinal plants seems to cut across most of the provinces in South Africa as evidenced from various works in other provinces such as the Eastern Cape **(DOLD and COCKS, 2002)**, Mpumalanga **(MANDER *et al.*, 2007; GQALENI *et al.*, 2007)**, and Gauteng **(BOTHA *et al.*, 2004)**. These studies confirm the increases in the trade of medicinal plants locally, regionally and internationally **(MARSHALL, 1998)**.

## 1.5 Conservation of medicinal plants

**OKIGBO *et al*. (2008)** summarized conservation as "*a process involved in the preservation and careful management of the environment as well as natural resources to prevent neglect, over-exploitation and destruction*". Most natural resources especially medicinal plants, are under strain due to increasing urbanization and increased demand by the population **(ZSCHOCKE *et al.*, 2000; OKIGBO *et al.*, 2008)**. Globally, the demand for medicinal plants is increasing leading to over-exploitation and indiscriminate harvesting of the natural flora within all ecosystems **(CUNNINGHAM, 1997)**. **JÄGER and VAN STADEN (2000)** identified the increased use of the non-renewable plant materials such as roots, bulbs and bark as the major contributing factor to the decline of most species in the wild. Furthermore, herbalists believe that the most potent principles are located in the underground plant parts, encouraging non-sustainable harvesting from the wild **(SHALE *et al.*, 1999)**. This problem is more pronounced in Africa considering that of the estimated 650 million hectares of closed forest areas, a loss of 1% is recorded annually **(OKIGBO *et al.*, 2008)**. **MPIANA *et al*. (2007)** emphasized the danger of the possible loss of the rich biodiversity of these ecosystems. Extinction of these plants before being investigated could cause an irreplaceable loss to future generations apart from the loss of benefits derived from such plants presently.

In South Africa, various workers **(VAN WYK *et al.*, 1997; ZSCHOCKE *et al.*, 2000; DOLD and COCKS, 2002)** have highlighted the rise in forest depletion due to the increase in the demand for medicinal plants by a rapidly growing population coupled with the non-sustainable and indiscriminate harvesting of these medicinal plants from the wild. Popular species such as *Warburgia salutaris* and *Siphonochilus aethiopicus* are apparently extinct locally and available only in highly protected areas **(MANDER, 1998)**. There is a correlation between over-exploitation and indiscriminate harvesting by a high number of untrained gatherers. This group consists mostly of unemployed persons who lack experience in plant material collection but are involved in the trade for the immediate economic gains **(MANDER *et al.*, 2007)**.

South Africa has a low success rate in the use of law enforcement for biodiversity conservation **(WIERSUM *et al.*, 2006)**. New approaches such as plant part

substitution as means for sustainable harvesting **(ZSCHOCKE *et al.*, 2000)** and stimulation of medicinal plant cultivation have been recommended **(VAN STADEN, 1999; WIERSUM *et al.*, 2006)**. For example, the non-destructive part of medicinal plants particularly the leaves should be investigated for possible bioactivity. Furthermore, traditional healers should be encouraged to use related plant species where similar biological activity has been demonstrated **(ZSCHOCKE *et al.*, 2000)**. This would reduce pressure on the popular threatened species and neccessiated the investigation of alternative plant parts in this study. Also, community based medicinal plant farming coupled with the provision of subsidy and basic techniques provided by government agencies would enhance conservation of medicinal plants. The domestication of medicinal plants will create new opportunities such as alternative/additional sources of income and help reduce the pressure on the wild natural medicinal plant population **(JÄGER and VAN STADEN, 2000)**.

## 1.6 Helminth infections

Helminths are divided into three groups based on their body segmentations, namely: trematodes (flukes), nematodes (roundworms) and cestodes (tapeworms). Helminths have multi-cellular bodies and complex life cycles involving maturation in a host organism. Required nutrients are derived from the host, causing the parasitic activity of most helminths. Helminth infections are one of the most prevalent diseases in developing and developed countries **(KROGSTAD and ENGLEBERG, 1998)**. Globally, an estimated 2 billion people are infected by intestinal nematodes **(WEN *et al.*, 2008)**. The increase in helminth infection and their growing resistance to most broad spectrum chemotherapeutics is a major problem facing human health **(JAMES and DAVEY, 2009)**. Also infections by gastrointestinal helminth parasites in livestock is one of the most common and economically important diseases of grazing livestock **(PERRY *et al.*, 2002)**. In addition, research on development of new treatment regimes against helminth infections has been relegated to the background by the western governments, researchers and the pharmaceutical industries due to poor economic prospects and the presumed low priority of the diseases that go with it **(GEARY *et al.*, 1999a; GILLES and HOFFMAN, 2002)**.

Helminth infections resulting to diseases such as ascariasis, hookworm infection and schistosomiasis constitute the bulk of the 13 diseases classified as neglected tropical diseases (NTDs) by the WHO **(HOTEZ *et al.*, 2007a)**. These incapacitating diseases have continued to inflict severe disability and often death. It is more pronounced among the impoverished population living in marginalized areas of the world. **HOTEZ *et al*. (2007b)** estimated that the global burden of helminth infections, in terms of disability-adjusted life years (DALYs), is 39 million life years which was comparable to that of tuberculosis (34.7 million DALYs) or malaria (46.5 million DALYs), the two major human infectious diseases associated with an high mortality rate.

In most developing countries, intestinal helminth infections are a major health concern because factors that pre-dispose humans to these infections abound in these areas **(IJAGBONE and OLAGUNJU, 2006)**. Factors that sustain the parasite life cycles and favour the proliferation of the disease vectors include poor sanitation, poverty, unsafe water, malnutrition and ignorance **(BROOKER *et al.*, 2006a)**. As a result, parasitic diseases generally have become indices of low levels of socio-economic status of the countries where the infections are prevalent. Children, especially those at a preschool age (less than five years), have been identified as the most vulnerable group with very high rates of infection **(SINNIAH, 1984; De SILVA *et al.*, 2003)**. Due to the asymptomatic nature of these diseases, they could remain undetected and children born in an endemic region may harbour the worms for the most part of their lives **(WHO, 1987)**.

South Africa has no reported official data on prevalence and intensity of helminth infections in the country as a whole **(SCHUTTE *et al.*, 1995; FINCHAM *et al.*, 1996)**, but various workers have reported a high prevalence of intestinal nematode infection in Mpumalanga **(EVANS *et al.*, 1987)**, the Western Cape **(FINCHAM *et al.*, 1996)** and KwaZulu-Natal **(KVALSVIG *et al.*, 1991; APPLETON *et al.*, 1999; MABASO *et al.*, 2004)**. Of particular interest is KwaZulu-Natal, the third poorest province in South Africa, which has a high mortality and prevalence of HIV/AIDS **(HIRSCHOWITZ and ORKIN, 1997; MASLAN, 2001)**. The transmission and epidemiology problems of helminth infections have remained relatively high over a long period of time **(JINABHAI *et al.*, 2001)**, and children have continued to be the most vulnerable group in KwaZulu-Natal **(TAYLOR *et al.*, 1995; SAATHOFF *et al.*, 2004)**.

The manifestation of most parasitic diseases is not only due to mechanical or chemical tissue damage but also the host responses to the presence of the parasite **(MURRAY *et al.*, 1998)**. Helminths consume nutrients from their host, thereby causing or aggravating malnutrition which results in retarded growth and physical development. Consequently, symptoms like retarded cognitive development, iron-deficiency anaemia, abdominal pains and related health problems are characteristic features of most heavy helminth infections **(CROMPTON and NESHEIM, 2002; KIRWAN *et al.*, 2009)**. Many researchers **(BROOKER *et al.*, 2004; BORKOW and BENTWICH, 2006)** have observed a decline in host immune status as a result of helminth infection thereby increasing the host susceptibility to other pathogens.

## 1.7 Aims and objectives

Helminth infections affect the immune system and enhance development of secondary infections such as microbial diseases. In South Africa, especially the rural areas, the prevalence rate is alarming because factors such as poor sanitation and malnutrition that pre-dispose humans to these infections abound. Also, there is growing resistance to the available chemotherapeutics with frequent cases of re-infection occurring. It is desirable to investigate some of the medicinal plants used in helminth infection treatment for efficacy and possible identification of new therapeutic compound(s). Medicinal plants provide mixtures of many chemical compounds possessing multiple biological activities. For this reason, it was deemed important to screen the medicinal plants studied for other biological activities especially those with symptoms associated with helminth infections. The findings of this work are expected to serve as a preliminary phase in validating investigated plants as anthelmintics, to provide a homecare herbal remedy against helminth infections and to contribute to the expanding ethnopharmacological field in South Africa.

The aims of this research were:

- to evaluate some of the pharmacological activities including anthelmintic, antimicrobial and anti-inflammatory of South African plants used as anthelmintics;
- to investigate some of the medicinal plants for possible plant part substitution to promote conservation; and
- to determine the phytochemical composition of the investigated plants.

# CHAPTER 2: PLANT COLLECTION AND EXTRACT PREPARATION

## 2.1 Introduction

Ethnopharmacology is the scientific study encompassing ethnic groups, their health in relation to their physical habits as well as the methodology in the creation and use of medicines. The main aim of this interdisciplinary field is the biological evaluation of the effectiveness of traditional medicine. An ethnopharmacological approach to drug discovery has been shown to be an effective and faster means of discovering new pharmaceuticals and bioactive compounds from higher plants **(FARNSWORTH, 1994)**. Traditional medicinal plant usage in various regions of the world has been extensively documented by various workers **(WATT and BREYER-BRANDWIJK, 1962; MAGASSOUBA *et al.*, 2007; HUSSAIN *et al.*, 2008a; HEINRICH *et al.*, 2009)**. The literature provides information such as the plant part used, preparation method and dosage that enhances the investigation process of the medicinal plants.

The collection, preservation, extraction and storage of the plant material are important steps in a scientific study. Generally, abiotic and biotic factors such as light, temperature, moisture and microbes affect the chemical composition of plant material. For instance, the plant chemical composition changes under variable conditions when necessary precautions are not taken during collection and preparative stages of plant material harvesting for scientific study **(MAKKAR, 2000)**. For most pharmacological and phytochemical analyses, extraction of dried plant material is preferred to using fresh plant material. The dried plant material is easier to handle and more stable because water content in fresh plant material changes over time causing a wide variation in plant chemical composition and properties **(MAKKAR, 2000)**.

The extraction of plant material using different solvents is based on the biological concepts of permeability of the plant cells. Dried plant material extraction can be achieved using different solvent systems. These include the use of a single solvent

or a series of solvents (such as hexane, ethyl acetate, and methanol) of increasing polarity either sequentially or non-sequentially. The dried plant material is extracted using different solvents to obtain the bioactive compound(s) present in it for pharmacological investigation. It is importance to use a similar extraction method employed by the local people for traditional medicinal plant research **(GURIB-FAKIM, 2006)**. This will enhance the extraction of the same natural bioactive product used by the people and probably help in validation of a medicinal plant.

## 2.2 Materials and methods

The general methods and techniques used for the collection, preservation, preparation and storage of the plant materials are detailed below.

### 2.2.1 Plant collection

A total of 11 plants **(Table 2.1)** that are used traditionally to treat helminth infections were selected from the available literature for investigation **(WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS *et al.*, 1996; VAN WYK *et al.*, 1997; POOLEY, 1998; OKUJAGU *et al.*, 2006)**. Plant material of these species was collected from the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg and Mount Gilboa (29º 15.450' S, 30º 20.289' E) between February and April 2009. Voucher specimens of the plants were deposited in the UKZN Herbarium, Pietermaritzburg.

### 2.2.2 Extract preparation

The plant material was oven dried at 50 ºC for 3-5 days depending on the moisture content, ground into fine powders through a 1 mm ring sieve using an Ultra Centrifugal Mill (ZM 200, Retsch®, Germany) and stored in airtight containers under cool dark conditions at room temperature.

Independently, 1 g of ground plant material was extracted with 10 ml of four different solvents namely; petroleum ether (PE), dichloromethane (DCM), ethanol (EtOH) and water. The ground plant material in the extracting solvent was placed on ice in a

sonication bath (Julabo GMBH, Germany) for 1 h. The extract was filtered under vacuum through Whatman No. 1 filter paper. Organic solvent extracts were concentrated *in vacuo* with a rotary evaporator (Büchi, Germany) at 30 ºC while the water extracts were collected into pre-weighed glass jars and freeze-dried using a freeze-drying unit. The concentrates obtained were transferred to pre-weighed glass pill vials and placed under a stream of cold air for complete dryness. The percentage yield of extract was determined in terms of the mass of the starting ground material. The prepared crude extracts were stored in clean airtight glass pill vials at 10 ºC in the dark until required for analysis.

## 2.3 Results and discussion

The family, plant part(s), and ethnopharmacological uses for each plant species selected for investigation are shown in **Table 2.1**. A total of nine families were selected with at least one species investigated from each family. For Crassulaceae, two varieties of *Cotyledon orbiculata* (var. *dactylosis* and *orbiculata*) which were readily available were selected for investigation.

The plant material was dried to prevent the fluctuations in chemical composition as a result of moisture content. Grinding of dried plant material to powders was to increase the surface area for greater extraction with the extracting solvents. The plant material was stored under dark conditions to prevent enzymatic degradation that could be stimulated by light and to limit microbial growth **(GURIB-FAKIM, 2006)**. The different solvents were used due to the difference in polarity to enhance extraction of all biologically active compounds in the plant material. Although, the solvent mainly used for herbal preparation in traditional medicine is water and a few preparations utilize water-alcohol for rapid results **(ZSCHOCKE and VAN STADEN, 2000; SPARG *et al.*, 2002)**, the solvents (PE, DCM, EtOH) were used for consistency and total extraction of potential bioactive compounds **(McGAW and ELOFF, 2008)**. Ice was added to the sonication bath to prevent breakdown of thermo-labile bioactive compound(s) in the plant materials during extraction.

**Table 2.1:** South African plants used as anthelmintics based on ethnobotanical information from the literature.

| Family | Plant species<br>Voucher number[a]<br>Plant part screened | Ethnopharmacological uses |
|---|---|---|
| Apocynaceae | *Acokanthera oppositifolia* (Lam.) Codd<br>A.AREMU 1NU<br>Leaves, Twigs | Dried leaves or roots and wood are used as anthelmintics **(HUTCHINGS *et al.*, 1996; VAN WYK *et al.*, 1997)**. |
| Asteraceae | *Felicia erigeroides* DC.<br>A.AREMU 3NU<br>Leaves, Stems | Hot leaf infusions are administered as enemas for intestinal parasites and abdominal pains and also as purgatives **(HUTCHINGS *et al.*, 1996; POOLEY, 1998)**. |
| Caesalpiniaceae (Fabaceae) | *Senna petersiana* (Bolle) Lock.<br>A.AREMU 7NU<br>Leaves | *Senna* species are used pharmaceutically in laxative preparations. Root and leaf infusion are used as a purgative to treat constipation, stomach-ache and intestinal worms **(VAN WYK *et al.*, 1997; HUTCHINGS *et al.*, 1996)**. |
| Crassulaceae | *Cotyledon orbiculata* var. *dactylopsis* Tölken<br>A.AREMU 12NU<br>Leaves, Stems | Leaves are eaten as vermifuge and applied as a hot poultice to treat boils, earache and inflammation **(WATT and BREYER-BRANDWIJK, 1962; VAN WYK *et al.*, 1997)**. |
| Crassulaceae | *Cotyledon orbiculata* var. *orbiculata*<br>A.AREMU 5NU<br>Leaves, Stems | Leaves are eaten as vermifuge and applied as a hot poultice to treat boils, earache and inflammation **(WATT and BREYER-BRANDWIJK, 1962; VAN WYK *et al.*, 1997)**. |
| Cyatheacea | *Cyathea dregei* Kunze<br>A.AREMU 2NU<br>Leaves, Roots | Dried roots used as anthelmintic **(HUTCHINGS *et al.*, 1996)**. |

**Table 2.1:** continued...

| Family | Plant species<br>Voucher number[a]<br>Plant part screened | Ethnopharmacological uses |
|---|---|---|
| Hypoxidaceae | *Hypoxis colchicifolia* Bak.<br>A.AREMU 10NU<br>Leaves, Corms | Used as purgatives and ascarifuges in unspecified parts of Africa **(WATT and BREYER-BRANDWIJK, 1962)**. |
| Hypoxidaceae | *Hypoxis hemerocallidea* Fisch. & C.A. Mey.<br>A.AREMU 11NU<br>Leaves, Corms | Plant decoctions have purging effects **(WATT and BREYER-BRANDWIJK, 1962)**. |
| Lamiaceae | *Ocimum basilicum* (L.)<br>A.AREMU 6NU<br>Leaves | Juice of leaves are used to expel worms; leaves are used for respiratory disorders, dysentery, constipation **(OKUJAGU *et al.*, 2006)**. |
| Rosaceae | *Leucosidea serice*a Eckl. & Zeyh.<br>A.AREMU 4NU<br>Leaves, Stems | Used as an astringent and an ingredient with other plants in a vermifuge; ground leaf paste is used for ophthalmia **(WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS *et al.*, 1996)**. |
| Verbenaceae | *Clerodendrum myricoides* (Hochst.) Vatke.<br>A.AREMU 8NU<br>Leaves, Stems | Root and leaf infusions are taken as anthelmintics; they are also used for pains in the chest, colds, bleeding of the gums and indigestion **(HUTCHINGS *et al.*, 1996)**. |

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

### 2.3.1 Ethnopharmacological approach for plant selection

The use of ethnopharmacological information in the selection of plants is based on the therapeutic use by an ethnic group **(SOUZA BRITO, 1996)** and has been established to provide a valuable short cut in discovering plants with good biological activities **(GENTRY, 1993)**. **KHAFAGI and DEWEDAR (2000)** highlighted the advantages and limitations of the different approaches to drug discovery and concluded that an ethnopharmacological approach as opposed to random screening yielded the best results. Recently, most pharmaceutical companies use this indigenous knowledge for identification of bioactive compound(s) that could be use in the synthesis of new drugs. It is believed that valuable medicinal plants are those with a long history of usage in different localities and are also presumed to be safer compared to plants with no ethnomedical records **(FABRICANT and FARNSWORTH, 2001)**.

A different set of metabolites is sometimes produced in different anatomical plant parts such as roots, corms, stems, leaves and flowers. Related plants (same genus) and different parts of the same plant (such as stems, roots, leaves, and twigs) have been reported to occasionally exhibit similar pharmacological properties **(JÄGER *et al.*, 1996; AMOO *et al.*, 2009a)**. In addition, **ZSCHOCKE *et al*. (2000)** emphasized the need to enhance conservation by investigating other plant parts especially leaves and twigs for similar pharmacological activity regardless of the part traditionally used. In this view and for higher chances of discovering of bioactive compound(s) as well as for comparative purpose, different parts and different species within the same genus were selected for investigation.

### 2.3.2 Effect of extracting solvents on percentage yield

The yields of extracts after extraction and the drying are given in **Table 2.2**. Generally; extracting with water resulted in the highest quantity of crude extract, while PE gave the least quantity of crude extract except for the leaves of the two *Cotyledon* species where EtOH had the lowest yield.

**Table 2.2:** Extract yield (% of dry weight) from plant parts investigated.

| Plant species | Plant part extracted | Yield (%) | | | |
|---|---|---|---|---|---|
| | | PE | DCM | EtOH | Water |
| *Acokanthera oppositifolia* | Leaves | 3.6 | 5.4 | 11.8 | 22.7 |
| | Twigs | 5.9 | 7.0 | 10.0 | 20.3 |
| *Clerodendrum myricoides* | Leaves | 3.2 | 3.5 | 4.4 | 30.3 |
| | Stems | 0.4 | 0.6 | 3.6 | 13.8 |
| *Cotyledon orbiculata* var. *dactylopsis* | Leaves | 2.6 | 3.3 | 1.8 | 30.8 |
| | Stems | 0.6 | 1.0 | 0.8 | 17.5 |
| *Cotyledon orbiculata* var. *orbiculata* | Leaves | 1.7 | 2.1 | 1.5 | 33.8 |
| | Stems | 0.5 | 0.7 | 3.0 | 15.6 |
| *Cyathea dregei* | Leaves | 4.0 | 4.4 | 6.4 | 6.8 |
| | Roots | 0.2 | 0.3 | 1.0 | 2.3 |
| *Felicia erigeroides* | Leaves | 1.5 | 4.5 | 6.4 | 20.2 |
| | Stems | 0.3 | 0.6 | 1.2 | 10.2 |
| *Hypoxis colchicifolia* | Leaves | 2.4 | 3.0 | 5.6 | 7.1 |
| | Corms | 0.3 | 0.5 | 10.6 | 24.4 |
| *Hypoxis hemerocallidea* | Leaves | 0.8 | 1.1 | 2.0 | 6.2 |
| | Corms | 0.2 | 0.4 | 33.4 | 35.0 |
| *Leucosidea sericea* | Leaves | 3.4 | 4.4 | 9.0 | 10.0 |
| | Stems | 0.4 | 0.5 | 1.7 | 3.0 |
| *Ocimum basilicum* | Leaves | 3.2 | 11.4 | 7.8 | 12.0 |
| *Senna petersiana* | Leaves | 3.5 | 3.4 | 8.4 | 35.2 |

Extracting solvent: PE = Petroleum ether, DCM = Dichloromethane, and EtOH = Ethanol.

The high polarity of water probably accounted for the high yield because both polar and some less polar compounds were extracted. The lowest yield (0.2%) was obtained in the PE root extract of *C. dregei* and corm extract of *H*. *hemerocallidea* (0.2%). A water extract of *S. petersiana* leaves gave the highest yield (35.2%). Beside PE and DCM extracts of *A. oppositifolia* twigs, the leaf part generally yielded more extracts than the other plant parts. In most plant species, the leaf is known to be more complex and probably contains more compounds than other plant organs **(KATERERE and ELOFF, 2008)**.

## 2.4 Conclusions

An ethnopharmacological approach was used for the selection of the plant species investigated. The plants were selected based on their documented anthelmintic property across Africa and availability in South Africa. As expected, the extract yield increased when using solvents of increasing polarity. The general trend of the yield obtained in ascending order was PE < DCM < EtOH < water. The extraction of active principles from the medicinal plants for pharmacological evaluation was to some extent dependent on the polarity of the solvents used in the extraction.

# CHAPTER 3: ANTHELMINTIC SCREENING

## 3.1 Introduction

Helminth diseases may arise as a result of infection of the body by parasitic worms **(MAIZELS and YAZDANBAKHSH, 2003)**. These infections have been with mankind for centuries and still remain prevalent in most regions of the world **(COX, 2002)**. An estimated 50% of the world's population is affected at some stage of their life, and the prevalence of gastrointestinal nematode infections have remained stable over the last 50 years **(CHAN, 1997)**. There is a higher prevalence in Asia, Latin America and sub-Saharan Africa as these regions have warmer and more humid climatic conditions that favour larval development and survival of most helminth species **(KAPPUS *et al.*, 1994)**. Studies in different regions have shown that factors such as surface temperature and high rainfall are important in the prevalence of soil-transmitted helminth (STH) infections **(APPLETON and GOUWS, 1996; GUNAWARDENA *et al.*, 2005; BROOKER *et al.*, 2006b)**.

The infecting helminth species, degree of infection and host age, determine the clinical symptoms of helminth infection **(WANG *et al.*, 2008)**. A number of factors determine the parasite pathogenicity and include: exposure and entry; adherence and replication; cell and tissue damage; disruption, evasion and inactivation of host defences **(MURRAY *et al.*, 1998)**. These factors distinguish parasite infections from other infections that are caused by fungi, bacteria and viruses. This could be linked to the chronic nature and long duration of most parasitic diseases in humans. Generally, helminth infections are regarded as a global problem of medical, educational and economic significance because of the long term chronic inflammatory disorders and disabling effects when left untreated **(STEPEK *et al.*, 2006; WANG *et al.*, 2008)**.

### 3.1.1 Human helminth infections

Humans are affected by approximately 342 helminth species **(CROMPTON, 1999)**, of which 197 are considered as primary inhabitants of the gastro-intestinal tract **(HORTON, 2003)**. Globally, only an estimated 20 species are known to cause diseases. Nematodes are regarded as the most important parasitic group as a result of their wide spread and global distribution **(HOTEZ *et al.*, 2008)**. Examples of the helminth species of greatest medical interest are *Ascaris lumbricoides*, *Necator americanus*, *Schistosoma* species, *Strongyloides stercoralis* and *Trichuris trichiura* **(HORTON, 2003; BETHONY *et al.*, 2006)**. The disease prevalence, infection patterns and clinical significance of three common types of helminth infections are discussed in the sections below.

#### 3.1.1.1 Ascariasis

Ascariasis is more pronounced in many developing countries in Africa, South America as well as Asia. An estimated 1.5 billion people are infected worldwide **(CROMPTON, 1999)**. Ascariasis (roundworm infection) is the most common helminth infection of humans and is caused by members of the genus *Ascaris*. *A*. *lumbricoides* is the largest intestinal roundworm and can grow to an estimated 20-35 cm in length in the intestine of human **(MURRAY *et al.*, 1998)**.

An individual can be infected through ingestion of food contaminated with faeces containing the *Ascaris* eggs. The eggs hatch in the jejunum, penetrate the mucosa and are carried through the hepatic circulation to the heart and lungs after which they re-enter the stomach via the tracheae and oesophagus before growing to adulthood in the small intestine. Adult worms live in the small intestine where they lay large numbers of eggs that are passed out with the faeces into the soil for further re-infection of new hosts **(PETERS and GILLES, 1995)**. After maturity and fertilization, the female can produce about 200 000 eggs per day for as long as a year. Fertilized eggs become infectious after approximately 2 weeks in the soil **(MURRAY *et al.*, 1998)**.

