An investigation of the medicinal properties of *Siphonochilus aethiopicus*

by

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

in the
School of Botany and Zoology
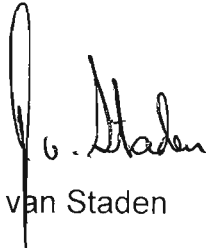University of Natal
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October 2002

DECLARATION

I hereby declare that this thesis, unless otherwise acknowledged to the contrary in the text, is the result of my own investigation, under the supervision of Professor J. van Staden and Dr. A.K. Jäger, in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg.

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ACKNOWLEDGEMENTS

Thank You Father God for the abilities you have given me, for Your all-sufficient grace and mercy, and Your love. All glory to You.

ABSTRACT

Siphonochilus aethiopicus (Schweinf.) B.L. Burtt (Zingiberaceae), commonly known as wild ginger, is a highly sought after plant for use in traditional medicine in South Africa. Over-exploitation of this medicinal plant has resulted in regional extinction in the wild. As a result, there is great interest in the medicinal properties of *S. aethiopicus*, and as a plant for small scale cultivation to increase the supply for use in traditional medicine.

Water, ethanol and ethyl acetate extracts were prepared from the leaves, rhizomes and roots of *S. aethiopicus*. These extracts were tested for *in vitro* anti-inflammatory activity in the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) assays, and in the microdilution antibacterial assay.

The aqueous extracts showed no significant prostaglandin synthesis inhibition in the COX-1 and COX-2 assays. The ethanol and ethyl acetate extracts of the leaves showed the highest levels of activity at a concentration of 250 μg ml^{-1} per test solution, in both the COX-1 and COX-2 assays. The ethanol and ethyl acetate extracts of the rhizomes and roots also had moderate levels of activity in the COX-1 assay. These results provide some evidence for the rational use of *S. aethiopicus* in traditional medicine for anti-inflammatory purposes.

In the microdilution antibacterial assay, no inhibitory activity against the test bacteria was detected with the aqueous extracts. The ethanol and ethyl acetate extracts tested showed greater antibacterial activity at minimal inhibitory concentrations ranging from 0.78 to 3.13 mg ml^{-1} against the Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) than the Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae*). No distinct differences were observed between the ethanol and ethyl acetate extracts, or between the different plant parts.

A serial extraction of *S. aethiopicus* rhizome material was conducted and the extracts were tested in the COX-1 assay and the microdilution assay as a preliminary investigation for a bulk extraction. The hexane and ethyl acetate extracts gave slightly higher COX-1 inhibition than the ethanol extract. No distinct differences were observed

in the microdilution assay.

A bulk ethyl acetate extract of *S. aethiopicus* rhizome material was prepared, yielding 6.3 g of a thin orange oil. Vacuum liquid chromatography (VLC) was used to fractionate ≈4 g of the extract. The VLC fractions were evaluated using thin layer chromatography (TLC) and a bioautographic assay, using *S. aureus* as a test organism. The fractions were also tested in the COX-1 assay. The bioautography revealed a number of compounds which exhibited antibacterial activity.

Fraction C was purified further using preparative TLC, and 24.9 mg of a pure compound from R_f 0.54 (toluene:ethyl acetate 93:7) was isolated. The structure of the compound was elucidated from nuclear magnetic resonance (NMR) spectra, and mass spectroscopy of the compound was also recorded. The compound was identified as the sesquiterpenoid furanoeremophil-2-en-1-one, which is structurally identical to the recently reported compound 4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-*b*]-furan-8-one. The compound showed only a very minimal bacteriostatic effect in the microdilution assay.

S. aethiopicus plants were harvested before and after seasonal senescence. Ethanol extracts were prepared from fresh or dried material of the leaves, rhizomes and roots, and tested in the COX-1 assay and the microdilution assay. TLC fingerprints of the various extracts were also prepared.

No noteworthy changes in COX-1 inhibition, due to senescence, were observed with extracts prepared from fresh material, although there did appear to be a slight decrease in activity in the α-roots and an increase in the β-roots after senescence (fresh and dry).

A decrease in the antibacterial activity of the leaves and an increase in the antibacterial activity of the α-roots was observed after senescence. These results suggest that the time of harvest may only have a minimal influence on the degree of anti-inflammatory and antibacterial activity.

PUBLICATIONS FROM THIS THESIS

LIGHT, M.E., McGAW, L.J., RABE, T., SPARG, S.G., TAYLOR, M.B., ERASMUS, D.G., JÄGER, A.K. and VAN STADEN, J. (2002). An investigation into the biological activity of *Siphonochilus aethiopicus* and the effect of seasonal senescence. *South African Journal of Botany* **68**:55-61.

CONFERENCE CONTRIBUTIONS FROM THIS THESIS

World Conference on Medicinal and Aromatic Plants
8-11 July 2001, Budapest, Hungary

Poster presentation:

Pharmacological activity of *Siphonochilus aethiopicus*

M.E. Light, L.J. McGaw, T. Rabe, S.G. Sparg, A.K. Jäger and J. van Staden

Bilateral (International) Scientific and Technological Cooperation: The screening of plant species for potential medicinal use
19 October 2001, Mol, Belgium

Oral presentation:

Pharmacological activity of *Siphonochilus aethiopicus*

M.E. Light, A.K. Jäger and J. van Staden

28th Annual Congress of SAAB
15-18 January 2002, Grahamstown, South Africa

Oral presentation:

Aspects of the pharmacological activity of *Siphonochilus aethiopicus*

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LIST OF ABBREVIATIONS

2-D	two dimensional
AA	arachidonic acid
AIDS	acquired immunodeficiency syndrome
AS	anisaldehyde/sulphuric acid reagent
ATCC	American Type Culture Collection
BSA	bovine serum albumin
COSY	correlation spectroscopy (^{1}H-^{1}H)
coxibs	selective COX-2 inhibitors
COX	cyclooxygenase
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid disodium salt
EtOAc	ethyl acetate
EtOH	ethanol
FW	formula weight
GC	gas chromatography
HETE	hydroxyeicosatetraenoic acid
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond correlations
HMQC	heteronuclear multiple quantum coherence
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
IC_{50}	median inhibitory concentration
ID_{50}	median inhibitory dose
INT	iodonitrotetrazolium chloride
LT	leukotriene
MBC	minimum bactericidal concentration
MH	Mueller-Hinton

MIC . . . . minimum inhibitory concentration

MLC . . . . minimum lethal concentration

MRSA . . . . methicillin-resistant *Staphylococcus aureus*

MS . . . . mass spectroscopy

MTT . . . . methylthaizoyltetrazolium chloride

NMR . . . . nuclear magnetic resonance

NOESY . . . . nuclear Overhauser exchange spectroscopy

NSAIDs . . . . non-steroidal anti-inflammatory drugs

PG . . . . prostaglandin

PGD_2 . . . . prostaglandin D_2

PGE_2 . . . . prostaglandin E_2

$PGF_{2\alpha}$ . . . . prostaglandin $F_{2\alpha}$

PGG_2 . . . . prostaglandin G_2

PGH_2 . . . . prostaglandin H_2

PGI_2 . . . . prostacyclin

PTLC . . . . preparative thin layer chromatography

R_f . . . . mobility relative to front

RNA . . . . ribonucleic acid

SD . . . . standard deviation

TLC . . . . thin layer chromatography

TPA 12-*O*-tetradecanoylphorbol-13-acetate

Tris . . . . 2-amino-2(hydroxymethyl)-1,3-propanediol (buffer)

TX . . . . thromboxane

TXA_2 . . . . thromboxane A_2

USA . . . . United States of America

UV . . . . ultraviolet

VLC . . . . vacuum liquid chromatography

1   Literature Review

1.1   INTRODUCTION

1.1.1 The medicinal value of plants

The recognition of the medicinal value of certain plants seems to have occurred in the history of all cultures. The sciences of medicine and botany have, for a long time, been very closely linked. One of the earliest records of the use of plants for medicinal purposes is found on clay tablets from Mesopotamia, written in cuneiform, and dating back to about 2600 BC (CRAGG and NEWMAN, 2001). Another ancient reference to the medicinal use of plants, which dates back to approximately 1550 BC, is found in the Ebers Papyrus discovered in 1873. In this Egyptian manuscript, some 700 herbal remedies are described, some of which are familiar today, such as opium and castor oil (WILLIAMS, 1947; PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997).

Two well developed systems of traditional medicine can be seen in Indian Ayurvedic medicine and Chinese traditional medicine, which have been extensively documented over centuries (CRAGG and NEWMAN, 2001). In the Indian system of Ayurvedic medicine, which has been in practice for centuries, about 2400 plant species are used for the treatment of human ailments. The properties and preparations of Indian medicinal plants have been well documented by ancient Indian scholars (BHAKUNI, 1986). The use of many crude plant extracts for the treatment of a wide range of ailments is also documented in well-respected pharmacopoeias, such as the European Pharmacopoeia and the United States Pharmacopoeia.

There are a number of well-known plants which have provided medical science with significant discoveries. Two highly valuable drugs for leukaemia chemotherapy were isolated from *Catharanthus roseus* (Madagascar periwinkle). Vinblastine is effective in the treatment of Hodgkins disease, and vincristine is a particularly effective treatment for leukaemia in children (ADDAE-MENSAH, 1992). Another well-known example is the isolation of digitoxin from *Digitalis purpurea*, the common purple foxglove, for the

treatment of certain kinds of heart disease. Many other examples of plant-derived drugs include aspirin, atropine, bulbocapnine, cocaine, codeine, colchicine, ephedrine, hyoscyamine, ipecac, morphine, papaverine, physostigmine, picrotoxin, pilocarpine, pseudoephedrine, quinidine, reserpine, scopolamine, strychnine, theophylline and *d*-tubocurarine (COX and BALICK, 1994; COX, 1995). A more recent example is taxol, a taxane diterpenoid from *Taxus brevifolia* (Pacific yew), which is used for the treatment of ovarian cancer (BALANDRIN, KINGHORN and FARNSWORTH, 1993).

1.1.2 Drug development from plants

The process of extracting and isolating active chemicals from plants forms an important part of modern drug development. In 1805 Sertürner isolated the first pure drug, morphine (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997), and in 1820 Caventou and Pelletier reported the isolation of quinine from the bark of *Cinchona*, for the treatment of malaria (CRAGG and NEWMAN, 2001). These early examples of the isolation of plant-derived drugs greatly extended this branch of science during the 19th century, and in recent years, we have seen an even greater expansion of research in the fields of phytochemistry, pharmacognosy and the study of medicinal plants. The field of pharmacognosy deals with studies of the chemistry of natural product drugs and has evolved from being a descriptive botanical subject to one having a more chemical and biological focus (KINGHORN, 2001).

The isolation of pure substances for pharmacological testing is often more desirable than the use of crude extracts, because the use of medicinal plants in their natural state can present several difficulties (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997). The content of the active constituents may vary with geographic locality, climatic and ecological conditions. The time of harvest and seasonal variation may also play an important role. Furthermore, the efficacy of raw plant material may deteriorate because of various collection, storage and preparation factors (COLEGATE and MOLYNEUX, 1993). The isolation of pure substances thus has the advantage that the administration of effective doses can be more accurate.

Another advantage to the extraction and isolation of a useful plant chemical is that the compound can be identified. From this identification, it may be possible to develop an economical synthesis of the chemical, thus avoiding the dependence on the plant as a source for the drug. Furthermore, chemists can gain valuable information from isolated natural products which could provide clues to structure/activity relationships and the design and development of novel drug compounds (COLEGATE and MOLYNEUX, 1993). It may also be possible to develop a drug with less side effects or improved efficacy. This is usually done through the chemical modification of biologically active substances (WILLIAMS, 1947). An example of this is the well-known, and probably most widely used drug, aspirin. Aspirin is a more effective analgesic and anti-pyretic drug from the synthetic acetyl derivative of salicylic acid, which occurs naturally in the bark of willow (*Salix alba*) and poplar (WILLIAMS, 1947).

Although modern chemistry has allowed for the development of many synthetic drugs, drugs and drug derivatives from plants still find extensive and increasing use. Natural products and their derivatives represent more than 50% of all drugs in clinical use in the world, with higher plants contributing about 25% to the total (CRAGG, NEWMAN and SNADER, 1997). Furthermore, BALANDRIN, KINGHORN and FARNSWORTH (1993) estimate that at least 85% of the world's estimated 250 000 species of higher plants have not been adequately surveyed for potentially useful biological activity. Many plant secondary metabolites are genus or species specific, and as a result, the chances of discovering new biologically active plant constituents are promising.

It is important to consider that the development of a clinical drug from a natural product is a formidable challenge and includes various hurdles such as toxicology, pharmacokinetics and metabolism studies. Few of the compounds which exhibit activity in laboratory tests become new drugs. Nevertheless, the discovery of biological activity and the subsequent isolation of the active principles are necessary first steps in the drug development process (COX and BALICK, 1994).

1.2 ETHNOBOTANY

1.2.1 Ethnobotanical approach to drug discovery

Drug discovery is generally based on three principal approaches: (1) the modification of structures of a known drug; (2) random screening; and (3) the synthesis of substances based on the knowledge of the biological process with which it is to interfere (LAURENCE and BENNETT, 1980). However, the incorporation of the information gained from traditional medicine and plant usage can greatly enhance the chances of discovering phytochemicals which may be medicinally useful. Ethnobotanical findings can be used for targeting plant materials in a more meaningful way, instead of relying on random screening procedures.

Ethnobotany is a broad term which refers to the study of plants by people, and encompasses the use of plants as foods, medicines, building materials or for other economic applications. Ethnomedicine (or ethnobotanical medicine) is a term which is used to refer to the use of plants by people for medicinal purposes (FARNSWORTH, 1994).

The ethnobotanical approach to drug discovery combines the analysis of indigenous knowledge and bioassay-guided evaluation of medicinally used plants (COX, 1995). Exploring the medicinal uses of plants by indigenous peoples and obtaining information of which plants are used in traditional medicine, how they are used, and for which ailments, potentially increases the chances for the discovery of novel therapeutic agents. This method of plant selection, based on their use in traditional medicine, has already provided a number of well-established drugs. FABRICANT and FARNSWORTH (2001) list 122 drugs from 94 plant species which have been discovered from ethnobotanical leads.

As discussed earlier, plants have long been used for various medicinal purposes by many cultures. The indigenous knowledge of the use of plants is often passed on orally from one generation to another, and in some cultures, through various written forms.

There is much concern that the loss of endemic cultures in many parts of the world, due to increased influence of other cultures, will result in the loss of empirical ethnobotanical knowledge that has been acquired over thousands of years (COLEGATE and MOLYNEUX, 1993). As a result, research in ethnobotanical medicine, the biological activity of medicinal plants and the active chemical principles have received renewed interest in recent years.

1.2.2 Traditional medicine in South Africa

In many developing countries traditional herbal remedies and traditional medicine play a major role in the health care of the people. As a result, the World Health Organization has estimated that 80% of the world's population rely on traditional medicine for their primary health care needs (FARNSWORTH, 1994). In such countries, most prescription medicines are either inaccessible, too expensive or not readily accepted by the people from a cultural perspective (VAN STADEN, 1999).

In South Africa, indigenous or traditional medicine forms an integral part of the culture and health care of many people. A report by MANDER (1998) on the trade of indigenous medicinal plants in KwaZulu-Natal, has estimated that there are some 27 million consumers of indigenous medicines in South Africa, and over 700 plant species which are actively traded. According to VAN WYK, VAN OUDTSHOORN and GERICKE (1997) approximately 3000 South African plants are used as medicines, with some 350 species being commonly used and traded as medicines.

In recent times, there has been a shift in traditional medicine, where not only the traditional herbalists (*izinyanga*) and the diviners (*izangoma*) are involved in the trade of medicinal plants. There is now an extensive trade network related to the sale of medicinal plants in rural and urban areas. Key components of this network include collectors, transporters, hawkers, wholesalers, retailers and traditional healers (MANDER, MANDER and BREEN, 1996). Many commercial gatherers, mostly women, also sell medicinal plant material at informal markets or supply formal-sector traders. Many of these gatherers know little about traditional healing and the sustainability of the

plant resources, but collect plants to generate income. Furthermore, most plant products are sold in the raw form, with little processing of the plants (CUNNINGHAM, 1991; VAN STADEN, 1999).

There has been an increased focus in recent years in ethnobotanical research and the study of medicinal plants. Part of the increased attention to this field in South Africa has been as a result of an understanding that there is a lack of detailed documentation on the use of medicinal plants, and the increased pressure for conservation (VAN WYK, VAN OUDTSHOORN and GERICKE, 1997). Most ethnobotanical knowledge is derived from traditional healers, whose understanding and knowledge of medicinal plants has been passed down orally through many generations. With rapid urbanisation, there is an increasing threat that much of this information will disappear (FABRICANT and FARNSWORTH, 2001).

1.2.3 Conservation of medicinal plants

Traditional healers are more numerous and more accessible than western doctors in most rural areas (CUNNINGHAM, 1988a). Furthermore, the demand for traditional medicine is considerably higher relative to the demand for western health care services, even in urban areas. There is therefore a great demand for medicinal plants in terms of number and the mass of plants used because traditional medicine is based almost entirely on the use of indigenous plants, which are collected from wild plant stocks (MANDER, 1998).

As is the case in many other countries, the harvesting of these wild stocks is not well managed in South Africa, and little cultivation takes place (MANDER, 1998). This high demand for popular plant species used for traditional medicine, and unrestricted collection, has resulted in the extinction of some species outside of protected areas, and the large scale decline of other species. Many of the most popular medicinal plants are slow-growing forest trees, from which bark is often destructively harvested, and bulbous and tuberous plants which are removed entirely. For example *Siphonochilus aethiopicus* (wild ginger) is now considered to be regionally extinct in KwaZulu-Natal and

is a protected species. Other protected species include *Ocotea bullata* (black stinkwood) and *Warburgia salutaris* (pepper bark tree) (SCOTT-SHAW, 1999). There is therefore an increasing need for conservation of many medicinal plant species through sustainable use of wild populations and enhanced supply through cultivation (MANDER, MANDER and BREEN, 1996).

The cultivation of medicinal plants, as suggested by MANDER, MANDER and BREEN (1996) and CUNNINGHAM (1988b), is one way in which to alleviate the intensive harvesting of wild stocks, and reduce the impact on biodiversity. However, for this to be successful there is a need for the development of greater understanding with respect to the cultivation and economics of producing useful indigenous plants (MANDER, MANDER and BREEN, 1996). Cultivation protocols and harvesting regimes for the different species need to be determined for successful cultivation of medicinal plants.

The cultivation of important medicinal plant species by farmers, gatherers and traditional healers is an important strategy for the conservation of endangered species. This would fulfil two functions, providing a means of employment and income, and an alternative source of medicinal plants which are in high demand (CUNNINGHAM, 1991). The propagation of medicinal plants by traditional healers and small scale farmers is receiving increasing attention and has the potential to alleviate some of the pressure on wild populations (JÄGER and VAN STADEN, 2000).

The possibility of plant substitution or plant-part substitution is a further means of alleviating the pressure placed on highly popular medicinal plants. ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN (2000) conducted a study to investigate the use of plant part substitution (i.e. the use of leaves instead of bark or underground parts) as a means of conservation through sustainable harvesting.

A further study by ZSCHOCKE and VAN STADEN (2000) was conducted on *Cryptocarya* species, the bark of which is used by traditional healers as an alternative to the very scarce *Ocotea bullata* (black stinkwood) bark. Results suggested that it may be possible to select alternative plant species to substitute for highly endangered

species. However, this is highly plant specific and would require the evaluation of any differences or similarities of chemical and pharmacological properties of the different plant parts. A further important aspect is that the traditional healers using the plants would need to be encouraged to use leaves or twigs instead of underground parts or bark, or use alternative plants (ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN, 2000).

1.3 *SIPHONOCHILUS AETHIOPICUS*

Siphonochilus aethiopicus (Schweinf.) B.L. Burtt, commonly known as wild ginger or Natal ginger, belongs to the monocotyledonous family Zingiberaceae (tribe Hedychieae). There has been some taxonomic confusion with the species *S. aethiopicus* and *S. natalensis*, however SMITH (1998) considers these two species to be synonymous. Synonyms of the species, as listed in the most recent taxonomic treatment, are *Cienkowskia aethiopica* Schweinf., *Kaempferia aethiopica* (Schweinf.) Benth., *K. ethelae* J.M.Wood, *K. natalensis* (Schlecht. and K.Schum.), *Siphonochilus natalensis* (Schlecht. and K.Schum.) J.M.Wood and Franks and *Cienkowskiella aethiopica* (Schweinf.) Y.K.Yam (SMITH, 1998). The plant is used extensively in traditional medicine in South Africa, and was therefore chosen as the focus for this investigation.

1.3.1 The Zingiberaceae

The Zingiberaceae is a family well known for its spice plants, including real ginger, *Zingiber officinale*; cardamom, *Elletaria cardamomum*; and turmeric, *Curcuma longa*. The exotic garden ginger *Hedychium* is another well known member of the Zingiberaceae. The species are usually caulescent aromatic herbs with thickened rhizomes and secretory cells with volatile oils (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996). Over 40 genera and about 1300 species are found in the Zingiberaceae. They occur mainly in the tropics of the Old World (Indomalasia), with some representatives in South and Central America (SMITH, 1998).

Many members of the Zingiberaceae are used in traditional medicine around the world. The CRC Ethnobotany Desk Reference (JOHNSON, 1999) documents over 100 different species of the Zingiberaceae which are used for a variety of medicinal purposes (see Appendix 1). There have been a number of investigations into the biological properties of many of these species, and the isolation of biologically active constituents.

1.3.1.1 Zingiberaceae: Anti-inflammatory activity

A variety of compounds which demonstrate anti-inflammatory activity have been isolated from a number of species of the Zingiberaceae. Hot aqueous extracts from the rhizomes of *Zingiber officinale* and *Alpinia officinarum* were investigated for their action against PG synthetase and 5-lipoxygenase, with gingerols and diarylheptanoids being identified as active compounds. The most active compound against PG synthetase was yakuchinone A, a diarylheptanoid from the fruit of *A. officinarum*, with an IC_{50} less than 1 µm (KIUCHI, IWAKAMI, SHIBUYA, HANAOKA and SANKAWA, 1992).

CLAESON, PANTHONG, TUCHINDA, REUTRAKUL, KANJANOPOTHI, TAYLOR and SANTISUK (1993) isolated three non-phenolic diarylheptanoids from hexane extracts of the rhizomes of *Curcuma xanthorrhiza*. These compounds showed significant anti-inflammatory activity in the assay of carrageenin-induced hind paw oedema in rats.

Bioassay-guided fractionation of extracts of the rhizomes of *Zingiber cassumunar* led to the isolation of three cassumunins with anti-inflammatory activity (MASUDA and JITOE, 1994). In further studies on *Z. cassumunar* by PONGPRAYOON, TUCHINDA, CLAESON, SEMATONG, REUTRAKUL and SOONTORNSARATUNE (1996), five compounds with topical anti-inflammatory activity were isolated from hexane extracts of the rhizome. These compounds gave ID_{50} values ranging from 2 to 62 µg/ear in the model of 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ear oedema in rats.

Two pimarane diterpenes have been isolated from *Kaempferia pulchra*, and were found to have ID_{50} values estimated at 330 and 50 µg/ear in the TPA induced rat ear oedema

bioassay (SEMATONG, REUTRAKUL, TUCHINDA, CLAESON, PONGPRAYOON and NAHAR, 1996).

Investigations on the constituents of the red rhizome variety of *Boesenbergia pandurata* yielded two cyclohexenyl chalcone derivatives which showed significant anti-inflammatory activity in the TPA induced rat ear oedema bioassay. The ID_{50} values of (-)-hydroxypanduratin A and (-)-panduratin A were determined as 84 and 12 μg/ear, respectively (TUCHINDA, REUTRAKUL, CLAESON, PONGPRAYOON, SEMATONG, SANTISUK and TAYLOR, 2002).

1.3.1.2 Zingiberaceae: Antibacterial activity

Antibacterial activity has been shown for a number of species of the Zingiberaceae. In a study by AHMAD, MEHMOOD and MOHAMMAD (1998), wherein 82 Indian medicinal plants were screened for antimicrobial properties, alcoholic extracts of the rhizomes of *Curcuma longa* showed activity against *Bacillus subtilis* and *Staphylococcus aureus*. Hexane extracts of the seeds of *Elettaria cardamomum* showed activity against *B. subtilis*, *Escherichia coli* and *S. aureus*, and alcoholic extracts showed activity against *B. subtilis*, *E. coli*, *Proteus vulgaris*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *S. aureus*. In the same study hexane and alcoholic extracts of *Zingiber officinale*, real ginger, showed antibacterial activity against *B. subtilis* and *S. aureus*.

The essential oil composition and antibacterial activity of oil from the seeds of *Aframomum citratum* was investigated in a study of the correlation between the chemical composition and antimicrobial activity of some African essential oils. The seed oil showed activity against *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *S. aureus* (CHALCHAT, GARRY, MENUT, LAMATY, MALHURET and CHOPINEAU, 1997).

The antibacterial activity of the fruit of *Aframomum melegueta* was studied by OLOKE, KOLAWOLE and ERHUN (1988). Volatile oils from the fruit pod and seeds and solvent extracts of the seeds were tested against a range of bacteria and showed activity against *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, *Pseudomonas*

fluorescens, Serratia marcescens, S. aureus and *Yersinia enterocolitica.*

The essential oils of *Kaempheria galanga* root and rhizome showed activity against *E. coli* and *S. aureus* (ARAMBEWELA, PERERA and WIJESUNDERA, 1999). In screening 13 species of *Alpinia, Costus* and *Zingiber*, most of the dichloromethane and methanol extracts showed some antibacterial activity against *B. subtilis*, methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa*. The strongest inhibitory activity of a dichloromethane extract was shown by *Alpinia mutica* with the minimum inhibitory dose of 125 µg per disc against both *B. subtilis* and MRSA (HABSAH, AMRAN, MACKEEN, LAJIS, KIKUZAKI, NAKATANI, RAHMAN and GHAFAR, 2000). Essential oils from fresh and dried rhizomes of *Alpinia galanga* also showed antibacterial activity against *B. subtilis* and *S. aureus* (JANSSEN and SCHEFFER, 1985).

The essential oil composition and antimicrobial activity of three Zingiberaceae species widely used as medicinal plants from S. Tomé e Príncipe were studied by MARTINS, SALGUEIRO, GONÇALVES, PROENÇA DA CUNHA, VILA, CAÑIGUERAL, MAZZONI, TOMI and CASANOVA (2001). The fruit of *Aframomum danielli* showed antibacterial activity in the agar diffusion assay against *E. coli, S. aureus, Staphylococcus epidermis, Streptococcus faecalis* and *P. vulgaris*. The zone diameter of inhibition of 15 µl of oil per disc ranged from 13 -19 mm. The oils of *C. longa* and *Z. officinale* also showed antibacterial activity against these bacteria. The oil of *Z. officinale* showed the most significant activity against *P. vulgaris* with an inhibition zone of 36 mm.

