SOUTH AFRICAN MEDICINAL ORCHIDS:

A PHARMACOLOGICAL AND PHYTOCHEMICAL EVALUATION

MAYASHREE CHINSAMY

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in the

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School of Biological and Conservation Sciences

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FACULTY OF SCIENCE AND AGRICULTURE
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Student Number: **202500097**

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

Student's Full Name: **MAYASHREE CHINSAMY**

Student Number: **202500097**

Thesis title: **South African Medicinal Orchids: A Pharmacological and Phytochemical Evaluation**

Regular consultation took place between the student and us 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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DR JF. FINNIE

ABSTRACT

The Orchidaceae makes up the largest and most diverse family of flowering plants. Orchids are popular, often expensive ornamentals, with a broad range of ethnobotanical applications. There is very limited documented information on South African medicinal orchid species; no formal pharmacopoeia outlining ethnobotanical uses; and ethnobotanical and distribution records are either scarce or inconsistent and plant populations are becoming gradually smaller. There have been significant developments in medicinal orchid research worldwide with medicinal use and corresponding pharmacological and phytochemical properties being extensively investigated. It is evident from the literature that there is no pharmacological research on South African medicinal orchids; hence the need to explore biological activity and chemical composition of South African medicinal orchid species. The ethnobotanical approach used to select the orchid species for pharmacological and phytochemical research elsewhere, yielded valuable biological compounds. Thus, a similar approach was applied to South African medicinal orchids.

There are approximately 20 000 species and 796 genera of orchids distributed across the world. In southern Africa, orchids are widely represented with 55 genera and 494 species. Approximately 75% are endemic to this region. As part of the current investigation a review of available ethnobotanical literature on South African medicinal orchids was prepared. The review revealed that an estimated 49 indigenous orchid species from 20 orchid genera are currently being informally traded and used in South African traditional medicine. They are used primarily for medicinal and cultural purposes, especially by the Zulu community in South Africa. Medicinal uses of orchid species include: treatment of inflammatory, intestinal, neurological and reproductive disorders and emetics are used to cause emesis. Non-medicinal uses of orchid species include: love, fertility, protective and lethal charms. Based on their ethnobotanical uses and endemism, South African orchids were considered to be one of the untapped sources of bioactive compounds that needed to be researched.

The current investigation addressed the broader aims of medicinal plant research by determining the efficacy, safety and chemical profile of seven indigenous orchid species used in South African traditional medicine and practices. The biological and toxic effects of orchid plant

extracts were assessed using established pharmacological bioassays. The phytochemical evaluation of the seven orchid plant extracts provided insight into the classes of chemical compounds present and their possible role in the observed biological activities. The potential of plant extracts from seven orchid species used in South African traditional medicine, as sources of natural bioactive products, are discussed. The current investigation determined the biological activity and chemical profile of seven orchid species commonly traded in KwaZulu-Natal herbal markets: *Ansellia africana* Lindl., *Bulbophyllum scaberulum* (Rolfe) Bolus, *Cyrtorchis arcuata* (Lindl.) Schltr., *Eulophia hereroensis* Schltr., *Eulophia petersii* (Rchb.f.) Rchb.f., *Polystachya pubescens* (Lindl.) Rchb.f. and *Tridactyle tridentata* (Harv.) Schltr.

Well established *in vitro* micro-dilution bioassays were used to determine the antibacterial, antifungal, anthelmintic activities of crude orchid extracts. A minimum inhibitory and/or lethal effect of organic and aqueous crude orchid extracts was observed against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Candida albicans* and *Caenorhabditis elegans*. *Tridactyle tridentata* aqueous root extract produced the most effective antibacterial activity against *S. aureus* (0.049 mg/ml). All *T. tridentata* organic root extracts produced significant inhibitory activities against *B. subtilis* and *S. aureus*. *Eulophia petersii* DCM pseudobulb extracts significantly inhibited all bacterial strains tested (0.39 mg/ml against *S. aureus* and 0.78 mg/ml against *B. subtilis*, *E. coli*, and *K. pneumoniae*). *Eulophia hereroensis* 80% EtOH root extract was the only other extract to exhibit significant inhibitory effects against *K. pneumoniae* (0.65 mg/ml). After 48 h *C. albicans* was most susceptible to *P. pubescens* aqueous pseudobulb extract (0.0816 mg/ml). *Eulophia petersii* DCM pseudobulb extract however, exhibited significant activity against *C. albicans* (0.65 mg/ml) over 72 h. *Cyrtorchis arcuata* leaf and root extracts were the most effective anthelmintic extracts with MLCs of 0.041 mg/ml for 80% EtOH leaf and root extracts; 0.049 mg/ml for aqueous leaf extracts and 0.78 mg/ml for aqueous and DCM root extracts. *Caenorhabditis elegans* was most susceptible to all *A. africana* and *T. tridentata* organic root extracts. A similar significant effect was observed for all *E. petersii* organic pseudobulb extracts, DCM extracts and organic root extracts of *B. scaberulum*. Only the DCM tuber and root extracts of *E. hereroensis* exhibited lethal effects on *C. elegans*. All of the *P. pubescens* extracts showed poor anthelmintic activity.

Similarly, *in vitro* enzyme based cyclooxygenase (COX) 1 and 2 and acetylcholinesterase (AChE) inhibitory bioassays, revealed significant inhibition of COX-1, COX-2 and AChE enzymes by crude organic and certain aqueous orchid extracts. Out of a total of 53 evaluated extracts, 21 and 13 extracts exhibited significant anti-inflammatory activity in the COX-1 and COX-2 assays respectively. The DCM tuber extract of *E. hereroensis* was the only extract to significantly inhibit both COX enzymes, 100.02±0.11% and 87.97±8.38% respectively. All *B. scaberulum* root extracts (DCM, EtOH and water) exhibited COX-2 selective inhibitory activity (100.06±0.01, 93.31±2.33 and 58.09±3.25%). Overall, the DCM root extract of *A. africana* was found to be the most potent extract (EC_{50} 0.25±0.10 mg/ml). The 80% EtOH root extract of *B. scaberulum* was the most potent in the COX-2 assay (EC_{50} 0.44±0.32 mg/ml). Generally the root extracts exhibited greater AChE inhibitory activity; where the most active extract was *B. scaberulum* DCM root extract (EC_{50} 0.02±0.00 mg/ml). All aqueous extracts, except that of *A. africana* roots and *B. scaberulum* pseudobulbs, showed poor or no COX-1 and COX-2 inhibition.

The antioxidant capacity of crude orchid extracts was determined using: hydrogen atom transfer (HAT) (β-carotene/linoleic acid assay) and single electron transfer (SET) (2,2'-diphenylpicrylhydrazyl (DPPH) free radical scavenging assay and ferric reducing antioxidant power (FRAP) assay) reaction-based assays. Potent antioxidant effects were observed for certain crude methanolic orchid extracts. Generally, there was a dose-dependent change in radical scavenging activities of crude extracts from which EC_{50} values were determined. The root extracts of all species, except that of *E. petersii*, had consistently more effective radical scavenging activity than that of other plant parts within each species. The pseudobulb extract of *E. petersii*, was the most potent extract (EC_{50} 1.32±0.86 mg/ml). In the β-carotene-linoleic acid assay, based on the oxidation rate ratio (ORR), the leaf extract of *T. tridentata* and the root extracts of *C. arcuata* and *E. hereroensis* exhibited the best antioxidant effects (0.02, 0.023 and -0.15 respectively). Similarly, the average antioxidant activity (%ANT) of these samples was greater than that of BHT (95.88±6.90%) and all other samples. *Bulbophyllum scaberulum* leaf, pseudobulb and root extracts, *E. petersii* pseudobulb extract and *T. tridentata* root extract also exhibited a greater capacity to prevent β-carotene oxidation when compared to BHT. All crude orchid extracts tested demonstrated a general dose-dependent response in the ferric reducing

power assay. The reducing power of ascorbic acid (0.08 mM) and BHT (0.05 mM), as measured as absorbance, was 1.12±0.12 and 0.73±0.08 respectively. At 6.25 mg/ml, *A. africana* root and *E. petersii* pseudobulb extracts were the most effective in reducing power activity.

The short-term bacterial reverse mutation Ames *Salmonella*/microsome mutagenicity (ASMM) assay, which makes use of mutant histidine-dependent *Salmonella typhimurium* strains, was used to determine the mutagenicity and toxicity of crude orchid extracts. In the presence of a mutagen *S. typhimurium* TA98 strain detects frameshift events while the TA100 and TA102 strains detect base-pair substitutions. In the absence of metabolic activation, mutagenic extracts were observed against the TA98 strain only. All *A. africana* DCM leaf and stem extracts tested, the DCM root extract (0.5, 0.05 mg/ml) and EtOH leaf, stem and root extracts at 5 mg/ml exhibited mutagenic effects. The EtOH root extracts (5, 0.5 mg/ml) of *B. scaberulum* exhibited mutagenic indices (MI) comparable to that of 4NQO (17.00 and 13.00, respectively). *Eulophia petersii* PE pseudobulb extract demonstrated mutagenic potential at 5 mg/ml. The ethanolic root extracts of *T. tridentata* showed mutagenic effects at 5 and 0.5 mg/ml. The mutagenicity index (MI) with metabolic activation (S9) was determined using only the TA98 strain; where no mutagenic effects were observed.

In the phytochemical evaluation of crude methanol orchid extracts, the Folin-Ciocalteu assay for total phenolics, butanol-HCl assay for condensed tannins, rhodanine assay for gallotannins and vanillin assay for flavonoids revealed a quantitative chemical profile of the tested samples. The correlation between observed biological effects and chemical compounds present was found to be generally significant. The significant antimicrobial, anthelmintic, anti-inflammatory and antioxidant activity of *E. petersii* pseudobulb extracts and *E. hereroensis* tuber and root extracts may be attributed to their high total phenolic content. Alternatively, the significant levels of gallotannin content in *E. hereroensis* may have contributed to the bioactivity. The flavonoid content of *B. scaberulum* and *T. tridentata* may explain the potent activity observed in the anti-inflammatory, antioxidant and acetylcholinesterase inhibitory assays; while the flavonoid content *C. arcuata* may have contributed to the potent anthelmintic and antioxidant activities. The significantly higher levels of gallotannin content may explain the significant anti-inflammatory and anthelmintic activity of *A. africana*.

A number of biologically active compounds have been isolated from certain Orchidaceae species around the world on the basis of their traditional medicinal uses. The traditional uses of these orchid species were scientifically validated. No pharmacological research has been previously conducted on South African medicinal orchids; therefore the current investigation has produced novel findings on the efficacy and safety of these orchid species and promotes the continued research of medicinal orchids in South Africa.

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If it is to be, it is up to me.

Ten of the simplest words, when put together, have a powerful meaning. Life is similar. Alone, experiences seem overwhelming. When put together, life has meaning.

The journey, that brings me to today has been nothing less than remarkable.

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"**We don't receive wisdom, we must discover it for ourselves after a journey that no one else can take for us or spare us**." Marcel Proust (1871-1922).

"**Science is organised knowledge. Wisdom is organised life**". Immanuel Kant (1724-1804).

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LIST OF ABBREVIATIONS

%ANT	Average antioxidant activity
2AA	2-Aminoanthracene
4NQO	Nitroquinoline-N-oxide
AA	Arachidonic acid
AChE	Acetylcholinesterase
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AR	Antibiotic resistance
ASMM	Ames *Salmonella*/microsome mutagenicity assay
ATCC	American type culture collection
ATCI	Acetylthiocholine iodide
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
CMV	Cytomegalovirus
CNS	Central nervous system
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CXM	Cycloheximide
DALYs	Disability adjusted life years
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5-Dithiobis-2-nitrobenzoic acid
EC_{50}	Effective concentration at 50%

E_{max}	Maximum effect
EtOH	Ethanol
FIPV	Feline infectious peritonitis virus
Folin C	Folin Ciocalteu
FRAP	Ferric reducing power assay
GABA	*γ*-Aminobutyric acid
$GABA_A$	*γ*-Aminobutyric acid A
GAE	Gallic acid equivalent
h	Hour
H_2O_2	Hydrogen peroxide
H4TG	Thioguanine-resistant rat hepatoma cells
HAT	Hydrogen atom transfer
HCl	Hydrochloric acid
HeLa	Henrietta Lacks
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC_{50}	Inhibitory concentration at 50%
IFIs	Invasive fungal infections
IFN-λ	Lymphocyte cytokines interferon λ
INT	*p*-Iodonitrotetrazolium chloride
IUCN	International Union for Conservation of Nature
JNK	c-Jun NH_2-terminal kinase
KA31T	Kirsten strain Moloney sarcoma virus-transformed 3T3 cells
LC	Least concern
LDL	Low density lipoprotein
LLD	5α-lanosta-24,24-dimethyl-9(11),25-dien-3β-ol
LTs	Leukotrienes
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
MAPKs	Mitogen-activated protein kinases
MBC	Minimum bactericidal concentration

MDCK	Madin-Darby Canine Kidney
MFC	Minimum fungicidal concentration
MH	Mueller-Hinton
MI	Mutagenic index
MIC	Minimum inhibitory concentration
MLC	Minimum lethal concentration
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NCCLS	National Committee for Clinical Laboratory Standards
NG	Nematode growth
NIH3T3	NIH Swiss mouse embryo fibroblasts
NO	Nitric oxide
NRF	National Research Foundation
NSAIDs	Non-steroidal anti-inflammatory drugs
NU	Natal University
O_2	Oxygen
$O^{-}_{2}{}^{\cdot}$	Superoxide anion
OD	Optical density
$OH^{\cdot}$	Hydroxyl radical
OPC	Oropharyngeal candidiasis
ORAC	Oxygen radical absorbance capacity
ORR	Oxidation rate ratio
PE	Petroleum ether
PFLP	Plant ferredoxin-like protein
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin F_2
PGH_2	Prostaglandin H_2
PGs	Prostaglandins
$Ph_3C^{\cdot}$	Triphenylmethyl radical

PHC	Primary health care
ppm	Parts per million
RCPGD	Research Centre for Plant Growth and Development
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSA	Radical scavenging activity
RSV	Respiratory syncytical virus
SAAB	South African Association of Botanists
SARS-CoV	Severe acute respiratory syndrome coronavirus
SEM	Standard error of the mean
SET	Single electron transfer
STH	Soil-transmitted helminths
TB	Tuberculosis
TCA	Tricarboxylic acid
TNF-α	Tumour necrosis factor
TXs	Thromboxanes
U5MR	Under five mortality rate
USSR	Union of Soviet Socialist Republics
UV	Ultraviolet
WHO	World Health Organisation
YM	Yeast Malt

CHAPTER 1
INTRODUCTION

1.1. INTRODUCTION

Humanity's evolution is a direct result of our valuable relationship with nature. The simultaneous development of science and research methods has contributed to a more knowledgeable civilisation. Despite this, a great majority of our population remain malnourished, ill, and merely surviving in diseased and poor living conditions. The situation is characterised by recurring infections, incidences of associated disorders and the emergence of new diseases. This, coupled with a lack of effective medicines and/or medicines with various side effects, reduces the quality of life for many generations, and impedes our growth and development as humans. Therefore our immediate and urgent attention is necessary.

How do we all progress further under such challenging conditions? While the answer at times seems impossible, programs which advocate for the provision of adequate, accessible, effective and safe healthcare is a step in the right direction. Such programs recognise the value of our human and natural resources and address the short and long term outcomes of healthcare. Primary or conventional healthcare is the current most recognised system in most countries, and favours the use of synthetically derived medicines. However, plant derived products have contributed greatly to this system.

Medicinal plant research has revealed the value of indigenous knowledge systems and is accomplished by a systematic evaluation of natural plant resources. The continued development of the human race depends on the efficient management of our human and natural resources, and recognition of the value of knowledge contained around and within them.

1.2. THE WAY FORWARD

„Health for All' is a World Health Organisation (WHO) initiative which has placed a great deal of importance on the access to healthcare. The campaign is based on the view that health is a

fundamental right (**PATEL, 1983**). Presently, conventional health care systems are inadequate and inaccessible to facilitate this right in various developing and developed regions. The WHO facilitates yet another programme which identifies traditional medicine (a medical system already in use in many developing parts of the world) as an integral health-care resource that can improve the health status in those areas (**HILLENBRAND, 2006**). On a global scale the promotion of the Traditional Medicine Programme places emphasis on the formulation of national policies on traditional medicine, increased investigations into potential therapeutic activity, toxicity, enhancing the knowledge of all stakeholders (which includes the traditional healers and conventional practitioners) and to activate educational efforts to enlighten the public in the member states (**WHO, 2002a**). The Declaration of Alma Ata in 1978 at the „International Conference on Primary Health Care' held in the USSR was a form of global recognition of traditional medicine and the potentially beneficial role it could play were it integrated into the primary healthcare system (PHC).

Traditional medicine, which comprises both indigenous knowledge and medical systems, has survived through the ages and remains to be the preferred medical system to conventional medicine (**WHO, 2002a**). With dependence and preference weighing heavily in favour of traditional medicine, the strategy to integrate the two medical systems in countries such as South Africa would greatly benefit patients (mostly indigenous people) who subscribe to traditional medicine. Traditional healers are trusted members of their communities and are therefore well positioned to educate their communities (**KING** and **HOMSY, 1997**). With formal registration, the advice and medication dispensed by traditional healers would be appropriately recognized, regulated and adequately supported by the different disciplines of science (**CLARKE, 1998; RICHTER, 2003**). Certain aspects of traditional medicine such as safety and efficacy of the medicine are still to be addressed.

In South Africa, the integration of the two medical systems requires more time, as traditional medicine needs to be adequately regulated (**SINDIGA et al., 1995**). By applying modern scientific techniques, such as pharmacological evaluation to traditional medicine, and continually developing our technologies we will be able to address issues of safety, availability and a have broader application of the natural plant products indigenous to our country **(DRAFT**

NATIONAL POLICY ON AFRICAN TRADITIONAL MEDICINE FOR SOUTH AFRICA, 2008). By formally documenting indigenous knowledge, conducting scientific investigations on medicinal and related plants to validate their use and providing adequate and suitable governmental support, African Traditional Medicine may become globally recognized as an established medical system **(DRAFT NATIONAL POLICY ON AFRICAN TRADITIONAL MEDICINE FOR SOUTH AFRICA, 2008)**.

1.3. PLANT DRUGS IN MODERN MEDICINE

Modern conventional medicine has had significant breakthroughs over the years. A number of herbal remedies have served as templates for some clinical drugs used in modern medicine (**FABRICANT** and **FARNSWORTH, 2001**). Today, a large proportion of clinically used drugs and new formulations are derivatives of natural plant products (**HARVEY, 2008; KINGSTON, 2010**). Familiar examples include: artemisinin, aspirin, atropine, bulbocapurine, cocaine, codeine, colchicines, ephedrine, hyoscyamine, ipecac, morphine, papverine, physostigmine, picrotoxin, pilocarpine, pseudoephedrine, quinidine, quinine, reserpine, scopolamine, strychnine, theophylline and d-tubocaranine (**COX, 1994; COX** and **BALICK, 1994; COX, 1995; VAN WYK et al., 1997**).

A report by **FABRICANT and FARNSWORTH (2001)** states that, 122 bioactive compounds from an estimated 94 plant species are currently consumed as clinical drugs. It was further established that approximately 80% of these drugs are derived from plants already in use for similar purposes in traditional medicine. In 1991, as reported by **O'NEILL** and **LEWIS (1993)**, clinical drugs from natural products and or their derivatives represented a major portion of the best selling pharmaceuticals in that year. In 1996, six of the top 20 dispensed drugs were natural products (**PHILLIPSON, 2001**). Other reports claim that, of the 119 plant-derived clinical drugs reported by **FARNSWORTH et al. (1985)**, 12 featured in the top 25 best selling pharmaceuticals (**VERPOORTE, 1998**).

It is obvious that modern medicine is closely linked to the continuous development of more effective and safe drugs. Judging from the list of familiar clinical drugs above, plants are an

important source of compounds that are ultimately used for drug development. High-value secondary metabolites have also been applied in pharmaceutical, food and agricultural industries. Therefore, the potential of medicinal plant research is immense.

1.4. MEDICINAL PLANT SPECIES OF INTEREST

An estimated 49 indigenous orchid species from 20 orchid genera are currently being informally traded and used in South African traditional medicine (**HUTCHINGS et al., 1996**). They are used primarily for medicinal and cultural purposes, especially by the Zulu community in South Africa (**GERMISHUIZEN** and **MEYER, 2003; HUTCHINGS et al., 1996**).

1.5. AIMS AND OBJECTIVES

The current investigation addressed the broader aims of medicinal plant research by determining the efficacy, safety and chemical profile of seven indigenous orchid species used in South African traditional medicine and practices.

The biological and toxic effects of orchid plant extracts were assessed using established pharmacological bioassays. The phytochemical evaluation of the seven orchid plant extracts provided insight into the classes of chemical compounds present and their possible role in the observed biological activities. The potential of plant extracts from seven orchid species produced in South African traditional medicine, as sources of natural bioactive products, are discussed.

CHAPTER 2
LITERATURE REVIEW

2.1. INTRODUCTION

2.1.1. Medicinal plants

Man's dependence on the Plant Kingdom seems to parallel our evolutionary history. Our basic, domestic, cultural and health needs have all been catered for by plants, and through the ages our dependence has hardly decreased. The medicinal value of plants was realised by early civilisations and the traditional use of plants as medicine is still in practice today (**VERPOORTE, 1998**). Some of the earliest indications of mankind's dependence on medicinal plants and their applications are documented in ancient pharmacopeias (**PAGE et al., 1997; CRAGG** and **NEWMAN, 2001**). **UNESCO (1996)** and **WHO (2002a)** reported widespread usage of medicinal plants within the practice of traditional medicine in developing countries. Historically, plants were the only source of medication and they continue to demonstrate their therapeutic usefulness by being a part of, possibly the only primary health care system in certain regions (**RIBEIRO et al., 2010**).

The more established traditional health care systems found in India, China, America and Europe, have extensive well preserved and detailed documentation in pharmacopoeia. For example, of the approximately 8 000 higher plant species catalogued as having medicinal uses in India, more than 1 500 are known to be used across the four major traditional medical systems practised in India (Ayurveda, Siddha, Tibetan and Unani) (**SUBRAT et al., 2002**). Similarly, the Chinese, Far East and South American nations still subscribe to their traditional medical systems. They are well integrated and widely studied in their respective countries and have made significant contributions to modern medicine (**CRAGG** and **NEWMAN, 2001**).

2.1.2. Drug discovery process

In traditional medical practices plant based medicines are administered in their crude form. Drug development, however, requires the extraction and isolation of pure bioactive compounds. The earliest record of compound isolation is that of the alkaloid morphine from *Papaver somniferum* in 1805 (Sertürner)**,** closely followed by quinine (bark of *Cinchona*) in 1820 (Caventon and Pelletier) (**RAY, 1960; BUTLER, 2005**).

Differences between crude and pure plant extracts include their consistency in activity. With crude extracts if individual constituents are active only when acting synergistically, then various factors such as physiological, environmental, geographic variation (among others) may impact on activity (**FIGUEIREDO et al., 2008**). Additional factors known to impact on the efficacy or potency post harvest include: collection techniques, storage and preparation conditions. When compared, there may be more variability and inconsistency in crude plant extracts than in pure compounds. Advantages of pure compound isolation include the ability to determine an accurate and effective dose and proper identification of compounds which could lead to the possible chemical synthesis of the compound. This has the added benefit of relieving stress on natural plant sources. Also, one would be able to trace how and where the compound may be involved in the different/ relevant biological processes (**CANNELL, 1998**). The chemical modification of bioactive compounds is carried out in an attempt to produce fewer side effects. A good example of an isolated compound is aspirin which is an effective analgesic and anti-pyretic. Salicylic acid (naturally occurring in the bark of willow (*Salix alba*) and the poplar tree was isolated and a synthetic acetyl derivative was produced from which aspirin was modeled (**COLEGATE** and **MOLYNEUX, 1993; HARBOURNE et al., 2009**). Upon isolation and or identification, active compounds need to undergo meticulous toxicological, pharmacokinetic and metabolic studies to determine their precise effectiveness and safety. These final steps in the drug development process often pose many problems and determine the outcome of studies into naturally derived plant products.

The extensive use of drugs such as artemisinin and taxol are indicative of their efficacy. It also draws attention to the therapeutic value and potential of plants as sources of novel bioactive

agents. Unfortunately, this potential is yet to be fully exploited (**KINGHORN, 2008).** In addition, **BALANDRIN et al. (1993)** and **KINGHORN (2008)** showed that plants already investigated, were tested for very few types of biological activity. **KINGHORN (2008)** cautioned that phytochemical studies alone may not reveal all potentially bioactive constituents. The advent of high-throughput bioassays has allowed for more in depth research (**HOUGHTON et al., 2007**). Some of the advantages include a smaller sample size and the ability to investigate many plants at a time. Bioassays, though convenient, need to be sensitive to the presence of bioactive agents while at the same time preventing false positives. There is the added possibility of false negative results when using low concentrations of crude extracts (**RASOANAIVO** and **RATSIMAMANGA-URVERG, 1993**). **RASOANAIVO** and **RATSIMAMANGA-URVERG (1993)** and **HOUGHTON et al. (2007)** caution that false negatives may also be a result of loss of synergistic effects when active compounds are separated out in methods such as bioassay guided fractionation. Therefore, as highlighted by **VERPOORTE (2000)**, a combined approach by the various disciplines would be advantageous. Often, however, very few laboratory tested active compounds appear on the shelves as clinical drugs.

Natural plant products are still widely used and the demand is steadily growing. However, only 94 plant species are represented in the 122 plant-derived pharmaceuticals used today (**FABRICANT** and **FARNSWORTH, 2001**). Based on the extensive historic and global use of plants as medicine, as well as their availability (≈250 000 higher plant species), there is a great possibility of uncovering useful bioactive compounds within the Plant Kingdom (**NEWMAN** and **CRAGG, 2007**). This in turn could result in the formulation of new drugs.

2.1.3. Secondary metabolites

Secondary metabolites are often considered to be the source of bioactive compounds within plants (**BALANDRIN et al., 1993**). The valuable role of secondary metabolites as bioactive agents has become more apparent over recent years. Unlike macromolecules, such as nucleic acids, proteins and carbohydrates, that are necessary for the proper functioning of the plant; secondary metabolites are known to be synthesised for various reasons including, in response to

a biological attack and lack of nutrients (**CANNELL, 1998**). They are also considered to be unique to certain species, with some serving a particular purpose such as antiherbivory effects, sexual stimuli and/or antibiotic effects; while others have no apparent purpose (**CANNELL, 1998**). These chemical structures may be produced at different stages of a plant's life with a variation in type and quantity. Such intra-specific variation may be due to differences in: genetic make-up, growth conditions, habitat, stage in life-cycle or the plant organ being investigated (**FIGUEIREDO et al., 2008**). In **1998, VERPOORTE** reported that there was in excess of 100 000 known secondary metabolites, however only a few of these have been studied. He further emphasised the immense potential of discovering bioactive compounds. New chemical structures may be present with different modes of action, better activity, or perhaps fewer side effects than those already known and used in clinical drugs. Therefore, there is significant potential for the discovery of substances with therapeutic value from traditionally used plants.

2.1.4. Traditional medicine

Traditional medicine can be defined as encompassing "***diverse health practices, approaches, knowledge and beliefs [that] incorporate plant and animal, and or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well being as well as to treat, diagnose or prevent illness***" (**WHO, 2002a**). Through the ages many cultural groups around the world have incorporated and practiced such medical practices into their cultures, which has been passed down to the next generation as part of their cultural heritage (**WHO, 2002a**). In developing countries traditional medicine is more widely practiced when compared to Western medicine. The World Health Assembly acknowledged the dependence of developing nations on traditional medicine for primary health care in their declarations. **UNESCO (1996)** reported a widespread usage of medicinal plants within the practice of traditional medicine in developing countries. It is estimated that up to 80% of the world's population depend on traditional medicine to cater for their primary health care (**ABELSON, 1990; HILLENBRAND, 2006**). However, traditional medicine should not be considered to be a primary healthcare alternative found only in developing countries. A later report showed an increased dependence by developed countries on natural plant products (in the

form of plant derived clinical drugs/herbal remedies) (**KUHN, 2002; RASKIN et al., 2002**). Twenty five percent of clinical drugs contain bioactive compounds extracted from plants (**FARNSWORTH, 1984; FARNSWORTH, et al., 1985; CAMERON et al., 2005**). The **WHO** reported in **2008** that little over 70% of European, North American and other developed countries' population has used various forms of alternative/complementary therapies (**VAN DER WATT et al., 2008**; **WHO 2008**).

Traditional medicine may be considered a more holistic system where both the physical and mental aspects of an illness are considered. The **WHO 2001** report, lists some of the advantages of traditional medicine over conventional medicine: accessibility, affordability, wider acceptance by the developing nations, increasing popularity in developed countries. In Africa the number of people using traditional medicine varies greatly with that of those that use conventional medicine. Take for example the relative ratio of patients to practitioners in Ghana: 224 people: 1 traditional healer compared to 21 000 people: 1 orthodox practitioner and in Swaziland: 110 people: 1 traditional healer compared to the 10 000 people: 1 orthodox practitioner (**RUKANGIRA, 2001**). According to Statistics South Africa, there are an estimated 200 000 traditional healers in South Africa (cited in **MOAGI, 2009**). This estimate includes diviners (sangoma) and herbalists (inyanga) as well as traditional birth attendants and surgeons (**WREFORD, 2007**).

With regard to African Traditional Medicine, a medicinal plant may also be consumed as food so there is no impression of it being toxic as it contains therapeutic properties. Unfortunately though, clinical drugs which are often produced synthetically with very specific and potent therapeutic activity have been associated with numerous, often severe, side effects. The iatrogenic effects of modern drugs made available to communities that rely on traditional medicine may be due to the lack of information on its use, misadministration of the drugs or even drug abuse. A medicinal plant on the other hand may not exhibit such high potency (as the active compound is present in its crude form) but there have been few reports of severe side effects (**TOMASSONI** and **SIMONE, 2001**; **BANDARANAYAKE, 2006**).

Natural products have played an integral role in treating infectious diseases (**CRAGG et al., 1997; MAHADY et al., 2008**). Earlier discoveries of therapeutic agents from natural resources are an indication of the value of natural products/nature. The vast number of illnesses that affect communities in developing countries like Africa can be attributed, in part, to unhygienic practices and living conditions and the lack of food and safe drinking water (**BLACK et al., 2003; WEISS** and **McMICHAEL, 2004; COKER et al., 2011**). **PELEG and HOOPER (2010)** call attention to the chronic worldwide crises of ineffective treatment for emerging and re-emerging infectious diseases and the constant development of antibiotic resistance (AR).

However, it makes more economic sense for pharmaceutical companies based in developed countries to conduct extensive research on ailments such as heart disease and cancer. This is often in response to market/population demands. So, with hardly any buying power, communities in the developing countries have not been able to influence/motivate for research initiatives towards ailments that most affect them (**SHAH, 2010**). With traditional medicine being a well supported industry and considering the wealth of natural resources, a more focused and meaningful approach to research should be to assess traditional medicinal plants for potential therapeutic compounds. This would allow for the validation of use of those species.

2.1.5. Traditional medicine in South Africa

The African continent is home to a multitude of habitats which comprise a wide range of plant species. The African medical system draws on this resource to treat a large proportion of the population that prefers to consult a traditional healer rather than a conventional medical practitioner. **RASKIN et al. (2002)** estimated that 75% of the population from developing countries depend on plant-based remedies.

A major sector of the South African population subscribe to the use of medicinal plants to attend to their health needs. The preference for traditional medicine over conventional medicine was thought to be due to medication being accessible and a cheaper option. According to **MANDER et al. (2007)** however, the use of traditional medicine is not limited to regions and/or persons that

are poor and uneducated. He goes on further to state that "traditional medicine was often more expensive than at local government clinics". This trend is not surprising though as, the practice and consumption of African Traditional Medicine is based primarily on the cultural beliefs and traditions of the African community. The Southern African region supports approximately 30 000 higher plant species of which an estimated 3 000 are used in traditional medicine (**VAN WYK et al., 1997**).

MANDER (1998) reported on the extent of traditional medicine usage in KwaZulu-Natal. Since then statistics on the trade of traditional medicines in South Africa have not changed much. **MANDER et al. (2007)** revealed similar estimates; with about 27 million consumers, more than 133 000 persons employed and over 700 plant species traded. Another report by **VAN WYK et al. (2009)** indicated the national position with around 3 000 plants being used for medicine, with only 350 species used more commonly/traded more often. **VAN WYK et al. (2009)** also reported that an estimated 200 000 indigenous traditional healers in South Africa consult with a large sector of the population (providing primary healthcare alternatives). It is because of this high number and their location, that they are more available and accessible to communities in rural and remote parts of South Africa (**LIVERPOOL et al., 2004**). Previously, the South African Medical Association estimated that in South African cities there is 1 orthodox medical practitioner to 700 patients. In rural areas however, that ratio is 1:10 000. These kinds of statistics reveal why there may be such a heavy reliance on traditional medicine at present. More recent data published by **BUSIA (2005)** show ratios of 1:1639 and 1:700-1200 for orthodox doctor:patient and traditional medical doctor:patient respectively.

South African traditional medicine, however, is at risk of losing vital indigenous knowledge. Unlike Indian and Chinese traditional medicine, knowledge has not been captured in documents or pharmacopeias detailing the use, preparation and administration of the medication. Instead, African cultural heritage, which includes knowledge about traditional medication, has been passed down by word of mouth from generation to generation (**MOROLONG, 2007**). This tradition of oral history has the risk of future misinterpretation and loss of information. However, there have been significant ethnobotanical contributions over the years by various authors which serve as the only available documented information on African traditional

medicine, at times detailing the uses, preparations and administration of South African plants in the African medical system.

It is mainly the elders of a rural community that hold significant knowledge of herbal medicine (**LALONDE, 1993**). However, **VAN WYK et al. (1997)** are quick to point out that, though traditional medicine is deeply rooted in ancient tradition, it is adaptable and open to change through modern developments. **SIMON and LAMIA (1991)** reported the dispensation of effective conventional medication such as penicillin by traditional healers. They have also shown keen interest in primary health care training programmes (**RICHTER, 2003**).
Another positive development is the renewed interest in the medicinal plants used. Recent research efforts address two main topics: the conservation of the more commonly used plants and their cultivation; and the pharmacological evaluation of the plants to verify their medicinal value and efficacy.

2.1.6. Conservation issues

There has been renewed interest in traditional practices, drugs originating from plants around the world, and plants used for ethnomedicinal uses since the 1990s. This renewed respect for the indigenous people and the wealth of knowledge they hold is mainly due to conservation concerns and the increased demand by the public for alternative medicines (**IDRC FACTSHEET**).
South Africa boasts great plant biodiversity, yet there are a number of factors contributing to their decline. One of these factors is the incorrect and/or over-harvesting of vulnerable medicinal plant species. Historically, plant material was harvested in a sustainable manner mostly by the healer himself (**VAN WYK et al., 1997**). It has however, become modern practice to purchase these plants from informal/untrained gatherers trying to generate an income. This impacts negatively on the natural/wild plant resources as there is no sustainable harvesting (**MANDER et al., 2007**) and it is an obvious threat to South Africa's plant biodiversity. The practice of traditional medicine is not widely accepted due to the dire conservation status of many medicinal plants. The harvesting of plant species for traditional medical practices is however, not considered the greatest threat to biodiversity. According to the Living Planet Index

(which “reflects the state of the planet’s ecosystems”) in the **LIVING PLANET REPORT (2008)** habitat loss, land changes and fragmentation due to agriculture are the chief anthropogenic factors that negatively impact on natural ecosystems. Other threats are grouped as either overexploitation, pollution, spread of invasive species or genes and climate change; in order of severity. Accordingly, **HINRICHSEN** and **ROWLEY (1999)** predict that up to 60 000 plants could become extinct by the year 2025. It has also been suggested that there is significant economic value attached to plant-based drugs and those that are yet to be discovered (**PRINCIPIE, 1996**).

Given the vast plant biodiversity and rich cultural heritage of South Africa, the renewed interest in medicinal plant research and conservation concerns; we are obliged to conduct exact and responsible scientific research. Medicinal plants and holders of indigenous knowledge are valuable resources to our society. While South Africa is unique in many ways, the environmental, social and economic difficulties we face are not new. Medicinal plant research presents a variety of avenues via which science can benefit society.

CHAPTER 3
ORCHIDACEAE

3.1. INTRODUCTION

The Orchidaceae make up the largest and most diverse family of flowering plants. They are popular, often expensive, ornamentals. They are widespread, with a broad range of ethnobotanical applications. Their medicinal use and corresponding pharmacological and phytochemical properties have been extensively investigated globally. However, the biological activity and chemical composition of South African medicinal orchid species is yet to be explored fully. There is very limited documented information on South African medicinal orchid species; no formal pharmacopoeia outlining ethnobotanical uses; and ethnobotanical and distribution records are either scarce or inconsistent and plant populations are becoming gradually smaller. In this Chapter the potential for South African species is highlighted by reviewing pharmacological and phytochemical research done globally on other orchid species.

3.2. THE FAMILY

The Orchidaceae is the largest Angiosperm family and boasts a worldwide distribution of an estimated 20 000 species and 796 genera (**CHASE et al., 2003; DRESSLER, 1993b**). More recent estimates are in the region of 35 000 species (**CRIBB et al., 2003**). Currently, there are five accepted subfamilies, Apostasioideae (earliest orchids), Cypripedioideae, Vanillioideae, Orchidioideae and Epidendrioideae (largest and most evolved) (**SMITH** and **READ, 2008a**). Most orchids are either epiphytic or terrestrial with some orchid species having other growth forms such as epilithic or lithophilic (attached to rocks) and saprophytic (feeding on dead decaying matter).

3.3. MORPHOLOGY AND REPRODUCTIVE BIOLOGY

The Orchidaceae are monocotyledonous plants with very distinguishing characteristics (**SEIDENFADEN** and **WOOD**, **1992; DRESSLER, 1993a; HTTP://WWW.KEW.ORG/SCIENCE/ORCHIDS/ORCHIDSTRUCTURE.HTML**). Morphologically, orchids are unique with zygomorphic flowers that contain a labellum or lip (highly modified and enlarged petal) and varying lengths of spurs (elongated perianth lobes or median and lateral sepals). The lip is considered to be an adaptation that facilitates cross pollination. Another distinctive feature of an orchid flower is the gynostemium (column) which is a combination of the pistil and remaining stamen/s (female and male reproductive organs). There is vast evolutionary history associated with this particular fused organ. The anther found at the tip of the column has a sticky mass – viscidium to which pollinia (separate masses of pollen) are attached via a stipe in most epiphytic orchids or a caudicle in most terrestrial orchids. The stigma is ventrally positioned in the column. Pollinia are generally transferred to the stigma via a rostellum (modified stigma lobe). Most orchid flowers undergo resupination at the inferior ovary, where the flowers rotate 180°, to ensure that the lip is positioned below the column and to allow more efficient and successful pollination. Resupination, however, seems to have been lost in some genera, while in some genera, the flower may rotate 360°. Post pollination, ovules develop into minute „dust-like' seeds which are suitable for wind dispersal. Other significant structures include the inflorescence (structure on which flowers are presented), pseudobulbs (modified stem that perform the role of a water storage organ), tubers (underground structures that endure the dry seasons), velamen (a protective layer found on the older section of the roots) and a symbiotic relationship with mycorrhiza at the root soil interface (which facilitates uptake of nutrients). Such valuable features have enabled the different genera to colonise many diverse habitats (**DRESSLER, 1993a**).

3.4. DISTRIBUTION

Orchids have adapted to a wide range of habitats around the world, occurring mainly in the tropical and subtropical regions and at altitudes ranging from sea level up to 5 000 m. They are

particularly dominant in a nutrient-deficient environment and more than half the world species are epiphytic. The tropics of South America support the highest number of speices (±6400), followed by Asia (±4000) and Africa (±1500) (**BRUMMIT, 2001; McMURTRY et al., 2008**). Comparatively, Africa is less well endowed, however, southern Africa accounts for a third of the 1500 orchid species and can therefore be considered rich in orchid diversity. In southern Africa, orchids are widely represented, with 55 genera and approximately 494 epiphytic and terrestrial species (**GERMISHUIZEN** and **MEYER, 2003**). A high frequency of endemism is also a feature with approximately 75% being endemic to the region, occupying a very restricted distribution range (**STEWART et al., 1982**). For those genera found in other regions, the majority of their species are endemic in southern Africa.

3.4.1. Distribution pattern in South Africa

An orchid distribution map posted on the *Plantzafrica* website shows the presence of orchid populations across the greater part of the country (**POWRIE** and **KURZWEIL, 2000**). The northwestern region however, reveals a much reduced occurrence of orchid species. In South Africa, 35 species are terrestrial, 17 epiphytic and 1 epilithic.

The species used for traditional medicine in South Africa are distributed mainly in the eastern and northern regions of the country (**Table 3.1. Chapter 3**). This distribution pattern is probably dictated by seasonal rainfall patterns. Species such as *Ansellia africana* Lindl., *Disa aconitoides* Sond., *Eulophia clavicornis* Lindl. var. c*lavicornis, E. ensata* Lindl. share similar distribution patterns across the south eastern coast towards the northern Provinces of South Africa. Some species are found across the border into neighbouring countries such as Zimbabwe and Mozambique (*E. tenella* Rchb.f. and *D. polygonoides* Lindl. respectively), or as far north as tropical Africa (*Diaphananthe xanthopollinia* (Rchb.f.) Summerh. Synonym: *Rhipidoglossum xanthopollinium* (Rchb.f.) Schltr. and *E. angolensis* (Rchb.f.) Summerh.).