Infections are usually asymptomatic, especially if the number of worms is small. The most common symptoms are diarrhoea and upper abdominal discomfort of varying degrees **(SHEOREY *et al.*, 2007)**. Infection may, however, be accompanied by inflammation and fever **(MURRAY *et al.*, 1998)**. Large numbers of adult worms can cause mechanical blockage of the intestinal tract, especially in children, and worms penetrating the host's intestinal wall can result in peritonitis **(SHEOREY *et al.*, 2007)**. During movement through the lungs, the larvae may provoke an immune-mediated hypersensitivity response due to metabolic excretions and secretions of adult worms **(CROMPTON, 2001)**. In addition, the worm's migration to other parts of the body can cause morbidity and sometimes death, by compromising nutritional status, affecting cognitive processes, inducing tissue reactions such as granuloma, and provoking intestinal obstruction or rectal prolapse **(MAGUIRE, 2005; SHEOREY *et al.*, 2007)**.

#### 3.1.1.2 Hookworm infection

Hookworm infection remains one of the most common chronic infections of humans with an estimate of 576-740 million individuals affected worldwide **(BETHONY *et al.*, 2006)**. It is predominantly found in areas of rural poverty in China and sub-Saharan Africa **(De SILVA *et al.*, 2003)**. *Necator americanus* and *Ancylostoma duodenale* are the two most common hookworm species known to affect humans **(BROOKER *et al.*, 2004)**.

In the human host, the third larval stage (L3) is the infective stage and it passes through the skin as a result of walking bare-footed in areas contaminated with faecal matter. *N*. *americanus* L3 larvae may invade the buccal epithelium when they enter through the mouth **(BROOKER *et al.*, 2004)**. The larvae are able to penetrate the skin of the foot and once inside the body, they migrate through the vascular system to the lungs and trachea. They pass down the oesophagus into the digestive system and eventually to the intestine where the larvae mature into adult worms. These worms feed by sucking blood from injured intestinal tissues. Egg laying starts 4-6 weeks after infection and may remain viable for as long as 5 years. Adult worms lay as many as 10 000-20 000 eggs per day which are released into the soil with faeces. As a result, the non-infective larvae hatch and within 2 weeks develop into L3 larvae **(MURRAY *et al.*, 1998; HOTEZ *et al.*, 2009)**.

A mild hookworm infection is asymptomatic and a common mild symptom that occurs soon after infection is the “ground-itch”. This is an allergic reaction at the site of parasite penetration and entry, common in patients infected with *N*. *americanus* **(BETHONY *et al.*, 2006)**. However, signs of advanced severe infection include anaemia and protein deficiency that lead to high morbidity associated with hookworm infection **(GILGEN *et al.*, 2001)**. In addition, scientific evidence has established a direct relationship between the number of adult hookworms in the human gut and the amount of intestinal blood loss **(STOLTZFUS *et al.*, 1997)**. Intestinal blood loss leads to severe iron-deficiency anaemia (IDA) reported to account for 20% of maternal deaths during pregnancy globally **(CROMPTON, 2000)**. Furthermore, intestinal sites may become susceptible to bacterial infection when the worms migrate along the intestinal mucosa **(MURRAY *et al.*, 1998)**.

#### 3.1.1.3 Schistosomiasis

Schistosomiasis, otherwise known as bilharziasis, or snail fever is a tropical parasitic disease caused by several types of fluke of the genus *Schistosoma*. The three main species associated with several human diseases are *S*. *mansoni*, *S*. *japonicum* and *S*. *haematobium* **(GRYSEELS *et al.*, 2006)**. In Africa, the two most common species are *S*. *haematobium* transmitted by *Bulinus* snails causing urinary schistosomiasis and *S*. *mansoni* transmitted by *Biomphalaria* snails causing hepatic schistosomiasis **(MURRAY *et al.*, 1998)**. The disease is endemic in 74 countries with an estimated 800 million individuals at risk and 200 million people infected **(MØLGAARD *et al.*, 2001)**. African countries account for 90% of the infections and in terms of the number of people at risk and infected, schistosomiasis ranks second (behind malaria) among the parasitic diseases **(STEINMANN *et al.*, 2006)**.

The parasite infective forms, known as cercariae, are released from the intermediate host (snail) and penetrate a suitable human host’s skin. The cercariae secrete enzymes that break down the skin’s protein to allow penetration of the cercarial head through the skin, leading to the formation of a migratory schistosomula stage. These immature forms of the parasitic schistosome may remain in the skin for 48 h before locating a suitable blood capillary. The schistosomula travel through the lungs, heart, and eventually to the liver, where they mature into male or female adult worm pairs

in about 45 days. *S*. *japonicum* migrates faster than *S*. *mansoni* and usually reaches the liver within 8 days of penetration. Juvenile worms of both species develop an oral sucker after arriving in the liver which the parasites use to feed on the red blood cells. Parasites reach maturity in 6-8 weeks and begin the production of eggs. *S*. *mansoni* produces up to 300 eggs while *S*. *japonicum* may produce up to 3000 eggs per day. Some of the produced eggs are passed out through urine and faeces which later hatch to release miracidia (an intermediate life cycle stage) that undergoes development and multiplication in the snail's digestive gland in 4-6 weeks. Worm pairs can live in the body for an average of four and half years, but may persist up to 20 years. About 50% of the worms become trapped in the mesenteric veins or are washed back and lodged in the liver. The trapped eggs mature normally and secrete antigens that elicit a vigorous immune response resulting in pathology classically associated with schistosomiasis **(MURRAY *et al.*, 1998)**.

Schistosomiasis is a chronic disease and acute pathology may occur a few weeks after initial infection by the parasite **(GRYSEELS *et al.*, 2006)**. Common symptoms associated with the acute state include abdominal pains, genital sores, fever and fatigue **(HOTEZ *et al.*, 2009)**. Sub-clinical symptoms such as mild anaemia and malnutrition are frequently observed among infected youth **(KING *et al.*, 2005)**. A study in northwest Tanzania carried out among pregnant woman revealed an increased risk of anaemia as a result of heavy infection with *S*. *mansoni* **(AJANGA *et al.*, 2006)**.

### 3.1.2 Treatment of helminth infections

The control of helminth infections requires a multi-faceted approach due to the complex life cycle of most causative organisms and environmental influences **(WHITFIELD, 1996)**. Proper health education, good hygiene and improved sanitation, as well as use of anthelmintic chemotherapy such as levamisole, albendazole and pyrantel, are the major control measures against helminth infections **(SAVIOLI *et al.*, 1992; STEPEK *et al.*, 2006)**. The use of broad spectrum chemotherapy is useful for cases of multiple worm infection in humans **(BOOTH *et al.*, 1998)**. However, only three classes of broad spectrum anthelmintics are

available and the chances of discovering novel anthelmintics are very low due to the limited number of anthelmintic drug research projects in academic laboratories and pharmaceutical industries globally **(GEARY *et al.*, 1999a)**.

The latest drug class development dates back to the early 1980s when ivermectin, a member of class 3 anthelmintic drugs, called macrocyclic lactones, was discovered. These drugs act by paralysis of body-wall muscles in nematodes **(BEHNKE *et al.*, 2008)**. Subsequently, other ivermectin analogues such as moxidectin, doramectin and abamectin, which have distinct properties for specific helminth infections were developed **(GEARY, 2005)**. Prior to the discovery of invermectin, two other classes of drugs were used. Levamisole, an example of the class 2 imidazothiazoles, acts by stimulating the nicotinic acetylcholine receptors and result in paralysis of the worms which can then be washed out the intestine by peristalsis **(STEPEK *et al.*, 2006)**. The class 1 drugs, known as the benzimidazoles (albendazole and mebendazole), bind to free *β*-tubulin, inhibiting its polymerization and thereby interfering with the microtubule-dependent glucose uptake by the parasite **(RANG *et al.*, 2003)**.

Despite the efficacy and relatively good safety margins of most available chemotherapies, there is still a growing concern about potential problems of acquired resistance as varying degrees of resistance in nematode populations in human and livestock have been reported globally **(ALBONICO *et al.*, 2003; JABBAR *et al.*, 2006)**. The problem of re-infection after chemotherapy **(KIRWAN *et al.*, 2009)**, adverse drug complications in certain groups of people such as hepatitis patients, pregnant and lactating woman **(SAVIOLI *et al.*, 2003)** as well as the lack of a potent and safe macrofilaricide **(WHITFIELD, 1996)** have necessitated the search for new anthelmintic substances. As the plant kingdom remains poorly explored **(RATES, 2001)**, more effort is now being directed to the discovery of new anthelmintics of plant origin **(ATHANASIADOU *et al.*, 2007)**.

For centuries, indigenous people have utilized plants and their extracts for the treatment of a variety of human and livestock gastro-intestinal parasites **(WALLER *et al.*, 2001)**. Some of the earliest known medicinal anthelmintic plants include *Carica papaya*, *Ficus* species and *Ananas comosus*. Until recently, most medicinal plant anti-parasitic activity was based on anecdotal evidence and there is currently

an increase in the number of scientific studies aiming to verify, validate and quantify, as well as determine the safety of such plant activity **(ATHANASIADOU *et al.*, 2007)**. Consequently, anthelmintic compounds such as santonin and filicic acids have been isolated from *Artemisia maritime* L. and *Dryopterix filix-mas* (Maxim.), respectively **(SETZER and VOGLER, 2006)**.

### 3.1.3 Anthelmintic bioassays

Most helminths belong to a very large group of organisms that are widely diverse in body structure and in parasitic mechanisms **(FENNELL *et al.*, 2004)**. In view of this, important factors such as the type of test organisms, plant extracting methods and seasonal variation in plant chemical composition, must be considered when evaluating the potential of medicinal plant use in parasite control **(ATHANASIADOU *et al.*, 2007)**.

*In vivo* and *in vitro* bioassays, as well as a number of test organisms, have been employed to screen plants for anthelmintic activity. Both test systems and the varieties of organisms employed, have various limitations **(JABBAR *et al.*, 2006)**. Most preliminary screening investigation of plants for anthelmintic activity use *in vitro* bioassays **(SIMPKIN and COLES, 1981)**. Generally, the main advantages of using *in vitro* bioassays over *in vivo* bioassays include the low cost involved and rapid turnover that allows for large-scale screening of plants, as well as the low quantity of extracts, required for most *in vitro* investigations **(WHITFIELD, 1996; GITHIORI *et al.*, 2006)**. However, *in vitro* bioassays should be followed by *in vivo* tests when validating the anthelmintic activity of medicinal plants **(ATHANASIADOU *et al.*, 2007)**. Examples of *in vitro* test systems include the larval arrested morphology assay (LAMA), larval motility assay (LMA), egg hatch test (EHT), colorimetric assays and adult development test (ADT) **(JABBAR *et al.*, 2006; KOPP *et al.*, 2008)**.

Although the *in vitro Caenorhabditis elegans* bioassay has long been employed for anthelmintic screening with limited success in discovery of valuable new leads **(GEARY *et al.*, 1999b)**, it remains the most suitable test organism for preliminary high-through put *in vitro* screening for broad spectrum anthelmintic activity **(GEARY and THOMPSON, 2001)**. In addition, *C. elegans* is a representative of a large

phylum with many parasites **(BÜRGLIN *et al.*, 1998)**, its sensitivity to most commercial anthelmintic drugs as well as the ease of culture growth and maintenance contribute to its widespread use for anthelmintic screenings **(SIMPKIN and COLES, 1981)**.

Many studies using *C*. *elegans* require measuring the survival and/or reproductive potential of the worms after incubating with the test extracts for a specific time. The effectiveness of the tested extracts is often measured based on the worms' viability (reproduction responses) or mobility (behaviour responses) after the incubation period **(McGAW *et al.*, 2000)**. These methods are subjective, often unreliable and time consuming. As a result, a visual, qualitative and quantitative worm viability assessment has been developed **(JAMES and DAVEY, 2007)**. This technique is a colorimetric assay that utilizes metabolic activity as a measure of the viability in the investigated organisms and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-formazan (MTT-formazan) is used as the indicator compound **(JAMES and DAVEY, 2007)**. The assay provides a simple method for viability testing in potential anthelmintic plant extracts against *C*. *elegans* and has the advantage of rapid evaluation.

## 3.2 Materials and methods

For evaluation of the plant material for anthelmintic activity, the techniques employed are detailed below.

### 3.2.1 Extract preparation

Extract preparation was carried out as described in **Section 2.2.2**. The stored dried plant extracts were redissolved at a concentration of 25 mg/ml for use in DMSO for organic solvent extracts (PE, DCM and EtOH) and 50 mg/ml for use in distilled water for aqueous extracts.

### 3.2.2 Maintenance and storage of *Caenorhabditis elegans* cultures

*Caenorhabditis elegans* var. Bristol (N2) was used as the test organism and was obtained from our laboratory stock culture. The nematodes were cultured on nematode growth (NG) agar **(Appendix 1)** seeded with *Escherichia coli* (ATCC 11775) serving as a food source at 20 ºC under dark conditions **(BRENNER, 1974)**. An overnight (18 h) culture of *E*. *coli* was made by inoculating a single colony in 5 ml of sterilized Mueller-Hinton (MH) broth (Oxoid Limited, England) in a sterile McCartney bottle and incubated at 37 ºC in a water bath with an orbital shaker for 18 h. For the sub-culturing, 5 ml of sterile M9 buffer **(Appendix 1)** were added to an old *C. elegans* culture plate to wash the nematodes off the plate. Fifty microlitres aliquot of the overnight *E. coli* were transferred to the new NG agar plates and spread across the plate using a glass rod. Thereafter, 500 µl of the old *C. elegans* culture in M9 buffer were transferred to the new NG agar plate already seeded with *E*. *coli*. The newly cultured NG agar plates were incubated at 20 ºC under dark conditions until required. The sub-culturing was repeated every 6 weeks to maintain the strength and viability of the nematodes.

### 3.2.3 *In vitro* anthelmintic bioassay

A rapid *in vitro* colourimetric assay used in the determination of nematode free-living larvae viability, as described by **JAMES and DAVEY (2007)**, with slight modifications, was employed in evaluating the minimum lethal concentration (MLC) values of plant extracts against *C*. *elegans*. A 3-day-old *C*. *elegans* culture was prepared by sub-culturing a stock nematode plate as described above **(Section 3.2.2)**. The NG agar plate was seeded with an autoclaved *E*. *coli* culture to serve as a food source for the nematodes, without resulting in a colour change to the indicator compound. The 3-day-old *C*. *elegans* culture was washed with 5 ml of M9 buffer into a sterile McCartney bottle and the optical density (OD) at 530 nm was measured using a UV-visible spectrophotometer (Varian Cary 50, Australia). Thereafter, 5 ml of M9 buffer were adjusted with the appropriate volume of the prepared stock *C*. *elegans* culture to obtain a mixture in the $OD_{530}$ range of 0.04-0.06, which was sufficient for a microtitre plate. Similar dilutions were made to obtain enough *C*. *elegans* culture required for the bioassay.

Sterile distilled water (100 µl) was added to each well in a 96-well microtitre plate (Greiner Bio-one GmbH, Germany). From the redissolved plant extracts, aliquots of 100 µl were added to the first well of the microtitre plate (row A) and diluted two-fold serially (column 1-12 downward: A to H). Levamisole (100 µl of 1 mg/ml) was also two-fold serially diluted with water and this served as the reference anthelmintic drug. From the prepared *C*. *elegans* mixture, aliquots of 50 µl (containing approximately 100 individuals of *C*. *elegans*) were added to the wells of the microtitre plate. DMSO, sterile water and *C*. *elegans* cultures were included in the test as controls. The final concentration of the extracts in the microtitre plate ranged from 0.065 to 8.33 mg/ml and 0.13 to 16.67 mg/ml for organic solvent and water extracts, respectively. To reduce evaporation and prevent contamination, the microtitre plates were covered with parafilm and incubated at 20 ºC for 48 h.

After incubation, 50 µl of 1.25 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) were added to all the microtitre plate wells and incubated further at 20 ºC for 24 h. Active organisms biologically reduce the colourless INT to a pink-red colour **(McGAW *et al.*, 2007)**. No colour change indicated inhibition of the nematodes and the concentration of the lowest clear well was recorded as the MLC value of the extract. Extracts were screened in duplicate and the experiment was performed twice.

### 3.3 Results and discussion

A total of 80 extracts were screened for anthelmintic activity and the MLC values of all the extracts are presented in **Table 3.1**. The MLC is defined as the lowest concentration that causes mortality in a liquid culture. The possible mechanism of action of the extract against the *C*. *elegans* can be considered to be a direct toxic effect as a result of the assay method employed for this study. INT was used as an indicator in place of MTT-formazan because it has been shown that INT is more stable over time **(ELOFF, 1998a)**.

In comparison to studies on antimicrobial activity, scientific evaluation or standards comparing the efficacy of plant extracts to commercial anthelmintics, as well as determining the level of anthelmintic activity, are limited. As a result, there is much

experimental variability that has led to an increased apparent conflict of results on anthelmintic activity by plant extracts **(ATHANASIADOU *et al.*, 2007)**. Most anthelmintic screening is reported as an estimate of the percentage of nematode survival, or the use of a scoring system, after incubation of the extracts and nematode culture for a certain period. In this study, three levels of anthelmintic activity were defined, MLC values less than 1 mg/ml were considered as high anthelmintic activity, from 1 mg/ml to less than 4 mg/ml as moderate activity while values greater than 4 mg/ml to 16.67 mg/ml were regarded as low anthelmintic activity. These definitions were based on factors such as the acceptable noteworthy level of crude extract concentration as used in other screening bioassay as well as the anthelmintic activity exhibited by various extract concentrations as reported in other preliminary anthelmintic screening assays **(McGAW *et al.*, 2007; VAN VUUREN, 2008)**.

### 3.3.1 Anthelmintic activity of investigated plant extracts

The MLC values ranged from 0.13 to 16.67 mg/ml, with MLC values greater than 16.67 mg/ml being regarded as having no activity at the screening concentration. The lowest MLC value of 0.13 mg/ml, corresponding to the best anthelmintic activity, was displayed by the DCM extract of *H*. *colchicifolia* leaves. Water extracts of *C*. *orbiculata* var. *dactylopsis* (leaves and stems), *C*. *orbiculata* var. *orbiculata* (leaves) and *F*. *erigeroides* (stems) showed no anthelmintic activity at the maximum screening concentration. In summary, 19 extracts had high anthelmintic activity, 34 extracts displayed moderate activity and 27 extracts had low or no anthelmintic activity.

Apart from *H*. *hemerocallidea*, all the plant species had at least one of its extract showing a high anthelmintic activity. The potential anthelmintic activity shown by these plants further confirms the importance of using an ethnopharmacological approach in screening plants as a source of novel pharmaceuticals. This approach provides a valuable short-cut by taking advantage of information available from traditional healers and ethnopharmacological records **(ELGORASHI and VAN STADEN, 2004)**.

**Table 3.1:** Minimum lethal concentration (MLC) values (mg/ml) of plant extracts against *C*. *elegans* in the *in vitro* anthelmintic bioassay. Values in bold (MIC < 1 mg/ml) indicate high anthelmintic activity.

| Plant species | Plant part | MLC (mg/ml) | | | |
|---|---|---|---|---|---|
| | | PE | DCM | EtOH | Water |
| *Acokanthera oppositifolia* | Leaves | **0.52** | 1.04 | **0.52** | 4.17 |
| | Twigs | 2.08 | 4.17 | 2.08 | 2.08 |
| *Clerodendrum myricoides* | Leaves | 2.08 | 1.04 | **0.26** | 1.04 |
| | Stems | 2.08 | 2.08 | 2.08 | 2.08 |
| *Cotyledon orbiculata* var. *dactylopsis* | Leaves | **0.52** | **0.52** | **0.26** | >16.67 |
| | Stems | 4.17 | 4.17 | 4.17 | >16.67 |
| *Cotyledon orbiculata* var. *orbiculata* | Leaves | **0.26** | 1.04 | **0.26** | >16.67 |
| | Stems | 4.17 | 4.17 | 8.33 | 8.33 |
| *Cyathea dregei* | Leaves | 1.04 | **0.52** | **0.52** | 1.04 |
| | Roots | 8.33 | 4.17 | 2.08 | 4.17 |
| *Felicia erigeroides* | Leaves | 1.04 | **0.52** | 4.17 | 16.67 |
| | Stems | 4.17 | 2.08 | 4.17 | >16.67 |
| *Hypoxis colchicifolia* | Leaves | **0.52** | **0.13** | **0.26** | 4.17 |
| | Corms | 4.17 | 2.08 | 2.08 | 2.08 |
| *Hypoxis hemerocallidea* | Leaves | 1.04 | 1.04 | 2.08 | 2.08 |
| | Corms | 2.08 | 4.17 | 2.08 | 1.04 |
| *Leucosidea sericea* | Leaves | **0.52** | **0.26** | **0.26** | 8.33 |
| | Stems | 2.08 | 4.17 | 2.08 | 8.33 |
| *Ocimum basilicum* | Leaves | 2.08 | **0.26** | 1.04 | 1.04 |
| *Senna petersiana* | Leaves | 1.04 | 1.04 | **0.52** | 8.33 |

Extracting solvent: PE = Petroleum ether, DCM = Dichloromethane, EtOH = Ethanol.

The MLC of the reference levamisole was 40 µg/ml.

The extracts of *C*. *dregei* leaves exhibited better anthelmintic activity than the root extracts. Interestingly, traditional healers use the dried roots of this fern as an anthelmintic **(HUTCHINGS *et al.*, 1996)**. The destructive harvesting of the roots for medicinal uses is probably responsible for the plant being listed as one of the threatened plants of southern Africa as in Lesotho and South Africa **(TALUKDAR, 2002)**. Similarly, the *Hypoxis* species leaf extracts had better anthelmintic activity than the underground corms. Indiscriminate and over-harvesting of underground parts such as roots and corms endangers plant survival **(JÄGER and VAN STADEN, 2000)**. Different compounds could be responsible for the difference in activity levels **(STEENKAMP *et al.*, 2006)** or a variation in the quantities of bioactive compounds in the two plant parts providing a possible explanation for the higher anthelmintic activity in the leaf extracts. Most importantly, these results support the ideal of investigating alternative plant parts as a substitute to ensure sustainable non-destructive plant harvesting **(ZSCHOCKE *et al.*, 2000)**.

Members of the genus *Acokanthera* are well known to be poisonous due to the presence of several toxic cardiac glycosides **(VAN WYK *et al.*, 2002)**. In a previous study, *A*. *oblongifolia* did not exhibit any anthelmintic activity against *C*. *elegans* in respect of both nematode mortality and reproductive ability in *in vitro* assays **(McGAW *et al.*, 2000)**. *A*. *oppositifolia* have been reported to have a considerable amount of phenolic compounds **(ADEDAPO *et al.*, 2008)**. In view of different reports, the high anthelmintic activity displayed by PE and EtOH extracts of *A*. *oppositifolia* leaves deserves further investigation for its safety as the extracts may well be toxic to humans over and above to their therapeutic value. Toxic principles such as tyledoside and three bufadienolide glycosides (orbicuside A, B and C) are present in *Cotyledon* species **(HUTCHINGS *et al.*, 1996)** and the noteworthy activity of this species might be as a result of the lethal effect of these principles on *C*. *elegans*.

The organic solvent extracts of *S*. *petersianna* displayed moderate to high anthelmintic activity while the water extract had low activity. **SPARG et al. (2000)** also reported a significant lethal activity of the water extract against the schistosomula at 6.25 mg/ml in an *in vitro* assay. The active constituents of the different parts of the plant have been identified as anthraquinone glycosides **(WATT and BREYER-BRANDWIJK, 1962)** and probably accounted for the anthelmintic

activity. The leaf extracts of *O*. *basilicum* contain many essential oils such as limonene and α-terpineol **(RUNYORO *et al.*, 2010)** which are known to possess pharmacological activities and may well be responsible for the high anthelmintic activity against *C*. *elegans* observed in this study.

As shown in **Figure 3.1**, the organic solvent extracts (PE, DCM and EtOH) had better anthelmintic activity than the water extracts. A total of 12 water extracts showed low or no anthelmintic activity. Although some herbal preparations employ alcohol for rapid action, water is the most common solvent used in traditional medicine, **(SPARG *et al.*, 2002)**. The use of water for extraction naturally limits the type and amount of compounds extracted from a plant **(ELOFF, 1998b)**. Non-polar principles are better extracted with organic solvents such as PE, DCM and EtOH, as used in this investigation. EtOH had the highest number (8) of extracts that exhibited high activity, indicating the best extraction of the anthelmintic principle(s) by this solvent.

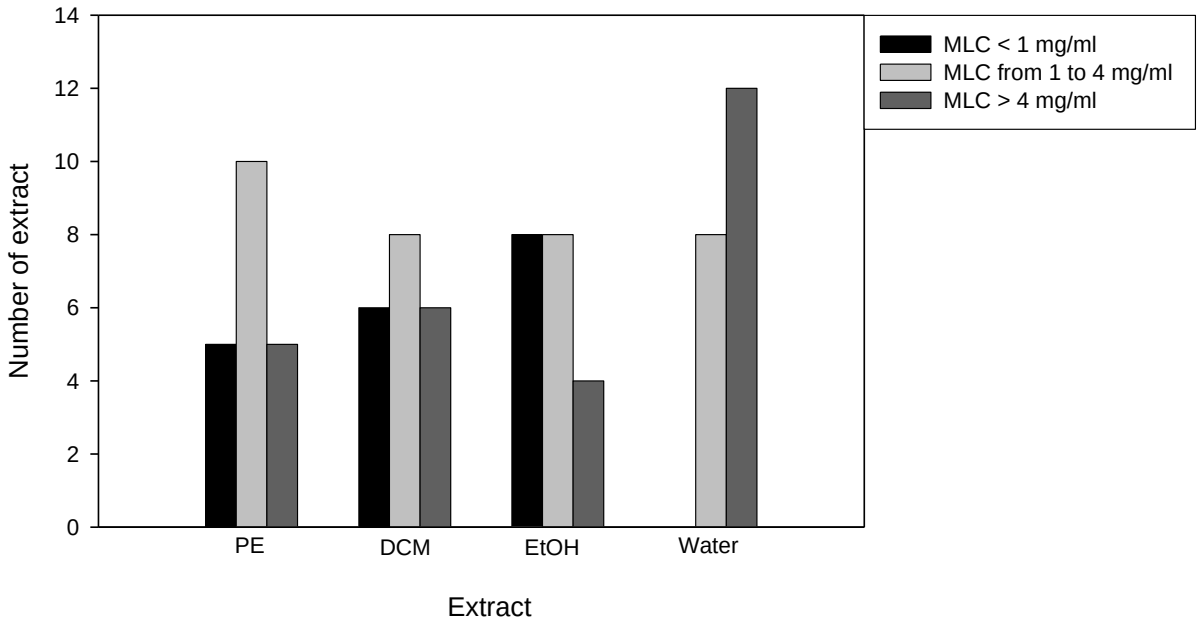


**Figure 3.1:** Number of extracts exhibiting different levels of anthelmintic activity as a result of the solvent types used for the plant extraction. PE = Petroleum ether, DCM = Dichloromethane, EtOH = Ethanol, MLC = Minimum lethal concentration.