The hydrodistilled essential oil from the leaves of *Aframomum stipulatum*, used as a medicinal plant in the Democratic Republic of Congo, showed antibacterial activity against a variety of bacteria in the agar diffusion assay. The zone diameter of inhibition of 5 µl of oil per disc ranged from 10 -17 mm against *B. subtilis, Citrobacter* sp., *E. coli, K. pneumoniae, P. vulgaris, P. aeruginosa, S. typhimurium, Shigella flexneri* and *S. aureus* (CIMANGA, KAMBU, TONA, APERS, DE BRUYNE, HERMANS, TOTTÉ, PIETERS and VLIETINCK, 2002).

Members of the Zingiberaceae have also yielded novel compounds with antibacterial

activity. In phytochemical studies of *Amomum aculeatum*, two new minor compounds showing antibacterial activity were isolated from petroleum ether extracts of the rhizome. The first compound, a dioxa-dispiroketal, gave MIC values of 16, 16 and 8 μg ml^{-1} against *Bacillus cereus*, *E.coli* and *S. epidermis,* respectively. The second compound, an alkenone, gave MIC values of 32, 64 and 16 μg ml^{-1} against the same range of organisms (HEILMANN, BRUN, MAYR, RALI and STICHER, 2001).

1.3.2 Distribution and habitat of *S. aethiopicus*

S. aethiopicus is widely distributed throughout Africa, occurring southwards from Senegal and Ethiopia to the northern and eastern parts of South Africa (GORDON-GRAY, CUNNINGHAM and NICHOLS, 1989). It is the only member of the Zingiberaceae that is indigenous to South Africa (MAKHUVHA, VAN WYK, VAN DER BANK and VAN DER BANK, 1997), although three species of *Hedychium* and *Alpinia zerumbet* have become naturalised in certain areas (SMITH, 1998). *S. aethiopicus* grows in savanna and coastal grasslands, and in the ecotone of forests and bush clumps in damp and partially shady sites (ONDERSTALL, 1978; SCOTT-SHAW, 1999).

In South Africa, it has a restricted distribution in Mpumalanga and the Northern Province, and has become regionally extinct in KwaZulu-Natal as a result of overexploitation (MANDER, 1998; SCOTT-SHAW, 1999). It was known to have occurred at Ngoye, Inanda, Dumisa and Umbambasa (Port Shepstone area) and near Lusikisiki in Pondoland (SCOTT-SHAW, 1999). In a survey conducted by WILLIAMS (1996), medicinal plant traders in the Witwatersrand area recognised the scarcity of the plants and regarded *S. aethiopicus* to be in short supply.

1.3.3 Morphology and reproductive biology of *S. aethiopicus*

S. aethiopicus is a perennial herb with large, erect, hairless leaves which develop annually from small cone-shaped rhizomes. The rhizomes are segmented and fleshy, and narrowly elongate tubers are present on the fibrous roots (Figure 1.1). The crushed rhizome and roots yield a pungent scent similar to that of real ginger, *Zingiber officinale*.

Between 4 and 8 leaves develop on an unbranched false stem up to 60 cm tall during or after flowering (GORDON-GRAY, CUNNINGHAM and NICHOLS, 1989; SMITH, 1998). Flowers are borne separately from the leafy shoot, and appear at ground level in early summer, from the end of October to early December, usually just as the leaves start to appear. They are broadly funnel-shaped, pink-purple and white in colour with a small yellow patch in the throat. Most plants are bisexual, and have larger flowers than female plants (GORDON-GRAY, CUNNINGHAM and NICHOLS, 1989).
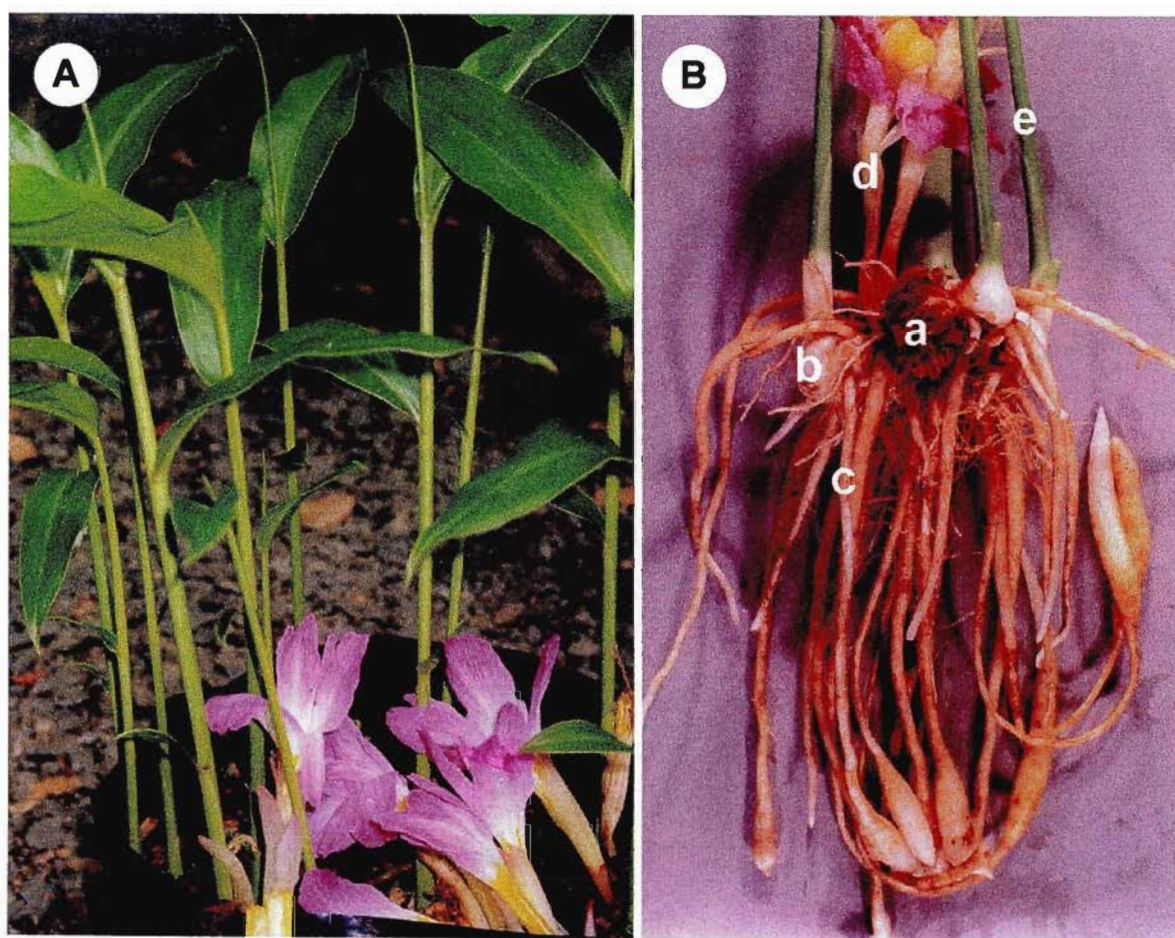
Figure 1.1 (A) *S. aethiopicus* plants with female flowers. (B) *S. aethiopicus* plant showing (a) rhizome from the previous year (α-rhizome); (b) current season rhizome segment (β-rhizome); (c) fleshy roots with small tubers; (d) female flowers; and (e) leaves.

This inconsistent polygamous production of hermaphroditic and female flowers, sometimes on the same rhizome (WOOD and FRANKS, 1911) has partly caused the confusion of the taxonomy of the species. Although as many as 20 flowers can develop from one plant, only one flower is fully open at a time and lasts for only one day (ONDERSTALL, 1978). Small, berry-like fruits are borne below or above the ground (GORDON-GRAY, CUNNINGHAM and NICHOLS, 1989).

Generally, little is known regarding the population biology of *S. aethiopicus*, however, GORDON-GRAY, CUNNINGHAM and NICHOLS (1989) have investigated some aspects of the floral and reproductive biology of the plant. The complete reproductive cycle is known for rhizomes that produce bisexual flowers, and details of the vegetative organs, flowering and growth cycle also apply to rhizomes that produce female flowers.

The rhizomes of *S. aethiopicus* grow either on the soil surface, or underground to a depth of 150 mm. During winter, no above ground parts are visible, but with the beginning of the spring rains the aerial shoots develop. These shoots continue to elongate after flowering, but growth gradually ceases after the December solstice. Yellowing of the leaves occurs quite quicky, usually during April, and by May the aerial parts have fully senesced (GORDON-GRAY, CUNNINGHAM and NICHOLS, 1989).

1.3.4 Volatile oils of *S. aethiopicus*

Volatile oils are generally used as medicines for their decongestant, antiseptic and diuretic effects (BRUNETON, 1995). It has been suggested that the monoterpenoids and sesquiterpenoids in the volatile oils in *S. aethiopicus* are responsible for the reported benefits against cough, colds and influenza (HUTCHINGS, 1989; VAN WYK, VAN OUDTSHOORN and GERICKE, 1997). However, anti-inflammatory activity has also been shown to be present in extracts of *S. aethiopicus* (McGAW, JÄGER and VAN STADEN, 1997; LINDSEY, JÄGER, RAIDOO and VAN STADEN, 1999; ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN, 2000).

In a study of the hydrodistilled essential oils of the roots and rhizomes of *S. aethiopicus*,

VILJOEN, DEMIRCI, BASER and VAN WYK (2002) identified 70 compounds in the root oil (representing 88% of the total oil composition) and 60 compounds in the rhizome oil (88% of total composition) using GC and GC-MS. The essential oils of the roots and rhizomes were very similar in composition, and showed only minor quantitative variations. However, none of the terpenoids of the oil of real ginger are present in the essential oil of S. aethiopicus.

The root and rhizome oils contain high quantities of a novel furanoterpenoid, $4a\alpha$H-$3,5\alpha,8a\beta$-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8-one, which represents approximately 20% of the oils (VILJOEN, DEMIRCI, BASER and VAN WYK, 2002; HOLZAPFEL, MARAIS, WESSELS and VAN WYK, 2002). Other major compounds in the oils are 1,8-cineole, (E)-β-ocimene and cis-alloocimene.

1.3.5 Medicinal uses of S. aethiopicus

S. aethiopicus is known in Zulu as indungulo/indungulu (WOOD and FRANKS, 1911) or isiphephetho (CUNNINGHAM, 1988b). Generally, the rhizome is dug up and used in traditional medicine (VAN WYK, VAN OUDTSHOORN and GERICKE, 1997). The rhizomes which are harvested during the growing season have roots on them which are also used. However, rhizomes which are harvested during the dormant period are leafless and do not have any roots. Usually, a mixture of crushed rhizomes and roots is drunk to relieve various ailments, or the rhizomes may also be chewed.

S. aethiopicus has been reported to be used for various respiratory ailments, febrile complaints, as well as nervous ailments (HUTCHINGS, 1989). It is also sometimes used as a charm for protection against snakes and lightening, or may be given to horses as prophylactics against horse sickness (WOOD and FRANKS, 1911; WATT and BREYER-BRANDWIJK, 1962; SMITH, 1998). Respiratory ailments include asthma, chest pain, coughs and sore throats. Febrile complaints include influenza, colds and fevers, including malaria and rheumatic fever (WATT and BREYER-BRANDWIJK, 1962). Nervous or psychological ailments include such things as hysteria, mental disturbance and nightmares. Other traditional uses include the treatment of

dysmenorrhoea (menstrual pain), pain, toothache, rheumatism and neuralgia (WATT and BREYER-BRANDWIJK, 1962; MANDER, MANDER, CROUCH, McKEAN and NICHOLS, 1995).

The use of *S. aethiopicus* for pain relief and inflammation has been supported through investigations into the inhibitory action against prostaglandin synthesis (McGAW, JÄGER and VAN STADEN, 1997; LINDSEY, JÄGER, RAIDOO and VAN STADEN, 1999; ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN, 2000). However, little is known about other pharmacological properties, and whether there is any seasonal influence on the levels of activity in *S. aethiopicus*. It is possible that plant material of different ages, or harvested at different times, may have varying levels of biological activity.

1.3.6 Popularity and trade of *S. aethiopicus*

In a report on the marketing of medicinal plants in KwaZulu-Natal, MANDER (1998) gives the trading (retail) price for *S. aethiopicus* as high as R450/kg, making it one of the most expensive plant products per kg. From the survey conducted, MANDER (1998) showed that 1.9 t of the plant was traded annually in the Durban Medicinal Trade. On a weight basis, *S. aethiopicus* ranked 9th of the popular species being sold. It was also frequently demanded by consumers, and ranked 8th according to the number of traders who identified the plants as important (MANDER, 1998).

1.3.7 Propagation and cultivation of *S. aethiopicus*

As mentioned earlier, *S. aethiopicus* has received the status of being regionally extinct in KwaZulu-Natal (SCOTT-SHAW, 1999). However, in recent years there has been significant interest in cultivating the plant, and successful attempts have been made at propagating *S. aethiopicus* on a small scale (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM, 1996; VAN WYK and GERICKE, 2000). Cultivation studies have been conducted to investigate the effect of propagule size, density and soil type on the yield (McCARTAN, GILLMER AND SYMMONDS, 1999).

S. aethiopicus is easily cultivated in the warmer parts of South Africa and large-scale production through tissue culture is possible (CROUCH and SYMMONDS, 2002). Furthermore, vegetative propagation, through rhizome division (simple splitting or longitudinal division), is a very efficient way of propagating *S. aethiopicus* as fruit set is generally poor, and seed are difficult to find (NICHOLS, 1989; CROUCH and SYMMONDS, 2002).

1.4   AIMS AND OBJECTIVES

Medicinal plant research is diverse and often involves a multi-disciplinary approach. CAVÉ (1986) describes the goals of research in this field as being (1) the identification of the active principles of medicinal plants (i.e. plants that "posess pharmacological activities of possible therapeutic use"), and the investigation of the extracts to ensure safety, effectiveness and constant activity; and (2) the isolation of the active principles and the determination of their structure, in order for synthetic development and structural modifications.

The aims of this study were to:
- Screen extracts of *S. aethiopicus* for anti-inflammatory and antibacterial activity;
- Investigate seasonal variation in anti-inflammatory and antibacterial activity;
- Investigate the effects of the state of plant material (fresh or dried) on anti-inflammatory and antibacterial activity; and
- Attempt to isolate and identify compounds exhibiting anti-inflammatory and/or antibacterial activity.

2 Investigation of Anti-inflammatory Activity

2.1 INTRODUCTION

2.1.1 Anti-inflammatory agents

Inflammation of living animal tissues occurs as a response to various kinds of "injuries". It involves a complex combination of effects such as enzyme activation, mediator release, extravasation of fluid, cell migration, tissue breakdown and repair (VANE and BOTTING, 1996). Drugs which are used to suppress inflammatory reactions fall into two main categories: the glucocorticoids and the non-steroidal anti-inflammatory drugs (NSAIDs) (RANG and DALE, 1987).

The NSAIDs are among the most widely used of all therapeutic agents and are used for a variety of musculoskeletal inflammatory conditions and pain. Large quantities of these drugs, such as aspirin and paracetamol, are bought over the counter for the treatment of headaches, toothaches and other minor complaints (RANG and DALE, 1987).

NSAIDs include a variety of different compounds from different chemical classes. Generally, they are classified into the following groups: salicylates (e.g. aspirin, diflunisal); acetic acids (e.g. indomethacin, diclofenac); propionic acids (e.g. ibuprofen, naproxen); fenamates (e.g. mefenamic acid, meclofenamate); pyrazoles (e.g. phenylbutazone); and oxicam (e.g. piroxicam) (MANTRI and WITIAK, 1994).

Most of the NSAIDs have three major types of effects, although not necessarily all to the same extent. These include the modification of the inflammatory reaction (anti-inflammatory effect), the reduction of certain types of pain (analgesic effect), and the lowering of a raised temperature (antipyretic effect). In general, these effects are all related to the primary action of the NSAIDs which is the inhibition of cyclooxygenase (COX) and thus the inhibition of the production of prostaglandins and thromboxanes (RANG and DALE, 1987). Many of the NSAIDs, however, also have various side effects in addition to their inhibition of COX. For example, aspirin has a particularly pronounced

action on platelets (RANG and DALE, 1987).

2.1.2 Prostaglandin pharmacology

The prostaglandins (PGs) and thromboxanes (TXs), along with leukotrienes (LTs), make up a group of substances known as the eicosanoids. They are implicated in the control of a wide variety of physiological processes and are among the most important mediators and modulators of inflammatory reactions (RANG and DALE, 1987). All mammalian cells, except red blood cells, produce these inflammatory mediators, whose physiological role involves the production of inflammation, pain and fever, the regulation of blood pressure and the induction of blood clotting (MANTRI and WITIAK, 1994).

The eicosanoids are generated in response to a wide range of stimuli. They are derived mainly from arachidonic acid (eicosatetraenoic acid) (Figures 2.1 and 2.2), although a small proportion may be derived from either dihomolinolenic acid or eicosapentaenoic acid. Arachidonic acid (AA) is present as a triglyceride ester in the phospholipids of cell membranes (MANTRI and WITIAK, 1994). Through the action of phospholipase A_2 (or phospholipase C and then diacylglycerol lipase), free arachidonic acid is liberated. Phospholipase A_2 action can also give rise to lyso-glyceryl-phosphorylcholine which is the precursor of platelet activating factor, another powerful mediator of inflammation (RANG and DALE, 1987). The free arachidonic acid is metabolised by two main pathways. The first is by the COX enzyme (prostaglandin H_2 [PGH_2] synthase) which initiates the biosynthesis of the prostaglandins (together referred to as the "prostanoids") and the thromboxanes. The second pathway, by various lipoxygenases (e.g. 5-lipoxygenase), initiates the synthesis of the leukotrienes, which are of importance as mediators of asthma (RANG and DALE, 1987; VANE and BOTTING, 1996). A summary of these pathways is seen in Figure 2.1.

COX is found bound to the endoplasmic reticulum and catalyses the formation of the cyclic endoperoxides, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) (Figure 2.1). This process incorporates both a cyclooxygenase and a peroxidase activity (VANE and BOTTING, 1996). This dual reaction occurs in almost every cell type in the body,

but the subsequent steps in the COX metabolic pathway differ depending on the cell type. This tissue-specificity is as a result of the selective presence of the enzymes which convert PGH_2 (MANTRI and WITIAK, 1994). In blood platelets the pathway leads to the synthesis of thromboxane A_2 (TXA_2), in vascular endothelium it leads to prostacyclin (PGI_2) and in macrophages prostaglandin E_2 (PGE_2) is mainly synthesized (RANG and DALE, 1987).

It has been found that the presence of the lipid peroxide enhances the COX activity and the formation of prostaglandins and thromboxanes. In normal tissue, peroxide levels are continually reduced by cellular peroxides, thereby limiting the production of prostanoids. However, in the presence of inflammatory signals, stimulated phagocytic cells generate superoxide and hydrogen peroxide, increasing the levels of peroxides which increase the synthesis of prostanoids (MANTRI and WITIAK, 1994; VANE, 1994).

The prostanoids PGE_2, PGI_2, $PGF_{2\alpha}$ and the thromboxane TXA_2 are probably the most important products of the COX pathway (Figures 2.1 and 2.2). Some of the main actions of the COX metabolites include the vasodilatation of blood vessels, effects on the aggregation of platelets, vasoconstriction and bronchodilatation in the lungs, and spasmogenic effects in the uterus. Their role in the production of pain in an area of inflammation is not a direct one. They have been shown to potentiate the effect of subthreshold concentrations of other pain-producing mediators such as histamine and bradykinin (RANG and DALE, 1987; VANE and BOTTING, 1996). Eicosanoids may also have a role in the pyretic effect of inflammation (i.e. fever). The antipyretic effect of NSAIDs is mainly due to their inhibition of the synthesis of PGE_1 in the hypothalamus, as a result of their inhibition of COX (RANG and DALE, 1987). The COX enzyme is inhibited by three different mechanisms: (1) reversible competitive inhibition; (2) mechanism based or time-dependent inactivation (irreversible inhibition); and (3) reversible noncompetitive inhibition (MANTRI and WITIAK, 1994).

Membrane-fixed phospholipids

Phospholipase A_2

Arachidonic acid

Cyclooxygenase   *5-Lipoxygenase*

PGG$_2$ **5-HPETE**

PGH$_2$ **LTA$_4$**   **5-HETE**

Prostacyclin synthase   *Thromboxane synthase*

PGI$_2$
Vascular endothelium, stomach mucosa

TXA$_2$
Platelets

LTB$_4$ **LTC$_4$**

6-keto-PGF$_{1\alpha}$ **TXB$_2$**   **LTD$_4$**

LTE$_4$

PGD$_2$
Brain tissue
Mast cells

PGE$_2$
Kidney

PGF$_{2\alpha}$
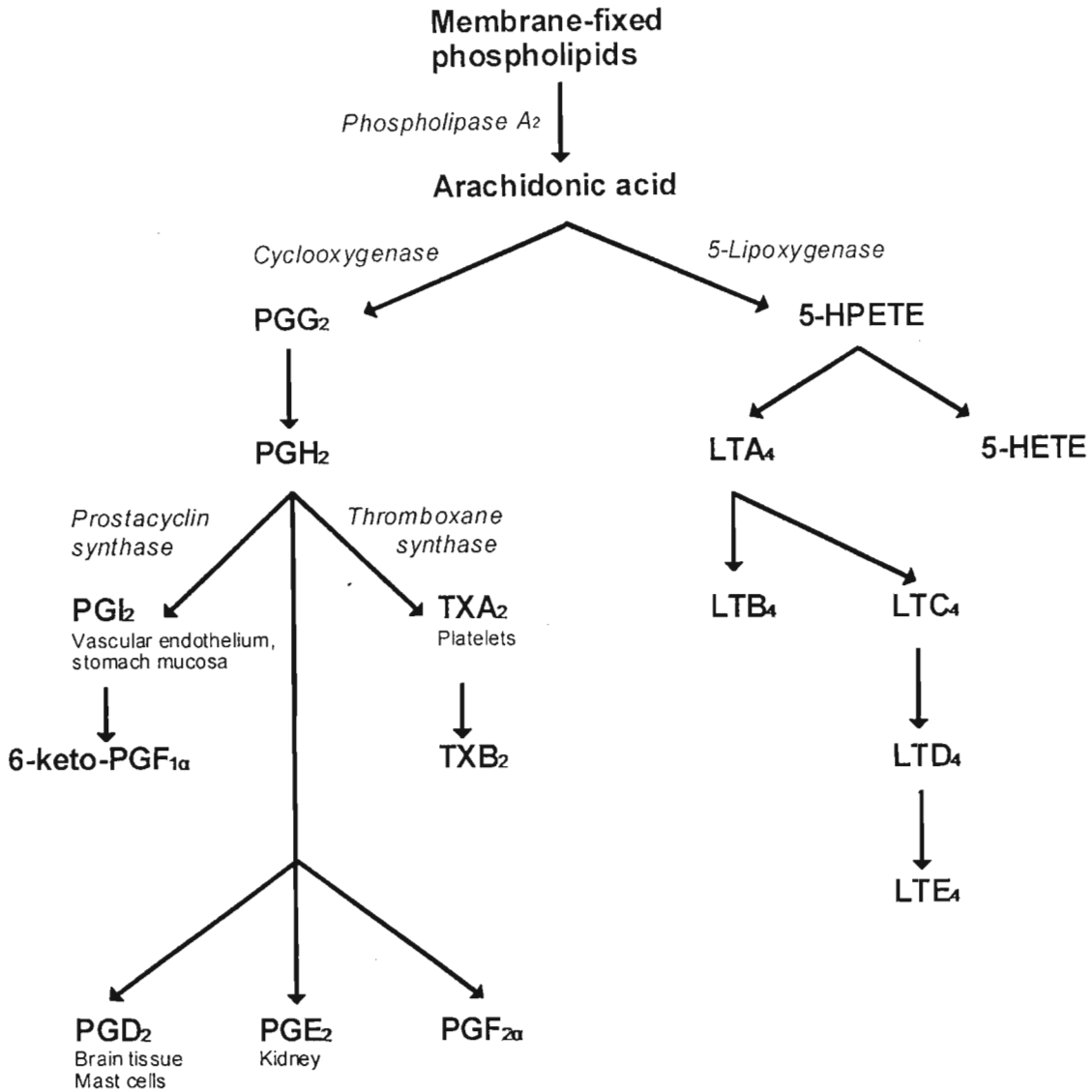
Figure 2.1   A schematic representation of the biosynthesis of arachidonic acid metabolites - prostaglandins and related eicosanoids. PG = prostaglandin; PGI_2 = prostacyclin; TX = thromboxane; LT = leukotriene; HPETE = hydroperoxyeicosatetraenoic acid; HETE = hydroxyeicosatetraenoic acid. (Adapted from RANG and DALE, 1987).