Seven species were investigated in this study; two belong to the pantropical terrestrial *Eulophia* genus, *E. petersii* and *E. hereroensis*. The epiphytic *Ansellia* genus which also occurs in these

habitats is considered rare due to overharvesting. *Cyrtorchis arcuata* and *Tridactyle tridentata*, two epiphytic species share similar distribution patterns across Africa. Another well represented genus in the southern African region is the epiphytic/lithophytic *Polystachya*. *Polystachya pubescens* is found more commonly in forest and bushland regions. *Bulbophyllum scaberulum*, another lithophyte can be found in large masses on trees and rocks.

3.5. ETHNOBOTANICAL USES OF ORCHIDS AROUND THE WORLD

3.5.1. Medicinal uses

It is a broadly known concept that orchids are used for medicinal purposes around the world. The medicinal value of orchids was realised by the early civilizations and they were subsequently used as therapeutic agents to treat common ailments. **BULPITT (2005)** described the widespread medicinal use of orchids. In early European history, Theophrastus (372-286 BC) derived the word orchid from the Greek word *orchis* (as the tubers of many European terrestrial orchids resembled testicles). Orchids were therefore used initially to treat testicular diseases and as an aphrodisiac (**BULPITT, 2005**). **LANGHAM (1579)** described anti-pyretic; anti-consumption; and anti-diarrhoeal effects. The mucilaginous nature of salep served as a demulcent and nutritive to sooth gastrointestinal irritations (**BULPITT, 2005**). The Americans make extensive use of the vanilla orchid as a flavourant and perfume, a tradition passed on from the ancient Aztecs to modern civilizations. The vanilla aroma is used to detect Alzheimer's disease (**FLADBY et al., 2004**). Vanillin (active compound isolated from vanilla) is also reported to have antimicrobial activity (**FITZGERALD et al., 2003**). The Australian aborigines and early settlers mainly used the *Cymbidium* and *Dendrobium* species to treat dysentery, as an oral contraceptive, to relieve pain/poultice, and to cure ringworm infection. Some species were consumed as a food source. **BULPITT (2005)** also makes reference to other regions such as India and China with records of orchid use in their pharmacopoeia.

In India, the Ayurvedic Pharmacopoeia currently consists of seven volumes and is a legal document of standards for the quality of Ayurvedic drugs used since the Vedic era. Tonics such as Ashtawarga which comprise of eight drugs include four orchid species – *Malaxis muscifera* (Lindl.)Kuntze, *M. acuminate* D.Don, *Habenaria intermedia* D.Don and *H. edgeworthi* Hook.F. There is record of 40 other species used in traditional Indian medicine (**SINGH et al., 2007; RAO, 2004**). These species represent approximately 21 genera; all identified as important medicinal sources: *Acampe, Bulbophyllum, Calanthe, Coelogyne, Cymbidium, Cypripedium, Dactylorhiza, Dendrobium, Epipactis, Eria, Eulophia, Filckingeria, Habenaria, Liparis, Luisia, Malaxis, Pecteilis, Pholidota, Rhynchostylis, Satyrium* and *Vanda* (**SINGH et al., 2007**). Chinese traditional medicine, which is just as ancient as Ayurvedic medicine, has many references to orchid use for medicinal treatment. **BULPITT et al. (2007)** highlighted the five major traditional medicines of which orchids are key ingredients – Shi-Hu (five *Dendrobium* species) used to treat deficiency disorders in the kidney, lung and stomach, Tian-Ma (*Gastrodia elata*) used to relieve dizziness, convulsions, hypertension and to treat stroke patients, Bai-Ji (*Bletilla striata*) used to treat haemorrhagic disorders as well as cancers; Jin-Xian-Lian (two *Anoectochilus* species) used mainly to treat nephritis, cystitis and pneumonia and Shan-Ci-Gu (*Cremastra appendiculata*) used to treat tonsillitis, hypertension and cancers.

On the African continent there are many examples for use; for instance Malawians use about 12 species for medicinal purposes, of which approximately nine species are used to treat stomach ailments and two more cater for fertility problems. *Cyrtorchis arcuata* is used to treat diabetes and skin infections; epilepsy is prevented by using *E. cucullata* and psychological disorders such as madness is treated with *T. tricuspis* (**BULPITT, 2005**). While there is an awareness of medicinally used orchids around the world; the extensive use of certain southern African orchids for medicinal and magical purposes is not known to most people, illustrated in **Table 3.1.** (**Chapter 3**). The African medical system lacks significant detail on the therapeutic uses, preparations and administration of South African orchid species.

3.5.2. Non-medicinal uses

Globally orchids are appreciated for their ornamental appeal and therefore considered as significant horticultural plants. **BULPITT (2005)** reported on some other economic and cultural uses of orchids. Salep (dried and powdered orchid tubers), famously known in European countries, was originally produced by 16th century Europeans for use in beverages and ice cream. It is a key ingredient in a popular Turkish dish – salep *dondurma* (orchid ice cream) and is considered to be nutritious.

In Africa, **KURZWEIL (2000)** confirmed the use of some orchids as food. The tubers of some *Disa* and *Satyrium* species in central and eastern Africa are common sources of food for communities in rural areas. Other South African species from these genera are used to produce a sweet juice from their roots and tubers while *Neobolusia tysonii* and *Eulophia* species. are sources of food (**KURZWEIL, 2000**).

3.6. ETHNOBOTANICAL USES OF ORCHIDS IN SOUTH AFRICA

An estimated 49 indigenous orchid species from 20 orchid genera are currently being informally traded and used in South African traditional medicine (**Table 3.1. Chapter 3**) (**HUTCHINGS et al., 1996**). They are used primarily for medicinal and cultural purposes, especially by the Zulu community in South Africa (**HUTCHINGS et al., 1996; GERMISHUIZEN** and **MEYER, 2003**).

Informal market surveys, in the greater Pietermaritzburg, Umlazi, and Nongoma regions of KwaZulu-Natal, indicated that a number of medicinal orchid species share the same vernacular name (e.g.: the vernacular *iphamba* refers to 12 different species *Cyrtorchis arcuata*, *Diaphananthe millarii*, *D. xanthopollinia*, *Eulophia ensata*, *E. ovalis*, *E. leontoglossa*, *Microcoelia exilis*, *Mystacidium capense*, *M. venosum*, *Polystachya transvaalensis*, *Tridactyle bicaudata*, *T. tridentata*). It is unclear if this relates to the shape of the plant organs being sold, locality from which it was collected, or for its claimed therapeutic use. Traders were interviewed

in their vernacular language and in English about the uses of plants listed in **Table 3.1.** (**Chapter 3**). Traders recognised plants when using the vernacular names and from pictures of dried herbarium samples. Interviews with plant traders rarely produce congruent answers; with some traders not even recognizing the plant or vernacular name at all. Plant traders may also sell substitutes for certain orchids; if there are alternative plants with the same/similar vernacular name or therapeutic value as the one requested, or if the plant requested is not available. None of the traders interviewed referred to the plant species by their scientific names.

Our observations, of KwaZulu-Natal herbal markets, have indicated that the rate of trade in medicinal orchids, as well as the volume traded is higher for species such as *A. africana* (whole plants); which are sold mainly for their aphrodisiac properties. *Eulophia streptopetala* (tubers and roots) is traded at a similar rate, but at a slightly lower volume. Species from the genus *Eulophia* are often sold interchangeably due to their appearance and common use in the treatment of infertility. *E. petersii* (whole plants) is sold frequently, in smaller volumes, as it is used both medicinally and culturally.

3.6.1. Medicinal uses

3.6.1.1. Inflammatory disorders

From the literature it is evident that approximately 14 orchid species from seven genera are used medicinally in South Africa (**Table 3.1: Section A Chapter 3**). Nearly 50% of these species belong to the genus *Eulophia*. Three *Eulophia* species and one *Polystachya* species are used to treat inflammatory related disorders in South African traditional medicine. In South Africa, the burnt tuber powder of *E. ovalis* is rubbed into incisions made on limbs affected by pain symptoms (**HUTCHINGS et al., 1996**). *Polystachya ottoniana* (plant part not specified) is used to produce a soothing effect for teething babies (**BATTEN** and **BOKELMANN, 1966**). The Malawians use cooked roots of *E. cucullata* as a poultice (**WATT** and **BREYER-BRANDWIJK, 1962**). In Zimbabwe, the tubers of *E. petersii* are prepared as an infusion and

used to treat dropsy (**GELFAND et al., 1985**). The Pedi community uses a root infusion of *A. africana* for children with cough symptoms (**WATT** and **BREYER-BRANDWIJK, 1962**).

3.6.1.2. Intestinal disorders

Polystachya ottoniana (plant part not specified) is used to produce a soothing or curative effect for patients with diarrhoea (**BATTEN** and **BOKELMANN, 1966**). For those patients diagnosed as having intestinal worms, a strong infusion of pounded *Satyrium bracteatum* tuberous roots mixed with milk is used as an enema-type anthelmintic (**HULME, 1954**).

3.6.1.3. Neurological disorders

For persons that have lost their speech mainly due to an illness (possibly a stroke), an infusion of the tubers of *D. polygonoides* is administered (**HULME, 1954**). The Xhosa people use a boiled liquid preparation (decoction) of the roots of *B. ovata* for people inflicted with symptoms of madness (**BATTEN** and **BOKELMANN, 1966**). Hysterial outbursts are treated with an emetic prepared from certain *Eulophia* species (plant part not specified) (**GERSTNER, 1941**). An infusion of the leaves and stem of *A. africana* is prepared by the Zambians in the Mpika district to treat symptoms of madness (**HUTCHINGS et al., 1996**). In certain South African regions the seeds of *P. ottoniana* are used as snuff (**BATTEN** and **BOKELMANN, 1966**). These seeds could be producing a psychoactive or hallucinogenic effect which is used in some aspects of African traditional culture.

3.6.1.4. Reproductive disorders

A root infusion of *D. aconitoides* is administered as an emetic for women; to promote conception (**HULME, 1954**). The Sotho use a cold water infusion of a few *Eulophia* species (*E. clavicornis* (plant part not specified) and *E. tenella* (tubers)) to treat infertility (**WATT** and **BREYER-BRANDWIJK**, **1962**). **JACOT GUILLARMOD (1971)** reported a similar use for tuber

infusions of *E. ovalis*. An *E. cucullata* bulbous root infusion is ingested in small doses by married couples if the wife is considered barren (**HULME, 1954**). Alternatively, a decoction of the bulbous root of *E. cucullata* is ingested by men to treat impotence (**BRYANT, 1966**). A decoction of *A. africana* is used as an aphrodisiac by women (**WATT** and **BREYER-BRANDWIJK, 1962**). **GELFAND et al. (1985)** documented a similar use for *A. africana* in Zimbabwe.

3.6.1.5. Other medicinal uses

The therapeutic information, administration and preparation of *E. ensata* is not entirely descriptive and the specific ailment for which this orchid is prescribed is not clear. However, it is mentioned that in the Transkei (Eastern Cape) infant ailments are treated with *E. ensata* (plant part not specified) (**BATTEN** and **BOKELMANN, 1966**). Emetics are prepared to cause emesis, that is to either induce vomiting or nausea. In African traditional medicine, emetics are widely used to facilitate the removal of what is thought to be the cause of the ailment. One would be expected to cough up phlegm or mucus that is produced. This practice is particularly common in the use of orchids for cultural purposes. Root infusions of *E. speciosa* are prepared as emetics for both humans and animals (**GERSTNER, 1941**). Similarly, root infusions of *H. epipactidea* are used to induce vomiting (**WATT** and **BREYER-BRANDWIJK, 1962**).

3.6.2. Non-medicinal uses

Love charms are used extensively in African traditional culture. Approximately 18 orchid species from 10 genera are used for such purposes (**Table 3.1: Section B Chapter 3**). Forty percent of these species belong to the genus *Eulophia*.

3.6.2.1. Love charms

A love charm emetic infusion of *Acrolophia cochlearis* roots is ingested by young men (**HULME, 1954**). *Aerangis mystacidii* (plant part not specified) is recorded as being used for a

similar purpose in traditional medicine (**CUNNINGHAM, 1988**). The roots of *A. africana* may be prepared as an infusion and administered as an emetic (**GERSTNER, 1941**), while the leaves are used by men as a courting charm (**HUTCHINGS et al., 1996**). *Cyrtorchis arcuata* (plant part not specified) is the key ingredient in an emetic that is administered by the man to the young lady to make her love him (**HULME, 1954**). The tuber of *E. angolensis* is prepared as an emetic infusion which is consumed by young men (**HULME, 1954**). *Eulophia cucullata* bulbous roots are prepared as an emetic infusion (**GERSTNER, 1941**). The tubers of *E. ensata* and *E. ovalis* are prepared as emetic infusions which are used by young men (**HULME, 1954**). The whole plant of *E. parviflora* is vaguely mentioned as a love or protective charm (**CUNNINGHAM, 1988**). The tubers of *E. petersii* are prepared as a sprinkling infusion love charm (**HUTCHINGS et al., 1996**). Other *Eulophia* love charm emetic infusions may be prepared from *E. streptopetala* (**HUTCHINGS et al., 1996**) and from tubers of *E. welwitschii*; which are used by young men (**HULME, 1954**). The tuber infusion prepared from *E. tenella* is used by young men to wash with and is used as a courting charm (**HUTCHINGS et al., 1996**). The preparation and administration of the following species is not entirely descriptive and lacks vital information. Two *Liparis* species, *L. bowkeri* and *L. remota* are used as love or goodluck charms (plant part not specified) in traditional medicine (**HUTCHINGS et al., 1996**). An infusion of *Microcoelia exilis* or an emetic of *Mystacidium capense* is also used as love charms (plant part not specified) (**HUTCHINGS et al., 1996**). A tuber infusion of *Oeceoclades mackenii* is taken by young men (**HULME, 1954**). Similarly, an emetic prepared from tubers of *S. parviflorum* is ingested by young men (**HULME, 1954**).

3.6.2.2. Fertility charms

More specific charms are prepared to either prevent or promote fertility. *Ansellia africana* is used by young men to prevent women from having children if their love is not returned (**WATT** and **BREYER-BRANDWIJK, 1962**). The tubers of two *Eulophia* species *E. clavicornis* (**HUTCHINGS et al., 1996**) and *E. ovalis* (**JACOT GUILLARMOD, 1971**) are used separately to create dolls that represent fertility. Whole plant decoctions of unspecified *Habenaria* species are ingested by the couple to ensure the birth of a son (**HULME, 1954**).

3.6.2.3. Protective charms

Protective charms may be used for bad dreams, to ward off evil, to protect one from lightening and to protect the home. *Ansellia africana* leaves are prepared as an infusion to treat persons experiencing bad dreams (**HUTCHINGS et al., 1996**). Alternatively one can inhale the smoke of its burning roots for the same purpose (**HUTCHINGS et al., 1996**). The tubers of certain orchids are used as an infusion that is sprinkled around the home to ward off evil: *B. ovata*, *Brachycorythis* species, *Eulophia clavicornis*, *S. longicauda* and *S. parviflorum* (**HULME, 1954**). Dried, burnt powder of whole plants of *H. epipactidea* is mixed with sheep fat which is applied to pegs put into the ground of a new homestead (**HULME, 1954**). Whole plant infusions may also be prepared from *D. stachyoides* and *D. versicolor* for sprinkling around the home to ward off evil, while the roots of *Corycium nigrescens* are prepared as an emetic infusion that is ingested (**HULME, 1954**). An enema-type tuber infusion from *E. tenella* is used to neutralise evil charms put in the food or drink (**HULME, 1954**). A whole plant infusion of *D. stachyoides*, a tuber infusion of *E. leontoglossa*, a root and stem infusion of *E. speciosa* or a bulb and leaf infusion of *H. dregeana* (**HULME, 1954**) may also be used to protect against lightening. Dried burnt powder from *H. epipactidea* whole plants mixed with sheep fat may also be applied to incisions cut into the wrists, foreheads and ankles of every member of the homestead as protection from storms (**HUTCHINGS et al., 1996**).

The preparation and administration of other orchid-derived protective charms is not clear e.g.: *E. streptopetala*. Similarly, the following species are merely listed as emetic protective or love charms: *M. venosum*, *P. ottoniana*, *P. pubescens*, *P. sandersonii*, *P. transvaalensis* (**HUTCHINGS et al., 1996**); while the following species are simply recorded as being used in traditional medicine as a charm in southern Africa, unless otherwise stated: *A. africana* in Zimbabwe (**GELFAND et al., 1985**), *E. clitellifera* (**CUNNINGHAM, 1988**), *D. millarii* (**HUTCHINGS et al., 1996**), *D. xanthopollinia* (**HUTCHINGS et al., 1996**), *Rangaeris muscicola* (**CUNNINGHAM, 1988**), *T. bicaudata*, *T. tridentata* and *Vanilla roscheri* (**HUTCHINGS et al., 1996**). Whole plants of *Bolusiella maudiae* are vaguely known to be used in traditional medical practice (**CUNNINGHAM, 1988**).

3.6.2.4. Lethal charms

A death charm, used to induce lethal effects, is prepared from the dried powder of *Habenaria dives* Rchb.f. (inhluthi yotshani) tubers, which is mixed with other herbs (**HULME, 1954**).

Table 3.1.: Description, distribution and conservation status of orchids used for medicinal, cultural and ritualistic purposes in southern Africa

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
(Section A) Medicinal Uses					
Ansellia africana Lindl.	Tough, stiff alternate leaves (250-1000 × 30 mm) with parallel veins; Yellow or yellow marked with reddish brown flowers (lip is brighter yellow) (30-50 mm wide) arranged on branching inflorescence (±500 mm) (Jun-Nov)	Imfe-nkawu	Aphrodisiac Madness Coughs	Tropical Africa (MLW, MOZ, ZAM, ZIM, BOT, NAM, SWZ) South Africa (NAT, TVL)	Declining
Brachycorythis ovata Lindl.	(egg-shaped) Perrenial herb; crowded sharp-tipped leaves (±70×22 mm); Pale pink to purple flowers with white keel and purple spotted lip; 3 lobed with no spur (±13 mm wide) arranged on a crowded inflorescence with lower flowers hidden by within long bracts (Oct-Jan)	Imfeyamasele yentaba	Madness	Tropical Africa (MLW, ZAM, ZIM, SWZ) South Africa (CPP, NAT, OFS, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Disa aconitoides Sond.	(monk's hood) Perennial herb; Many overlapping leaves; 15-70 white to pale mauve with darker spots flowers with a thick upward pointing spur (±10 mm) that is almost as broad as the hood. (Oct-Jan)	Ihlamvu Umashushu	Conception	Ethiopia, Tropical Africa (MLW, MOZ, ZAM, ZIM, SWZ) South Africa (CPP, NAT, OFS, TVL)	LC
Disa polygonoides Lindl.	(knot-grass)Perennial herb; Erect clasping leaves (±150×15 mm); Red orange flowers on dense spikelike inflorescence (±170×20 mm) with slender spur (± 4,5 mm) that faces down. (Oct-May)	Ihlamvu elibomvu Umklakleshe	Lost speech	MOZ, ZIM, LES, SWZ South Africa (CPP, NAT, OFS, TVL)	LC
Eulophia clavicornis Lindl. var. c*lavicornis*	(Horn) Perennial herb; Partly to fully developed leaves at flowering (50-730 mm); green to purplish brown sepals with white to pale pink or pale blue petals and a lip as long as the median sepal (9-18 mm); cylindrical spur (14-89 mm) with tall crests till end of lip (Aug-Sep)	Elihlaza Imfe yamasele (encani)	Infertility	ZIM, MOZ, SWZ South Africa (CPP, NAT, TVL)	Not Available

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia cucullata (Afzel. ex Sw.) Steud.	(Hooded) Perennial herb; Leaves may be absent or partly grown at flowering and ±300×10 mm at maturity; 3-15 large flowers (±30 mm diameter) and petals and sepals (±27 mm) with bright pink to white tinged pale purplish pink petals and lip, inner surface of lip yellow with orange and purple spots; purplish brown sepals (Sep-Jan)	Amabelejongosi Uhlamvu lwabafazi Umabelejongosi Undwendweni	Impotence Infertility Poultice	Tropical Africa (MWL, MOZ, ZAM, ZIM) Madagascar South Africa (NAT)	LC
Eulophia ensata Lindl.	(Sword-shaped) Perennial herb; Stiff, erect, pleated, tapering partly to fully grown leaves at flowering (±900×15 mm); 6-30 pure yellow flowers (±20 mm) with 3 lobed blunt lip (±25×12 mm) and slender spur (±7 mm) (Sep-Feb)	Iphamba yentaba Mahlane	Infant ailments	MOZ, SWZ South Africa (CPP, NAT, TVL	LC
Eulophia ovalis Lindl. subsp. ovalis	(Oval) Perennial herb; Fully developed leaves at flowering (±600×30 mm); ±18 smaller flowers with white petals tinged yellow or purple, with fleshy lip and peg-like outgrowths and 1-5 mm spur (Oct-Apr)	Iphamba	Sore limbs/pain Infertility	ZIM, LES, SWZ South Africa (CPP, NAT, OFS, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia petersii (Rchb.f.) Rchb.f.	Perennial herb; Thick, spreading leaves with rough margins (±400×44 mm); flowers arranged on well spaced inflorescence with branching stem, large flowers, sepals (19-33 mm), petals and sepals curled back, greenish purplish brown, white lip (±30×15 mm) with purplish pink crests; 2-8 mm spur (Nov-Apr)	Isaha/isaka	Dropsy / Heart congestive disorder	Eritrea, Arabian Peninsula MLW, MOZ, ZAM, ZIM, SWZ, South Africa (NAT, TVL)	Vulnerable (**GORDON-GRAY, 2003**) LC
Eulophia species		Umahayiza Amabelejongosi Umabelejongosi Undwendweni Imabeleyongosii Mfe yamasele empofu	Hysteria Infertility	Not Applicable	Not Applicable

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia speciosa (R.Br. ex Lindl.) Bolus	(Beautiful) Perennial herb; Sti smooth and thick mature leaves flowering (±600×20 mm); 10-3 flowers arranged on inflorescence wi small pale green sepals, large spreadir bright yellow petals (±20×15 mm) ar a blunt spur (Aug-Jan)	Amabelejongosi Umlunge omhlope	Emetic	Ethiopia - Arabian Peninsula MLW, MOZ, ZAM, ZIM, BOT, NAM, SWZ South Africa (NAT, TVL)	Lower Risk – Near Threatened Declining
Eulophia tenella Rchb.f.	(Tender) Perennial herb; leaf dimensions (100-420×2-6 mm); Small flowers with green to brownish purple sepals (±9 mm) and creamy yellow petals, midlobe of lip is bright yellow with stout spur (2-3 mm) (Nov-Jan)	Untongazibomvana	Infertility	South Tropical Africa (MOZ, ZIM) South Africa (CPP, NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Habenaria epipactidea Rchb.f.	(Similar to *Epipactus*) Perennial herb; overlapping ribbed leaves that clasp the stem and grade into bracts (±100×20 mm); creamy green flowers with white lip arranged on dense inflorescence (±120 mm) with erect bracts ±40×10 mm, midlobe of lip ±15 mm hair-like side lobes, ±30 mm slender spur with thickened tip; ± 10 mm stalk (Nov-Feb)	Umabelebuca omkhulu	Emetic	Ethiopia, ZIM, BOT, LES, NAM, SWZ South Africa (CPP, TVL)	LC
Polystachya ottoniana Rchb.f.	Perennial herb; Epiphyte; Vertically flattened pseudobulb and tapering to one end (±25×15 mm); 2-4 blunt notched leaves (±80×10 mm); 1-6 white to pinkish or yellow flowers (10-14 mm wide) on slender, erect inflorescence, 3 lobed, curled lip with yellow mid stripe with pink streak in triangular side sepals (Aug-Dec)	Amabelejongosi	Teething babies Diarrhoea Snuff	South Tropical Africa (MOZ, ZIM, SWZ) South Africa (NAT, CPP, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Satyrium bracteatum (L.f.) Thunb.	Plants have a foetid smell	Ubani lwenkangala	Intestinal worms	LES South Africa (CPP, NAT, TVL)	LC
(Section B) Cultural and Ritualistic Uses					
Acrolophia cochlearis (Lindl.) Schltr. & Bolus	(shell-like) Perrenial herb, 4-9 leaves arranged in 2 ranks; Small (<10 mm wide) flowers coloured greenish brown, white with tinge of purple arranged on a widely branced inflorescence (Aug – Jan)	Impimpi	Love charm	Southern Cape Province – KwaZulu-Natal	LC
Aerangis mystacidii (Rchb.f.) Schltr.	Flat leathery, oblong/ wider at bilobed tip, dark green leaves arranged in a fan; 2-6 (±280 mm) inflorescence with 4-14 (up to 24) flowers (±20×25 mm wide) that are white tinged pink; Spur 60-80 mm (Feb-Jun)	Not Available	Love or protective charm	South-west Tanzania, MLW, MOZ, ZAM, ZIM, SWZ South Africa (CPP, NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Ansellia africana Lindl.	Tough, stiff alternate leaves (250-1000 × 30 mm) with parallel veins; Yellow or yellow marked with reddish brown flowers (lip is brighter yellow) (30-50 mm wide) arranged on branching inflorescence (±500 mm) (Jun-Nov)	Imfe-nkawu	Courting charm Bad dreams Love charm Bad dreams Charms	Tropical Africa (MLW, MOZ, ZAM, ZIM, BOT, NAM, SWZ) South Africa (NAT, TVL)	Lower Risk – Near Threatened Declining
Bolusiella maudiae (Bolus) Schltr.	Rare, thin short roots; Short stems; 3-10 dark green fleshy, succulent leaves that overlap at base in a fan arrangement (Feb) (20-30×5-8 mm); 20-30mm wide flowers partially hidden by bracts; with lip 3 lobed at base and short rounded spur.	Not Available	Used in traditional medical practice	Tropical Africa (MLW, ZAM) South Africa (NAT)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Brachycorythis ovata Lindl.	(egg-shaped) Perrenial herb; crowded sharp-tipped leaves (±70×22 mm); Pale pink to purple flowers with white keel and purple spotted lip; 3 lobed with no spur (±13 mm wide) arranged on a crowded inflorescence with lower flowers hidden by within long bracts (Oct-Jan)	Imfeyamasele yentaba	Ward off evil	Tropical Africa (MLW, ZAM, ZIM, SWZ) South Africa (CPP, NAT, OFS, TVL)	LC
Brachycorythis species		Ilabatheka elikhulu	Ward off evil	Not Applicable	Not Applicable
Corycium nigrescens Sond.	Perennial herb; Erect (±200 mm) overlapping leaves; small rounded brown to black flowers in dense inflorescence, lateral sepals mostly joined, lip appendage (±4 mm); Bracts have long slender points (Dec-May)	Umabelebuca	Ward off evil	Southern Tanzania, LES, SWZ South Africa (CPP, NAT, OFS, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Cyrtorchis arcuata (Lindl.) Schltr.	(bent like a bow) Perrenial herb; epiphyte; with whitish roots with greenish orange tips; ±400×10 mm wide stems; strap-shaped leathery wide stiff leaves (110-160×25-30 mm); Large cream flowers (±50 mm wide) with 40-60 mm spur arranged on 2-6 hanging inflorescence (80-100 mm) (Sep-May)	Iphamba Imfeyenkawu Umbambela	Love charm	Tropical Africa (MLW, MOZ, ZAM, ZIM, SWZ) South Africa (CPP, NAT, TVL)	LC
Diaphananthe millarii (Bolus) H.P.Linder	Perennial herb; epiphyte; Rare; (4-5mm) diameter Roots grey- green with elongated white dots; ±25 mm stems; 3-10, 70-120×15-17 mm stiff, velvety leaves with conspicuous veins; 1-2 hanging inflorescence (±27 mm) with 7-13 (±12 mm) diameter flowers with rounded sepals, green anther caps, spur with broad mouth, slender tip (Dec-Jan)	Iphamba	Protective charm	South-eastern Cape Province – KwaZulu-Natal	Endangered (B1B2 abc) Vulnerable

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Diaphananthe xanthopollinia (Rchb.f.) Summerh. Synonym: *Rhipidoglossum xanthopollinium* (Rchb.f.) Schltr.	(yellow pollinia) Perennial herb;Epiphtye; branched stems (120 mm); ±16 (40-100×6-9 mm) leathery, thin, straight leaves with rounded lobes at tip; 10-25 yellowish green flowers (6-12 mm diameter) with very broad lip, slightly curved lip (±5 mm) on horizontal or hanging inflorescence (Feb-May)	Iphamba	Protective charms	Uganda, MLW, MOZ, ZAM, ZIM South Africa (CPP, NAT)	LC
Disa stachyoides Rchb.f.	(Wound wort) Perennial herb; pointed leaves (±80×10 mm); Small purple flowers with white lip, 2-6 mm broad, flat almost horizontal spur; arranged on dense inflorescence (±100 mm) (Nov-Jan)	Ihlamvu elimpofu Lasenkangala	Ward off evil and lightening	LES, SWZ South Africa (CPP, NAT, OFS, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Disa versicolor Rchb.f.	(Changing colour) Perennial herb; 3-5 folded basal leaves (±200×20 mm) from separate shoot; sheathing stem leaves (±100×15 mm); dull red to pink or fading greenish brown flowers with hook spur (5-7 mm) arranged on dense inflorescence (±150 mm) with bracts longer than flowers; vanilla scented (Dec-Feb)	Ihalmvu elibomvu	Ward off evil	Southern Tropical Africa (MOZ, ZIM, LES, SWZ) South Africa (CPP, NAT, OFS, TVL)	LC
Eulophia angolensis (Rchb.f.) Summerh.	Perennial herb; Stiff, erect, pleated leaves (±900×50 mm); 4-10 bright lemon yellow with purplish brown or olive tinged flowers (± 26 mm) with large blunt tipped sepals and poorly developed spur arranged on ±300 mm inflorescence; sweetly scented (Oct-Apr)	Amabelejongosi	Love charm	Tropical Africa (MLW, MOZ, ZAM, ZIM, BOT) South Africa (CPP, NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia clavicornis Lindl. *var. clavicornis*	(Horn) Perennial herb; Partly to fully developed leaves at flowering (50-730 mm); green to purplish brown sepals with white to pale pink or pale blue petals and a lip as long as the median sepal (9-18 mm); cylindrical spur (14-89 mm) with tall crests till end of lip (Aug-Sep)	Elihlaza, Imfe yamasele (encani)	Ward off evil Fertility charm	ZIM, MOZ, SWZ South Africa (CPP, NAT, TVL)	Not Available
Eulophia clitellifera (Rchb. f.) Bolus	(Small pack saddle) Perennial herb; Leaves may be absent or ±40 mm at flowering with mature thick, stiff leaves (±240×13 mm); 5-25 small white to pale yellow with reddish purple lined flowers, petals and sepals spreading (4-9 mm), bright yellow crest on lip with ±4 mm spur (Jul-Nov)	Not Available	Used as a charm in traditional medicine	Tropical Africa (MOZ, ZAM, ZIM) Madagascar South Africa (CPP, NAT, OFS, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia cucullata (Afzel. ex Sw.) Steud.	(Hooded) Perennial herb; Leaves may be absent or partly grown at flowering and ±300×10 mm at maturity; 3-15 large flowers (±30 mm diameter) and petals and sepals (±27 mm) with bright pink to white tinged pale purplish pink petals and lip, inner surface of lip yellow with orange and purple spots; purplish brown sepals (Sep-Jan)	Amabelejongosi Uhlamvu lwabafazi Umabelejongosi Undwendweni	Love charm	Tropical Africa (MWL, MOZ, ZAM, ZIM) Madagascar South Africa (NAT)	LC
Eulophia ensata Lindl.	(Sword-shaped) Perennial herb; Stiff, erect, pleated, tapering partly to fully grown leaves at flowering (±900×15 mm); 6-30 pure yellow flowers (±20 mm) with 3 lobed blunt lip (±25×12 mm) and slender spur (±7 mm) (Sep-Feb)	Iphamba yentaba Mahlane	Love charm	MOZ, SWZ South Africa (CPP, NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia leontoglossa Rchb f.	(Tongue like a lion) Perennial herb; Stiff; erect; partly to fully grown leaves at flowering (±450×9 mm); 7-35 (±16 mm) white to lemon yellow to pink tinged green flowers; pale yellow, pink, or white petals; yellow to brown crests on lip; slender spur (3-5 mm) with overlapping bracts (Aug-Feb)	Iphamba	Protection from storms	LES South Africa (CPP, NAT, OFS, TVL)	LC
Eulophia ovalis Lindl. *subsp. ovalis*	(Oval) Perennial herb; Fully developed leaves at flowering (±600×30 mm); ±18 smaller flowers with white petals tinged yellow or purple, with fleshy lip and peg-like outgrowths and 1-5 mm spur (Oct-Apr)	Iphamba	Love charm Infertility charm	ZIM, LES, SWZ South Africa (CPP, NAT, OFS, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia parviflora (Lindl.) A.V. Hall	(With small flowers) Perennial herb; Partly developed leaves at flowering, leathery, and (250×16 mm) at maturity; 5-30 flowers with blunt petals and sepals, (7-20 mm), sepals are brownish green on the outside and orange-brown on the inside and pale yellow petals with brownish red veins, yellow midlobe with broad fleshy ridges and 2-5 mm spur (Aug-Dec)	Not Available	Love or protective charm	Southern Tropical Africa (MOZ, ZIM, SWZ) South Africa (CPP, NAT, TVL)	LC
Eulophia petersii (Rchb.f.) Rchb.f.	Perennial herb; Thick, spreading leaves with rough margins (±400×44 mm); flowers arranged on well spaced inflorescence with branching stem, large flowers, sepals (19-33 mm), petals and sepals curled back, greenish purplish brown, white lip (±30×15 mm) with purplish pink crests; 2-8 mm spur (Nov-Apr)	Isaha Isaka	Love charm	Eritrea, MLW, MOZ, ZAM, ZIM, SWZ, Arabian Peninsula South Africa (NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia species		Umahayiza Amabelejongosi Umabelejongosi Undwendweni Imabeleyongosi Imfe yamasele empofu	Love charm Protective charm	Not Applicable	Not Applicable
Eulophia speciosa (R.Br. ex Lindl.) Bolus	(Beautiful) Perennial herb; Stiff, smooth and thick mature leaves at flowering (±600×20 mm); 10-30 flowers arranged on inflorescence with small pale green sepals, large spreading bright yellow petals (±20×15 mm) and a blunt spur (Aug-Jan)	Amabelejongosi Umlunge omhlope	Protection from storms	Ethiopia, MLW, MOZ, ZAM, ZIM, BOT, NAM, SWZ, Arabian Peninsula South Africa (NAT, TVL)	Data deficient (**GORDON-GRAY, 2003**) Declining

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Eulophia streptopetala Lindl.	(Twisted petals) Perennial herb; Thin pleated partially developed leaves at flowering (±750×70 mm); 30-50 flowers arranged on simple or branching inflorescence with green sepals mottled purplish brown (±20 mm) and yellow roundish petals, side lobes of lip are streaked red-brown, with stout spur (1-2 mm) (Sep-Feb)	Amabelejongosi	Love charm Protective charm	Eritrea, MLW, MOZ, ZAM, ZIM, NAM, SWZ, South-west Arabian Peninsula South Africa (CPP, TVL)	Vulnerable (**GORDON-GRAY, 2003**) LC
Eulophia tenella Rchb.f.	(Tender) Perennial herb; leaf dimensions (100-420×2-6 mm); Small flowers with green to brownish purple sepals (±9 mm) and creamy yellow petals, midlobe of lip is bright yellow with stout spur (2-3 mm) (Nov-Jan)	Untongazibomvana	Ward off evil Courting charm	Southern Tropical Africa (MOZ, ZIM) South Africa (CPP, NAT, TVL)	LC
Eulophia welwitschii (Rchb.f.) Rolfe	Perennial herb; Stiff, erect, pleated leaves (±700×23 mm); 4-5 large, pale creamy lemon to bright yellow flowers (25(46)×12 mm), petals and sepals not spreading, side lobes of lip reddish purple with slender spur (3-7 mm) (Nov-Feb)	Umlunge	Love charm	Zaïre, ZAM, SWZ South Africa (CPP, NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Habenaria dives Rchb.f.	(Rich) Perennial herb; Stiff leaves, prominent veins and clasping at base (±150×20 mm); small white flowers with green veined sepals arranged dense inflorescence (±300 mm), 3 lobed lip with 8-15 mm spur; short slender stalks (Dec-Mar)	Inhluthi yotshani	Death charm	LES, SWZ South Africa (CPP, NAT, OFS, TVL)	LC
Habenaria dregeana Lindl.	Perennial herb; Two basal leaves (±85 mm), leaves on stem taper to hair-like tip (±20 mm); hooded greenish flowers arranged on a dense inflorescence, 3 lobed lip with a slightly longer middle lobe, wide mouthed spur which is thicker at tip (±10 mm) (Oct-Apr)	Intelezi ye-zulu	Protection from storms	Uganda, ZIM, LES, SWZ South Africa (CPP, NAT, OFS, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Habenaria epipactidea Rchb.f.	(Similar to *Epipactus*) Perennial herb; overlapping ribbed leaves that clasp the stem and grade into bracts (±100×20 mm); creamy green flowers with white lip arranged on dense inflorescence (±120 mm) with erect bracts ±40×10 mm, midlobe of lip ±15 mm hair-like side lobes, ±30 mm slender spur with thickened tip; ± 10 mm stalk (Nov-Feb)	Umabelebuca omkhulu	Protection Protection from storms	Ethiopia, ZIM, BOT, LES, NAM, SWZ South Africa (CPP, TVL)	LC
Habenaria species		Ihlamvu	Birth of a son	Not Applicable	Not Applicable
Liparis bowkeri Harv.	Perennial herb; epiphytic or terrestrial; soft green pseudobulbs in closely packed surface groups (20-70 mm); 2-4 pale green soft leaves with side veins (±70×60 mm); 4-12 translucent yellowish green fading brown flowers on a slender stem with flat side sepals (±12 mm) and very narrow petals, 6-8 mm broad lip (Dec-Mar)	Not Available	Love Goodluck charm	Ethiopia, MLW, ZAM, ZIM, SWZ South Africa (CPP, NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Liparis remota J.Stewart & Schelpe	Similar to *Liparis bowkeri* with an extended rhizome between pseudobulbs	Not Available	Love / Goodluck charm	Southern Cape Province – Swaziland	LC
Microcoelia exilis Lindl.	(Thin/Weak) Perennial herb; Epiphyte; Leafless; 20-80 tiny white flowers (±2 mm) on numerous slender inflorescences (60-120 mm), flower throughout the year.	Iphamba	Love charm	Kenya, MLW, MOZ, ZAM, ZIM – KwaZulu-Natal, Southern Madagascar	LC
Mystacidium capense (L.f.) Schltr.	(Of the Cape) Perennial herb; Epiphyte; Pale grey with elongated white dots on roots (4-6 mm diameter); Long, flat, stiff leaves (30-130×10-15 mm) unequally bilobed; 6-14 white flowers (±23 mm) widely spaced on 2-4 hanging inflorescences (60-100 mm), pale green spur (40-60 mm) (Sep-Jan)	Iphamba	Love charm	SWZ South Africa (CPP, NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Mystacidium venosum Harv. ex Rolfe	(Having veins) Perennial herb, Epiphyte; Stemless; Grey-green mass of roots with white streaks and green tips (4-5 mm diameter); Leaves absent or stunted with distinctive veins if present (30-50×8-11 mm); 4-7 white flowers (15-20 mm diameter) on 1-2 hanging inflorescences; 30-50 mm spur (Apr-Jul)	Iphamba	Protective/love charm	MOZ, SWZ South Africa (CPP, NAT, TVL)	LC
Oeceoclades mackenii (Rolfe ex Hemsl.) Garay & P.Taylor synonym: *Oeceoclades maculata* (Lindl.) Lindl.	Rare Perennial herb; Dark purplish green pseudobulbs; thick horizontal, blade-like leaves (±150×15 mm) on short mottled grey-green stalk; ±30 pink and cream flowers arranged on single or branched inflorescence, side lobes of lip marked pinkish red and 2 red blotches in front, club-shaped spur (Feb-Apr)	Impimpi encane	Love charm	Africa (MLW, MOZ, ZIM) – introduced in Tropical America South Africa (NAT)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Polystachya ottoniana Rchb.f.	Perennial herb; Epiphyte; Vertically flattened pseudobulb and tapering to one end (±25×15 mm); 2-4 blunt notched leaves (±80×10 mm); 1-6 white to pinkish or yellow flowers (10-14 mm wide) on slender, erect inflorescence, 3 lobed, curled lip with yellow mid stripe with pink streak in triangular side sepals (Aug-Dec)	Amabelejongosi	Protective/love charm	South Tropical Africa (MOZ, ZIM, SWZ) South Africa (CPP, NAT, TVL)	Vulnerable (**GORDON-GRAY, 2003**) LC
Polystachya pubescens (Lindl.) Rchb.f.	(Downy/ Slightly hairy); Clustered pseudobulbs (20-60×1-15 mm); Leaves are not fully developed until after flowering 2-3 (±100×17 mm); 8-20 golden yellow flowers (±23 mm) with side sepals (±12 mm wide) streaked with dark red lines, red veined lip, lobes covered with white silky hairs (Sep-Jan)	Amabelejongosi	Protective/love charm	ZIM, SWZ South Africa (CPP, NAT, TVL)	Vulnerable (**GORDON-GRAY, 2003**) LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Polystachya sandersonii Harv.	Short pseudobulbs (±30×15 mm); 2-3 small leaves (±100×15 mm); 6-12 yellowish green and brownish orange flowers(±13 mm wide), narrowly triangular side sepals with a velvety smooth outer surface, 3 lobed creamy white lip (heavily spotted with maroon in basal half) (Oct-Jan)	Amabelejongosi	Protective/love charm	SWZ South Africa (CPP, NAT, TVL)	LC
Polystachya transvaalensis Schltr.	2-6 Blunt blade-like leaves (±150×20 mm); ±12 yellowish green tinged reddish brown on the outer surface flowers arranged on short erect inflorescence (±60 mm), median sepal ±12 mm, white lip with faint purple streaks on the side lobes only (Oct-Jan)	Iphamba	Protective/love charm	Cameroon – Kenya, MLW, ZAM, ZIM, SWZ South Africa (NAT, TVL)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Rangaeris muscicola (Rchb.f.) Summerh.	(Growing on / near mosses) Perennial herb; Epiphyte; 4-6 mm grey roots; woody stem ±25×10 mm; Erect, deeply keeled, curving leaves with unequally bilobed tips (70-120×10-13 mm); 5-15 white flowers (±25 mm diam) with a triangular lip and pointed tip, spur 50-90 mm (Dec-Mar)	Not Available	Protective/love charm	Tropical Africa (MLW, MOZ, ZAM, ZIM) South Africa (CPP, NAT)	LC
Satyrium longicauda Lindl. *var.longicauda x Satyrium neglectum* Schltr. *subsp. woodii* (Schltr.) A.V.Hall	(Long-tailed) Perennial herb; 1-2 basal leaves(±200 mm) prostrate / partly erect found on a separate shoot from the flowering stem or may be absent; ±60 flowers (±20 mm) on ±200 mm inflorescence, deep pink to red flowers, hairy flap above lip entrance, slender spur ±46 mm (Sep-Apr)	Ilabatheka elibomvu	Ward off evil	Suspected hybrid – KwaZulu-Natal and Eastern Region (**POOLEY, 1998**)	LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Satyrium parviflorum Sw.	(Small flowers) Perennial herb; 2-3 basal leaves (±300×100 mm) from separate shoot or at base of flowering stem; ±100 small densely packed yellowish green tinged olive to dark maroon flowers on ±300 mm inflorescence, spur ±15 mm (Sep-Mar)	Ilabatheka elikhulu elibomvu, Impimpi enkulu	Ward off evil Love charm	LES, SWZ South Africa (CPP, NAT, OFS, TVL)	LC
Tridactyle bicaudata (Lindl.) Schltr.	(Two-tailed lip) Perennial herb; Lithophyte; grey roots and rough stems; Leaf dimensions (60-160×10-15 mm); 12-16 flowers (12-16 mm diam), arranged in 2 rows on ±6 stems (60-80 mm), 3 lobed lip with fringed side lobes spur (10-18 mm) (Oct-Apr)	Iphamba	Protective charm	Tropical Africa (MLW, MOZ, ZAM, ZIM, SWZ) South Africa (CPP, NAT, TVL)	Vulnerable (**GORDON-GRAY, 2003**) LC

Species	Botanical description	Vernacular term	Use	General distribution range	Conservation status
Tridactyle tridentata (Harv.) Schltr.	(Three-toothed); Perennial herb; Epiphyte; forms large dense masses; grey roots; round stems (±3 mm); 10-14 narrow, round and fleshy leaves (50-100×2-4 mm) which are widely spaced, pointed and slightly grooved on upper surface; Flowers (5-8 mm) arranged in spikes (3-5 flowers/ spike) opposite leaves, coloured pale waxy cream, 3-toothed lip, narrow spur (±8 mm) (Nov-Mar)	Iphamba	Protective charm	Tropical Africa (MLW, MOZ, ZAM, ZIM) South Africa (CPP, NAT)	LC
Vanilla roscheri Rchb.f.	Perennial herb; liana; Imperfectly developed leaves, scale-like and papery brown (±30 mm); Creamy white flowers are arranged spirally on terminal or axillary inflorescences; succulent stems ±200 mm; roots arise at nodes that clasp vegetation or absorb nutrients from the soil	Not Available	Protective charm	South-west Ethiopia, MOZ North-east KwaZulu-Natal	Endangered (B1B2 abc) NT

BOT - Botswana, CPP - Cape Provinces, LES - Lesotho, LC – Least concern, MWL - Malawi, MOZ - Mozambique, NAT - Natal, NT - Not Threatened, OFS - Orange Free State, SWZ - Swaziland, TVL - Transvaal, ZAM - Zambia, ZIM - Zimbabwe
Description and Vernacular name reference: **HUTCHINGS et al. (1996)**
Distribution references (unless otherwise stated): **BRUMMIT (2001)**; **GOVAERTS (2003)**
Conservation status (unless otherwise stated): **RAIMONDO et al. (2009)**

3.7. PHARMACOLOGICAL AND PHYTOCHEMICAL RESEARCH OF ORCHIDS

There are a number of marketable high-value secondary metabolites currently used in the pharmaceutical, food and agricultural industries (**RAO** and **RAVISHANKAR, 2002**). Various research initiatives are geared towards maintaining reliable sources of these and other useful primary and secondary metabolites. These include the continuous search for novel compounds, identification of biosynthetic pathways and improvement in synthetic production; making use of chemical, biotechnological and molecular research techniques. Medicinal plant research forms an integral part of this greater scheme of things as the chemistry of the majority of plant species are yet to be determined, novel compounds are often identified and; their therapeutic and commercial potential is made apparent.