MLC < 1 mg/ml, MLC ≥ 1 to ≤ 4 and MLC > 4 mg/ml indicate high, moderate and low anthelmintic activity, respectively.

## 3.4 Conclusions

In this investigation, 19 plant extracts exhibited high anthelmintic activity against *C*. *elegans*, thereby supporting their use as anthelmintic plants in traditional medicine. Conversely, the absence of significant (low or no) anthelmintic activity in other extracts could be due to factors such as insufficient active principle(s) and antagonistic effects by other compounds against the plant extracts active principle(s) and not necessarily the inactivity of the plant extracts **(TAYLOR *et al.*, 2001)**.

The *in vitro* bioassay served as a means of rapidly screening for potential anthelmintic activity in the plant extracts as well as providing evidence for the possible mechanism of the active compound(s) on the test organism (*C*. *elegans*). However, in view of the differences between *in vitro* and *in vivo* environments, these results remain indicative of the anthelmintic potential of the plant extracts and further investigations involving *in vivo* studies and parasitic helminth organisms would be necessary for valid conclusions. In addition, bioactivity-guided isolation and safety of the active compound(s) would be necessary, and non-toxic and very potent active compound(s) could serve as leads for the development of new anthelmintic drugs for animals as well as humans.

# CHAPTER 4: ANTIMICROBIAL SCREENING

## 4.1 Introduction

Infectious diseases represent a serious health problem and are part of the leading causes of death globally. The increasing microbial resistance to antibiotics is gradually aggravating this situation over time **(BANDOW *et al.*, 2003; KUETE *et al.*, 2009)**. Recently, developing countries have witnessed an increased mortality rate due to infectious diseases such as diarrhoea and dysentery, mainly caused by intestinal infection **(LONGANGA OTSHUDI *et al.*, 2000)**. Common organisms associated with intestinal infection include viruses, bacteria, protozoa and helminths. Scientific evidence also suggests that both synergism and antagonism between helminth infections and other pathogenic infections are increasing **(COX, 2001)**. Parasites can either reduce host immunity or boost the immune system against certain other diseases such as autoimmune disorders (for example: Crohn's disease) **(HUNTER and McKAY, 2004)**. Parasites such as helminths do not always cause obvious diseases but the host's health integrity could be compromised due to the metabolic activities of the parasites such as nutrition, excretion and reproduction **(DEACON, 2006)**. This probably accounts for the increased susceptibility and enhances the progression of bacterial diseases such as tuberculosis and other opportunistic infections among helminth hosts **(BENTWICH *et al.*, 1995; BROOKER *et al.*, 2004)**. In addition, microorganisms such as bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*) and fungi (*Candida albicans*) have been implicated in the increased infection rate in immune-compromised patients globally **(SLEIGH and TIMBURG, 1998)**.

The importance of pharmacological screening of plant extracts for multiple biological activities has been extensively documented **(HOUGHTON *et al.*, 2005)**. This importance has been demonstrated using diseases such as Alzheimer's, diabetes and wound healing. The use of multiple bioassays gives a clearer indication of the effect of the extracts in relation to the disease state **(HOUGHTON *et al.*, 2007)**. According to **GURIB-FAKIM (2006)**, medicinal plants contain complex mixtures of compounds that may act individually, additively or in synergy for health improvement.

For instance, a medicinal plant containing phenolic compounds may act as antioxidant and as an anti-inflammatory agent. Similarly, **McGAW *et al.* (2001)** studied the pharmacological activity of different *Combretum* species and four species demonstrated a significant bioactivity in more than one bioassay. In another study, antibacterial and anti-inflammatory activity of five species in the family Sterculiaceae was reported, as a result a variety of compounds were isolated from the plant species **(REID *et al.*, 2005)**. Moreover, there is a risk of non-discovery of other potentially useful bioactivity of medicinal plants when only a single biological activity is investigated **(RATES, 2001)**.

Consequently, antimicrobial activity of the selected South African plants used as anthelmintics was investigated. Furthermore, as helminth and microbial infections have some common symptoms such as diarrhoea, abdominal pains and inflammation, there is a possibility of discovering antimicrobial activity in the investigated plants species.

### 4.1.1 Effect of microbial infections on human health

Although only a small fraction of the thousands of microorganism species are harmful, the impact of these pathogenic microorganisms is of great concern in human medicine. Most microbes are beneficial, playing a crucial role in the ecosystem as decomposers and in the production of antibiotics against human diseases **(BLACK, 2002)**. Unfortunately, the living conditions of the majority of people in the third-world and most developing countries are characterized by poor sanitation, low-levels of hygiene and overcrowded environments **(LONGANGA OTSHUDI *et al.*, 2000; HOTEZ *et al.*, 2007a)**. These factors enhance the growth of a wide range of microorganisms, especially the virulent pathogens present within the community **(SLEIGH and TIMBURG, 1998)**. Due to lower immunity caused by these factors, the human skin, mouth, urinary tract, respiratory organs and gastro-intestinal tract becomes more susceptible to infection by these microorganisms **(EISENSTEIN and SCHAECHTER, 1998)**. Most helminth infections have been attributed to the shift in the host-parasite relationship in favour of the parasite, thereby causing serious health problems **(SLEIGH and TIMBURG, 1998)**.

#### 4.1.1.1 Bacterial infections

Bacterial infections are caused by a wide range of organisms resulting in mild infections to life threatening diseases. For instance, *Bacillus subtilis* is an aerobic, rod-shaped, motile, and endospore-forming Gram-positive bacterium. It is a soil inhabiting saprophytic organism **(SLEIGH and TIMBURG, 1998)**. Although harmless, it occasionally causes some opportunistic infections such as conjunctivitis **(MURRAY *et al.*, 1998; BUWA and VAN STADEN, 2006)**. *Staphylococcus aureus*, a Gram-positive coccus bacterium, is part of the normal flora of human skin and mucous membranes **(MURRAY *et al.*, 1998)**. This organism is responsible for human staphylococcal skin infections (wounds and impetigo), soft tissues (septic arthritis) and pneumonia **(SLEIGH and TIMBURG, 1998)**. *Escherichia coli*, a Gram-negative rod-shaped bacterium is mostly found inhabiting the human gastrointestinal tract **(ROSS and PEUTHERER, 1987)**. The organism has been implicated in most bacterial infections including urinary tract infection, travellers' diarrhoea, bacteraemia and pneumonia **(DuPONT, 2006)**. *Klebsiella pneumoniae* is a non-motile, rod-shaped Gram-negative bacterium. The organism is easily observed in culture as it forms large colonies. It is also part of the human intestinal and colon flora, having a prominent polysaccharide capsule that provides resistance against host defence mechanisms **(HUGO, 1992)**. Common human *Klebsiella* infections include community acquired pneumonia, urinary tract infection, lower and upper respiratory tract infections **(EINSTEIN, 2000)**.

Other examples of medically important bacterial groups, known to cause various infectious diseases in humans, include mycobacterium (tuberculosis), vibrio (cholera), neisseria (gonorrhoea) and spirochaetes (syphilis) **(SLEIGH and TIMBURG, 1998)**. In South Africa, these infectious bacterial diseases, especially tuberculosis are increasing due to the increasing drug resistance and high incidence of HIV/AIDS infection amongst the population **(ELDEEN *et al.*, 2005; NAIDOO, 2008)**.

#### 4.1.1.2 Fungal infections

Fungal and yeast infections have a major influence on human health causing diseases ranging from mild superficial problems to potentially lethal systemic disorders (infections invading internal organs) **(KOBAYASHI and MEDOFF, 1998)**. The number of diseases due to fungal infections is however, lower than bacterial infections. Approximately 200 of the thousands of fungal species are implicated in human diseases **(DEACON, 2006)**. Recently, pathogenic fungal infections have been increasing, mostly due to a compromised immune system as humans have a high degree of innate immunity to most fungi with exception of the dermatophytic type **(MURRAY *et al.*, 1998; DEACON, 2006)**. Most alterations in human immune system status are associated with the acquired immune-deficiency syndrome (AIDS), increased use of immune-suppressive therapy (cancer chemotherapy, organ or bone marrow transplant, X-ray irradiation) and a compromised immune system due to parasitic infections such as helminths and protozoans **(WALSH and GROLL, 1999; FLEMING *et al.*, 2002)**.

Opportunistic fungal pathogens such as *Candida*, *Mucor* and *Aspergillus* are the major causes of morbidity and mortality in humans **(GARBINO *et al.*, 2001)**. For instance, *C*. *albicans* which is part of the normal flora of the upper respiratory, gastrointestinal and female genital tracts is responsible for 90% of fungal infections in immuno-compromised patients globally **(SLEIGH and TIMBURG, 1998)**. Likewise, **SHAI *et al*. (2008)** reported a high occurrence of candidiasis among South African HIV/AIDS immune compromised patients. In KwaZulu-Natal, a similar trend with associated symptoms such as severe inflammatory diarrhoea and resistance to antifungal chemotherapy was observed **(MOTSEI *et al.*, 2003)**.

### 4.1.2 Treatment of microbial infections

The development of natural products such as antibiotics created a major breakthrough in the fight against pathogenic microorganisms. The discovery by Alexander Fleming in 1929 of *Penicillium nonatum* (penicillin) inhibition of *Staphylococci* cultures ushered in the antibiotic era and subsequently led to the development of other antibiotics such as streptomycin and tetracycline **(SLEIGH and**

**TIMBURG, 1998; MURRAY *et al.*, 1998)**. Unfortunately, the emergence of drug resistant bacteria and the elimination of useful bacterial flora leading to host susceptibility to other pathogenic infections, are today major problems associated with most antibacterial chemotherapy **(GUARNER and MALAGELADA, 2003)**.

The number of effective chemotherapies against fungal infections is relatively small when compared to antibacterial drugs. Common antifungal drugs include Amphotericin B, fluconazole and griseofulvin. Amphotericin B is mostly used against fungi causing systemic diseases. It binds to the sterols causing disruption in fungus cell membrane and finally leads to death of the organism **(DEACON, 2006)**. Most antifungal chemotherapeutic agents are toxic in high dosage and have limited therapeutic values because of problems with solubility, stability, and absorption by the human body **(RAPP, 2004)**. Furthermore, *Candida* species are resistant to all antibacterial antibiotics because fungi are eukaryotic microorganisms **(SLEIGH and TIMBURG, 1998)**.

Globally, there is a need for new strategies against the increasing microbial infections due to development of resistance to conventional drugs and the adverse effects of most available chemotherapy. Fortunately, plants are described as the best “combinatorial chemists”, and have the potential to provide novel compounds which may turn out to be useful drugs **(SAKLANI and KUTTY, 2008)**.

## 4.2 Materials and methods

In the quest to evaluate antimicrobial activity, factors such as plant collection, bioassay methodology and standardization, as well as various intricacies of antimicrobial investigation need to be defined **(VAN VUUREN, 2008)**. These parameters as applied in this study are detailed below.

### 4.2.1 Extract preparation

Extract preparation was carried out as described in **Section 2.2.2**. The stored dried extracts were redissolved at a concentration of 50 mg/ml for use in water for aqueous extracts and DMSO for use with organic solvent extracts (PE, DCM and EtOH).

### 4.2.2 Microbial culture storage and maintenance

Although it is ideal to test against specific target microorganisms, taxonomical representative species were used in this preliminary antimicrobial screening to avoid handling numerous pathogenic microorganisms **(TAYLOR *et al.*, 2001)**. The test organisms used for the *in vitro* antibacterial investigation were; Gram-positive bacteria (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus*, ATCC 12600) and Gram-negative bacteria (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883). *Candida albicans* (ATCC 10231) was used for *in vitro* antifungal investigation of the plant extracts.

The microorganism stocks were preserved in sterile cryovials (Greiner, Germany) containing glycerol solution **(Appendix 2)** and stored in an ultra-freezer at -70 ºC until required. Mueller-Hilton (MH) agar (Merck, South Africa) was prepared, sterilized and poured into sterile plastic Petri dishes and allowed to gel. These MH agar plates were stored at 4 ºC for 24 h before the stock bacteria were sub-cultured on the MH agar plates. The inoculated plates were incubated at 37 ºC overnight. After incubation, the cultured bacterial growth was controlled by storing at 4 ºC until required for bioassays. For maintenance of the bacterial strength and viability, bacteria were sub-cultured every 42 days **(WISTREICH, 1997)**. *C*. *albicans* culture was prepared and maintained in a similar manner as the bacteria with the only difference being the use of Yeast Malt (YM) agar (Becton Dickinson, USA) in place of MH agar **(WISTREICH, 1997)**.

### 4.2.3 *In vitro* antibacterial bioassay

The antibacterial activity of the plant extracts was evaluated by determining the minimum inhibitory concentration (MIC) using the microplate dilution technique **(ELOFF, 1998a)**. An overnight (18 h) culture of the four bacteria (*B*. *subtilis*, *S*. *aureus*, *E*. *coli* and *K*. *pneumonia*) were prepared by inoculating a single colony of each bacterium in 5 ml of sterilized Mueller-Hinton (MH) broth (Oxoid, England) in a sterile McCartney bottle and incubated at 37 ºC in a water bath with an orbital shaker. The absorbance of each bacterial overnight culture was measured using a UV-visible spectrophotometer (Varian Cary 50, Australia) at 600 nm. The overnight

bacterial suspension cultures at the following concentrations; 4.8 x $10^9$ CFU/ml (*B*. *subtilis*), 2.7 x $10^9$ CFU/ml (*S*. *aureus*), 7.0 x $10^{10}$ CFU/ml (*E*. *coli*) and 2.5 x $10^9$ CFU/ml (*K*. *pneumoniae*) were diluted with sterile broth (1:100) and used in the screening.

Sterile water (100 µl) was added to each well in a 96-well microtitre plate (Greiner Bio-one GmbH, Germany). From the redissolved plant extracts (50 mg/ml), aliquots of 100 µl were added to the first well of the microtitre plate (row A) and diluted two-fold serially (column 1-12 downward: A to H). Neomycin (Sigma-Aldrich, Germany) was prepared by diluting 20 µl (10 mg/ml) into 480 µl sterile water in an Eppendorf tube. A similar two-fold serial dilution of this prepared neomycin (100 µl) was used as the reference drug. From the prepared bacteria cultures, aliquots of 100 µl were added to the wells of the microtitre plate. DMSO, sterile water, bacteria-free MH broth and bacterial culture were included as the controls. The final concentration of the extracts and neomycin in the wells of the microtitre plate ranged from 0.098 to 12.50 mg/ml and 0.003 to 100 µg/ml, respectively. To reduce evaporation and prevent contamination, the microtitre plate was covered with parafilm and incubated at 37 ºC for 24 h.

After incubation, 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT; Sigma-Aldrich, Germany) was added to the wells of the microtitre plate and incubated further for 1 h at 37 ºC. Active microorganisms biologically reduce the colourless INT to a pink-red colour **(McGAW *et al.*, 2007)**. No colour change showed that inhibition had occurred and the concentration of the lowest clear well was recorded as the MIC value of the plant extract. The experiments were performed in duplicate and repeated independently three times.

### 4.2.4 *In vitro* antifungal bioassay

The microplate dilution technique **(ELOFF, 1998a)** as modified for the antifungal assay **(MASOKO *et al.*, 2007)** with slight modifications **(MOTSEI *et al.*, 2003)** was used in plant extract antifungal activity determination. Both the MIC and minimum fungicidal concentration (MFC) values were determined.

An overnight (18 h) *C. albicans* culture was prepared by inoculating a colony of *C. albicans* from the cultured YM plate into 5 ml of sterilized Yeast Malt (YM) broth (Sigma-Aldrich, Germany) in a sterile McCartney bottle and incubated at 37 °C in a water bath with an orbital shaker. For the *in vitro* bioassay, 400 µl of the overnight fungal culture was diluted with 4 ml of sterile saline solution (0.85% NaCl) in a sterilized McCartney bottle. The absorbance of the mixture was determined using a UV-visible spectrophotometer at 530 nm. This absorbance was adjusted with sterile saline solution to match a 0.5 McFarland standard solution at a range of 0.2500 to 0.2800. From the prepared culture, a 1:1000 dilution with sterile YM broth was made to obtain a concentration of 5 x $l0^5$ CFU/ml.

Sterile water (100 µl) was added to each well in a 96-well microtitre plate (Greiner Bio-one GmbH, Germany). From the redissolved plant extracts (50 mg/ml), aliquots of 100 µl were added to the first well microtitre plate (row A) and diluted two-fold serially (column 1-12 downward: A to H). Amphotericin B (0.25 mg/ml; Sigma-Aldrich, Germany) was prepared as the reference drug and aliquot (100 µl) was diluted two-fold serially. From the prepared culture, aliquots of 100 µl were added to the wells of the microtitre plate. DMSO, sterile water, fungal-free YM broth and *C. albicans* culture were included as controls. This gave a final concentration ranging from 0.098 to 12.50 mg/ml for the plant extracts and 4.80 to 62.50 µg/ml for Amphotericin B. The mixture in the microtitre plate was covered with parafilm to limit evaporation and prevent contamination.

The microtitre plate was incubated overnight at 37 ºC after which 50 µl of 0.02 mg/ml *p*-iodonitrotetrazolium chloride (INT) was added as a fungal growth indicator. The plant extract MIC values were recorded as the concentration of the lowest well without colour change after 48 h and growth was indicated by the reddish-pink colour. Furthermore, MFC values were determined by adding sterile YM broth (50 µl) to all clear wells then incubated for another 24 h at 37 ºC. The extract MFC values were recorded as the concentration in the lowest wells of the microtitre plate in which there was no colour change. Extracts were screened in duplicate and the experiment was performed three times.

## 4.3 Results and discussion

The extract MIC values (antibacterial and antifungal) and MFC values (antifungal) in the bioassay are presented in **Table 4.1**. A total of 80 extracts from the 11 plants were screened for their antimicrobial activity. The plant extract evaluation was quantified in terms of the MIC required for inhibition of the test microorganism (bacteria and fungus) growth as well as the extract's lethal effect (MFC) against the test fungus in the *in vitro* bioassay. The MIC is defined as the extract's lowest concentration that produces an almost complete microbial growth inhibition in a liquid culture while MFC is defined as the extract's lowest concentration that completely causes the death of the test fungus in the *in vitro* bioassay.

### 4.3.1 Antibacterial activity of the investigated plant extracts

From the evaluation, most investigated plant species showed some activity at the maximum screening concentration (12.50 mg/ml) against one of the test bacteria, with the exception of water extracts of *C*. *orbiculata* (var. *orbiculata* and *dactylopsis*) leaves and stems. As indicated in **Table 4.1**, extract MIC values ranged from 0.025 to 12.50 mg/ml with MIC values greater than 12.50 mg/ml considered as not active in this study.

As presented in **Figure 4.1**, twenty five (31%) extracts had antibacterial MIC values less than 1 mg/ml. From the four extracting solvents, more ethanol extracts had noteworthy antibacterial activity while water extracts were the least active. Gram-negative bacteria were more resistant to the plant extracts than the Gram-positive bacteria. Generally, *S*. *aureus* was the most susceptible while *E*. *coli* was the most resistant bacteria to the plant extracts tested. Using an MIC value of less than 1 mg/ml as the benchmark of noteworthy antibacterial activity **(ALIGIANNIS *et al.*, 2001; KATERERE and ELOFF, 2008)**, seven extracts exhibited a broad spectrum of activity, three extracts were active against Gram-negative bacteria and 13 extracts inhibited Gram-positive bacteria growth only.

**Table 4.1:** Antimicrobial activity (MIC) of plant extracts determined using the microplate dilution techniques in *in vitro* bioassays. Values in bold (MIC < 1 mg/ml) are considered noteworthy antimicrobial activity.

| Plant species | Plant part screened | Extract[c] | Antibacterial activity MIC[a] (mg/ml) | | | | Antifungal activity *Candida albicans* | |
|---|---|---|---|---|---|---|---|---|
| | | | Bacteria[d] | | | | | |
| | | | *B.s.* | *S.a.* | *E.c.* | *K.p.* | MIC[a] (mg/ml) | MFC[b] (mg/ml) |
| *Acokanthera oppositifolia* | Leaves | PE | 3.13 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | DCM | 3.13 | 3.13 | 3.13 | 1.56 | 3.13 | 6.25 |
| | | EtOH | 6.25 | 3.13 | 3.13 | 1.56 | 6.25 | 6.25 |
| | | Water | 12.50 | 6.25 | 6.25 | 6.25 | 12.50 | >12.50 |
| | Twigs | PE | 6.25 | 6.25 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | DCM | 3.13 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | EtOH | 6.25 | 6.25 | 6.25 | 3.13 | 6.25 | 6.26 |
| | | Water | 12.50 | 6.25 | 6.25 | 6.25 | 12.50 | 12.50 |
| *Clerodendrum myricoides* | Leaves | PE | 3.13 | 6.25 | 3.13 | 3.13 | 3.13 | 3.13 |
| | | DCM | 1.56 | 3.13 | 3.13 | **0.78** | 3.13 | 3.13 |
| | | EtOH | 1.56 | 1.56 | 3.13 | **0.78** | 3.13 | 6.25 |
| | | Water | >12.50 | 12.50 | 6.25 | >12.50 | >12.50 | >12.50 |
| | Stems | PE | 3.13 | 1.56 | 3.13 | 3.13 | 3.13 | 3.13 |
| | | DCM | 6.25 | 6.25 | 6.25 | 3.13 | 3.13 | 3.13 |
| | | EtOH | **0.78** | 1.56 | 3.13 | 3.13 | 3.13 | 3.13 |
| | | Water | >12.50 | 1.56 | 6.25 | >12.50 | 12.50 | >12.50 |

**Table 4.1:** continued...

| Plant species | Plant part screened | Extract[c] | Antibacterial activity MIC[a] (mg/ml) | | | | Antifungal activity *Candida albicans* | |
|---|---|---|---|---|---|---|---|---|
| | | | Bacteria[d] | | | | | |
| | | | *B.s.* | *S.a.* | *E.c.* | *K.p.* | MIC[a] (mg/ml) | MFC[b] (mg/ml) |
| *Cotyledon orbiculata* var. *dactylopsis* | Leaves | PE | 3.13 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | DCM | 3.13 | 1.56 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | EtOH | 1.56 | 1.56 | 1.56 | 3.13 | 6.25 | 6.25 |
| | | Water | >12.50 | >12.50 | >12.50 | >12.50 | >12.50 | >12.50 |
| | Stems | PE | 6.25 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | DCM | 6.25 | 3.13 | 3.13 | 3.13 | 3.13 | 6.25 |
| | | EtOH | 6.25 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | Water | >12.50 | >12.50 | >12.50 | >12.50 | >12.50 | >12.50 |
| *Cotyledon orbiculata* var. *orbiculata* | Leaves | PE | 3.13 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | DCM | 3.13 | 1.56 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | EtOH | 1.56 | 1.56 | 1.56 | 3.13 | 6.25 | 6.25 |
| | | Water | >12.50 | >12.50 | >12.50 | >12.50 | >12.50 | >12.50 |
| | Stems | PE | 6.25 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | DCM | 6.25 | 3.13 | 3.13 | 3.13 | 3.13 | 6.25 |
| | | EtOH | 6.25 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | Water | >12.50 | >12.50 | >12.50 | >12.50 | >12.50 | >12.50 |
| *Cyathea dregei* | Leaves | PE | 6.25 | 3.13 | 6.25 | 6.25 | 6.25 | 6.25 |
| | | DCM | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 6.25 |
| | | EtOH | 3.13 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | Water | 12.50 | 3.13 | 3.13 | 6.25 | 6.25 | >12.50 |

**Table 4.1:** continued...

| Plant species | Plant part screened | Extract[c] | Antibacterial activity MIC[a] (mg/ml) | | | | Antifungal activity *Candida albicans* | |
|---|---|---|---|---|---|---|---|---|
| | | | Bacteria[d] | | | | | |
| | | | *B.s.* | *S.a.* | *E.c.* | *K.p.* | MIC[a] (mg/ml) | MFC[b] (mg/ml) |
| *Cyathea dregei* | Roots | PE | **0.39** | **0.78** | 3.13 | 3.13 | 3.13 | 6.25 |
| | | DCM | 1.56 | 1.56 | 3.13 | 3.13 | 1.56 | 3.13 |
| | | EtOH | 1.56 | **0.39** | 3.13 | 3.13 | 6.25 | 6.25 |
| | | Water | 3.13 | 3.13 | 6.25 | 6.25 | 12.50 | >12.50 |
| *Felicia erigeroides* | Leaves | PE | 1.56 | 3.13 | 3.13 | 3.13 | 6.25 | 6.25 |
| | | DCM | 1.56 | 1.56 | 1.56 | 1.56 | 6.25 | 6.25 |
| | | EtOH | **0.78** | **0.78** | 3.13 | 3.13 | 12.50 | >12.50 |
| | | Water | >12.50 | 12.50 | >12.50 | >12.50 | 1.56 | 3.13 |
| | Stems | PE | **0.78** | **0.78** | 3.13 | 3.13 | 6.25 | 6.25 |
| | | DCM | **0.78** | 1.56 | 1.56 | 1.56 | 6.25 | 6.25 |
| | | EtOH | 1.56 | 1.56 | 3.13 | 3.13 | 12.50 | >12.50 |
| | | Water | >12.50 | 12.50 | 12.50 | 12.50 | 12.50 | >12.50 |
| *Hypoxis colchicifolia* | Leaves | PE | **0.78** | **0.78** | 3.13 | 3.13 | **0.78** | **0.78** |
| | | DCM | **0.78** | **0.78** | 1.56 | 1.56 | 1.56 | 6.25 |
| | | EtOH | **0.78** | **0.78** | 1.56 | **0.78** | 6.25 | 12.50 |
| | | Water | >12.50 | 12.50 | 6.25 | 12.50 | 12.50 | >12.50 |
| | Corms | PE | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 | >12.50 |
| | | DCM | 6.25 | 3.13 | 6.25 | 3.13 | 6.25 | 6.25 |
| | | EtOH | 3.13 | 3.13 | 6.25 | 3.13 | 6.25 | 6.25 |
| | | Water | 1.56 | 3.13 | 6.25 | 3.13 | 12.50 | >12.50 |