(A) Arachidonic acid

(B) PGD$_2$

(C) PGE$_2$

(D) PGF$_{2\alpha}$

(E) PGI$_2$ (Prostacyclin)
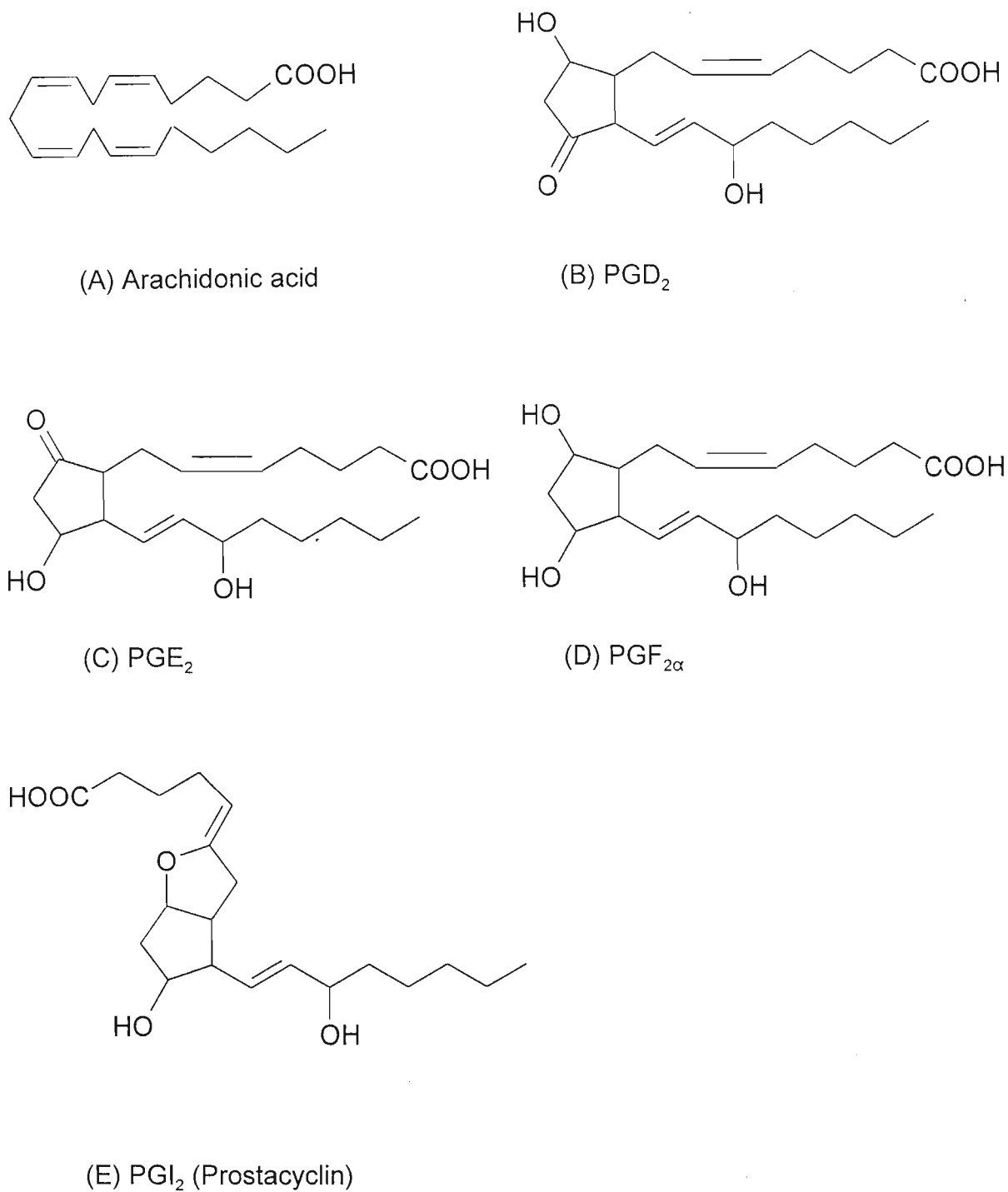
Figure 2.2 The chemical structures of (A) Arachidonic acid; (B) PGD$_2$; (C) PGE$_2$; (D) PGF$_{2\alpha}$; and (E) PGI$_2$. (From RANG and DALE, 1987).

2.1.3 Cyclooxygenase enzymes: COX-1 and COX-2

The COX enzyme exists in two isoforms, COX-1 and COX-2 (MANTRI and WITIAK, 1994; VANE, 1994). COX-1 is constitutively expressed in most tissues, and is necessary for the regulation of normal cell activity ("housekeeping") (VANE and BOTTING, 1996). For example, the production of prostacyclin (PGI_2) by the endothelium provides an antithrombogenic function (VANE, 1994), and in the gastric mucosa it is cytoprotective (WHITTLE, HIGGS, EAKINS, MONCADA and VANE, 1980). COX-2, however, is synthesized *de novo* in a number of cells, such as neutrophils and mast cells, by inflammatory stimuli (e.g. bacterial endotoxins) and/or cytokines (e.g. tumor necrosis factor and interleukin 1β) (XIE, ROBERTSON and SIMMONS, 1992). A summary of the actions of the two isoforms is given in Figure 2.3.
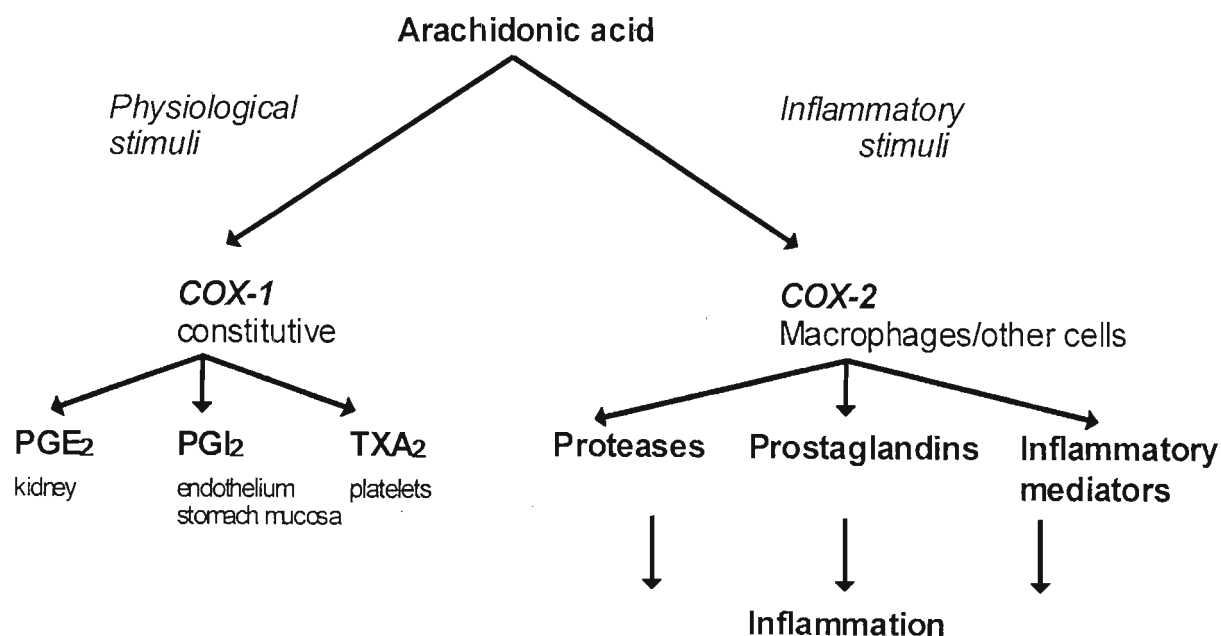
Figure 2.3   Actions of the two cyclooxygenase isoforms COX-1 and COX-2. (Adapted from VANE and BOTTING, 1996).

It has been suggested that the anti-inflammatory action of most NSAIDs is due to the inhibition of COX-2, and that many of the unwanted side-effects (such as gastric bleeding and ulceration, reduced platelet aggregation, and toxic effects on the kidney)

are due to the inhibition of COX-1 (VANE, 1994). The activities of NSAIDs against the two enzymes differ, ranging from a high selectivity towards COX-1, through to equal activity (VANE, 1994). For example, NSAIDs such as aspirin and indomethacin are more potent inhibitors of COX-1 than COX-2 (MEADE, SMITH and DE WITT, 1993). This range of activities can explain the variation in the side-effects observed with many NSAIDs.

The difference in activities against COX-1 and COX-2 can be expressed as a ratio of the IC_{50} values. A high ratio indicates more potent inhibition of COX-1. For example, aspirin and indomethacin have been shown to have COX-1/COX-2 ratios of 166 and 60 respectively (VANE and BOTTING, 1996). However, the published COX-1/COX-2 ratios can vary according to the test system used and are not ideal for predicting in vivo activity (VANE and BOTTING, 1996).

In more recent years, there has been increased attention at finding NSAIDs which selectively inhibit COX-2 only, and do not interfere with COX-1. Compounds which are selective inhibitors of COX-2 are potentially anti-inflammatory and nonulcerogenic and would therefore be of considerable interest for therapeutic use (MANTRI and WITIAK, 1994; VANE, 1994). These compounds should have fewer side effects and cause less gastric irritation. However, in more recent studies, as reviewed by SAUTEBIN (2000), it seems that the role of COX-1 and COX-2 is more complex than the original hypothesis which was first put forward. It has also been shown that both COX-1 and COX-2 are expressed within the normal adult kidney, indicating that COX-2 does not exist purely as an inducible enzyme (KÖMHOFF, GRÖNE, KLEIN, SEYBERTH and NÜSING, 1997).

To date, two highly selective COX-2 inhibitors (Coxibs) have been launched for therapeutic use: rofecoxib and celecoxib (EMERY, 2001). These two coxibs have been shown to be safe and effective in the treatment of rheumatoid arthritis, osteoarthritis and produced no inhibition of gastric mucosa PG production, in contrast to conventional NSAIDs. Furthermore, thromboxane synthesis and platelet aggregation is not inhibited by COX-2 inhibitors. However, some of the side effects of NSAIDs are still observed

with these coxibs, such as fluid retention and dyspepsia (EMERY, 2001; SCHNITZER, 2001).

2.1.4 Testing COX inhibition

Plants potentially provide a useful source of new effective anti-inflammatory agents, and the results gained from preliminary *in vitro* studies are necessary in order to subsequently perform targeted *in vivo* studies. However it should be remembered that molecules which exhibit good *in vitro* activity are not always selective in *in vivo* systems (SAUTEBIN, 2000). Non-steroidal natural products which have shown inhibition against COX are found among the flavonoids, phenolic phenylpropane derivatives, naphthoquinones, alkylamides, diaryl heptane derivatives and tannins (BOHLIN, 1995).

There are a variety of models which have been developed to test the activity of NSAIDs, as well as crude plant extracts, for inhibitory action against COX-1 and COX-2. The use of whole cell systems is an important method which has been developed, because the penetration of cell membranes is an important aspect of the activity of NSAIDs (VANE and BOTTING, 1996). However, for the purpose of assessing the relative selectivity of plant extracts on COX-1 and COX-2, it is often simpler to use purified enzymes or microsomal preparations in *in vitro* systems. Furthermore, these type of *in vitro* bioassays are advantageous in bioassay-guided fractionation of active extracts where a large number of fractions need to be tested along with phytochemical separation work (KIUCHI, IWAKAMI, SHIBUYA, HANAOKA and SANKAWA, 1992).

The inhibition of prostaglandin synthesis can be evaluated using an *in vitro* enzyme assay adapted from an assay originally described by WHITE and GLASSMAN (1974) to detect activity of COX. This assay, with various modifications, has been used extensively for the evaluation of plant extracts. Ovine seminal vesicles are usually used as a source for the microsomal preparation of the COX-1 enzyme.

Indomethacin (Figure 2.4) is a potent inhibitor of COX in *in vitro* tests. Although it is clinically effective, it has been shown to have high incidences of side effects (RANG and

DALE, 1987). However, it is a highly suitable compound for use as a reference standard when testing for anti-inflammatory action of plant extracts in the COX assay. Similarly, the selective COX-2 inhibitor, nimesulide, can be used as a reference standard in the COX-2 assay (Figure 2.4). The arylsulfonamide NS-398 has also been used successfully as a COX-2 standard (NOREEN, RINGBOM, PERERA, DANIELSON and BOHLIN, 1998).

(A) indomethacin   (B) nimesulide
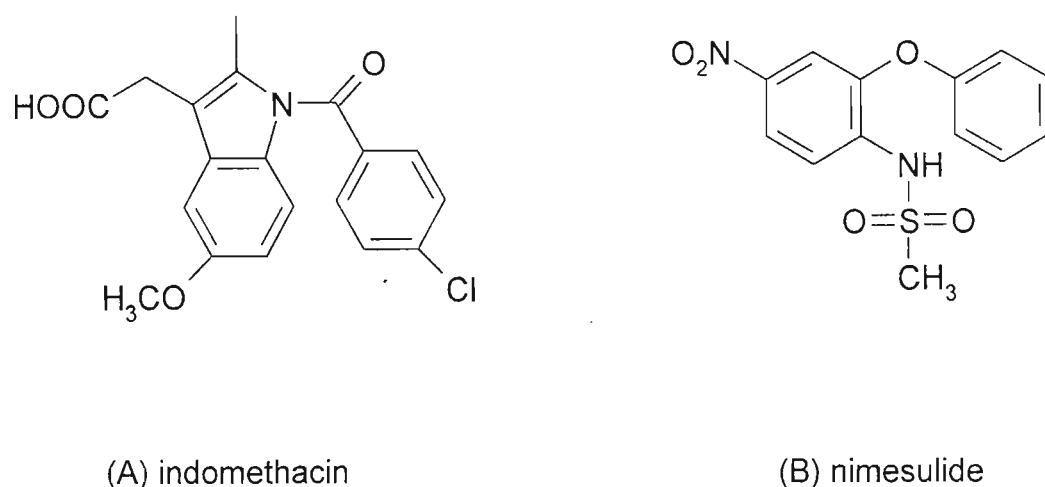
Figure 2.4   The chemical structures of (A) indomethacin (FW = 357.8) and (B) nimesulide (FW =308.3).

The presence of polyphenols, saponins, certain pigments or fatty acids in crude plant extracts can result in false positives in enzyme assays. It is often preferable to remove such undesirable compounds from plant extracts prior to screening, although it is usually easier to first test the crude extracts, and then discriminate between false and true positive results at a later stage (O'NEILL and LEWIS, 1993).

2.2   MATERIALS AND METHODS

2.2.1   Extract preparation

S. aethiopicus plants were obtained from stock plants at the University of Natal Botanical Garden, Pietermaritzburg. A voucher specimen was deposited in the

University of Natal Herbarium (*Light 17* NU). Mature plants were harvested in January (summer) and divided into leaves, rhizomes and roots. This material was cut into smaller pieces, placed in brown paper bags, and dried at 50 °C for two days. Dried material was stored in the dark at room temperature until extraction.

Dried material was finely ground using an A10 analysis mill (Janke & Kunkel, IKA®-Labortechnik). The material (10 g) was extracted in 100 ml solvent (water, ethanol or ethyl acetate) in an ultrasound bath (Branson 5210) for 60 min. The extracts were vacuum filtered through Whatman No. 1 filter paper, and dried under vacuum at 35 °C.

The residues from the plant extracts were redissolved to a concentration of 10 mg ml^{-1} in water (for aqueous extracts) or ethanol (for ethyl acetate or ethanol extracts). Each extract was tested for *in vitro* prostaglandin synthesis inhibition in both the COX-1 and COX-2 assays.

2.2.2 Testing COX-1 inhibition

2.2.2.1 Bioassay protocol

The COX-1 assay was performed as described by JÄGER, HUTCHINGS and VAN STADEN (1996). The COX-1 enzyme (10 µl microsome suspension from sheep seminal vesicles, 0.35 µg protein) was activated with 50 µl of co-factor solution (0.3 mg ml^{-1} L-adrenaline and 0.3 mg ml^{-1} reduced glutathione in 0.1 M Tris buffer, pH 8.2) on ice for 15 min. The enzyme solution (60 µl) was added to the sample solution (2.5 µl ethanolic or aqueous plant extract and 17.5 µl water) and preincubated at room temperature for 5 min. The plant extracts were tested at a concentration of 10 mg ml^{-1}, giving a final concentration of 250 µg ml^{-1} per test solution. Indomethacin (5 µM) was tested as a positive control. For each assay, a duplicate set of samples were tested. The reaction was started by adding 20 µl ^{14}C-arachidonic acid (30 µM, 16 Ci mol^{-1}) (Amersham). Samples were incubated in a water bath for 10 min at 37 °C. The reaction was terminated by adding 10 µl 2 N HCl.

Prostaglandins and unmetabolized arachidonic acid were separated by column chromatography over silica gel, after addition of 4 µl 0.2 mg ml^{-1} unlabeled prostaglandins (PGE$_2$:PGF$_{2\alpha}$ 1:1) (Sigma) as a carrier solution. Small silica columns were prepared by packing silica gel (silica gel 60, 0.063-0.200 mm, Merck) to a height of 3 cm in Pastueur pipettes stoppered with glass wool. The test solution was applied to the column with 1 ml of hexane:1,4-dioxan:glacial acetic acid (70:30:0.2). The arachidonic acid was eluted first with 4 ml of the hexane:1,4-dioxan:glacial acetic acid. The prostaglandin products were then eluted with 3 ml of ethyl acetate:methanol (85:15) and collected in scintillation vials. After mixing with 4 ml of scintillation fluid, the samples were counted in a Beckman LS 6000LL scintillation counter. Percentage inhibition of the test solutions was calculated using the following formula:

$$\% \text{ Inhibition} = \left[1 - \left[\frac{\left(\text{RADIOACTIVITY}_{\text{Extract}} - \text{RADIOACTIVITY}_{\text{Background}} \right)}{\left(\text{RADIOACTIVITY}_{\text{Solvent Blank}} - \text{RADIOACTIVITY}_{\text{Background}} \right)} \right] \right] \times 100$$

Inhibition refers to reduction of PGE$_2$ formation in comparison to an untreated sample (2.5 µl ethanol in 17.5 µl water).

2.2.2.2 Enzyme preparation

The COX-1 enzyme was prepared from fresh sheep seminal vesicles. The urinary organs were obtained from freshly slaughtered rams directly from the slaughter line at the Ladysmith Abattoir. These were kept on ice and transported to the laboratory where the microsomal preparation was conducted.

Eight seminal vesicles (± 4 cm in diameter) were trimmed free from fat and connective tissue, and cut into smaller pieces on a glass petri dish placed on a layer of ice. Fifty grams of the seminal vesicles were placed in a beaker with 150 ml 0.1 M potassium phosphate buffer (pH 7.4) with 1 mM EDTA (ethylene diamine tetra-acetic acid disodium salt). The beaker was kept on ice, and the mixture homogenized using an Ultra-Turrax T25 homogenizer (Janke & Kunkel, IKA$^{®}$-Labortechnik) at medium speed.

The homogenate was centrifuged at 4000 g for 15 min (Sorvall RC-5 Centrifuge) to remove cell debris. The supernatant was decanted and centrifuged at 17 000 g for 10 min to remove the mitochondria. The microsomes were isolated from the supernatant by ultracentrifugation of 60 ml at 100 000 g for 1 h (Beckman L-80 Ultracentrifuge). The resulting pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4).

The protein concentration was determined using the Bio-Rad Protein Assay Kit. The standard procedure was slightly modified by pipetting 20 μl of each standard or sample solution and 1 ml of the diluted dye reagent directly into a cuvette. A standard curve was constructed using bovine serum albumin (BSA) as a protein standard at concentration range from 0.2 to 1.2 mg ml^{-1} (Figure 2.5). The protein concentration of the enzyme preparation was diluted with 0.1 M potassium phosphate buffer (pH 7.4) to approximately 10 mg ml^{-1}, by comparing appropriate dilutions against the standard curve. Aliquots of 10, 100 and 1000 μl were pipetted into Eppendorf tubes and stored at -70 °C (JÄGER, HUTCHINGS and VAN STADEN, 1996).
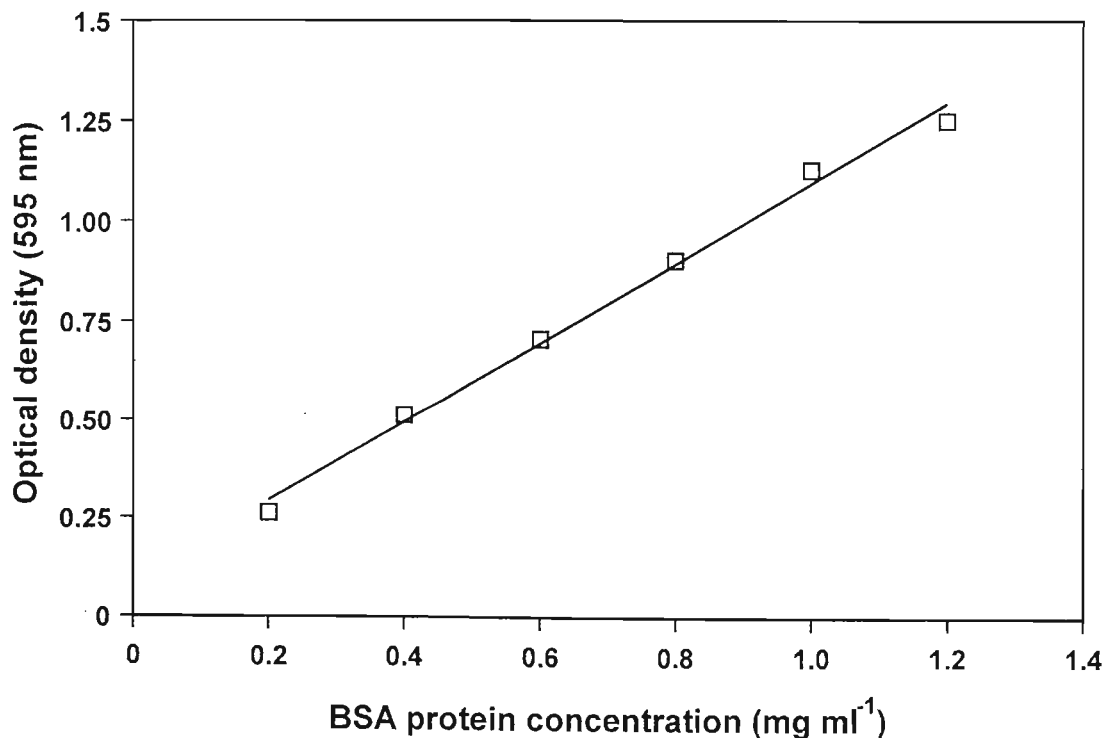
Figure 2.5   BSA standard curve for the determination of the protein concentration of the microsomal preparation.

2.2.2.3 Optimization of the bioassay

The optimum concentration and incubation time of the enzyme preparation for use in the COX-1 assay was determined by testing various enzyme concentrations, followed by testing a range of incubation times.

2.2.2.4 Enzyme concentration

The enzyme preparation was tested in the COX-1 assay at various dilutions ranging from 0 to 1%. Solvent blanks (2.5 μl ethanol and 17.5 μl water) were used as the test solutions, and the incubation time at 37 °C was 10 min. From the results obtained, a suitable enzyme concentration for use in the COX-1 assay was determined, by choosing a dilution giving approximately a third of the total radioactivity of the ^{14}C-arachidonic acid (i.e. 300-360 kBq).

2.2.2.5 Optimal incubation time

The optimal incubation time for the arachidonic acid and enzyme at 37 °C was tested using enzyme concentrations of 0.3, 0.35 and 0.4% at a range of incubation times from 0 to 20 min.

2.2.2.6 Indomethacin standard curve

Indomethacin solutions, ranging between 10^{-7} M to 10^{-5} M, were assayed for activity in the COX-1 assay. From the results obtained, a dilution curve was constructed, and the IC_{50} value determined using regression analysis of the log concentrations.

2.2.3 Testing COX-2 inhibition

2.2.3.1 Bioassay protocol

The COX-2 assay was performed as described by NOREEN, RINGBOM, PERERA,

DANIELSON and BOHLIN (1998), with slight modifications (ZSCHOCKE and VAN STADEN, 2000). The COX-2 assay follows a very similar protocol as the COX-1 assay. Purified COX-2 enzyme from sheep placental cotyledons was purchased from Cayman Chemicals. The enzyme (10 µl containing 3 units) was activated with 50 µl co-factor solution (0.6 mg ml^{-1} L-adrenaline, 0.3 mg ml^{-1} reduced glutathione and 1 µM hematin in 0.1 M Tris buffer, pH 8.0) on ice for 5 min. The enzyme solution (60 µl) was added to the sample solution (2.5 µl ethanolic or aqueous plant extract and 17.5 µl water) and preincubated at room temperature for 5 min. The plant extracts were tested at a concentration of 10 mg ml^{-1}, giving a final concentration of 250 µg ml^{-1} per test solution. Positive control measurements were carried out with indomethacin and nimesulide at concentrations of 200 µM. For each assay, a duplicate set of samples were tested. The reaction was started by adding 20 µl ^{14}C-arachidonic acid (30 µM, 16 Ci mol^{-1}) (Amersham). Samples were incubated in a waterbath for 10 min at 37 °C. The reaction was terminated by adding 10 µl 2 N HCl. Prostaglandins and unmetabolized arachidonic acid were separated and COX-2 inhibition was determined as described for the COX-1 assay.

2.2.3.2 Indomethacin standard curve

Indomethacin solutions, ranging between 10^{-5} M to 10^{-3} M, were assayed for activity in the COX-2 assay. From the results obtained, a dilution curve was constructed, and the IC$_{50}$ value determined using regression analysis of the log concentrations.

2.3   RESULTS AND DISCUSSION

2.3.1 Enzyme preparation and bioassay optimization

The results of testing different enzyme concentrations are shown in Figure 2.6. A concentration of 0.4% gave a value of approximately 240 kBq. This value was slightly less than a third of the radioactivity of the ^{14}C-arachidonic acid substrate, which is the maximum level of conversion that is within the linear range. Testing of an enzyme concentration of 0.5% resulted in a very steep curve, and further testing to determine

the optimal incubation time was therefore conducted at enzyme concentrations of 0.3, 0.35 and 0.4%.
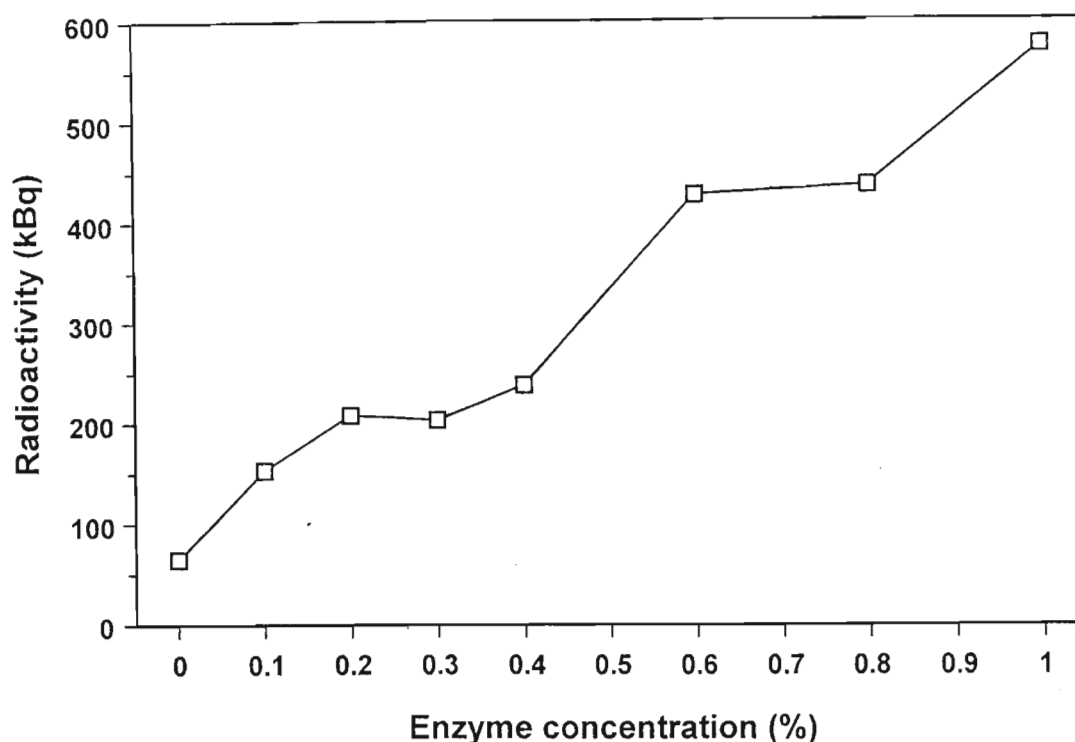
Figure 2.6   Standard curve for the determination of the optimal enzyme concentration for the COX-1 assay.