The information on South African orchid species has been extensive in certain areas of interest, such as biology, classification, and conservation of orchids (**LINDER** and **KURZWEIL, 1999**). Botanical research in this region has achieved significant progress over the years, with distribution patterns, morphology and orchid-environment relationships for numerous species being established (**LINDER** and **KURZWEIL, 1999**). The same authors however, pointed out the lack of information in certain aspects, such as pollination biology and ecology of South African orchids. In addition, with the recent taxonomic revisions in orchid classification, complete updated information on some genera is difficult to locate.

Taxonomic relationships within the Orchidaceae family are often described in relation to their chemical profiles with specific reference to certain classes of compounds such as alkaloids (**LAWLER** and **SLAYTOR, 1969**) or flavonoids (**WILLIAMS, 1979**). More recently, the chemicals and molecular mechanisms involved in the diverse pollination systems have been of interest; where chemical mimicry or deceit is used to encourage visits from pollinators (**NILSSON, 1992; BRODMANN et al., 2008; SCHLÜTER** and **SCHIESTL, 2008; TSAI et al., 2008**). Research has also focused on factors that affect germination and propagation of orchids (**SMITH** and **READ, 2008b**); as well as how biotechnology can be applied to produce and recover significant metabolites from orchids (**WALTON et al., 2000**).

3.7.1. Global research

International pharmacological and phytochemical research has elucidated a number of active compounds from exotic orchids. Extensive phytochemical research has been conducted on orchids originating from India resulting in the isolation of polyphenols, ligans, alkaloids, monoterpenes, triterpenes and stilbenoids such as bibenzyls and phenanthrenes (**GARO et al., 2007**). At times, further studies to determine the biological activity of isolated compounds validated the traditional uses of the plant species. For other compounds such determinations are lacking. **MAJUMDER et al. (1987)** for example isolated two bibenzyl derivatives – amoenylin and isoamoenylin as well as moscatilin (4,4'-hydroxyl-3,3',5-trimethoxybibenzyl) from the orchid *Dendrobium amoenum*. For certain bibenzyl derivatives, **PETTIT et al. (1988)** reported antimitotic activity for certain bibenzyl derivatives; while other bibenzyl derivatives are documented as being effective endogenous plant growth regulators (**GORHAM, 1980**). Such activity is yet to be determined and/or confirmed for amoenylin and isoamoenylin as well as moscatilin (4,4'-hydroxyl-3,3',5-trimethoxybibenzyl).

Summarised below are pharmacological and phytochemical findings on orchid species used for traditional medicinal purposes.

3.7.1.1. Anti-inflammatory/immunomodulatory activity

Over expression of tumour necrosis factor alpha, interleukins and nitric oxide could result in various inflammatory disorders and autoimmune diseases. *D. chrysanthum* is commonly used as an antipyretic and immunoregulatory plant in Chinese traditional medicine (**BARLOCCO, 2006**). Ethyl acetate stem extracts produced phenanthrene dendrochrysanene and eight other known compounds. The anti-inflammatory effects of dendrochrysanene were confirmed by a concentration dependent inhibition of mRNA expression of interleukins and free radicals (**YANG et al., 2006**). Of the two phenanthraquinones isolated from *D. moniliforme* stems, moniliformin and denbinobin, the latter exhibited strong anti-inflammatory effects (**LIN et al., 2001**). Denbinobin has been isolated from *D. nobile*, *Ephemerantha lonchophylla* and *E.*

fimbriata with cytotoxic effects (**TALAPATRA et al., 1982; TEZUKA et al., 1991; TEZUKA et al., 1993; LEE et al., 1995**). Denbinobin (1 µg/ml) significantly inhibited TNF-α and prostaglandin E_2 (PGE_2) formation by 62 and 43% respectively, while affecting the release of histamine and ß-glucuronidase in mast cells. These results validate the traditional uses of *D. moniliforme* to treat colds, fever and heat stroke.

Scaphyglottis livida and *Maxillaria densa* dichloromethane:methanol (1:1) whole plant extract produced antinociceptive and anti-inflammatory effects. These plants are used traditionally in Mexico to treat inflammatory disorders. Both extracts demonstrated significant antinociceptive effects in the writhing test, however only *S. livida* extract produced significant antinociceptive effects, in terms of thermal latency, in the hot plate test. A concentration dependent inhibitory effect was noted in the carrageenan-induced paw edema inflammation test for *S. livida* extract but not for *M. densa* extract. Of the four compounds, triterpenoid LLD (5α-lanosta-24,24-dimethyl-9(11),25-dien-3β-ol), cyclobalanone, gigantol (3,4'-dihydroxy-5,5'-dimethoxybibenzyl) and DTB, isolated from *Scaphyglottis livida* extract, only LLD and DTB exhibited antinociceptive and anti-inflammatory effects. Fimbriol A and erianthridin, isolated from *M. densa* extract, produced similar antinociceptive effects to that of LLD and gigantol in the writhing test. Gigantol and LLD (25-100 mg/kg) exhibited significant anti-inflammatory effects; which was concentration dependent for gigantol (**DÉCIGA-CAMPOS et al., 2007**).

Sesquiterpene glycosides dendroside A and dendronobilosides A and B were detected in *D. nobile* stem extracts. An immunostimulatory effect was observed for dendroside A and dendronobiloside A where, dendroside A stimulated the production of T lymphocytes at 10^{-7} M and both dendroside A and dendronobiloside A produced a significantly higher stimulatory effect on B lymphocyte production (at 10^{-5} and 10^{-6} M). Alternatively, dendronobiloside B exhibited inhibitory effects on T lymphocyte production at concentrations between 10^{-7} and 10^{-5} M (**ZHAO et al., 2001**).

Comparatively, three polysaccharides from *D. aphyllum* (Roxb.) C.E. Fischer stem extract produced immunostimulatory activity (**ZHAO et al., 1994**). There has been biological development of carbohydrates and their application in the pharmaceutical industry. Two species,

Cyrtopodium andersonii R. Br. and *C. cardiochilum* are used traditionally in Brazil to treat chest colds, tuberculosis (TB) and haemoptysis (**BARRETO** and **PARENTE, 2006**). *Cyrtopodium cardiochilum* hot water pseudobulb extract produced a polysaccharide referred to as *Cyrtopodium cardiochilum* polysaccharide (CCP). It was found to significantly increase the phagocytic index when compared to the standard (Zymosan) and exhibited similar significant anti-inflammatory effects when compared to Indomethacin (10 mg/kg body weight). An increase in vascular permeability is one of the early signs of an inflammatory response. CCP glucomannan suppressed vascular permeability by approximately 20% of the control (**BARRETO** and **PARENTE, 2006**).

Chinese Materia Medica mention Shi-Hu traditionally used to treat throat inflammation, fever, curing cataract or as a tonic that promotes production of body fluid. Various primary and secondary metabolites have been isolated and their pharamacological activities determined. Hot water stem extract of *D. huoshanense* produced a polysaccharide isolate that stimulated the immune response by increasing macrophage cytokine tumour necrosis factor alpha (TNF-α) and lymphocyte cytokines interferon λ (IFN-λ). At 200 µg/ml, polysaccharide HPS-1B23, enhanced TNF-α production up to 1130.4 pg/ml and stimulated IFN-λ release close to 1300 pg/ml (**ZHA et al., 2007**).

Traditionally used to treat inflammatory disorders such as rheumatism, *G. elata* EtOH extract demonstrated considerable anti-angiogenic effects in the chorioallantoic membrane (CAM) assay (vascular development inhibition of 55.0 and 71.7% at 1 and 3 µg/egg extract) while the *n*-butanol fraction showed dose-dependent activity at IC_{50} 0.47 µg/egg. *In vivo* vascular permeability, an early sign of inflammatory response, was significantly inhibited; while strong analgesic and anti-inflammatory activity was observed. It was postulated that such activity was a result of inhibited NO production and cyclooxygenase-2 expression. Compounds such as 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde, isolates of *G. elata*, are thought to be responsible for the effects (**AHN et al., 2007**).

Nitric oxide, a free radical gaseous signalling molecule in the body, is involved in the maintainence of blood vessels by relaxing smooth muscle (resulting in vasodilation), inhibiting

platelet aggregation and leukocyte adhesion to the endothelium. Nitric oxide is often associated with physiological and pathological processes such as chronic or acute inflammation. Occasionally, in the event of an infection there is an increased production of NO. The result is excessive vasodilation and hypotension which often progresses to septic shock. The inhibition of NO, therefore, is considered to be of therapeutic benefit.

Bulbophyllum odoratissimum contains bulbophyllispiradienone which inhibits NO release from macrophages (**YAO et al., 2005a; b; CHEN et al., 2008b**). Both dendroflorin and nobilone isolated from *D. nobile* stem extracts exhibited inhibitory effects (IC_{50} 13.4 and 38.1 μM respectively), with dendroflorin being more active than resveratrol (23.5 μM). Traditional medicines such as Shi Hu could be producing curative effects via antioxidant effects or inhibition of NO production. (**ZHANG et al., 2007**).

3.7.1.2. Neuroprotective and antioxidant activity

Anti-inflammatory effects are also related to various neuroprotective and antioxidant effects. Neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease have often been associated with mitochondrial oxidative damage and effects of reactive oxygen species (ROS) (**BEAL, 2004; HARMAN, 1994**).

Dendroflorin, moscatilin and nobilone, a 2,7-dihydroxy-4-methoxy-9-fluorenone, were isolated from *D. nobile* 60% EtOH stem extracts (**ZHANG et al., 2007**). The antioxidant activity was determined using DPPH free radical scavenging and ORAC assays. Dendroflorin and moscatilin exhibited significant scavenging activities when compared to for Vitamin C (IC_{50} 16.2±0.2 and 14.5±0.3) in the DPPH assay. There was markedly higher peroxyl radical scavenging activity for dendroflorin, nobilone and moscatilin than for Vitamin C in the ORAC assay (0.596±0.003, 0.432±0.005 and 0.625±0.013 μM Trolox equivalent/μM). EtOH

Of the 70 herbal medicines tested for their active-oxygen scavenging activity *D. plicatile* methanol stem extract was one of those that demonstrated potent activity (72% inhibition) against superoxide anion radical ($\cdot O_2^-$) (**OHSUGI et al., 1999**). The rhizome of *Gymnadenia*

conopsea R. Br. found in China and Nepal, traditionally referred to as Wangla, has been used in Tibetan medicine for coughs, asthma and as a tonic in Chinese medicine. Antioxidant and anti-hepatitis B effects have been established (**PCT INT. APPL. WO 2004, 2004, CAI et al., 2006**). Dichloromethane extract of the aquatic orchid *H. repens* yielded habenariol (bis-*p*-hydroxybenzyl-2-isobutylmalate). A structurally similar 2-[1-methylpropyl]malate ester was isolated from the rhizomes of the non-aquatic orchid, *Galeola faberi* (**WILSON et al., 1999; LI et al., 1993**). Habenariol has been characterized as an antifeedant (**WILSON et al., 1999**). Like most phenolic compounds, habenariol has also demonstrated antioxidant properties in the human low density lipoprotein (LDL) lipid peroxidation model. The model records the delay in the onset of conjugated lipid hydroperoxide formation. When compared to the more effective α-tocopherol, habenariol (25 μM) produced a maximum oxidation rate of 0.0048 with a lag-phase time of 180 minutes and a concentration dependent inhibition of copper-induced lipid hydroperoxide production (IC_{50} 35 μM) (**JOHNSON et al., 1999**).

Active constituents of *G. elata* rhizome, gastrodin and *p*-hydroxybenzyl alcohol, have proven antioxidant (free radical scavenging) activities which is believed to be responsible for their neuroprotective effects (**LIU** and **MORI, 1992; 1993; KIM et al., 2001** and **YU et al., 2005**). The effect of ageing on memory and performance is often associated with neurodegenerative processes. Increased production of free radicals is believed to be the main cause of such processes. The impairment of any one of the major aspects of memory; acquisition, consolidation and retrieval, leads to undesirable effects. In each phase, the various neurotransmitter levels are varied.

Memory consolidation impaired with a protein synthesis inhibitor, cycloheximide (CXM), was positively affected by gastrodin (50 mg/kg) and *p*-hydroxybenzyl alcohol (1-25 mg/kg). Memory retrieval impaired with a dopaminergic receptor agonist, apomorphine (APO), was also positively affected by gastrodin (1-25 mg/kg) and *p*-hydroxybenzyl alcohol (0.1-5 mg/kg). The effect of *p*-hydroxybenzyl alcohol on memory and learning was found to be significantly superior to that of gastrodin. Gastrodin is known to pass through the blood-brain barrier and decompose into *p*-hydroxybenzyl alcohol. The mechanisms via which the different compounds facilitate these processes include decreasing dopamine and other monoamine concentrations and

prevention of DNA degradation (**HSIEH et al., 1997**). Other studies propose alternative mechanisms (**SUN et al., 2004**). The learning ability and memory retention in rats was positively affected by *G. elata* extract; where the number of electric strokes in rats performing the Y-maze test was significantly reduced ($P < 0.01$) after the second and third month. *Gastrodia elata* extract also prevented damage to the ultrastructure of the rat hippocampus (**HU et al., 2003a; b**).

Anticonvulsant properties are believed to be linked to the antioxidant effects on lipid peroxidation in the brain, and a result of antioxidant enzymes (**LIU** and **MORI, 1992; 1993; MORI et al., 2004**). Also known to treat convulsive disorders like epilepsy, the significant inhibition of lipid peroxide production *in vivo*, as well as the significant free radical scavenging properties observed *in vitro*, suggests antioxidant activity to be responsible for the antiepileptic result (**LIU** and **MORI, 1992**). In addition to a reduction in epileptic seizures and free radicals, there is microglia activation and apoptosis which suggest a protective effect against neuronal damage (**HSIEH et al., 2005**).

Steamed and dried tubers of *G. elata* (Tian-ma) are traditionally used in China to treat rheumatism, paralysis and neuralgic diseases (**CHIANG SU NEW MEDICAL COLLEGE AND SHANGHAI SCIENTIFIC TECHNOLOGIC, 1977**). *p*-Hydroxybenzyl alcohol and vanillin, constituents of *G. elata*, demonstrated antioxidant and pro-oxidant effects in various cellular and molecular models which suggest their contribution to the overall antioxidant effect (**LIU** and **MORI, 1993**). Vanillin and *p*-hydroxybenzyl significantly inhibited calcium influx and apoptosis, which is suggestive of its effect on the calcium signaling pathway (**LEE et al., 1999**). In terms of anti-epileptic research, the anticonvulsant effect of gastrodin is believed to act on the *γ*-aminobutyric acid (GABA) metabolic pathway by, inhibiting the GABA shunt; thereby increasing the concentration of GABA in the brain (**AN et al., 2003**). The inhibition of GABA transaminase enzyme prevents the degradation of GABA into the neurotransmitter glutamate. Elevated levels of glutamate cause excitotoxicity in cells whereby an increase in calcium influx activates enzymes that can damage cell structures. 4-Hydroxybenzaldehyde, from *G. elata*, significantly inhibited GABA transaminase (<0.05 moles/mg protein/h NADPH) when compared to a positive control, Valproic acid (±0.1 moles/mg protein/h NADPH). Vanillin was shown to

significantly scavenge free radicals (**LIU** and **MORI, 1993**); and increased binding of GABA at the $GABA_A$ receptor complex (**OJEMANN et al., 2006**). Seeing that the regulation of GABA levels in the brain is related to antiepileptic and anticonvulsant effects, such inhibitory activity highlight the potential of such compounds (**HA et al., 2000**).

Other studies found an increased expression of antioxidant proteins (disulfide isomerase, 1-Cys peroxiredoxin) following transient focal cerebral ischemia, in the presence of both *G. elata* crude extract and active compound *p*-hydroxybenzyl alcohol. This suggests a neuroprotective role (**YU et al., 2005**). Gastrodin is another neuroprotective compound present in *G. elata* (**LI et al., 2003**). When tested in an Alzheimer's disease cellular model, gastrodin was found to promote cerebral cortical and hippocampal cell survival and significantly decrease lactate dehydrogenase production which is suggestive of a neuroprotective effect (**LIU et al., 2005**). Alzheimer's is characterized by senile plaques, cerebrovascular amyloidisis, neurofibrillary tangles, and selective neuronal loss (**KIM et al., 2003**). The insoluble amyloid ß-peptide is thought to be responsible for the neuronal loss or cell death. The ethyl ether fraction of *G. elata*, significantly reduced neuronal cell death by 92% (**KIM et al., 2003**). Vanillin (4-hydroxy-3-methoxybenzaldehyde), a constituent of Tian ma, also contributes to the observed pharmacological activities (**WU et al., 1989**).

Apoptosis or programmed cell death is a natural developmental process within cells. It is associated with neuronal cell death in various neurodegenerative disorders. The process is activated by the lack of certain serum or nerve growth factors. Mitogen-activated protein kinases (MAPKs) are well-known in cell signaling pathways within cells. Methanol extracts (75%) of *G. elata* were found to inhibit cell apoptosis in serum deprived rat pheochromocytoma (PC12) naïve cells as observed in the MTT assay and under Hoechst staining. The protective effect observed was a result of the activation of the serine/threonine kinase dependent pathway and a suppression of stress activated c-Jun NH_2-terminal kinase (JNK) activity (**HUANG et al., 2004**)

The enzyme acetylcholinesterase breaks down the neurotransmitter acetylcholine. A shortage of acetylcholine and its transmission is characteristic of Alzheimer's disease. Therefore the restoration of acetylcholine levels, by inhibiting the action of AChE in the brain, is the current

treatment for the disease. 4-Hydroxybenzaldehyde, an active compound isolated from *G. elata* exhibit potent antioxidant activity and GABA transaminase inhibitory effects but weak AChE inhibitory activity. Researchers, looking for compounds with more extensive biological activities, produced a new class of active and selective AChE inhibitors by introducing amino acid moieties into the backbone of 4-hydroxybenzaldehyde. The resultant compounds exhibited more potent AChE inhibitory activity. Two compounds 4b (IC_{50} 0.19 μM) and 4i (IC_{50} 0.28 μM) performed better than galanthamine (IC_{50} 0.55 μM), and 4i was selective for AChE (**WEN et al., 2007**).

Monoamine oxidases are involved in the breakdown of neurotransmitters such as serotonin, dopamine, adrenalin and nor-adrenalin. Found in the mitochondrial membrane of neuronal and non-neuronal cells, they catalyse the oxidative deamination of primary, secondary and tertiary amines to imines and release by-product hydrogen peroxide (H_2O_2). The two isoforms MAO-A and MAO-B have different substrate preferences, inhibitor specificity and tissue distribution. The inhibition of MAO-B increases dopamine levels which is therapeutically beneficial in anxiety disorders and other neurological diseases such as Alzheimer's and Parkinson's. When compared to 27 different plant extracts *G. elata* tuber extract (50% EtOH) exhibited poor MAO-A and MAO-B inhibitory effects with IC_{50} (<50 mg/ml) and had showed no selectivity to MAO-B (**LIN et al., 2003**).

A sedative effect can be defined as a general reduction in the activity of the central nervous system (CNS). The dried rhizome of *Gastrodia elata* is used for such a purpose in Chinese, Japanese and Korean medicine, during paralysis, epilepsy, vertigo and tetanus. It is considered to be an essential ingredient in the formulation that is prepared (**JUNG et al., 2006**). *Gastrodia elata* EtOH (75%) root extracts were found to exhibit strong antidepressant activity in animal behavioural models. At 300 mg/kg the extract significantly decreased the time of immobility and was similar to, if not better than, the effect of fluoxetine (20 mg/kg) (**ZHOU et al., 2006**).

3.7.1.3. Anti-angiogenic / Anti-tumour

Anti-angiogenic drugs are currently used to inhibit growth of blood vessels in cancer cells. In the presence of certain growth factors the growth of blood vessels may be stimulated. Currently, cancer research looks into the use of what is termed angiogenesis-based tumour therapy – using angiogenesis inhibitors from both natural and synthetic sources. Also, the production and development of endothelial cells is associated with tumour growth and development. The inhibition of angiogenesis prevents neoplasms (abnormal tissue growth) and inflammation, therefore, angiogenesis research is also vital for cardiovascular and inflammatory disorders.
Erianin, isolated from the Chinese medicinal orchid *D. chrysotoxum*, exhibited antitumour activity (**ZHANG et al., 2005**). In determining the various mechanisms involved, significant *in vivo* and *in vitro* anti-angiogenic effects, selective inhibition of endothelial cell generation (EC_{50} 34.1±12.7 nM) and cytoskeletal disruption of endothelial cells were observed (**GONG et al., 2004**).

Very similar potent antitumour and anti-angiogenesis effects were noted for moscatilin isolated from *D. loddigesii* (**TSAI et al., 2010**.) Research on compounds such as moscatilin; with known anti-inflammatory, cytotoxic, anti-platelet aggregation and anti-proliferative properties; and erianin, provide insight into the mechanisms involved in cancer progression. Antitumour activities have been detected in compounds from the *Bulbophyllum* genus (**YAO et al., 2005a; b**), with *B. odoratissimum*, in particular, containing cytotoxic phenolics (**CHEN et al., 2008b**). Calanquinone A isolated from *Calanthe arisanensis* also exhibited cytotoxic activity (**LEE et al., 2008**). Whole plant extracts of *D. chrysotoxum* are used in Chinese traditional medicine. *Dendrobium chrysotoxum* EtOH stem extracts as well as isolated compounds such as erianin, chrysotoxine and confusarin produced antitumour activities (**WANG et al., 1997; GONG et al., 2006**); while the aerial parts of *D. nobile* were found to contain antitumoral phenanthrenes (*in vitro* and *in vivo*) (**LEE et al., 1995**). Moscatilin, a bibenzyl compound, demonstrated potential antimutagenic activity (**MIYAZAWA et al., 1999**). *Dendrobium cariniferum* DCM whole plant extracts revealed dendronone, a phenanthrequinone (**CHEN et al., 2008a**). Another *Dendrobium* species (*D. thyrsiflorum*) exhibited significant cytotoxic effects due to the presence of bi and tri-cyclic compounds (**ZHANG et al., 2005**).

Cremastra appendiculata is the only orchid species to have a triphenanthrene isolated from its tubers. *Cremastra appendiculata* is used in Chinese medicine to treat various cancers (**XUE et al., 2006**). The ethanolic extracts of the tubers were found to be inactive against various human cancer cell lines in cytotoxic assays (IC_{50} > 5 µg/ml). Pure compounds such as cirrhopetalanthrin however were selectively active against lung (17.8 µM), ovarian (13.2 µM), hepatoma (8.4 µM), stomach (10.5 µM), colon (11.2 µM) and breast (12.5 µM) cancer cell lines.

3.7.1.4. Antiviral and antimicrobial activity

In vitro antiviral activity of lectins from a *Cymbidium* hybrid, *Listera ovata* and *Epipactis helleborine* against severe acute respiratory syndrome coronavirus (SARS-CoV) and feline infectious peritonitis virus (FIPV) were determined to be (EC_{50}) 4.9±0.8, 2.2±1.3 and 1.8±0.3 µg/ml against SARS-CoV and 4.5+2.6, 0.7±0.3 and 1.6±0.9 µg/ml against FIPV. *Listera ovata* mannose-specific lectin produced the most effective antiviral activity against coronavirus (FIPV) (**KEYAERTS et al., 2007**). **KEYAERTS et al. (2007)** established that, of the 33 plant lectins investigated, mannose-binding lectins produced the strongest anti-coronavirus activity. This effect could be a result of the sugar specificity of these lectins (**VAN DAMME et al., 1998; KEYAERTS et al., 2007**). Previous *in vitro* studies on mannose-specific plant lectins from these three species revealed antiviral activities against human immunodeficiency virus (HIV) type 1 and type 2 in MT-4. Considerable antiviral activity was also noted in HEL, HeLa and MDCK cells when testing against anti-human cytomegalovirus (CMV), respiratory syncytial virus (RSV) and influenza A virus (**BALZARINI et al., 1992**).

Carbohydrate binding proteins or glycoproteins = lectins or agglutinin are well documented and characterised. Mannose binding lectins are common in monocotyledons. Their high specificity/ affinity gives rise to their characteristic biological activities which are being investigated to be applied against viruses, cancer cells and in crop protection. As mentioned earlier, antiviral properties differ based on the carbohydrate specificity of the lectin (**TIAN et al., 2008**).

Cypripedium macranthos var. *rebunense* seeds demonstrated antifungal properties at various growth phases as a mechanism to facilitate germination (**SHIMURA et al., 2007**). *Dendrobium officinale* agglutinin 2 demonstrated significant antifungal properties against *Gibberella zeae* which suggests its possible use for crop plants (**CHEN et al., 2005**). French botanist, Noel Bernard observed acquired immunity in *Orchis morio* and *Loroglossum hircinum* against *Rhizoctonia repens*, an endomycorrhizal fungus. Phytoalexins are defined as low molecular weight antimicrobial compounds synthesised and accumulated in plants post-exposure to microorganisms (**STOESSL** and **ARDITTI, 1984**). Orchinol (2,4-dimethoxy-7-hydroxy-dihydrophenanthrene) isolated from *O. militaris*, loroglossol and hircinol (2,5-dihydroxy-4-methoxy-dihydrophenanthrene) isolated from *L. hircinum* exhibited antimicrobial properties (**BÖLLER et al., 1957; HARDEGGER et al., 1963**). Hircinol and orchinol were considered to be non-specific antimicrobials as they produced varying activities against a range of fungi and bacteria (**GÄUMANN et al., 1960; URECH et al., 1963; FISCH et al, 1973**). Loroglossol, an isomer of orchinol, was found to exhibit similar effects. (**WARD et al., 1975**).

The amino acid sequence of gastrodianin, the major antifungal component of *G. elata*, shared strong homology to that of monomeric mannose-binding proteins of orchids such as *E. helleborine* and *L. ovata*. Such mannose-binding proteins have proven *in vitro* antifungal activity; therefore, gastrodianin-like proteins are considered a novel group of antifungal proteins (**WANG et al., 2001**). It was established that gastrodianin has a stable structure with potent inhibitory activity against certain fungal strains (**XU et al., 1998**).

Dengue viral infection is common in tropical and subtropical regions. The infection can progress to haemorrhagic fever and dengue shock syndrome which are lethal. Heparan sulfate proteoglycan found on the surface of all mammalian cells has a heparan sulfate chain that facilitates infection. Sulfated polysaccharides are currently being researched as potential anti-dengue drugs. The sulfated derivatives of polysaccharides WGEW and AGEW (two α-D-glucans) from *G. elata* exhibited potent anti-dengue virus activities with WSS45 being selective for type-2 dengue virus. Also, it was observed that with an increase in the degrees of substitution there was an increase in activity (**QIU et al., 2007**).

The aromatic molecule, vanillin (4-hydroxy-3-methoxybenzaldehyde) has been extensively studied and applied in the food, chemical, pharmaceutical and cosmetic industries (**WALTON et al., 2003**). This versatile aromatic compound is used widely and more commonly in the culinary industry as a flavourant (**CHOMCHALOW, 1996**). It is naturally obtained during the ageing and alcoholic extraction (fermentation) of green vanilla pods (*Vanilla planifolia* Andrews, syn. *V. fragrans* Ames). Vanillin exhibits antioxidant and antimicrobial properties against certain Gram-positive and Gram-negative bacteria known to spoil food and is therefore used as a food preservative (**KRUEGER** and **KRUEGER, 1983; BURRI et al., 1989; CERRUTTI, et al., 1997; DAUGSCH** and **PASTORE, 2005**).

Genetic modification is a relatively new method used to maintain orchids commonly traded as potted plants for a longer period. This is accomplished by conferring antimicrobial properties into orchid species like *Oncidium* by introducing a plant ferredoxin-like protein (PFLP) (**LIAU et al., 2003**).

3.7.1.5. Phytotoxic activity

Erianthridin and gymnopusin, two phenanthrene derivatives from *Maxillaria densa* methanol:chloroform (1:1) whole plant extract, contributed to the moderate phytotoxic effect (growth inhibition, electrolyte leakage, and chlorophyll reduction) observed on duckweed tissue. Gymnopusin was found to exhibit a more potent effect over a shorter period. Growth inhibitory activity, measured by inhibition of radical elongation of *Amaranthus hypochondriacus*, was observed for gymnopusin and erianthridin (IC_{50} 330 and 58.2 μM respectively). The overall results suggested a non-specific mechanism; however specific lysis of the tonoplast membrane by gymnopusin implied a different and specific mode of action. The herbicidal potential of gymnopusin is weak as it displayed cytotoxic effects *in vitro* against four mammalian cell lines thioguanin-resistant rat hepatoma cells (H4TG) (IC_{50} 13.0±0.9 μM),MDCK (IC_{50} 11.0±0.5 μM), NIH Swiss mouse embryo fibroblasts (NIH3T3) (IC_{50} 12.0±1.0 μM), and Kirsten strain of Moloney sarcoma virus-transformed 3T3 cells (KA31T) (IC_{50} 21.0±0.5 μM) (**VALENCIA-ISLAS et al., 2002**). Interestingly, other researchers had previously determined various approaches to synthetically produce gymnopusin (**WANG** and **SNIECKUS, 1991**).

Collectively, such information can benefit further research efforts dealing with bioherbicides and their safety.

WILSON et al. (1999) were the first to identify the defense mechanism in the form of the phenolic compound habenariol from a perennial freshwater orchid *H. repens*. Phenolics are generally shown to possess antioxidant activity with curative and preventative properties (**LIU et al., 1992**). The lipophilic secondary metabolite habenariol is produced naturally by the plant to prevent omnivorous crayfish from grazing on the water plant. A further example of a feeding deterrent was discovered in *Phalaenopsis* hybrids in the form of two 1,2-saturated pyrrolizidine monoesters (**FRÖLICH et al., 2006**). Investigators established that the young and developing tissues contained the highest concentration of these alkaloids, thus serving as a preventative measure (**FRÖLICH et al., 2006**). Pyrrolizidine alkaloids, potent antifeedants for most herbivores, are highly conserved alkaloids found only in five plant families. In Orchidaceae, these are represented in eight genera: *Liparis*, *Malaxis*, *Cysis*, *Phalaenopsis*, *Vanda*, *Vandopsis* (**HARTMANN** and **WITTE, 1995**), *Pleurothallis* (**BORBA et al., 2001**), and *Cremastra* (**IKEDA et al., 2005**). Taxonomically speaking, these genera belong to five tribes within the subfamily Epidendroideae only (**CAMERON, 2004**); which emphasises the conserved nature of this alkaloid occurrence. Pyrrolizidine alkaloid phalaenopsine T from *Phalaenopsis* hybrids is also known to exhibit growth inhibitory properties (**FUJIEDA et al., 1988**).

3.7.1.6. Spasmolytic activity

The spasmolytic effect or inhibition of smooth muscle contraction by natural plant extracts has been widely used in traditional medicine to treat hypertension, stomachache and prevent abortion. In Mexico, *M. densa* and *S. livida* are used for such purposes. Five compounds isolated from *S. livida* demonstrated concentration-dependent spasmolytic effects *in vitro* similar to that of papaverine. Researchers also determined that the NO/cGMP pathway is the mechanism via which 3,4'-dihydroxy-5,5'-dimethoxybibenzyl acts (**ESTRADA et al., 1999**).

Gigantol and 3,7-dihydroxy-2,4-dimethoxyphenanthrene also isolated from *S. livida* were found to have concentration-dependent vasorelaxing activity. Mechanisms thought to mediate their

activity include increased levels of NO and or cGMP (**ESTRADA-SOTO et al., 2006**). The spasmolytic activity of stilbenoids isolated from *M. densa* (2,5-dihydroxy-3,4-dimethoxyphenanthrene, fimbriol-A, nudol, gymnopusin and erianthridin) was found to be similar to that of papaverine. However, activity was established to be not as a result of nitric oxide mechanism or calcium influx (**ESTRADA et al., 2004**).

Laelia autumnalis (Lex.) Lindley methanol extract demonstrated vasorelaxant properties (IC_{50}) 34.61±1.41 µg/mL (E_{max}) 85.0±4.38% in intact rat aortic endothelium rings. In endothelium-denuded rings the extract induced relaxation at IC_{50} 45.11±4.17 µg/mL (E_{max} 80.0±12.1%). Also, a significant decrease in systolic and diastolic blood pressure and heart rate in spontaneously hypertensive rats was observed. Antihypertensive and vasorelaxant effects were found to be a result of calcium (Ca^{2+}) channel blockage (**VERGARA-GALICIA et al., 2008**). Similar vasorelaxant effects were observed for *L. autumnalis* used in traditional Mexican medicine for hypertension and related diseases, validating their traditional uses (**AGUIRRE-CRESPO et al., 2005**).

Gigantol and 3,7-dihydroxy-2,4-dimethoxyphenanthrene isolated from *S. livida* used to treat stomachache and prevent abortion, exhibited vasorelaxant properties as a result of more than one mechanism (**ESTRADA-SOTO et al., 2006**). The vasorelaxant effect reduces hypertension and promotes muscle relaxation, which supports the traditional uses of this plant.

Of the five muscarinic acetylcholine receptor subtypes, M3 directs the contraction of smooth muscle and mucous secretion in mucous glands. Selective blockage of M3 receptors is suggested to offer relief in respiratory disorders such as chronic obstructive pulmonary disease, gastrointestinal disorders such as irritable bowel syndrome and urinary tract disorders such as urinary incontinence. There is a need for selective receptor subtype substitutes to prevent adverse side effects found in current medication. *Cremastra appendiculata* is used in Chinese medicine to treat various cancers (**XUE et al., 2006**). The bulb extract was originally found to be active, and further investigation found cremastrine, a pyrrolizidine alkaloid, to selectively inhibit muscarinic M3 receptors (IC_{50} 594 nM (K_i=126 nM)). (**IKEDA et al., 2005**).

3.7.1.7. Other activities

3.7.1.7.1. Anti-platelet aggregation effects

The Chinese prepare a (Yin) tonic from various *Dendrobium* stems which is used to treat fever symptoms, increase body fluid production and as nourishment. *Dendrobium densiflorum* DCM stem extracts produced anti-platelet aggregation active compounds such as gigantol, moscatilin, homoeriodictyol, scoparone and scopoletin; *in vitro*. Scoparone proved to be the most effective and was used as a positive control. When compared to the control, at 100 μM, percentage inhibition for gigantol, moscatilin, homoeriodictyol, scoparone and scopoletin was, 31, 36, 50, 64, 21, respectively. (**FAN et al., 2001**).

Methanol stem extract of *D. loddigesii* Rolfe inhibited platelet aggregation. Three compounds (moscatilin, moscatin and moscatilin diacetate) obtained from a fraction of the extract also exhibited potent concentration dependent antiplatelet aggregation activities. Moscatilin, moscatin and moscatilin diacetate inhibited arachidonic acid (AA)-induced platelet aggregation at IC_{50} 61.8, 37.2, 11.2 μM, respectively. Thromboxane A_2, a signalling molecule that triggers platelet aggregation and is produced in the presence of AA, may have been the target for moscatilin and moscatin, while moscatilin diacetate may have had a more direct intracellular effect (**CHEN et al., 1994**).

3.7.1.7.2. Inhibition of cell-surface glycoside cleavage

Various classes of flavonoids such as anthocyanins, flavonols and *C*-glycosylflavones have been isolated from orchids. *C*-glycosylflavones are known to inhibit cell-surface glycoside cleavage (**TOVAR-GIJÓN et al., 2006**). Glycosides contribute to pigments; form the basis of certain antibiotics and cardiac glycosides. Glycosides are converted into glycosyl (carbohydrate) and aglycone components by hydrolytic cleavage (addition of water). By inhibiting glycoside breakdown, plant pigments are preserved; and mechanisms involved in glycoside production and storage can be determined.

3.7.1.7.3. Inhibition of kainic acid binding to brain glutamate receptors

S-(4-hydroxybenzyl)-glutathione, isolated from aqueous extracts of *G. elata*, was found to inhibit kainic acid from binding to glutamate receptors (IC_{50} 2 x 10^{-6} M) with less effectiveness than glutamate (IC_{50} 2 x 10^{-7} M) and glutathione (IC_{50} 8 x 10^{-7} M) (**ANDERSSON et al., 1995**). Kainic acid is considered an excitatory neurotoxin (**MCGRAW-HILL CONCISE DICTIONARY OF MODERN MEDICINE, 2002**) and is effective when binding and upon activation of the glutamate receptor subtype found on neurons.