**Table 4.1:** continued...

| Plant species | Plant part screened | Extract[c] | Antibacterial activity MIC[a] (mg/ml) | | | | Antifungal activity *Candida albicans* | |
|---|---|---|---|---|---|---|---|---|
| | | | Bacteria[d] | | | | | |
| | | | *B.s.* | *S.a.* | *E.c.* | *K.p.* | MIC[a] (mg/ml) | MFC[b] (mg/ml) |
| *Hypoxis hemerocallidea* | Leaves | PE | 1.56 | 1.56 | 3.13 | 3.13 | 6.25 | 12.50 |
| | | DCM | **0.78** | **0.78** | 1.56 | 3.13 | 1.56 | 6.25 |
| | | EtOH | 3.13 | 1.56 | 3.13 | 1.56 | 1.56 | 3.13 |
| | | Water | >12.50 | 3.13 | 3.13 | 3.13 | >12.50 | >12.50 |
| | Corms | PE | 3.13 | 3.13 | 6.25 | 3.13 | 6.25 | 12.50 |
| | | DCM | 3.13 | 1.56 | 6.25 | 3.13 | 6.25 | 12.50 |
| | | EtOH | 3.13 | 3.13 | 6.25 | 6.25 | 6.25 | 6.25 |
| | | Water | >12.50 | 3.13 | 3.13 | 3.13 | >12.50 | >12.50 |
| *Leucosidea sericea* | Leaves | PE | **0.025** | **0.098** | **0.39** | **0.39** | 1.56 | 1.56 |
| | | DCM | **0.098** | **0.025** | **0.39** | **0.39** | 3.13 | 3.13 |
| | | EtOH | **0.098** | **0.195** | **0.39** | **0.39** | 6.25 | 6.25 |
| | | Water | **0.78** | 1.56 | 3.13 | 3.13 | 12.50 | >12.50 |
| | Stems | PE | **0.39** | **0.39** | 1.56 | **0.78** | 3.13 | 6.25 |
| | | DCM | **0.78** | **0.78** | 1.56 | 1.56 | 6.25 | 6.25 |
| | | EtOH | **0.78** | **0.78** | 1.56 | 1.56 | 6.25 | 6.25 |
| | | Water | 3.13 | 3.13 | 6.25 | 6.25 | >12.50 | >12.50 |
| *Ocimum basilicum* | Leaves | PE | 1.56 | **0.78** | 3.13 | 3.13 | 3.13 | 6.25 |
| | | DCM | 1.56 | **0.78** | 1.56 | **0.78** | 3.13 | 6.25 |
| | | EtOH | 3.13 | 1.56 | **0.78** | **0.78** | 3.13 | 6.25 |
| | | Water | >12.50 | 3.13 | >12.50 | 3.13 | **0.78** | 1.56 |

**Table 4.1:** continued...

| Plant species | Plant part screened | Extract[c] | Antibacterial activity MIC[a] (mg/ml) | | | | Antifungal activity *Candida albicans* | |
|---|---|---|---|---|---|---|---|---|
| | | | Bacteria[d] | | | | | |
| | | | *B.s*. | *S.a*. | *E.c*. | *K.p*. | MIC[a] (mg/ml) | MFC[b] (mg/ml) |
| *Senna petersiana* | Leaves | PE | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 | 3.13 |
| | | DCM | 3.13 | **0.78** | 3.13 | 1.56 | 1.56 | 3.13 |
| | | EtOH | **0.39** | **0.39** | **0.78** | **0.39** | 1.56 | 1.56 |
| | | Water | 3.13 | 3.13 | 6.25 | 6.25 | >12.50 | >12.50 |
| Neomycin (µg/ml) | - | - | 1.56 | 1.56 | 0.39 | 0.78 | - | - |
| Amphotericin B (µg/ml) | - | - | - | - | - | - | 0.15 | 9.80 |

[a]MIC = Minimum inhibitory concentration

[b]MFC = Minimum fungicidal concentration

[c]Extract: PE = Petroleum ether, DCM = Dichloromethane, EtOH = Ethanol

[d]Bacteria: *B.s*. = *Bacillus subtilis*, *S.a*. = *Staphylococcus aureus*, *E.c*. = *Escherichia coli* and *K.p*. = *Klebsiella pneumoniae*

- = not tested

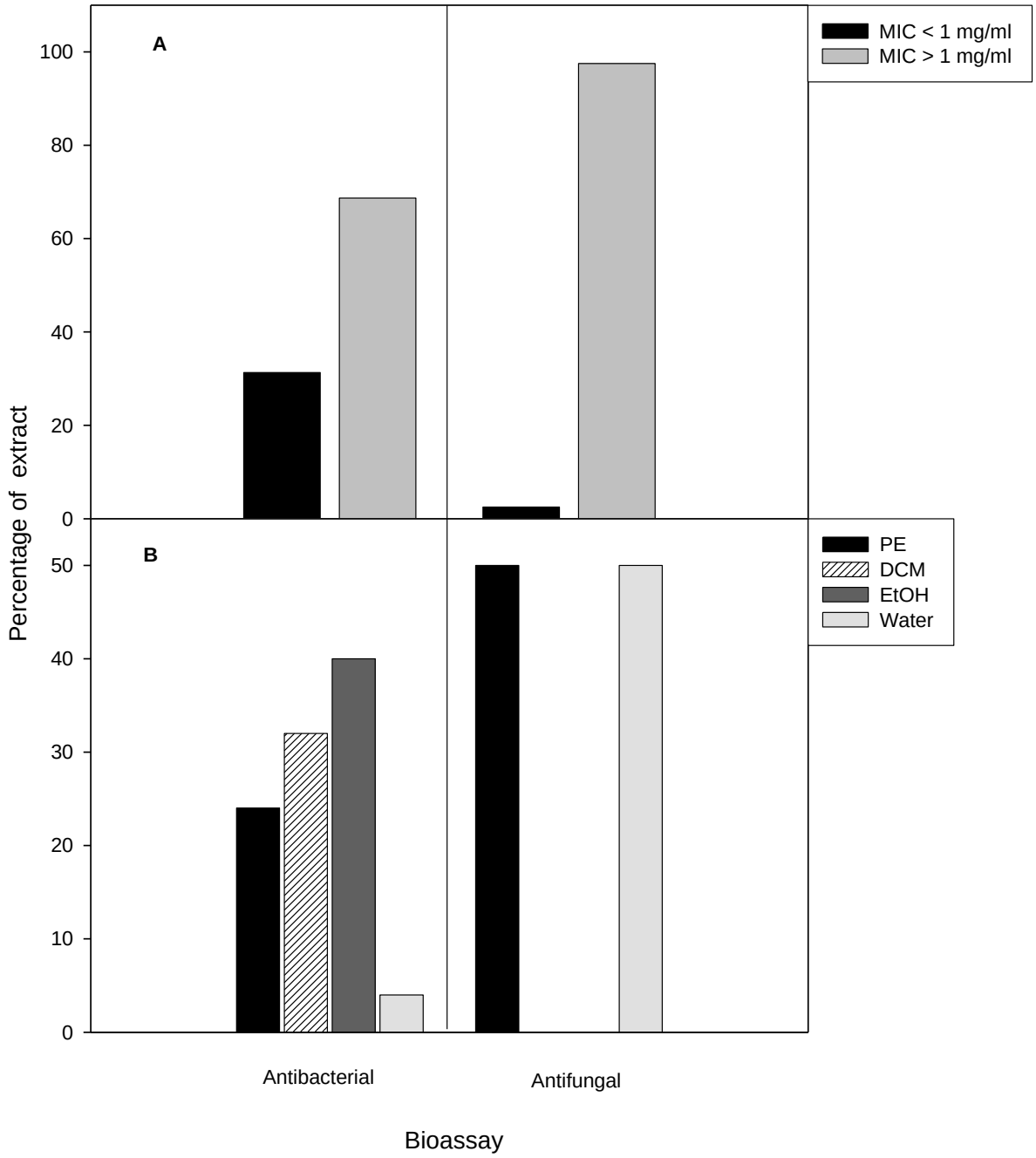


**Figure 4.1:** Percentage of extracts showing (A) antibacterial and antifungal MIC levels (MIC < 1 mg/ml = noteworthy and MIC > 1 mg/ml = non-significant) and (B) extracting solvents with noteworthy MIC (MIC < 1 mg/ml) in the antimicrobial bioassay**.** PE = Petroleum ether, DCM = Dichloromethane, EtOH = Ethanol, MIC = Minimum inhibitory concentration.

At least one of the four solvent extracts of *L*. *sericea* (leaves and stems) had an MIC values less than 1 mg/ml demonstrating noteworthy antimicrobial activity against the four test bacteria. The best antibacterial inhibitory activity of 0.025 mg/ml was exhibited by the PE and DCM extracts of *L*. *sericea* (leaves) against *B*. *subtilis* and *S*. *aureus*, respectively. Pharmacological and phytochemical studies on this plant are limited and it is the only species in the genus *Leucosidea* **(BOSMAN *et al.*, 2004)**. Further investigation for the possible isolation of bioactive compound(s) will be necessary.

Besides the water extract of *L*. *sericea* leaves (MIC: 0.78 mg/ml against *B*. *subtilis*), no other investigated water extracts had any noteworthy antibacterial activity. Generally, water extracts exhibit poor or non-detectable antibacterial activity in most antimicrobial studies **(RABE and VAN STADEN, 1997; FAWOLE *et al.*, 2009a)**. However, a few studies have reported good antibacterial activity in some plant water extracts **(BUWA and VAN STADEN, 2006; MULAUDZI *et al.*, 2009)**. The water extracts may have dose-dependant antibacterial activity or be effective against other pathogenic microbial species/strains not included in this screening **(SHALE *et al.*, 1999)**. In contrast, ethanol extracts were more active with ten (40%) extracts **(Figure 4.1)** having an MIC less than 1 mg/ml. An investigation of South Africa remedies used against various infections showed that ethanol extracts more compounds from plant material and possess better antibacterial activity than water extracts **(JÄGER, 2003)**. In addition, some traditional medicinal plant remedies are prepared using alcohol (ethanol) for rapid results **(SPARG *et al.*, 2000)**. Although water is more polar and provided higher yields **(**see **Table 2.2)** than ethanol, ethanolic extracts showed better antimicrobial activity **(Figure 4.1)**. This was probably due to greater affinity of bioactive compound(s) in the plant materials to ethanol during extraction. Likewise, **TAYLOR and VAN STADEN (2001)** observed that ethanol has a better affinity for lipophilic compounds from plant material.

The ethanol extract of *H*. *colchicifolia* leaves exhibited broad spectrum antibacterial activity, although no noteworthy activity was detected in extracts of the corms. Likewise, the DCM extract of *H*. *hemerocallidea* leaves was active against the Gram-positive bacteria (*B*. *subtilis* and *S*. *aureus*; MIC < 1 mg/ml). Although the corm extracts of the *Hypoxis* species showed no noteworthy antibacterial inhibition,

antibacterial activity of the ethanol extract of *H*. *hemerocallidea* corms against *E*. *coli* grown on blood plates has been reported **(STEENKAMP *et al.*, 2006)**. Also, a previous study on *H*. *hemerocallidea* showed antimicrobial activity of the leaves, suggesting possible phytochemical similarity in corms and leaves **(KATERERE and ELOFF, 2008)**. Consequently, the leaf extracts of *Hypoxis* species that exhibited noteworthy antibacterial activity justifies the need to further investigate alternative plant parts, especially the leaves for possible similar pharmacological activity. In addition, the use of leaves for treatment of infections enhance the conservation of the plant, particularly the endangered species that are extensively harvested **(ZSCHOCKE *et al.*, 2000; LEWU *et al.*, 2006)**.

The ethanol extract of *S*. *petersiana* leaves showed broad spectrum of antibacterial inhibitory activity with MIC less than 1 mg/ml. The ethanol extract of *S*. *petersiana* seeds has been reported to possess broad spectrum of antibacterial activity and luteolin was isolated from the seeds **(TSHIKALANGE *et al.*, 2005)**. Previous work by **SATO *et al*. (2000)** had also demonstrated the antibacterial activity of luteolin. The presence of this compound in the leaves of *S*. *petersiana* cannot be ruled out and may possibly be responsible for the observed antimicrobial activity in the present investigation.

The organic solvent extracts (PE, DCM and EtOH) of *C*. *dregei* roots as well as *F*. *erigeroides* leaves and stems displayed noteworthy antibacterial activity against the Gram positive bacteria (*B*. *subtilis* and *S*. *aureus*). **SALIE *et al*. (1996)** reported the antibacterial activity of methanol extract of *F*. *erigeroides* leaves against *Pseudomonas aeruginosa* and detected the presence of secondary metabolites such as flavonoids and saponins. Flavonoids have been reported to exhibit diverse pharmacological activities such as antibacterial and anti-inflammatory activity **(CROZIER *et al.*, 2006)**. The presence of flavonoids probably accounted for the observed antimicrobial activity in this study.

The most susceptible bacterium was *S*. *aureus* while *E*. *coli* was the most resistant as shown in **Figure 4.2**. The presence of unique lipopolysaccharides on the outer membrane of Gram-negative bacteria makes them impermeable to most antibacterial compounds **(CLEMENTS *et al.*, 2002)**. This can explain the low number

of active plant extracts against *E*. *coli* and *K*. *pneumoniae*. The observed trend is in agreement with previous research findings by various workers **(LIN *et al.*, 1999; TSHIKALANGE *et al.*, 2005; AMOO *et al.*, 2009b)**. Gram-negative bacteria however, are responsible for most infectious diseases such as gastro-intestinal disorder (dysentery and diarrhoeal) and continue to be a serious health problem globally **(SLEIGH and TIMBURG, 1998)**. Therefore, selective Gram-negative bacteria inhibitory activity exhibited by the leaf extracts of *C*. *myricoides* (DCM and EtOH) and *O*. *basilicum* (EtOH) was noteworthy (MIC values < 1 mg/ml) and deserves further investigation for possible isolation of compound(s) with potential therapeutic value.

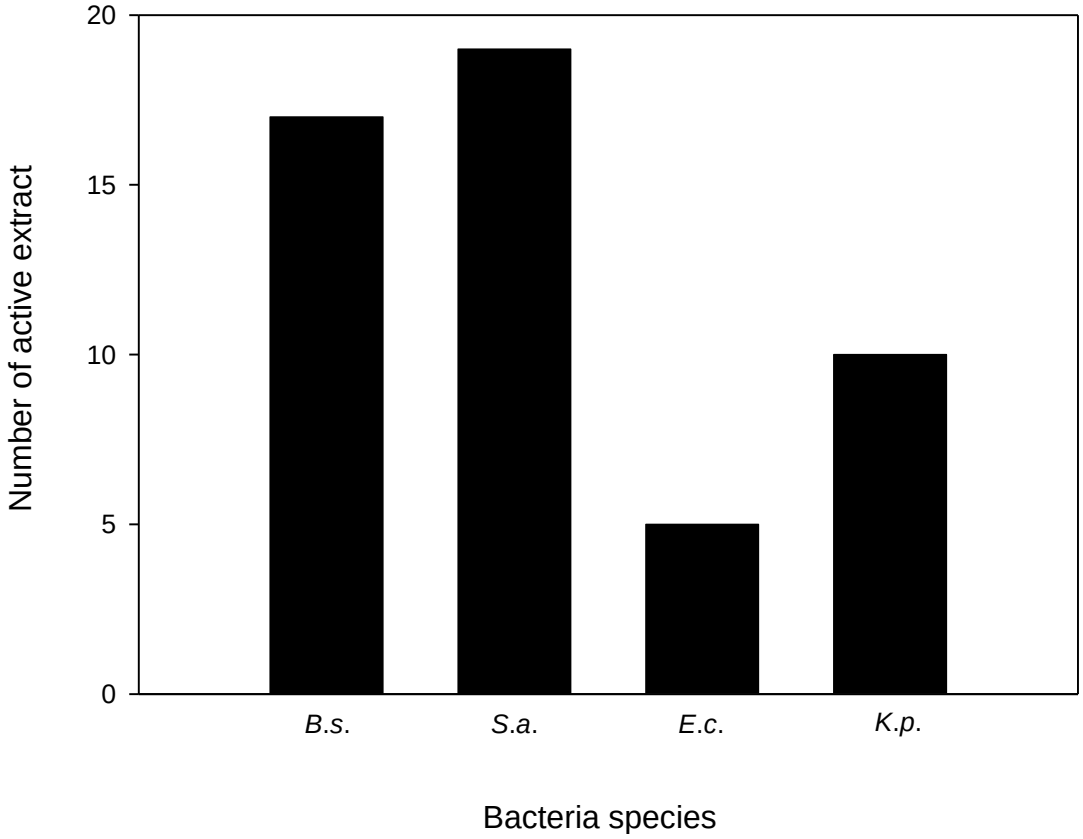


**Figure 4.2:** Bacterial susceptibility to the investigated plant extracts at an MIC value of less than 1 mg/ml. *B.s.* = *Bacillus subtilis*, *S.a.* = *Staphylococcus aureus*, *E.c.* = *Escherichia coli*, *K.p.* = *Klebisella pneumonia*.

### 4.3.2 Antifungal activity of the investigated plant extracts

A total of 80 plant extracts were screened and 71 extracts inhibited *C. albicans* growth with MIC values ranging from 0.78 to 12.50 mg/ml **(**see **Table 4.1)**. Although 89% of the extracts showed some level of fungistatic activity at various concentrations tested, generally, majority of the plant extracts exhibited rather weak antifungal inhibitory activity. A total of 60 extracts (75%) exhibited varying levels of fungicidal effects on *C*. *albicans* with MFC values ranging from 0.78 to 12.50 mg/ml. Only 20 extracts (25%) showed no fungicidal activity at the highest screening concentration (12.50 mg/ml) in the bioassay.

As shown in **Figure 4.1**, only two extracts (2.5%) had MIC values less than 1 mg/ml and are considered as having noteworthy antifungal activity. The PE extract of *H*. *colchicifolia* leaves exhibited both fungistatic and fungicidal activity. Also, the water extract of *O*. *basilicum* leaves had an MIC value of 0.78 mg/ml against the test fungal strain. Previous studies on *O*. *basilicum* revealed its antimicrobial activity which was attributed to the presences of phenolic and aromatic compounds **(GUTIERREZ *et al.*, 2008; HUSSAIN *et al.*, 2008b)**.

The plants investigated were selected based on their ethnobotanical usage as anthelmintics and probably accounted for the relatively lower number of plant extracts with noteworthy antifungal activity (MIC/MFC < 1 mg/ml). Also, pathogenic fungi infection are more resistant to most therapies used against other infections such as bacteria **(SLEIGH and TIMBURG, 1998)**. Antifungal activity screening of plant extracts by other workers also showed that pathogenic fungi are quite resistant to most plant extracts **(MOTSEI *et al.*, 2003; BUWA and VAN STADEN, 2006)**. Furthermore, factors such as low concentration of bioactive compound(s), antagonistic effects of other compound(s), and susceptibility of the test fungal strain are possible reasons for the rather poor activity observed in this study.

## 4.4 Conclusions

Due to the immune-suppressing activity of helminth infection, leading to the development of microbial diseases, and the importance of investigating medicinal plants for potential pharmacological activities, the plant extracts were screened for antimicrobial activity. Eight plant species exhibited noteworthy antimicrobial activity. Extracts from *L*. *sericea* had the best antibacterial activity in this study. It exhibited broad spectrum antibacterial activity with the lowest MIC value of 0.025 mg/ml against *B*. *subtilis* and *S*. *aureus*. *H*. *colchicifolia* and *O*. *basilicum* were the only plant species with noteworthy antifungal activity both having an MIC value of 0.78 mg/ml. General, ethanol extracts had best antimicrobial activity while the water extracts showed low or no antimicrobial activity at the various concentrations tested.

Considering that the plants were selected based on their recorded anthelmintic ethnobotanical usage, the antimicrobial activity of the plant extracts was remarkable. These results further justify the validity of investigating plant extracts in a variety of *in vitro* bioassays, especially during preliminary tests, because plants contain numerous chemicals with potential pharmacological activity. Further investigation using *in vivo* bioassay as well as determining the safety of the active plant extracts will also be necessary for valid conclusion to be reached on their potential as sources of novel antimicrobial agents.

# CHAPTER 5: SCREENING FOR CYCLOOXYGENASE INHIBITORY ACTIVITY

## 5.1 Introduction

Cyclooxygenase (COX) enzymes catalyse the conversion of arachidonic acid to any of the three main groups of prostanoids namely: prostaglandins, prostacyclins, and thromboxanes, which are involved in inflammatory responses **(DuBOIS *et al.*, 1998; KAM and SO, 2009)**. Inflammation is a localized protective reaction of body cells or tissues in response to physical trauma, chemical irritations such as acids, ultraviolet radiations and burns as well as infections caused by bacteria and parasites **(IWALEWA *et al.*, 2007)**. Generally, inflammatory processes are complex, involving a variety of enzyme activation, chemical mediator release, tissue breakdown and repair **(VANE and BOTTING, 1995; FERRERO-MILIANI *et al.*, 2007)**. Lipoxygenase (LOX) is also capable of metabolizing arachidonic acid to produce leukotrienes in an alternative pathway **(SVEINBJÖRNSSON *et al.*, 2008)**. The LOX pathway is equally important because the inhibition of leukotriene synthesis prevents inflammation that causes asthma and bronchitis **(CELOTTI and LAUFER, 2001)**.

Parasitic infections such as helminthiasis are associated with gastrointestinal inflammation and exhibits various symptoms such as abdominal pain, heat and swelling **(PARHAM, 2000)**. In many infected hosts, parasitism may lead to severe immuno-pathological complications such as granulomatous disease and organ failure **(MAIZELS and YAZDANBAKHSH, 2003)**. In addition, it has been established that a number of parasitic organisms produce prostaglandins in the same way as their mammalian hosts, and by similar enzymatic mechanisms **(BELLEY and CHADEE, 1995)**. This probably accounts for the pathogenesis of many parasitic diseases **(LIU and WELLER, 1990; BELLEY and CHADEE, 1995)**. Many plants traditionally used as medicines have been shown to posses multi-pharmacological activity and most disease states occur with multiple symptoms **(LAUPATTARAKASEM *et al.*, 2003; HOUGHTON *et al.*, 2007)**.

### 5.1.1 Overview of inflammation

The occurrence of inflammatory diseases is increasing and affecting more people in both developed and developing countries **(JÄGER and VAN STADEN, 2005)**. Inflammatory responses are major contributing factors to the exacerbation of many disease states such as arthritis, asthma and cardiovascular disease that constitute a serious health problem globally **(CARRETERO *et al.*, 2008)**. Inflammation arises as a result of an organism attempting to remove injurious stimuli, as well as to initiate the healing process of infected cells and tissues **(FERRERO-MILIANI *et al.*, 2007)**. These are classified as either acute or chronic. Acute inflammation is characterized by rapid onset and short duration involving exudation of fluids and plasma proteins to the infected area. Chronic inflammation has a delayed response and prolonged duration with the presence of lymphocytes and macrophages leads to fibrosis and tissue necrosis **(IWALEWA *et al.*, 2007)**.

During inflammation, arachidonic acid is released from cell membranes by phospholipase $A_2$ ($PLA_2$) under stimulation **(SCOTT *et al.*, 1999)**. Once released, arachidonic acid is initially oxygenated by COX or LOX to form unstable intermediate prostanoids (by inserting molecular oxygen at carbon 5) and finally catalysed by COX or LOX enzyme to form the eicosanoid products **(BELLEY and CHADEE, 1995)**. Chemical mediators such as prostaglandins (PG), nitric oxide (NO), cytokines and leukotrienes (LK) are released from mast cells, platelets, neutrophils and macrophages or as plasma proteins. They bind to specific target receptors on the stimulated cells and tissues, and may increase vascular permeability, promote neutrophil chemotaxis, stimulate smooth muscle contraction, increase direct enzymatic activity, induce pain and mediate oxidative damage **(COLEMAN, 2002)**. During cyclooxygenase reaction, two molecules of oxygen are added to arachidonic acid, forming a bicyclic endoperoxide with a further hydroperoxy group in position 15 that leads to the formation of an intermediate prostaglandin $G_2$ ($PGG_2$). The hydroperoxide is then reduced by a functionally coupled peroxidase reaction to form prostaglandin $H_2$ ($PGH_2$), an unstable intermediate from which all other prostanoids are derived by a variety of enzymatic reactions **(SMITH and SONG, 2002)**.

Chemical mediators are also referred to as pro-inflammatory fundamental factors because they determine the severity of inflammation based on the duration of the inflammatory stimulants. The release of some of the cytokines such as Interleukin (IL) -3 ,-4, -5, -6, -10, -13 act as anti-inflammatory mediators within the cells and are beneficial to the body **(ESCH and STEFANO, 2002)**. Cytokines such as IL-17A and IL-7F significantly contribute to psoriasis in humans **(TRIGONA *et al.*, 2009)**. In view of the beneficial and detrimental activity of these chemical mediators, they present an important pathway target through which body inflammatory disorders can be eradicated or ameliorated **(ESCH and STEFANO, 2002; YEDGAR *et al.*, 2007)**.