Testing the different enzyme concentrations at varying incubation times did not result in a curve with a clear linear relationship as expected (Figure 2.7). However, at an enzyme dilution of 0.35% and an incubation time of 10 min the enzyme activity was approximately steady, and the radioactivity sufficiently high (300-360 kBq). This concentration and incubation time was therefore chosen for use in the COX-1 assay, in order to fulfill the requirements of a repeatable assay in which many samples can be tested together (NOREEN, RINGBOM, PERERA, DANIELSON and BOHLIN, 1998).

Results from the tests to optimize the enzyme concentration and time duration for the COX-1 assay illustrate the sensitive nature of the assay system. Although it has been used successfully for studying *in vitro* inhibition of prostaglandin synthesis, it is important that it is performed carefully and accurately to obtain repeatable results.
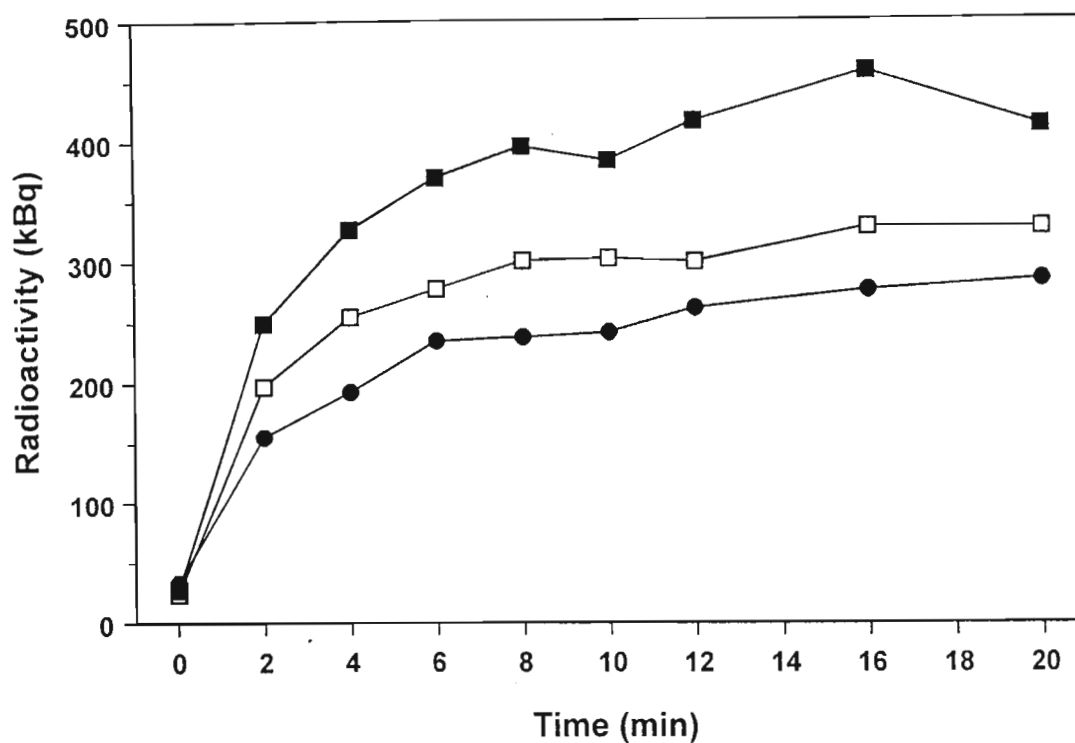
Figure 2.7 Standard curves for the determination of the optimal incubation time and enzyme concentration for the COX-1 assay. (Enzyme dilutions: ● 0.3%; □ 0.35%; ■ 0.4%).

2.3.2 Indomethacin standard curves

The inhibition of COX-1 and COX-2 by various concentrations of indomethacin are shown in Figures 2.8 and 2.9. The IC_{50} for indomethacin in the COX-1 assay was calculated to be 0.9 µM and in the COX-2 assay to be 5.3 µM. Although indomethacin is not a COX-2 specific inhibitor, it does show inhibition of COX-2 at higher concentrations than against COX-1.
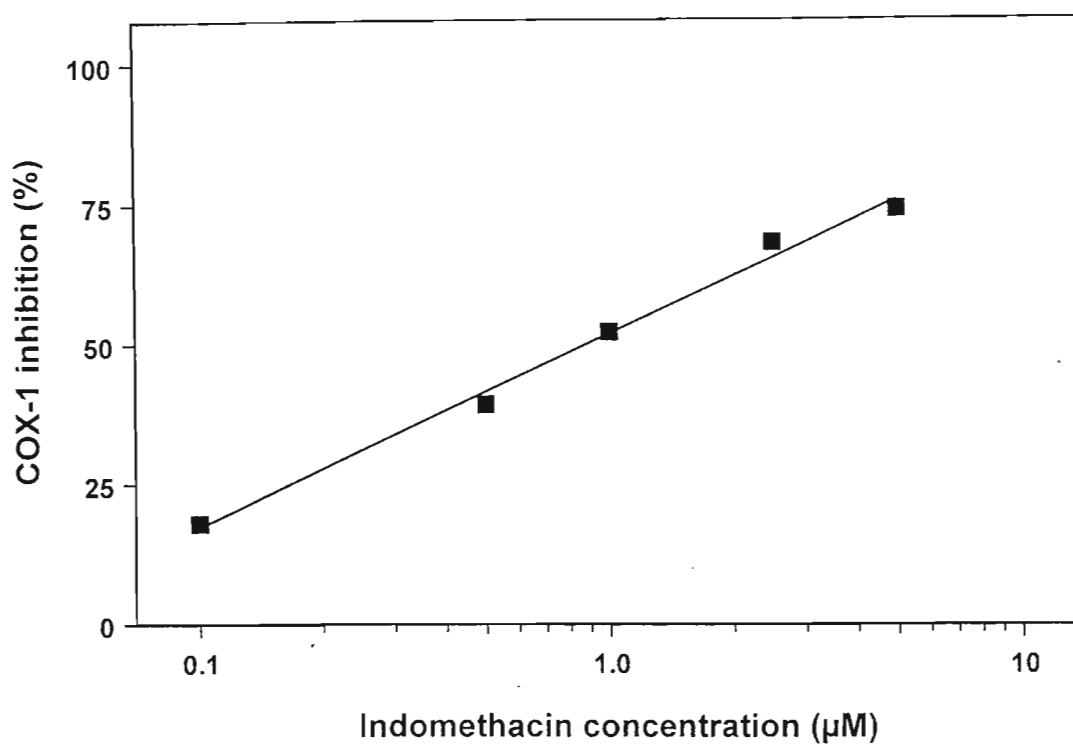
Figure 2.8   Standard curve for the inhibition of COX-1 by indomethacin.
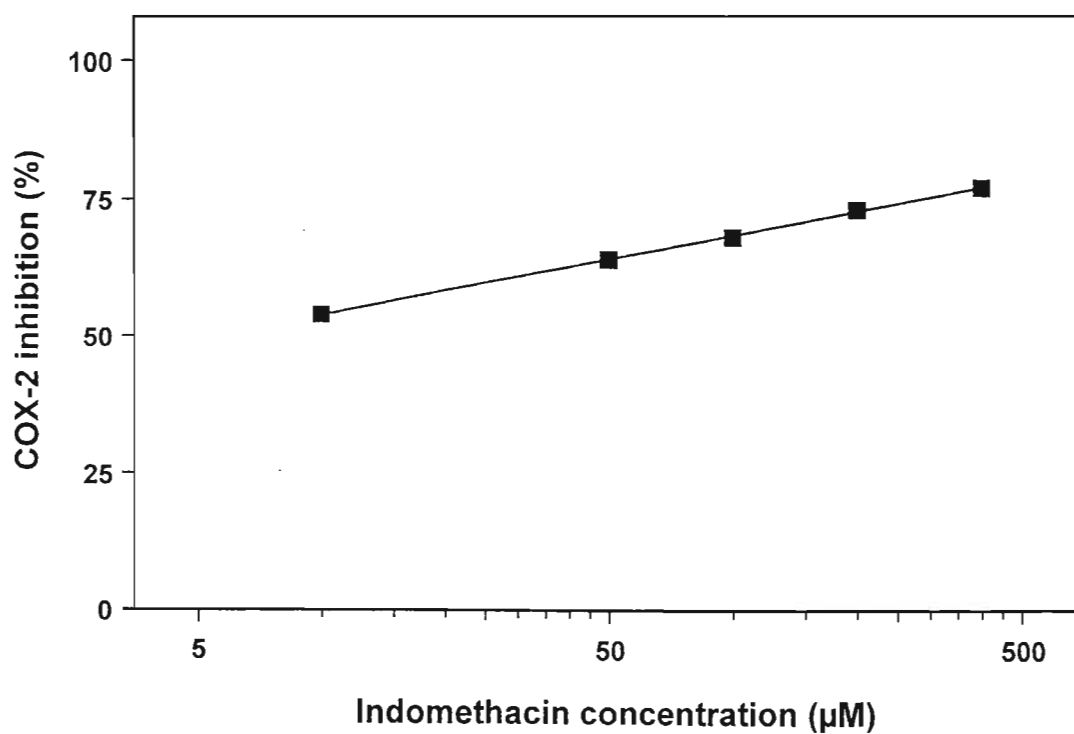
Figure 2.9   Standard curve for the inhibition of COX-2 by indomethacin.

2.3.3 COX-1 inhibition by *S. aethiopicus* extracts

The results for the COX-1 assay are shown in Figure 2.10. Aqueous extracts showed no noteworthy activity. Ethanol and ethyl acetate extracts of the leaves showed high levels of COX-1 inhibition. These results confirm the findings of ZSCHOCKE, RABE, TAYLOR, JÄGER and VAN STADEN (2000) that the leaves of *S. aethiopicus* showed the highest levels of COX-1 inhibition. The rhizome and root extracts showed lower levels of activity, with the ethyl acetate extracts having slightly higher activity than the ethanol extracts. Furthermore, the level of activity in the rhizomes was lower than in the roots.
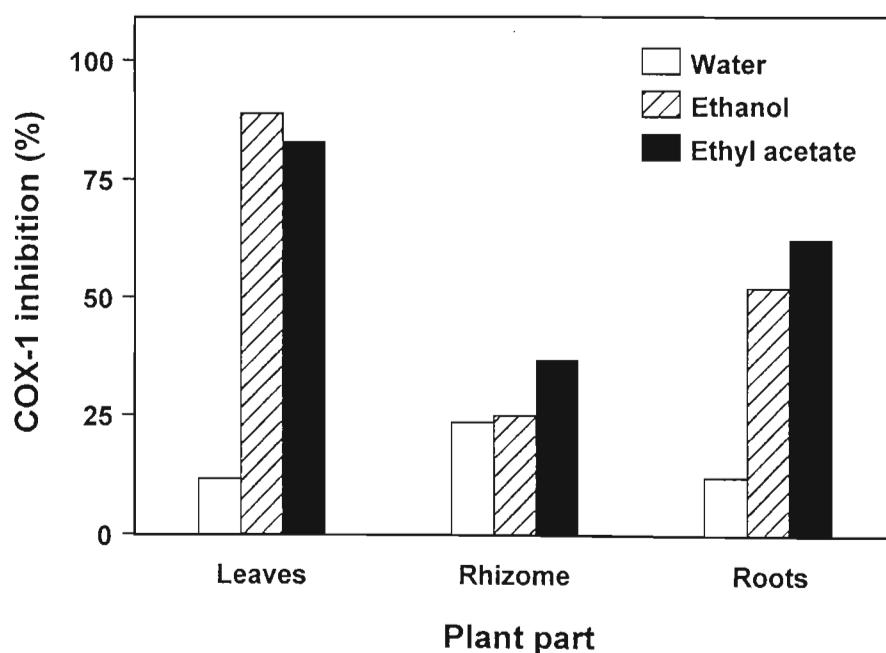
Figure 2.10 COX-1 inhibition of water, ethanol and ethyl acetate extracts of various plant parts of *S. aethiopicus*. Extracts were tested at a final concentration of 250 µg ml⁻¹ per test solution. Values represent the mean of double determinations. The indomethacin standard inhibited the synthesis of prostaglandins by 71%.

These results do provide some evidence for the rational use of this plant in traditional medicine for anti-inflammatory purposes. However, activity was mainly observed with organic solvent extracts, and not with aqueous extracts. Traditional healers would most likely prepare hot aqueous extracts of the rhizomes or roots, whereas the aqueous extracts prepared in this study were cold extractions. A hot extract would also extract some of the volatile oils, possibly responsible for some of the anti-inflammatory action (HUTCHINGS, 1989; VAN WYK, VAN OUDTSHOORN and GERICKE, 1997).

2.3.4 COX-2 inhibition by *S. aethiopicus* extracts

Inhibition of the various extracts in the COX-2 assay are shown in Figure 2.11.
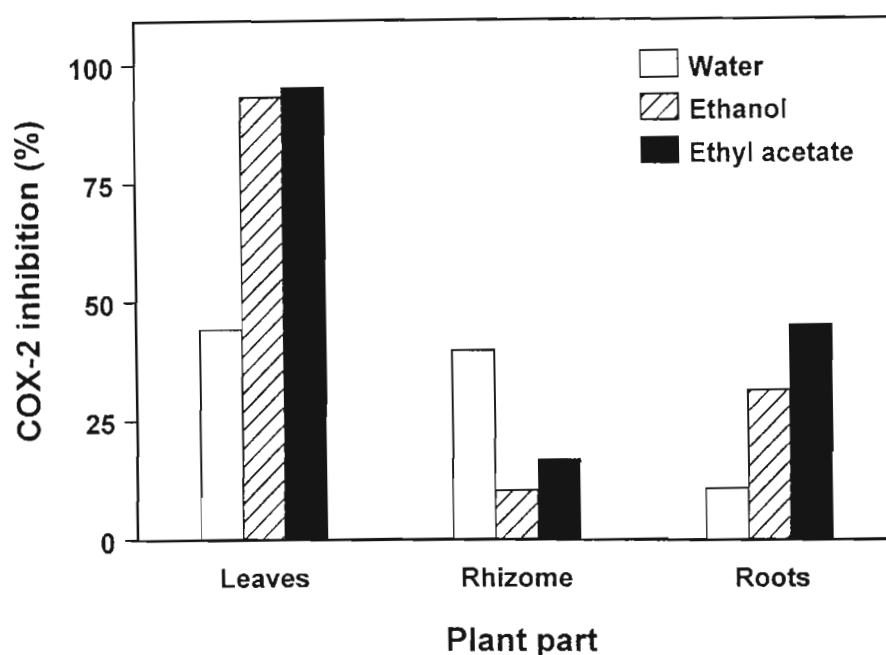
Figure 2.11 COX-2 inhibition of water, ethanol and ethyl acetate extracts of various plant parts of *S. aethiopicus*. Extracts were tested at a final concentration of 250 µg ml^{-1} per test solution. Values represent the mean of double determinations. Indomethacin and nimesulide standards inhibited the synthesis of prostaglandins by 54% and 33% respectively.

As was seen with the COX-1 assay, the ethanol and ethyl acetate extracts of the leaves showed the highest levels of activity. Similarly, the rhizome and root extracts showed lower levels of activity with the ethyl acetate extracts having slightly higher activity than the ethanolic extracts. The aqueous extracts showed no noteworthy activity, although those of the rhizomes and leaves were slightly higher than the root extract.

In this investigation, the use of an *in vitro* enzyme assay to test for inhibition against the COX-1 or COX-2 enzyme provided an appropriate method for determining the presence of inhibitory activity by crude plant extracts. It also provided a means for comparing levels of activity between different extracts. *In vitro* bioassay systems are suitable for the investigation of biological activities of extracts, fractions and isolated compounds. They are advantageous for activity guided fractionation, where a large number of samples require testing (KIUCHI, IWAKAMI, SHIBUYA, HANAOKA and SANKAWA, 1992).

Similar bioassays have been used successfully to isolate compounds from *Zingiber officinale* which have shown activity against COX-1 and 5-lipoxygenase (KIUCHI, IWAKAMI, SHIBUYA, HANAOKA and SANKAWA, 1992). Although such studies have shown *in vitro* activity of compounds from *Z. officinale*, no significant effect of ginger extract could be demonstrated in a randomized, placebo-controlled, cross-over study with ibuprofen in patients with osteoarthritis (BLIDDAL, ROSETZSKY, SCHLICHTING, WEIDNER, ANDERSEN, IBFELT, CHRISTENSEN, JENSEN and BARSLEV, 2000). This does not necessarily negate the possible positive effect of this popular herbal remedy. However, it does highlight the necessity for further *in vivo* studies and clinical trials of herbal extracts and isolated compounds which show promising activity.

2.4 SUMMARY

- Water, ethanol and ethyl acetate extracts were prepared from the leaves, rhizomes and roots of *S. aethiopicus*, and tested in the COX-1 and COX-2 assays.

- Aqueous extracts showed no significant prostaglandin synthesis inhibition.

- Ethanol and ethyl acetate extracts of the leaves showed the highest COX-1 and COX-2 inhibition.

- Ethanol and ethyl acetate extracts of the rhizomes and roots also showed moderate levels of activity in the COX-1 assay.

3 Investigation of Antibacterial Activity

3.1 INTRODUCTION

3.1.1 Antibacterial agents

The important discovery of the ability of penicillium fungi to suppress the growth of various bacterial cultures, by Fleming in 1928, promoted great interest in the discovery and development of chemotherapeutic agents in the twentieth century (LAURENCE and BENNETT, 1980). Programmes for the screening of fungi, actinomycetes and bacteria for antibiotic production were conducted following the success of penicillin for the treatment of casualties during the Second World War. These screening programmes led to the successful isolation of streptomycin, the tetracyclines, erythromycin and other antibiotics (LAURENCE and BENNETT, 1980).

By definition, the term antibiotic refers to substances produced by micro-organisms that, in high dilution, destroy or inhibit the growth of other micro-organisms (LAURENCE and BENNETT, 1980), although in practice it has become synonymous with antibacterial/antimicrobial agents. These are terms which are used to describe chemotherapeutic agents used against micro-organisms, and includes compounds such as sulphonamides, isoniazid and quinine (not true antibiotics).

The term chemotherapy refers to the drug treatment of various infections (bacteria, viruses, protozoa, fungi, worms), in which the agent of infection is removed or destroyed without injuring the host or affecting host cells. The term also includes the therapy of cancer (LAURENCE and BENNETT, 1980).

3.1.2 Action of antibacterial agents

Bacteria are generally classified as either Gram-positive or Gram-negative. This classification is based on whether the organisms do or do not stain with Gram's stain. Although based on a simple staining reaction, Gram-positive and Gram-negative

bacteria are different in several respects. With regard to the action of antibiotics, these differences play an important role (RANG and DALE, 1987).

The cell wall of a Gram-positive organism is a relatively simple structure which forms no appreciable barrier to the entry of antibiotics. It contains many layers of peptidoglycan, between 15-50 nm thick, and can be easily separated from the plasma membrane. The surface is highly polar and carries a negative charge (RANG and DALE, 1987; PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997).

Gram-negative organisms have a more complex cell wall structure. From the plasma membrane outwards it consists of: (1) a periplasmic space containing enzymes and other components; (2) a 2 nm peptidoglycan layer, often linked to outward projecting lipoprotein molecules; (3) an outer membrane consisting of a lipid bilayer with protein molecules and complex lipopolysaccharides on its outer surface. These polysaccharides are different in different strains, and are the main determinants of the antigenicity of the organism. They form the "endotoxins" which trigger various aspects of the inflammatory reaction of a bacterial infection (RANG and DALE, 1987).

Many antibiotics are less active against Gram-negative than Gram-positive bacteria, probably as a result of the complex outer layer which is more difficult to penetrate. However, proteins in the outer membrane of Gram-negative bacteria form transmembrane water-filled channels, called "porins", through which hydrophilic antibiotics can move freely (RANG and DALE, 1987).

Antibacterial agents can be generally divided into two groups, those which act primarily by stopping bacterial growth (bacteriostatic) and those which act by killing the bacteria (bactericidal). However, some drugs are bacteriostatic or bactericidal according to their concentration, and most bacteriostatic drugs can be shown to be bactericidal at high concentration. These differences do, however, have relevance in relation to combined therapy (LAURENCE and BENNETT, 1980).

The lowest concentration at which an antibacterial agent inhibits bacterial growth is

usually referred to as the minimum inhibitory concentration (MIC), and the lowest concentration which is required to kill the test bacterium is known as the minimum bactericidal concentration (MBC) or the minimum lethal concentration (MLC). In many cases, the MBC is 2-8 times that of the MIC (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997).

Antibacterial agents are generally classified according to their mechanism of action (site of action). These sites of action include: (1) the cell wall; (2) the cytoplasmic membrane; (3) the inhibition of protein synthesis; (4) nucleic acid metabolism; and (5) intermediary metabolism (LAURENCE and BENNETT, 1980; GILLHAM, PAPACHRISTODOULOU and THOMAS, 1997).

Two important classes of antibiotics that inhibit bacterial cell wall synthesis are β-lactams and glycopeptides. β-Lactams act by inhibiting the cross-linkage of the peptide chains of the bacterial cell wall. They include penicillins, cephalosporins, carbapenems and monobactams. Glycopeptides are high molecular weight drugs consisting of sugars and amino acids (e.g. vancomycin) (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997). Isoniazid, a synthetic drug, inhibits the biosynthesis of the mycolic acid found in the outer membrane of mycobacteria (GILLHAM, PAPACHRISTODOULOU and THOMAS, 1997).

Polymyxins act by disrupting the cytoplasmic membrane functioning of Gram-negative bacteria. However, due to their toxicity, they are mainly used for topical therapy of Gram-negative infections (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997).

There are several major classes of antibiotics that act mainly by inhibiting bacterial protein synthesis. They exhibit selective toxicity by inhibiting bacterial protein synthesis to a greater extent than the host cell protein synthesis. Antibiotics with this mode of action include aminoglycosides (e.g. gentamicin, streptomycin), amphenicols (e.g. chloramphenicol), lincosamides, macrolides (e.g. erythromycin), streptogramins and tetracyclines (GILLHAM, PAPACHRISTODOULOU and THOMAS, 1997; PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997).

Other classes of antibiotics act as inhibitors of nucleic acid synthesis. They include quinolones and nitroimidazoles which inhibit the synthesis of bacterial DNA, and rifamycins which inhibit bacterial RNA synthesis. Drugs such as sulphonamides interfere with the bacterial folate synthetic pathway, thus interfering with the intermediary metabolism (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997).

3.1.3 Bacterial resistance to drugs

The use of antibacterial drugs in the twentieth century has dramatically changed the course of many illnesses. However, partly because of their excellent safety profiles, they are among the most over-prescribed drugs. As a result, this has contributed to the expanding problem of resistance by a variety of bacteria (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997). For example, in recent years there has been an increase of tuberculosis in the USA due to the development of multiple-drug resistance by Mycobacterium tuberculosis (GILLHAM, PAPACHRISTODOULOU and THOMAS, 1997).

Antibiotic resistance can be classified as either innate (intrinsic) or acquired. Innate resistance refers to the inherent resistance of an organism due to the mechanism of the drug. Acquired resistance, however, refers to the acquisition of a resistance gene in an organism that is not intrinsically resistant to the drug. The major stimulus for the development of acquired antibiotic resistance in bacteria is due to the use of antibacterial drugs themselves. Such use exerts selective pressure on the bacteria to develop resistance in order to survive. However, the probability of developing resistance does depend on the specific drug and bacterium involved (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997).

Drug resistance can be divided into two types: (1) drug tolerant, where the organism is able to grow in the presence of the antimicrobial agent; and (2) drug destroying, where the organism is able to inactivate the drug (e.g. penicillinase-producing staphylococci) (LAURENCE and BENNETT, 1980).

There are three main biochemical mechanisms of acquired resistance: (1) reduced bacterial permeability in the cell membrane of Gram-negative bacteria; (2) the production of bacterial enzymes that alter the structure of the antibiotic (e.g. β-lactamase); and (3) alteration in the target site, where the antibiotic normally binds (e.g. methicillin-resistant staphylococci) (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN, 1997).

A recent study in Bristol has shown that healthy 7-year-old children may carry strains of *Staphylococcus aureus*, *Haemophilus* spp., *Branhamella catarrhalis*, group A β-haemolytic streptococci and *Escherichia coli* with acquired resistance to antibiotics to which they have not been exposed (MILLAR, WALSH, LINTON, ZHANG, LEEMING, BENNETT and the ALSPAC Study Team, 2001). This study illustrates the extent to which resistant bacteria are circulating in healthy children and highlights the potential for increasing problems related to bacterial resistance. There is therefore a great need to continue the search for compounds which could potentially lead to the development of safe and effective antimicrobial agents. There is also a great need for new, more effective and less toxic antibiotics for the treatment of opportunistic infections related to acquired immunodeficiency syndrome (AIDS). Patients with AIDS are particularly susceptible to certain opportunistic infections, for which conventional antibiotic therapy is not always suitable (CLARK and HUFFORD, 1993).

3.1.4 Testing antibacterial activity

HEWITT and VINCENT (1989) describe a microbiological assay as the "technique whereby the potency or concentration of a chemical substance may be determined by its effect on the growth of a microorganism". Microbiological assays are important techniques for the detection of antimicrobial substances and for the development of effective antimicrobial agents, and are essential for process and product control in the pharmaceutical industry (HEWITT and VINCENT, 1989).

Assays used for testing antibacterial activity can be generally classified into 3 groups: (1) diffusion methods; (2) dilution methods; and (3) bioautographic assays (RIOS,

RECIO and VILLAR, 1988). The choice of technique to be used will depend on the extract or compound to be tested, and it is important to understand the advantages and limitations of each method. There are many factors which can influence the results obtained and the great variation often observed in testing crude plant extracts in antibacterial assays. These include the assay technique, the culture medium, the strain of bacteria used for testing, the botanical source of the plant, the age of the plant, the state of the plant material used (fresh or dried) and the quantity of extract tested (JANSSEN, SCHEFFER and BAERHEIM SVENDSE, 1987; THOMAS, 1989). Furthermore, there is no standardized method for expressing the results of antibacterial testing (RIOS, RECIO and VILLAR, 1988).