3.7.1.7.4. Skincare

Scientific research of exotic orchids have also allowed for the expansion of the cosmetic industry. The utilization of orchid extracts and other such natural cosmetic products are becoming more popular among the American population (**MINTEL COSMETIC RESEARCH**). Some companies attribute the beautifying effects to compounds found in the roots of certain orchid species (**GUERLAIN COSMETIC COMPANY**). The identities of the orchids are not often revealed however, the emphasis is on the protective properties of the extracts such as: fighting free radicals, increase in skin immunity, enrichment in naturally occurring minerals, and anti-ageing properties.

3.7.1.7.5. Vanillin

Synthetically produced vanillin is used for chemical and pharmaceutical applications as the natural extraction process is approximately 250 times more expensive. Uncured beans of the vanilla orchid were used medicinally by the Mayan civilization to treat venomous insect bites and to heal wounds. Studies on vanillin revealed additional biological activities which include anti-carcinogenic (ability to inhibit tumour formation), anti-mutagenic effects and anti-clastogenic (inhibition of chromosomal damages) (**KESHAVA et al., 1998; ZABKOVA et al., 2006**). Vanillin's curative properties have also been showcased against sickle cell disease – as an effective inhibitor of red blood cell sickling. **ZHANG et al. (2004)** provided details on the

vanillin prodrug MX-1520, which was synthesized to treat red blood cell sickling with vanillin, *in vivo*.

It is evident from the literature that there is no pharmacological research on South African medicinal orchids (**CHINSAMY et al., 2011**). However, there have been significant developments in medicinal orchid research worldwide. The ethnobotanical approach used to select the abovementioned species for pharmacological and phytochemical research yielded valuable biological compounds. A similar approach can therefore also be applied to South African medicinal orchids, with potentially favourable results.

3.8. ORCHID CONSERVATION

The conservation status of medicinal plants is often associated with indiscriminate harvesting and demand when used for traditional medicine practices (**ARNOLD et al., 2002**). According to **HILTON-TAYLOR (1996)**, the reason a large number of species became extinct in the southern African region was attributed to the large number of persons using them for traditional medicines. Generally, orchids have been fiercely protected on the basis that they are „rare' and under threat from excessive collection. However, according to **McMURTRY et al. (2008)**, the collection of plants for cultivation and or horticultural purposes was the original threats to orchid populations. Currently orchid populations are most affected by the loss of suitable habitats due to industrial practices (timber plantations, mining and agriculture) and urbanization (**DIXON et al., 2003; McMURTRY et al., 2008**). Comparatively, the collection of orchids for herbal trade features as one of the lesser threats to orchid populations. In the South African context, the conservation status of most medicinally used orchid species (Least Concern – LC) is indicative of this (**Table 3.1. Chapter 3**). **DIXON et al. (2003)** mention that medicinal orchids often have small flowers and are of little horticultural value – therefore the threat to various medicinal species needs to be separated from that imposed by collection for horticultural purposes. The conservation status of South African medicinal orchid species (**Table 3.1. Chapter 3**) could either reflect a negligible impact by collection for medicinal and cultural uses or, it could indicate a lack of knowledge on such orchids. Earlier it was mentioned that there are „gaps' in

our knowledge of orchids (**CRIBB et al., 2003**). The authors also allude to the fact that orchid conservation is „multi-faceted' – ethnobotanical, pharmacological and phytochemical studies of medicinally used orchids in South Africa will contribute positively to the conservation goals by presenting data that add value to medicinal and related orchid species. Also, the increased protection of orchids will in turn allow for the protection of other species that occur in the same regions. The conservation status of *Vanilla roscheri* (Endangered – **Table 3.1. Chapter 3**) in South Africa could be associated with their use in African traditional practices. According to **BRUMMIT (2001)**, the presence of *V. roscheri* is prolific from south-west Ethiopia to north-east KwaZulu-Natal. Therefore it would be more reasonable to assume that the conservation status, with respect to local IUCN Red List ratings of *V. roscheri*, is due to a lack of availability of suitable habitat within South Africa. While certain orchids are valued for their horticultural appeal, certain species often hold medicinal, cultural, commercial, and or nutritional value in the regions where they occur and deserve the necessary scientific attention and protection.

3.9. QUO VADIS

It is evident that medicinal research of orchids has surged ahead in some regions. Often their indigenous uses are validated, novel compounds are identified and potential uses in industries are discussed. The ethnobotanical uses of orchids in South Africa (**Table 3.1. Chapter 3**) have not been exploited pharmacologically (**CHINSAMY et al., 2011**). Scientific validation is essential as the uses are indigenous and unique to South African orchids.

Common reasons for the lack of research on South African medicinal orchids include; orchids have mostly been appreciated for their horticultural appeal and medicinal orchids mostly have insignificant/ unpretentious flowers – reducing their appeal for further study. Also, as mentioned earlier, the generally accepted conservation status of most orchid species could discourage researchers from studying these specific species. Only a small percentage of South African orchid species is used in African traditional practices (49 out of ±500 in southern Africa). Often there are some morphological and ecological clues as to the chemistry of certain plants. Colourful flowers are suggestive of high flavonoid content. In the case of South African orchids

a considerable number of species are white or green which should suggest their lack of certain secondary metabolites – often responsible for observed pharmacological effects. Also, secondary metabolites are responsible for attracting pollinators, in the case of South African orchids most are pollinated by moths, which do not require a chemical stimulus. Therefore, the few species actually used in traditional medicine may represent a refined list of most active species or genera with bioactive compounds. Finally, the use of traditional medicine is still not fully recognised in South Africa and abroad, and the medicinal plant research field is still establishing itself in our country, therefore orchids have not featured as yet.

Literary resources on ethnobotanical uses of South African (**Table 3.1. Chapter 3**) have provided the initial impetus for medicinal orchid research in South Africa. The relatively small number of species found in South Africa (±500) is said to be a result of biology and not taxonomic bias. Taxonomic bias could either be an underestimate or an inflation of species number. South African orchid species numbers are not a result of taxonomic bias. Inflation of species number is a problem in regions like Europe and the distribution and occurrence of orchid species in South Africa is relatively well known. Endemism is the result of very limited factors that makes a habitat suitable for particular organisms or plants. Given that geographical location of species is one of the determining factors in the type and quantity of secondary metabolites produced; and that 75% of orchids in the southern African region are endemic (**STEWART et al., 1982**) it could be assumed that their unique geographical location would have some influence on the chemical composition of these species. The potential of revealing bioactive compounds that are unique to South African orchids is great.

Scientific validation is necessary as these species are still being used for traditional medicine. During preliminary market surveys, a constant trade and demand for certain orchids was observed. This demand could imply therapeutic or beneficial effects. The toxicology of medicinal plants needs equal consideration in medicinal plant research. Most of the orchid species used for cultural practices are administered as emetics. It would be important to know what the effects of these orchid-derived medicines are on the human body and more especially their safety, in terms of toxicity. Pharmacological and phytochemical investigations of South African medicinal orchids may reveal bioactive compounds that could add value to medicinal

and related orchid species. These could be further developed into much needed therapeutic agents or be developed into viable commercial products in different industries.

CHAPTER 4
ANTIMICROBIAL EVALUATION

4.1. INTRODUCTION

The earliest inhabitants of the earth, bacteria, are ubiquitous mainly due to their adaptable nature. It was not until the late 17th century that they became more apparent to the scientific community. They are morphologically diverse and while some benefit the ecosystem others are pathogenic. This chapter investigates the effect of crude orchid extracts on various test organisms like bacteria and fungi which are known to cause widespread diseases.

One third of all deaths of children under five occur in sub-Saharan Africa (**HILL et al., 1999**). The annual death toll of this group of the world's population is an estimated 11 million. Over 90% of these deaths occur in developing countries; nearly 70% result from communicable diseases, such as respiratory infections, malaria and diarrhoea (**HILL** and **AMOUZOU, 2006**). **PINNER et al. (1996)** revealed that between 1981 and 1992 infectious diseases had moved from the 5th to the 3rd leading cause of mortality. A later report by **IWU et al. (1999)** showed that half of all deaths in tropical countries were a result of infectious diseases. According to the Millennium Development Goals a reduction of 4.3% of the U5MR, between 1990 and 2015, is required in the sub-Saharan region. Contrary to common belief, even populations in developed countries experience under five mortalities due to communicable diseases (**BLACK et al., 2010**).

Worldwide, 33 million persons are infected with HIV (**WILLIAMS, 2010**). Two million die every year (**WILLIAMS, 2010**). In 2008 more than 70% of all AIDS-related deaths worldwide, occurred in the sub-saharan African region (**UNAIDS, 2009**). With the nearly two million new infections in 2008 there are approximately 22.4 million persons (67% of the global infected population) living with HIV in this region. By 2007 it was established that the largest population of persons living with HIV (5.7 million) occurred in South Africa (**UNAIDS, 2009**). Immunodeficient persons are more susceptible to opportunistic infections (**KAPLAN et al., 1998; SASSI, 2011**).

Immunodeficient populations include transplant recipients, elderly and HIV-infected persons. **KAPLAN et al. (1998)** and **GOUWS** and **KARIM (2010)** report TB as the most prevalent opportunistic infection. In immunocompromised transplant recipients, infections such as invasive candidiasis, TB and resistant Gram-negative bacteria are prevalent. Aged persons are more prone to post-operation infection and during long periods in hospital. In recent times, the most common AIDS-related diseases include pneumonia, diarrhoea, TB and hepatitis C (**SASSI, 2011**). According to **SASSI (2011)** 90% of all AIDS-related deaths were a result of opportunistic infections.

Fungi from the genus *Candida* form part of our natural microflora found on skin, mucous membranes and the gastrointestinal tract, yet *C. albicans* is the main contributor of mucosal infections and systemic mycoses (**MORSCHHÄUSER, 2002; WROBLEWSKA et al., 2002**). *Candida albicans* can turn pathogenic and cause oral, vaginal and systemic infections (**De BACKER et al., 2000**). According to hospital admissions, the rate of invasive fungal infections (IFIs) in America almost doubled in just 10 years (1980- 1990) (**BECK-SAGUÉ** and **JARVIS, 1993**). In Europe a similar pattern emerged with a 1.6% frequency in the period 1978-1982 to a 4.1% in 1988-1992 (**GROLL et al., 1996**). Such statistics are indicative of the alarming increase in frequency of severe IFIs in organ and bone marrow transplant recipients as well as patients infected with HIV (**MEYERS, 1990**).

In the case of persons who are immunocompromised *Candida* species cause opportunistic infections (**WROBLEWSKA et al., 2002**). Mucosal and systemic fungal infections are prevalent in patients with AIDS (**LOEFFLER** and **STEVENS, 2003**). The colonization of the mouth, pharynx and esophagus (oral candidiasis) may be present in up to 84% of patients suffering from HIV (**MAENZA et al., 1997; MARTINS et al., 1997**). In immunocompromised patients there is the threat of superficial infections such as oropharyngeal candidiasis (OPC) and more serious systemic infections (**MORSCHHÄUSER, 2002**). Cancer patients most commonly present with *C. albicans* fungal infections (**WINGHARD, 1995**), while, *C. albicans* and *C. parapsilosis* feature as the most common IFIs in pediatric and neonatal cases (**BLYTH et al., 2007**). The most prevalent mycosis in hospitals is candidiasis (**WROBLEWSKA et al., 2002**). There is a notable increase in the prevalence of *Candida* and *Aspergillus* infections.

VISCOLI and **CASTAGNOLA (1999)** list some common problems encountered when treating patients presenting with fungal infections: i) difficult to confirm diagnosis until late stage of the disease; ii) some fungi have intrinsic or acquired resistance to antifungal drugs; and iii) there is the risk of drug toxicity.

4.1.1. Antibacterial agents

Considerable effort has been made to reduce infection rates. Conventional medicine had a major breakthrough in the fight against infectious diseases with the serendipitous discovery of penicillin by Fleming (1928). This perhaps was the basis for the original definition of an antibiotic – "***substance produced by one microorganism which is capable of inhibiting growth or killing another microorganism***". Subsequent antibiotic discoveries included the isolation of erythromycin and the family of tetracyclines (among others) from various microorganisms (**LAURENCE** and **BENNETT, 1980**). Over the years this definition has been modified to accommodate other antibiotic sources that have been discovered. For a while in the 20th century the advent of antibacterial drugs were considered one of the most crucial therapeutic contributions to the healthcare system of man and animals (**PAGE et al., 1997**). An antibacterial drug (or chemotherapeutic agent) may be defined as a drug that is used to treat an infectious disease by either removing or destroying the infective agent without harming the host.

The action of an antibiotic may be described as either bacteriostatic (inhibition of bacterial growth) or bactericidal (ability to kill the bacterium) (**PAGE et al., 1997**). Many of the major antibacterial drugs have specific target sites (a) bacterial cell wall biosynthesis; (b) protein synthesis; (c) DNA replication and repair. Antibiotics are therefore further classified according to their mode of action, for example, β-lactams and glycopeptides represent 2 classes of antibiotics that inhibit bacterial growth by targeting bacterial cell wall synthesis. Other major classes of antibiotics involve certain modes of action such as: inhibition of protein synthesis; nucleic acid metabolism; intermediary metabolism and the disruption of cytoplasmic membranes (**LAURENCE** and **BENNETT, 1980**).

4.1.2. Antifungal agents

Antifungal drugs are often prescribed in both conventional and traditional medical systems. **ROGERS (2002; 2006)** highlights the concern over limited clinical drugs currently available, whilst it is clear that there is an increasing incidence of IFIs especially in immunocompromised patients. Antifungal agents have been used extensively in HIV-positive patients for treatment of OPC and cryptococcosis. There are different classes of antifungal agents (**KONTOYIANNIS** and **LEWIS, 2002; ROGERS, 2002**):

- **Polyenes** – this group includes the broad spectrum acting Amphotericin B and nystatin drugs. Polyenes target fungi by interacting with ergosterol which is the major sterol in the fungal plasma membrane. This action creates pores and channels in the plasma membrane which allows for the leakage of potassium and other cell constituents and ultimately cell death (**VANDEN BOSSCHE et al., 1994**). Amphotericin B may be administered orally to produce a topical effect which decreases intestinal colonisation in addition to the treatment of OPC and gastrointestinal candidiasis. Amphotericin B, however, is known to cause nephrotoxicity and its use has therefore been limited. Nystatin, which is considered even more toxic, has been prescribed only for topical infections.
- **Azoles** – included in this group are the triazoles, fluconazole and itraconazole as well as the imidazoles. These also act by affecting the sterols in the plasma membrane but particularly inhibit ergosterol biosynthesis. Fluconazole has exhibited specific activity against *C. albicans* and *Cryptococcus neoformans* but not against *Aspergillus* species. Whereas itraconazole, which exhibited more broad spectrum activity, is less palatable. This group has shown both fungistatic and fungicidal activity *in vivo*.
- **Fluoropyrimidines –** Flucytosine acts by inhibiting DNA and RNA synthesis as it transgresses the fungal cell membrane using a cytosine permease. These drugs have shown activity against *Candida* species and *Cryptococcus neoformans*. They are more commonly used in combination therapy with polyenes like Amphotericin B (**FRANCIS** and **WALSH, 1992).** This combination therapy is either to increase potency or to counter Amphotericin B toxicity. Combination therapy of Amphotericin B and

flucytosine has the risk of developing flucytosine-related toxicity (**FRANCIS** and **WALSH, 1992).** Alternatively, flucytosine could be combined with azoles to reduce toxicity while preventing drug resistance (**HOSPENTHAL** and **BENNETT, 1998**). Flucytosine resistance in *C. albicans* isolates has ruled out the option of using this drug in monotherapy for fatal yeast infections.

- Other groups include: **Allylamines** (Lamisil) which target the precursory steps of ergosterol biosynthesis while **echinocandins** (Cancidas) inhibit beta-(1,3)-glucan synthase. Both classes have shown significant potential in their respective applications.

The triazole fluconazole is the fourth most prescribed antifungal drug in the world (**DENNING et al., 1995**). The new triazoles are now considered effective treatment (**GOA** and **BARRADELL, 1995**). They are fungistatic (inhibit fungal growth) and are considered to be less toxic than the polyene Amphotericin B and more active against yeasts, yeast-like fungi and dimorphic fungi. This however, often results in a recurrent infection (**REX et al., 1995**). **MORSCHHÄUSER (2002)** states that, the emergence of the fluconazole-resistant *C. albicans* strain is the result of long-term, low dose fluconazole treatment of recurring OPC in AIDS patients (**ODDS, 1993; HUNTER et al., 1998**; **LOPEZ et al., 2001**).

There is a worldwide incidence of rising severe fungal infections (**GOLDMAN et al., 2004**). The development of resistance in previously susceptible *Candida* strains has been reported for patients with AIDS. It has been hypothesised that the systemic use of antifungal drugs has affected the epidemiology of fungal infections, of which drug resistance is a direct result (**ROGERS, 2002**). In light of the number of persons with HIV and AIDS suffering from opportunistic fungal infections, there is an urgent requirement for effective antifungal agents (**GEORGOPAPADAKOU, 1998**). Unfortunately, there are only a few classes of antifungal drugs available to treat *Candida* infections (**MORSCHHÄUSER, 2002**).

4.1.3. Drug resistance and contributing factors

All things considered, antibiotics are the preferred drug when treating infectious diseases. **PAGE et al. (1997)**, however, pointed out that the over-prescription and excessive use of antibiotics has resulted in the promotion and development of antibacterial drug resistance. Various bacterial strains have become resistant to available antibacterial drugs. The structure of microbes affects their reaction to antimicrobial agents (**WHITE** and **McDERMOTT, 2001**). Gram-positive bacteria lack the outer membrane present in Gram-negative bacteria; they are therefore, generally more vulnerable to antibacterial agents. Alternatively, as a consequence of certain antimicrobial mode of activity, Gram-negative bacteria may exhibit greater susceptibility than Gram-positive bacteria (**WHITE** and **McDERMOTT, 2001**).

PAGE et al. (**1997**) classified AR as being a result of one of 2 mechanisms: innate (or intrinsic) AR, which depends on the properties of the drug whereas acquired AR depends on the genetic adaptation of the bacteria concerned. All presently available antibiotics are susceptible to acquired AR (**KIMBALL, 2008a**). **LAURENCE** and **BENNETT (1980)** observed 2 general types of drug resistance: (a) drug tolerance (infective organism multiplies (growth) in the presence of the drug) and (b) drug destroying (bacteria able to inactivate the drug). **PELEG** and **HOOPER (2010)** present a summary of the seven known resistance mechanisms found in Gram-negative bacteria and their effects on the antibiotics: loss of porins, use of β-lactamases, transmembrane efflux pumps, antibiotic-modifying enzymes, mutation of target sites, ribosomes and lipopolysaccharides and metabolic bypass mechanisms.

Clinical antifungal drugs may be susceptible to drug resistance; however, other contributing factors such as: impaired drug adsorption; accelerated drug metabolism; antagonism, high fungal load and a constant case of being immunocompromised may be involved. HIV-positive patients with OPC are known to be affected by the latter two factors (**REX et al., 1995; MAENZA et al., 1996**). Like with AR, the mechanism of resistance may either be intrinsic or acquired. Some mechanisms known to be used by infecting fungi against triazoles include: i) The use of efflux pumps to reduce drug accumulation; ii) Alteration of membrane sterol composition and; iii) Alteration of concentration and or structure of target fungal proteins (**SANGLARD** and **ODDS,**

2002). The most recent resistance mechanism to be discovered was the use of bacterial efflux systems (**POOLE, 2007**). It was observed as tetracycline resistance in *E. coli*. Efflux mechanisms are both drug-specific and multidrug which causes them to impact on the treatment of infectious diseases.

The potentially fatal mycoses commonly observed in patients with immunocompromised conditions occur systemically not superficially. In 1998 reports by **REVANKAR et al. (1998)** and **MARTINS et al. (1998)** showed a decrease in rate of oral candidiasis in AIDS patients using antiretrovirals and the result is a decrease in azole-resistant isolates from these patients. *In vitro* and clinical antifungal resistance may be defined differently. *In vitro* resistance is determined by establishing the degree of susceptibility of fungal species to certain antifungal drugs. The fungi can be further classified as possessing either intrinsic or acquired resistance. However, if a fungal infection persists while the patient is on a regulated antifungal drug treatment, then it is considered as clinical resistance.

With an increasing number of patients presenting with infectious diseases and the alarmingly high incidences of AR in recent years, the search for new therapeutic drugs is a necessary measure. The available conventional medication is under pressure to treat the new and re-emerging diseases such as the increasing number of opportunistic infections often found in patients with AIDS. The „National Committee for Clinical Laboratory Standards (NCCLS)' produced a revised and certified *in vitro* susceptibility test that determines the minimum inhibitory concentration of antifungal agents against *Candida* species. Currently there are numerous studies investigating the molecular mechanisms involved in antifungal drug resistance. Once these mechanisms elucidate the mode of action of fungi in drug resistance, more specific recommendations can be made during drug development and design.

4.1.4. Antibacterial evaluation techniques

HEWITT and **VINCENT (1989)** defined a microbiological assay as a "***technique whereby the potency or concentration of a chemical substance may be determined by its effect on the***

growth of a microorganism". They went on further to stress that such assays are essential tools for detecting and developing suitable antimicrobial agents/ drugs.

VALGAS et al. (2007), CHOMA and **GRZELAK (2010)** describe three broad groups of bioassays suitable when testing for antibacterial activity (a) diffusion; (b) dilution; (c) bioautographic:

- (a) Diffusion – a common technique which is suitable for preliminary evaluation especially of pure compounds. The disc-diffusion bioassay allows for a higher throughput of crude plant extracts to be tested against various bacteria. The bioassay does not require an immediate homogenous dispersion of extract however; if the extract is insoluble in water then the technique is not suitable as there would be poor extract diffusion. Results are scored based on inhibition zones around the test extract. There are certain limiting factors which include the solubility or molecular size of the sample or the growth rate of the test microorganism that may not allow for sensitive/ accurate reproducible results for crude plant extracts.
- (b) Microdilution – is an ideal preliminary test when using a few test organisms and testing crude extracts. The technique requires the homogenous dispersion of a small quantity of test sample in water. It is a more rigorous and precise evaluation of a crude plant extract and enables one to determine either the minimum inhibitory concentration (MIC) or minimum bactericidal or lethal concentration (MBC; MLC) i.e. the concentration value necessary to inhibit bacterial growth or to kill bacteria respectively for a given microorganism. **PAGE et al. (1997)** indicate that there may be a 2-8 times difference between the abovementioned determinants. It is quick, sensitive and reproducible (**ELOFF, 1998**). The use of indicators *p*-iodonitrotetrazolium violet (INT) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) to visualize inhibition zones is a beneficial feature (**HOSTETTMANN et al., 1995; GIBBONS** and **GRAY, 1998).**
- (c) Bioautography – is a more rapid approach. It is an ideal technique where the use of bioassay-guided fractionation allows for easy detection of those compounds which exhibit biological activity. The use of indicators to visualize inhibition zones is an added advantage (**HOSTETTMANN et al., 1995; GIBBONS** and **GRAY, 1998**).

VALGAS et al. (2007) and **CHOMA** and **GRZELAK (2010)** cited external factors such as extraction procedure, volume of inoculum used, culture medium, composition and pH of the medium and incubation temperature that may influence MIC values. The authors **CHOMA** and **GRZELAK (2010)** also impress that when using any evaluation technique to test for antibacterial activity in crude extracts the results may vary. In addition to the factors mentioned above, other factors include the actual assay technique, bacterial strain used, the source of the plant material, the age of the plant, condition of the plant material (fresh/dried), and the concentration of extract tested (**JANSSEN et al., 1987**). Due to the high number of factors that may result in variability the formulation of a standard protocol remains difficult.

During the early stages of drug development, crude extract concentration is very important. One may assume that an extract is inactive while using a concentration that is too low. There is the possibility that the active ingredient is present at very low concentrations in the plant and should be tested at a higher concentration. However, in traditional medicine, plant material cannot be ingested in bulk to obtain the benefits of an active constituent that is present at low concentrations. This investigation therefore simply serves as a means to validate the use of orchids in traditional medicine. Extracts that lacked any significant activity may in fact show greater activity at higher concentrations. Also, scientific evaluations often reveal crude plant extracts that have potential antimicrobial activity. A suitable dose can thereafter be determined for a therapeutic drug for use in the future.

4.2. MATERIALS AND METHODS

4.2.1. Plant collection

Leaves, stems and roots of *Ansellia africana* Lindl. (MC 01NU); leaves, pseudobulbs and roots of *Bulbophyllum scaberulum* (Rolfe) Bolus (MC 02NU); leaves and roots of *Cyrtorchis arcuata* (Lindl.) Schltr. (MC 03NU); tubers and roots of *Eulophia hereroensis* Schltr. (MC 04NU); leaves, stems, pseudobulbs and roots of *Eulophia petersii* (Rchb.f.) Rchb.f. (MC 05NU); pseudobulbs and roots of *Polystachya pubescens* (Lindl.) Rchb.f. (MC 06NU); leaves and roots

of *Tridactyle tridentata* (Harv.) Schltr. (MC 07NU) were purchased from herbal markets in Nongoma, Pietermaritzburg and Umlazi (KwaZulu-Natal). Voucher specimens were deposited at the University of KwaZulu-Natal Herbarium.

4.2.2. Extraction of plant extracts

For the current investigation, all plant material was dried at 37 °C in an incubator, for 72 h and subsequently macerated into fine powders using an electric blender. The powdered plant material was stored in air-tight containers and stored in the dark at room temperature, until further use. Four solvents of varying polarity (PE, DCM, 80 % EtOH and water) were used in a sequential extraction process. In order of increasing polarity, 5 g powdered plant material were soaked in 100–200 ml cold solvent, sonicated for 1 h in an ultra-sound water bath, left to saturate overnight, and then filtered under vacuum using Whatman No. 1 filter paper. The resultant extract was concentrated using either a rotary evapourator or freeze-drier.

4.2.3. Resuspension of plant extracts

Antibacterial assay: Plant material extracted in PE, DCM and 80 % EtOH were re-suspended in 80 % EtOH to a concentration of 50 mg/ml. Plant material extracted with distilled water was resuspended in such to produce an aqueous extract.

Antifungal assay: Plant extracts were re-suspended in dimethyl sulfoxide (DMSO) and distilled water to produce organic solvent and aqueous extracts respectively to a concentration of 100 mg/ml.

4.2.4. Test organisms used in biological assays

Plant extracts were screened for activity against four bacterial strains and one fungal strain obtained from the American type culture collection (ATCC). Two Gram-positive bacteria: *Staphylococcus aureus* (ATCC 12600) and *Bacillus subtilis* (ATCC 6051), and two Gram-negative bacteria: *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883) were employed. These cultures were maintained on Mueller-Hinton (MH) nutrient agar and

stored at 4°C. Cultures of the fungal strain *Candida albicans* (ATCC 10231) were, maintained on Yeast Malt (YM) agar and stored at 4°C.

4.2.5. Microdilution antibacterial assay

This method is more sensitive than the previously used disc-diffusion bioassay. The aim of the assay is to determine the Minimum Inhibitory Concentration (MIC) values (mg/ml) of plant extracts over a period of 24 h (**ELOFF, 1998**). The micro-dilution assay was used to test the antibacterial activity of orchid plant material. An added advantage of this method is that it could be used for bioassay guided fractionation (post-purification).

Materials prepared in advance under aseptic conditions included McCartney bottles (2 bottles for each bacterial strain), MH broth, a steel trough covered in foil, pipette tips, Eppendorf tubes and 100 ml distilled water. Each bacterial strain was aseptically prepared in MH broth and kept overnight in an orbital shaker at 37 °C. The following day, optical density readings of the overnight bacterial cultures were taken with a VARIAN® CARY 50 Conc Spectrophotometer. Overnight bacterial cultures were diluted with MH broth 1:100 (i.e. 200 µl in 19.8 ml). Sterile distilled water (100 µl) was added to each well of a 96-well microplate. To this, 100 µl of each plant extract were added and serially diluted in a two-fold dilution. Subsequently, 100 µl of the dilute bacterial cultures were added to the respective wells of the microplate. Additional microplates filled with just MH broth or water and no bacteria, with no extract and with just solvents served as the negative controls. Neomycin served as a positive control and was prepared by adding 20 µl of neomycin to 480 µl of distilled water in an Eppendorf tube to make a 500 µl solution. One hundred microlitres of this solution were used to prepare a 2-fold serial dilution to test for the MIC of each bacterial strain being tested. The microplates were then sealed with parafilm and stored in an incubator overnight at 37°C. After 24 h a 0.2 mg/ml stock solution of the (Sigma®) INT indicator was prepared and 50 µl was added to each well. The microplates were again incubated at 37 °C for 1 h and observed. The indicator is reduced to a red colour (tetrazolium salt) when acted upon by biologically active organisms. This indicates a lack of antibacterial activity. Antibacterial activity is recorded as the minimum inhibitory concentration (MIC); which is the last well not to exhibit a colour change.

4.2.6. Antifungal bioassay

A microdilution protocol (where MIC values (mg/ml) were determined) was performed, however a combination of **ELOFF (1998); MOTSEI et al. (2003)** and **MASOKO et al. (2007)** protocols was used as fungal growth differs from bacterial growth. Cultures of *Candida albicans* (ATCC 10231) fungus were prepared in Yeast Malt (YM) broth and incubated overnight in a water bath set at 37 °C. Subsequent to this, the absorbance readings of 1 ml 0.5 McFarland barium sulphate standard solution was taken, followed by, 1 ml of 4 ml sterile saline:400 µl of a 24 h old fungal culture mixture. The percentage absorbance was read at 530 nm. The absorbance reading for the *Candida* saline solution was adjusted with sterile saline to match that of the McFarland standard (0.2500 – 0.2800). Once this was accomplished the resultant fungal solution was further diluted in YM broth 1:1000 (i.e. 10 µl stock yeast culture: 10 ml broth) (**MOTSEI et al., 2003**).

According to the technique used by **ELOFF (1998)**, 100 µl of sterile distilled water was added to each well of a 96-well microplate. Plant extracts (100 µl) were added and a serially diluted in a two-fold dilution. Thereafter, 100 µl of the dilute fungal cultures were added to each well. Samples containing just YM broth or water and no fungus, with no extract and with just solvents served as the negative controls. The positive control was performed as a serial dilution of 25 µl antibiotic Amphotericin B (25 mg/ml) (prepared by weighing out 5 mg of Amphotericin B into an Eppendorf and dissolving it in 195 µl of DMSO) inoculated with fungi. Fifty microlitres (50 µl) of growth indicator, (Sigma®) INT, was added during the assay – to serve as an indicator of fungal growth after 24 and 48 h periods. Microplates were sealed with parafilm and incubated overnight at 37°C. Minimum inhibitory concentrations were recorded after 48 h, after which, 40 µl of YM broth was administered to each well to determine if there was fungicidal/fungistatic activity. Final fungicidal/fungistatic concentrations were recorded after a further 24 h.

4.3. RESULTS

Results for the antibacterial and anti-candidial activity of crude extracts for seven orchid species are shown in **Table 4.1.** (**Chapter 4**). Significant inhibitory and lethal effects were scored at less than 1 mg/ml. *Tridactyle tridentata* aqueous root extract produced the most effective antibacterial activity against *S. aureus* (0.049 mg/ml). All *T. tridentata* organic root extracts produced significant inhibitory activities against both Gram positive bacteria. *Eulophia petersii* DCM pseudobulb extracts significantly inhibited all bacterial strains tested (0.78 mg/ml against *B.s.*, *E.c.*, and *K.p.* and 0.39 mg/ml against *S.a.*). *Eulophia hereroensis* 80% EtOH root extract was the only other extract to exhibit significant inhibitory effects against Gram-negative bacteria, specifically *K.p.* (0.65 mg/ml). After 48 h *C. albicans* was most susceptible to *P. pubescens* aqueous pseudobulb extract (0.0816 mg/ml). *Eulophia petersii* DCM pseudobulb extract however, demonstrated significant activity against *C. albicans* with MIC and MFC values of 0.65 mg/ml.

Table 4.1.: Antibacterial and antifungal activity of crude extracts from seven South African medicinal orchid species against standard bacterial and fungal strains from ATCC.

Species	Plant part	Extraction solvent	Bacterial strains (MIC mg/ml)				*Candida albicans* (mg/ml)	
			B.s.	*S.a.*	*E.c.*	*K.p.*	MIC (48 h)	MFC(72 h)
Ansellia africana	Leaves	PE	5.21	4.72	4.69	4.17	12.5	>12.50
		DCM	2.15	2.67	2.86	2.6	8.33	12.5
		Ethanol	2.05	1.85	2.6	2.08	12.5	12.5
		Water	>12.50	>12.50	>12.50	>12.50	6.25	6.25
	Stems	PE	2.03	3.44	3.75	2.19	6.25	6.25
		DCM	**0.39**	2.19	2.81	2.81	6.25	6.25
		Ethanol	2.34	5.63	4.38	2.5	12.5	12.5
		Water	>12.50	>12.50	>12.50	>12.50	>12.5	>12.5
	Roots	PE	-	-	3.13	-	12.5	12.5
		DCM	**0.46**	3.12	2.08	2.08	2.08	2.08
		Ethanol	1.95	2.34	2.34	1.95	3.13	4.17
		Water	>12.50	>12.50	>12.50	>12.50	2.08	>12.50
Bulbophyllum scaberulum	Roots	DCM	**0.15**	**0.0735**	4.69	4.69	**0.78**	3.13
		Ethanol	**0.26**	**0.26**	6.25	4.17	1.56	6.25
		Water	6.25	6.25	>12.50	12.5	>12.50	>12.50
Cyrtorchis arcuata	Leaves	PE	1.56	1.56	3.13	1.56	6.25	6.25
		DCM	**0.78**	1.56	3.13	3.13	3.13	4.69
		Ethanol	2.08	3.13	3.13	2.08	6.25	6.25
		Water	>12.50	2.08	12.5	3.13	3.13	3.13
	Roots	PE	2.34	1.56	3.13	3.13	6.25	6.25
		DCM	**0.59**	**0.59**	3.13	3.13	6.25	6.25
		Ethanol	2.6	3.13	3.13	4.17	6.25	6.25
		Water	>12.50	1.95	>12.50	3.13	>12.50	>12.50
Eulophia hereroensis	Tubers	PE	3.13	1.56	3.13	3.13	-	-
		DCM	2.08	3.13	1.56	3.13	-	-
		Ethanol	4.17	6.25	3.13	3.13	-	-
		Water	>12.50	12.5	12.5	10.42	>12.50	>12.50
	Roots	DCM	**0.39**	1.56	6.25	6.25	-	-
		Ethanol	**0.13**	**0.42**	1.3	**0.65**	-	-

Species	Plant part	Extraction solvent	Bacterial strains (MIC mg/ml)				*Candida albicans* (mg/ml)	
			B.s.	*S.a.*	*E.c.*	*K.p.*	MIC (48 h)	MFC(72 h)
Eulophia petersii	Leaves	PE	1.56	**0.39**	3.13	1.56	>12.50	>12.50
		DCM	3.13	3.13	3.13	3.13	1.56	6.25
		Ethanol	2.6	**0.91**	3.13	3.13	3.13	3.13
		Water	-	-	-	-	8.6	>12.50
	Stems	PE	**0.78**	**0.39**	3.13	3.13	12.5	12.5
		DCM	1.56	1.56	1.56	1.56	**0.78**	**0.78**
		Ethanol	1.3	1.56	6.25	2.08	2.6	10.42
		Water	>12.50	>12.50	>12.50	>12.50	>12.50	>12.50
	Pseudobulbs	PE	**0.098**	**0.2**	3.13	3.13	3.13	3.13
		DCM	**0.78**	**0.39**	**0.78**	**0.78**	**0.65**	**0.65**
		Ethanol	1.56	2.08	6.25	6.25	**0.78**	6.25
		Water	>12.50	3.13	6.25	5.21	>12.50	>12.50
	Roots	PE	**0.78**	**0.39**	3.13	3.13	>12.50	>12.50
		DCM	**0.33**	**0.2**	1.3	1.3	1.04	1.3
		Ethanol	3.13	4.17	3.13	4.17	3.13	12.5
		Water	>12.50	>12.50	12.5	12.5	>12.50	>12.50
Polystachya pubescens	Pseudobulbs	PE	**0.78**	1.56	1.56	1.56	3.13	6.25
		DCM	**0.29**	**0.39**	3.13	3.13	3.13	3.13
		Ethanol	1.56	2.08	3.13	2.08	3.13	6.25
		Water	-	-	-	-	**0.0816**	3.13
	Roots	DCM	1.17	1.17	3.13	3.13	6.25	6.25
		Ethanol	1.56	1.56	3.13	2.6	1.56	3.13
		Water	6.25	3.13	12.5	3.13	>12.50	>12.50
Tridactyle tridentata	Roots	PE	**0.2**	**0.59**	3.13	2.34	6.25	6.25
		DCM	**0.0653**	**0.0816**	5.21	5.21	2.6	5.21
		Ethanol	**0.65**	**0.91**	3.13	3.13	3.13	3.13
		Water	3.13	**0.049**	>12.50	12.5	3.13	3.13
Neomycin (µg/ml)			**0.012**	**0.78**	**0.39**	**0.098**	-	-
Amphotericin B (µg/ml)			-	-	-	-		**49.0**

B.s. - *Bacillus subtilis*; E.c. - *Escherischia coli*; K.p. - *Klebsiella pneumoniae*; S.a. - *Staphylococcus aureus.* The values presented are averages (n=3). Values in bold indicate those extracts that exhibited significant activity (< 1 mg/ml).

4.4. DISCUSSION

The current list of medicinally and culturally used orchid species in South African traditional practices (**Table 3.1. Chapter 3**) may represent a refined list of species that were observed by the traditional healers and traders to be most therapeutically beneficial (**CHINSAMY et al., 2011**). Certain classes of compounds have specific biological activity, which may have been harnessed when used in traditional medical practices.

Phytoalexins are defined as low molecular weight antimicrobial compounds synthesised and accumulated in plants post-exposure to microorganisms (**STOESSL** and **ARDITTI, 1984**). This is in accordance with the observation made by French botanist, Noel Bernard; who revealed an acquired immunity in orchid species *O. morio* and *L. hircinum* against *R. repens*, an endomycorrhizal fungus. Other orchid species with functional uses of their antifungal properties include *C. macranthos* var. *rebunense* seeds which demonstrated antifungal properties at various growth phases as a mechanism to facilitate germination (**SHIMURA et al., 2007**). *Dendrobium officinale* agglutinin-2 demonstrated significant antifungal properties against *G. zeae* which suggests its possible use for crop plants. (**CHEN et al. 2005**).

The pseudobulb aqueous extract of *P. pubescens*, which shares the vernacular name with *P. ottoniana* (*amabelejongosi*) and may be sold a replacement, significantly inhibited *C. albicans* after 48 h (0.0816 mg/ml). This was the only aqueous extract to have such an effect on *C. albicans*. The stem and pseudobulb DCM extract of *E. petersii* exhibited a more moderate but persistant antifungal effect. While the antimicrobial use of *Polystachya* species in African traditional medicine is validated; a more comprehensive evaluation of the antifungal properties and chemistry of *Polystachya* species of South Africa may reveal novel phenanthrene and dihydro phenanthrene compounds with possibly novel modes of action. Similar studies on *E. petersii* may uncover interesting results.

Phenanthrenes and their dihydro phenanthrene derivatives constitute the major phytoalexins in orchids (**KOVÁCS et al., 2008**). Orchinol (2,4-dimethoxy-7-hydroxy-dihydrophenanthrene) isolated from *O. militaris* and loroglossol and hircinol (2,5-dihydroxy-4-methoxy-

dihydrophenanthrene) isolated from *L. hircinum* exhibited antimicrobial properties (**BÖLLER et al., 1957; HARDEGGER et al., 1963**). Hircinol and orchinol were considered to be non-specific antimicrobials as they produced varying activities against a range of fungi and bacteria (**GÄUMANN et al., 1960; URECH et al., 1963; FISCH et al., 1973**). Hircinol (100 ppm) exhibited fungistatic effects against *C. lipolitica* over three days; while loroglossol was found to be inactive. Orchinol (100 ppm) demonstrated a more potent antifungal effect by completely inhibiting growth of *C. lipolitica* over six days (**KOVÁCS et al., 2008**).

Alternatively, future studies should look into the antifungal activity of lectins. Mannose binding lectins are commonly found in higher plant families; Orchidaceae, Amaryllidaceae, Alliaceae, Araceae, Iridaceae and Liliaceae. They are involved in recognition of high-mannose type glycans of foreign microorganisms or predators. Orchid species from which mannose binding lectins have been isolated include *L. ovata*, *E. helleborine*, and *Cymbidium* hybrid (**VAN DAMME et al., 2000**). Gastrodianin, a stable structure with potent inhibitory activity against certain fungal strains (**XU et al., 1998**) was isolated from *G. elata* as the main antifungal compound. It was found to be closely related to the antifungal mannose-binding proteins of orchids such as *E. helleborine* and *L. ovata*; therefore, gastrodianin-like proteins are considered a novel group of antifungal proteins (**WANG et al., 2001**).

According to **KOVÁCS et al. (2008)** phytoalexins such as phenanthrenes and dihydrophenanthrenes are the defence compounds present in orchid species to counter microbial attack. *Bulbophyllum scaberulum* is one of the less frequently sold orchids in herbal markets. There is no record of *B. scaberulum* being used in African traditional medicine in the available ethnobotanical literature. The DCM root extract was most effective against *S.a.* (**Table 4.1 Chapter 4**). The organic root extracts were particulary active against Gram-positive bacteria. A large number of phenanthrenes have been isolated from the genus *Bulbophyllum* (**KOVÁCS et al., 2008**). These include: bulbophyllanthrin, cirrhopetalanthrin, coelonin, confusarin, erianthridin, fimbriol-B, flavanthrin, flavanthrinin, gymnopusin, isoreptanthrin, nudol, reptanthrin. A more extensive evaluation of *Bulbophyllum* species in South Africa may uncover known and/or novel phenanthrenes with potent antimicrobial effects.