### 5.1.2 Cyclooxygenase (COX) enzymes

COX enzyme is also known as prostaglandin endoperoxide synthase (PGHS). It is the main enzyme involved in the production of prostaglandins from the substrate arachidonic acid **(KAM and SO, 2009)**. Two distinct isoforms have been identified namely: COX-1 and COX-2. In 2002, a splice variant of COX-1 was discovered in canine brain cells and given various names such as COX-3, COX-1b or COX-1v **(BOTTING, 2006; KAM and SO, 2009)**. Due to the important and distinct roles that COX-1 and COX-2 enzymes play in inflammatory processes **(WILLIAMS and DuBOIS, 1996)**, both enzymes were considered in this investigation.

COX-1 enzyme has a molecular weight of 71 kDa and was first isolated from sheep seminal vesicles **(HEMLER and LANDS, 1976)**. This enzyme is present at relatively constant levels in many tissues and cells, and is regarded as a “house-keeping” gene **(CROFFORD, 1997; MORITA, 2002)**. The COX-1 enzyme concentration slightly increases between 2- to 4-fold in response to stimulants such as hormones and growth factors **(DeWITT, 1991)**. COX-1 enzyme produces prostaglandins involved in the regulation of normal kidney and stomach functions as well as vascular homeostasis **(KLIMP *et al.*, 2001)**. It is also present in platelet aiding in thromboxane $A_2$ ($TXA_2$) production which prevents inappropriate bleeding **(FUNK *et al.*, 1991)**. Consequently, the prostanoids produced by COX-1 are important for the maintenance of regular cellular physiological functions **(BOTTING, 2006)**.

The COX-2 enzyme has a molecular weight of 71 kDa and was discovered in 1988 by Daniel Simmons **(BOTTING, 2006)**. During stable conditions, the COX-2 enzyme is present in low concentrations in most cells and is regulated by factors such as cytokines, intracellular messengers and substrate (arachidonic acid) availability. The COX-2 enzyme is particularly responsive to chemical mediators of inflammation **(MORITA, 2002)**. This enzyme is a source of prostaglandins in most acute inflammation and is mainly present in the nuclear envelope, brain and endoplasmic reticulum **(VANE *et al.*, 1998)**.

### 5.1.3 Treatment of inflammation

Anti-inflammatory refers to the property of a substance or treatment to reduce inflammation. Anti-inflammatory agents are categorised into two groups namely: the non-steroidal and steroidal or glucocorticoid drugs. The non-steroidal anti-inflammatory drugs (NSAIDs) have been used by humans in various forms for over 3500 years **(VANE, 2000)**. The inhibitory activity of NSAIDs is responsible for the anti-inflammatory, antipyretic and analgesic properties exhibited by these agents. These drugs act by inhibiting the activity of COX enzymes, the rate-limiting step in the conversion of arachidonic acid to prostaglandins **(VANE and BOTTING, 1995)**. For instance, NSAIDs such as aspirin inhibit COX enzymes by irreversible acetylation of serine 530 in the COX-1 active site or serine 516 in the COX-2 active site **(VAN DER OUDERAA *et al.*, 1980)**. Glucocorticoids also inhibit the two main products (prostaglandins and leukotrienes) of inflammatory processes. These are available in most vertebrate animal cells and functions by binding to the glucocorticoid receptor (GR). The formation of the GR complex leads to the synthesis of anti-inflammatory proteins that block the expression of pro-inflammatory proteins in the cytosol **(RHEN and CIDLOWSKI, 2005)**.

Both steroidal and non-steroidal anti-inflammatory chemotherapy presently used in treatment of inflammation have various limitations **(CARRETERO *et al.*, 2008)**. For instance, steroids such as prednisone, prednisolone, and hydrocortisone which are naturally secreted by the adrenal glands or can be chemically synthesized are associated with side effects such as immunosuppression, muscle breakdown, glaucoma, increased blood pressure and retarded growth in children. NSAIDs such

as ibuprofen and aspirin have been implicated in gastro-toxicity, anti-thrombotic action as well as Reye's syndrome in children **(WILSON, 1991; BOTTING, 2006)**.

Studies have shown that medicinal plants potentially provide a useful source of new effective anti-inflammatory agents **(TAYLOR *et al.*, 2001)**. Globally, many plants have been successfully screened as potential sources of anti-inflammatory agents **(TUNÓN *et al.*, 1995; LAUPATTARAKASEM *et al.*, 2003; KAILEH *et al.*, 2007; MENICHINI *et al.*, 2009)**. Likewise, many South African medicinal plants have also shown promising results as sources of potential anti-inflammatory agents **(TAYLOR *et al.*, 2001; IWALEWA *et al.*, 2007)**. Plant secondary metabolites such as flavonoids, alkaloids, essential and volatile oils have the tendency to modify activities of inflammatory cells **(IWALEWA *et al.*, 2007)**. Prostaglandin synthesis inhibition can be evaluated using the COX bioassays, an example of mechanism-based assays utilizing sub-cellular structures for inhibition detection **(HAMBURGER and HOSTETTMANN, 1991)**. The inhibitory effect of plant extracts against COX enzyme as a target of the inflammatory process was evaluated.

## 5.2 Materials and methods

Extract preparation and the bioassays (COX-1 and -2) were performed as detailed below.

### 5.2.1 Extract preparation

Plant extraction was carried out as described in **Section 2.2.2.** For each plant sample investigated, dried materials were redissolved in 80% ethanol for organic solvent extracts (PE, DCM and EtOH) and in water for aqueous extracts. The extracts were redissolved at 10 mg/ml with the above mentioned solvents.

### 5.2.2 Enzyme and substrate preparation

COX-1 and COX-2 enzymes were obtained from Sigma-Aldrich, USA. COX-1 was isolated from ram seminal vesicles while human recombinant COX-2 was isolated from a *Baculovirus* over-expression system in *Sf* 21 cells. The enzyme activity

concentration was 5838 units/ml for COX-1 and 625 units/ml for COX-2. The COX enzymes were diluted with Tris(hydromethyl)aminomethane (TRIS) storage buffer (pH 8.0) **(Appendix 3)** to obtain 50 µl of 75 units enzyme concentration per aliquot. The prepared COX enzymes (50 µl, 75 units) were stored in an ultra-freezer at -70 ºC until required.

The substrate $^{14}$C-arachidonic acid with a specific activity of 57 mCi/mmol and concentration of 0.1 mCi/ml was obtained from Amersham, GE Healthcare, UK. One hundred microlitres of the radio-labelled arachidonic acid was diluted with 6.75 µl of unlabelled arachidonic acid (Sigma-Aldrich, Germany) to obtain the final concentration (16 Ci/mol, 30 µM) required for the bioassay.

### 5.2.3 *In vitro* COX-1 inhibitory bioassay

The COX-1 inhibitory activity of the plant extracts was evaluated according to the method of **WHITE and GLASSMAN (1974)** as modified by **JÄGER et al. (1996)** with slight modifications. For the organic extracts, an aliquot of 2.5 µl from the 10 mg/ml extract was diluted with 17.5 µl distilled water in Eppendorf tubes while an aliquot of 20 µl water extracts were added directly to the Eppendorf tubes. The background and solvent blanks contained 17.5 µl distilled water and 2.5 µl of 80% ethanol while a reference anti-inflammatory drug was prepared by adding 2.5 µl of 200 µM indomethacin (Sigma-Aldrich, Germany) with 17.5 µl distilled water. The controls and test samples were prepared in duplicate for the bioassay. Thereafter, 10 µl of 2 N hydrogen chloride (HCl) was added to the background samples to acidify the solution and denature the enzymes.

A co-factor solution was prepared by adding 3 mg *L*-epinephrine (Sigma-Aldrich, Germany) and 3 mg reduced glutathione (Sigma-Aldrich, USA) into 10 ml of 0.1 M TRIS buffer (pH 8.0). One hundred microlitres hematin solution (Sigma-Aldrich, Germany) **(Appendix 3)** were added to the mixture and maintained on ice. The prepared aliquot of 50 µl COX-1 enzyme containing 75 units stored at -70 °C was dissolved in 200 µl of 0.1 M TRIS buffer (pH 8.0) **(Appendix 3)** and activated with 1250 µl co-factor solution. This resultant solution (1500 µl) was pre-incubated on ice at room temperature for 5 min. After incubation, 60 µl of the resultant solution were

added to each prepared test extract sample and controls contained in the Eppendorf tubes and pre-incubated at room temperature for 5 min.

The reaction substrate was prepared by mixing 5 µl of $^{14}$C-arachidonic acid (16 Ci/mol, 30 µM), 5 µl of 0.2% $Na_2CO_3$ and 490 µl of 0.1 M phosphate buffer (pH 7.4) **(Appendix 3)**. Thereafter, the enzymatic reaction was initiated by adding 20 µl of the prepared arachidonic acid (substrate) solution into the enzyme-sample as well as the controls and incubating for 10 min in a water bath at 37 ºC. The final concentrations in the bioassay were 250 µg/ml for the organic solvent extracts and 2 mg/ml for aqueous extracts while the indomethacin was at a final concentration of 5 µM. Each test solution contained 3 units of COX-1 enzyme and 6 µM of the arachidonic acid in the final volume. After incubation, the reaction was terminated by adding 10 µl of 2N HCl to all the reaction tubes with the exception of background test samples. Following this, 4 µl of 0.2 mg/ml unlabelled prostaglandins ($PGE_2$:$PGF_{2\alpha}$ 1:1) (Sigma-Aldrich, Germany) and 1 ml of hexane:1,4-dioxan:acetic acid at a ratio of 70:30:0.2 (v/v/v) was added to each sample.

Prostaglandins and unmetabolized arachidonic acid were separated by column chromatography over silica gel. The silica columns were prepared by packing silica gel 60 (Merck, Germany) with particle size of 0.063-0.200 mm in hexane:1,4-dioxan:acetic acid (70:30:0.2 v/v/v) mixture into Pasteur pipettes reaching a height of 3 cm and stopped at the base with glass wool. The test solutions were applied to individual Pasteur pipettes and the unmetabolized arachidonic acid was eluted with 4 ml of hexane:1,4-dioxan:acetic acid (1 ml added at a time). The prostaglandin products were eluted with 3 ml ethyl acetate:methanol (85:15 v/v) and collected from each column into individual scintillation vials. Scintillation fluid (4 ml) (Beckman Coulter$_{TM}$, USA) was added to the eluate in the scintillation vials and the radioactivity of resultant cocktail was measured using a Multi Purpose Scintillation Counter (Beckman Coulter$_{TM}$ LS 6500, USA). The experiment was repeated twice and replicates of each test solution were used for each experiment. The percentage of prostaglandin synthesis inhibition by the plant extracts were evaluated by comparing the amount of radioactivity present in the sample to that in the solvent blank and background samples using the equation given below:

$$\text{COX inhibition (\%)} = \left\{1 - \left(\frac{DPM_{extract} - DPM_{background}}{DPM_{solvent\ blank} - DPM_{background}}\right)\right\} \times 100$$

where DPM = disintegrations per minute; solvent blank = 17.5 µl water + 2.5 µl EtOH (80%); background = 17.5 µl water + 2.5 µl EtOH (80%) with the added COX enzyme inactivated by HCl before the addition of arachidonic acid (substrate).

### 5.2.4 *In vitro* COX-2 inhibitory bioassay

A similar experimental protocol as described for the COX-1 bioassay with slight modifications, was used for the COX-2 enzyme **(ZSCHOCKE and VAN STADEN, 2000)** The co-factor was prepared by adding 0.3 mg *L*-epinephrine and 0.6 mg reduced glutathione into 10 ml TRIS buffer (pH 8.0), followed by addition of 100 µl hematin solution. The reference anti-inflammatory drug was prepared by mixing 2.5 µl of 8 mM indomethacin and 17.5 µl filtered water which resulted in a final concentration of 200 µM in the bioassay. Further technique employed and evaluation of inhibition (%) is as described for COX-1 above **(Section 5.2.3)**.

In addition, plant extracts that exhibited an inhibition of 70% and above for COX-2 enzyme inhibitory activity were further tested at lower extract concentrations of 5 mg/ml (final concentration: 125 µg/ml) and 2.5 mg/ml (final concentration: 62.5 µg/ml) against both COX-1 and -2 enzymes as described above **(Sections 5.2.2 and 5.2.3)**.

### 5.2.5 Statistical analysis

The level of significant differences in the activity of the investigated plant extracts was determined statistically. The statistical analysis was performed using SPSS software package for Windows (SPSS® version 10.0, Chicago, USA). Analysis was performed within individual extracting solvents and compared at the highest screening concentration of 250 µg/ml and 2 mg/ml for organic and water extracts, respectively.

## 5.3 Results and discussion

A total of 80 extracts were screened for their ability to inhibit both COX-1 and COX-2 enzymes. These results were analyzed using a scheme devised by **TUNÓN *et al*. (1995)** where four levels of inhibitory activity were defined as: below 20% was considered “insignificant”, between 20 and 40% was “low” activity, from 40 to 70% was “moderate” activity, and above 70% was regarded as “high” inhibition at the extract concentration tested.

### 5.3.1 COX-1 inhibitory activity of the investigated plant extracts

The COX-1 inhibition (%) by the 80 plant extracts are presented in **Table 5.1**. Based on activity level definition **(TUNÓN *et al.*, 1995)**, 46 extracts displayed a high COX-1 inhibitory activity, 16 extracts exhibited moderate activity, and 7 extracts had low activity while for 11 extracts inhibition was considered insignificant. Although, the highest COX-1 inhibition (%) was exhibited by the DCM extract of *A*. *oppositifolia* leaves with 99.98% inhibition, it was not significantly different from inhibition of the DCM extracts of *Cotyledon* species (leaves), *C*. *dregei* (roots) *F*. *erigeroides* (Leaves), *Hypoxis* species (corms) and *L*. *sericea* (leaves and stems). A total of 8 extracts namely: *A*. *oppositifolia* twigs (PE and EtOH), *C*. *myricoides* leaves (EtOH and water), *C*. *orbiculata* var. *orbiculata* stems (PE), *H*. *colchicifolia* corms (EtOH) as well as *H*. *hemerocallidea* corms and leaves (EtOH) displayed no COX-1 inhibitory activity (0%) against the COX-1 enzyme.

Non-polar organic solvent extracts showed higher COX-1 inhibition (%) than the polar solvent extracts. The extracting solvents (PE, DCM, EtOH and water) yielded 20 extracts each which were investigated and a total of 16 DCM and 13 PE extracts as well as 11 EtOH extracts exhibited high COX-1 inhibition while only 6 water extracts displayed high COX-1 inhibition. Similar COX inhibition investigations have shown that non-polar solvent extracts generally possess better COX inhibition than water extracts **(JÄGER *et al.*, 1996; FAWOLE *et al.*, 2009b)**. The observed inhibitory activity of the non-polar extracts could be attributed to better extraction of the active principles by the extracting organic solvents.

**Table 5.1:** COX-1 inhibition (%) of the plant extracts in the COX-1 bioassay which was tested at a final concentration of 250 µg/ml and 2 mg/ml for the organic and water extracts, respectively. Values are presented as mean ± standard error, where n = 4.

| Plant species | Plant part | Inhibition (%) | | | |
|---|---|---|---|---|---|
| | | PE | DCM | EtOH | Water |
| *Acokanthera oppositifolia* | Leaves | 98.24±4.55$^{a}$ | 99.98±1.79$^{a}$ | 97.81±6.98$^{a}$ | 48.05±0.73$^{f}$ |
| | Twigs | 0.00$^{f}$ | 31.50±2.67$^{g}$ | 0.00$^{g}$ | 37.69±1.30$^{h}$ |
| *Clerodendrum myricoides* | Leaves | 51.52±2.67$^{e}$ | 76.69±2.13$^{deh}$ | 0.00$^{g}$ | 0.00$^{m}$ |
| | Stems | 68.87±0.75$^{c}$ | 84.36±2.17$^{cd}$ | 10.43±2.66$^{f}$ | 16.26±0.92$^{k}$ |
| *Cotyledon orbiculata* var. *dactylosis* | Leaves | 70.11±3.90$^{c}$ | 97.96±1.47$^{abc}$ | 98.00±1.31$^{a}$ | 27.32±1.55$^{j}$ |
| | Stems | 68.47±2.49$^{c}$ | 58.88±1.09$^{f}$ | 26.45±2.72$^{e}$ | 44.30±0.87$^{fg}$ |
| *Cotyledon orbiculata* var. *orbiculata* | Leaves | 97.54±3.40$^{a}$ | 93.32±3.46$^{a}$ | 67.38±4.00$^{c}$ | 15.07±0.69$^{l}$ |
| | Stems | 0.00$^{f}$ | 62.59±4.96$^{f}$ | 97.11±4.23$^{a}$ | 42.12±0.81$^{gh}$ |
| *Cyathea dregei* | Leaves | 98.03±0.52$^{a}$ | 73.75±2.88$^{e}$ | 99.21±0.80$^{a}$ | 68.01±0.99$^{d}$ |
| | Roots | 91.78±2.69$^{a}$ | 96.06±0.92$^{abc}$ | 77.93±0.12$^{bc}$ | 84.15±1.21$^{bc}$ |
| *Felicia erigeroides* | Leaves | 93.63±1.75$^{a}$ | 99.43±1.12$^{a}$ | 87.59±3.17$^{ab}$ | 61.07±2.14$^{e}$ |
| | Stems | 88.04±1.65$^{a}$ | 71.59±2.28$^{e}$ | 36.14±4.78$^{d}$ | 32.99±1.27$^{i}$ |
| *Hypoxis colchicifolia* | Leaves | 99.29±0.84$^{a}$ | 35.44±2.54$^{g}$ | 98.42±1.16$^{a}$ | 68.06±1.14$^{d}$ |
| | Corms | 93.60±1.76$^{a}$ | 96.40±1.19$^{abc}$ | 0.00$^{g}$ | 63.37±1.20$^{de}$ |
| *Hypoxis hemerocallidea* | Leaves | 60.65±1.31$^{d}$ | 78.38±2.94$^{de}$ | 0.00$^{g}$ | 59.56±1.23$^{e}$ |
| | Corms | 97.13±3.03$^{a}$ | 98.38±1.31$^{ab}$ | 0.00$^{g}$ | 82.97±1.30$^{c}$ |
| *Leucosidea sericea* | Leaves | 96.56±0.66$^{a}$ | 96.44±1.08$^{abc}$ | 90.15±0.29$^{ab}$ | 95.97±1.78$^{a}$ |
| | Stems | 95.40±3.90$^{a}$ | 95.70±2.02$^{abc}$ | 89.67±2.46$^{ab}$ | 89.00±0.86$^{abc}$ |
| *Ocimum basilicum* | Leaves | 61.97±5.89$^{d}$ | 76.74±2.22$^{de}$ | 94.07±2.37$^{ab}$ | 83.58±0.60$^{c}$ |
| *Senna petersiana* | Leaves | 77.62±1.23 $^{b}$ | 85.53±1.87$^{bcd}$ | 99.29±1.23$^{a}$ | 92.47±0.41$^{ab}$ |

Mean values in the same column followed by different letter(s) are significantly different ($P$ = 0.05) according to Duncan's Multiple Range Test.

Extracting solvents: PE = Petroleum ether, DCM = Dichloromethane and EtOH = Ethanol.

COX-1 inhibition (%) by indomethacin at final bioassay concentration of 5 µM was 75.50±2.34.

Organic solvent extracts of the leaves of *A*. *oppositifolia* showed a high COX-1 enzymes inhibitory activity. Previous screening of some Zulu medicinal plants for prostaglandin-synthesis inhibitors yielded a high COX enzyme inhibitory (78%) activity for the EtOH extract of *A*. *oppositifolia* roots **(JÄGER *et al.*, 1996)**. The potential anti-inflammatory activity by the leaf extracts is very important for the sustainable harvesting of the plant for medicinal use. *A*. *oppositifolia* is a member of the family Apocynaceae which are noted for their high alkaloid content accounting for their potent pharmacological activity **(GURIB-FAKIM, 2006)**. Likewise, *L*. *sericea* is a member of the family Rosaceae which are known for a high tannin content. Members of this family have been used as remedies for many diseases in traditional medicine **(TUNÓN *et al.*, 1995)**. All the extracts of the leaves and stems of *L*. *sericea* displayed high COX-1 inhibition. **IWALEWA *et al.* (2007)** identified alkaloids and tannins as examples of plant secondary metabolites with inhibitory activity on the molecular targets of pro-inflammatory mediators in inflammatory responses. Thus, tannins and alkaloids probably accounted for the observed high COX-1 activity in *A*. *oppositifolia* and *L*. *sericea* extracts tested in this study.

Although ethanol and water extracts of *H*. *hemerocallidea* corms displayed no significant inhibitory activity in this study, previous studies of both extracts had demonstrated promising anti-inflammatory activity both *in vitro* **(JÄGER and VAN STADEN, 2005)** and *in vivo* **(OJEWOLE, 2006)** investigations, respectively. Factors such as seasonal variation in chemical composition of plant species **(TAYLOR *et al.*, 2001)** as well as the discrepancies that occur between *in vitro* and *in vivo* extract biological activity **(HOUGHTON *et al.*, 2007)** are possible reasons for the non-activity in *H*. *hemerocallidea* corm extracts in this study.

Both water and organic solvent extracts of *O*. *basilicum* leaves yielded high to moderate COX-1 inhibition. Recent *in vitro* studies on methanolic extracts of *O*. *basilicum* demonstrated anti-inflammatory effects by inhibiting the pro-inflammatory cytokines and mediators in a mitogenic lymptocyte proliferation assay **(SELVAKKUMAR *et al.*, 2007)**. Similarly, significant anti-inflammatory activity by the extract of *O*. *basilicum* have been demonstrated in an *in vivo* test against carrageenan and different other mediator-induced paw oedema in experimental rats

**(SINGH, 1999)**. The COX inhibition of *O*. *basilicum* in this study further confirms the anti-inflammatory activity potentials of the plant extract.

### 5.3.2 COX-2 inhibitory activity of the investigated plant extracts

The COX-2 inhibition (%) of the 80 plant extracts are presented in **Table 5.2**. According to inhibitory activity definition **(TUNÓN *et al.*, 1995)**, 32 extracts displayed high COX-2 inhibitory activity, 22 extracts exhibited moderate inhibitory activity, 10 extracts had low activity and16 extracts had inhibitory activity that was considered insignificant. The most active extract was the DCM extract of *F*. *erigeroides* (stems) with 99% inhibition. Generally, the inhibitory activity against COX-2 enzyme was lower than that of COX-1. This can be attributed to the COX-1 active site being smaller than that of COX-2 **(MORITA, 2002)**. Hence, the smaller active site of COX-1 is more easily inhibited than the larger COX-2 site **(BOTTING, 2006)**.

As observed in the COX-1 bioassay, the non-polar solvent extracts (PE and DCM) inhibited the COX-2 enzyme better than the more polar solvent extracts. High COX-2 inhibition was exhibited by 12 PE and DCM extracts while only 7 EtOH and 1 water extracts displayed similar high COX-2 inhibition. A total of 7 had zero COX-2 inhibition and they were all polar solvent extracts. This observed activity at the low screening concentration (250 µg/ml) was noteworthy because lipophilic compounds which are extracted by non-polar solvents have better resorption through the cell membrane **(ZSCHOCKE *et al.*, 2000)**. Lipophilic compounds are not likely to occur in higher concentrations in water extracts **(TUNÓN *et al.*, 1995)** and are possibly responsible for the high number of non-significant as well as the low activity displayed by most polar solvent extracts.

As observed with the COX-1 bioassay, non-polar solvent extracts of *C*. *myricoides* stems had high inhibitory activity against the COX-2 enzyme. Many species of the genus *Clerodendrum* such as *C*. *trichotomum, C*. *serratum* and *C*. *phlomidis* have been reported to exhibit potent inhibitory effect on pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and Interleukin-1-beta (IL-1*β*) responsible for various inflammatory conditions **(SHRIVASTAVA and PATEL, 2007)**.