The disc-diffusion assay is one of the most common forms of testing for antibacterial activity, and does not require homogenous dispersion of the extract. A filter paper disc, impregnated with the test substance or extract, is placed on an inoculated medium. There are some variations of this method which use a hole in the agar medium, or a cylinder for the sample reservoir. After incubation the inhibition zone around the disc, hole, or cylinder is measured. It is also possible to keep the inoculated system at a low temperature before incubation to allow for greater diffusion of the test substance through the culture medium (RIOS, RECIO and VILLAR, 1988). This method is not suitable when the sample is not soluble in water, as this would result in poor diffusion through the agar, resulting in small inhibition zones, even if the substance is highly active.

Dilution techniques generally require a homogeneous dispersion of the sample in water. They are often used to determine the MIC or MLC values of an extract, essential oil or pure substance, but can also be used for the preliminary screening of crude plant extracts for antibacterial activity (RIOS, RECIO and VILLAR, 1988). As is the case with the diffusion assays, there are different variations of the dilution technique. The most common method is a liquid dilution method, although there are other methods which incorporate agar in the mixture.

The use of bioautography is a useful procedure for testing antibacterial activity and for bioassay-guided fractionation of antibacterial compounds. Developed thin layer chromatography (TLC) plates are allowed to dry before being sprayed with a mixture of the test bacteria and liquid nutrient medium (direct bioautography), or the plate may be covered with an agar medium containing the test bacteria (overlay assay). The plates are incubated in a humid atmosphere, resulting in inhibition zones where growth is prevented by the active components. These inhibition zones are usually visualized more clearly by the use of tetrazolium salts such as iodonitrotetrazolium chloride (INT) or methylthiazoyl-tetrazolium chloride (MTT) (BEGUE and KLINE, 1972; HOSTETTMANN, MARSTON and WOLFENDER, 1995; GIBBONS and GRAY, 1998).

It is often useful to include a known antibiotic in the assay as a positive control. This is useful for establishing the sensitivity of the test organism (RIOS, RECIO and VILLAR, 1988). However, a comparison of the antibacterial activity of the samples and the standard antibiotic cannot be drawn (JANSSEN, SCHEFFER and BAERHEIM SVENDSE, 1987).

In this investigation two Gram-positive organisms and two Gram-negative organisms were used as test bacteria. These were the Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, and the Gram-negative *Escherichia coli* and *Klebsiella pneumoniae*. *S. aureus* is an important pathogen responsible for wound infections, conjunctivitis, toxic food poisoning and various superficial infections (pustules, boils, abscesses etc.) and deep infections (septicaemia, endocarditis, pyaemia). A normal inhabitant of the human and animal intestine, *E. coli*, is mainly responsible for wound and urinary infections, and gastroenteritis. Other infections include peritonitis, septicaemia and neonatal meningitis. *K. pneumoniae* is also found in the human and animal intestine, and is similarly responsible for urinary infections and other forms of sepsis (RANG and DALE, 1987; SLEIGH and TIMBURY, 1998).

3.2 MATERIALS AND METHODS

3.2.1 Extract preparation

Extracts of the leaves, rhizomes and roots of *S. aethiopicus* were prepared as described in section 2.2.1. The residues from the plant extracts were redissolved to a concentration of 50 mg ml^{-1} in water (for aqueous extracts) or ethanol (for ethyl acetate or ethanol extracts) for antibacterial testing.

3.2.2 Testing antibacterial activity

3.2.2.1 The microdilution bioassay

Antibacterial activity was assessed using the microdilution bioassay, as described by ELOFF (1998). Each extract was tested against bacterial strains obtained from the bacterial collection of the Microbiology Department, University of Natal, Pietermaritzburg and maintained on Mueller-Hinton (MH) nutrient agar (Biolab) at 4 °C. The bacteria tested were *Bacillus subtilis* and *Staphylococcus aureus* (Gram-positive), and *Escherichia coli* and *Klebsiella pneumoniae* (Gram-negative).

For each test bacterium, 100 µl of redissolved extract was serially diluted two-fold with 100 µl sterile distilled water in a sterile 96-well microtitre plate. A similar two-fold serial dilution of neomycin (100 µg ml^{-1}) was used as a positive control for each bacterium. Extract and bacteria-free negative controls were also included.

Suspension cultures of bacteria were inoculated in Mueller-Hinton (MH) broth (Oxoid) from stock cultures and incubated overnight at 37 °C in a waterbath on an orbital shaker. Prior to use in the bioassay, the saturated suspension cultures were diluted 1:100 with sterile MH broth. To each of the wells containing the test or control solutions, 100 µl of the bacterial cultures were added. The plates were covered and incubated overnight at 37 °C.

To indicate bacterial growth, 40 µl of 0.2 mg ml^{-1} iodonitrotetrazolium chloride (Sigma), were added to each well, and the plates were incubated for a further 30 min. The wells which displayed no change in colour represented antibacterial activity. The MIC was taken as the lowest concentration of plant extract to elicit a bacteriostatic or bactericidal effect against the tested bacterium (i.e. first well free from colour change).

3.3　RESULTS AND DISCUSSION

3.3.1　Antibacterial activity of *S. aethiopicus* extracts

Results for the screening of extracts from the different plant parts for antibacterial activity are shown in Table 3.1. No inhibitory activity against the test bacteria was detected with the aqueous extracts. The ethanol and ethyl acetate extracts tested showed greater antibacterial activity against the Gram-positive bacteria than the Gram-negative bacteria. No distinct differences were observed between the activity obtained with the ethanol and ethyl acetate extracts of the different plant parts, or between the activity of the different plant parts.

Table 3.1 Minimum inhibitory concentrations (mg ml^{-1}) of *S. aethiopicus* extracts against test bacteria.

		Bacterium[a]			
Plant part	Extract	*B.s.*	*S.a.*	*E.c.*	*K.p.*
Leaves	Water	> 12.5	> 12.5	> 12.5	> 12.5
	Ethanol	3.13	1.56	3.13	3.13
	Ethyl acetate	1.56	1.56	3.13	6.25
Rhizome	Water	> 12.5	> 12.5	> 12.5	> 12.5
	Ethanol	1.56	1.56	3.13	6.25
	Ethyl acetate	1.56	1.56	6.25	6.25
Roots	Water	> 12.5	> 12.5	> 12.5	> 12.5
	Ethanol	1.56	0.78	6.25	6.25
	Ethyl acetate	0.78	1.56	6.25	12.5
Neomycin standard (µg ml^{-1})		0.1	0.1	1.56	0.39

Abbreviations: [a] *B.s.* - *B. subtilis*; *S.a.* - *S. aureus*; *E.c.* - *E. coli*; *K.p.* - *K. pneumoniae*.

As discussed in section 1.3.4, the rhizome and roots contain a large proportion of volatile oils, the major constituents of which are 4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-*b*]-furan-8-one, 1,8-cineole, (*E*)-β-ocimene and *cis*-alloocimene (VILJOEN, DEMIRCI, BASER and VAN WYK, 2002). Many studies have been conducted on the antibacterial effects of essential oils, and their components. Table 3.2 gives an indication of the antibacterial activity of some of the essential oil components identified in *S. aethiopicus* root and rhizome oils (VILJOEN, DEMIRCI, BASER and VAN WYK, 2002).

Table 3.2   Some essential oil components from *S. aethiopicus* root and rhizome oil (and percentage composition) (VILJOEN, DEMIRCI, BASER and VAN WYK, 2002) and related antibacterial activity.

Compound	% Roots	% Rhizomes	Comments	Reference
bornyl acetate	0.08	-	Inactive against *S. aureus*, *E. coli* and *P. aeruginosa*; Moderately active against a variety of organisms	HINOU, HARVALA and HINOU, 1989; KNOBLOCH, PAULI, IBERL, WEIGAND and WEIS, 1989
camphor	0.03	-	Very slight activity against a variety of organisms	KNOBLOCH, PAULI, IBERL, WEIGAND and WEIS, 1989
1,8-cineole	9.63	16.11	Little or no activity	JANSSEN and SCHEFFER, 1985
p-cymene	0.43	0.22	Little or no activity against a variety of organisms	KNOBLOCH, PAULI, IBERL, WEIGAND and WEIS, 1989
limonene	1.68	1.17	Little or no activity	JANSSEN and SCHEFFER, 1985
linalool	0.4	0.84	Active against *S. aureus* and *E. coli*; Active against a variety of organisms	HINOU, HARVALA and HINOU, 1989; KNOBLOCH, PAULI, IBERL, WEIGAND and WEIS, 1989
myrcene	1.80	1.15	Did not show activity on its own, but enhanced activity when combined with geranial or neral	ONAWUNMI, YISAK and OGUNLANA, 1984
(*E*)-nerolidol	0.09	0.05	Inactive against *S. aureus*, *E. coli* and *P. aeruginosa*	HINOU, HARVALA and HINOU, 1989

Compound	% Roots	% Rhizomes	Comments	Reference
neryl acetate	-	0.04	Active against *S. aureus* and *P. aeruginosa*	HINOU, HARVALA and HINOU, 1989
(*E*)-β-ocimene	7.61	6.11	Slight activity	KNOBLOCH, PAULI, IBERL, WEIGAND and WEIS, 1989
α-pinene	0.84	0.72	Little or no activity; Inactive against *S. aureus*, *E. coli* and *P. aeruginosa*; Slight activity against a variety .of organsims	JANSSEN and SCHEFFER, 1985; HINOU, HARVALA and HINOU, 1989; KNOBLOCH, PAULI, IBERL, WEIGAND and WEIS, 1989
β-pinene	1.96	1.86	Little or no activity; Active against *S. aureus*	JANSSEN and SCHEFFER, 1985; HINOU, HARVALA and HINOU, 1989
piperitone	0.04	0.01	Active against *S. aureus*; Active against a variety of organisms	HINOU, HARVALA and HINOU, 1989; KNOBLOCH, PAULI, IBERL, WEIGAND and WEIS, 1989
sabinene	5.52	4.52	Active against *S. aureus*	HINOU, HARVALA and HINOU, 1989
α-terpineol	1.01	1.71	Weak activity against a variety of test bacteria; Active against *S. aureus* and *E. coli*	JANSSEN and SCHEFFER, 1985; HINOU, HARVALA and HINOU, 1989
terpinolene	0.58	0.22	Terpinolene active against *S. aureus* and *E. coli*, slight activity against *P. aeruginosa*	HINOU, HARVALA and HINOU, 1989
α-terpinyl acetate	0.55	0.5	Active against *S. aureus* and *E. coli*	HINOU, HARVALA and HINOU, 1989

Some studies have shown that the compounds in the greatest proportions are not necessarily responsible for the greatest total activity, and that the less abundant constituents can also play an important role (CIMANGA, KAMBU, TONA, APERS, DE BRUYNE, HERMANS, TOTTÉ, PIETERS and VLIETINCK, 2002). For example, a study of the antimicrobial activity of *Eucalyptus* oils showed that there was no direct relationship between the 1,8-cineole content and the inhibition zones obtained (DELLACASSA, MENENDEZ, MOYNA and CERDEIRAS, 1989).

In a study on the antibacterial constituents of the essential oil of *Cymbopogon citratus* (lemon grass), it was observed that myrcene did not show antibacterial activity on its own against *B. subtilis* and *E. coli*. However, it enhanced the activities of either of the other two main components, α-citral (geranial) and β-citral (neral) when combined (ONAWUNMI, YISAK and OGUNLANA, 1984).

It has also been observed that the chemical configuration of essential oils can have an effect on the antibacterial activity. For example, in a study which screened 32 common constituents of essential oils, it was seen that β-pinene was more active than its isomer α-pinene (HINOU, HARVALA and HINOU, 1989).

There has been much investigation into the method of determining antibacterial activity for essential oils. In some cases, depending on the method used (diffusion or dilution), different results can be obtained. A very important factor related to the antibacterial activity of essential oils and their components is their solubility in water. Numerous terpenoid compounds were tested against various bacteria and it was found that their potency was related to their solubility in water. However, some substances (eg. thymol, carvacrol and eugenol) which appeared less water soluble showed strong antibacterial properties. In general, essential oils caused damage to biological membranes due to their lipophilic properties, although specific functional groups are also effective (KNOBLOCH, PAULI, IBERL, WEIGAND and WEIS, 1989).

3.4 SUMMARY

- Water, ethanol and ethyl acetate extracts were prepared from the leaves, rhizomes and roots of *S. aethiopicus*, and tested in a microdilution antibacterial assay.
- No antibacterial activity was detected with the aqueous extracts.
- Ethanol and ethyl acetate extracts showed greater activity against Gram-positive bacteria than Gram-negative bacteria.
- No distinct differences were observed between the ethanol and ethyl acetate extracts, or between the extracts from the different plant parts.

4   Isolation of an Antibacterial Compound

4.1   INTRODUCTION

4.1.1 Bioassay-guided fractionation

The isolation of biologically active natural compounds is often limited because they may be present in very minute quantities. Furthermore, such substances may be thermally or hydrolytically unstable or have unfavourable solubility properties (KINGHORN, 1992). The isolation of an active constituent from a crude plant extract may seem somewhat daunting in light of the fact that a plant may contain hundreds or even thousands of compounds with a variety of different biological properties (HOSTETTMANN, 1986). However, despite the difficulties which may be encountered, there are numerous separation techniques which can be employed to obtain pure compounds.

In the process of the separation of complex mixtures, the aim is to have maximum yield with a minimum of effort in order to reduce the time and cost involved. Various types of solid phase chromatography (thin layer chromatography, column chromatography, low-, medium and high pressure chromatography) and liquid-liquid chromatography are among some of the systems which are used in compound isolation. Each method has advantages and limitations, and usually several separation steps involving different techniques, when used in combination, make the isolation of pure compounds possible (HOSTETTMANN, 1986; HOSTETTMANN, MARSTON and WOLFENDER, 1995).

Bioassay-guided fractionation (also known as "bioassay-directed" or "activity-directed" fractionation) is a useful technique for the isolation of unknown substances which are biologically active. Extracts which exhibit a particular biological activity of interest are purified chromatographically. Periodic evaluation with one or more bioassay systems, results in the eventual isolation of one or more biologically active constituents (KINGHORN, 1992).

Appropriate bioassays for the initial screening of biological activity and for monitoring

the progress of the separation procedure are crucial to the isolation of compounds with biological activity. In order to cope with the number of extracts or fractions from various fractionation steps, the bioassays should ideally be simple, rapid, reproducible and relatively inexpensive. If the active constituents are only present at low concentrations in the crude extracts, or the fractions, it is important that the biological test systems be sensitive enough for the detection of active principles. Furthermore, it is important to reduce the possibilities of false positive or false negative results (McCHESNEY, 1993; HOSTETTMANN, MARSTON and WOLFENDER, 1995; HOSTETTMANN, WOLFENDER, RODRIGUEZ and MARSTON, 1996).

Bioautography, as discussed in section 3.1.4, is a useful method for the bioassay-guided fractionation of compounds with antimicrobial activity. Both fungi and bacteria may be investigated using these methods. However, some compounds show poor migration through the agar overlay and may not be detected (GIBBONS and GRAY, 1998). In such cases, it is often better to use a liquid broth medium.

According to COUZINIER and MAMATAS (1986), there are four possible outcomes from the attempted determination of the active principle from active fractions: (1) no activity is confirmed; (2) one or more type of activity is observed, but cannot be linked to any particular active principle as a result of synergistic effects; (3) activity is observed, and is attributed to an already known substance; and (4) a new chemical is discovered, potentially leading to the development of a new drug, or a new structural concept which chemists can try to optimize.

4.1.2 Preparative thin layer chromatography

Thin layer chromatography (TLC) is a versatile technique which can be used for the qualitative separation of simple mixtures. It can also be used as a powerful separation tool for the quantitative analysis of complex mixtures (JORK, FUNK, FISCHER and WIMMER, 1990). Although a number of other techniques exist which can be used for compound isolation (eg. HPLC and countercurrent chromatography), many natural products are still isolated by conventional preparative TLC (PTLC). It is still a useful

isolation method in many cases due to its simplicity, relatively low cost and speed. PTLC is nearly always used as a final purification step of mixtures which have been partially purified using other chromatographic methods, such as column chromatography, flash chromatography or vacuum liquid chromatography (VLC). Of course, the isolation of some compounds using PTLC is not always the most suitable method, and there is poor detection and control of elution compared to HPLC (GIBBONS and GRAY, 1998).

There are a variety of factors which are important for the separation and subsequent isolation of compounds using PTLC. The choice of a suitable separation system (sorbent and solvent system), form of development (isocratic, multiple or step-gradient) and the use of suitable detection reagents need to be considered (GIBBONS and GRAY, 1998). An example of a useful universal reagent for natural products, that makes colour differentiation possible, is anisaldehyde-sulfuric acid. It can be used for the detection of antioxidants, steroids, prostaglandins, carbohydrates, phenols, glycosides, sapogenins, essential oil components or terpenes, antibiotics and mycotoxins (JORK, FUNK, FISCHER and WIMMER, 1990). It must be remembered, however, that the use of spray reagents is chemically destructive and cannot be used directly for identifying compounds for isolation purposes.

4.1.3 Nuclear magnetic resonance spectroscopy

Following the isolation of constituents with biological activity, it is necessary to identify the individual compounds and determine the structure of these compounds. Nuclear magnetic resonance (NMR) spectroscopy allows for the unambiguous structural identification of extremely complex plant constituents with just a few milligrams of material (DEROME, 1989; PHILLIPSON, 1995). A further advantage of NMR spectroscopy is due to the non-destructive nature of the technique which allows for the recovery of the substance for further biological or chemical testing (AQUINO, DE SIMONE, DE TOMMASI, PIACENTE and PIZZA, 1995).

NMR spectroscopy essentially measures the magnetic moments of its hydrogen atoms

(^{1}H NMR) and the small magnetic moments generated by ^{13}C atoms. Differently substituted carbon atoms give shifts within specific ranges and provide information on the nature of the carbon skeleton of the molecule, whereas the information from proton NMR provide a record of the number of hydrogen atoms attached to different groups (HARBORNE, 1984).

Strategies for structure elucidation based on two-dimensional (2-D) NMR correlations were developed in the mid 1980's. A typical strategy using 2-D NMR data, is to link the ^{1}H-^{13}C resonances by a heteronuclear COSY experiment and to establish the ^{1}H-^{1}H correlations by a homonuclear COSY experiment. From these correlations it is possible to infer C-C connectivities and establish part structures. Long range connectivities can be established using heteronuclear multiple bond correlations (HMBC). The derivation of a planar structure is possible with these correlations. The 2-D nuclear Overhauser exchange spectroscopy (NOESY) or the measurement of proton coupling constants can then be used to ascertain the relative stereochemistry of the structure (DEROME, 1989; HANSON, 2001).

4.1.4 Mass spectroscopy

Mass spectroscopy (MS) yields a complex fragmentation pattern, according to mass, from the degradation of trace amounts of an organic compound. The sample is bombarded with high-energy electrons which causes fragmentation of the chemical bonds resulting in positively charged ions. Acceleration of these ions into a magnetic field causes dispersion of the ions, and allows for the measurement of the size and relative abundance of these ions (FOX and WHITESELL, 1997). In many cases, some of the parent compound will survive the process and is recorded as a molecular ion peak. The technique of MS requires small amounts of material and can provide an accurate molecular weight. Furthermore, the fragmentation pattern is often characteristic of a particular compound, and can be used for identification purposes (HARBORNE, 1984).

4.2   MATERIALS AND METHODS

4.2.1   Preliminary serial extraction of *S. aethiopicus* rhizome material

A serial extraction was performed on *S. aethiopicus* rhizome material using a Soxhlet apparatus.   Rhizomes were harvested from stock plants at the University of Natal Botanical Garden, Pietermaritzburg.   Mature plants were harvested in July (following senescence) and the rhizomes cut into smaller pieces and dried at 50 °C for two days. The dried ground rhizome material (4 g) was placed in a Whatman cellulose extraction thimble and covered with glass wool.   The extraction solvent (150 ml) was heated to boiling and maintained for 4 h.   This was allowed to cool and drain, before being filtered through Whatman No. 1 filter paper and dried under vacuum at 35 °C.   This procedure was repeated for successive solvent extractions using the same rhizome material and thimble.   The solvents used were hexane, followed by ethyl acetate and then ethanol. A Soxhlet extraction using only ethyl acetate or ethanol was performed on 4 g of ground rhizome material.

The extracts were analysed using TLC separation, using a solvent system of hexane:ethyl acetate (2:1).   The extracts (0.25 mg) were loaded on the TLC plate (silica gel 60 F_{254}, 0.25 mm, 10 cm x 10 cm, Merck) in 1 cm bands.   The TLC plates were developed over 7.5 cm.   The solvent front was marked and the plates were allowed to dry before visualisation under UV_{254} and UV_{366}.   The plates were stained with anisaldehyde-sulphuric acid reagent (AS) (0.5 ml anisaldehyde, 10 ml sulphuric acid, 85 ml ethanol and 5 ml concentrated sulphuric acid, mixed in this order).   The stain was poured over the plates and heated for 5-10 min at 110 °C.

The extracts were redissolved in ethanol and tested in the COX-1 assay, as described in section 2.2.2.1, at a concentration of 10 mg ml^{-1} (giving a final concentration of 250 µg ml^{-1} per test solution).   The extracts were also tested in the microdilution antibacterial assay, as described in 3.2.2.1, against *B. subtilis* (ATCC 6051), *S. aureus* (ATCC 12600), *E. coli* (ATCC 11775), *K. pneumoniae* (ATCC 13883).

4.2.2 Bulk extraction of *S. aethiopicus* rhizome material

A bulk extract of rhizome material was prepared using 6 Soxhlet extractors. In each case dried ground rhizome material (15 g) was placed into a Whatman cellulose extraction thimble and covered with glass wool. Ethyl acetate was used as the extraction solvent (150 ml/apparatus). The ethyl acetate was heated to boiling and the extraction was carried out for 4 h. The extract was allowed to cool and drain, before being filtered through Whatman No. 1 filter paper and dried under vacuum at 35 °C.

4.2.3 Bioassay-guided fractionation

The isolation of a compound which exhibited antibacterial activity in a bioautographic assay, using *S. aureus* as a test organism, was conducted according to the outline shown in Figure 4.1.

Bulk extraction of *S. aethiopicus* rhizome material
(Ethyl acetate)

↓

Vacuum liquid chromatography
(Hexane:ethyl acetate gradient)

↓

Fractions A-M
TLC and Bioautography (*S. aureus*)

↓

Fraction C

↓

Preparative TLC
(Toluene:ethyl acetate 93:7)

↓

NMR and mass spectroscopy of isolated compund
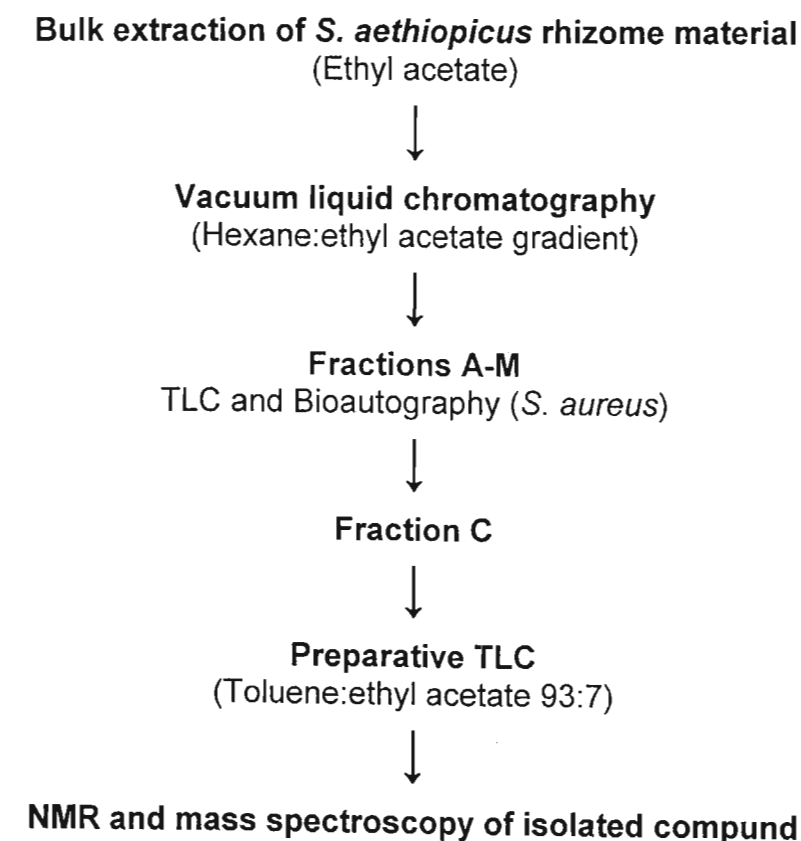
Figure 4.1 Summary of steps used in the isolation of an antibacterial compound using bioassay-guided fractionation.

4.2.3.1 Vacuum liquid chromatography

Dry silica gel 60 (150 g) (0.040-0.063 mm, Merck) was placed in a large vacuum liquid chromatography (VLC) column (5 x 27 cm). The extract ($\approx$4 g) was adsorbed onto a small amount of the silica gel and allowed to dry before being placed as a top layer on the silica gel in the column. A hexane:ethyl acetate gradient (hexane proportions: 100%; 85%; 80%; 75%; 70%; 65%; 60%; 50%; 40%; 30%; 20%; 10%; 0%) was used and the solvent mixtures (400 ml) were eluted on the column under vacuum. The fractions were collected and dried under vacuum at 35 $^{\circ}$C.

4.2.3.2 Evaluation of VLC fractions

To evaluate the chromatographic separation of the vacuum liquid chromatographic fractions (A-M), and to ascertain the presence of compounds showing antibacterial activity, TLC separation and a bioautographic assay were performed. Fractions A-M (0.125 mg) were spotted onto 2 large TLC plates (silica gel 60 F_{254}, 0.25 mm, 20 cm x 20 cm, Merck). The plates were developed simultaneously in hexane:ethyl acetate (2:1), and developed over 17.5 cm. The solvent front was marked and the plates were allowed to dry before visualisation under UV_{254} and UV_{366}.

For the bioautographic assay, S. aureus (ATCC 12600) was used as a test organism (MARTINI and ELOFF, 1998). An overnight culture was prepared in MH broth medium (2 x 20 ml). The cultures were centrifuged at 3000 g for 10 min and the supernatant medium discarded. The pellets of bacterial cells were combined and resuspended in 10 ml of fresh MH broth. This broth culture was then sprayed onto one TLC plate and incubated overnight at 37 $^{\circ}$C in 100% humidity. After incubation, the plate was allowed to dry slightly before spraying with a 2 mg ml^{-1} solution of iodonitrotetrazolium chloride (INT) (Fluka). The plate was then re-incubated for a further 30 - 60 min to allow for colour development. Zones of inhibition appeared as white spots against a pink coloured background (BEGUE and KLINE, 1972). The reference plate was stained with AS reagent and heated for 5-10 min at 110 $^{\circ}$C.