With regard to the current investigation, *E. petersii* is used by the Zimbabweans as a tuber infusion to treat edema (**GELFAND et al., 1985**); the pseudobulb DCM extract significantly affected all test organisms (**Table 4.1. Chapter 4**); while the PE and DCM root extracts significantly inhibited only Gram-positive bacteria. While this may not be a direct correlation with the use of the plant, active constituents in this non-polar extract may have other protective properties not previously known. The total phenolic content of the pseudobulb was significantly higher than other extracts (**Figure 6.3 A**). The therapeutic effect of this plant may include prevention of opportunistic infections. Different species of the *Eulophia* genus are traded under one vernacular name. Such substitution might imply similar uses, therapeutic effect and/or lack of knowledge on the different species. The use of *E. hereroensis* (syn. *E. junodiana* Kraenzl., *E. undulata* Rolfe and *E. pillansii* Bolus) in African traditional medicine has never been previously reported. The DCM and 80% EtOH root extracts exhibited significant antibacterial activity against certain test organisms (**Table 4.1. Chapter 4**). This was the only other species tested (other than *E. petersii*) that exhibited a significant effect against a Gram-negative bacterium. A more complete phytochemical and broad pharmacological assessment of the South African *E. petersii* species, and *Eulophia* genus, might reveal similarities and/or differences to that of the Yemen species.

A phytochemical report by **BLITZKE et al. (2000)** revealed five phenanthrenes and several phytosterols from the roots of *E. petersii* collected in Yemen. **KOVÁCS et al. (2008)** reviewed the phytochemical and pharmacological research on phenanthrenes from orchids. Of the phenanthrenes found in *E. petersii* (coelonin, lusianthridin, lusianthrin, eulophiol) lusianthridin was found to be active against Gram-positive bacteria but with poor effect against certain fungi (**KOVÁCS et al., 2008**). In Chinese medicine, *Bletilla striata* is used to treat „pneumonorrhagia' and „pneumonopthisis'. Gram-positive bacteria were found to be susceptible to lusianthridin, isolated from the methanol extract of *B. striata*. The same compound was also found to have cytotoxic activity *in vitro* and *in vivo*, anti-tumour effects and increase contractions (**KOVÁCS et al., 2008**). Biphenanthrenes, blestriarene A, B and C were particularly active against *S. aureus* and *Streptococcus nutans*; with blestriarene B exhibiting the most potent antibacterial effects (**YAMAKI et al., 1989**). Lusianthridin was isolated from another *Eulophia* species – *E. boothii* (**KOVÁCS et al., 2008**).

Tridactyle tridentata is the most difficult species to locate in herbal markets. Very few traders are familiar with this „*iphamba'*. While it is mostly used as a protective charm (**HUTCHINGS et al., 1996**), all crude root extracts were consistently active against Gram-positive bacteria (except *B.s.* for aqueous extract). The use of related species or sale of previously unrecorded „medicinal' species is an indication of a lack of in-depth records, and lack of interest in orchids as a medicinal plant. Without such information, the change in availability of these species, and subsequently, the conservation status would go unnoticed. With regard to the genus *Tridactyle*, determining the presence of structurally related compounds in related and unrelated species would be of interest. Since all organic and aqueous extracts (even after a sequential extraction process) produced significant antibacterial effects; a more extensive investigation of *T. tridentata* root extracts may reveal significant bioactive compounds.

Current antibiotics tend to exhibit less activity against Gram-negative than Gram-positive bacteria possibly due to the complex cell wall structure of the former (**WHITE** and **McDERMOTT, 2001**). **McGAW et al. (2000)** reported a tendency for plant extracts to be more active against Gram-positive than Gram-negative bacteria**.** This perhaps could explain the current results obtained where more significant activity was observed against Gram-positive bacteria (**Table 4.1. Chapter 4**). Vanillin, a constituent of *V. planifolia* Andrews (syn. *V. fragrans* Ames), exhibits antimicrobial properties against certain Gram-positive and Gram-negative bacteria known to spoil food and is therefore used as a food preservative (**KRUEGER** and **KRUEGER, 1983; BURRI et al., 1989; CERRUTTI et al., 1997; DAUGSCH** and **PASTORE, 2005**).

In terms of validating the ethnobotanical use of South African orchids in traditional medicine, the results obtained show bateriostatic, fungistatic and fungicidal activity in all extracts. *Tridactyle tridentata* aqueous root extract which produced the most effective antibacterial activity against *S. aureus* (0.049 mg/ml) and *E. petersii* DCM pseudobulb extracts which significantly inhibited all test organisms could be of particular interest, in terms of isolation and identification of active constituents. The standard strain of *C. albicans* (ATCC 10231) proved to be resistant to most plant extracts tested but most susceptible to *P. pubescens* aqueous pseudobulb extract. Generally, the DCM extracts showed better activity than the PE and EtOH

extracts which merits further investigation into the identification of the compounds present in these crude extracts. There is no information on the use of orchid species as antifungal agents in African traditional medicine.

CHAPTER 5
ANTHELMINTIC EVALUATION

5.1. INTRODUCTION

Persons living in tropical and subtropical areas of sub-Saharan Africa, Asia and Latin America are especially susceptible to soil-transmitted helminth (STH) infection (**HOTEZ et al., 2003; BETHONY et al., 2006**). The major infective agents that cause STH include: *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms. Parasitic worm infestations of the intestine, most commonly by nematodes (round worms) are known to cause considerable distress to both animals and humans. Statistics show a total of close to 2 billion people affected by parasitic worm infections (**HOTEZ et al., 2003; WHO, 2004**). Over 1 billion people are thought to be infected with *A. lumbricoides*, 795 million with *T. trichiura*, and approximately 740 million with hookworms (*Ancylostoma duodenale* and *Nector americanus*) (**BETHONY et al., 2006**). Nematodes are free-living organisms, known to be ubiquitous in nature. Those that do find their way into a human gastrointestinal tract only manifest as helminthiasis when the nematode load is too high (**LEWIS** and **ELVIN-LEWIS, 1977**).

Helminthiasis is one of the most common tropical diseases, responsible for high morbidity and mortality especially in the poorer regions of developing countries (**WHO, 2002b**). The tropical climate is favourable for the completion of the nematode life cycle in the soil. (**HOTEZ et al., 2003**). Humans contract the infection by either ingesting the eggs from contaminated soils or the larve in the soil actively penetrate the skin. Parasitic worm infections seldom lead to death (**HOTEZ et al., 2006**), however; a persistent infection has serious consequences. Infections, which are more prevalent in school children, result in a feeling of general weakness, signs of malnutrition, depression, iron-deficiency anaemia (caused mainly by hookworm infection – chronic intestinal blood loss), and noticeable impaired physical and intellectual development (**WHO, 2002b**). While the highest infection rates occur in the 5 – 15-year-old age group (**WHO, 2002b**); the long-term effects will unfortunately only manifest later in their lives. In addition, it has been suggested that untreated helminthiasis could compromise the individual's

immune system, leaving them vulnerable to unrelated infections such as malaria, HIV and TB (**HOTEZ et al., 2006; KEISER** and **UTZINGER, 2008**).

In his review **BROOKER (2010)** discusses the development of the standard measurement of burden of disease; disability adjusted life years (DALYs). The effect of diseases such as helminthiasis are felt more in children and impact negatively on their physical and cognitive development. Also, symptoms of helminthiasis are often "non-specific and chronic" **BROOKER (2010)**; allowing the disease to continue undetected. According to **BETHONY et al. (2006)**, as a result of helminthiasis; an estimated 39 million DALYs are considered lost each year. As a comparison this estimate is more than the value estimated for malaria infections (36 million) and close to those DALYs lost to TB (44 million). Environmental and human (social habits, diet, poverty, hygiene) factors are major contributors to the persistence of these infections (**HOTEZ et al., 2006**).

The United Nations held a „General Assembly's Special Session on Children' in May 2002 from which a document entitled „A World fit for Children' was produced (**WHO, 2002b**). Contained within were the resolutions by world leaders to "reduce the incidence of intestinal parasites" and to "reduce by one-third the prevalence of anaemia, including iron deficiency anaemia, by 2010". Similarly, it is one of the World Health resolutions to provide continuous treatment against STH and schistosomiasis to approximately 75% of all school children in affected areas by the year 2010. One success story from a rural community in Cape Town, South Africa showed a decrease in infection (95%-20%) in 11 000 school children infected from 12 primary schools; following regular deworming treatment (**WHO, 2002b).**

5.1.1. Anthelmintic drugs: Modes of action

SIMPKIN and **COLES (1981)** and **KEISER** and **UTZINGER (2008)** mention two major groups of anthelmintics: benzimidazoles (albendazole and thiabendazole) and non-benzimidazoles (levamisole, pyrantel and ivermectin). **PRICHARD (1994); MARTIN (1997)** and **KEISER** and **UTZINGER (2008)** maintain that it is the excessive use of anthelmintic drugs

in veterinary medicine that has contributed to the current problem of drug resistance. Alternatives include the use of drugs with different modes of action on resistant parasites to effectively treat the infection. Some important considerations (i.e. sufficient biological information on the infecting organism as well as the drugs available) should be made when selecting an anthelmintic agent for treatment (**FRAYHA et al., 1997**). It is therefore necessary to be aware of the different modes of action of available anthelmintic drugs.

FRAYHA et al. (1997) compiled a reference of all the available anthelmintic drugs for humans. Drugs were grouped according to the mechanism of action on helminths.

- Drugs that affect the biochemical processes of the helminths include: antimony organic compounds; niclosamide; bithionol; cyanine dyes; benzimidazoles; and nitroimidazoles.
    - Antimony drugs compete with fructose-6-phosphate for the same site on the enzyme involved in glycolysis (**BUEDING, 1969**). By inhibiting the glycolysis pathway, antimony drugs cut off the supply of ATP, thereby killing the parasite. The earlier use of antimony organic compounds (trivalent and pentavalent) has recently been opposed due to its toxic effects; with pentavalent compounds showing less toxicity than their trivalent counterparts (**JAMES** and **GILLES, 1985**). Use of these drugs however, has resulted in the development of resistant parasites. The mechanism of this is not fully understood (**ONELETTE** and **PAPADOPOULOU, 1993**). **ONELETTE** and **PAPADOPOULOU (1993)** provided another target of antimony drugs: enzymes involved in fatty acid ß-oxidation where, the mechanism of action as of yet is unknown.
    - Niclosamide drugs are primarily used to treat human tapeworm infections and trematode parasites. The drug is thought to remove the protective substance against proteolytic enzymes of the host found in parasites **ROLLO (1970)**. It may also affect the metabolism of ATP by inhibiting the process. This may affect the uptake of glucose either directly or indirectly (**JAMES** and **GILLES, 1985**).
    - Bithionol has been replaced by praziquantel in the treatment of certain parasitic infections (**JAMES** and **GILLES, 1985**). The mechanism of action is similar to that of other drugs in this group where ATP production is inhibited (**JAMES** and **GILLES, 1985**).
    - There are two cyanines: dithiazanine (no longer in use due to its toxicity) and pyrvinium (still used against certain helminths). They have two modes of action: interference with

respiration and inhibition of oxygen uptake; and obstruction of glucose uptake in the nematode's intestine, thereby affecting its motility (**FRAYHA et al., 1997**).

- The benzimidazoles include: mebendazole, albendazole, flubendazole and thiabendazole. This group is more widely used in both human and veterinary medicine. **COOK (1990; 1991)** reported on the susceptibility of certain systemic and intestinal nematodes to one or more of these drugs. Mebendazole prevents the uptake of glucose, which compels the parasite to use its reserve glycogen. Consequently, its source of energy is depleted and its ability to survive and reproduce is hindered (**FRAYHA et al., 1997**). **LACEY (1990)** provides an additional mode of action; mebendazole has an affinity to tubulin, a structural protein found in microtubules, and therefore prevents the formation of microtubules. However, albendazole, an effective broad spectrum anthelmintic, is fast replacing mebendazole (**FRAYHA et al., 1997**). Its mode of action is found to affect the glucose uptake in larval and adult stages of the parasite, thereby consuming the glycogen reserves and reducing ATP production (**FRAYHA et al., 1997**). Albendazole also inhibits microtubule formation. Flubendazole and thiabendazole have similar activities on parasites (**FRAYHA et al., 1997**).
- Nitroimidazoles consist of two groups: metronidazole (low toxicity) and benznidazole (some side effects reported – **De RAADT (1989)**). With metronidazole, subsequent derivatives have shown promising results with a prolonged effect, thereby reducing the number of doses required. Whilst being a broad spectrum drug, it has an even better effect in combination with other drugs. Benznidazole affects the polymerases and DNA templates involved in protein and RNA synthesis – inhibiting the process (**FRAYHA et al., 1997**).

- Another familiar group of anthelmintics are the antimalarials which among other include: quinine, chloroquine and amodiaquine, mefloquine, halofantrine, primaquine and atovaquone (**FRAYHA et al., 1997**).
- Other anthelmintic drugs are known to target the reproductive system and in so doing, affect the multiplication of the parasite (**FRAYHA et al., 1997**).
- Alternatively, some anthelmintic drugs affect the neuromuscular physiology of the susceptible parasite (**FRAYHA et al., 1997**).

- The reference anthelmintic drug used in this investigation as a standard, levamisole, along with other drugs like pyrantel and morantel are considered to be agonists at the nicotinic acetylcholine receptors of nematode muscle which results in spastic paralysis (**MARTIN**, **1997**). The primary action affects the cholinergic receptors at the neuromuscular junctions that paralyse the nematodes, which are then easily excreted (**JAMES** and **GILLES, 1985**). A secondary action targets energy production by inhibiting the enzyme succinodehydrogenase that catalyses the conversion of fumarate to succinate in the TCA cycle (**JAMES** and **GILLES, 1985**).
- Other drugs such as piperazine cause flaccid paralysis by acting as agonists of nematode muscles, while avermectins cause paralysis of pharyngeal pumping by adjusting the glutamate-gated chloride channels (**MARTIN**, **1997**).
- Praziquantel causes rapid muscle contraction and paralysis by increasing the permeability of the tegument and the influx of calcium (**MARTIN**, **1997**).

Currently there are various preventative measures in place, such as: provision of proper sanitation, vector control and chemotherapy. Chemotherapy has been the treatment of choice since ancient times, when plants with anthelmintic activity were used to prevent and treat parasitic infections (**FRAYHA et al., 1997**). Presently, chemotherapy with broad spectrum anthelmintics (**NJOKU et al., 1996**) or the use of specific drugs is the standard treatment for those already infected. Anthelmintics like other clinical drugs need to be effective against the infecting organism but safe for the patient to ingest.

5.1.2. Anthelmintic resistance

Anthelmintic resistance has been most notable in commercially important livestock in certain regions including South Africa (**GEERTS et al., 1997**) and has seriously affected the livestock industry. **GEERTS** and **GRYSEELS, (2001)** highlight the growing concern of drug resistance in human helminthes and stress that the signs of drug resistance are in their early stages and could develop into a serious situation. Drawing on their knowledge **GEERTS et al. (1997)** and **GEERTS** and **GRYSEELS (2000)** suggest that tolerance traits already found in some human

helminthes might become dominant under selective drug pressure. **GEERTS et al. (1997)** caution against some of the methods proposed to treat helminth infections in humans. A more specific group of individuals should be targeted for treatment, instead of mass therapy to avoid the threat of drug resistance (**GEERTS** and **GRYSEELS, 2001**). They advise against using short term, low-dosage treatment to prevent the development of resistance to existing anthelmintics. They do however; acknowledge that resistance may develop more slowly in helminths compared to other infective organisms. It has been hypothesised that broad-spectrum anthelmintics used in mass therapy may encounter resistance in the long-term (**GEERTS et al., 1997**). The WHO recommended the use of anthelmintic treatments for schistosomiasis during dry, low transmission periods **(WHO, 1992)**. However, **GEERTS** and **GRYSEELS (2001)** advise a cautious assessment of each case before commencing with treatment campaigns.
The current threat of AR has fatal consequences. This scenario could be just as dire for anthelmintics. With concerns of rising drug resistance rates and the limited modes of action, of drugs available, there is a need for the development of, and research into, safe and effective anthelmintic drugs. An added concern is the inability of infected individuals to develop immunity to the infective agent once successfully treated. Therefore, the search for anthelmintic drugs to be used in worldwide treatment programs will be a necessity until environmental and social factors that contribute to helminthiasis are corrected, thereby reducing the risk of re-infection. A review by **TAYLOR et al. (2002)** outlines some of the common *in vitro* tests used to detect resistance. **KWA et al. (1994)** describe the molecular mechanisms (mutation in ß-tubulin) involved in resistance to benzimidazoles in helminths. Such studies could reveal the mechanisms used by resistant parasites which would aid in the search for new, effective and safe drugs.

Records of natural products with anthelmintic activity being used by mankind date back to ancient times. Extensive pharmacological studies have been carried out on some South African medicinal plants using the *in vitro* bioassay which makes use of the free-living nematode *C. elegans* (**McGAW et al., 2000**). Such studies revealed some effective anthelmintic compounds in South African medicinal plants.

5.2. MATERIALS AND METHODS

5.2.1. Re-suspension of plant extracts

Plant extracts were prepared as mentioned in **Section 4.2.2**. Organic and aqueous extracts were prepared by reconstituting the PE, DCM, 80% EtOH extracts in 5% DMSO and water extracts in distilled water at a concentration of 25 mg/ml.

5.2.2. Anthelmintic bioassay

Crude plant extracts were evaluated using an eight-day *in vitro* rapid colourimetric assay for quantification of *Caenorhabditis elegans* viability. *Caenorhabditis elegans* nematodes that were maintained on Nematode Growth (NG) agar at 20°C were used. On day one an overnight culture of *E. coli* in 5 ml of MH broth was prepared, along with M9 buffer (6 g Na_2HPO_4; 3 g KH_2PO_4; 5 g NaCl, 0.25 g $MgSO_4.7H_2O$ in 1 liter distilled water). On day two overnight *E. coli* culture was autoclaved and new plates of *C. elegans* nematodes were prepared by washing old cultures with 5 ml of M9 buffer and adding 100 µl of neomycin (0.5 mg/ml) to the old plates. New NG agar plates were seeded with 50 µl of the autoclaved *E. coli* culture, after which 500 µl of the neomycin-treated nematodes were added. New plates were incubated in the dark for 72 h at 20°C. On day five distilled water, a stainless steel trough (covered in foil), 2 McCartney bottles, tips and 2 Eppendorf tubes were autoclaved before the assay. A nematode suspension was prepared by washing 72 h old cultures with 5 ml sterile M9 buffer. The optical density of the suspension was determined at 530 nm using a VARIAN® CARY 50 Conc Spectrophotometer. M9 buffer was used to zero the spectrophotometer and make a more diluted suspension that produced an optical density value in the range of 0.04 – 0.06. Crude plant extracts were prepared to a concentration of 25 mg/ml while the positive control, levamisole, was tested at a concentration of 1 mg/ml. In a 96-well microtitre plate 100 µl of sterile distilled water was added to each well. To this, 100 µl of each sample (crude plant extract or control substance) was added to the first row and a two-fold serial dilution was followed. Thereafter, 50 µl of the diluted nematode suspension were added to each well. Microplates were covered with parafilm and incubated in the dark at 20°C for 48 h. On day seven, 50 µl of INT (2 mg/ml) were added to each well and plates were incubated for a further 24 h. The indicator is reduced to a red colour

(tetrazolium salt) when acted upon by biologically active organisms. This indicates a lack of anthelmintic activity. The minimum lethal concentration (MLC) was determined as the last well not to exhibit a colour change.

5.3. RESULTS

Anthelmintic effects of extracts of seven indigenous orchid species are presented in **Table 5.1**. *Cyrtorchis arcuata* leaf and root extracts were the most effective anthelmintic extracts with MLCs of 0.041 mg/ml for 80% EtOH leaf and root extracts; 0.049 mg/ml for aqueous leaf extracts and 0.78 mg/ml for aqueous and DCM root extracts. All *A. africana* and *T. tridentata* organic root extracts significantly affected *C. elegans*. A similar significant effect was observed for all *E. petersii* organic pseudobulb extracts and DCM extracts. The organic root extracts of *B. scaberulum* exhibited lethal effects on *C. elegans*. Only the DCM tuber and root extracts of *E. hereroensis* displayed significant activities. All of the *P. pubescens* extracts showed poor activity.

Table 5.1.: Anthelmintic (MLC mg/ml) activity of crude extracts from seven South African medicinal orchid species against the nematode *Caenorhabditis elegans*

Species	Plant part analysed	Extraction solvent	MLC (mg/ml) (72 h)
Ansellia africana	Leaves	PE	3.13
		DCM	**0.20**
		Ethanol	1.56
		Water	6.25
	Stems	PE	1.56
		DCM	2.34
		Ethanol	1.56
		Water	6.25
	Roots	PE	**0.20**
		DCM	**0.10**
		Ethanol	**0.16**
		Water	3.13
Bulbophyllum scaberulum	Leaves	Water	>6.25
	Pseudobulbs	Water	>6.25
	Roots	DCM	**0.39**
		Ethanol	**0.39**
		Water	3.13
Cyrtorchis arcuata	Leaves	Ethanol	**0.041**
		Water	**0.049**
	Roots	DCM	**0.78**
		Ethanol	**0.041**
		Water	**0.78**
Eulophia hereroensis	Tubers	DCM	**0.20**
		Ethanol	1.56
		Water	>6.25
	Roots	DCM	**0.39**
		Ethanol	1.56
Eulophia petersii	Leaves	PE	1.30

Species	Plant part analysed	Extraction solvent	MLC (mg/ml) (72 h)
		DCM	**0.39**
		Ethanol	1.04
		Water	3.13
	Stems	PE	1.56
		DCM	**0.39**
		Ethanol	**0.78**
		Water	>6.25
	Pseudobulbs	PE	**0.78**
		DCM	**0.39**
		Ethanol	**0.78**
		Water	6.25
	Roots	PE	**0.78**
		DCM	**0.29**
		Ethanol	1.56
		Water	6.25
Polystachya pubescens	Pseudobulbs	Ethanol	1.56
		Water	>6.25
	Roots	Ethanol	1.56
Tridactyle tridentata	Leaves	Water	3.13
	Roots	PE	**0.78**
		DCM	**0.78**
		Ethanol	**0.20**
		Water	3.13
Levamisole® (µg/ml)			**40.0**
DMSO			**2.08**
Water			**3.13**

Figures in bold indicate crude extracts with significant anthelmintic activities (scored at <1 mg/ml). The values presented are averages (n=3)

5.4. DISCUSSION

There are no previous reports of anthelmintic activity for South African orchid species. The seven species were selected based on their use and availability in herbal markets in KwaZulu-Natal. Traditionally, crude plant extracts are made using cold or hot water extraction to produce infusions, decoctions and creams. When determining the general pharmacological activity of medicinal plants, solvents of varying polarities are used to extract different groups of compounds. All orchid crude extracts exhibited broad-spectrum anthelmintic activity.

Except for *C. arcuata*, only the organic extracts of most other orchid species exhibited significant lethal effects on *C. elegans*. **RHEE et al. (1982)** (cited in **PÉREZ GUTIÉRREZ, 2010**) isolated a substance from the ethyl ether tuber extract of *B. striata* found in Gangweon-do in the Korean peninsula. The substance was soluble in PE and demonstrated anthelmintic effects *in vitro* on various stages of *Clonorchis sinensis* (cercaria, excysted metacercaria and adult stages). **TAYLOR** and **VAN STADEN (2001)** mention that solvents such as EtOH extract lipophilic compounds from plant material. These non-polar principles may contribute to the anthelmintic activity observed.

The presence of certain classes of compounds may influence biological activity in medicinal plants. Phytochemical analysis revealed *C. arcuata* methanol leaf extracts to have the highest condensed tannin (syn. proanthocyanidin) content (1.36 mg leucocyanidin equivalent/g dry matter) compared to other extracts. Recent veterinary parasitology studies have focused on the use of proanthocyanidin-rich diets for direct and indirect anthelmintic effects (**IQBAL et al., 2007**). Similarly, *C. arcuata* methanol root extracts recorded the highest flavonoid content (1.24 mg catechin equivalent/g dry matter). Anatomical studies of *Encyclia calamara* roots revealed the presence of flavonoid compounds (**OLIVEIRA** and **SAJO**, **1999**); while the comprehensive survey by **WILLIAMS (1979)** illustrates the distribution of leaf flavonoids in Orchidaceae. **KIM et al. (2009)** reported on the lethal anthelmintic effects of flavonoids; where the natural flavonoid flavone inhibited embryogenesis in *C. elegans*. Such reports provide credence to the significant anthelmintic effects observed in all *C. arcuata* extracts tested.

Eulophia petersii methanol pseudobulb extract was found to contain the highest total phenolic content (24.73± 0.57 mg gallic acid equivalent GAE/g dry matter – **Figure 6.3A Chapter 6**) compared to other orchid extracts. All DCM extracts of *E. petersii* significantly affected *C. elegans*. The tubers of *E. nuda*, found in the Himalayas, are used to treat various ailments including, as a vermifuge to treat worm infestation. The methanol extract and isolated phenolic compound of the tubers exhibited cytotoxic potential (**SHRIRAM et al., 2010**). The anthelmintic effect of the extract may be due to the cytotoxic effect of phenolic compounds. Hydrolysable tannin (gallotannin) content varied among species; with *E. hereroensis* methanol root extract containing the highest amount (1.40 mg GAE/g dry matter). **MOHAMED et al. (2000)** explored the lethal effects of gallo- and condensed tannins specifically against *C. elegans*. Hydrolysable tannins (gallic acids) are of lower molecular weight than condensed tannins and are more easily absorbed in the digestive tract of ruminants; and are therefore considered more toxic (**HOSTE et al., 2006**). Studies on the effect of pure compounds isolated from *Rhus niveus* on the plant-parasitic nematode (*Meloidogyne incognita*) revealed that gallic acid was one of the more potent nematicidal compounds present (**SULTANA et al., 2010**). **MOHAMED et al. (2000)** concluded that natural tannins could possibly contribute to the search for novel sources of anthelmintics and that the use of tannin-rich plants could help control nematode infection in animals.

KEISER and **UTZINGER (2008)** put into context the current situation of anthelmintic drug efficacy in treatment of soil-transmitted helminthiasis. They recommend research and development of new anthelmintics with different modes of action that could be used in combination with existing drugs. While **BETHONY et al. (2006)** agree with this, they suggest the development of vaccines to control helminthiasis. They discuss ongoing clinical studies on hookworm vaccines. However, in the long term, chemotherapeutic strategies to prevent and treat soil-transmitted helminthiasis would best be accompanied by various environmental, socio-economic and educational programmes (**WHO, 2004**).

This is the first report on anthelmintic activities of *A. africana, B. scaberulum, C. arcuata, E. hereroensis, E. petersii, P. pubscens* and *T. tridentata* currently sold in herbal markets in KwaZulu-Natal. *Caenorhabditis elegans* was most susceptible to organic and aqueous extracts

of *C. arcuata*. The significant lethal effects of the crude 80% EtOH leaf and root extracts as well as the aqueous leaf extract of *C. arcuata* were comparable to that of Levamisole®. The presence of proanthocyanidins and flavonoids in *C. arcuata* leaf and root extracts may have contributed to the significant anthelmintic effects.

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CHAPTER 6
ACETYLCHOLINESTERASE INHIBITORY, ANTI-INFLAMMATORY AND ANTIOXIDANT EVALUATION

6.1. INTRODUCTION

Common degenerative diseases such as asthma, heart disease, inflammatory bowel disease and rheumatoid arthritis are often associated with inflammatory processes. The development of inflammatory, cardiovascular and neurodegenerative disorders are also commonly linked with an over-production of oxidants such as ROS during an inflammatory and/or immunological response (**WINROW et al., 1993**). The current chapter explores the role of inflammatory processes and oxidative stress in the development of Alzheimer's disease. The search for potential anti-inflammatory and antioxidant natural compounds for treating common inflammatory and pain-related disorders are discussed.

Alzheimer's, described as a progressive neurodegenerative disorder in aging persons, is characterized by an accumulation of extracellular deposits of β-amyloid peptides in senile plaques and intracellular formation of neurofibrillary tangles of hyperphosphorylated *tau* protein (**HENEKA, 2006**). An examination of the brain of an Alzheimer's patient revealed a reduction in the size of regions responsible for learning and memory. Also, senile plaques and neurofibril tangles were present in these regions and emotional centres (entorhinal cortex, hippocampus, basal forebrain and amygdala). The number of synapses and neurons (associated with the neurotransmitters glutamate and acetylcholine) are reduced or damaged in affected brain regions (**MATTSON, 2004**). These conditions in an aged brain lead to loss of memory, personality changes and impacts on quality of life in patients (**MANDAIRON** and **DIDIER, 2010**).

Alzheimer's patients are usually older than 60 years of age (**HENEKA, 2006**). The prevalence of the disorder has increased from 15 million to approximately 30 million cases globally (**FRANCIS et al., 1999; SELKOE, 2005; HENEKA, 2006**). Populations are growing older, and as a result there is an increase in the risk of developing a neurodegenerative disorder

(**SELKOE, 2001**). Future Alzheimer's incidence estimates are in the region of 90 million (**MATTSON, 2004; HENEKA** and **O'BANION, 2007**). There is no known cure for Alzheimer's due to the complexity of its pathogenicity.

6.1.1. Acetylcholinesterase and Alzheimer's

Based on the research conducted by **PERRY et al. (1978)** there appears to be a relationship between the cholinergic system and the progression of Alzheimer's disease. This observation gave rise to the „cholinergic hypothesis' where a decrease in the neurotransmitter acetylcholine is associated with the Alzheimer's disorder (**TABET, 2006**). The review by **BARTUS et al. (1982)** addressed the validity of the cholinergic hypothesis. At the time, certain studies showed a significant relationship between changes in the cholinergic system, the aged brain and memory loss. The changes in the cholinergic system were linked to the loss of neurons. Artificially altered cholinergic systems in younger subjects produced comparable results to that of old subjects and those experiencing dementia. Of particular interest was the positive effect of cholinergic drugs on aged subjects. Nearly two decades later, while still trying to ascertain the exact cause of Alzheimer's, the hypothesis was re-examined by **FRANCIS et al. (1999)**. The authors addressed more recent developments in Alzheimer's research and concluded that while a breakdown in cholinergic function is not the cause of Alzheimer's it indirectly affects cognitive performance (**FRANCIS et al., 1999**). **BUCCAFUSCO (2004)** reiterated that there is a relationship between aging, cholinergic dysfunction and breakdown in cognitive function.

The cholinergic hypothesis led to the treatment of Alzheimer's using acetylcholinesterase (AChE) inhibitors. The three most common AChE inhibitors are galanthamine, donepezil and rivastigmine (**STUCHBURY** and **MÜNCH, 2005; TABET, 2006**). **FRANCIS et al. (1999)** reviewed the effect of these three cholinesterase inhibitors; all licensed in Europe for the symptomatic treatment of cognitive decline in Alzheimer's patients. The authors also included a schematic representation of how AChE inhibitors act. **GIACOBINI (2004)** discussed briefly the pharmacology of cholinesterase inhibitors (donepezil, galanthamine, huperzine A, metrifonate, physostigmine, rivastigmine and tacrine). The only compound to exhibit irreversible inhibition

is the organophosphate metrifonate. Pseudo-irreversible inhibition was observed for the carbamate compounds physostigmine and rivastigmine. The widely used drug galanthamine has a phenanthrene structure and demonstrates a reversible inhibition of enzyme AChE. Galanthamine also affects nicotinic acetylcholine receptors which may provide an alternative non-cholinergic route to treat symptoms of Alzheimer's (**GIACOBINI, 2004**). As there is still no cure for Alzheimer's, the focuses of recent research initiatives are preventative strategies and treatment of early-onset symptoms. While the direct cause of Alzheimer's is unknown, processes involved in the development of Alzheimer's now also include inflammatory systems and oxidative stress on neurons (**DeKOSKY, 2003**).

6.1.2. Inflammation

Inflammation is a cells' reaction to tissue damage as a result of infection, chemical or physical trauma, or even radiation. Inflammation is often a symptom of other serious illnesses and associated characteristics of an inflammatory response include pain, fever and even a rapid heart rate. **VANE** and **BOTTING (1996)** describe the inflammatory process as a complex combination of steps that include enzyme activation, mediator release, extravasation of fluid, cell migration, tissue breakdown and repair.

The inflammatory process is described simply as the biosynthesis of AA to form various mediators including primary and intermediate prostaglandins (PGs) (eicosanoids) which manifest as the "pain symptom". Other biosynthetic metabolites of AA include thromboxanes (TXs) and leukotrienes (LTs) (**RANG and DALE, 1987**). Eicosanoids are a family of compounds often implicated as mediators of basic physiological processes (**CAMPBELL** and **HALUSHKA, 2001**). They are produced throughout the body and are responsible for the control and maintenance of physiological processes such as inflammation, blood pressure regulation and initiation of blood clotting. Based on the specific function of the tissue, only certain enzymes may be present in the cell, resulting in only certain PGs being produced (**VANE, 1971; MANTRI** and **WITIAK, 1994**).

Aside from the functions discussed above, eicosanoids are also responsible for the pain and fever (pyretic) symptoms of inflammation. The metabolic conversion of AA to PGs is catalysed by the enzyme prostaglandin endoperoxide synthase, which has two constituents – cyclooxygenase and peroxidase. Therefore the inhibition of the catalysing cyclooxygenase (COX) enzyme (**VANE, 1971; SALMON** and **HIGGS, 1994**) seems an acceptable measure when treating painful and fever-related medical conditions.

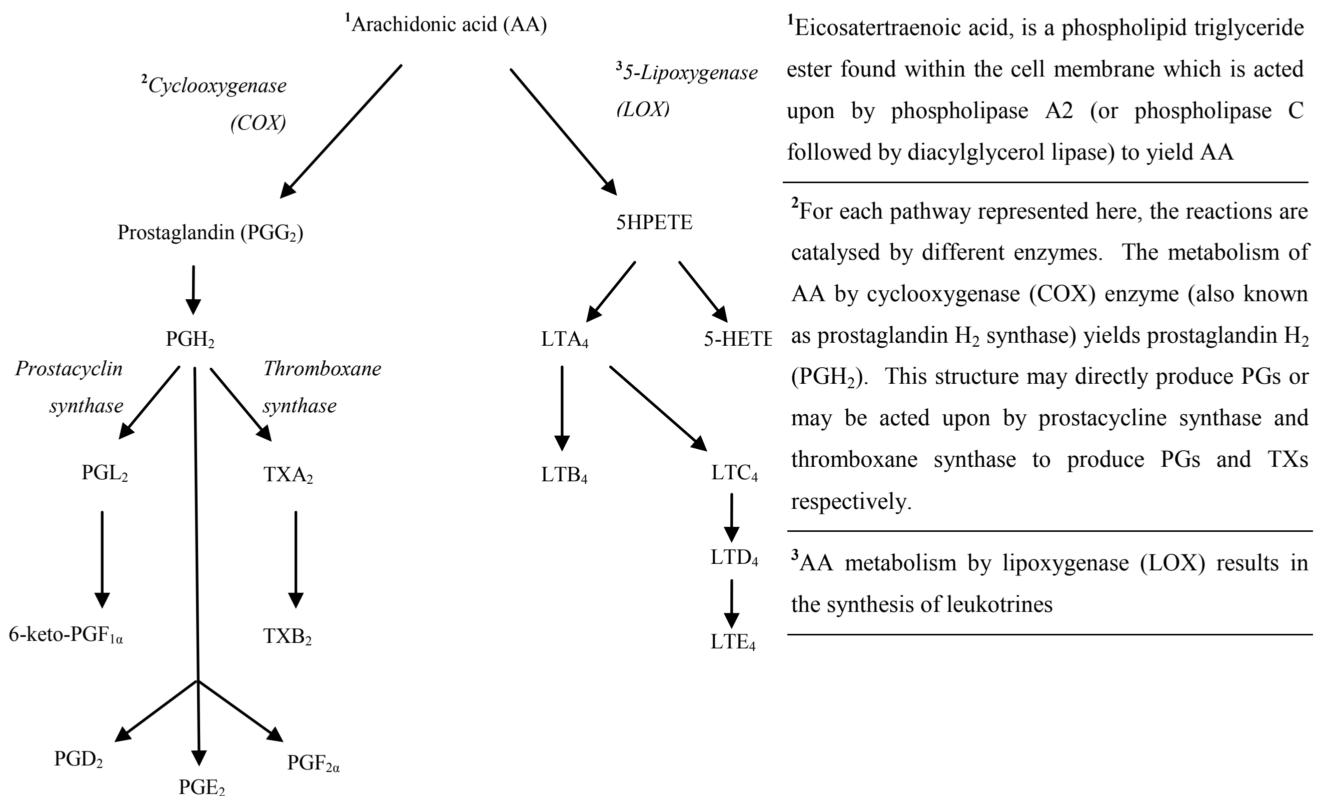


Figure 6.1.: An annotated schematic representation of the metabolic pathway/s of arachidonic acid (AA) synthesis (adapted from **DeRUITER, 2002**)

The COX enzyme is made up of two isozymes COX-1 and COX-2. In recent years there has been much debate over the number of isozymes present, their function, and their exact role and pathway during the inflammation process. Initial reports described the COX-1 isozyme as a constitutive enzyme with a number of homeostatic cellular functions. COX-2, on the other hand, was considered to be inducible by inflammation. Recent reports contain conflicting views; with

COX-2 now being considered by the majority to be a constitutive enzyme. It was recently argued in a review by **SAUTEBIN (2000)** that COX-2 is not an inducible isozyme but rather a constitutive one (**KÖMHOFF et al., 1997**). In their review, **PATRIGNANI et al. (2005)** cite publications that refer to COX-1 as an inducible enzyme in the developmental phases and COX-2 as a constitutive enzyme in the brain tissue. When treating pain-related symptoms, COX inhibition may be achieved via one of three mechanisms: (a) competitive inhibition – described as the reversible competition for the COX active site (otherwise occupied by arachidonate); (b) non-competitive inhibition – described as an irreversible binding of the inhibiting bioactive compound to the COX active site; and (c) time-dependant inactivation – may be described as the result of an enzyme –inhibitor complex formation (**MANTRI** and **WITIAK, 1994; SMITH** and **DEWITT, 1995**).

Drugs that inhibit the inflammatory process are either glucocorticoids or non-steroidal anti-inflammatory drugs (NSAIDs); with the latter exhibiting the non-competitive inhibition mechanism (**RANG** and **DALE, 1987**). NSAIDs are commonly used as therapeutic agents. They are further divided into: salicylates (aspirin, diflunisal); acetic acids (indomethacin, diclofenac); propionic acids (ibuprofen, naproxen); fenamates (mefenamic acid, meclofenamate); pyrazoles (phenylbutazone); and oxicam (piroxicam) (**RANG** and **DALE, 1987; MANTRI** and **WITIAK, 1994**). NSAIDs may have an anti-inflammatory effect (affecting the inflammatory process); an analgesic effect (decrease in pain); or an antipyretic effect (a decrease in high body temperature – fevers) all of which relate to the action of NSAIDs on COX enzyme (**RANG** and **DALE, 1987**).

Almost all NSAIDs used to treat chronic inflammatory diseases are COX-1 inhibitors while other COX inhibitors are able to inhibit both COX-1 and COX-2 with different selectivity. Current anti-inflammatories cause side effects (diarrhoea, bloatedness, heartburn and ulceration (a result of chronic use)) of varying severity due to the effect on the homeostatic functioning of the COX-1 enzyme. This has led to the assumption that NSAIDs are inhibiting both isozymes. The problem may lie in the 70% molecular homology of the isozymes and though they are products of different genes on separate chromosomes, they have a similar affinity to convert AA

to PGs (**FÉLÉTOU et al., 2011**). The ambiguity may also involve cellular and molecular mechanisms.

6.1.2.1. Role of inflammatory systems in Alzheimer's disease

The most obvious characteristic of Alzheimer's is the decline in cognitive performance. The burden of disease affects quality of life for patients and care-givers. The actual causes and mechanisms of progression of the disease are yet to be fully elucidated. Identifying the complex set of systems involved in the development of Alzheimer's has been the focus of many neurological and geriatric studies. Neuroinflammation has been closely linked to Alzheimer's disease progression (**MOORE** and **O'BANION, 2002**). **HENEKA** and **O' BANION (2007)** reviewed the role of inflammatory processes in the development of Alzheimer's. The authors discussed how initial neurodegenerative processes induce neuroinflammatory responses.

Work on prostaglandins by **SAMUELSSON (1964)** pioneered research of the CNS and associated diseases. Since the detection of prostaglandins in the brain, the function, distribution and expression of all constituents including COX in the inflammatory process have been researched (**KAUFMANN et al., 1997**). While a more comprehensive understanding is needed, the role of anti-inflammatory agents in the prevention or treatment of Alzheimer's is the focus of current research (**MOORE** and **O'BANION, 2002**). According to **RICH et al. (1995)** and **DeKOSKY (2003)** certain NSAIDs when administered over a long term period exhibited positive results (decreased risk of Alzheimer's). The varying effects of different classes of anti-inflammatory agents in different trials suggest the use of anti-inflammatories as a preventative measure and not as a treatment (**DEKOSKY, 2003**). The conflicting reports may be suggestive of neuroprotective pathways other than COX-inhibition (**STUCHBURY** and **MÜNCH, 2005**). NSAIDs which primarily inhibit the COX enzyme may affect alternative pathways of Alzheimer's progression and delay the onset of symptoms (**HENEKA** and **O'BANION, 2007**). **MONTINE et al. (1999)** demonstrated that while COX activity in persons susceptible to Alzheimer's was comparable to that of control persons; concentration levels of PGE_2 in cerebrospinal fluid of susceptible patients had increased five times. This study demonstrated the potential of COX-inhibitors in the treatment of Alzheimer's.