**Table 5.2:** COX-2 inhibition (%) of the plant extracts in the COX-2 bioassay which was tested at a final concentration of 250 µg/ml and 2 mg/ml for the organic and water extracts, respectively. Values are presented as mean ± standard error, where n = 4.

| Plant species | Plant part | Inhibition (%) | | | |
|---|---|---|---|---|---|
| | | PE | DCM | EtOH | Water |
| *Acokanthera oppositifolia* | Leaves | 84.71±0.39$^{abc}$ | 80.98±0.26$^{bc}$ | 68.13±2.37$^{c}$ | 0.00$^{f}$ |
| | Twigs | 38.45±3.39$^{h}$ | 72.60±1.05$^{de}$ | 29.62±3.12$^{g}$ | 16.86±0.72$^{c}$ |
| *Clerodendrum myricoides* | Leaves | 6.82±1.13$^{j}$ | 32.78±2.43$^{k}$ | 42.47±0.83$^{f}$ | 0.00$^{f}$ |
| | Stems | 74.50±0.67$^{cde}$ | 70.92±0.55$^{def}$ | 54.22±1.38$^{de}$ | 0.00$^{f}$ |
| *Cotyledon orbiculata* var. *dactylosis* | Leaves | 58.75±2.80$^{fg}$ | 72.58±4.02$^{def}$ | 73.05±3.83$^{bc}$ | 10.45±4.26$^{d}$ |
| | Stems | 53.01±0.91$^{g}$ | 58.50±2.07$^{hı}$ | 32.35±6.95$^{g}$ | 29.17±1.86$^{b}$ |
| *Cotyledon orbiculata* var. *orbiculata* | Leaves | 85.15±2.33$^{abc}$ | 80.78±0.69$^{bc}$ | 66.78±1.21$^{c}$ | 0.00$^{f}$ |
| | Stems | 32.53±3.16$^{i}$ | 46.73±3.08$^{j}$ | 71.76±2.01$^{bc}$ | 25.14±0.98$^{bc}$ |
| *Cyathea dregei* | Leaves | 55.87±1.11$^{fg}$ | 61.10±1.42$^{gh}$ | 71.68±0.99$^{bc}$ | 55.09±0.69$^{a}$ |
| | Roots | 70.08±4.32$^{de}$ | 74.34±1.73$^{cde}$ | 48.56±3.53$^{def}$ | 70.15±2.38$^{a}$ |
| *Felicia erigeroides* | Leaves | 85.39±1.54$^{abc}$ | 87.34±1.73$^{b}$ | 69.38±1.68$^{c}$ | 0.51±0.01$^{f}$ |
| | Stems | 98.27±2.69$^{a}$ | 99.79±2.07$^{a}$ | 78.68±1.93$^{ab}$ | 3.35±0.98$^{e}$ |
| *Hypoxis colchicifolia* | Leaves | 90.05±1.95$^{ab}$ | 49.90±1.05$^{j}$ | 83.51±0.11$^{a}$ | 51.47±3.83$^{a}$ |
| | Corms | 80.96±2.57$^{bcd}$ | 76.17±3.75$^{cde}$ | 0.00$^{i}$ | 7.89±0.59$^{d}$ |
| *Hypoxis hemerocallidea* | Leaves | 37.43±2.10$^{ij}$ | 55.21±1.28$^{i}$ | 19.87±0.33$^{h}$ | 2.70±0.86$^{e}$ |
| | Corms | 74.16±2.47$^{cde}$ | 69.12±1.33$^{ef}$ | 0.00$^{i}$ | 25.71±1.18$^{bc}$ |
| *Leucosidea sericea* | Leaves | 91.10±2.56$^{ab}$ | 86.58±1.32$^{b}$ | 55.82±1.59$^{d}$ | 19.99±4.43$^{bc}$ |
| | Stems | 90.76±1.83$^{ab}$ | 87.10±2.32$^{b}$ | 72.27±2.00$^{bc}$ | 0.00$^{f}$ |
| *Ocimum basilicum* | Leaves | 94.42±0.77$^{ab}$ | 67.10±2.32$^{fg}$ | 85.72±2.43$^{a}$ | 30.19±0.99$^{b}$ |
| *Senna petersiana* | Leaves | 65.15±2.27$^{ef}$ | 77.74±0.13$^{cd}$ | 47.75±3.80$^{ef}$ | 56.80±1.57$^{a}$ |

Mean values in the same column followed by different letter(s) are significantly different ($P$ = 0.05) according to Duncan's Multiple Range Test.

Extracting solvents: PE = Petroleum ether, DCM = Dichloromethane and EtOH = Ethanol.

COX-2 inhibition (%) by indomethacin at final bioassay concentration of 200 µM was 69.10±2.44.

The *in vivo* anti-inflammatory activity of the EtOH extract of *C*. *serratum* roots has also been demonstrated in previous studies **(NARAYANAN *et al.*, 1999)**. Closely related plants such as different plant species of the same genus are often known to possess the same chemical compounds **(JÄGER *et al.*, 1996)**, the presence of similar bioactive compound(s) in these different species in the genus *Clerodendrum* possessing anti-inflammatory potentials cannot be ruled out. This probably accounted for the high COX-2 inhibition exhibited by the non-polar extracts of *C*. *myricoides* stems.

Organic solvent extracts of *S*. *petersiana* exhibited high to moderate COX-2 enzyme inhibition activity. Studies by **TSHIKALANGE *et al*. (2005)** reported the presence of luteolin in *S*. *petersiana* which was subsequently isolated from the seeds. Flavonoids particularly luteolin have been reported to inhibit pro-inflammatory enzymes which probably contribute to their anti-inflammatory activity **(LÓPEZ-LÁZARO, 2009)**. According to **JÄGER *et al.* (1996)**, a species containing a known anti-inflammatory compound has the potential to inhibit inflammatory processes. The presence of flavonoids such as luteolin in *S*. *petersiana* probably accounted for the high COX-2 inhibitory activity observed in the DCM extract of this species.

The high to moderate inhibitory activity exhibited by most leaf extracts against COX-2 inhibition is important for conservation of medicinal plant species. For example, extracts from the leaves of plant species such as *H*. *colchicifolia* and *C. dregei* exhibited better COX-2 inhibition activity than the underground parts. Leaves can be harvested sustainably while utilizing these plants as medicine without the inherent survival threat associated with destructive harvesting of underground parts such as corms and roots. This observed high COX-2 inhibitory activity of the leaf extracts was in line with the suggestion for the need to investigate non-destructive medicinal plant parts for possible noteworthy similar pharmacological activity as present in the underground parts **(ZSCHOCKE *et al.*, 2000)**.

A high number of extracts inhibited both the COX-1 and -2 enzymes. This denotes the potentials of these extracts to reduce inflammation, and they are probably employed in traditional medicine to reduce pain which is associated with helminth infections. Traditional medicine lack scientific investigations to determine the cause

of diseases, as a result, the common symptoms that are observed provide a clue on plant material used and other treatment regimes employed **(HEWSON, 1998)**.

### 5.3.3 Dose responses of plant extracts in COX-1 and -2 bioassays

The COX percentage inhibition in both COX-1 and COX-2 bioassays of the extracts at three concentrations are depicted in **Figures 5.1** and **5.2**, respectively. The dose responses of the only water extract (*C*. *dregei* roots) with high (> 70%) COX-2 inhibition at maximum screening was not included in the figures. Water extracts were screened at 2, 1 and 0.5 mg/ml and *C*. *dregei* water displayed 84, 59 and 30% COX-1 inhibition while COX-2 was 70, 50 and 0%. Generally, a dose-dependant inhibitory activity was exhibited by the plant extracts. A similar trend was reported for the anti-inflammatory investigation of twelve medicinal plants used against gastro-intestinal ailments in South Africa **(FAWOLE *et al.*, 2009b)**.

Apart from *F*. *erigeroides* leaves and stems, and *L*. *sericea* leaves, all PE extracts exhibited a dose-dependant COX-1 enzyme inhibitory activity as depicted in **Figure 5.1 (A)**. For the DCM extracts, *S*. *petersiana* (leaves) and *C*. *dregei* (roots) had approximately the same level of COX-1 inhibition regardless of the extract concentration. All the other DCM extracts were dose-dependant as shown in **Figure 5.1 (B)**. The COX-1 inhibition of the ethanol extracts varied with changes in concentration of extracts as depicted in **Figure 5.1 (C)**.

In the COX-2 bioassay, with the exception of the DCM extract of *S*. *petersiana* (leaves), all other extracts exhibited a dose-dependant COX-2 inhibitory activity as shown in **Figure 5.2**. At the lowest concentration (62.5 µg/ml) assayed, only five extracts namely: leaves of *C*. *orbiculata* var. *dactylopsis* (EtOH), *H*. *colchicifolia* (PE), *L*. *sericea* (PE), *F*. *erigeroides* (DCM), and *S*. *petersiana* (DCM) showed high inhibition.

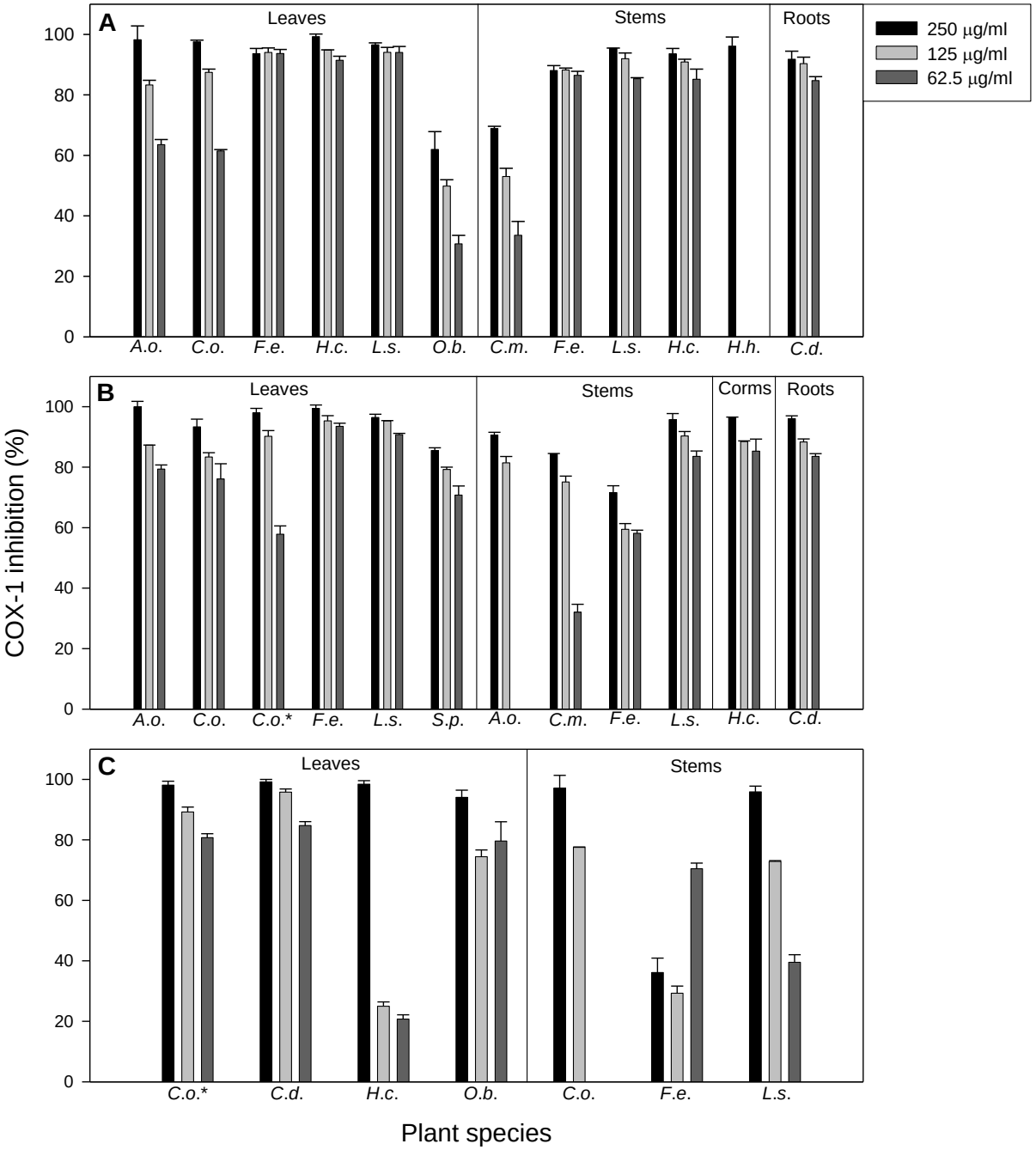


**Figure 5.1:** COX-1 inhibitory activity (%) at three concentrations of the plant extracts that had high (> 70%) COX-2 inhibition at the highest screening concentration; (A) petroleum ether, (B) dichloromethane and (C) ethanol extracts. COX-1 inhibition (%) of indomethacin was 78.50±3.38.

*A.o.* - *Acokanthera oppositifolia*, *C.m.* - *Clerodendrum myricoides*, *C.o.* - *Cotyledon orbiculata* var. *orbiculata*, *C.o.** - *Cotyledon orbiculata* var. *dactylopsis*, *C.d.* - *Cyathea dregei*, *F.e.* - *Felicia erigeroides*, *H.c.* - *Hypoxis colchicifolia*, *H.h.* - *Hypoxis hemerocallidea*, *L.s.* - *Leucosidea sericea*, *O.b.* - *Ocimum basilicum*, *S.p.* - *Senna petersiana*.

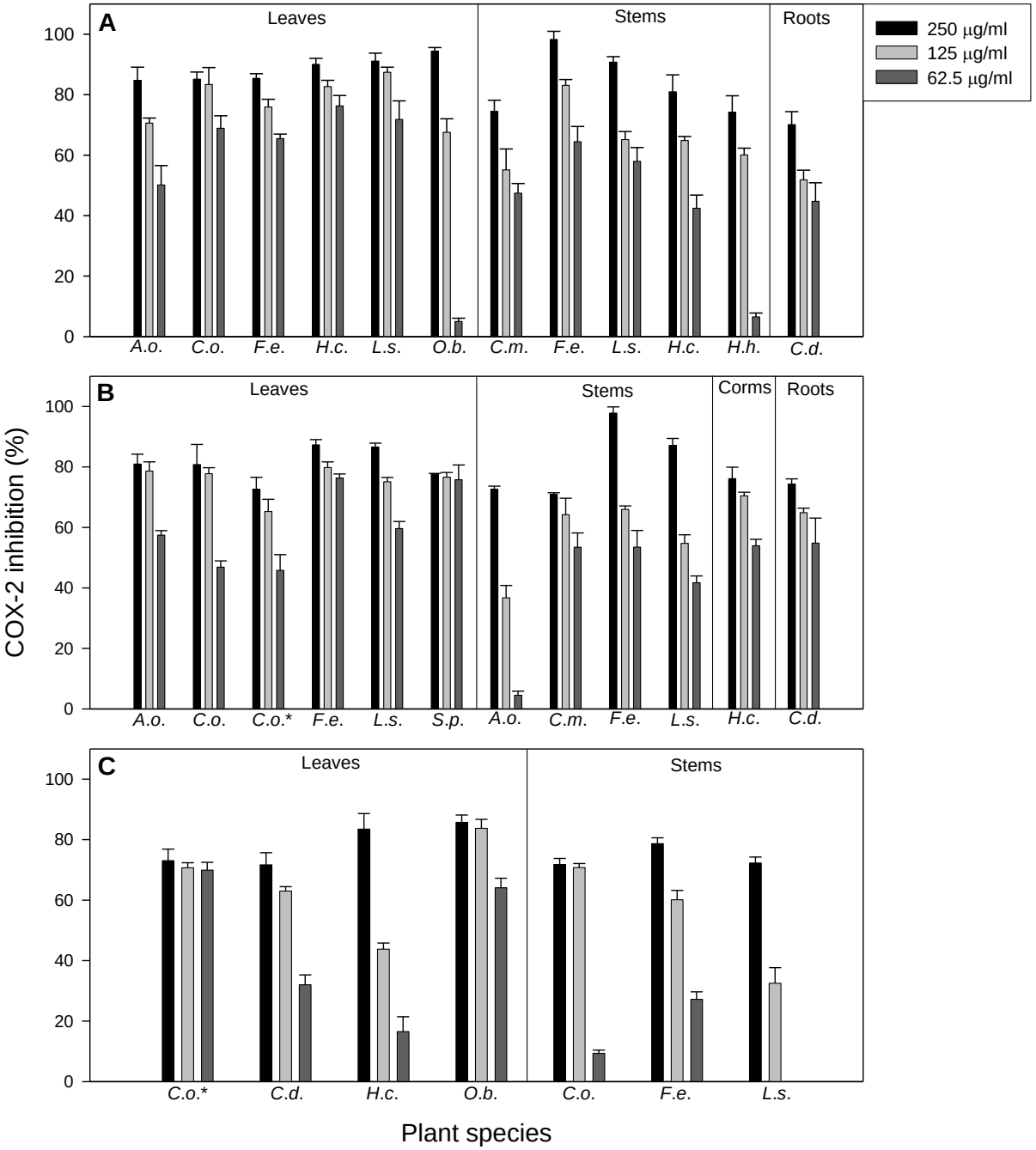


**Figure 5.2:** COX-2 inhibitory activity (%) at three concentrations of the plant extracts that had high (> 70%) COX-2 inhibition at the highest screening concentration; (A) petroleum ether, (B) dichloromethane and (C) ethanol extracts. COX-2 inhibition (%) of indomethacin was 65.10±3.54.

*A.o.* - *Acokanthera oppositifolia*, *C.m.* - *Clerodendrum myricoides*, *C.o.* - *Cotyledon orbiculata* var. *orbiculata*, *C.o.** - *Cotyledon orbiculata* var. *dactylopsis*, *C.d.* - *Cyathea dregei*, *F.e.* - *Felicia erigeroides*, *H.c.* - *Hypoxis colchicifolia*, *H.h.* - *Hypoxis hemerocallidea*, *L.s.* - *Leucosidea sericea*, *O.b.* - *Ocimum basilicum*, *S.p.* - *Senna petersiana*

## 5.4 Conclusions

Generally, the extracts inhibited the COX-1 enzyme better than the COX-2. The non-polar extracts were more active than polar extracts for both enzymes. Organic solvent extracts of *A*. *oppositifolia* leaves, *C*. *orbiculata* var. *dactylopsis* leaves, *C*. *dregei* leaves and roots, *F*. *erigeroides* leaves, *H*. *hemerocallidea* corms, *L*. *sericea* leaves and stems, *O*. *basilicum* leaves and *S*. *petersiana* leaves had three or more extracts inhibiting COX-1 by greater than 70% in the COX-1 bioassay. Similar high COX-2 percentage inhibition was obtained for organic solvent extracts of *C*. *dregei* roots, *F*. *erigeroides* stems, and *L*. *sericea* stems in the COX-2 bioassay.

The inhibitory activity of most extracts were dose dependent as lower activity was observed at lower extract concentrations. Organic solvent extracts of *C*. *dregei* roots, *F*. *erigeroides* leaves and stems as well as *L*. *sericea* leaves and stems showed the most promising COX inhibitory activity. Further investigations will be required to eliminate the possibility of false positive results by these extracts. The removal of tannins from the plants might be necessary as these compounds are known to denature or destroy the enzymes **(TUNÓN *et al.*, 1995)**.

COX-2 inhibition is attributed with fewer side effects and is important for inflammation treatment associated with many diseases **(MORITA, 2002; BOTTING, 2006)**. Hence, possible isolation and identification of active compounds of plant species such as *F*. *erigeroides*, *L*. *sericea* and *O*. *basilicum* that exhibited significant inhibitory activity against the COX-2 enzyme will be necessary. Inflammation is a complex process that involves other enzymes and reactions **(VANE and BOTTING, 1998)**. Thus, the COX enzymes inhibition by the plant extracts give an indication of the anti-inflammatory potentials of the plant. The effectiveness of these species and isolated compound(s) against other enzymes such as LOX involved in the LOX pathway during the inflammatory syndrome also requires investigation.

# CHAPTER 6: PHYTOCHEMICAL ANALYSIS

## 6.1 Introduction

Phytochemicals are chemicals that naturally occur in plants **(BRIELMANN *et al.*, 2006)**. In plants, the metabolic processes can be classified into primary and secondary metabolism, which are often interconnected to one another **(GURIB-FAKIM, 2006)**. Primary metabolites such as phyto-sterols, nucleotides, amino acids and organic acids are required for basic life processes **(WINK, 2003)**. In addition, plants synthesize and accumulate a vast group of compounds known as secondary metabolites that are expressed in terms of ecological and biochemical differentiation as well as species diversity **(GURIB-FAKIM, 2006; LEWINSOHN and GIJZEN, 2009)**. Although, secondary metabolites are mostly present in low quantities and have no recognized role in basic life processes such as photosynthesis, and the growth and development that are associated with primary metabolites, they are mainly involved in the plant's interaction and survival in their environment **(CROZIER *et al.*, 2000; NAMDEO, 2007)**. The biological roles of most secondary metabolites remain obscure **(LEWINSOHN and GIJZEN, 2009)**, however, studies have shown that they protect plants against ultra violet radiations, serve as attractants for pollinators, and impediments against plant herbivores and pathogens **(WINK, 1999; DEWICK, 2002)**.

Plants are characterized by the production of a vast and highly diverse reservoir of secondary metabloites **(WINK, 2003)**. Although these chemicals are present in most plants, similar metabolites may be found in highly divergent plant groups while others may be peculiar to only a species, genus or family. This irregular occurrence and chemical variation among plants has been linked to the evolutionary relationship that existed between the plants and their associated herbivores and pathogens **(NYMAN and JULKUNEN-TIITTO, 2005)**. In addition, the production and accumulation of these metabolites are regulated in space and time, as well as being specific to certain cells and organelles **(WINK, 1999)**.

### 6.1.1 Medicinal activity of plant secondary metabolites

For centuries, mankind has utilized plant secondary metabolites as dyes, fragrances, stimulants, insecticides, vertebrate and human poisons, as well as for their therapeutic values **(CROZIER *et al.*, 2006)**. Plants are currently seen as potential sources of novel natural drugs and antibiotics **(CROZIER *et al.*, 2000; DEWICK, 2002)**. Many of the secondary metabolites are biologically active having phyto-pharmaceutical uses. Pharmaceutically significant secondary metabolites include alkaloids, glycosides, flavonoids, volatile oils, tannins and resins **(NAMDEO, 2007)**.

Based on their biosynthetic origins, plant secondary metabolites are broadly divided into three major groups, namely phenolics, alkaloids and saponins (steroidal or triterpenoid glycosides) **(CROZIER *et al.*, 2006)**. These metabolites have been exploited for their therapeutic values in several ways. Characteristics, distribution and therapeutic application of these metabolite groups are discussed below.

#### 6.1.1.1 Phenolics

Phenolic compounds are characterized by the presences of at least one aromatic ring with one or more hydroxyl groups attached. Phenolics are widely represented in the plant kingdom and approximately 8000 phenolic structures have been identified **(STRACK, 1997)**. The phenolics, as a group, include flavonoids and other related polyphenolic compounds (tannins). Flavonoids are the most common of the phenolics occurring throughout the plant kingdom **(HARBORNE, 1993)**. Flavonoids are further divided into sub-groups such as flavones, flavonols, flavan-3-ols and anthocyanidins **(CROZIER *et al.*, 2006)**. Tannins are common to vascular plants and exist primarily within woody tissues, but may also be present in leaves, flowers and seeds. Tannins are found either as condensed or hydrolysable forms **(BRIELMANN *et al.*, 2006)**. Condensed tannins are the most abundant polyphenols found in many plant families and may constitute up to 50% of the dry weight of the leaves **(LEVIN, 1976)**. Most tannins are extremely astringent rendering plant tissues inedible. Due to their astringency, they precipitate or bind to proteins causing detrimental effects in the digestive tract of livestock and are referred to as anti-nutritional compounds **(GITHIORI *et al.*, 2006)**.

Pharmacological activities of flavonoids such as anti-inflammatory, antimicrobial, antioxidant and anticancer activities have been extensively reported **(TAPAS *et al.*, 2008; LÓPEZ-LÁZARO, 2009)**. Flavonoids such as hesperidin and rutin are used for decreasing capillary fragility, and quercetin is commonly utilized for its anti-diarrhoeal property **(GURIB-FAKIM, 2006)**. A recent study also reported the anthelmintic activity of flavones isolated from *Struthiola argentea* **(AYERS *et al.*, 2008)**. The medicinal properties of tannins, especially their anti-parasitic activity against gastro-intestinal nematodes **(ATHANASIADOU *et al.*, 2001; HOSTE *et al.*, 2006)**, antimicrobial **(AKIYAMA *et al.*, 2001; FUNATOGAWA *et al.*, 2004)**, as well as antioxidant and anti-human-immunodeficiency-virus (HIV) activities **(NOTKA *et al.*, 2004)** have been extensively documented.

### 6.1.1.2 Saponins

Saponins are a chemically diverse group widely distributed in over 100 vascular plant families **(YOSHIKI *et al.*, 1998)**. Saponins are structurally related compounds containing a steroid or triterpenoid aglycone (sapogenin). They are linked to one or more oligosaccharide moieties by a glycosidic linkage **(MAKKAR *et al.*, 2007)**. The steroidal saponins are mostly found in monocotyledonous angiosperms while triterpenoid saponins are mainly associated with dicotyledonous angiosperms **(BRUNETON, 1999)**. The presence of both polar (sugar) and non-polar (steroid or triterpene) groups is responsible for the strong surface-active properties of saponins **(MAKKAR *et al.*, 2007)**. The presence of these groups and the variability of their structures as well as the attachment positioning has been studied with respect to the physiological, immunological and pharmacological properties of saponins **(FRANCIS *et al.*, 2002)**.

Most of the biological potential of saponins is attributed to their action on cell membranes as they possess the ability to form pores in membranes resulting in their frequent use in physiological research **(PLOCK *et al.*, 2001; FRANCIS *et al.*, 2002)**. Saponins have been reported to possess antimicrobial, antioxidant and molluscicidal activities as well as having haemolytic potential **(FRANCIS *et al.*, 2002; MAKKAR *et al.*, 2007)**.

#### 6.1.1.3 Alkaloids

Alkaloids are a diverse group of low molecular weight organic bases containing secondary, tertiary or cyclic amines **(MAKKAR *et al.*, 2007)**. As a result, alkaloids are highly soluble in most acidic solutions to form water soluble salts. Alkaloids form the largest single class of plant secondary metabolites, occurring in approximately 20% of all plant species **(ZULAK *et al.*, 2006; MAKKAR *et al.*, 2007)**. Many of the earliest compounds isolated from plants such as morphine, cocaine and quinine are alkaloids.

Many of the approximately 12 000 known alkaloids have been exploited as pharmaceuticals, stimulants, narcotics and poisons **(WINK, 1998)**. Plants containing alkaloids are well known for their pharmacological activity. Cocaine, morphine, quinine and strychnine are examples of plant-derived alkaloids with potent pharmacological activity **(BRIELMANN *et al.*, 2006)**. Cocaine, from *Erythroxylum coca*, is used as a local anesthetic and as a potent central nervous system stimulant, while strychnine, derived from *Strychnos* bark, is a nerve stimulant. Papaverine, which is obtained from opium, is a smooth muscle relaxant **(POLYA, 2003)**. Nicotine, a product from *Nicotiana tabacum*, is used as a powerful insecticide, while quinine, which was isolated from the bark of *Cinchona* species, as well as artemisinin, obtained from *Artemisia annua* are well known anti-malarial agents **(BRIELMANN *et al.*, 2006)**.