The VLC fractions were also tested in the COX-1 assay as described in section 2.2.2.1, at a concentration of 10 mg ml^{-1} (giving a final concentration of 250 μg ml^{-1} per test solution).

4.2.3.3 Preparative TLC

From the results obtained with the bioautographic assay of the VLC fractions, it was decided to use fraction C for further purification and compound isolation using preparative TLC.

Approximately 25 mg of VLC fraction C was applied to a large TLC plate (silica gel 60 F_{254}, 0.25 mm, 20 cm x 20 cm, Merck) in a long band. Five plates were developed simultaneously in toluene:ethyl acetate (93:7), which gave a better separation than the previous solvent system used. The solvent front was marked and the plates were allowed to dry before visualisation under UV_{254} and UV_{366}. The 1.2 cm wide band at R_f 0.54, which showed up as a dark bank under UV_{254}, was scraped off each plate. Two small strips were cut from one plate for bioautographic verification and for staining with AS reagent. The combined silica gel scrapings were eluted 3 times with 20 ml ethyl acetate in an ultrasound bath (Branson 5210) for 30 min. The solvent was filtered through a 0.22 μm Millipore filter and dried under vacuum at 35 $^\circ C$.

4.2.4 NMR and mass spectroscopy

NMR spectra (^1H, ^{13}C, COSY, HMBC, HMQC and NOESY) of the isolated compound were recorded in $CDCl_3$ using a Varian Inova 500 MHz NMR spectrometer at the School of Chemical and Physical Sciences, University of Natal, Pietermaritzburg. Analysis of the NMR spectra was performed with the help of Professor D.A. Mulholland of the School of Pure and Applied Chemistry, University of Natal, Durban.

MS were run on a Hewlett-Packard gas chromatographic mass spectrometer (HP 5988A), at the School of Chemical and Physical Sciences, University of Natal, Pietermaritzburg.

4.2.5 MIC determination

The MIC of the isolated compound was determined using the microdilution bioassay described in section 3.2.2.1. The compound was dissolved in ethanol at a concentration of 12.5 mg ml^{-1} (giving a starting concentration of 3.13 mg ml^{-1}) and tested against *B. subtilis* (ATCC 6051), *S. aureus* (ATCC 12600), *E. coli* (ATCC 11775), *K. pneumoniae* (ATCC 13883). Due to a limited amount of pure compound, only two replicates were performed.

4.3   RESULTS AND DISCUSSION

4.3.1 Serial extraction of *S. aethiopicus* rhizome material

The residue yields for the serial extraction of 4 g of *S. aethiopicus* rhizome material, and the ethyl acetate and ethanol extracts are given in Table 4.1. The TLC separation of the extracts developed in hexane:ethyl acetate (2:1) can be seen in Figure 4.2.

Table 4.1   Residue yields for serial extraction of *S. aethiopicus* rhizome material using Soxhlet extraction.

Extract	Extraction Solvent	Residue (g)
A	Hexane	0.234
B	Ethyl acetate (only)	0.280
C	Ethanol (only)	0.387
D	A ➜ Ethyl acetate	0.049
E	D ➜ Ethanol	0.074
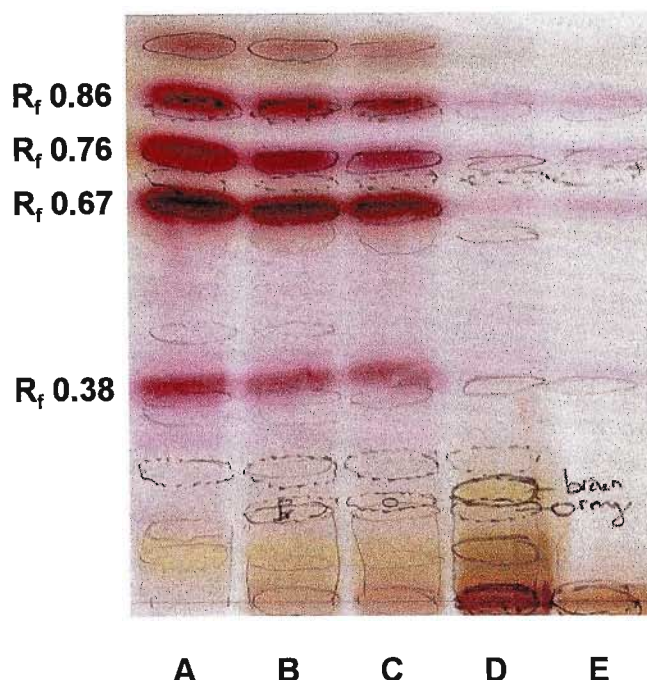
Figure 4.2 Chromatogram of Soxhlet extracts of *S. aethiopicus* rhizome material, developed in hexane:ethyl acetate 2:1 and stained with AS reagent. (A) Hexane; (B) EtOAc; (C) EtOH; (D) A ➜ EtOAc; (E) D ➜ EtOH.

From the TLC separation it can be seen that the hexane, ethyl acetate and ethanol extracts (A-C) appeared to be quite similar. There were a number of compounds which stained bright pink with AS reagent at R_f 0.38, 0.67, 0.76 and 0.86. For the serial extracts (D,E), an initial extraction in hexane removed a large proportion of the extractable compounds resulting in lower amounts of residue and fewer visible bands on the TLC plate.

The inhibition of prostaglandin synthesis in the COX-1 assay by the Soxhlet extracts is shown in Figure 4.3, and the antibacterial activity is given in Table 4.2. The hexane and ethyl acetate extracts gave slightly higher activity than the ethanol extract, and the ethyl acetate extract following a hexane extraction also showed good activity. However, the final extract (E) showed very little COX-1 inhibition. No noteworthy differences were observed between the different extracts in the microdilution assay, with the exception of the final extract (E) which showed slightly lower activity against the Gram-positive bacteria than the other extracts (indicated by a higher MIC value). In general, the

extracts were more active against the Gram-positive bacteria (*B. subtilis* and *S. aureus*) than the Gram-negative organisms.

Extracts prepared using a Soxhlet apparatus are subjected to high temperatures in the solvent compartment of the apparatus. As the extracts exhibited activity in the COX-1 bioassay, and the microdilution antibacterial assay, it appeared that the heat did not have an deleterious effect on the compounds responsible for the activity. These results from the small-scale Soxhlet extractions showed that it would be feasible to conduct a bulk extraction of the rhizome material of *S. aethiopicus* for bioassay-guided fractionation using this method.
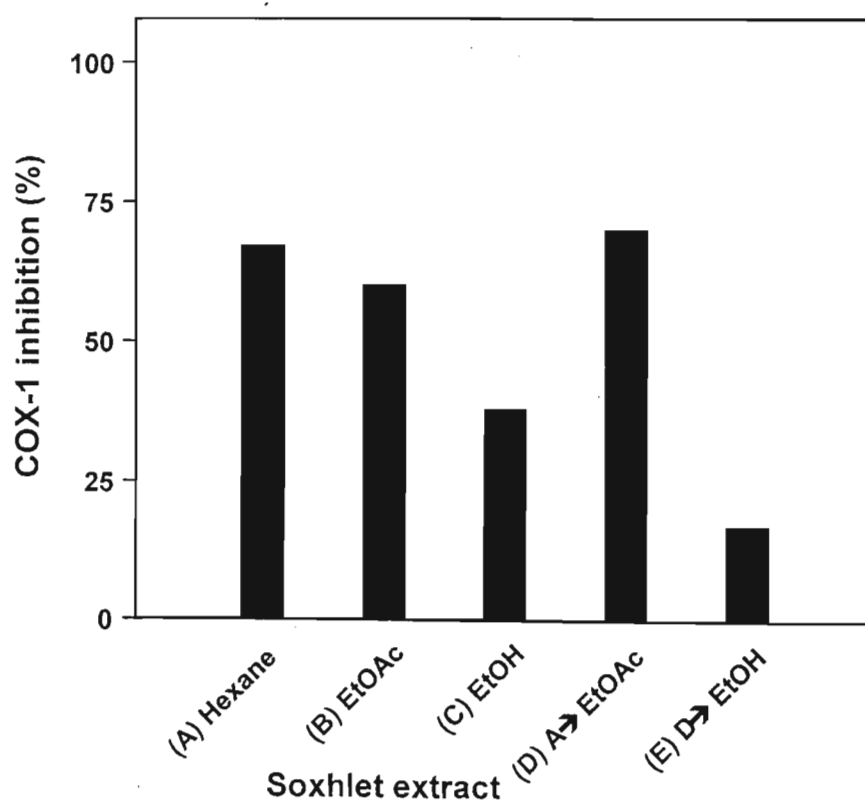
Figure 4.3   COX-1 inhibition of Soxhlet extracts of *S. aethiopicus* rhizome material. Extracts were tested at a final concentration of 250 µg ml^{-1} per test solution. Values represent the mean of double determinations. The indomethacin standard inhibited the synthesis of prostaglandins by 73%.

Table 4.2   Minimum inhibitory concentrations (mg ml^{-1}) of Soxhlet extracts of *S. aethiopicus* rhizome material against test bacteria (ATCC cultures).

Extract	Bacterium[a]			
	B.s.	*S.a.*	*E.c.*	*K.p.*
(A) Hexane	0.39	0.39	6.25	6.25
(B) Ethyl acetate (only)	0.39	0.39	6.25	6.25
(C) Ethanol (only)	0.39	0.39	6.25	3.13
(D) A ➜ Ethyl acetate	0.39	0.39	3.13	3.13
(E) D ➜ Ethanol	6.25	6.25	3.13	3.13
Neomycin standard (µg ml^{-1})	0.025	0.78	0.78	0.78

Abbreviations: [a] *B.s.* - *B. subtilis*; *S.a.* - *S. aureus*; *E.c.* - *E. coli*; *K.p.* - *K. pneumoniae*.

4.3.2 Bulk extraction of *S. aethiopicus* rhizome material

The ethyl acetate extract obtained from the bulk extraction of the rhizome material resulted in a clear bright yellow extract. It dried down to a thin orange oil (6.309 g) with a strong "ginger-like" aroma. This residue represents 7.01% of the dry material and 1.85% of the fresh material used. In comparison, hydrodistilling fresh roots and rhizomes for three hours yielded a yellow oil which was 0.1% of the wet weight (VILJOEN, DEMIRICI, BASER and VAN WYK, 2002).

4.3.3 Bioassay-guided fractionation

4.3.3.1 Vacuum liquid chromatography

The residue yields for the fractions obtained from the VLC of ≈4 g of the ethyl acetate bulk extract of *S. aethiopicus* rhizomes are given in Table 4.3. Fraction C yielded the highest amount (1.236 g) and fraction M the lowest amount (0.018 g).

Table 4.3   Residue yields for the fractions obtained from the VLC of the ethyl acetate extract of *S. aethiopicus* rhizome material.

Fraction	Hexane:EtOAc	Residue (g)	Fraction	Hexane:EtOAc	Residue (g)
A	100:0	0.388	H	50:50	0.113
B	85:15	1.008	I	40:60	0.357
C	80:20	1.236	J	30:70	0.125
D	75:25	0.087	K	20:80	0.167
E	70:30	0.069	L	10:90	0.037
F	65:35	0.379	M	0:100	0.018
G	60:40	0.066			

4.3.3.2 Evaluation of VLC fractions

The TLC separation of the VLC fractions and the corresponding bioautographic assay (*S. aureus*) can be seen in Figure 4.4. The chromatogram which was stained with AS reagent gave a clear indication that the VLC was a successful method of initial separation of the bulk extract. Extracts A to G showed little overlap of similar compounds, although fractions H to M were quite similar in nature (this is also evident in the bioautography). The AS stained plate also served as a reference plate for the bioautographic assay.

The bioautography showed a number of clear zones against a pink background. Inhibition zones were visible in fractions: B and C at R_f 0.75; C and D at R_f 0.6; D at R_f 0.56; E at the origin; and H to M along the origin. There was also a large clear zone in fraction G which did not correspond with any compound visible on the reference plate (or visible with UV). The bioautography was repeated and similar results were obtained, thus eliminating the possibility of an error in the technique with the first attempt. Fraction C was chosen for further purification using preparative TLC.

These clear zones give an indication of antibacterial activity by compounds present in these areas. However, lipophilic compounds may also result in clear zones because the innoculated nutrient broth may not sufficiently wet the area due to the hydrophobic nature of the compounds (JORK, FUNK, FISCHER and WIMMER, 1990).
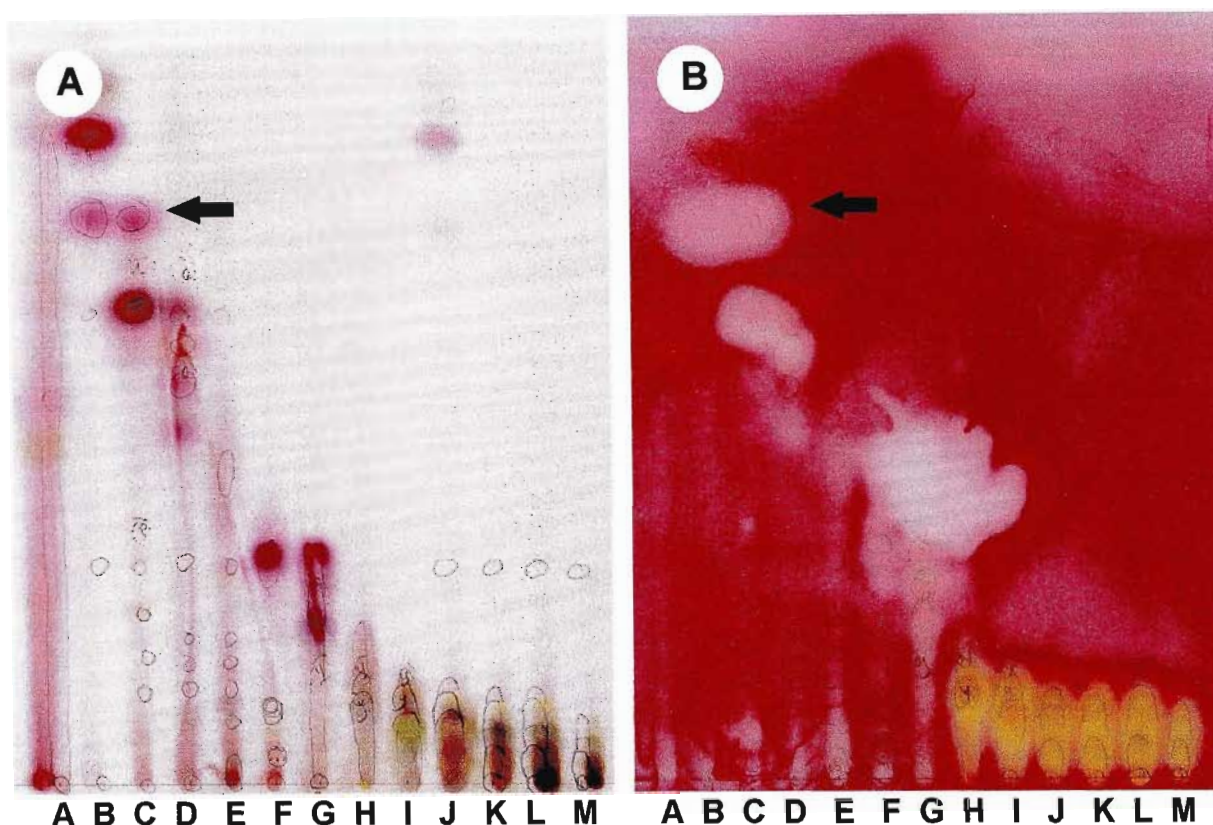
Figure 4.4 Chromatogram of vacuum liquid chromatography fractions A-M, developed in hexane:ethyl acetate 2:1: (A) stained with AS reagent, and (B) the corresponding bioautographic assay using *S. aureus* as a test organism. Arrows indicate corresponding zones.

The results for the COX-1 assay of the VLC fractions, tested at 10 mg ml^{-1}, are shown in Figure 4.5. The highest COX-1 inhibition was obtained with fractions A (86%), E (76%), G (87%) and H (82%). COX-1 inhibition with the remaining fractions ranged from 38% to 57%. From these results, it may be possible to further purify the more active fractions.
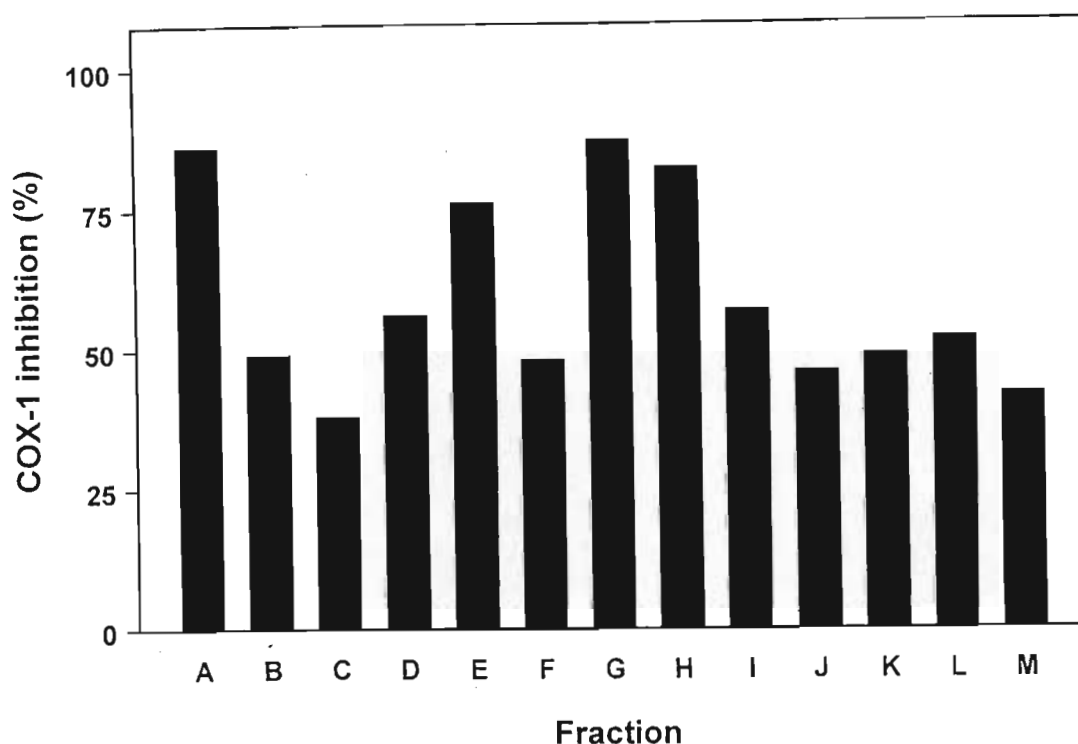
Figure 4.5 COX-1 inhibition of VLC fractions. Extracts were tested at a final concentration of 250 µg ml^{-1} per test solution. Values represent the mean of double determinations. The indomethacin standard inhibited the synthesis of prostaglandins by 73%.

4.3.3.3 Preparative TLC

Fraction C was chosen for further separation due to the high yield of this fraction and the apparent separation of the compound showing antibacterial activity from other compounds in the fraction with TLC. Using PTLC, 24.8 mg of the compound marked with arrows in Figure 4.4 and visible as a dark band under UV$_{254}$ at R$_f$ 0.54 (toluene:ethyl acetate 93:7), was isolation. Two narrow strips from one of the TLC plates were used for staining with AS reagent, and for a bioautographic assay. The band stained bright pink with AS reagent, and corresponded with a clear zone in the bioautographic assay (*S. aureus*).

It is important to verify the presence of a compound with staining reagents and/or bioautographic assays, because the resolution of a compound separated on an analytical plate can change significantly when the amount of mixture is increased for PTLC. GIBBONS and GRAY (1998) suggested that this is possibly attributable to sorbent particle size.

4.3.4 NMR and mass spectroscopy

Furanoeremophil-2-en-1-one ($C_{15}H_{18}O_2$, FW = 230.13) was isolated as an amorphous compound. The presence of 15 carbon resonances in the ^{13}C NMR spectrum indicated that this compound was a sesquiterpenoid. At the time of the investigation, this compound had not been reported previously in the chemical literature and a full assignment of the NMR spectra was undertaken to determine the structure (Table 4.4). The NMR spectra appear in Appendix 2.

The relative stereochemistry could be assigned by means of the NOESY spectrum and is given in the structure shown in Figure 4.6. The COSY spectra show the H-H coupling and the HMBC spectra show 3 -bond coupling (and occasionally 2 and 4 bond coupling) and are reported here as C→H correlations. The NOESY spectra show correlations through space and show hydrogen atoms close to one another, allowing for the determination of the stereochemistry.
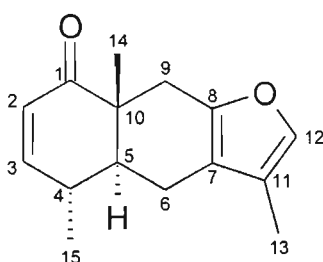
Figure 4.6 Structure of the furanosesquiterpenoid furanoeremophil-2-en-1-one isolated from the rhizome of *S. aethiopicus*.

Table 4.4 ^{1}H, ^{13}C, COSY, HMBC and NOESY NMR data for the compound isolated from *S. aethiopicus* (CDCl$_3$, 500 MHz).

Carbon	^{1}H	^{13}C	COSY	HMBC C→H	NOESY
1	-	204.14	-	3, 5, 14	-
2	5.94 d 10.1	126.56	2, 4	15w	3
3	6.77 dd 10.1, 2.1	154.31	3	4, 15	2, 4, 15
4	2.42 m	34.2	5, 15	2, 3, 5, 6a, 15	3, 6β, 14, 15
5	1.84 td 5.2, 10.7	44.91	4, 2H-6	2, 9α, 9β, 14, 15	6β, 9β, 15
6	β 2.15 m	22.51	5, 6α	5	4, 6α, 14
	α 2.64 m		5, 6β		*
7	-	114.62	-	6β, 6α, 9α, 9β, 13	-
8	-	149.24	-	6β, 6α, 9α, 9β, 13w	-
9	α 2.76 dd 1.3, 16.9	31.93	9β	14, 5	*
	β 2.67 d 16.9		9α		*
10	-	44.92	-	3, 6β, 6α	-
11	-	119.04	-	6α, 13	-
12	7.05	137.48	13	13	13w
13	1.93 d 1.2	8.08	12	-	6?, 12, 15
14	1.05 s	16.56	-	5, 9α, 9β	6β, 4, 9β
15	1.23 d 7.2	18.08	4	3, 4, 5	4, 5, 6α

* Resonances superimposed – can't distinguish correlations.

The results for the GC-MS 70eV, *m/z* (% rel. int.), are: 230 (100), 215 (96), 197 (18), 187 (75). The structure of the isolated compound (from NMR) and the GC-MS results correlated with the recently reported data for the compound 4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-*b*]-furan-8-one which was isolated from the essential oils of *S. aethiopicus* (HOLZAPFEL, MARAIS, WESSELS and VAN WYK, 2002).

4.3.5 MIC determination

The results for the MIC determination of the isolated compound are shown in Table 4.5. Only a very minimal bacteriostatic effect was observed in the microdilution antibacterial assay (0.1 to 3.13 mg ml^{-1}). There was slightly higher activity against the Gram-positive bacteria than the Gram-negative bacteria. However, the levels of activity of the isolated compound seen in this assay did not differ from activity obtained with the crude extracts (section 3.3.1, Table 3.1).

The crude extracts were tested against bacterial cultures obtained from the Microbiology Department, University of Natal, Pietermaritzburg (section 3.2.2.1), whereas the isolated compound was tested against ATCC culture strains. A comparison of the MIC values of the neomycin control (section 3.3.1, Table 3.1; Table 4.5) gives an indication that the ATCC strains are more resistant than the cultures obtained from the Microbiology Department. The MIC values of the neomycin against the ATTC strain of *S. aureus* was 62-fold higher, and the ATCC strain of the *B. subtilis* was 8-fold higher than obtained against the Microbiology Department cultures. However, the bioautographic assay was performed using the more resistant ATCC strain of *S. aureus* and did present zones of inhibition and apparent compound "activity".

Table 4.5 Concentrations (mg ml^{-1}) at which the isolated compound from *S. aethiopicus* rhizome showed a bacteriostatic effect against test bacteria (double determination).

	Bacterium[a]			
	B.s.	S.a.	E.c.	K.p.
Sesquiterpenoid	0.78	0.1	3.13	3.13
Neomycin standard (μg ml^{-1})	0.78	6.25	1.56	1.56

Abbreviations: [a] *B.s. - B. subtilis; S.a. - S. aureus; E.c. - E. coli; K.p. - K. pneumoniae.*

Following the microdilution assay, the mixture from one of the wells of the 96-microtitre plate was removed and dried under nitrogen. This residue was spotted on a TLC plate and developed in hexane:ethyl acetate (2:1) to verify the presence of the compound, in comparison to an untested sample. From this simple procedure it was ascertained that the compound was present, and had not degraded (a possible explanation for the low activity in the assay). As mentioned earlier, it is possible that the lipophilic nature of the compound may have resulted in the clear zone observed with the bioautography.

4.4   SUMMARY

- A preliminary serial extraction of *S. aethiopicus* rhizome material was conducted using a Soxhlet apparatus.

- Extracts were tested in the COX-1 assay and microdilution antibacterial assay.

- Hexane and ethyl acetate extracts gave slightly higher COX-1 inhibition than the ethanol extract.

- No distinct differences were observed in the microdilution assay.

- A bulk ethyl acetate extract of *S. aethiopicus* rhizome material was prepared.

- VLC of ≈4 g of extract was performed, yielding 13 fractions.

- The VLC extracts were evaluated using TLC and a bioautographic assay, and tested in the COX-1 assay.

- The bioautography, using *S. aureus* as a test organism, revealed a number of compounds present which exhibited antibacterial activity.

- Fraction C was purified further using PTLC, and 24.8 mg of a pure compound from R_f 0.54 (toluene:ethyl acetate 93:7) was isolated.

- NMR spectra and GC-MS spectra for the isolated compound were recorded.

- The compound was identified as the sesquiterpenoid furanoeremophil-2-en-1-one, which is structurally identical to the recently reported compound 4aαH-3,5α,8aβ-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-*b*]-furan-8-one.

- The compound showed only a very minimal bacteriostatic effect in the microdilution assay.