The metabolism of AA by COX to form pro-inflammatory mediator PGE_2 is one of many pathways in the inflammatory response. The implications of this pathway regarding treatment of inflammatory disorders are vast. The exact role of both COX isozymes in neuroinflammation is still unclear (**MOORE** and **O'BANION, 2002**). As mentioned earlier, current anti-inflammatory drugs (NSAIDs) inhibit the constitutive COX-1 which contributes to reported side-effects. By selectively inhibiting the inducible COX-2, one could possibly produce an efficient and tolerable anti-inflammatory that could be used to treat symptoms of common inflammatory disorders such as pain and fever; and that could be used to prevent the onset and/or treat symptoms of diseases affecting the CNS.

According to **BOROVIKOVA et al. (2000)** there is an established link between the cholinergic system and inflammation, with acetylcholine playing a role in cytokine release. **TABET (2006)** reported on evidence that AChE inhibitors have an anti-inflammatory role through action against free radicals, amyloid toxicity and a reduction in release of cytokines from activated microglia in the brain and blood.

6.1.3. Oxidative stress

Free radicals and other ROS are constantly formed in the human body. The superoxide anion ($O^-_{2\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^\cdot$) radicals are produced as natural metabolic by-products. The simplest free radical is a hydrogen atom. Interest in free radicals began with the work of Moses Gomberg (1900) who demonstrated the existence of the triphenylmethyl radical ($Ph_3C^\cdot$). A free radical is any molecule (capable of independent existence) that possesses one or more unpaired electrons (**MARKESBURY** and **CARNEY, 1999**). Consequently, radicals are less stable compounds (**CHAUHAN** and **CHAUHAN, 2006**). Certain control mechanisms are in place to reduce the effect of oxidative damage naturally, thereby protecting the cells and tissues. **MARKESBURY** and **CARNEY (1999)** however, explained how the uncontrolled production of ROS can lead to damaged cells and tissues – otherwise known as oxidative stress.

Compounds such as antioxidants prevent oxidative stress (**HELEN et al., 2000**). Antioxidants help prevent and/or reduce the risk of degenerative diseases (**AGUDO et al., 2007**). They act as reducing agents, hydrogen donators, singlet oxygen quenchers and may perhaps have metal chelating properties (**RICE-EVANS et al., 1996**). Synthetic antioxidants, such as 2- and 3-*tert*-butyl-4-methoxyphenol (butylated hydroxyl-anisole (BHA)) and 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene (BHT)) have long been used as food additives acting as protective agents.

6.1.3.1. Oxidative stress in Alzheimer's

Oxidative stress has been associated with several degenerative disorders; which include cancer, arteriosclerosis, inflammatory disorders and neurodegenerative diseases. Processes such as lipid peroxidation have been implicated in neurodegenerative diseases such as Alzheimer's (**MARKESBURY** and **CARNEY, 1999**). The ageing process has also been associated with oxidative stress (**MARKESBURY** and **CARNEY, 1999**).

CHAUHAN and **CHAUHAN (2006)** produced a comprehensive review on oxidative stress and its role in the development of Alzheimer's. **MARKESBURY** and **CARNEY (1999)** mention that the brain is at risk of oxidative stress due to the high lipid content (membrane) and oxygen consumption rate. Beta-amyloid plaques in the brain, characteristic in Alzheimer's patients, have been shown to promote oxidative damage via lipid peroxidation, protein oxidation and formation of free radicals (**DeKOSKY, 2003**). During the process of neuroinflammation, free radical products of microglia and astrocytes such as O^{-}_{2} and NO are produced, in addition to ROS generated via the COX pathway (**STUCHBURY** and **MÜNCH, 2005**).

TABET (2006) suggested that AChE inhibitors indirectly increase the production of antioxidants in the brain by inhibiting the release of cytokines from microglia and monocytes. The author also suggested that the anti-inflammatory effect of antioxidant compounds has a positive effect in Alzheimer's patients (**TABET, 2006**). **SELKOE (2005)** suggested the use of antioxidants and free radical scavengers as possible treatment options for certain features of Alzheimer's.

The toxicity of synthetic antioxidant compounds has lead to the development and/or isolation of natural antioxidants from botanical sources. While this potential source of antioxidant compounds sounds promising, their use would be limited without relevant pharmacological and toxicological information. **STUCHBURY** and **MÜNCH (2005)** mentioned that natural antioxidant compounds generally have a relatively low toxicity; which supports the research of these compounds**.**

When discussing future treatment options for neuroinflammatory disorders such as Alzheimer's, **SELKOE (2001)** cited the use of anti-inflammatory drugs. **SELKOE (2001)** further suggested a continued search for anti-inflammatory compounds that target alternative mechanisms in the inflammatory process. Natural plant products have been used as poultices and or anti-inflammatories for years. Aspirin was one of the first plant drugs to become a common prescription anti-inflammatory drug. **BOHLIN (1995)** listed flavonoids, naphthoquinones, alkylamides, phenolic phenyl-propane derivatives, among others, as those compounds responsible for COX inhibition in certain natural products. In the current investigation, seven indigenous orchid species were evaluated for their anti-inflammatory, antioxidant and AChE inhibitory activity.

6.2. MATERIALS AND METHODS

6.2.1. Anti-inflammatory evaluation

Anti-inflammatory activity of crude orchid extracts was determined using an enzyme-based COX assay described by **JÄGER et al. (1996)** with slight modifications as per **NOREEN et al. (1998)** and **ZSCHOCKE** and **VAN STADEN (2000)**. Plant extracts that were previously dried and stored (**Section 4.2.2**) were re-suspended in either 80% EtOH or water to yield ethanolic or aqueous extracts respectively to a concentration of 10 mg/ml.

6.2.1.1. COX-1 Bioassay

Cyclooxygenase (COX) 1 enzyme, isolated from ram seminal vesicles, was purchased from Sigma Aldrich®. The enzyme (10 µl) was activated on ice with 50 µl of co-factor solution (3 mg L-epinephrine and 3 mg reduced glutathione in solution with 10 ml Tris buffer (pH 8.0)) and 100 µl hematin solution. The enzyme/co-factor solution (60 µl) was added to a) 20 µl of aqueous plant extract, b) 2.5 µl EtOH plant extract and 17.5 µl water or c) 2.5 µl indomethacin standard (5 µM) and 17.5 µl water. After an incubation period of five minutes at room temperature 20 µl of [^{14}C] labeled AA (16 Ci/mol, 3 mM), was added to each sample. The reaction mixture was then incubated in a water bath for 10 minutes set at 37°C. The reaction was terminated by the addition of 10 µl 2 N hydrochloric acid (HCl). To the background standard containing 2.5 µl EtOH and 17.5 µl water, 10 µl of 2 N HCl were added to inactivate the enzyme prior to the addition of the enzyme/co-factor solution and the AA. This was kept on ice until the above assay was completed. Thereafter, 4 µl (0.2 mg/ml) of unlabelled prostaglandin carrier solution (precursors: PGE_2:PGF_2) were added in a 1:1 v/v ratio to all Eppendorf tubes.

6.2.1.2. COX-2 Bioassay

The same basic procedure as for the COX-1 assay was followed. Cyclooxgenase-2 (human recombinant) enzyme was purchased from Sigma Aldrich®. Aliquots (10 µl) of enzyme (3 U) were prepared and activated with 50 µl co-factor solution (6 mg L-epinephrine, 3 mg reduced glutathione, 100 µl of 1 µM hematin and 0.1 M Tris buffer (pH 8)) for 5 minutes on ice. The enzyme/co-factor solution (60 µl) was added to a) 20 µl of aqueous plant extract, b) 2.5 µl EtOH plant extract and 17.5 µl water or c) 2.5 µl indomethacin standard (200 µM) and 17.5 µl water. After an incubation period of five minutes at room temperature 20 µl of [^{14}C] labeled AA (16 Ci/mol, 3 mM), was added to each sample. The reaction mixture was then incubated in a water bath for 10 minutes at 37 °C. The reaction was terminated by the addition of 10 µl 2 N HCl. To the background standard containing 2.5 µl EtOH and 17.5 µl water, 10 µl of 2 N HCl were added to inactivate the enzyme prior to the addition of the enzyme/co-factor solution and the AA. This

was kept on ice until the above assay was completed. Thereafter, 4 µl (0.2 mg/ml) of unlabelled prostaglandin carrier solution (precursors: PGE_2:PGF_2) were added in a 1:1 v/v ratio to all Eppendorf tubes.

The samples from the two abovementioned assays were then subjected to silica column chromatography in an attempt to separate the [^{14}C] labelled PGs synthesised in the Eppendorf tubes during the assay from any unmetabolised [^{14}C] AA. Eluent 1 (hexane:dioxane:acetic acid, 350:50:1 v/v/v) was mixed with silica gel (0.063-0.200 mm particle size, Merck) and set in Pasteur pipettes 3 cm high. Eluent 1 (1 ml) was added to each assayed sample and introduced to separate columns. A further 4 ml of eluent 1 were added to each column. This was responsible for extracting the unmetabolised AA and was discarded. Labelled PGs were then eluted into scintillation vials with 3 ml of eluent 2 (ethyl acetate: methanol, 425:75 v/v). Scintillation fluid (4 ml) was added to the extracted PGs and the radioactivity of the mixture was measured using a scintillation counter (Beckman LS 6000LL scintillation counter).

The effect of the plant extracts, that is percentage inhibition of PG synthesis, was determined by comparing the amounts of radioactivity (disintegrations per minute (DPM)) in a solvent blank to those of the samples. Inhibition was indicated by a decline in PGE_2 formation. This was calculated using the following formula:

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

Three controls were run for this assay: the solvent blank; the background sample where the enzyme was inactivated with HCl before the addition of labeled AA; and the positive control which was the indomethacin sample. The EC_{50} (mg/ml) of extracts considered significantly active were also determined using extracts within a range of 250 – 4.625 µg/ml.

6.2.2. Antioxidant evaluation

Antioxidant capacity of crude orchid extracts was determined using hydrogen atom transfer (HAT) and single electron transfer (SET) reaction-based assays (**HUANG et al., 2005**). Plant extracts (prepared as mentioned in **Section 4.2.2**) were resuspended in methanol at different concentrations (0.065 – 50 mg/ml).

6.2.2.1. 2,2'-Diphenylpicrylhydrazyl (DPPH) free radical scavenging assay

The 2,2'-diphenylpicrylhydrazyl (DPPH) free radical scavenging assay is based on the SET reaction. The DPPH radical is an organic nitrogen radical commercially available and has a deep purple colour (**PRIOR et al., 2005**). The assay is simple, rapid and sensitive; and measures the antioxidant potential of compounds based on their ability to donate hydrogen atoms to the stable DPPH radical. The deep purple colour (absorption maximum at 517 nm) fades during reduction of the DPPH radical by antioxidant molecules. The DPPH free radical quenching generates a 2,2-diphenyl-1-hydrazine product which is colourless. Ultraviolet spectrophotometry (UV absorption at 517 nm) is used to determine antioxidant activity; where high antioxidant activity is measured by a decline in the absorbance of the DPPH solution. It is important to note that this particular assay measures free radical scavenging activity and not pro-oxidant activity.

The free radical scavenging antioxidant capacity of crude orchid extracts were determined using the protocol outlined in **KARIOTI et al. (2004)** with slight modifications. The DPPH radical used in this assay is a substrate that can be reduced to DPPH-H by antioxidants that have the ability to donate a hydrogen atom to the DPPH radical. Methanolic plant samples were prepared at different concentrations (0.065 – 50 mg/ml). Each sample (15 µl) was diluted with 735 µl of methanol in a glass test tube to which 750 µl of a methanolic DPPH solution (0.1 mM) (**SHARMA** and **BHAT, 2009**), prepared under green light conditions, was added. The effective concentration of DPPH in the assay was 50 µM. A separate set of samples, referred to as correction factor solutions, were prepared without the methanolic DPPH solution (DPPH solution was substituted with methanol). Two antioxidants, ascorbic acid and BHT, were

prepared as the positive controls (5 – 80 μM). Methanol served as the negative control. All reaction vessels were incubated for 30 minutes at room temperature in the dark. The absorbance of each sample was measured spectrophotometrically at 517 nm (Varian Cary 50, Australia). The percentage free radical scavenging activity (RSA) of crude plant extracts was calculated according to the formula:

$$RSA (\%) = \left[1 - \left(\frac{A_E - A_{blank\ solution}}{A_D} \right) \right] \times 100$$

Where, A_E is expressed as the absorbance value of plant extracts and standard antioxidants prepared without the DPPH solution subtracted from the absorbance value of corresponding samples prepared with DPPH solution and; A_D is the absorbance value of the negative control.
The concentration of extract required to scavenge 50% of the DPPH radical (EC_{50}) for each sample was determined from a logarithmic non-linear regression curve using GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA). A one-way analysis of variance (ANOVA) and Duncan Multiple Range Test of mean±standard error of mean (SEM) was used to determine differences and significant differences among means; using SPSS software version 10 (SPSS Inc., Chicago, USA). $P = 0.05$ indicated significantly different samples.

6.2.2.2. β-Carotene/Linoleic acid assay

The β-carotene-linoleic acid (linoleate) assay is based on the HAT reaction. Lipid peroxidation is the oxidative degradation of lipids. It is a well known mechanism where unsaturated fatty acids (linoleic acid, linolenic acid, AA) are acted upon by ROS such as $O^{-}_{2}{}^{\cdot}$, singlet oxygen and a $HO^{\cdot}$ radical. Reactive oxygen species remove a hydrogen atom off the methylene side-chain of unsaturated fatty acids to form free radicals. Heat induced oxidation of the β-carotene-linoleic emulsion was used as an antioxidant assay. In the presence of ROS and oxygen (O_2) linoleic acid is oxidised and forms a peroxyl free radical. Beta carotene reacts with the peroxyl radical, loses a hydrogen atom to the free radical and produces stable β-carotene epoxides (**KENNEDY**

and **LEIBLER, 1991**). Due to the lost hydrogen atom, β-carotene molecules lose their characteristic orange colour. Antioxidants stop the breakdown of β-carotene by neutralising the effect of free radicals. The β-carotene-linoleic bioassay explores the antioxidant capacity of a compound by testing its ability to donate with the resulting peroxyl radical.

The capacity of crude methanolic orchid extracts to prevent or reduce the coupled oxidation of β-carotene and linoleic acid in an emulsified aqueous system (**PAJERO et al., 2002**) was determined using the protocol outlined by **AMAROWICZ et al. (2004)** with modifications. The entire assay was carried out under green light conditions as β-carotene is light sensitive. β-carotene was prepared in a dark 500 ml Schott bottle by dissolving 10 mg in 10 ml of chloroform to give a final concentration of 0.1 g/ml. Excess chloroform was evapourated under vacuum, leaving a thin film of β-carotene, to which 200 µl of linoleic acid and 2 ml of polyoxyethylene sorbitan monolaurate (Tween 20) were added. The total volume was made up to 500 ml with aerated distilled water with the final concentration of β-carotene in the assay being 20 µg/ml. The mixture was saturated with oxygen by vigorous agitation and formed an orange emulsion. The β-carotene emulsion (4.8 ml) was dispensed into glass test tubes, followed by 200 µl of either plant samples or positive control: BHT (6.25 mg/ml). The final effective concentration of samples and positive controls was 250 µg/ml in the reaction mixture. The negative control consisted of 50% methanol. Each sample was prepared and tested in triplicate. The absorbance of each reaction mixture was measured (t = 0) at 470 nm using a spectrophotometer (Varian Cary 50, Australia). Reaction vessels were thereafter incubated for 2 h at 50 °C in the dark. The absorbance of each sample was measured every 30 minutes during the incubation period at 470 nm. Tween 20 was used to blank the spectrophotometer.

Antioxidant capacities were expressed as average antioxidant activity (%ANT) and oxidation rate ratio (ORR). The rate of β-carotene bleaching was calculated according to first order kinetics using the formula:

$$\text{Rate of β-carotene bleaching} = \left[\text{Ln}\left(\frac{A_{t=0}}{A_{t=t}}\right)\right] \times 1/t \qquad (1)$$

where $A_{t=0}$ is the absorbance of the emulsion at 0 min; and $A_{t=t}$ is the absorbance at time t (30, 60, 90 minutes). The average rate of β-carotene bleaching was calculated based on rates at 30, 60 and 90 minutes. The calculated average rates were used to determine the %ANT of the respective extracts, and expressed as percent inhibition of the rate of β-carotene bleaching in relation to the negative control using the formula:

$$\% \text{ ANT} = \left[\frac{\left(R_{\text{control}} - R_{\text{sample}} \right)}{R_{\text{control}}} \right] \times 100 \qquad (2)$$

where R_{control} and R_{sample} represent the respective average β-carotene bleaching rates for the standard antioxidant and plant extracts, respectively. Antioxidant activity was further expressed as the ORR based on the formula:

$$\text{ORR} = \left(\frac{R_{\text{sample}}}{R_{\text{control}}} \right) \qquad (3)$$

6.2.2.3. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing power assay (FRAP) is also based on a SET reaction. It is a rapid and sensitive redox-linked colorimetric method. Antioxidants serve as reductants of the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form; producing a distinctive blue colour. All antioxidants donating electrons contribute to the total reducing or antioxidant activity.

The protocol by **LIM et al. (2009)** was used to determine the ability of crude plant extracts to reduce the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form. Using a microtitre plate, 30 µl of millipore water was added to each well; followed by 30 µl of methanolic plant extract (6.25 mg/ml), ascorbic acid (0.8 mM) or BHT (0.5 mM) into the respective wells in row (A). Each test sample was serially diluted in a 2-fold dilution. Thereafter, 40 µl of 0.2 M phosphate buffer (pH

7.2) and 40 µl of potassium ferricyanide (1% w/v in potassium phosphate buffer) were added consecutively to each well. Test solutions were incubated for 20 minutes at 50 °C in an incubator. Following the incubation, 40 µl of trichloroacetic acid (10% w/v in millipore water), 150 µl of distilled water, and 30 µl of ferric chloride (0.1% w/v in potassium phosphate buffer) were added to each well. Samples were prepared and tested in triplicate. Samples were incubated for a further 30 minutes at room temperature in the dark. The absorbance was read at 630 nm using a spectrophotometer (Varian Cary 50, Australia); where an increase in absorbance indicated a greater reducing power.

6.2.3. Acetylcholinesterase (AChE) inhibitory microplate assay

The capacity of crude orchid extracts to inhibit AChE enzyme was determined using the protocol described by **ELLMAN et al. (1961)**. Acetylcholinesterase enzyme activity was measured by spectrophotometric observation of the increase in a yellow colour produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. Three buffers (A, B, C) were prepared with Millipore water for use in the assay. Buffer A: 50 nm Tris-HCl, pH 8; Buffer B: 0.5 g (0.1%) bovine serum albumin (BSA) in 500 ml Buffer A; Buffer C: 2.92 g NaCl and 2.03 g $MgCl_2.6H_2O$ in 500 ml Buffer A. The substrate acetylthiocholine iodide (ATCI), 5,5'-dithiobio-(2-nitrobenzoic acid) (DTNB) or Ellman's reagent, enzyme AChE and galanthamine were purchased from Sigma Aldrich®.

Millipore water (25 µl) was dispensed into all wells of a 96-well microtitre plate; except wells H11 and H12. Samples (25 µl) prepared at a concentration of 10 mg/ml and the solvent blank methanol were added in triplicate to the bottom row (H) and serially diluted in a 2-fold dilution up the plate till row B. The effective concentration of samples in the assay was 0.0313, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml. Fifty microlitres of positive control galanthamine (20 µM) was introduced to wells H11 and H12 and serially diluted in a 2-fold dilution up the plate till row B. This was followed by 25 µl of substrate ATCI (15 mM), 125 µl of DTNB Ellman's reagent prepared at a concentration of 3 mM in Buffer C) and 50 µl of Buffer B in sequence to all wells. Microtitre plates were kept on ice. The absorbance of the reaction mixture was read at 405 nm

every 45 s using an ELISA microplate reader (Opsys MR™ microplate reader, Dynex Technologies Inc.). Each plate was read three times to obtain a stable background or baseline value, after which, 25 µl of AChE enzyme (0.2 U/ml in Buffer A) was added to each well. The absorbance was read once again at 405 nm at 45 s intervals, five times.

The percentage inhibition of the enzyme AChE by each sample was calculated using the formula:

$$\text{Inhibition (\%)} = \left[1 - \left(\frac{\text{Reaction rate}_{\text{sample}}}{\text{Reaction rate}_{\text{blank}}}\right)\right] \times 100$$

The ANOVA and Duncan Multiple Range Test of mean±SEM was used to determine differences and significant differences among means; using SPSS software version 10 (SPSS Inc., Chicago, USA). $P = 0.05$ indicated significantly different samples. The EC_{50} value for each sample was determined using a logarithmic non-linear regression GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA).

6.2.4. Phenolic compound analysis

Plant phenolic compounds were extracted according to the protocol outlined in **MAKKAR (1999)**. Phenolic compounds were extracted in 50% methanol in a sonicator for 30 minutes in cold water. The mixture was filtered under vacuum using Whatman No.1 filter paper.

6.2.4.1. Folin-Ciocalteu assay for total phenolics

Total phenolic content was determined using the protocol outlined in **MAKKAR (1999)**, with slight modifications. Methanolic plant extracts (50 µl) were transferred to glass test tubes and made up to 1 ml by adding 950 µl distilled water. Thereafter, 500 µl of Folin C reagent (1 N)

(diluted with distilled water and stored in the dark) and 2.5 ml of 2% sodium carbonate were added consecutively. A similar procedure was followed for the negative control (50% methanol) and positive control (standard gallic acid solution (0.1 mg/ml)). Test mixtures were incubated for 40 minutes at room temperature. The absorbance of each sample was read at 725 nm using a UV spectrophotometer (Varian Cary 50, Australia). Total phenolic content was expressed as GAE per gram of extract. The ANOVA and Duncan Multiple Range Test of mean±SEM were used to determine differences and significant differences among means; using SPSS software version 10 (SPSS Inc., Chicago, USA). $P = 0.05$ indicated significantly different samples. The EC_{50} value for each sample was determined using a logarithmic non-linear regression GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA).

6.2.4.2. Butanol-HCl assay for condensed tannins

Condensed tannin content was determined using the protocol outlined by **PORTER et al. (1986)**. Methanolic plant extracts (500 µl) were dispensed into glass test tubes set into a rack. This was followed by 3 ml of butanol-HCl (95:5 v/v) reagent and 100 µl ferric reagent (2 g of ferric ammonium sulphate dissolved in 2 N HCl (16.6 ml concentrated HCl made up to 100 ml with distilled water)). Test solutions were mixed by vortexing (Chiltern) and heated for 60 minutes in a water bath set at 100 °C. For the negative control a similar procedure was followed using each plant extract, however these test solutions were not heated. After a period of 60 minutes the absorbance of each sample and negative control was read at 550 nm using a UV spectrophotometer.

Condensed tannin content was recorded as leucocyanidin equivalents according to the formula:

$$\frac{[A_{550nm} \text{ x } 78.26 \text{ x dilution factor}]}{[\% \text{ dry matter}]}$$

The ANOVA and Duncan Multiple Range Test of mean±SEM were used to determine differences and significant differences among means; using SPSS software version 10 (SPSS Inc., Chicago, USA). $P = 0.05$ indicated significantly different samples. The EC_{50} value for

each sample was determined using a logarithmic non-linear regression GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA).

6.2.4.3. Rhodanine assay for gallotannins

Total gallotannin content was determined according to the protocol outlined in **MAKKAR (1999)**. Methanolic plant extracts (50 µl) were dispensed into glass test tubes and made up to 1 ml with distilled water. Thereafter, 100 µl of 0.4 N sulphuric acid and 600 µl of 0.667% rhodanine solution (w/v in methanol) were added consecutively to each sample. Samples were incubated for 5 minutes at room temperature, after which, 200 µl of 0.5 N potassium hydroxide was added. Samples were incubated at room temperature for a further 2.5 minutes, after which 4 ml of distilled water was added to each sample. This was followed by a final incubation period of 15 minutes at room temperature. Different concentrations of gallic acid (0.1, 0.05, 0.025, 0.0125 mg/ml) were prepared and served as the positive controls, while a reaction mixture containing 50% methanol served as the negative control. Test solutions were vortexed briefly and the absorbance of each test solution was read at 520 nm using a UV spectrophotometer. Gallotannin content was expressed as GAE per gram of extract. The ANOVA and Duncan Multiple Range Test of mean±SEM were used to determine differences and significant differences among means; using SPSS software version 10 (SPSS Inc., Chicago, USA). $P = 0.05$ indicated significantly different samples. The EC_{50} value for each sample was determined using a logarithmic non-linear regression GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA).

6.2.4.4. Vanillin assay for flavonoids

Total flavonoid content was determined according to the protocol outlined in **MAKKAR (1999)**. Methanolic plant extracts (50 µl) were dispensed into glass test tubes and made up to 500 µl with 450 µl glacial acetic acid. Thereafter, 2.5 ml 8% HCl (v/v in glacial acetic acid) were added to each sample, followed by 2.5 ml vanillin reagent (1% vanillin in glacial acetic acid). After an incubation period of 20 minutes at 30 °C the absorbance of each sample was read at 500 nm using a UV spectrophotometer. A pink colouration of reaction mixtures indicated the presence

of flavonoids. Different concentrations of the standard catechin (4, 2, 1, 0.5 mg/ml in glacial acetic acid) served as the positive control, while a reaction mixture containing 50% methanol was used as the negative control. Flavonoid content was expressed as catechin equivalents per gram of extract.

6.3. RESULTS

6.3.1. Anti-inflammatory activity

Table 6.1. (**Chapter 6**) presents the anti-inflammatory activity of different crude extracts of seven orchid species. Anti-inflammatory activity above 70% was considered significant (**TAYLOR** and **VAN STADEN, 2001**). Out of a total of 53 evaluated extracts, 21 and 13 extracts exhibited significant anti-inflammatory activity in the COX-1 and COX-2 assays respectively. All aqueous extracts, except that of *A. africana* roots and *B. scaberulum* pseudobulbs, showed poor or no COX-1 and COX-2 inhibition. The DCM tuber extract of *E. hereroensis* was the only extract to significantly inhibit both COX enzymes, 100.02±0.11% and 87.97±8.38% respectively. Organic extracts of *A. africana* were selectively effective against COX-1. Similarly, certain organic extracts of *E. petersii* exhibited COX-1 selective inhibitory activity. *Polystachya pubescens* pseudobulb EtOH and *T. tridentata* organic root extracts were selectively active against COX-1. *Tridactyle tridentata* aqueous leaf extract and all *B. scaberulum* organic root extracts exhibited COX-2 selective inhibitory activity.

Table 6.1.: The percentage inhibition of cyclooxygenase (COX) – 1 and 2 enzymes by crude organic and aqueous extracts of seven indigenous South African orchid species at 250 µg/ml

Species	Plant part	Extraction solvent	Percent inhibition (%)	
			COX-1	COX-2
Ansellia africana	Leaves	PE	ND	66.03±13.64
		DCM	ND	57.48±20.80
		Ethanol	**104.11±1.62**	0.00±0.00
		Water	30.66±9.75	19.51±4.73
	Stems	PE	ND	**83.38±3.16**
		DCM	**106.11±1.29**	78.52±0.97
		Ethanol	43.99±3.46	0.00±0.00
		Water	7.91±6.05	0.00±0.00
	Roots	PE	ND	16.08±11.03
		DCM	**99.36±1.35**	17.21±1.50
		Ethanol	**100.15±0.36**	0.00±0.00
		Water	71.15±0.37	51.56±3.78
Bulbophyllum scaberulum	Leaves	Water	ND	32.81±2.14
	Pseudobulbs	Water	ND	79.86.±10.70
	Roots	DCM	17.73±0.39	**100.06±0.01**
		Ethanol	18.12±3.08	**93.31±2.33**
		Water	5.02±11.53	58.09±3.25
Cyrtorchis arcuata	Leaves	Ethanol	89.19±0.18	76.75±11.02
		Water	44.80±8.04	ND
	Roots	Ethanol	94.05±5.67	72.26±3.35
		Water	54.02±0.95	ND
Eulophia hereroensis	Tubers	PE	ND	**98.39±6.04**
		DCM	**100.02±0.11**	**87.97±8.38**
		Ethanol	0.00±0.00	5.89±3.32
		Water	18.70±3.05	0.00±0.00
	Roots	DCM	84.78±3.19	73.54±2.27
		Ethanol	60.82±0.89	54.19±0.00
		Water	0.00±0.00	0.00±0.00
Eulophia petersii	Leaves	PE	**109.88±1.48**	27.30±9.78
		DCM	ND	22.16±13.85
		Ethanol	74.71±9.98	74.27±10.14
		Water	0.00±0.00	0.00±0.00
	Stems	PE	**107.22±0.94**	**32.89±6.13**
		DCM	**106.35±4.39**	56.24±3.93
		Ethanol	4.96±13.82	18.03±0.62
		Water	37.60±12.30	39.85±1.97
	Pseudobulbs	PE	**96.94±3.22**	10.71±0.00
		DCM	**106.49±2.94**	64.69±8.03

Species	Plant part	Extraction solvent	Percent inhibition (%)	
			COX-1	COX-2
		Ethanol	89.23±9.23	17.43±11.89
		Water	34.80±3.92	0.00±0.00
	Roots	PE	**100.83±0.93**	77.29±3.02
		DCM	**109.65±0.00**	84.17±4.83
		Ethanol	**99.72±2.43**	28.57±12.33
		Water	10.16±12.50	0.00±0.00
Polystachya pubescens	Pseudobulbs	DCM	ND	24.91±0.86
		Ethanol	55.26±2.59	0.00±0.00
		Water	0.00±0.00	15.05±8.06
	Roots	Ethanol	30.16±2.67	25.51±7.43
Tridactyle tridentata	Leaves	Water	4.56±1.02	33.06±6.20
	Roots	DCM	**100.18±0.25**	65.07±1.29
		Ethanol	89.64±1.16	46.27±21.05
		Water	26.24±24.52	14.63±2.81

Values are the mean (±) standard deviation (n = 3). ND – not determined. Anti-inflammatory activity below 20% was considered insignificant, 20-40% low, 40-70% moderate and above 70% was considered significant (**TAYLOR** and **VAN STADEN, 2001**); values in bold indicate those extracts where an EC_{50} was determined.

Table 6.2. (**Chapter 6**) presents the concentration of crude orchid extracts at which 50% inhibition of COX-1 and COX-2 is achieved (EC_{50}). Based on the anti-inflammatory activity presented in Table 6.1, the EC_{50} of 14 crude extracts were determined in the COX-1 assay; and six crude extracts were determined in the COX-2 assay. Overall, the DCM root extract of *A. africana* was found to be the most potent extract (0.25±0.10 mg/ml). This extract was the most potent extract in the COX-1 assay, while the 80% EtOH root extract of *B. scaberulum* was the most potent in the COX-2 assay (0.44±0.32 mg/ml).

Table 6.2.: Estimated EC_{50} (mg/ml) for cyclooxygenase (COX) – 1 and 2 inhibition of crude organic extracts of five South African orchid species.

Species	Plant part	Extraction solvent	EC_{50} (mg/ml)	
			COX-1	COX-2
Ansellia africana	Leaves	Ethanol	1.64 ± 0.25^{cd}	ND
	Stems	PE	ND	1.50 ± 0.15^{a}
		DCM	1.77 ± 0.09^{d}	ND
	Roots	DCM	$\mathbf{0.25 \pm 0.10^{a}}$	ND
		Ethanol	0.42 ± 0.09^{ab}	ND
Bulbophyllum scaberulum	Roots	DCM	ND	1.43 ± 0.86^{a}
		Ethanol	ND	$\mathbf{0.44 \pm 0.32^{a}}$
Eulophia hereroensis	Tubers	PE	ND	1.12 ± 0.33^{a}
		DCM	0.87 ± 0.28^{abc}	1.17 ± 0.15^{a}
Eulophia petersii	Leaves	PE	1.32 ± 0.18^{cd}	ND
	Stems	PE	2.79 ± 0.04^{e}	7.91 ± 1.11^{b}
		DCM	1.49 ± 0.05^{cd}	ND
	Pseudobulbs	PE	0.99 ± 0.51^{abcd}	ND
		DCM	0.87 ± 0.12^{abc}	ND
	Roots	PE	1.17 ± 0.06^{bcd}	ND
		DCM	1.41 ± 0.64^{cd}	ND
		Ethanol	0.99 ± 0.38^{abcd}	ND
Tridactyle tridentata	Roots	DCM	1.47 ± 0.89^{cd}	ND

Values are the mean (±) standard deviation ($n = 3$). ND – not determined. Different letters represent significant differences between EC_{50} means for each enzyme (same column), $P = 0.05$. Values in bold indicate the most potent anti-inflammatory extracts.

6.3.2. Antioxidant activity

6.3.2.1. DPPH radical scavenging assay

Table 6.3. (**Chapter 6**) presents the percent free radical scavenging activity of crude extracts of seven orchid species as measured in the DPPH radical scavenging assay. While the principle of the assay does not stipulate the meaning of negative percentage radical scavenging activity (as calculated in **Section 6.2.2.1**), the use of a wide range of concentrations ensured the determination of an EC_{50} value. There was a dose-dependent change in radical scavenging activities of crude extracts from which EC_{50} values were determined; and are presented in **Table 6.3.** (**Chapter 6**). In terms of EC_{50}, the methanolic root extracts of all species, except that of *E. petersii*, had consistently more effective radical scavenging activity than that of other plant parts within each species. The methanolic pseudobulb extract of *E. petersii*, was the most potent extract (1.32±0.86 mg/ml).

Table 6.3.: The DPPH radical scavenging capacity of crude methanol extracts of seven indigenous South African orchid species

Species	Plant part	Extract concentration (mg/ml)				EC_{50} (mg/ml)
		0.78	**1.56**	**3.125**	**6.25**	
Ansellia africana	Leaves	-61.36	-43.49	-23.01	25.68	$11.63±1.37^{de}$
	Stems	-46.90	26.74	3.75	26.08	$8.13±0.83^{bcde}$
	Roots	0.65	17.70	64.54	96.97	$2.54±0.35^{ab}$
Bulbophyllum scaberulum	Leaves	-50.06	-38.71	-37.39	-15.11	$26.25±1.14^{f}$
	Pseudobulbs	-36.86	-1.35	26.91	68.66	$4.86±0.62^{abc}$
	Roots	-24.34	15.70	50.04	75.52	$3.39±0.0595^{ab}$
Cyrtorchis arcuata	Leaves	-40.42	-5.10	18.47	56.97	$6.18±0.12^{abcd}$
	Roots	-34.63	-13.99	26.44	56.94	$5.30±0.10^{abc}$
Eulophia hereroensis	Tubers	-56.91	-61.04	-44.03	-30.23	$44.45±4.05^{g}$
	Roots	-55.51	-51.37	-41.25	-36.50	ND
Eulophia petersii	Leaves	-60.79	-27.76	18.43	52.01	$5.96±0.1^{abcd}$
	Stems	-59.03	-40.70	-20.72	12.75	$12.71±0.66^{e}$
	Pseudobulbs	-15.38	53.73	68.54	83.80	$\mathbf{1.32±0.86}^{a}$
	Roots	-38.53	-26.65	-11.68	13.45	$10.90±0.11^{cde}$
Polystachya pubescens	Pseudobulbs	-28.15	-34.06	-10.91	19.91	$10.15±0.28^{cde}$
	Roots	-33.09	-34.50	23.74	66.95	$4.99±0.0295^{abc}$
Tridactyle tridentata	Leaves	-53.18	-49.58	-44.71	-25.78	$254.85±6.15^{h}$
	Roots	-17.70	4.07	31.77	73.77	$4.51±0.25^{abc}$

Values; EC_{50} values are the mean (±) standard error (n = 2), values in bold indicate those extracts with most significant antioxidant activity. In DPPH assay EC_{50} of ascorbic acid (0.55 µg/ml) and BHT (5.20 µg/ml). Different letters represent significant differences between EC_{50} means, P = 0.05. ND – not detected.

6.3.2.2. β-Carotene/Linoleic acid assay

Table 6.4 (**Chapter 6**) presents the antioxidant capacity of crude extracts of seven orchid species as measured in the β-carotene-linoleic acid assay. Beta carotene bleaching occurs because of oxidation of linoleic acid; this coupled oxidation was observed by a decrease in absorbance over time. Based on ORR, the leaf extract of *T. tridentata* and the root extracts of *C. arcuata* and *E. hereroensis* exhibited the best antioxidant effects (0.02, 0.023 and -0.15 respectively). Similarly, the %ANT of these samples was greater than that of BHT (95.88±6.90%) and all other samples. Eight out of a total of 18 samples exhibited a greater capacity to prevent β-carotene oxidation in the assay when compared to BHT.

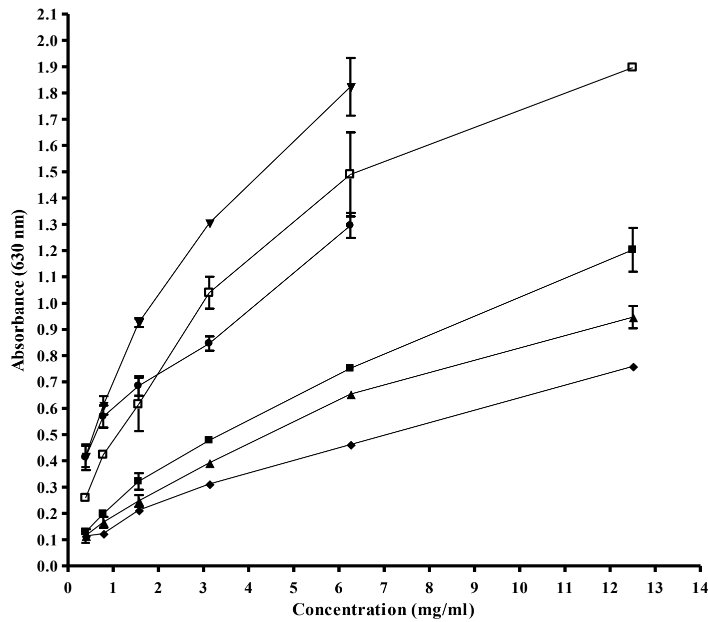
Table 6.4.: The effect of crude methanol extracts of seven indigenous South African orchid species on rate of β-carotene bleaching

Species	Plant part	ORR	% ANT
Ansellia africana	Leaves	0.42±0.31[a]	87.87±2.74[bc]
	Stems	0.31±0.01[a]	90.61±5.25[bc]
	Roots	ND	ND
Bulbophyllum scaberulum	Leaves	0.20±0.61[a]	106.19±55.61[cd]
	Pseudobulbs	0.1±0.001[a]	104.85±5.23[cd]
	Roots	1.22±1.37[b]	106.02±1.33[cd]
Cyrtorchis arcuata	Leaves	0.35±0.49[a]	29.45±4.22[a]
	Roots	**0.023**±0.04[a]	**120.63±10.48**[cd]
Eulophia hereroensis	Tubers	0.63±0.44[ab]	63.96±22.98[b]
	Roots	**-0.15±0.10**[a]	**122.64±8.41**[cd]
Eulophia petersii	Leaves	0.31±0.18[a]	84.96±3.10[bc]
	Stems	0.28±0.02[a]	92.18±2.10[bc]
	Pseudobulbs	0.22±0.07[a]	98.62±0.50[bcd]
	Roots	0.19±0.10[a]	92.42±0.001[bc]
Polystachya pubescens	Pseudobulbs	0.28±0.14[a]	92.75±2.78[bc]
	Roots	0.23±0.09[a]	90.12±2.89[bc]
Tridactyle tridentata	Leaves	**0.02±0.18**[a]	**133.79±25.74**[d]
	Roots	0.10±0.01[a]	96.66±1.00[bcd]

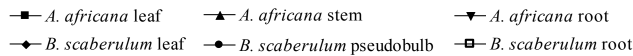
Values are the mean (±) standard error (n = 2); Values in bold indicate those extracts with most significant antioxidant activity. ORR = Average β-carotene bleaching rates as compared to the negative control (ORR for BHT = 0.18±0.07) and %ANT = average antioxidant activity represented as percent inhibition; %ANT for BHT (95.88±6.90%). Different letters in each column represent significant differences between means, P = 0.05.

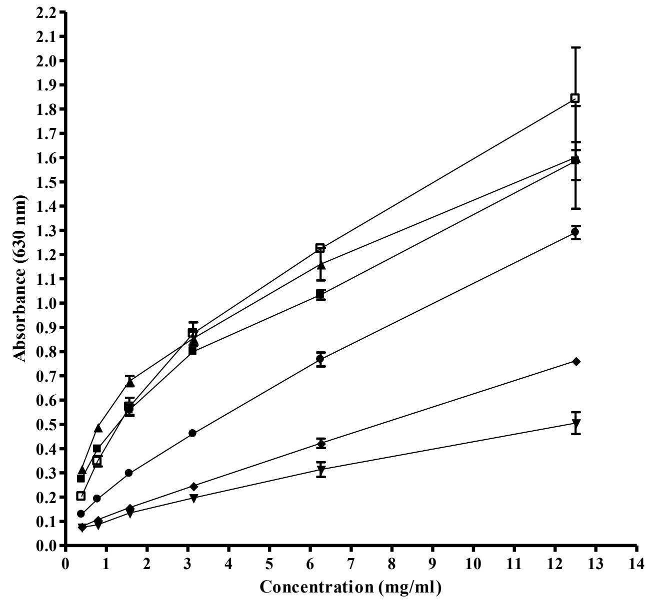
6.3.2.3. Ferric reducing power assay

Figure 6.2 presents the antioxidant activity of crude extracts of seven orchid species as measured in the ferric reducing power assay. All crude orchid extracts tested demonstrated a general dose-dependent response; where an increase in extract concentration resulted in an increased reducing power activity. The reducing power of ascorbic acid (0.08 mM) and BHT (0.05 mM), as measured as absorbance, was 1.12±0.12 and 0.73±0.08 respectively. At 6.25 mg/ml, methanolic *A. africana* root and *E. petersii* pseudobulb extracts were the most effective in reducing power activity.