### 6.1.2 Application of Thin Layer Chromatography (TLC) for chemical profiling

Thin layer chromatography (TLC) is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents **(WAGNER *et al.*, 1984)**. It is a quick, inexpensive micro-scale technique that can be used to analyse and compare the components present in mixtures such as plant extracts and drugs. The chemical composition of a mixture can be identified when compared with a known standard compound. In addition, the resultant chemical profiles can be visualized under ultra-violet radiation where the displayed migration distances as well as intensities of characteristic zones can be use for identification of possible compounds in the mixture.

## 6.2 Materials and methods

Plant material was collected as described in **Section 2.2.1**. Due to the high diversity in chemical composition of secondary metabolites, different methods of extraction and assays were employed to determine the metabolites. To prevent deterioration and decomposition of the metabolites, fresh extracts were used for each assay.

### 6.2.1 Extract preparation

Phenolic compounds were extracted from dry plant material as described by **MAKKAR (2000)**. Ground plant materials (2 g) were extracted with 20 ml of 50% aqueous methanol (MeOH) by sonicating in water for 20 min. Extracts were filtered *in vacuo* through Whatman No.1 filter paper and used in the assays outlined below.

For TLC chemical profiling, 1 g of the ground plant material was extracted in 10 ml acetone in a sonication bath for 30 min. The extracts were filtered *in vacuo* through Whatman No.1 filter paper and air-dried under a stream of cold air. Acetone extract compounds across a wide polarity range from plant material **(ELOFF, 1998b)**.

### 6.2.2 Folin-Ciocalteu assay for total phenolics

The Folin-Ciocalteu (Folin-C) assay, using gallic acid as a standard, was employed for the evaluation of total phenolics **(MAKKAR, 2000)**. Folin-C phenol reagent (2N) (Sigma-Aldrich, Germany) was made up with an equal volume of distilled water in a dark bottle, to obtain a 1N Folin-C reagent required for the assay. Gallic acid (Sigma-Aldrich, USA) was prepared in water at 0.1 mg/ml standard solution. In triplicate, 50 µl of MeOH extracts were added to 950 µl distilled water in glass test tubes, followed by 500 µl Folin-C reagent (1 N) and 2.5 ml sodium carbonate (2%). Similarly, a blank that contained 50% aqueous MeOH (50 µl) in place of the extract was also prepared. The test mixtures were incubated for 40 min at room temperature and the absorbance at 725 nm was measured using a UV-visible spectrophotometer (Varian Cary 50, Australia). A standard curve was used to convert the measured absorbance readings to total phenolic concentration, expressed as gallic acid equivalents (GAE) per gram dry matter.

### 6.2.3 Rhodanine assay for gallotannins

Gallotannin content was determined using the rhodanine assay as described by **MAKKAR (2000)**. The reagent rhodanine (Fluka, Germany) was prepared in methanol, and gallic acid (0.1 mg/ml) prepared in 0.2 N sulphuric acid was used as the standard solution. In triplicate, 50 µl of extracts were added to 950 µl distilled water in glass test tubes, followed by the addition of 100 µl sulphuric acid (0.4 N) and 600 µl rhodanine. After 5 min at room temperature, 200 µl potassium hydroxide (0.5 N) was added to the reaction mixture. The mixture was further incubated for 2½ min at room temperature after which 4 ml distilled water was added. The reaction mixture was incubated a further 15 min at room temperature. The blank contained water in place of 50 µl extract. Absorbance was measured at 520 nm using a UV-visible spectrophotometer. The gallotannin contents in the extracts were expressed as gallic acid equivalents (GAE) per gram dry matter.

### 6.2.4 Butanol-HCl assay for condensed tannins

The condensed tannin content was evaluated using the butanol-HCl assay as described by **MAKKAR (2000)** and modified by **NDHLALA *et al.* (2007)**. In triplicate, 3 ml of butanol-HCl reagent (95:5 v/v) was added to 500 µl of each extract, followed by 100 µl of ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The reaction mixture was thoroughly mixed using a vortex and incubated in a water bath for 1 h at 100 ºC. A blank test containing 500 µl extract, 3 ml butanol-HCl reagent and 100 µl ferric reagent was prepared for each extract, but without heating. After the incubation period, absorbance at 550 nm was measured using a UV-visible spectrophotometer against each extract blank. Condensed tannin contents (percentage of dry matter) were evaluated and expressed as leucocyanidin equivalents (LCE) using the formula developed by **PORTER *et al.* (1985)**:

Condensed tannin (%) = $(A_{550} \times 78.26 \times \text{dilution factor})/(\%\ \text{yield})$,

where $A_{550}$ = absorbance of sample at 550 nm, 78.26 = molecular weight of leucocyanidin, dilution factor = 1 (if no water was added) or = 500 µl/volume of water added (when water was added).

Water was added to prevent the absorbance from exceeding 0.6.

### 6.2.5 Vanillin assay for flavonoids

The flavonoid content was evaluated as described by **HANGERMAN (2002)** with modifications. In triplicate, 50 µl of each extract were diluted with 950 µl of glacial acetic acid, followed by the addition of 2.5 ml of 4% HCl in methanol (v/v) and 2.5 ml vanillin reagent (4% vanillin in glacial acetic acid, w/v), after which the reaction mixture was incubated for 20 min at room temperature. The presence of flavonoids was indicated by a pink colouration. After the incubation period, absorbance at 500 nm was measured using a UV-visible spectrophotometer against a blank consisting of water instead of the extract. Flavonoid content was expressed as catechin equivalents (CTE) per gram dry matter.

### 6.2.6 Saponin determination

In duplicate, 5 ml distilled water were added to 0.1 g of ground plant material in test tubes. The test tubes were corked and vigorously shaken for 2 min. The appearance of a stable foam or froth on the liquid surface after 45 min indicated the presence of saponins. The presence of saponins was further confirmed by taking 2 ml of the aqueous extract of each sample into a test tube, followed by the addition of olive oil (10 drops). The test tube was corked and vigorously shaken. The formation of an emulsion confirmed the presence of saponins **(TADHANI and SUBHASH, 2006)**.

### 6.2.7 Alkaloid determination

From the 50% methanol extracts, 5 ml were transferred to a glass test tube and a few drops of ammonium solution were added. The mixture was shaken and examined for formation of a precipitate after approximately 2 min **(MAKKAR and GOODCHILD, 1996)**.

### 6.2.8 Thin Layer Chromatography (TLC) chemical profiling

The acetone extracts were redissolved at 50 mg/ml, and 10 µl of each were loaded on a TLC plate (5 x 20 cm, Silica gel 60 $F_{254}$) (Merck, Germany). The plates were developed using chloroform:ethyl acetate:formic acid (CEF) (20:16:4 v/v/v). After

developing the chemical profiles over a distance of 7.5 cm (solvent front), the TLC plates were dried and visualized under ultraviolet light (254 and 366 nm) and photographs of the plates were taken to compare the chemical profiles of the various extracts. The TLC plates were sprayed with anisaldehyde reagent **(Appendix 4)** and incubated at 110 ºC for approximately 5 min. Thereafter the plates were visualized and photographed using a digital camera.

## 6.3 Results and discussion

The total phenolic content, including tannins and flavonoids were quantitatively analysed while qualitative tests were used to indicate the presence of saponins and alkaloids in the plant species investigated.

### 6.3.1 Total phenolic content

The assay was based on the oxidation-reduction principle using the Folin-C reagent. This method provides a simple test for determination of total phenolic content of plants due to its high sensitivity and reproducibility **(MAKKAR *et al.*, 2007)**. The total phenolic content (mg GAE/g dry matter) of the investigated plant extracts are presented in **Figure 6.1**. All the plants had varying concentrations of phenolic compounds in the extracts of the different plant parts. The largest amount (56.70 mg GAE/g dry matter) of total phenolics was found in *O*. *basilium* leaves while the lowest quantity (1.70 mg GAE/g dry matter) was found in leaves of *C*. *orbiculata* var. *dactylopsis*. Although all the plants investigated contained phenolics, it was observed that plants such as *Hypoxis* species, *L*. *sericea* and *O*. *basilicum,* that had phenolic concentrations above 25 mg GAE/g dry matter, exhibited the most interesting pharmacological activities (anthelmintic, antimicrobial and COX inhibition) in this study. For instance, *H*. *colchicifolia* had the best anthelmintic activity against *C*. *elegans*. The PE and DCM extracts of *L*. *sericea* exhibited the best antibacterial activity while *O*. *basilicum* displayed a high COX-1 and -2 percentage inhibition. This possibly suggests that the quality of the phenolic compounds also plays a major role in the pharmacological activities exhibited by the extracts of plants investigated.

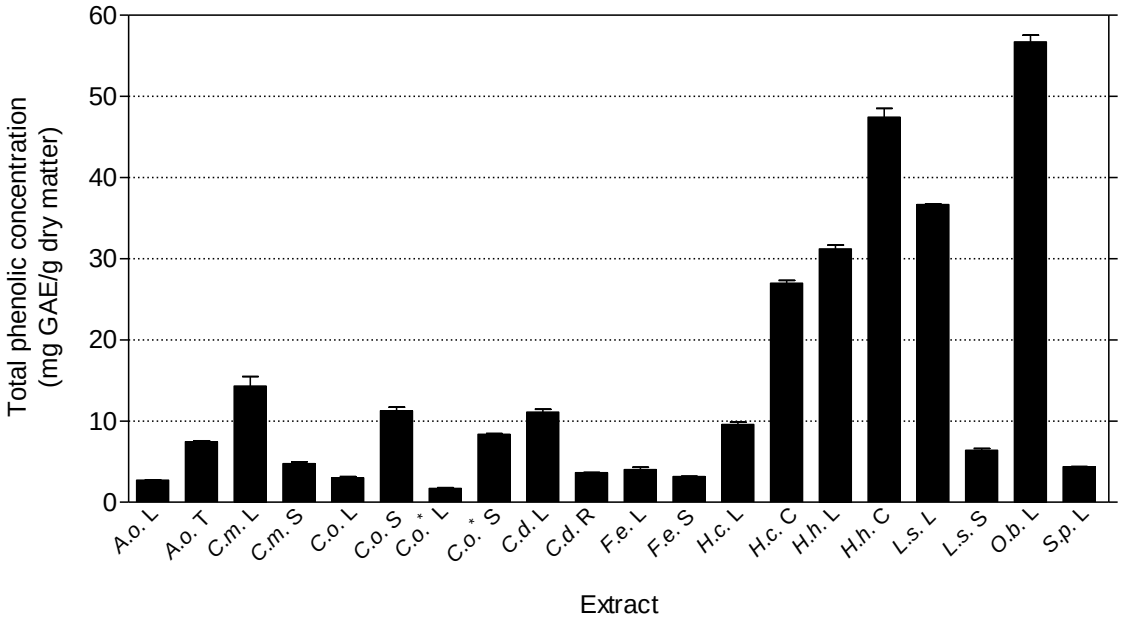


**Figure 6.1:** Total phenolic content determined using the Folin-C assay, of South African plants used as anthelmintics.

*A.o*. L - *Acokanthera oppositifolia* (leaves), *A.o*. T - *A. oppositifolia* (twigs), *C.m*. L - *Clerodendrum myricoides* (leaves), *C.m*. S - *C. myricoides* (stems), *C.o*. L - *Cotyledon orbiculata* var. *orbiculata* (leaves), *C.o*. S - *C. orbiculata* var. *orbiculata* (stems), *C.o*.* L - *C. orbiculata* var. *dactylopsis* (leaves), *C.o*.* S - *C. orbiculata* var. *dactylopsis* (stems), *C.d*. L - *Cyathea dregei* (leaves), *C.d*. R - *C. dregei* (roots), *F.e*. L - *Felicia erigeroides* (leaves), *F.e.* S - *F. erigeroides* (stems), *H.c.* L - *Hypoxis colchicifolia* (leaves), *H.c*. C - *H. colchicifolia* (corms), *H.h*. L - *H. hemerocallidea* (leaves), *H.h*. C - *H. hemerocallidea* (corms), *L.s*. L - *Leucosidea sericea* (leaves), *L.s*. S - *L. sericea* (stems), *O.b*. L - *Ocimum basilicum* (leaves), *S.p*. L - *Senna petersiana* (leaves).

### 6.3.2 Gallotannin content

The rhodanine assay is based on the hydrolysis of gallotannins to form gallic acid which reacts with the rhodanine to give an intense pink colour which is subsequently measured spectrophotometrically **(MAKKAR *et al.*, 2007)**. The amount of gallotannins determined in the plant materials are shown in **Figure 6.2**. The gallotannin concentration ranged from 45.36 µg GAE/g dry matter determined in *O. basilicum* (leaves) to 1.36 µg GAE/g dry matter in *C. orbiculata* var. *dactylopsis* (leaves). Due to their chemical differences, gallotannin has been shown to have a lower anti-nutritive effect than condensed tannins in ruminants **(ROCHFORT *et al.*, 2008)**. As a result, hydrolysable tannin containing plants are preferred to those

containing condensed tannins. Gallotannins have been reported to possess biological activities such as anti-inflammatory, anticancer, antimicrobial and antioxidant activities **(ERDÈLYI *et al.*, 2005)**. In this study, most of the plant extracts that displayed interesting pharmacological properties also contained a substantial concentration of gallotannins. For instance, organic solvent extracts of *O*. *basilicum* leaves had noteworthy antibacterial activity as well as a high COX percentage inhibition. The presence of high amounts of gallotannin could have contributed to this observed pharmacological activity.

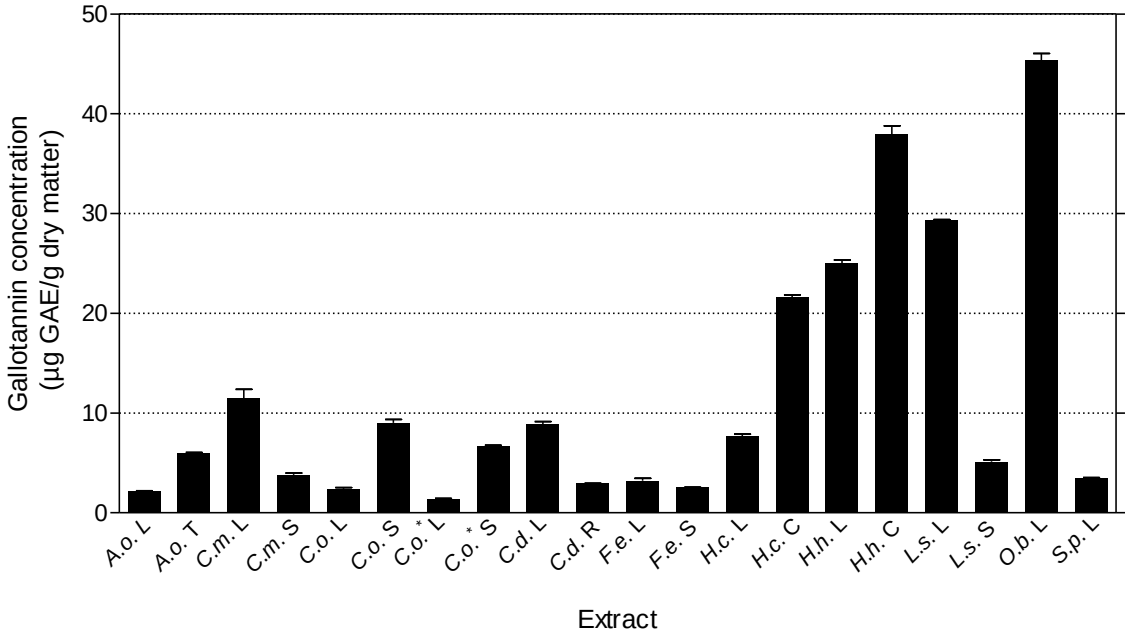


**Figure 6.2:** Gallotannin content determined using the rhodanine assay, of South African plants used as anthelmintics.

*A.o*. L - *Acokanthera oppositifolia* (leaves), *A.o*. T - *A*. *oppositifolia* (twigs), *C.m*. L - *Clerodendrum myricoides* (leaves), *C.m*. S - *C*. *myricoides* (stems), *C.o*. L - *Cotyledon orbiculata* var. *orbiculata* (leaves), *C.o*. S - *C*. *orbiculata* var. *orbiculata* (stems), *C.o.** L - *C*. *orbiculata* var. *dactylopsis* (leaves), *C.o.** S - *C*. *orbiculata* var. *dactylopsis* (stems), *C.d*. L - *Cyathea dregei* (leaves), *C.d*. R - *C*. *dregei* (roots), *F.e*. L - *Felicia erigeroides* (leaves), *F.e.* S - *F. erigeroides* (stems), *H.c.* L - *Hypoxis colchicifolia* (leaves), *H.c*. C - *H*. *colchicifolia* (corms), *H.h*. L - *H*. *hemerocallidea* (leaves), *H.h*. C - *H*. *hemerocallidea* (corms), *L.s*. L - *Leucosidea sericea* (leaves), *L.s*. S - *L*. *sericea* (stems), *O.b*. L - *Ocimum basilicum* (leaves), *S.p*. L - *Senna petersiana* (leaves).

### 6.3.3 Condensed tannin content

The butanol-HCl assay is based on the oxidative cleavage of interflavan bonds of condensed tannins in the presence of mineral acids in alcoholic solution at approximately 100 ºC. This results in the production of pink-coloured anthocyanidins which are subsequently measured spectrophotometrically **(MAKKAR *et al.*, 2007)**. The condensed tannin (percentage LCE/g dry matter) in the 50% aqueous MeOH extracts of the plant material investigated are depicted in **Figure 6.3**.

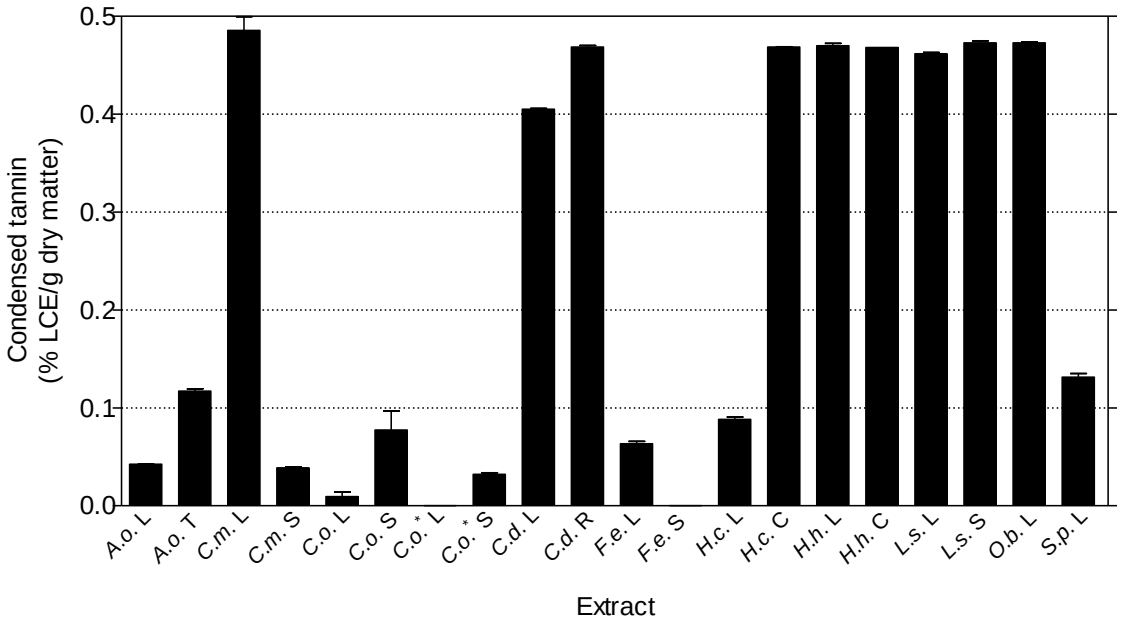


**Figure 6.3:** Condensed tannin content determined using the butanol-HCl assay, of South African plants used as anthelmintics.

*A.o*. L - *Acokanthera oppositifolia* (leaves), *A.o*. T - *A. oppositifolia* (twigs), *C.m*. L - *Clerodendrum myricoides* (leaves), *C.m*. S - *C. myricoides* (stems), *C.o*. L - *Cotyledon orbiculata* var. *orbiculata* (leaves), *C.o*. S - *C. orbiculata* var. *orbiculata* (stems), *C.o.** L - *C. orbiculata* var. *dactylopsis* (leaves), *C.o.** S - *C. orbiculata* var. *dactylopsis* (stems), *C.d*. L - *Cyathea dregei* (leaves), *C.d*. R - *C. dregei* (roots), *F.e*. L - *Felicia erigeroides* (leaves), *F.e.* S - *F. erigeroides* (stems), *H.c.* L - *Hypoxis colchicifolia* (leaves), *H.c*. C - *H. colchicifolia* (corms), *H.h*. L - *H. hemerocallidea* (leaves), *H.h*. C - *H. hemerocallidea* (corms), *L.s*. L - *Leucosidea sericea* (leaves), *L.s*. S - *L. sericea* (stems), *O.b*. L - *Ocimum basilicum* (leaves), *S.p*. L - *Senna petersiana* (leaves).

Apart from *C. orbiculata* var. *dactylopsis* leaves and *F. erigeroides* stems, all the plant extracts had varying amounts of condensed tannins. The condensed tannin

ranged from 0.009% LCE/g dry matter in *C*. *orbiculata* var. *orbiculata* leaf extract to 0.4856% LCE/g dry matter in *C*. *myricoides* leaf extract. Condensed tannins have the ability to denature protein and as the cuticles of nematode larvae are rich in glycoprotein, **THOMPSON and GEARY (1995)** postulated that this ability might be responsible for the death of nematodes when incubated with extracts rich in condensed tannins. Although, an extract showing noteworthy pharmacological activity involves interaction of various compounds, the presence of condensed tannins in most of the plants investigated probably contributed to the observed interesting anthelmintic activity. Other studies have also showed that condensed tannins account for anthelmintic activity in medicinal plants **(JACKSON and MILLER, 2006; ATHANASIADOU *et al.*, 2007)**.

### 6.3.4 Flavonoid content

The flavonoid concentration in the 50% aqueous MeOH extracts of the investigated plants are presented in **Figure 6.4**. All the extracts contained flavonoids at varying concentrations. This was especially evident for the different plant parts, as well as among the plant species. The leaf extract of *L*. *sericea* had the highest flavonoid concentration of 0.654 µg CTE/g dry matter while the lowest flavonoid concentration of 0.008 µg CTE/g dry matter was found in the leaves of *C*. *orbiculata* var. *orbiculata*.

Several flavonoids have been identified as potential inhibitors of COX and LOX enzymes in inflammatory processes **(POLYA, 2003; HIRATA *et al.*, 2005)**. Besides *A*. *oppositifolia* and *Cotyledon* species, all the plants that exhibited high to moderate inhibitory activity against COX enzymes also had flavonoid concentrations of approximately 0.1 mg CTE/g dry matter **(**see **Table 5.1 and 5.2)**. The flavonoid content in these plants probably contributed to their observed interesting COX enzyme inhibitory activity. In addition, different classes of flavonoids have also been reported to possess other pharmacological activities such as antimicrobial and anti-parasitic activity. Most of these flavonoids have low toxicity to animal cells **(HAVSTEEN, 2002)**

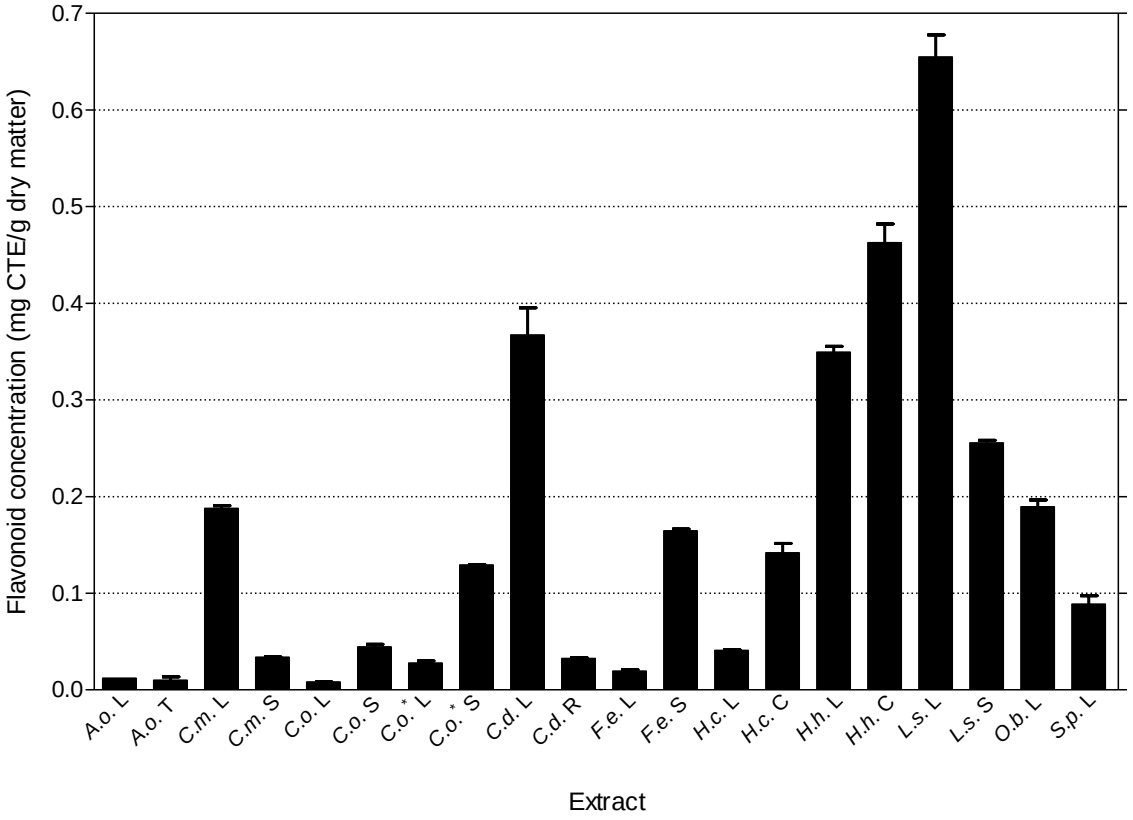


**Figure 6.4:** Flavonoid content determined using the vanillin assay, of South African plants used as anthelmintics.

*A.o*. L - *Acokanthera oppositifolia* (leaves), *A.o*. T - *A*. *oppositifolia* (twigs), *C.m*. L - *Clerodendrum myricoides* (leaves), *C.m*. S - *C*. *myricoides* (stems), *C.o*. L - *Cotyledon orbiculata* var. *orbiculata* (leaves), *C.o*. S - *C*. *orbiculata* var. *orbiculata* (stems), *C.o.** L - *C*. *orbiculata* var. *dactylopsis* (leaves), *C.o.** S - *C*. *orbiculata* var. *dactylopsis* (stems), *C.d.* L - *Cyathea dregei* (leaves), *C.d*. R - *C*. *dregei* (roots), *F.e*. L - *Felicia erigeroides* (leaves), *F.e.* S - *F. erigeroides* (stems), *H.c.* L - *Hypoxis colchicifolia* (leaves), *H.c*. C - *H*. *colchicifolia* (corms), *H.h*. L - *H*. *hemerocallidea* (leaves), *H.h*. C - *H*. *hemerocallidea* (corms), *L.s*. L - *Leucosidea sericea* (leaves), *L.s*. S - *L*. *sericea* (stems), *O.b*. L - *Ocimum basilicum* (leaves), *S.p*. L - *Senna petersiana* (leaves).