5 Effect of Senescence and Post-harvest Handling on Anti-inflammatory and Antibacterial Activity

5.1 INTRODUCTION

An important aspect of the bioactivity of medicinal plants stems from the fact that plants do not consistently produce the same chemicals, in the same quantities. As a result, the potency of medicinal plants is affected by a number of factors, such as the biochemical factors within individual species and external factors such as climate, geographical location, season and other ecological and growth conditions (PRANCE, 1994). Variation of the activity can also occur due to treatment after collection (storage and preparation) (COLEGATE and MOLYNEUX, 1993; HOUGHTON and RAMAN, 1998).

In a study of the essential oils of *Tagetes minuta* harvested at different locations at precise growth stages, CHALCHAT, GARRY and MUHAYIMANA (1995) showed that the oil yield varied during plant growth, being higher in younger plants (before flowering). Although the study showed no differences in oil composition between samples obtained from cultivated or wild plants from different locations in France, the French oils did show higher levels of (Z)-β-ocimene and tagetenone compared to the samples from Rwanda.

Seasonal variation in the schizontocidal effect of leaf extracts of *Morinda lucida*, a plant commonly used against fever in Nigeria, was shown by MAKINDE, AWE and SALAKO (1994). Their study illustrated that samples collected in March showed highest activity on an early infection and on established infections of *Plasmodium berghei berghei* in mice, and least activity from samples collected in December.

The seasonal variation in growth and chemical constituents of real ginger, *Zingiber officinale*, cultivated in Egypt was investigated by EL-GENGAIHI and WAHBA (1995). The volatile oil constituents of the shoots showed some variation over the course of an annual growth cycle.

In a study of the hypericin and pseudohypericin content of *Hypericum perforatum* (St. John's Wort) over two seasons, SOUTHWELL and BOURKE (2001) demonstrated the seasonal variation in the narrow and broad leaf biotypes. Their investigation showed that the total-hypericin concentration in the soft tops of the plants of both biotypes were lowest during the winter months and reached maximum values in summer. Furthermore, the narrow leaf biotype consistently showed higher levels of total-hypericins than the broadleaf biotype.

In a study on the essential oils from fresh and dried rhizomes of *Alpinia galanga* (Zingiberaceae), JANSSEN and SCHEFFER (1985) showed that the oil from dried material generally had greater activity than the fresh oil against various micro-organisms (fungi, yeast and bacteria).

The examples discussed above indicate that seasonal changes can indeed have an effect on the biological activity of certain plants. It is therefore an important consideration to examine any possible seasonal effects which may influence the biological activity of a plant under investigation.

S. aethiopicus plants are summer growing and become dormant in winter (GORDON-GRAY, CUNNINGHAM and NICHOLS, 1989). In the spring, new rhizomes develop from the mother rhizome, and new plants develop. This part of the investigation on the medicinal properties of *S. aethiopicus* was undertaken to determine if any differences in anti-inflammatory and antibacterial activity could be detected between plants harvested in summer, and once the plants had fully senesced. Extracts prepared from fresh material and from dried material were also tested to investigate the effect of the state of the material on biological activity.

5.2   MATERIALS AND METHODS

5.2.1 Extract preparation

Plants of *S. aethiopicus* were harvested at the end of summer, prior to senescence

(green leaves), and after the plants had fully senesced (Figure 5.1). For each set of extracts, plants were divided into leaves, rhizomes and roots, differentiating between α- and β-rhizomes and roots. The mature rhizomes, and associated roots, from the previous year's growth were classified as α-rhizomes and roots (i.e. mother rhizome and roots), and the younger rhizomes, and roots, which develop from the α-rhizome at the start of the growing season were classified as β-rhizomes and roots (i.e. daughter rhizome and roots) (Figure 5.1).
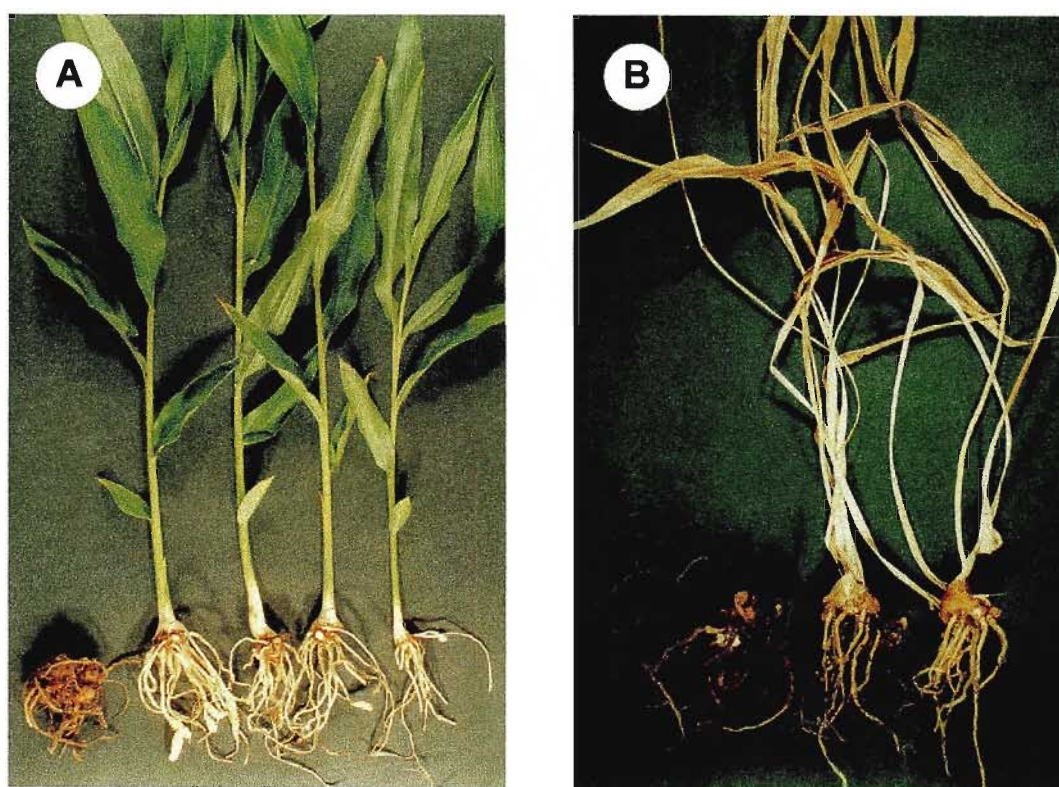
Figure 5.1   *S. aethiopicus* plants harvested (A) before senescence, and (B) after senescence, showing α- and β-rhizomes.

Portions (10 g) of the freshly harvested material was extracted in 100 ml ethanol using a Waring blender, and then placed in an ultrasound bath (Branson 5210) for 60 min. The remaining material was dried at 50 °C for 2 days prior to extraction in the same manner. The extracts were vacuum filtered through Whatman No. 1 filter paper, and dried under vacuum at 35 °C. Two sets of extracts were prepared for plants harvested before and after senescence.

5.2.2 TLC fingerprinting

TLC fingerprints of the different extracts were prepared by loading 0.5 mg of each extract in a 1 cm band onto 10 x 10 cm plastic-backed TLC plates (silica gel 60 F_{254}, 0.25 mm, Merck). The plates were developed in hexane:ethyl acetate (2:1), and developed over 7.5 cm. The plates were allowed to dry, and following examination under UV_{254nm} and UV_{366nm}, were stained with AS reagent and heated for 5 - 10 min at 110 °C.

5.2.3 Testing COX-1 inhibition

The extracts were redissolved in ethanol and tested in the COX-1 assay, as described in section 2.2.2.1, at a concentration of 10 mg ml^{-1} (giving a final concentration of 250 µg ml^{-1} per test solution).

5.2.4 Testing antibacterial activity

Antibacterial activity was assessed using the microdilution bioassay as described in section 3.2.2.1. The residues from the plant extracts were redissolved in ethanol at a concentration of 50 mg ml^{-1}. Each extract was tested against bacterial strains obtained from the bacterial collection of the Microbiology Department, University of Natal, Pietermaritzburg and maintained on Mueller-Hinton nutrient agar (Biolab) at 4°C. The bacteria used were *B. subtilis*, *S. aureus* (Gram-positive) and *E. coli*, *K. pneumoniae* (Gram-negative).

5.3   RESULTS AND DISCUSSION

5.3.1 TLC fingerprinting

The TLC fingerprints for extracts prepared from the different plant parts before and after senescence, and from fresh or dried material are shown in Figures 5.2-5.4.

Visible -
AS reagent

A →
B →

UV 254 nm

A →
B →

UV 366 nm

A →
B →

L(g)   L(g)   L(s)   L(s)
F   D   F   D
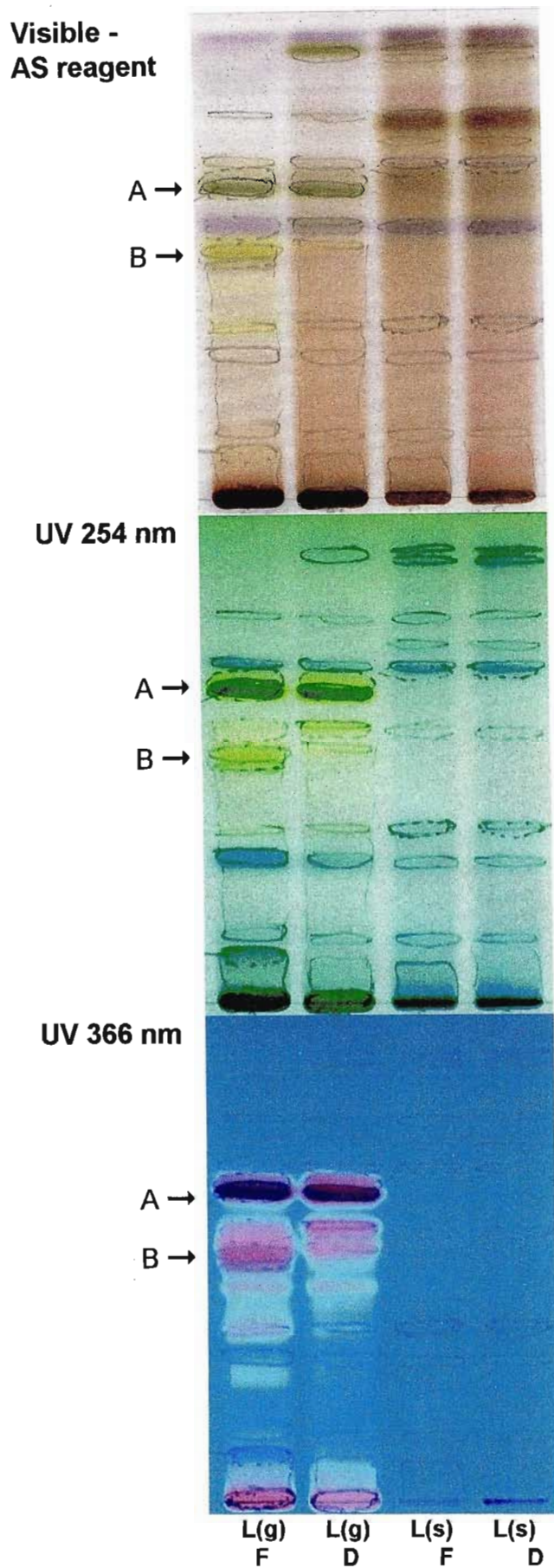
Figure 5.2 TLC fingerprints of green and senesced leaves of S. aethiopicus. L(g) = green leaves; L(s) = senesced leaves; F = fresh material; D = dried material. TLC plates developed in hexane:ethyl acetate 2:1.
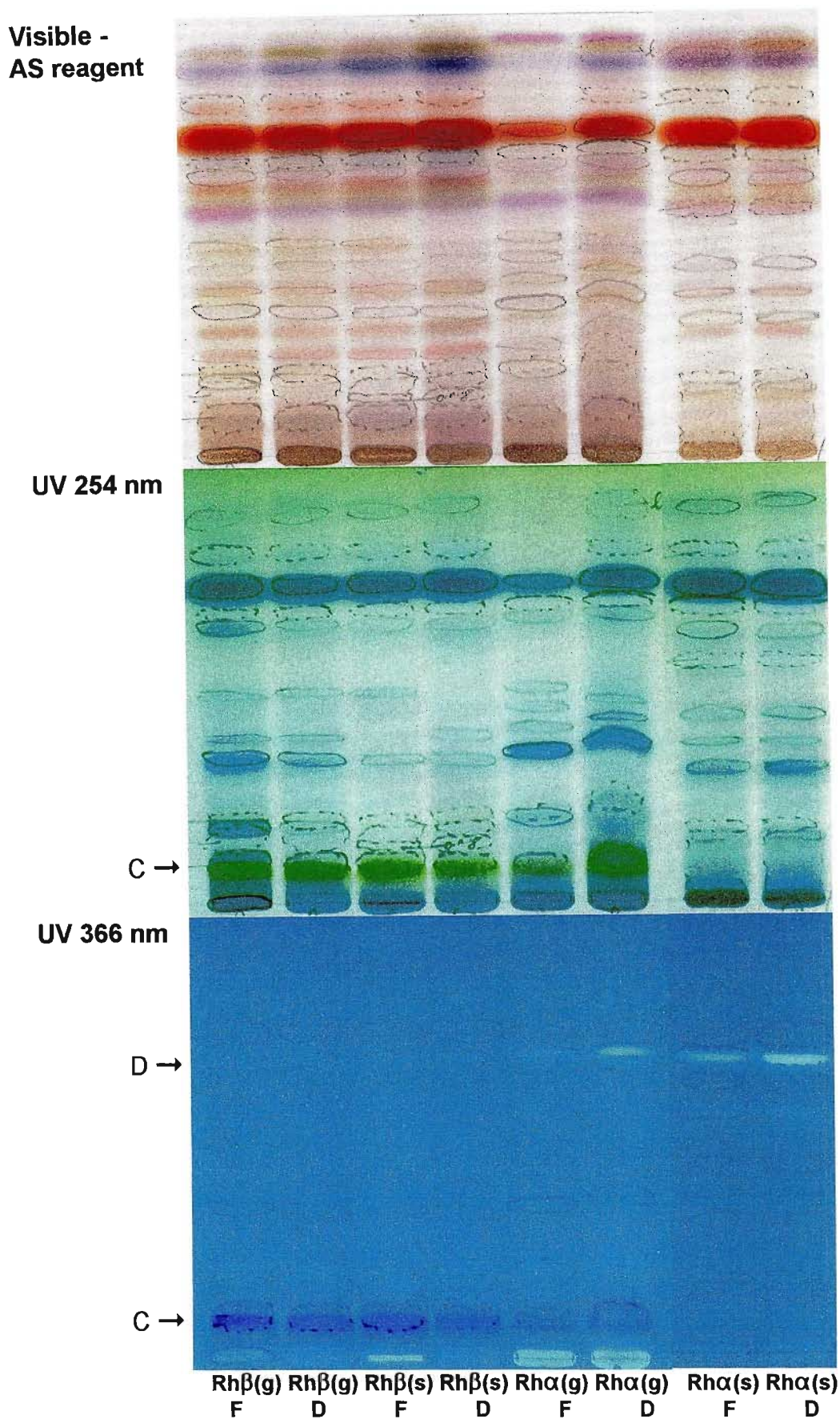
Figure 5.3 TLC fingerprints of rhizomes of *S. aethiopicus* before and after senescence. Rhα = α-rhizome; Rhβ = β-rhizome; (g) = rhizomes before senescence; (s) = rhizomes after senescence; F = fresh material; D = dried material. TLC plates developed in hexane:ethyl acetate 2:1.
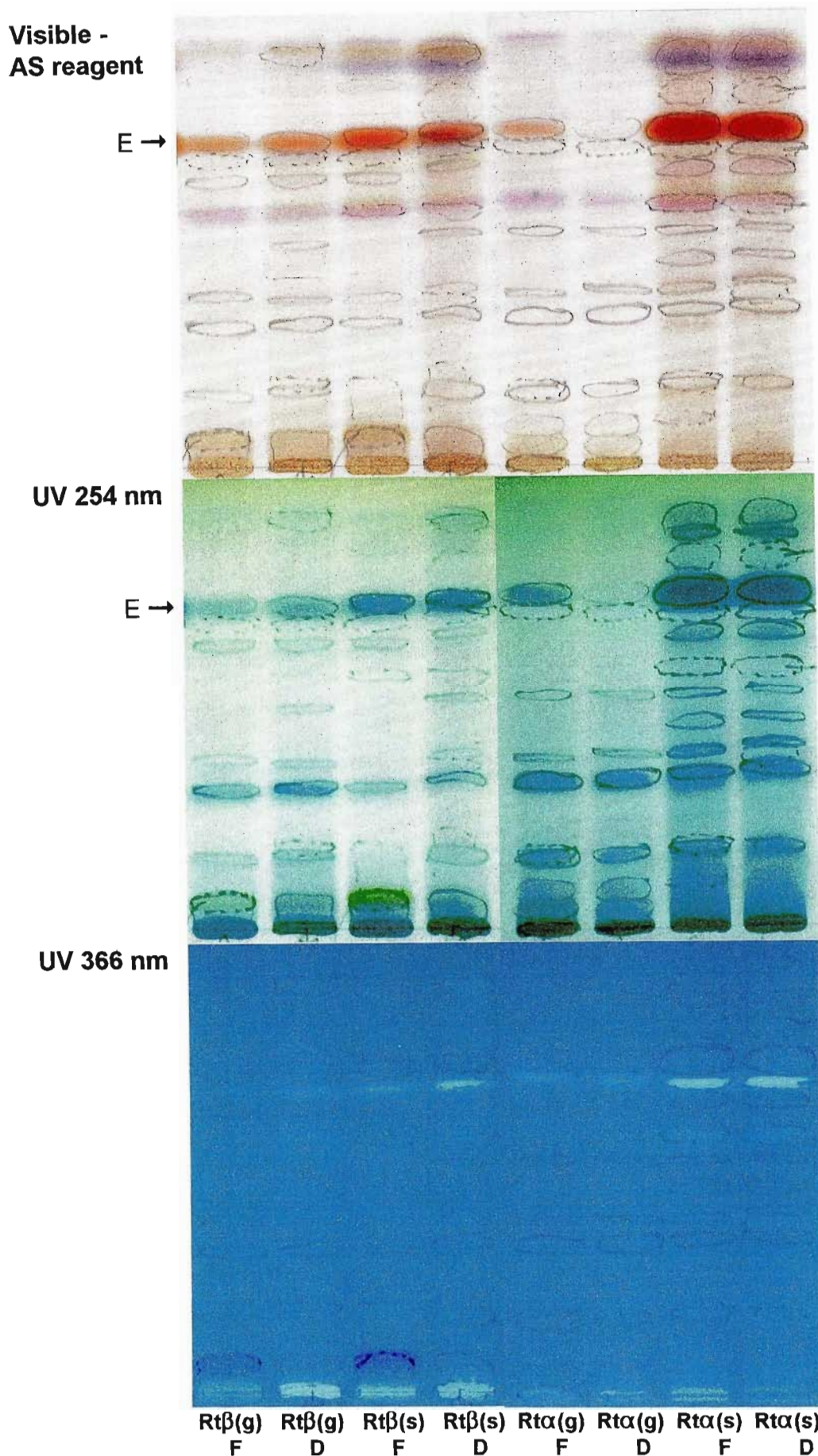
Figure 5.4 TLC fingerprints of roots of *S. aethiopicus* before and after senescence. Rtα = α-roots; Rtβ = β-roots; (g) = roots before senescence; (s) = roots after senescence; F = fresh material; D = dried material. TLC plates developed in hexane:ethyl acetate 2:1.

The senesced leaves showed a loss of photosynthetic pigments compared with the green leaves, as would be expected. Examples of this can be seen in bands A (R_f 0.63) and B (R_f 0.43) which are only present in the first two lanes (extracts from green material) (Figure 5.2). In the rhizome extracts, the dark band C (R_f 0.07), visible under UV light, was not seen in the α-rhizomes after senescence. However, the white fluorescing band D (R_f 0.64) did appear brighter in the α-rhizome extracts (Figure 5.3). The main difference observed in the root extracts appeared to be an increase in the compound seen in band E (R_f 0.74) in the α-rhizomes after senescence (Figure 5.4).

Although minor differences were observed in the TLC analysis of the various extracts prepared, it is not possible to conclude whether these changes are in any way related to changes in activity. However, TLC fingerprinting does provide a simple method of illustrating differences in the chemical composition of plant extracts. For quantitative analysis of TLC fingerprints it is important to employ consistent extraction methods, even loading of the extracts on the TLC plate and replicable development systems.

5.3.2 COX-1 inhibition by *S. aethiopicus* extracts

Results for the study of seasonal effects on COX-1 inhibition are shown in Figure 5.5. As observed with the initial screening (see section 2.3.3), the leaves showed the highest levels of activity, with the exception of senesced leaf material that was dried prior to extraction. No differences were observed between the activity of the α- and β-rhizomes, before and after senescence. For the α-roots, there was a slight decrease in activity following senescence. This was seen in both the fresh and dried material. The β-roots showed an opposite trend, in that there was an increase in activity following senescence.
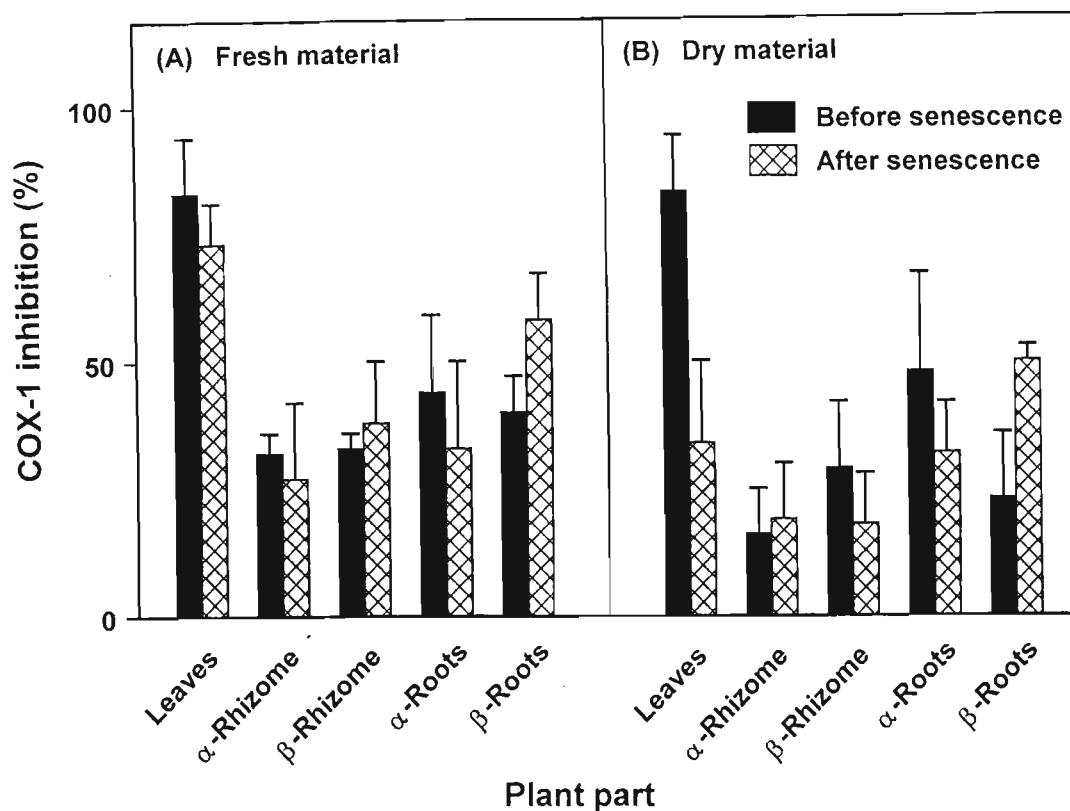
Figure 5.5 COX-1 inhibitory activity (± SD) of ethanol extracts of various plant parts of *S. aethiopicus* before and after senescence. Extracts were tested at a final concentration of 250 µg ml⁻¹ per test solution. Values represent the mean of double determinations from two experiments. The indomethacin standard inhibited the synthesis of prostaglandins by 54 ± 5.7%.

5.3.3 Antibacterial activity of *S. aethiopicus* extracts

Results for the study of seasonal effects on antibacterial activity are shown in Table 5.1. Little difference was observed between the extracts prepared from fresh and dry material for activity against *S. aureus*. Extracts prepared from the leaves gave a MIC value of 0.2 (fresh) and 0.1 (dry) mg ml⁻¹ before senescence and 3.13 mg ml⁻¹ after senescence, indicating a loss in activity. Extracts prepared from α-roots gave MIC values of 3.13 and 1.56 mg ml⁻¹ before senescence and 0.1 and 0.2 mg ml⁻¹ after senescence, indicating an increase in activity.

Table 5.1   Minimum inhibitory concentrations (mg ml^{-1}) of *S. aethiopicus* ethanol extracts against *S. aureus*.

Extract	Plant part	Before senescence	After senescence
Fresh material	Leaves	0.2	3.13
	Rhizome - α	1.56	1.56
	Rhizome - β	6.25	3.13
	Roots - α	3.13	0.1
	Roots - β	3.13	1.56
Dry material	Leaves	0.1	3.13
	Rhizome - α	3.13	3.13
	Rhizome - β	6.25	6.25
	Roots - α	1.56	0.2
	Roots - β	0.78	0.78
Neomycin standard (μg ml^{-1})		0.39	0.39

The method of drying plant material at temperatures of 40-50 °C can cause the breakdown of certain compounds or a change in the concentration of volatile components, leading to a potential loss of activity. It is therefore important to consider the effects of the preparation method and storage on the biological activity of medicinal plants. Although fresh material may be more pharmacologically active, dried material is less likely to decay due to bacterial or fungal infection and can be kept for longer periods.

5.4 SUMMARY

- *S. aethiopicus* plants were harvested before senescence and after senescence.
- Ethanol extracts were prepared from fresh or dried material of the leaves, rhizomes and roots, and tested in the COX-1 assay and the microdilution antibacterial assay.
- TLC fingerprints of the extracts were prepared.
- No noteworthy changes in COX-1 inhibition, due to senescence, were observed from extracts prepared from fresh material.
- There appeared to be a slight decrease in COX-1 inhibition with the α-roots and an increase in the β-roots after senescence.
- A decrease in antibacterial activity of the leaves after senescence was observed.
- An increase in antibacterial activity of the α-roots was observed after senescence.
- The time of harvest, and the effect of drying plant material, may only have a minimal influence on the degree of anti-inflammatory and antibacterial activity in the material.

6   General Conclusions

Within the plant kingdom there is great potential for the discovery of biologically active natural products for the development of new effective therapeutic agents (BALANDRIN, KINGHORN and FARNSWORTH, 1993). The discovery of biological activity and the subsequent isolation of active principles are necessary first steps in the drug development process (COX and BALICK, 1994). Successful research in this field requires a rigorous and methodical multi-disciplinary approach, incorporating analytical chemistry, bioactive compound discovery, bioassay method development, clinical studies, cultivation of medicinal plants, ethnobotany, organic synthesis, pharmacology, phytochemistry, the standardization of traditional medicines, taxonomy, tissue culture, and numerous other disciplines (KINGHORN, 2001).