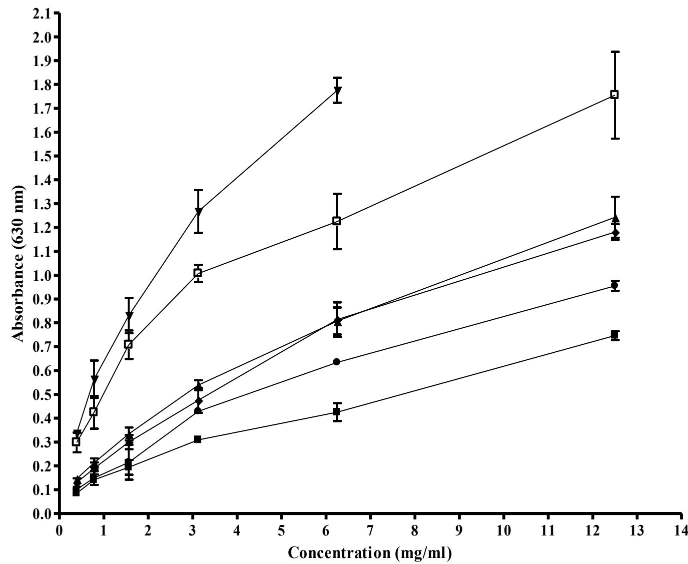
A





B

C. arcuata leaf
C. arcuata root
E. hereroensis tuber
E. hereroensis root
P. pubescens pseudobulb
P. pubescens root



C

E. petersii leaf
E. petersii stem
E. petersii pseudobulb
E. petersii root
T. tridentata leaf
T. tridentata root

Figure 6.2.: Ferric reducing capacity of methanolic extracts from seven indigenous South African orchid species.

6.3.3. Acetylcholinesterase inhibitory activity

Table 6.5. (**Chapter 6**) presents the AChE inhibitory capacity of different crude extracts of seven orchid species. Generally it was the root extracts which exhibited greater AChE inhibitory activity. The lowest EC_{50} value was observed in *B. scaberulum* DCM root extract (0.02±0.00 mg/ml). The most active extract of *A. africana* was the ethanolic root extract. For *C. arcuata* the aqueous root extract proved to be the most potent. The most effective extracts of *E. hereroensis* and *E. petersii* were the EtOH root and DCM stem extracts respectively. The DCM root extract was the most potent extract for *P. pubescens*, while the most active extract of *T. tridentata* was the EtOH root extract.

Table 6.5.: Acetylcholinesterase inhibitory activity (EC_{50}) of crude extracts of seven indigenous South African orchid species

Species	Plant part	Extraction solvent	EC_{50} (mg/ml)
Ansellia africana	Leaves	PE	2.19 ± 0.83^{jk}
		DCM	0.44 ± 0.02^{abc}
		Ethanol	0.47 ± 0.22^{abcd}
	Stems	PE	1.86 ± 0.12^{ij}
		DCM	0.50 ± 0.10^{abcde}
		Ethanol	0.95 ± 0.04^{bcdefgh}
	Roots	PE	0.33 ± 0.03^{ab}
		DCM	0.34 ± 0.14^{ab}
		Ethanol	0.24 ± 0.03^{ab}
Bulbophyllum scaberulum	Roots	DCM	$\mathbf{0.02\pm0.00}^{a}$
		Ethanol	0.26 ± 0.007^{ab}
Cyrtorchis arcuata	Leaves	DCM	1.89 ± 0.19^{jk}
		Ethanol	0.55 ± 0.03^{abcde}
		Water	0.72 ± 0.07^{abcdef}
	Roots	PE	2.21 ± 0.16^{jk}
		DCM	1.77 ± 0.41^{ij}
		Ethanol	0.84 ± 0.07^{bcdefgh}
		Water	0.47 ± 0.04^{abcd}
Eulophia hereroensis	Tubers	PE	1.20 ± 0.24^{cdefghi}
		DCM	0.23 ± 0.16^{ab}
		Ethanol	0.68 ± 0.06^{abcdef}
	Roots	DCM	0.29 ± 0.05^{ab}
		Ethanol	0.23 ± 0.07^{ab}
Eulophia petersii	Leaves	PE	2.72 ± 0.01^{k}

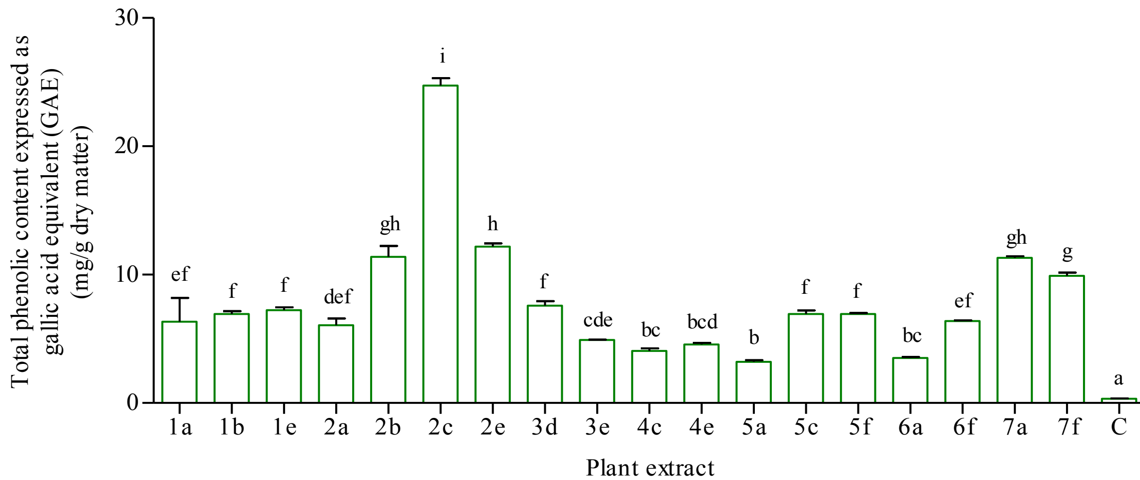
Species	Plant part	Extraction solvent	EC_{50} (mg/ml)
		DCM	$0.56±0.004^{abcde}$
		Ethanol	$1.57±0.17^{hij}$
	Stems	PE	$0.56±0.16^{abcde}$
		DCM	$0.39±0.04^{ab}$
		Ethanol	$1.20±0.19^{defghi}$
	Pseudobulbs	PE	$1.32±0.07^{fghi}$
		DCM	$0.51±0.14^{abcde}$
		Ethanol	$1.37±0.23^{fghi}$
	Roots	PE	$2.22±0.62^{jk}$
		DCM	$0.51±0.05^{abcde}$
		Ethanol	$1.47±0.12^{ghi}$
Polystachya pubescens	Pseudobulbs	PE	$0.48±0.21^{abcd}$
		DCM	$0.95±0.04^{bcdefgh}$
		Ethanol	$1.24±0.23^{efghi}$
	Roots	DCM	$0.27±0.02^{ab}$
		Ethanol	$0.78±0.05^{abcdefg}$
Tridactyle tridentata	Roots	DCM	$0.30±0.03^{ab}$
		Ethanol	$0.25±0.07^{ab}$
		Water	$0.46±0.01^{abcd}$

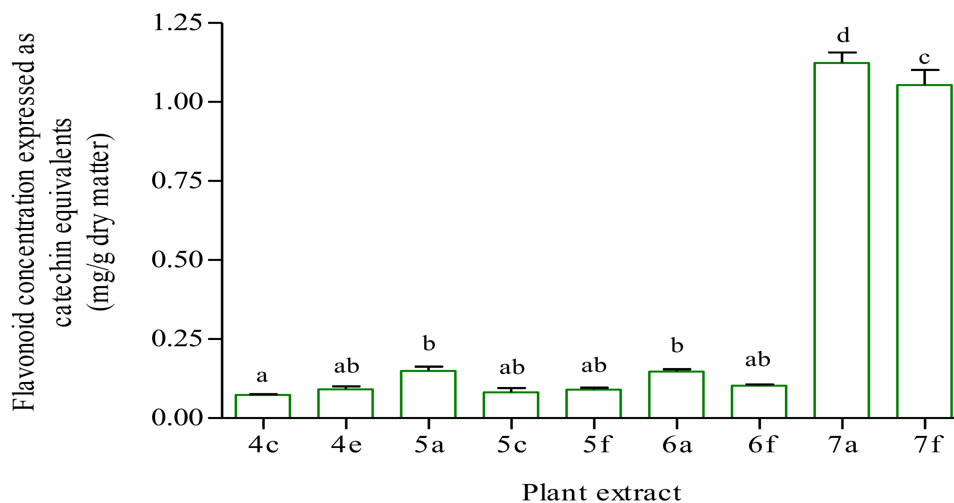
Values are the mean (±) standard deviation (n = 3); Different letters represent significant differences between EC_{50} means, $P = 0.05$, EC_{50} values in bold indicate those extracts with most significant AChE inhibitory activity. EC_{50} value of galanthamine (0.44±0.10 μM).

6.3.4. Phytochemical evaluation

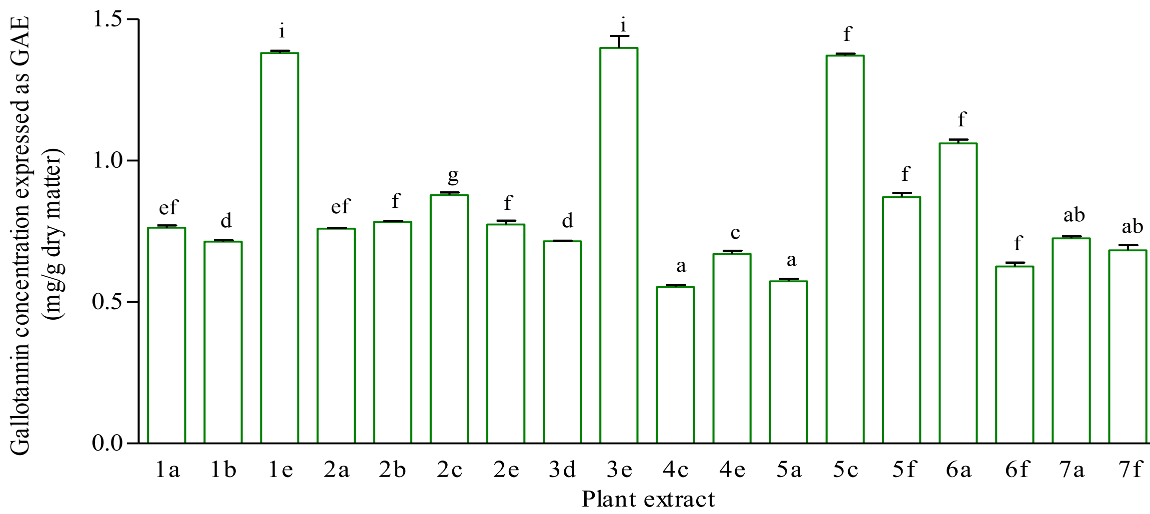
Figure 6.3 presents the total phenolic (A), flavonoid (B), gallotannin (C) and condensed tannin (D) content of different methanol extracts of seven orchid species. Flavonoids were not detected in *A. africana*, *E. hereroensis* and *E. petersii*. *Eulophia petersii* pseudobulb extract contained a significantly higher content of total phenolics; while the leaf and stem/root extracts of *C. arcuata* contained significantly higher levels of flavonoids. *Ansellia africana* and *E. hereroensis* root extracts had significantly high levels of gallotannin content. *Eulophia hereroensis* root, *B. scaberulum* stem/root and *C. arcuata* leaf extracts contained the highest levels of condensed tannins compared to other extracts.

A

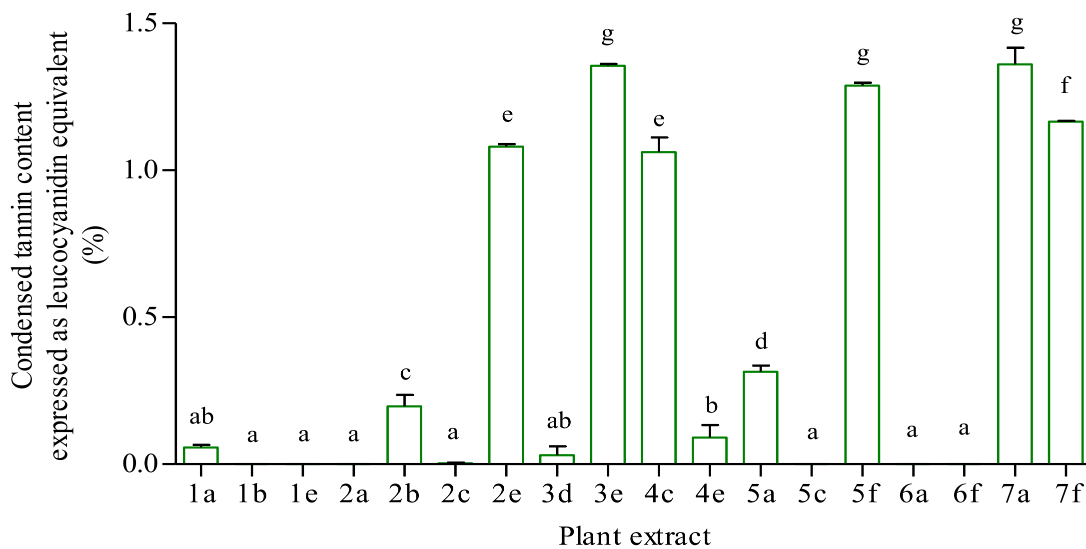




B



C



D

Figure 6.3.: Phytochemical evaluation of seven indigenous orchid species bought at herbal markets in KwaZulu-Natal total phenolic content (A), flavonoid content (B), gallotannin content (C), and condensed tannin content (D). For each graph, means with different letters are significantly different at $P = 0.05$.

[1]*A. africana*, [2]*E. petersii*, [3]*E. hereroensis*, [4]*P. pubescens*, [5]*B. scaberulum*, [6]*T. tridentata*, [7]*C. arcuata*

[a]Leaves, [b]Stems, [c]Pseudobulbs, [d]Tubers, [e]Roots, [f]Stem and root, [C]Control

6.4. DISCUSSION

Inflammatory responses such as pain and fever are commonly associated with severe microbial infections or with an assault on the immune system. Approximately five species of South African orchids are used to treat inflammatory conditions. *Polystachya ottoniana* to soothe pain experienced in teething babies and to treat diarrhoea. *Ansellia africana* is administered as an antimicrobial while *Eulophia* species such as *E. cucullata* and *E. ovalis*, are used primarily to relieve pain.

Anti-inflammatory activity above 70% was considered significant (**TAYLOR** and **VAN STADEN, 2001**). Based on this range, 40 and 25% of the extracts tested exhibited significant anti-inflammatory activity in the COX-1 and COX-2 assays respectively. On the assumption that users of traditional medicine would consume an aqueous concoction of plant material, aqueous extracts were expected to exhibit significant biological activity. Instead, all aqueous extracts, except that of *A. africana* roots and *B. scaberulum* pseudobulbs, showed poor or no COX-1 and COX-2 inhibition (**Table 6.1. Chapter 6**). Higher levels of activity were achieved with the organic extracts (PE, DCM and EtOH) (**Table 6.1. Chapter 6**). Alternatively, traditional healers could be using hot water extracts (as opposed to our cold water extracts); a procedure that could possibly extract the less water-soluble compounds when using water as the extractant.

Plant compounds such as flavonoids, naphthoquinones, alkylamides and phenolic phenyl-propane derivatives represent the usual compounds found in certain natural products that are responsible for COX inhibition (**BOHLIN, 1995**). Certain flavonoids are also known to be involved in the breakdown of glycosides (pigment-preservation compounds) (**TOVAR-GIJÓN et al., 2006**). The flavonoid content of *B. scaberulum* and *T. tridentata* may explain the potent activity observed in the anti-inflammatory and AChE inhibitory assays. The flavonoid content of other orchid species investigated, presented in **Figure 6.3 B** did not necessarily correspond to the biological activity of those species. The medicinal value of flavonoids includes anti-inflammatory, antifungal, antioxidant and wound healing. The wound healing efficacy of *Oncidium flexuosum*, an epiphytic orchid used in Brazilian traditional medicine for inflammation and wounds, was attributed to the presence of flavonoids and tannins (**De GASPI et al., 2011**).

When comparing significant anti-inflammatory activity to phytochemical content it was observed that *A. africana*, and *E. hereroensis* methanol root extracts had significantly higher levels of gallotannin content (**Figure 6.3 C**). *Eulophia hereroensis* root and *B. scaberulum* stem/root extracts contained one of the higher levels of condensed tannins compared to other extracts (**Figure 6.3 D**). All three species shared similar levels of total phenolic content (**Figure 6.3 A**).

The results of the current investigation indicate the presence of active compounds in certain polar and non-polar extracts. Natural plant constituents such as polyphenols, saponins, certain pigments and even fatty acids are often extracted when using non-polar solvents (**LOWER, 1985**). The EtOH extract of *G. elata*, traditionally used to treat inflammatory disorders such as rheumatism, demonstrated potent anti-angiogenic effects in the CAM assay; while the *n*-butanol fraction showed dose-dependent activity at IC_{50} 0.47 μg/egg. *In vivo* vascular permeability, an early sign of inflammatory response, was significantly inhibited; while strong analgesic and anti-inflammatory activity was observed. **AHN et al. (2007)** postulated that such activity was a result of inhibited NO production and COX-2 expression. Compounds such as 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde, isolates of *G. elata*, are thought to be responsible for the anti-inflammatory effects (**AHN et al., 2007**).

Primary metabolites such as carbohydrates are commonly extracted with water (**ZHA et al., 2007**). *Cyrtopodium cardiochilum* hot water pseudobulb extract produced the CC polysaccharide. It was found to significantly increase the phagocytic index when compared to the standard (Zymosan) and exhibited a similar significant anti-inflammatory effect when compared to Indomethacin (10 mg/kg body weight). An increase in vascular permeability is one of the early signs of an inflammatory response. The CCP glucomannan suppressed vascular permeability by approximately 20% of the control (**BARRETO** and **PARENTE, 2006**).

When testing methanolic crude extracts in two different SET reaction-based assays, the *E. petersii* pseudobulb extract, was the most potent radical scavenging extract; and one of two extracts (*A. africana* root**)** that were most efficient in reducing power capacities (**Table 6.3. Chapter 6** and **Figure 6.2**). Alternatively, when using the β-carotene-linoleic acid assay, a HAT reaction-based assay, the ORR revealed the leaf extract of *T. tridentata* and the root extracts of

C. arcuata and *E. hereroensis* to have the best antioxidant effects (**Table 6.4. Chapter 6**). Similarly, the overall %ANT of these three samples to prevent the coupled oxidation of β-carotene and linoleic acid were greater than that of BHT and all other samples. The total phenolic content of *E. hereroensis* pseudobulb extracts may contribute to the antioxidant activity; while the flavonoid content in the leaf and root extracts of *T. tridentata* and *C. arcuata*, respectively, may play a role in the antioxidant activity observed in the HAT reaction-based assay. An alternative mechanism may have been affected due to the presence of the flavonoids.

Antioxidant activity of orchid extracts and their isolates using various antioxidant assays has been previously established. Dendroflorin, moscatilin and nobilone, a 2,7-dihydroxy-4-methoxy-9-fluorenone, were isolated from *D. nobile* 60% EtOH stem extracts (**ZHANG et al., 2007**). The antioxidant activity was determined using DPPH free radical scavenging and ORAC assays. Dendroflorin and moscatilin exhibited significant scavenging activities when compared to Vitamin C (IC_{50} 16.2±0.2 and 14.5±0.3) in the DPPH assay. There was markedly higher peroxyl radical scavenging activity for dendroflorin, nobilone and moscatilin than for Vitamin C in the ORAC assay (0.596±0.003, 0.432±0.005 and 0.625±0.013 μM Trolox equivalent/μM). Of the 70 herbal medicines tested for their active-oxygen scavenging activity *D. plicatile* methanol stem extract was one of those that demonstrated potent activity (72% inhibition) against the superoxide anion radical ($\cdot O_2^-$) (**OHSUGI et al., 1999**). The rhizome of *G. conopsea* found in China and Nepal region, traditionally referred to as Wangla, has been used in Tibetan medicine for coughs, asthma and as a tonic in Chinese medicine. Antioxidant and anti-hepatitis B effects have been established (**PCT INT. APPL. WO 2004, 2004; CAI et al., 2006**).

Dichloromethane extract of the aquatic orchid *H. repens* yielded habenariol (bis-*p*-hydroxybenzyl-2-isobutylmalate). A structurally similar 2- [1-methylpropyl]malate ester was isolated from the rhizomes of the non-aquatic orchid, *G. faberi* (**LI et al., 1993; WILSON et al., 1999**). Habenariol has been characterized as an antifeedant (**WILSON et al., 1999**). Like most phenolic compounds, habenariol has also demonstrated antioxidant properties in the human LDL lipid peroxidation model. When compared to the more effective α-tocopherol, habenariol (25 μM) produced a maximum oxidation rate of 0.0048 with a lag-phase time of 180 minutes and a

concentration dependent inhibition of copper-induced lipid hydroperoxide production (IC_{50} 35 μM) (**JOHNSON et al., 1999**).

Two orchid species currently traded in South African herbal markets yet not mentioned in any South African ethnobotanical literature as being used for medicinal purposes were found to exhibit interesting anti-inflammatory effects. The ethanolic root extract of *B. scaberulum* exhibited the most potent inhibitory effect on COX-2 (**Table 6.2. Chapter 6**). While the DCM tuber extract of *E. hereroensis*, was the only extract to significantly inhibit both COX enzymes (**Table 6.2. Chapter 6**). Interestingly, *B. scaberulum* DCM root extract was also the most effective crude orchid extract to inhibit AChE 18 times greater activity than galanthamine (**Table 6.4. Chapter 6**). As an example of how science can enhance the effectiveness of a natural compound: 4-hydroxybenzaldehyde, an active compound isolated from *G. elata* has exhibited potent antioxidant activity and GABA transaminase inhibitory effects but weak AChE inhibitory activity. Researchers, looking for compounds with more extensive biological activities, produced a new class of active and selective AChE inhibitors by introducing amino acid moieties into the backbone of 4-hydroxybenzaldehyde. The resultant compounds exhibited more potent AChE inhibitory activity. Two compounds 4b (IC_{50} 0.19 μM) and 4i (IC_{50} 0.28 μM) performed better than galanthamine (IC_{50} 0.55 μM); and 4i was selective for AChE (**WEN et al., 2007**). Orchid extracts that performed well in both anti-inflammatory and AChE inhibitory assays may be potential natural plant product targets for inflammatory disorders. Extracts include: *A. africana* EtOH root, *B. scaberulum* DCM root, *E. petersii* DCM stem and *T. tridentata* DCM root extracts. The distinct COX-2 selective inhibition by *B. scaberulum* root extracts and *T. tridentata* aqueous leaf extracts are indications of potential drug targets with alternative mechanisms of action without the side-effects commonly associated with COX-1 inhibition.

The *Bulbophyllum* and *Eulophia* genera are not only traded in South African herbal markets for medicinal uses, they also feature in a list of 21 genera listed in Ayurvedic literature as being used for medicines (**SINGH et al., 2007**). The medicinal usefulness of the *Bulbophyllum* and *Eulophia* genera is supported by the pharmacological and phytochemical assessment of the genus *Vanda* which also features on this list of common medicinal plant sources in Indian traditional medicine (**SINGH et al., 2007**). **SIMMLER et al. (2010)** evaluated the skin photoprotective

effect of *Vanda coerulea* stilbenoids based on the widespread use of orchid extracts in the cosmetic industry. Ultraviolet - induced damage often results in oxidative stress and associated inflammatory processes. Ultraviolet B (UV_B) radiation is known to increase PGE2 production (due to the upregulation of COX-2 expression) and ROS; resulting in skin inflammation. **SIMMLER et al. (2010)** mention natural photoprotective polyphenol compounds such as proanthocyanidins and resveratrol with proven antioxidant and related anti-inflammatory activities. Stem methanolic solutions of isolated stilbenoids from *V. coerulea* performed better than "hydro-alcoholic" solutions in the *in vitro* DPPH/·O radical scavenging and Parameter™ PGE-2 enzyme immunological assays. Imbricatin, a stilbenoid isolated previously from *Bulbophyllum* and other orchid genera, was one of three isolated stilbenoids recommended for use as skin photoprotectants based on antioxidant, anti-inflammatory and immunomodulatory effects (**SIMMLER et al., 2010**). The neuroprotective, antioxidant and anti-inflammatory potential for South African orchid extracts especially for *Bulbophyllum scaberulum* root extracts are therefore great.

The potent anti-inflammatory and antioxidant effect of *E. hereroensis* and *E. petersii* supports the use of species from this genus for inflammatory-related symptoms (**Table 3.1 Chapter 3**) in South African traditional medicine. The overall %ANT of *P. pubescens* pseudobulb and root extracts was greater than 90%, which might validate the use of species from this genus as substitutes to *P. ottoniana*, to treat certain inflammatory disorders. The current investigation quantitatively determined the presence of flavonoids in the pseudobulbs and roots of *P. pubescens*. During the survey of leaf flavonoid content in Orchidaceae, **WILLIAMS (1979)** isolated xanthones, mangiferin and isomangiferin from five species of *Polystachya* and *Maxillaria*. The author also observed that there was no pattern of flavonoid distribution within the family Orchidaceae, and geographical location played a significant role in the presence of flavonoid compounds (**WILLIAMS, 1979**). All four species, that did register some flavonoid content, shared similar distribution ranges; and are all epiphytic species.

The use of *Prosthechea michuacana*, a Mexican medicinal orchid used for diabetes, renal disease and inflammation was validated in the study by **PÉREZ GUTIÉRREZ** and **SOLIS (2009)**. Similarly, the medicinal uses of *C. arcuata* (treatment of diabetes and skin infections) and *T.*

tridentata (treatment of psychological disorders such as madness) may be validated based on their significant anti-inflammatory and antioxidant effects (**Table 6.1, 6.2, 6.4 Chapter 6**) (**BULPITT, 2005**). The anti-inflammatory and antioxidant capacity demonstrated in this investigation may suggest cellular and neuroprotective effects. These two species also share a similar distribution pattern, which may result in the accumulation of similar bioactive compounds and account for the comparable antioxidant effects. The use of the same vernacular name for *C. arcuata*, various *Eulophia* species and *Tridactyle* species, may be explained by their comparable positive anti-inflammatory and antioxidant effects in this investigation.

The COX assay was not designed to detect compounds with pro-inflammatory effects (therefore extracts that produced negative inhibitory values were designated as 0% activity). Similarly, an EC_{50} value was only determined for extracts (10 mg/ml) which exhibited inhibitory effects greater than 95 and 80% in the COX-1 and 2 assays respectively. However, an EC_{50} value was not determined for extracts that lacked a concentration-dependant inhibitory response. When evaluating antioxidant potential of natural products, there is the possibility of discovering prooxidant effects. Common natural antioxidants and food additives have the capacity to exert prooxidant effects by promoting free radical damage. The antioxidant α-tocopherol demonstrated concentration-dependent prooxidant effects *in vitro* (**CILLARD et al., 1980**). A similar concentration-dependent trend was observed for ascorbic acid, where prooxidant and antioxidant effects were observed at low and high concentrations respectively (**ERDMAN** and **KLEIN, 1982**).

Different classes of well established anti-inflammatories have been shown to exhibit varied even limited effects in patients with neuroinflammatory disorders such as Alzheimer's. With prolonged use, other NSAIDs have demonstrated a positive response (**DeKOSKY, 2003**). Such varied responses to anti-inflammatory drugs, is indicative of alternative neuroprotective pathways other than COX-inhibition (**STUCHBURY** and **MÜNCH, 2005**). This observation, and the potential of COX-inhibitors in treatment of Alzheimer's, is further supported by the report by **MONTINE et al. (1999)** where COX activity in persons susceptible to Alzheimer's was comparable to that of control persons; while concentration levels of PGE_2 in cerebrospinal fluid of susceptible patients had increased five times. The current study demonstrated the anti-

inflammatory, antioxidant and AChE inhibitory activities of various orchid crude extracts. Such biological activity further demonstrates the potential of identifying novel treatment options for Alzheimer's. Non-steroidal anti-inflammatory drugs often relieve inflammation and associated pain by inhibiting specific enzymes (**RANG** and **DALE, 1987**). The effectiveness of anti-inflammatory and antioxidant drugs in treating inflammatory and degenerative disorders has been widely documented (**HOWES** and **HOUGHTON, 2003**; **HOUGHTON et al., 2007**).

Generally the results obtained in this study validate the use of certain orchid species in South Africa traditional medicine for pain-related ailments. Other pharmacological conditions linked to inflammation were also investigated to establish other potential activities of the medicinal plant extracts. A more comprehensive assessment of the chemistry of South African orchids would allow one to more confidently assert a relationship between chemical profiles, interaction between different classes of compounds, biological activity and influence of geographical location. The results obtained in this study provide scientific information that could aid in the isolation of potential pharmacologically active compounds with fewer side-effects from some of these medicinal plants in future research.

CHAPTER 7
MUTAGENICITY AND TOXICITY EVALUATION

"***All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy.*" Paracelsus**

7.1. INTRODUCTION

The toxicity of a drug is directly related to the dose of the drug being administered. Toxicological evaluations form an integral part of the drug discovery process, where an effective, yet safe dose is determined. With the advent of pure chemicals as medication, toxic substances were also found to act as a result of metabolic activation within the body. While this is a mechanism adopted by the body to excrete the compound, it produces certain reactive chemical groups that bind to and change the structure of DNA thus causing a mutation. It is important to draw the distinction between a mutagen and a carcinogen. A mutagen causes a change in the sequence or structure of DNA, while a carcinogen promotes the development of cancerous cells (**KIMBALL, 2008b**). The Ames test, a short-term bacterial reverse mutation assay, determines the mutagenic potential of a substance that may not be a carcinogen. The test is used extensively in the food and pharmaceutical industry to check for mutagenic properties (**KIMBALL, 2008b**).

7.1.1. Carcinogens

With a higher incidence of cancer reports in recent years there is a need to search for new and improved chemopreventative drugs (**KUNDU et al., 2005**). A carcinogen is a cancer causing agent that acts by changing the information that the cell receives and often prevents the differentiation into a functional cell (**KIMBALL, 2008b**). Unfortunately, such agents are present in our everyday lives and environment. The sun's UV rays are one source, while substances in our food or even the oxygen we breathe can induce malignant cell formation (**KIMBALL, 2008b**). From a historical perspective, cancer has only recently been linked to the exposure to carcinogenic agents. It was during the 18th century that Sir Percival Potts made the connection. *In vitro* mutagenic activity as well as toxic and carcinogenic activity has been shown in plants used for medicinal purposes (**CARDOSO et al., 2006**).

Selecting plants based on their use in traditional medicine is a good starting point as the majority of currently used clinical drugs are derived from plants known to be used in traditional medicine (**FARNSWORTH, 1984**). However, studies such as those conducted by **FOURIE et al. (1992)** showed that 9% of plants investigated exhibited toxic effects. In addition, **VERSCHAEVE** and **VAN STADEN (2008)**, highlight the lack of toxicological studies on traditional medicinal plants as compared to pharmaceutical compounds. It is generally accepted that the ancient use of these plants renders them safe to use. African traditional medicine has not been captured in pharmacopoeia and there is fear of misinterpretation of indigenous knowledge by the younger generations; where the misadministration of traditional medicines could lead to poisoning and/or severe side effects. There is no pharmacological record of South African medicinal orchids being used as anticancer treatment or having toxic side effects. This lack of information in the literature needs to be addressed, as it creates the perception that plant extracts in these age-old remedies have no harmful effects.

Most pharmacological and toxicological investigations are carried out to determine, respectively, the efficacy and safety of medicinal plants. The relationship between the dose of a compound and its effect on a living organism forms the basis of toxicological studies. **VARANDA et al. (2002)** reported on mutagenic furocoumarins in the root bark of *Brosimum gaudichaudii*, while **RIETJENS et al. (2005)** described certain flavonoids that exhibited mutagenic properties. There have been previous investigations into the mutagenic potential of a number of South African medicinal plants with only a few species exhibiting significant mutagenic properties (**ELGORASHI et al. 2003**). However, mutagenic plants have also benefited the medical system. Compounds such as taxol and vinblastine are known mutagens that are used as anticancer drugs (**GLASS-MARMOR** and **BEITNER, 1999**; **2003**; **SRIVASTAVA et al., 2007**). This beneficial aspect of mutagenic research and the need to assess other medicinal plants for mutagenic properties is highlighted by such investigations.

7.1.2. Mutagenicity evaluation techniques

The Ames *Salmonella*/ microsome mutagenicity (ASMM) assay is a useful assay that detects various chemical substances that cause genetic damage and ultimately gene mutations. It is a short-

term bacterial reverse mutation assay that makes use of some mutant histidine-dependent *Salmonella typhimurium* strains. The use of *S. typhimurium* as a test organism is not ideal as it does not represent the human body system; however it is a rapid *in vitro* model that can be adapted to incorporate eukaryotic cells. When the *Salmonella* mutagenicity test was revised in 1983 (**MARON** and **AMES, 1983**) the tester strains TA98 and TA100 were retained.

A bacterial model is useful as gene mutations are measured by a noticeable/visible change in growth requirements (**MORTELMANS** and **ZEIGER, 2000**). These bacterial strains contain different mutations in genes in the histidine operon which serve as targets for mutagens that cause DNA damage. *Salmonella typhimurium* may be manipulated to being a histidine mutant by either exposing it to radiation or chemicals. This mutation renders the bacteria unable to produce the amino acid, histidine (**MORTELMANS** and **ZEIGER, 2000**). This essential amino acid is necessary for the bacteria to grow and form new colonies. In the presence of a mutagen, new mutations occur near or at the site of the original mutation that restore the function of the gene and thus the synthesis of histidine. The bacteria are therefore able to grow and form colonies in the absence of histidine in the media, referred to as revertant colonies. The Ames test is therefore also known as the reversion assay (**MORTELMANS** and **ZEIGER, 2000**). A reverse mutation works such that the function of a mutated gene is returned. The deletion mutation (*hisD3052*) carried by the TA98 strain (also shared by TA1538 strain) affects a CG repetitive sequence which is deteriorated back to the wild-type by frameshift events (**ISONO** and **YOURNO, 1974**). The TA100 strain carries a deletion mutation at *hisG46* gene where a leucine codon is replaced with a proline combination. A base-pair substitution, by a mutagen, at the one GC pair, reverts the strain to the wild-type strain. When using the TA102 strain, one is able to detect mutagens that act via transitions/transversions (**MORTELMANS** and **ZEIGER, 2000**). At the *hisG428* mutation site there are AT instead of GC base-pairs. An ochre mutation, described as an alteration of a codon to a stop codon, causes premature termination of a polypeptide chain. Since the mutation is at the *hisG* gene, in the presence of a mutagen, all likely base-pair changes are possible; resulting in transitions and transversions (**MORTELMANS** and **ZEIGER, 2000**).

The Ames test is quantitatively scored. The mutagenic effect of a substance will cause histidine-requiring strains of *S. typhimurium* (His^-) to regain the ability to grow in the absence of histidine, thus forming colonies (**MORTELMANS** and **ZEIGER, 2000**). These colonies are different from

the spontaneous colonies that form a background. Therefore the presence of colonies is an indication of a reverse mutation of the His^+ gene to its active form. When compared to the spontaneous mutations found in the negative controls, the substance can be regarded as either mutagenic or non-mutagenic.

In other words, only *Salmonella* strains that are histidine independent will be able to form colonies. In the assay some bacterial mutants (histidine dependent) are able to revert spontaneously to histidine independence, and form colonies (**MORTELMANS** and **ZEIGER, 2000**). This value is relatively constant; therefore, when a mutagen is introduced, the resultant number of revertant colonies is clearly visible. However, some substances could be promutagens; that is they require some biochemical stimulation to be converted to mutagenic products (**MORTELMANS** and **ZEIGER, 2000**). This phenomenon is common in humans, and without a suitable modification the Ames test will not identify such substances. Often, a mixture of liver enzymes (S9) is incorporated to mimic the enzymatic conversion of a promutagen to a mutagen, taking into consideration the effect of body metabolism on substances and allowing for the detection of such substances using the Ames test. The S9 microsomal mixture contains a cytochrome-based P450 oxidation system.

The Ames assay was initially performed as a spot test and progressed to the more sensitive and quantitative plate incorporation assay (**MORTELMANS** and **ZEIGER, 2000**). An alternative assay that determines the toxicity more quantitatively is the „treat and plate' suspension assay. The plate incorporation assay has an advantage over this procedure in that there are fewer steps involved and the presence of trace amounts of histidine with the plant extract allows for the bacteria to become sensitized to mutagenesis by undergoing some cell division (**MORTELMANS** and **ZEIGER, 2000**). There have been some modifications to the plate incorporation assay to allow for the investigation of gases and volatile chemicals.

7.1.3. Toxicity

The toxicity of crude plant extracts were determined by investigating the background „lawn' of the minimum glucose plates. The overnight *Salmonella* culture contains mainly histidine-dependent bacteria and relatively few histidine-independent bacteria. With a trace amount of histidine present

in the top agar all the bacteria go through a few cell divisions until the histidine is exhausted. Colonies that are clearly visible are the revertant colonies while microcolonies of histidine-dependent bacteria form the hazy background lawn. Toxicity is therefore characterised as the thinning of the background lawn (usually a decrease in number of revertant colonies is also observed); absence of background lawn; or the production of pinpoint non-revertant colonies (**MORTELMANS** and **ZEIGER, 2000**). With regard to the toxicity of a compound, the dose of the compound may determine just how toxic it is. Initially, three concentrations are tested in the Ames assay to assess the toxic effect of the crude plant extracts. Usually the response is expected to follow a dose-dependent curve. A wider range of concentrations can be tested for those extracts that show mutagenic and toxic effects.

Possible errors that could result in either false positives or false negatives have to be guarded against. For example, the top agar which contains 0.05 mM histidine requires special attention during preparation. Too little histidine may not support the growth of a uniform background lawn, resulting in a plant sample being incorrectly considered to be toxic. This error is easily identified by observing the negative control which should produce a uniform background lawn. Alternatively, with the addition of too much histidine, heavy growth could make visibility difficult (**MORTELMANS** and **ZEIGER, 2000**).

The Ames test is normally used as a preliminary test to determine the potential mutagenic activity of new chemicals and drugs. The ability of a chemical substance to induce a mutation is an essential, almost standard, consideration when conducting a safety analysis. Also, the continuous formulation of new medication is necessary due to the undesirable side effects or a reduced effectiveness of current medication.

7.2. MATERIALS AND METHODS

7.2.1. Re-suspension of plant extracts

A dilution series of the crude plant extracts (prepared as described in **Section 4.2.2**) was prepared by re-suspending extracts in 10% DMSO. Adequate sample was prepared to run three replicates of three concentrations (5; 0.5; 0.05 mg/ml).

7.2.2. Ames *Salmonella*/microsome mutagenicity assay (Ames test)

An *in vitro* mutagenicity assay was carried out to determine the mutagenic activity of crude plant extracts of seven orchid species using three *S. typhimurium* bacterial strains (**MARON and AMES, 1983**). Bacterial strains were maintained at -70°C (stock bacteria). Using sterile bench technique, overnight cultures of TA98, TA100 and TA102 were prepared by inoculating 10 ml of Oxoid nutrient broth No. 2 with 100 µl of each stock bacterium. The cultures were incubated on an orbital shaker over a 16 h period at 37°C. A dilution series of the crude plant extracts was prepared by re-suspending extracts in 10% DMSO. A positive control, 4-nitroquinoline-1-oxide (4NQO), was prepared from a stock solution of 2 mg/ml to a concentration of 2 µg/ml by mixing 10 µl of 4NQO (stock) with 990 µl DMSO. From this, 100 µl of 4NQO (20 µg/ml) was added to 900 µl distilled water to produce a 2 µg/ml solution.