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### 6.3.5 Detection of saponins

The presence of saponins in the plants investigated is presented in **Table 6.1**. The amount of foams formed varied from 0.5 to 2 cm and remained stable for approximately 45 min. Saponins dissolve in water to form colloidal solutions that foam upon vigorous shaking **(FRANCIS *et al.*, 2002)**. Saponins were detected in all plant species with the exception of *Cotyledon* species, *O*. *basilicum* and *S*. *petersiana*. Pharmacological activities such as anti-inflammatory, antimicrobial and molluscicidal as well as anti-parasitic activity in many medicinal plant species has been attributed to the presence of saponins **(SPARG *et al.*, 2004)**. The presence of saponins in the plant extracts could have contributed to the observed pharmacological activity of the plants investigated.

### 6.3.6 Detection of alkaloids

The detection of alkaloids in the plant material investigated is presented in **Table 6.1**. It was indicated as the formation of precipitates in the sample test tubes. Apart from leaf extracts of *A*. *oppositifolia*, *C*. *orbiculata* var. *orbiculata*, *Hypoxis* species and *L*. *sericea*, alkaloids were not detected in the other plant extracts investigated. Because the type of alkaloid that may be present in a plant is unknown, it is often difficult to detect alkaloids using a single assay, especially a preliminary qualitative test as used in this study. Factors contributing to this difficulty include the wide range of solubility, numerous different groups and the highly heterogeneous chemical properties of alkaloid compounds **(MAKKAR *et al.*, 2007)**. This could be responsible for the low number of plant extracts with alkaloids observed in this study. Although angiosperm families are rich in alkaloid bases, the distribution is very uneven and many families lack them altogether **(REID *et al.*, 2005)**. For instance, a study on the phytochemical composition of four species in the family Asteraceae detected the presence of alkaloids in only *Arctotis auriculata* **(SALIE *et al.*, 1996)**. Likewise, *F*. *erigeroides* is a member of the family Asteraceae known for high alkaloids, but alkaloids was not detected in this species extracts in this study.

**Table 6.1:** Presence of saponins and alkaloids in the plant extracts investigated.

| Plant species | Plant part | Saponins | Alkaloids |
|---|---|---|---|
| *Acokanthera oppositifolia* | Leaves | - | + |
| | Twigs | + | - |
| *Clerodendrum myricoides* | Leaves | - | - |
| | Stems | + | - |
| *Cotyledon orbiculata* var. *dactylopsis* | Leaves | - | - |
| | Stems | - | - |
| *Cotyledon orbiculata* var. *orbiculata* | Leaves | - | + |
| | Stems | - | - |
| *Cyathea dregei* | Leaves | + | - |
| | Roots | + | - |
| *Felicia erigeroides* | Leaves | + | - |
| | Stems | + | - |
| *Hypoxis colchicifolia* | Leaves | - | + |
| | Corms | + | - |
| *Hypoxis hemerocallidea* | Leaves | - | + |
| | Corms | + | - |
| *Leucosidea sericea* | Leaves | - | + |
| | Stems | + | - |
| *Ocimum basilicum* | Leaves | - | - |
| *Senna petersiana* | Leaves | - | - |

+ = detected, - = not detected

### 6.3.7 Thin Layer Chromatography (TLC) chemical profiles

The TLC technique is based on the differential distribution of the plant extract constituents between the stationary phase and the mobile phase. The solvent system allows the different compounds in the plant extract to move at different rates across the stationary phase. The chemical profiles of the plants investigated are shown in **Figure 6.5** and **Figure 6.6**. The leaf extracts of the plant material showed more bands than the other parts and is an indication of the presence of more compounds in the leaf extracts. The chemical profiles of the plants observed after spraying with the universal indicator anisaldehyde and incubating at 110 ºC for 5 min showed the presence of more compounds than when visualized with UV-radiation alone **(Figures 6.5 C)** and (**Figure 6.6 C)**. Some plant extracts such as *A*. *oppositifolia*, *Cotyledon* species **(Figure 6.5 A, B, C)** as well as *L*. *sericea* **(Figure 6.6 A, B, C)** had similar compounds in both the leaves and stems/twigs. In the pharmacological investigation of these plants, the leaf extracts showed better bioactivity than the stem extracts. It is possible that the concentration of these same compounds was lower in the stems/twigs, or other factors such as synergism and antagonist of other compounds, were responsible for the differences in the plant parts pharmacology.

Essential oils are mixtures of many substances, predominantly terpenes and phenylpropane derivatives **(WAGNER *et al.*, 1984)**. Essential oils are known for their pharmacological activities such as antimicrobial and antioxidant activity **(REICHLING, 1999; RUNYORO *et al.*, 2010)**. They appear as dark zones under 254 nm because they contain at least two conjugated double bonds. After spraying with anisaldehyde reagents, components of essential oils give a strong blue, green and brown colouration **(WAGNER *et al.*, 1984)**. As a result, essential oils and their derivative are likely to be present in many plant species such as *C*. *dregei*, *F*. *erigeroides*, *Hypoxis* species **(Figure 6.5 C)** and *O*. *basilicum* **(Figure 6.6, C)**. This probably accounted for some of the antimicrobial activity that was observed in these plant extracts.

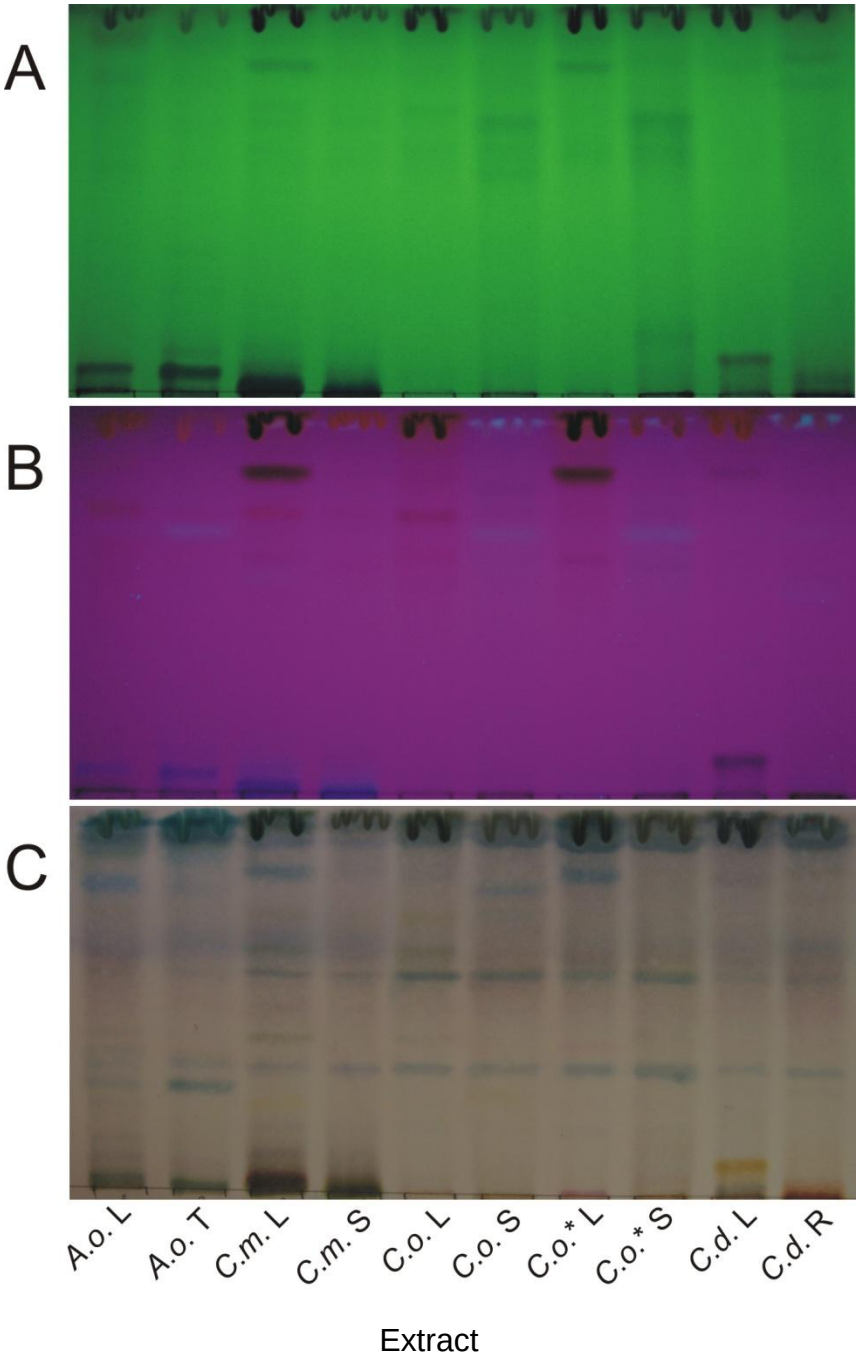


**Figure 6.5:** TLC profiles of plant extracts visualized under (A) UV λ=254 nm (B) UV λ= 366 nm fluorescence and (C) visible light after spraying with anisaldehyde universal indicator and heating at 110 ºC for 5 min. TLC solvent system used was: Chloroform:ethyl acetate:formic acid (CEF 20:16:4 v/v/v).

*A.o*. L - *Acokanthera oppositifolia* (leaves), *A.o*. T - *A. oppositifolia* (twigs), *C.m*. L - *Clerodendrum myricoides* (leaves), *C.m*. S - *C. myricoides* (stems), *C.o*. L - *Cotyledon orbiculata* var. *orbiculata* (leaves), *C.o*. S - *C. orbiculata* var. *orbiculata* (stems), *C.o.** L - *C. orbiculata* var. *dactylopsis* (leaves), *C.o.** S - *C. orbiculata* var. *dactylopsis* (stems), *C.d*. L - *Cyathea dregei* (leaves), *C.d*. R - *C. dregei* (roots).

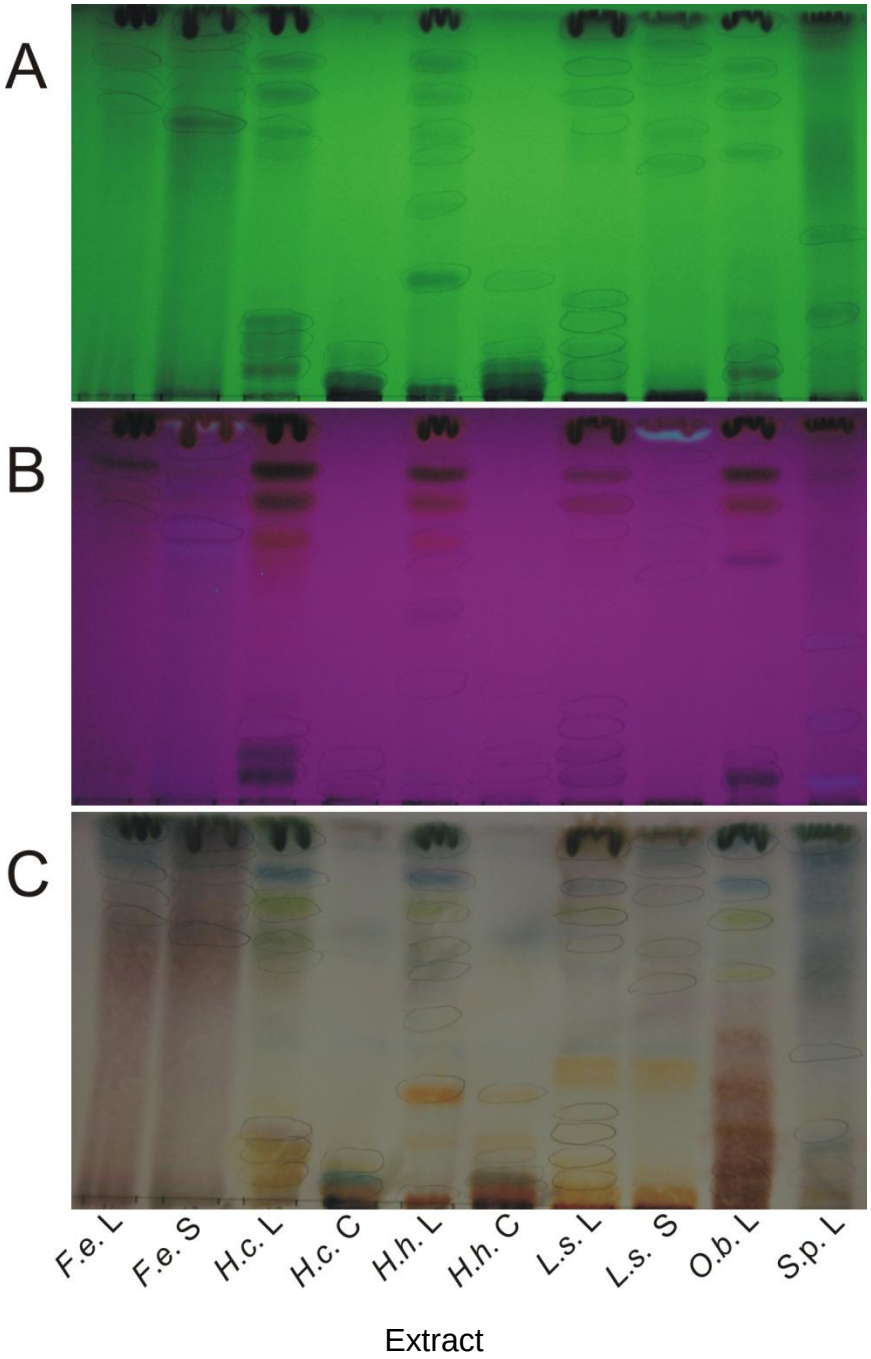


**Figure 6.6:** TLC profiles of plant extracts visualized under (A) UV λ=254 nm (B) UV λ= 366 nm fluorescence and (C) visible light after spraying with anisaldehyde universal indicator and heating at 110 ºC for 5 min. TLC solvent system used was: Chloroform:ethyl acetate:formic acid (CEF 20:16:4 v/v/v).

*F.e*. L- *Felicia erigeroides* (leaves), *F.e*. S - *F. erigeroides* (stems), *H.c*. L - *Hypoxis colchicifolia* (leaves), *H.c*. C - *H. colchicifolia* (corms), *H.h*. L - *H. hemerocallidea* (leaves), *H.h*. C - *H. hemerocallidea* (corms), *L.s*. L - *Leucosidea sericea* (leaves), *L.s*. S - *L. sericea* (stems), *O.b*. L - *Ocimum basilicum* (leaves), *S.p*. L - *Senna petersiana* (leaves).

## 6.4 Conclusions

Although many of the plant secondary metabolites investigated were found in more than one part of the plant, the leaf extracts showed more complexity as depicted in the TLC chemical profiles. The results obtained from the phytochemical screening provide preliminary information on some important groups of secondary metabolites that are present in the plants investigated. This also suggests the types of bioactive compounds that may be responsible for the biological activities exhibited by the plant extracts. Further studies to isolate and identify the plant bioactive principles present in these medicinal plants will be necessary.

# CHAPTER 7: GENERAL CONCLUSION

## 7.1 Introduction

In South Africa, the use of plants for treatment against diseases has been in existence for centuries and constitutes part of the culture of a majority of the people **(VAN STADEN, 2008)**. Although the country is endowed with a rich plant biodiversity that is used in the traditional medicine, the plants have been generally scientifically under-studied for efficacy and safety. Recently, a number of South African medicinal plants have been studied for pharmacological properties such as antimicrobial, anti-cancer and anti-HIV activities. Investigation of anthelmintic activity, however, of most of these plants has not received much attention. This neglect calls for more research, as helminth infections affect a large proportion of the population **(JÄGER, 2005)**, especially those living in informal and rural settlements across the country. Helminth diseases are common among the poor, who are the major users of traditional medicine. As a result, a number of plants are used by traditional healers to treat these diseases. An ethnopharmacological approach was used in the present study to select 11 South African plants used against helminth infections. The plants were investigated for their pharmacological efficacy as well as their phytochemical composition. In addition, antimicrobial as well as COX-1 and -2 enzyme inhibitory activities of these plants were also investigated, because of the association of microbial infections and inflammation with helminthiasis.

## 7.2 Pharmacological activities

In the anthelmintic screening, many of the plant extracts investigated showed activity against *C*. *elegans*. From the 80 extracts investigated, 19 and 34 extracts exhibited high and moderate anthelmintic activity, respectively. The most noteworthy anthelmintic activity (MLC = 0.13 mg/ml) was exhibited by the DCM extract of *H*. *colchicifolia* leaves. Organic solvent extracts of the leaves of *C*. *myricoides*, *Cotyledon* species, *L*. *sericea* and *O*. *basilicum* also displayed noteworthy anthelmintic activity. In this study, the organic solvent extracts showed better anthelmintic activity than the water extracts.

Helminth infections lead to the lowering of the human body immunity with increased susceptibility to pathogenic microorganisms such as bacteria and fungi. As a result, the plant extracts were also subjected to antimicrobial (antibacterial and antifungal) screening. A number of the plant extracts showed high antimicrobial activity against the test microorganisms, especially against *S*. *aureus*. *C*. *albicans* was less sensitive to the plant extracts and only the leaf extracts from *H*. *colchicifolia* (PE) and *O*. *basilicum* (water) exhibiting noteworthy antifungal activity (MIC < 1 mg/ml). The non-polar (PE and DCM) extracts from *L*. *sericea* leaves exhibited the most interesting antibacterial activity (MIC = 0.025 mg/ml) against the Gram-positive bacteria tested. Likewise, organic solvent extracts from *C*. *dregei* roots, *F*. *erigeroides* leaves and stems, *Hypoxis* species leaves, *O*. *basilicum* leaves and *S*. *petersiana* leaves also exhibited noteworthy antibacterial activity (MIC < 1 mg/ml). Although the water extract of *L*. *sericea* leaves had noteworthy antibacterial activity against *B*. *subtilis*, the other water extracts generally exhibited poor antibacterial activity (MIC > 1 mg/ml).

Inflammation is also often associated with helminth infections. The COX enzymes are the most important enzymes involved in this complex inflammatory pathway. The ability of a plant extract to inhibit COX enzymes indicates the potential of such an extract to act as an anti-inflammatory agent. Although the majority of the organic solvent extracts exhibited high COX-1 and -2 inhibition at the highest screening concentration (250 µg/ml), the inhibition decreased with reduced extract concentration of 125 and 62.5 µg/ml. Both organic solvent and water extracts were more effective in inhibiting the COX-1 enzyme than the COX-2 enzyme. As the inhibition of COX-2 enzyme is generally associated with fewer side effects **(BOTTING, 2006)**, the interesting COX-2 inhibitory activity exhibited by the organic solvent (PE, DCM and EtOH) extracts of leaves of *F*. *erigeroides*, *L*. *sericea*, *O*. *basilicum* and *S*. *petersiana* could make these plants potential sources of novel anti-inflammatory compounds.

## 7.3 Phytochemical studies

Chemical compounds found in plants, including secondary metabolites, have various functions ranging from defence against herbivores and microorganisms to ecological adaptations. Recent studies have reported a direct correlation between some plant secondary metabolites and pharmacological activities exhibited **(CROZIER *et al.*, 2006)**. As a result, the phytochemical composition of the plants was investigated so as to have an idea of possible secondary metabolites responsible for the observed pharmacological activity. Although present in varying amounts, phenolics, including gallotannin and flavonoids, were present in all the plants investigated. Higher amounts of these compounds were found in the 50% aqueous MeOH extracts from *Hypoxis* species (leaves and corms), *L*. *sericea* (leaves and stems), *O*. *basilicum* (leaves) and *S*. *petersiana* (leaves). The plant extracts, as well as *C*. *myricoides* (leaves) and *C*. *dregei* (leaves and roots) extracts, also had a higher concentration of condensed tannins while *C*. *orbiculata* var. *dactylopsis* leaf and *F*. *erigeroides* stem extracts yielded no condensed tannins. Compared to phenolics, saponins and alkaloids were qualitatively tested and were relatively scarce in the plant extracts. The presence of these secondary metabolites in the plants investigated probably contributed to the observed pharmacological activities. TLC plates showing the chemical fingerprints of the acetone extracts of the plants investigated also revealed some similarities between extracts of the different plant parts as well as among the plant species.

## 7.4 Conclusion and recommendations

*In vitro* screening is important in validating the traditional use of medicinal plants and for providing leads in the search for new active principles. Although noteworthy pharmacological activity identified by an *in vitro* test does not directly confirm that a plant extract is an effective medicine, or a suitable candidate for drug development, it does provide a basic understanding of the efficacy of a medicinal plant in traditional medicine and its potential use as a source of novel chemotherapy. Furthermore, active extracts should be subjected to *in vivo* investigation as well as the isolation and identification of bioactive compounds and toxicity testing to determine their safe use as medicines.

The anti-parasitic effect of many plants has been attributed to plant secondary metabolites which can also have an anti-nutritional effect and further studies will be necessary to clearly understand the effect of these metabolites *in vivo* **(JACKSON and MILLER, 2006)**. Any beneficial effect of secondary metabolites should be higher than any resultant side effects for it to be of therapeutic value and use against helminth infections.

The following recommendations could be considered for future pharmacological studies for effective helminth infection control:

- During pharmacological investigation of plant extracts, other biological activities related to the disease state for which the medicinal plant is used should also be tested. This would eliminate the potential loss of discovery of other possible bioactive compounds from plant extracts.
- It is important to investigate other plant parts and related species for similar pharmacological activity to enhance plant conservation. This is especially important for endangered medicinal plants and in cases where the underground parts are used for medicine or plant species are over-harvested due to popular demand.

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# APPENDIX 1

**Nematode Growth (NG) agar**

| | |
|---|---|
| NaCl | 3 g |
| peptone | 2.5 g |
| agar | 17 g |

dissolved in 975 ml distilled water

***After autoclaving***

1 ml cholesterol in ethanol (5 mg/ml)

1 ml 1M $CaCl_2$

1 ml 1M $MgSO_4$

25 ml potassium phosphate buffer (pH 6.0)

were added in order

**Potassium phosphate buffer (pH 6.0)**

| | |
|---|---|
| $KH_2PO_4$ | 12.014 g |
| $K_2HPO_4$ | 2.807 g |

in 100 ml distilled water

**M9 buffer**

| | |
|---|---|
| $Na_2HPO_4$ | 6 g |
| $KH_2PO_4$ | 3 g |
| NaCl | 5 g |
| $MgSO_4.7H_2O$ | 0.25 g |

per litre

# APPENDIX 2

**Glycerol solution**

65% glycerol $^{v}/_{v}$

0.1 M $MgSO_4$

0.025 M Tris pH 8

Make Tris and add $MgSO_4$ (to make 0.1 M)

Add glycerol to make it up to 65%

(For 100 ml, make 35 ml Tris/$MgSO_4$ and add 65 ml glycerol)

Autoclave and store in fridge at 4 ºC

Keep sterile

**Microorganism stock cultures**

Transfer 800 µl of liquid culture to an autoclaved cryovial

Add 800 µl of 65% glycerol solution, mix gently and tap.

Store at -70 ºC

# APPENDIX 3

**TRIS storage buffer (pH 8.0)**

| | |
|---|---|
| Tris(hydroxymethyl)aminomethane (TRIS) 80 mM | 9.7 g |
| TWEEN 20 (0.1 %) | 1.0 g |
| Sodium diethyldithiocarbamate (trihydrate) 300 µM | 0.0676 g |
| Distilled water | 800 ml |
| Hydrogen chloride (HCl) | Adjust to pH 8.0 |
| Distilled water | Make up to 1000 ml |

**TRIS buffer (pH 8.0) used in the bioassay**

| | |
|---|---|
| TRIS | 12.1 g |
| Distilled water | 800 ml |
| Hydrogen chloride | Adjust to pH 8.0 |
| Distilled water | Make up to 1000 ml |

**Hematin Stock solution**

| | |
|---|---|
| Hematin | 1.26 mg |
| TRIS | 96.9 mg |
| Distilled water | 10 ml |

**Phosphate buffer (pH 7.4)**

| | |
|---|---|
| $KH_2PO_4$ | 2.6 g |
| $K_2HPO_4.3H_20$ | 18.5 g |
| Distilled water | Make up to 1000 ml |

# APPENDIX 4

**Anisaldehyde reagent**

| | |
|---|---|
| Ethanol | 465 ml |
| Glacial acetic acid | 5 ml |
| Sulphuric acid (concentrated) | 13 ml |
| *p*-anisaldehyde | 13 ml |

Cold ethanol measured into a Schott bottle and placed on ice

In above order, other solvents were added and maintained under cold conditions

The prepared anisaldehyde reagent was kept in a freezer after use