Minimum glucose plates were prepared in advance using 90 mm Petri dishes. To make a litre of agar: 15 g of Difco agar and a magnetic stir bar was added to 930 ml of distilled water and autoclaved for 20 mins at 121°C. Thereafter, 20 ml of sterile Vogel-Bonner medium (50X VB) (10 g of $MgSO_4.7H_2O$; 100 g citric acid monohydrate; 500 g K_2HPO_4; 175 g $NaHNH_4(PO_4.4H_2O)$ were added consecutively to 670 ml warm distilled water and adjusted to one litre) and 50 ml of 40% glucose was added to the liquid media. The media were stirred thoroughly and 30 ml was dispensed into each Petri dish, which was allowed to set. On day two, two water baths were filled with sufficient water and set at 37°C and 50°C. Culture tubes were transferred onto racks (nine tubes per crude extract, three for the positive control and five tubes for the negative controls). The assay was conducted under sterile conditions where an ample supply of 70% EtOH is available and a constant flame was present. One hundred microlitres of each concentration of sample and control substance were dispensed into the respective culture tubes, followed by 500 µl of sterile 0.2 M sodium phosphate buffer (60 ml of 0.2 M $NaH_2PO_4.H_2O$ (13.8 g/500 ml) added to 40 ml 0.2 M Na_2HPO_4 (14.2 g/ 500 ml) adjusted to pH 7.4 with 0.2 M $NaH_2PO_4.H_2O$). One hundred millilitre aliquots of sterile top agar (6 g Difco agar, 5 g NaCl in one litre distilled water, were dispensed into glass Schott bottles and stored at 4°C) were defrosted in a microwave oven and placed in the 50°C water bath to cool. While waiting, 100 µl of overnight bacterial culture (2 x 10^8 cell/ml) were added to each tube. Minimum agar plates were labelled. Thereafter, 10 ml of 0.5 mM histidine/biotin

solution (12 mg D-biotin and 10.5 mg L-histidine / 100 ml sterile distilled water) were added to the liquid top agar. From this enriched top agar, with a trace of histidine, 2 ml were added to 18 culture tubes at a time (to prevent the agar from solidifying). Culture tubes were immediately placed in the 37°C water bath, while the contents of each tube were vortexed and poured onto the respective minimum glucose plates. The same procedure was carried out for the remaining culture tubes. Minimum glucose plates were allowed to set, inverted and thereafter incubated in a 37 °C oven for 48 h. A similar procedure was used when determining mutagenic potential with S9 metabolic activation. An S9 mixture (5% (v/v) S9 fraction sourced from Sprague-Dawley male rats (Sigma-Aldrich, Co., St Louis) in mixed enzymic cofactors with NADP) kept on ice was used in place of the phosphate buffer (500 µl). A separate positive control 2-aminoanthracene (2AA) was used. At the end of the incubation period, the number of revertant colonies was counted using an electronic counter. Toxicity was measured by examining the background of the glucose plates. Samples were compared to the control plate, which is very dense. Therefore a less dense glucose plate could indicate a toxic sample. A crude extract was considered to be mutagenic if the number or revertant colonies were twice the number found in the negative controls and showed a dose-dependent curve. The mutagenic index (MI) was also determined; where a MI ≥ 2 and a dose-dependent response was considered mutagenic (**VARELLA et al., 2004**).

7.3. RESULTS

Table 7.1. (**Chapter 7**) shows the average number of induced revertant colonies, the standard deviation and the MI at different concentrations (5, 0.5, 0.05 mg/ml) of crude orchid extracts using three *S. typhimurium* strains (TA98, TA100 and TA102). A crude extract was considered to be mutagenic if the number or revertant colonies were twice the number found in the negative controls and showed a dose-dependent response. Mutagenic extracts were observed for the TA98 strain only. All concentrations of *A. africana* DCM leaf and stem extracts tested, the DCM root extract (0.5, 0.05 mg/ml) and EtOH leaf, stem and root extracts at 5 mg/ml exhibited mutagenic effects. The EtOH root extracts (5, 0.5 mg/ml) of *B. scaberulum* exhibited mutagenic indices comparable to that of 4NQO (17.00 and 13.00, respectively). *Eulophia petersii* PE pseudobulb extract demonstrated mutagenic potential at the highest concentration tested (5 mg/ml). The ethanolic root extracts of *T. tridentata* showed mutagenic effects at 5 and 0.5 mg/ml.

Table 7.1.: Mutagenicity of crude orchid extracts as determined in the *Salmonella*/microsome assay (without metabolic activation) expressed as average revertants per plate and mutagenicity index.

Species	Plant part	Extraction solvent	Concentration (µg/ml)	*Salmonella typhimurium* strains					
				TA98		TA100		TA102	
				Revertants per plate	Mutagenicity index	Revertants per plate	Mutagenicity index	Revertants per plate	Mutagenicity index
Ansellia africana	Leaves	DCM	5000	47.34±12.3	**3.95**	174.00±19.80	1.66	656.00±67.88	1.57
			500	36.67±7.1	**3.06**	158.00±8.49	1.50	354.00±76.37	0.85
			50	32.67 ± 1.9	**2.72**	162.00±14.15	1.54	476.00±28.28	1.14
		Ethanol	5000	25.34±10.4	**2.11**	112.00±0.00	1.07	430.00±36.77	1.03
			500	20.17±4.9	1.68	84.00±1.10	0.80	414.00±25.46	0.99
			50	12.67±1.2	1.06	106.00±36.77	1.01	512.00±11.31	1.22
	Stem	DCM	5000	41.00±6.6	**3.42**	68.00±5.66	0.65	572.00±30.55	1.37
			500	26.00±1.4	**2.17**	120.00±28.28	1.14	322.00±14.14	0.77
			50	34.00±6.1	**2.83**	140.00±28.28	1.33	396.00±56.57	0.95
		Ethanol	5000	27.00±9.9	**2.25**	154.00±25.46	1.47	560.00±27.72	1.34
			500	22.84±6.8	1.90	122.00±48.08	1.16	ND	ND
			50	18.17±1.2	1.51	114.00±2.83	1.09	560.00±14.00	1.34
	Root	DCM	5000	36.50±12.02	**3.04**	146.00±19.80	1.39	428.00±11.31	1.02
			500	31.50±9.19	**2.63**	118.00±14.14	1.12	420.00±152.7	1.00
			50	22.50±3.54	1.88	106.00±31.11	1.01	338.00±127.3	0.81
		Ethanol	5000	31.50±9.19	**2.63**	154.00±19.80	1.47	360.00±16.97	0.86
			500	ND	ND	146.00±2.82	1.39	ND	ND
			50	ND	ND	110.00±19.80	1.05	ND	ND
Bulbophyllum scaberulum	Root	DCM	5000	20.50±6.36	1.71	143.00±9.90	1.36	175.50±6.36	0.42
			500	18.50±9.19	1.54	101.00±4.24	0.96	253.50±0.71	0.61
			50	18.00±9.90	1.50	127.00±7.07	1.21	280.50±17.68	0.67
		Ethanol	5000	204.00±31.1	**17.00**	118.00±4.95	1.12	189.00±26.87	0.45
			500	156.00±28.28	**13.00**	ND	ND	184.00±8.49	0.44
			50	11.50±4.95	0.96	ND	ND	199.00±4.24	0.48
Cyrtorchis arcuata	Leaf	PE	5000	18.00±4.24	1.50	168.00±5.66	1.60	226.00±42.43	0.54
			500	15.50±7.78	1.29	154.00±8.49	1.47	274.00±70.71	0.66
			50	13.50±0.71	1.13	116.00±5.66	1.10	350.00±59.40	0.84
		DCM	5000	16.00±5.66	1.33	146.00±14.14	1.39	342.00±53.74	0.82
			500	11.50±4.95	0.96	130.00±2.82	1.24	318.00±121.6	0.76
			50	11.00±5.66	0.92	128.00±33.94	1.22	358.00±36.77	0.86
		Ethanol	5000	9.00±5.66	0.75	154.00±19.80	1.47	352.00±28.28	0.84
			500	7.50±0.71	0.63	160.00±62.23	1.52	288.00±22.63	0.69
			50	12.50±2.12	1.04	166.00±25.46	1.58	338.00±19.80	0.81
	Roots	DCM	5000	7.50±6.36	0.63	122.00±8.46	1.16	390.00±98.99	0.93

Species	Plant part	Extraction solvent	Concentration (µg/ml)	*Salmonella typhimurium* strains					
				TA98		TA100		TA102	
				Revertants per plate	Mutagenicity index	Revertants per plate	Mutagenicity index	Revertants per plate	Mutagenicity index
			500	8.50±0.71	0.71	72.00±11.31	0.69	410.00±65.05	0.98
			50	13.00±1.41	1.08	148.00±0.00	1.41	330.00±70.71	0.79
		Ethanol	5000	9.00±2.83	0.75	128.00±16.97	1.22	186.00±36.77	0.44
			500	11.50±2.12	0.96	124.00±22.63	1.18	248.00±33.94	0.59
			50	13.50±4.95	1.13	120.00±11.31	1.14	358.00±25.46	0.86
Eulophia hereroensis	Tuber	Ethanol	5000	15.00±4.24	1.25	132.00±4.24	1.26	226.00±22.63	0.54
			500	14.50±2.12	1.21	89.00±0.00	0.85	217.50±45.96	0.52
			50	15.50±4.95	1.29	124.00±9.90	1.18	301.00±24.04	0.72
	Roots	DCM	5000	11.50±0.71	0.96	116.00±22.63	1.10	ND	ND
			500	15.00±2.82	1.25	98.00±8.49	0.93	387.00±21.21	0.93
			50	18.00±0.00	1.50	140.00±0.00	1.33	384.00±50.91	0.92
		Ethanol	5000	17.50±2.12	1.46	147.00±31.11	1.40	347.50±28.99	0.83
			500	11.00±1.41	0.92	124.50±0.71	1.19	322.00±15.56	0.77
			50	14.00±5.66	1.17	119.00±12.73	1.13	329.50±43.13	0.79
Eulophia petersii	Leaf	DCM	5000	22.00±0.00	1.83	ND	ND	540.00±5.66	1.29
			500	26.00±0.00	**2.17**	ND	ND	342.00±104.7	0.82
			50	ND	ND	ND	ND	422.00±127.3	1.01
		Ethanol	5000	14.00±4.24	1.17	118.00±19.80	1.12	242.00±25.46	0.58
			500	9.00±1.41	0.75	168.00±5.66	1.60	326.00±8.49	0.78
			50	11.00±5.66	0.92	120.00±11.31	1.14	324.00±0.00	0.78
	Stem	DCM	5000	14.50±0.71	1.21	170.00±25.46	1.62	234.00±42.43	0.56
			500	21.50±2.12	1.79	164.00±11.31	1.56	460.00±164.1	1.10
			50	15.00±5.66	1.25	140.00±0.00	1.33	368.00±33.94	0.88
		Ethanol	5000	18.50±9.19	1.54	106.00±14.14	1.01	624.00±11.31	1.49
			500	19.50±3.54	1.63	160.00±0.00	1.52	716.00±175.4	1.71
			50	15.00±4.24	1.25	116.00±22.63	1.10	492.00±22.63	1.18
	Pseudobulb	PE	5000	26.00±4.24	**2.17**	ND	ND	434.00±53.74	1.04
			500	19.50±0.71	1.63	ND	ND	308.00±62.23	0.74
			50	16.00±2.82	1.33	ND	ND	418.00±132.9	1.00
		DCM	5000	13.00±2.82	1.08	116.00±0.00	1.10	206.00±14.14	0.49
			500	16.50±3.54	1.38	88.00±0.00	0.84	390.00±76.37	0.93
			50	9.00±2.82	0.75	124.00±5.66	1.18	404.00±164.1	0.97
		Ethanol	5000	21.50±0.71	1.79	156.00±5.66	1.49	370.00±116.0	0.89
			500	17.50±4.95	1.46	168.00±10.61	1.60	340.00±22.63	0.81
			50	15.00±1.41	1.25	144.00±39.60	1.37	274.00±8.49	0.66
	Root	DCM	5000	15.50±0.71	1.29	116.00±50.91	1.10	483.00±26.87	1.16

Species	Plant part	Extraction solvent	Concentration (µg/ml)	*Salmonella typhimurium* strains					
				TA98		TA100		TA102	
				Revertants per plate	Mutagenicity index	Revertants per plate	Mutagenicity index	Revertants per plate	Mutagenicity index
			500	11.50±6.36	0.96	146.00±8.49	1.39	194.00±42.43	0.46
			50	16.00±1.41	1.33	134.00±42.43	1.28	322.00±76.37	0.77
		Ethanol	5000	14.50±0.71	1.21	94.00±14.14	0.90	340.00±28.28	0.81
			500	16.50±2.12	1.38	152.00±62.23	1.45	322.00±48.08	0.77
			50	16.00±1.41	1.33	148.00±45.26	1.41	236.00±45.25	0.56
Polystachya pubescens	Pseudobulb	PE	5000	11.50±3.54	0.96	135.00±12.73	1.29	214.50±4.95	0.51
			500	6.00±2.82	0.50	120.00±0.00	1.14	243.50±16.26	0.58
			50	16.50±6.36	1.38	114.00±28.28	1.09	228.00±28.28	0.55
		DCM	5000	12.00±1.41	1.00	126.00±42.42	1.20	127.50±33.23	0.31
			500	12.00±4.24	1.00	112.00±0.00	1.07	226.50±7.78	0.54
			50	9.50±2.12	0.79	108.00±22.63	1.03	204.50±16.26	0.49
		Ethanol	5000	17.00±1.41	1.42	132.00±50.91	1.26	184.00±22.63	0.44
			500	16.00±1.41	1.33	204.00±50.91	1.94	247.00±24.04	0.59
			50	18.50±3.54	1.54	108.00±16.97	1.03	278.00±8.49	0.67
	Root	PE	5000	14.50±9.19	1.21	123.00±7.07	1.17	193.50±2.12	0.46
			500	15.50±0.71	1.29	ND	ND	240.00±28.28	0.57
			50	12.00±5.66	1.00	120.00±14.14	1.14	266.50±36.06	0.64
		DCM	5000	13.00±5.66	1.08	ND	ND	204.00±16.97	0.49
			500	14.00±1.41	1.17	ND	ND	193.50±44.55	0.46
			50	11.50±2.12	0.96	ND	ND	160.00±52.33	0.38
		Ethanol	5000	11.00±4.24	0.92	88.00±11.31	0.84	242.50±2.12	0.58
			500	13.00±1.41	1.08	112.00±5.66	1.07	228.00±57.98	0.55
			50	13.50±0.71	1.13	90.00±2.83	0.86	260.00±22.63	0.62
Tridactyle tridentata	Roots	PE	5000	12.50±0.71	1.04	143.00±9.90	1.36	139.50±16.26	0.33
			500	15.50±2.12	1.29	123.00±13.44	1.17	244.00±4.24	0.58
			50	15.00±0.00	1.25	136.00±4.95	1.30	187.00±21.21	0.45
		DCM	5000	16.00±0.00	1.33	134.00±7.78	1.28	56.50±4.95	0.14
			500	16.00±1.41	1.33	131.00±3.54	1.25	164.50±10.61	0.39
			50	16.50±2.12	1.38	131.00±8.49	1.25	260.00±45.26	0.62
		Ethanol	5000	ND	ND	132.00±10.61	1.26	154.00±5.66	0.37
			500	94.50±9.19	**7.88**	129.00±3.54	1.23	255.00±33.94	0.61
			50	41.50±3.54	**3.46**	ND	ND	204.00±33.94	0.49
Positive control				196.00±2.83	**16.33**	1868.0±231.9	**17.79**	1679.0±175.4	**4.02**
Negative control				12.00±0.71	1.00	105.00±13.43	1.00	418.00±11.31	1.00

Values are the mean (±) standard deviation (n = 3); values in bold indicate those extracts with most significant mutagenic index. ND – not determined.

Table 7.2. (**Chapter 7**) shows the average number of induced revertant colonies, the standard deviation and the MI at different concentrations (5, 0.5, 0.05 mg/ml) of crude orchid extracts using *S. typhimurium* strain TA98 with metabolic activation. A crude extract was considered to be mutagenic if the number of revertant colonies were twice the number found in the negative controls and showed a dose-dependent response. None of the extracts tested demonstrated mutagenic effects.

Table 7.2.: Mutagenicity of crude orchid extracts as determined in the *Salmonella*/microsome assay (with metabolic activation) expressed as average revertants per plate and mutagenicity index.

Species	Plant part	Extraction solvent	Concentration (µg/ml)	*Salmonella typhimurium* strain (TA98)	
				Revertants per plate	Mutagenicity index
Ansellia africana	Stem	DCM	5000	16.50±4.95	0.67
			500	12.50±2.12	0.51
			50	11.50±2.12	0.47
	Root	DCM	5000	11.50±3.54	0.47
			500	13.00±2.82	0.53
			50	9.00±2.82	0.37
Eulophia hereroensis	Roots	DCM	5000	19.00±0.00	0.78
			500	21.00±2.82	0.86
			50	25.50±2.12	1.04
		Ethanol	5000	11.50±2.12	0.47
			500	16.00±2.82	0.65
			50	16.50±2.12	0.67
Eulophia petersii	Stem	DCM	5000	11.00±0.00	0.45
			500	14.00±0.00	0.57
			50	15.50±0.71	0.63
		Ethanol	5000	16.00±8.49	0.65
			500	10.50±0.71	0.43
			50	13.00±1.41	0.53
	Root	DCM	5000	11.50±2.12	0.47
			500	18.00±4.24	0.73
			50	11.50±2.12	0.47
Tridactyle tridentata	Roots	PE	5000	15.00±1.41	0.61
			50	18.00±0.00	0.73
		DCM	50	8.50±6.36	0.35
Positive control				141±9.19	**5.76**
Negative control				24.50±2.12	1.00

Values are the mean (±) standard deviation (n = 3); values in bold indicate those extracts with most significant mutagenic index.

7.4. DISCUSSION

Mutagenicity assessment programs have increased dramatically especially due to cancer concerns. There are two major concerns regarding mutagens, these are: their ability to either induce cancer or to affect the germ line and future generations. The Ames test is commonly used to determine the potential mutagenic property of a substance. It is assumed that a mutagenic substance may be carcinogenic. However, not all cancer causing substances show positive results in the Ames test. A positive result indicates the ability of a substance to produce a change in DNA structure; that is, gene point mutations or the gain or loss of whole chromosomes. However, further analysis is required to determine the carcinogenic potential of the substance. In industry the *Salmonella* mutagenicity (Ames) test, which detects gene mutations, (**VERSCHAEVE et al., 2004**) is used extensively as a preliminary indicator to identify possible carcinogens and/or the mutagenic potential of crude plant extracts and clinical drugs. **ELGORASHI et al. (2003)** reported on the possible mutagenic effects of 51 South African medicinal plant species. **REID et al. (2006)** evaluated 42 other South African species. There has been extensive research into the mutagenic and anti-mutagenic potential of medicinal plants used in the South African traditional medical system (**ELGORASHI et al., 2002**).

This investigation is the first report of mutagenic properties of seven medicinal orchids of South Africa. Plants were selected on the basis of their ethnobotanical use and availability. A plant extract was considered mutagenic when a MI of ≥2 for any concentration of that extract was observed; together with a dose-dependent response (**VARGAS et al., 1993**). An increase in number of His^+ revertant colonies over the negative control indicated genotoxic effects. Of the seven orchid species considered in this investigation, *C. arcuata* root extract contained the highest flavonoid content (1.24 mg catechin equivalent/g dry matter). Organic leaf and root extracts of *C. arcuata* produced no genotoxic effects (**Table 7.1. Chapter 7**). Food derived flavonols such as quercetin, kaempferol and myricetin have been reported to affect the cell cycle and growth of cancer cells, thereby reducing the risk of cancer (**VERMA et al., 1988**; **YOSHIDA et al., 1990**). The non-genotoxic effects of *C. arcuata* coupled with the high flavonoid content necessitates further investigation. **ZHANG et al. (2005)** also established that dimerised phenanthrenes are required to inhibit cancer cell growth. Research on compounds such as moscatilin; with known anti-inflammatory, cytotoxic, anti-platelet aggregation and anti-proliferative properties; and erianin,

provide insight into the mechanisms involved in cancer progression. Similary, orchid extracts that demonstrate significant biological activities should be researched further for other biological activities.

The ASMM assay revealed that mutagenic extracts were observed for the TA98 strain only. Without metabolic activation, certain organic extracts of *A. africana* leaf, stem and root extract at various concentrations tested (0.5 and 0.05 mg/ml) exhibited genotoxic effects; while the EtOH root extracts (5 and 0.5 mg/ml) of *B. scaberulum* exhibited mutagenic indices comparable to that of 4NQO. A more comprehensive chemical and toxicological profile is required for *B. scaberulum*. Bioactive *E. petersii* PE pseudobulb extract demonstrated mutagenic potential at the highest concentration tested (5 mg/ml). The ethanolic root extracts of *T. tridentata* showed mutagenic effects at 5 and 0.5 mg/ml. With metabolic activation using TA98, no mutagenic effects were observed for all crude orchid extracts tested.

The primary aim of mutagenicity testing of bioactive natural plant extracts is to determine their safety. The herbicidal potential of gymnopusin, a phenanthrene derivative from *M. densa*, was weak as it displayed cytotoxic effects *in vitro* against four mammalian cell lines H4TG (IC_{50} 13.0±0.9 µM), MDCK (IC_{50} 11.0±0.5 µM), NIH3T3 (IC_{50} 12.0±1.0 µM) and KA31T (IC_{50} 21.0±0.5 µM) (**VALENCIA- ISLAS et al., 2002**). Interestingly, other researchers have previously determined various approaches to synthetically produce gymnopusin (**WANG** and **SNIECKUS, 1991**). Collectively, such information can benefit further research efforts dealing with bioherbicides and their safety.

A fairly large number of phenanthrenes have been reported from the Orchidaceae family; particularly the *Dendrobium*, *Bulbophyllum*, *Eria*, *Maxillaria*, *Bletilla*, *Coelogyna*, *Cymbidium*, *Ephemerantha* and *Epidendrum* genera. Denbinobin, a phenanthraquinone isolated from *D. nobile*, *E. lonchophylla* and *E. fimbriata*, exhibited cytotoxic effects (**TALAPATRA et al., 1982; TEZUKA et al., 1991; TEZUKA et al., 1993; LEE et al., 1995**). Orchid extracts are key ingredients in the five major traditional medicines used in Chinese traditional medicine (**Chapter 3 Section 3.5.1.**). Those used in the treatment of cancers include: Bai-Ji (*Bletilla striata*) used to treat haemorrhagic disorders and cancers and Shan-Ci-Gu (*Cremastra appendiculata*) used to treat tonsillitis, hypertension and cancers (**XUE et al., 2006**; **BULPITT et al., 2007**). Phenanthrenes

were mainly isolated from whole plants, cortex, tubers or stems and a large number where isolated from *B. striata* and *B. vaginatum* (**KOVÁCS et al., 2008**). Lusianthridin, isolated from *B. striata* had no activity on a leukaemic P388 cell line *in vitro* but displayed antitumor activity at 20 µg/kg (**TAKAGI et al., 1983**). In the current study, *B. scaberulum* ethanolic root extracts exhibited mutagenic properties comparable to 4NQO (**Table 7.1. Chapter 7**). Further chemical and pharmacological investigation of other organs of this species could reveal phenanthrenes and antitumor properties of *B. scaberulum*. Taxol and vinblastine, well known mutagens, are used as anticancer drugs. Orchid extracts that exhibit mutagenic effects (*A. africana* organic leaf, stem and root, *B. scaberulum* EtOH root, *E. petersii* PE pseudobulb and *T. tridentata* EtOH root extracts) should be examined further to determine their full potential. Such compounds may effectively treat cancers; in addition to providing insight into the mechanisms involved in its progression.

There are a number of antitumor, antimutagenic and anti-angiogenesis reports for the *Dendrobium* genus (**LEE et al., 1995; WANG et al., 1997; MIYAZAWA et al., 1999; GONG et al., 2004; ZHANG et al., 2005; GONG et al., 2006; CHEN et al., 2008a; KOVÁCS et al., 2008; TSAI et al., 2010**). Similarly, antitumour activities have been detected in the *Bulbophyllum* genus (**YAO et al., 2005a**), with *B. odoratissimum*, in particular, containing cytotoxic phenolics (**CHEN et al., 2008b**). Glycosides were reported in *D. chrysanthum* [denchryside-A] and *Bletilla striata*. **KOVÁCS et al. (2008)** referred to the anticancer potential of these compounds due to their cytotoxic properties. Sesquiterpene glycosides dendroside-A and dendronobilosides A and B were also detected in *D. nobile* stem extracts. Calanquinone-A isolated from *C. arisanensis* also exhibited cytotoxic activity (**LEE et al., 2008**). Ephemeranthol-A and fimbriol-A, isolated from *E. rigidum* exhibited potent phytotoxicity against *A. hypochondriacus* (IC_{50} 0.12 µM and 5.90 µM, respectively) (**HERNÁNDEZ-ROMERO et al., 2005**). Compounds with phytotoxic properties may be further investigated for their herbicidal potential.

Toxicity was determined by examining the density of the background lawn of spontaneous colonies, compared to that of the negative control. The resultant population of microcolonies of His^+ bacteria, form the hazy background lawn (**MORTELMANS** and **ZEIGER, 2000**). A thinning or absent lawn indicates toxicity. The results of this technique revealed minimal toxic effects for all crude extracts when compared to the 10% DMSO control. In order to determine a definite

relationship between the lack of mutagenic potential and toxicity, other toxicity tests need to be conducted.

The toxicology and mutagenicity of medicinal plants needs equal consideration in medicinal plant research. Most of the orchid species used for cultural practices is administered as emetics. It would be important to know what the effects of these orchid-derived medicines are on the human body and more especially their safety. The detection of mutagenic substances has become a necessary measure in most industries' safety evaluations (**MORTELMANS** and **ZEIGER, 2000**). There has been a recent upsurge of mutagenicity and toxicological studies mainly due to the possibility of mutagenic substances being cancer causing agents (**MORTELMANS** and **ZEIGER, 2000**). Other toxicological tests such as the Micronucleus Test (MNT), Comet assay, Umu-C and VITOTOX® tests and antimutagenic assays could be used; where significant results warrant further investigation to determine the potential of plant compounds. Qualitative and quantitative HPLC analysis could also be performed on mutagenic extracts.

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSIONS

It is well known that orchids are used for medicinal purposes around the world. The medicinal value of orchids was realised by early civilizations and they were subsequently used as therapeutic agents to treat common ailments. **BULPITT (2005)** described the widespread medicinal use of orchids. In early European history, Theophrastus (372-286 BC) derived the word orchid from the Greek word *orchis* (as the tubers of many European terrestrial orchids resembled testicles). The Americans made extensive use of the vanilla orchid as a flavourant and perfume, a tradition passed on from the ancient Aztecs to modern civilizations. The Australian aborigines and early settlers mainly used *Cymbidium* and *Dendrobium* species to treat dysentery, as an oral contraceptive, to relieve pain, and to cure ringworm infection. Some species were consumed as a food source. In the Ayurvedic pharmacopoeia of India, over 40 orchid species from 21 genera were identified as important medicinal sources: *Acampe, Bulbophyllum, Calanthe, Coelogyne, Cymbidium, Cypripedium, Dactylorhiza, Dendrobium, Epipactis, Eria, Eulophia, Filckingeria, Habenaria, Liparis, Luisia, Malaxis, Pecteilis, Pholidota, Rhynchostylis, Satyrium* and *Vanda* (**SINGH et al., 2007**). In Chinese traditional medicine **BULPITT et al. (2007)** highlighted the five major traditional medicines of which orchids are key ingredients – Shi-Hu (five *Dendrobium* species) used to treat deficiency disorders in the kidney, lung and stomach, Tian-Ma (*Gastrodia elata*) used to relieve dizziness, convulsions, hypertension and to treat stroke patients, Bai-Ji (*Bletilla striata*) used to treat haemorrhagic disorders as well as cancers; Jin-Xian-Lian (two *Anoectochilus* species) used mainly to treat nephritis, cystitis and pneumonia and Shan-Ci-Gu (*Cremastra appendiculata*) used to treat tonsillitis, hypertension and cancers. On the African continent, Malawians use approximately nine species to treat stomach ailments and two more for fertility problems. *Cyrtorchis arcuata* is used to treat diabetes and skin infections; epilepsy is prevented by using *Eulophia cucullata* and psychological disorders such as madness is treated with *Tridactyle tricuspis* (**BULPITT, 2005**). While there is an awareness of medicinally used orchids around the world, the extensive use of certain southern African orchids for medicinal and magical purposes is not well documented.

Based on an extensive literature search, compared to the South African perspective, it is evident that medicinal orchid research has surged ahead in other countries. Pharmacological studies of orchids

have revealed anti-angiogenic, anti-cancer, anti-inflammatory, antioxidant, antimicrobial, anti-red blood cell sickling, anti-tumour, antiviral, cytotoxic, immunomodulatory, phytotoxic, spasmolytic and neuroprotective activities. Phytochemical research of orchid species has elucidated a number of bioactive compounds generally grouped as polyphenols, lignans, alkaloids, monoterpenes, triterpenes, stilbenoids, bibenzyls and phenanthrenes (**GARO et al., 2007**). Compounds of particular interest include: gigantol, which demonstrated significant anti-inflammatory and vasorelaxant properties, 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde which exhibited considerable anti-angiogenic, analygesic, anti-inflammatory, antioxidant and memory consolidation properties, gastrodin which positively affected memory retention and learning abilities, exhibited antioxidant, anticonvulsant effects and neuroprotective properties in Alzheimer's research. The aromatic vanillin, originally isolated from *Vanilla* species, has demonstrated the greatest medicinal potential despite its more common use in the culinary industry as a flavourant. Vanillin exhibited antioxidant, prooxidant, apoptosis inhibitory effects, neuroprotective, antimicrobial, anti-carcinogenic, anti-mutagenic and anti-clastogenic properties. Furthermore, **ZHANG et al. (2004)** provided details on the vanillin prodrug MX-1520, which was synthesized to treat red blood cell sickling with vanillin, *in vivo*. These significant developments in medicinal orchid research worldwide resulted from an ethnobotanical approach used to select the various orchid species which yielded valuable bioactive compounds.

The current investigation revealed for the first time, the pharmacological potential of seven indigenous orchid species of South Africa: *Ansellia africana* Lindl., *Bulbophyllum scaberulum* (Rolfe) Bolus, *Cyrtorchis arcuata* (Lindl.) Schltr., *Eulophia hereroensis* Schltr., *Eulophia petersii* (Rchb.f.) Rchb.f., *Polystachya pubescens* (Lindl.) Rchb.f. and *Tridactyle tridentata* (Harv.) Schltr. The phytochemical evaluation of these species provided insight into the classes of chemical compounds present and their possible role in the observed biological activities.

According to **KOVÁCS et al. (2008)** phenanthrenes and their dihydro-phenanthrene derivatives constitute the major phytoalexins in orchids. A phytochemical report by **BLITZKE et al. (2000)** revealed five phenanthrenes and several phytosterols from the roots of *Eulophia petersii* collected in Yemen. Lusianthridin, one of the phenanthrenes, demonstrated antimicrobial properties, cytotoxic activity *in vitro* and *in vivo*, anti-tumour effects and increased muscle contractions (**KOVÁCS et al., 2008**). With a number of South African orchid species demonstrating antimicrobial properties

(**Section 4.4.**) future research should focus on determining the presence and biological activity of phenanthrenes in orchid species. The organic root extracts of *Eulophia hereroensis* and organic and pseudobulb extracts of *E. petersii*, respectively, exhibited significant antimicrobial effects (**Table 4.1. Chapter 4**). A more focused project such as a complete phytochemical and pharmacological assessment of the South African *Eulophia* species might reveal results that could be compared to that of other *Eulophia* species.

The presence of certain classes of compounds may influence biological activities in medicinal plants. Phytochemical analysis revealed *C. arcuata* methanol root extracts to have the highest flavonoid content. Anatomical studies of *Encyclia calamara* roots revealed the presence of flavonoid compounds (**OLIVEIRA** and **SAJO**, **1999**), while the comprehensive survey by **WILLIAMS (1979)** illustrates the distribution of leaf flavonoids in Orchidaceae. **KIM et al. (2009)** reported on the lethal anthelmintic effects of flavonoids, where the natural flavonoid flavone inhibited embryogenesis in *Caenorhabditis elegans*. Such reports provide credence to the significant anthelmintic effects observed in all *C. arcuata* extracts tested.

Cyrtorchis arcuata methanol leaf extracts contained the highest condensed tannin content, while *E. hereroensis* methanol root extract contained the highest amount of hydrolysable tannin. Recent veterinary parasitology studies have focused on the use of proanthocyanidin-rich diets for direct and indirect anthelmintic effects (**IQBAL et al., 2007**). **MOHAMED et al. (2000)** explored the lethal effects of gallo- and condensed tannins specifically against *C. elegans*. Hydrolysable tannins (gallic acids) are of lower molecular weight than condensed tannins and are more easily absorbed in the digestive tract of ruminants. They are therefore considered more toxic (**HOSTE et al., 2006**). Studies on the effect of pure compounds isolated from *Rhus niveus* on the plant-parasitic nematode (*Meloidogyne incognita*) revealed that gallic acid was one of the more potent nematicidal compounds present (**SULTANA et al., 2010**). **MOHAMED et al. (2000)** concluded that natural tannins could possibly contribute to the search for novel sources of anthelmintics and that the use of tannin-rich plants could help control nematode infection in animals.

Eulophia petersii methanol pseudobulb extract was found to contain the highest total phenolic content. All DCM extracts of *E. petersii* significantly affected *C. elegans*. The tubers of *Eulophia nuda*, found in the Himalayas, are used to treat various ailments including, as a vermifuge to treat

worm infestation. The methanol extract and isolated phenolic compounds of the tubers exhibited cytotoxic potential (**SHRIRAM et al., 2010**). The anthelmintic effect of the extract may be due to the cytotoxic effect of phenolic compounds. Further analysis of *Cyrtorchis* and *Eulophia* species may reveal phenolic, flavonoid and tannin-based anthelmintic compounds that could be used in a more practical application in both veterinary and human nematode infections.

The medicinal value of flavonoids include: anti-inflammatory, antifungal, antioxidant and wound healing properties. The wound healing efficacy of *Oncidium flexuosum*, an epiphytic orchid used in Brazilian traditional medicine for inflammation and wounds, was attributed to the presence of flavonoids and tannins (**De GASPI et al., 2011**). The flavonoid content of *B. scaberulum* and *T. tridentata* may explain the potent activity observed in the anti-inflammatory and AChE inhibitory assays. The ethanolic root extract of *B. scaberulum* exhibited the most potent inhibitory effect on COX-2 and AChE; while the DCM tuber extract of *E. hereroensis*, was the only extract to significantly inhibit both COX enzymes. The flavonoid content in the leaf and root extracts of *T. tridentata* and *C. arcuata*, respectively, may play a role in the antioxidant activity observed in the HAT reaction-based assay. The presence of the flavonoids in certain orchid species may require further testing for antioxidant properties using alternative antioxidant mechanisms to that used in this investigation.

When testing for skin photoprotective effects, isolated stilbenoids from *Vanda coerulea* performed better than “hydro-alcoholic” solutions in the *in vitro* DPPH/·O radical scavenging and Parameter™ PGE-2 enzyme immunological assays. Imbricatin, a stilbenoid isolated previously from *Bulbophyllum* and other orchid genera, was one of three isolated stilbenoids recommended for use as skin photoprotectants based on antioxidant, anti-inflammatory and immunomodulatory effects (**SIMMLER et al., 2010**). The antioxidant and anti-inflammatory potential for South African orchid extracts is therefore great. Science can also enhance the effectiveness of a natural compound. Researchers produced a new class of active and selective AChE inhibitors by introducing amino acid moieties into the backbone of 4-hydroxybenzaldehyde. The resultant compounds exhibited more potent AChE inhibitory activity which performed better than galanthamine and demonstrated selectivity for AChE (**WEN et al., 2007**). Orchid extracts that performed well in both anti-inflammatory and AChE inhibitory assays may be potential natural plant product targets for inflammatory disorders. Extracts include: *A. africana* EtOH root, *B.*

scaberulum DCM root, *C. arcuata* EtOH root, *E. petersii* DCM stem and *T. tridentata* DCM root extracts.

The current investigation quantitatively determined the presence of flavonoids in the pseudobulbs and roots of *P. pubescens*. In the survey of leaf flavonoid content in Orchidaceae, **WILLIAMS (1979)** revealed xanthones, mangiferin and isomangiferin isolated from five species of *Polystachya* and *Maxillaria*. The author also observed that there was no pattern of flavonoid distribution within the family Orchidaceae, and geographical location played a significant role in the presence of flavonoid compounds (**WILLIAMS, 1979**). All four species, that did yield some flavonoid content, shared similar distribution ranges; and are all epiphytic species. The anti-inflammatory and antioxidant activity demonstrated in the current investigation was suggestive of cellular and neuroprotective effects of orchid extracts. *Cyrtorchis arcuata* and *T. tridentata* also share a similar distribution pattern, which may result in the accumulation of similar bioactive compounds and account for the comparable antioxidant effects. The use of the same vernacular name for *C. arcuata*, various *Eulophia* species and *Tridactyle* species, may be explained by their comparable positive anti-inflammatory and antioxidant effects detected during this investigation. A more comprehensive assessment of the chemistry of South African orchids would allow more confident assertions on relationships between chemical profiles, interaction between different classes of compounds, biological activity and influence of geographical location.

Determining the biological safety of natural plant products is as important as determining biological efficacy. Mutagenicity assessment programs have increased dramatically especially due to cancer concerns. There are two major concerns regarding mutagens, these are: their ability to either induce cancer or to affect the germ line in future generations. Compounds that affect mechanisms involved in the cell cycle and growth of cancer cells have long been targets in cancer research. Food derived flavonols such as quercetin, kaempferol and myricetin have been reported to reduce cancer risk by such mechanisms (**VERMA et al., 1988**; **YOSHIDA et al., 1990**). The non-genotoxic effects of *C. arcuata* coupled with the high flavonoid content necessitates further investigation in anti-cancer research. Primary compounds found in orchid extracts have also shown promise in anti-angiogenesis research; by reducing vascular permeability, one of the early signs of an inflammatory response and thus, one of the focus areas of cancer research. In the current investigation *B. scaberulum* roots exhibited mutagenic indices comparable to that of 4NQO. The

phytochemical profile of *B. scaberulum* stem/root extracts revealed phenolics, tannins and flavonoids. Antitumour activities have been detected in the *Bulbophyllum* genus (**YAO et al., 2005a**), with *B. odoratissimum*, in particular, containing cytotoxic phenolics (**CHEN et al., 2008b**). Phenanthrenes were mainly isolated from whole plants, cortex, tubers or stems and a large number where isolated from *Bulbophyllum vaginatum* (**KOVÁCS et al., 2008**). A more comprehensive chemical, toxicological, antimutagenic and pharmacological investigation of other organs of this species could reveal phenanthrenes, antitumor, anti-angiogenic, cytotoxic and phytotoxic properties of South African *Bulbophyllum* species. Compounds with phytotoxic properties may also be further investigated for their herbicidal potential.

The determination of the chemistry of orchid species may be beneficial to other avenues of orchid research. Taxonomic relationships within the Orchidaceae family are often described in relation to their chemical profiles with specific reference to certain classes of compounds such as alkaloids (**LAWLER** and **SLAYTOR, 1969**) or flavonoids (**WILLIAMS, 1979**). More recently, the chemicals and molecular mechanisms involved in the diverse pollination systems have been of interest, where chemical mimicry or deceit is used to encourage visits from pollinators (**NILSSON, 1992; BRODMANN et al., 2008; SCHLÜTER** and **SCHIESTL, 2008; TSAI et al., 2008**). Biotechnology, commonly used as an alternative method of orchid propagation (**SMITH** and **READ, 2008b**), can be applied to produce and recover significant metabolites from orchids (**WALTON et al., 2000**).

Recent medicinal plant research efforts address two main topics: the conservation of the more commonly used plants and their cultivation; and the pharmacological evaluation of the plants to verify their medicinal value and efficacy. Given the vast plant biodiversity and rich cultural heritage of South Africa and the renewed interest in medicinal plant research and conservation concerns, medicinal plants and holders of indigenous knowledge are valuable resources in our society. African traditional medicine is deeply rooted in ancient tradition and it is mainly the elders of a rural community that hold significant knowledge of herbal medicine (**LALONDE, 1993**). According to **VAN WYK et al.** (**1997**) such traditions are adaptable and open to change through modern developments. **SIMON and LAMIA (1991)** reported the dispensation of effective conventional medication such as penicillin by traditional healers. They have also shown keen interest in primary health care training programmes (**RICHTER, 2003**). Such partnerships should

be encouraged in future medicinal plant research. Furthermore, by formally documenting indigenous knowledge, conducting scientific investigations on medicinal and related plants to validate their use and providing adequate and suitable governmental support, African traditional medicine may become globally recognized as an established medical system

Plants are an important source of compounds that are ultimately used for drug development. High-value secondary metabolites have also been applied in cosmetic, food and agricultural industries. For previously identified high-value compounds, research has been channeled towards maintaining reliable sources. Current research is geared towards a continuous search for novel compounds, identification of biosynthetic pathways and determining the safety and commercial potential. Historically, plants were the only source of medication and they continue to demonstrate their therapeutic usefulness by being a part of, or possibly the only primary health care system in certain regions (**RIBEIRO et al., 2010**). In regions such as India, The Far East and South America, traditional medicinal practices are well integrated and plants used in these systems are widely studied in their respective countries and have made significant contributions to modern medicine (**CRAGG and NEWMAN, 2001**). A large percentage of modern medicines were derived from bioactive compounds isolated from plants already in use in traditional medicine.

The use of African traditional medicine is still not fully recognised in South Africa and abroad, and the medicinal plant research field is still establishing itself in South Africa, therefore orchids have not featured as yet. A number of biologically active compounds have been isolated from certain Orchidaceae species around the world on the basis of their traditional medicinal uses. Often their indigenous uses are validated, novel compounds are identified and potential uses in industries are discussed. The ethnobotanical uses of orchids in South Africa (**Table 3.1. Chapter 3**) have not been exploited pharmacologically (**CHINSAMY et al., 2011**). Scientific validation is essential as the uses are indigenous and unique to South African orchids. Also, given that the geographical location of plant species is one of the determining factors in the type and quantity of secondary metabolites produced; and that 75% of orchids in the southern African region are endemic (**STEWART et al., 1982**) it could be assumed that their unique geographical location would have some influence on the chemical composition of medicinally used species. There is therefore, great potential for revealing bioactive compounds which are unique to South African orchids.

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