Although the goal of modern therapeutic phytochemistry is the discovery of new active principles and their improvement, the use of plants and plant extracts will continue in traditional medicinal systems. It is important, however, to evaluate the safety and efficacy of plants used in traditional medicine.

In South Africa, there is a need for systematic documentation of traditional plant use and the evaluation of the medicinal plants used in traditional medicine (VAN WYK, 2002). Furthermore, with the increasing problem of HIV infection, there is a need for therapeutic agents which can be used against opportunistic infections related to AIDS. The emerging problem of bacterial resistance to drugs also necessitates the discovery and development of novel antibacterial agents.

The results presented in this thesis provide an example for approaching investigations of medicinal plants, including pharmacological screening of plant extracts, followed by bioassay-guided fractionation leading to the isolation of pure constituents (HOSTETTMANN, MARSTON and WOLFENDER, 1995). The first aspect of the investigation comprised the testing of extracts from the different parts of *S. aethiopicus* for *in vitro* anti-inflammatory activity in the COX-1 and COX-2 assays, and for antibacterial activity in the microdilution assay. The isolation and identification of a

compound showing activity in a bioautographic assay was also conducted using various chromatographic and spectroscopic techniques.

The final part of the investigation involved testing for COX-1 inhibitory activity and antibacterial activity of *S. aethiopicus* plants before and after senescence to determine if senescence has any effect on the medicinal properties of the plant. Minimal differences were observed between green and senesced plants, which suggests that plants could be harvested at either stage. However, the roots of fully senesced plants decay during the winter, and thus it would be more useful to harvest plants which still have healthy roots.

This thesis also provides a starting point for future, more detailed studies on *S. aethiopicus*, as an investigation of this nature cannot fully evaluate all aspects of the medicinal properties of the plant. Activity against COX-1 and COX-2 can be evaluated further for the potential isolation of active compounds. The bioautographic assay showed a number of antibacterial zones which can be further investigated, leading to the possible isolation of active principles.

Extracts from *S. aethiopicus* have also been screened for activity in a number of other bioassays in a further investigation of the pharmacological properties of the plant. No activity was observed in a simple anthelmintic bioassay, using *Caenorhabditis elegans* free-living nematodes as test organisms, and in an antischistosomal bioassay. Furthermore, no significant activity against the herpes simplex virus types 1 and 2, and the influenza A virus were observed. However, some cytotoxicity was observed with the aqueous extracts of the rhizome, indicative of potential harm (LIGHT, McGAW, RABE, SPARG, TAYLOR, ERASMUS, JÄGER and VAN STADEN, 2002). This is one aspect which certainly requires further investigation.

S. aethiopicus represents a valuable indigenous resource which has potential for future profit in small-scale agriculture for use in traditional medicine and in the more sophisticated pharmaceutical industry.

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APPENDIX 1: Medicinal uses of the Zingiberaceae

Plants of the Zingiberaceae, their occurrence and medicinal uses (JOHNSON, 1999).

Species name	Distribution	Action	Used to treat
Aframomum granum-paradisi	Nigeria		tumor
Aframomum melegueta	Egypt, Ghana, Liberia, Nigeria	aphrodisiac, astringent, carminative, excitant, stimulant, stomachic, tonic	dysentry, enteritis, gastritis, malaria, tumor
Alpinia antillarum			carcinoma
Alpinia chinensis			
Alpinia galanga	USA, India, Java, Turkey	cardiodepressant, carminative, digestive, expectorant	bronchitis, cancer, catarrh, parturition, ringworm, splenomegaly
Alpinia globosa			
Alpinia intermedia			
Alpinia japonica			
Alpinia malaccensis	Java		sore
Alpinia officinarum	USA, East Indies, Egypt, India, Southeast Asia, Turkey	aphrodisiac, bactericide, carminative, nervine, stimulant, stomachic, tonic	cancer, candida, cough, diabetes, halitosis, malaria, persistent flatulence
Alpinia purpurata	Haleakala National Park, Hawaii, Volcanoes National Park, Samoa		sore
Alpinia pyrmaidata			
Alpinia sp.	Caroline Islands, Great Britain, Philippines		nausea, wen

Species name	Distribution	Action	Used to treat
Alpinia speciosa	Dominica, Haiti	diuretic	common cold, fever, flatulence, flu, indigestion
Amomum aromaticum			
Amomum cardamom	Europe		sclerosis (uterus)
Amomum coccineum	Sumatra	vermifuge	
Amomum compactum			
Amomum costatum			
Amomum dealbatum			parturition
Amomum globosum			
Amomum gracile	Serkkom		dyspepsia, nausea
Amomum granum-paradisi	Europe		tumor (liver)
Amomum hochreutineri	Kihitir		lumbago
Amomum kepulaga	Malaya		common cold, cough, halitosis, hepatosis, insanity, rheumatism
Amomum krevanh	Cambodia		tumor (uterus)
Amomum melegueta	Gabon, Trinidad	antispasmodic, carminative, pectoral	cancer (nose), colic, common cold, dyspepsia, fever, stomach ache
Amomum sp.	Philippines		cough
Amomum squarrosum	Malaya		giddiness
Amomum subulatum	Nepal	stomachic	neuralgia
Amomum uliginosum	Pahang	vermifuge	
Amomum villosum			

Species name	Distribution	Action	Used to treat
Amomum xanthioides	Malaya	stomachic	
Costus villosissimus	Panama		to alleviate pain after childbirth
Curcuma aeruginosa	Java, malaya	purgative	asthma, cough, dermatosis, insanity, parturition, scabies, scurf
Curcuma amada		carminative, stomachic	contusion, sprains
Curcuma angustifolia	India, Turkey	demulcent	diarrhoea, fever, gravel
Curcuma aromatica	USA, India	bactericide, carminative, stimulant	candida
Curcuma caesia			tumor (joint)
Curcuma domestica	Egypt, Haiti, Hawaii, India, Japan, Malaya, Philippines, Samoa, Tonga, Trinidad, Turkey	alterative, anodyne, antiseptic, carminative, cholagogue, deobstruent, diuretic, emetic, lactogogue, stimulant, tonic, vermifuge, vulnerary	anthrax, asthma, atrophy, blindness, bronchitis, bruises, cancer, carbuncles, chills, cholera, common cold, conjunctivitis, consumption, cough, depurative, dermatitis, dyspepsia, fever, fistula, gall, hepatitis, hypotherbia, hysteria, inflammation, insect bites, jaundice, lockjaw, ophthalmia, parturition, pimples, polyps, prolapse, puerperium, rash, rhagades, rheumatism, scabies, slurring, sore, sprains, stomach ache, yaws
Curcuma heyneana	Java	deodorant	obesity, wounds

Species name	Distribution	Action	Used to treat
Curcuma longa	Asia, Haleakala National park, Java, Malaya, Nepal, Philippines	balsamic, diuretic, lactogogue, stomachic, tonic, urogenital, vulnerary	abscesses, amenorrhea, athlete's foot, common cold, conjunctivitis, depurative, dermatosis, diarrhoea, dysentery, gonorrhea, gravel, hepatosis, impetigo, jaundice, parturition, pyuria, rashes of infants, scabies, skin sores, sore, swelling, wounds
Curcuma mangga	Java, Malaya	stomachic	fever
Curcuma pallida	Vietnam		cancer (stomach)
Curcuma petiolata	Java, Vietnam	synergist	cancer (stomach)
Curcuma purpurascens	Koenengpengang, Koenengtinggang		boils, cough, fever, itch, scabies, wounds
Curcuma sp.	Great Britain		tumor (belly), wen
Curcuma xanthorrhiza	Java, Singapore	choleretic, emmenagogue	amenorrhea, constipation, dyspepsia, gallstones, hepatosis, parturition, rheumatism
Curcuma zeodoaria	Egypt, Iran, Java, Malaya	antidote, carminative, stomachic, tonic	bruises, convalescence, dyspepsia, parturition, sprains, tumor
Curcuma zerumbet			
Elettaria cardamomum	Cambodia, Egypt, India, Sri Lanka, Turkey	aromatic, carminative, digestive, diuretic, stimulant, stomachic	asthma, bronchitis, consumption, cough, flatulence, indigestion, phthisis, tumor (uterus)
Elettariopsis sp.	Malaya		parturition
Elettariopsis sumatrana	Sumatra		scorpion sting
Geanthus cevuga	Fiji	decongestant	

Species name	Distribution	Action	Used to treat
Globba aurantiaca	Malay		parturition
Globba malaccensis	Malaya		parturition
Globba panicoides	Indonesia, Malaya, Malaysia, Vietnam		fever, gonorrhea, parturition, rheumatism
Globba perakensis	Malaya		sore
Globba uliginosa	Java, Malaya	vermifuge	
Hedychium coccineum	India		evil-eye
Hedychium coronarium	Haleakala National Park, Hawaii, India, Indonesia, Java, Kalaupapa National Historic Park, Moluccas	excitant	halitosis, rhinitis, sore throat, swelling, tonsilitis, tumor
Hedychium flavescens	Haleakala national Park, Hawaii Volcanoes National Park, Kalaupapa National Historic Park		
Hedychium gardnerianum	Haleakala National Park, Hawaii Volcanoes National park		
Hedychium longecornutum	Malaya	vermifuge	earache, syphilis
Hedychium philippense	Philippines		sore
Hedychium spicatum	India	bactericide	candida
Hornstedtia macrocheilus	Kelantan		fever
Kaempferia galanga	Asia, India, Java, Malay, Malaysia, New Guinea, Philippines, The Moluccas	carminative, cicatrizant, entheogen, expectorant, stimulant	cancer, cough, dandruff, dyspepsia, eye infection, fever, headache, malaria, mastitis, ophthalmia, otosis, rheumatism, sore throat, swelling, veterinary chills

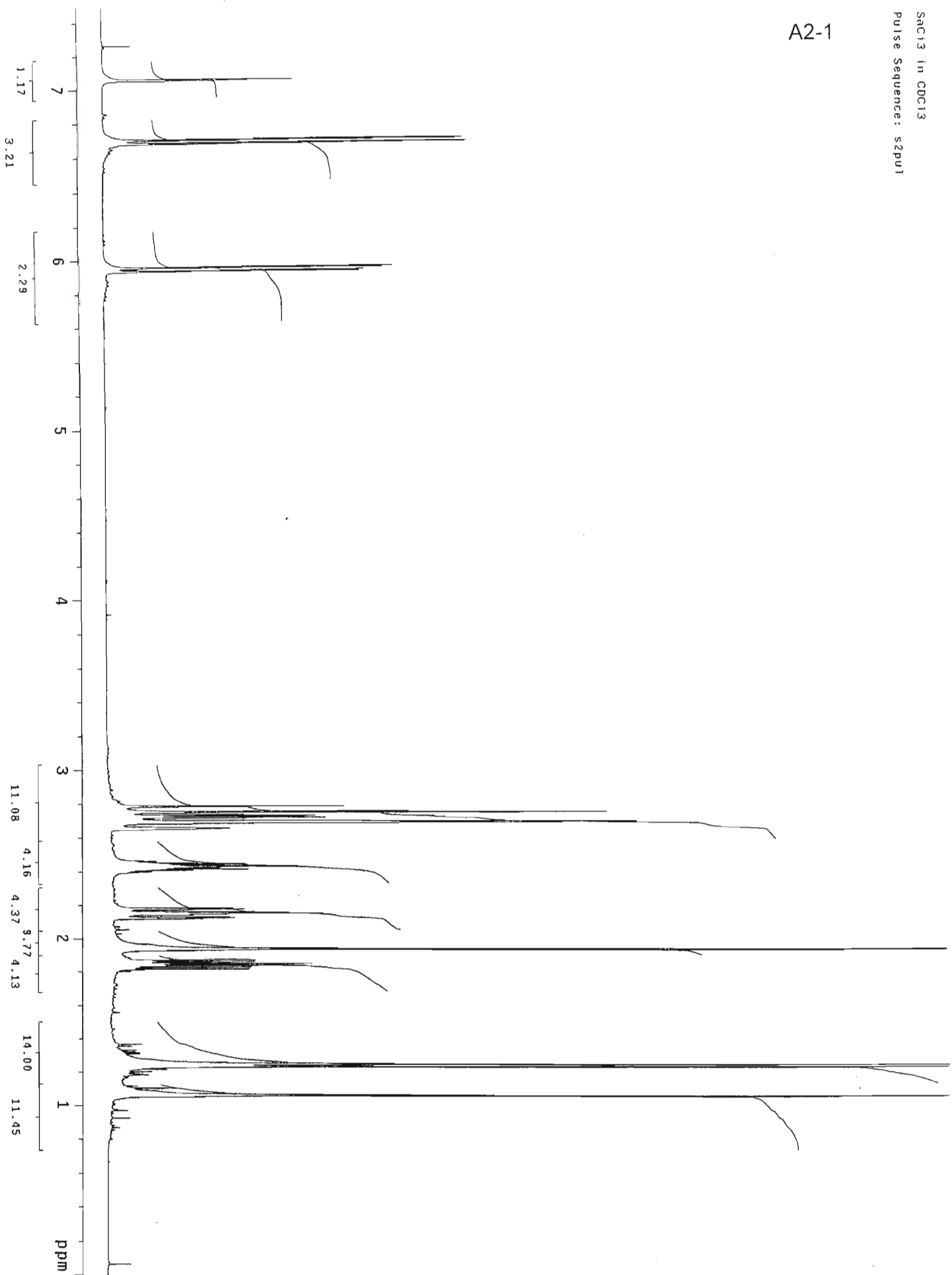| Species name	Distribution	Action	Used to treat
Kaempferia pandurata			
Kaempferia pulchra			
Kaempferia rotunda	Java, Malaya		stomach ache
Kaempferia sp.	Great Britain		wen
Languas cannifolia	Malaya		fever
Languas conchigera	Malaya		ostealgia, parturition, rheumatism
Languas malaccensis			
Languas mutica	Penang	stomachic	
Languas rafflesiana	Malacca		boils
Languas scabra	Perak		vertigo
Languas sp.	Malaya	pediculicide	
Nicolaia speciosa	Java, Malaya	deodorant	earache, wounds
Phaeomeria magnifica	Malaya		spice
Renealmia antillarum	Dominican Republic, Haiti	diuretic	rheumatism
Renealmia aromatica	Guatemala, Panama	deodorant	inflammation
Renealmia cernua	Panama		fever, typhoid
Renealmia domingensis	Brazil, Mexico	emmenagogue	piles
Renealmia exaltata	Brazil, Trinidad	antiseptic	cancer, depurative, marasmus, sore, sprains, swelling
Renealmia occidentalis	Venezuela	resolvent	excrescence, tumor
Renealmia pedicellaris	Guiana		fever

Species name	Distribution	Action	Used to treat
Renealmia sylvestris	Venezuela		tumor
Roscoea purpurea	Nepal		veterinary medicine
Zingiber amada			
Zingiber amaricans	Java	apertif, tonic	cramps, fever, infection, numbness, puerperium, stomach ache
Zingiber amomum	Europe		tumor (spleen)
Zingiber aromaticum	Java, Malaya	tonic	biliousness, chlorosis, cholecystosis, gout, parturition, pertussis
Zingiber cassumunar	Borneo, India, Java, Malay	anodyne, vermifuge	ague, anasarca, anemia, anthrax, ascites, asthma, bronchitis, colic, constipation, cramps, dropsy, fever, flatulence, gonorrhea, headache, jaundice, malaria, numbness, parturition, phthisis, stomach ache
Zingiber chrysostachys	Perak		fever
Zingiber elatum			
Zingiber griffithii	Malay		asthma, enteritis, fever, giddiness
Zingiber mioga	Japan		spice
Zingiber nigrum			

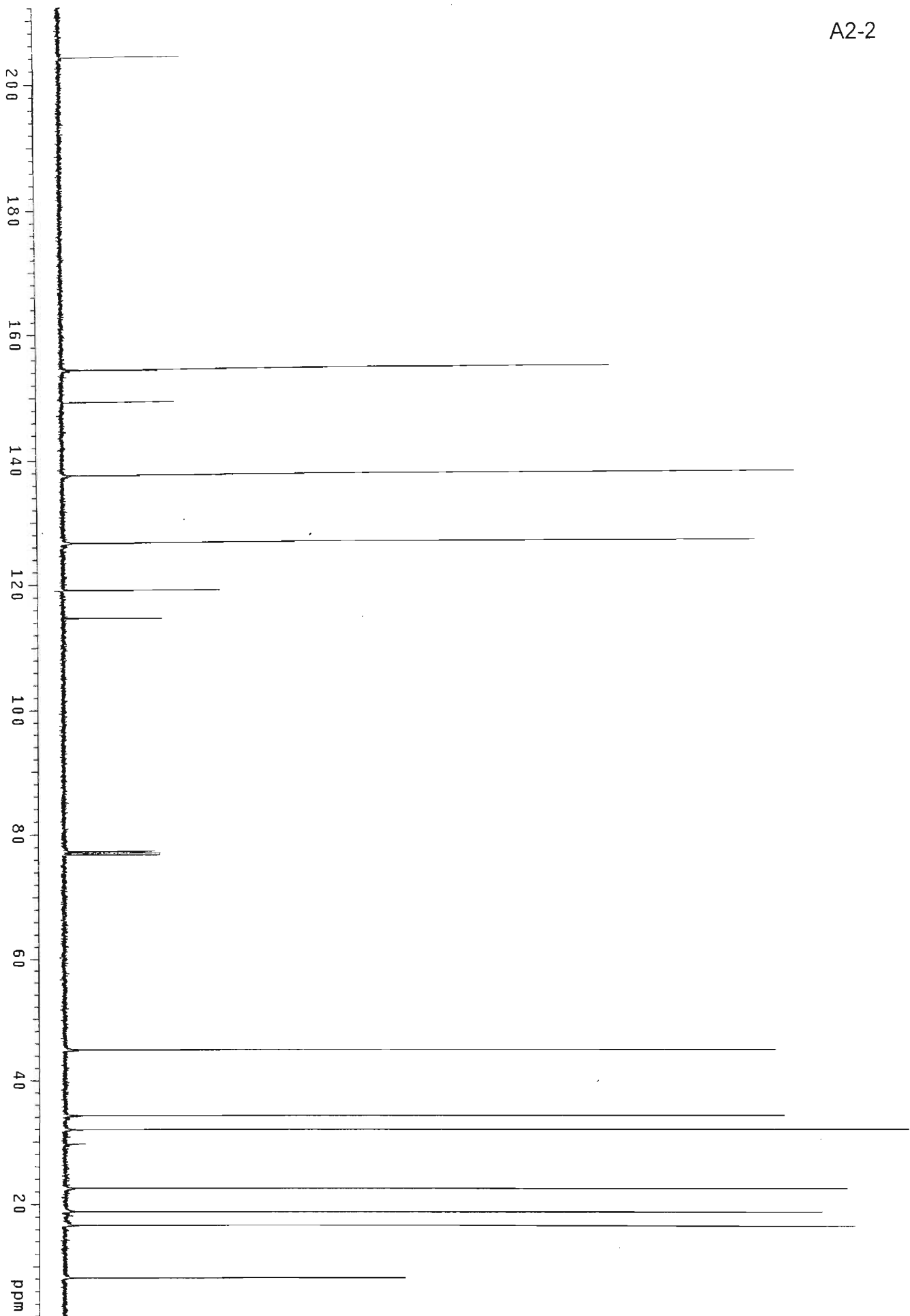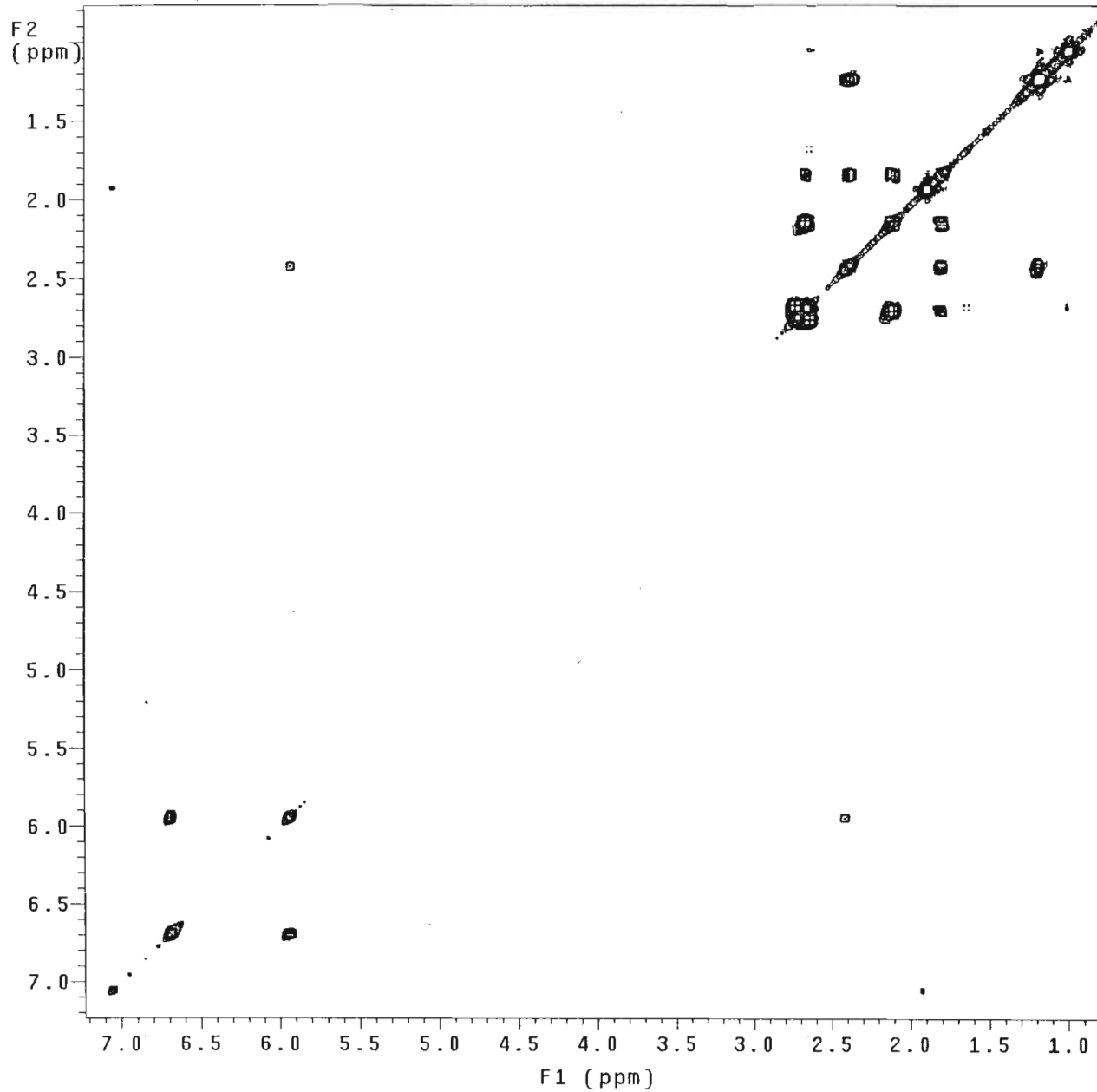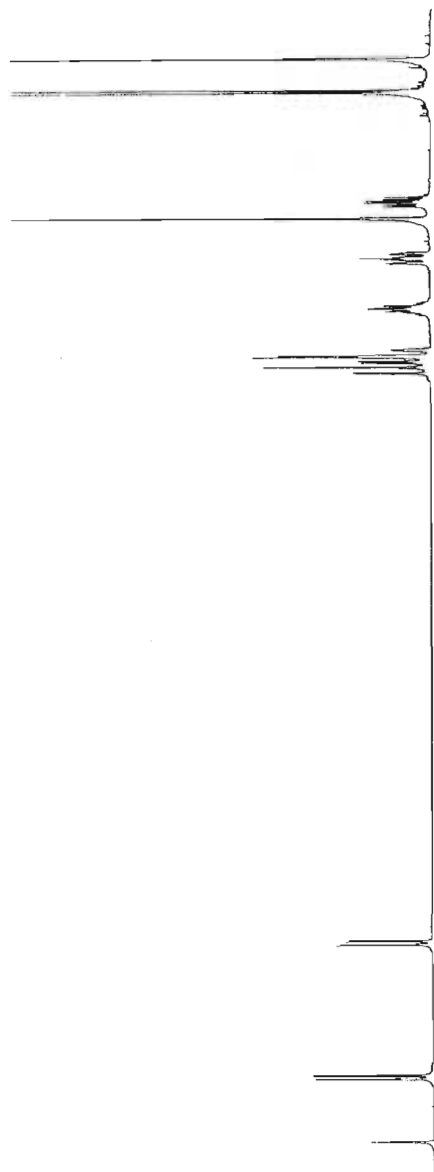| Species name	Distribution	Action	Used to treat
Zingiber officinale	USA, Asia, Burkina Faso, China, Dominican Republic, Egypt, Ethiopia, Great Britain, Haiti, India, Indonesia, Java, Malay, Mexico, New Guinea, Philippines, Trinidad, Turkey, Venezuela, West Indies	anodyne, antidote (scorpion), antiseptic, apertif, aphrodisiac, astringent, carminative, digestive, expectorant, panacea, pediculicide, rubefacient, sialogogue, sternutatory, stimulant, stomachic, sudorific, tonic	ague, amenorrhea, asthma, backache, bronchitis, cancer (breast), cataplasm, cough, diarrhoea, dog bites, dysentery, dyspepsia, fatigue, fever, fistula, flatulence, flu, gingivosis, gout, head colds, headache, hepatosis, indigestion, infection, intestinal gas, laryngitis, malaria, morning sickness, nausea, paralysis, parturition, phthisis, poor circulation, puerperium, rabies, rheumatism, rhinosis, snake bite, sore, stomach ache, suppressed menstruation, swelling, syphilis, tetanus, toothache, tumor (hand), wen
Zingiber officinalis			
Zingiber ottensii	Java, Perak, Sumatra	sedative	convulsions, cramps, lumbago, parturition
Zingiber sp.	Japan, Malaya		cancer
Zingiber spectabile	Sumatra		conjunctivitis, swelling
Zingiber zedoaria			
Zingiber zerumbet	Asia, Egypt, Fiji, Haleakala National Park, Hawaii, India, Java, Kalaupapa National Historic Park, nature Preserve of American Samoa, New Guinea, Philippines, Samoa, Tonga	antiseptic, apertif, vermifuge	asthma, biliousness, bronchitis, burns, cough, dermatitis, diarrhoea, dysentery, dysuria, gallstones, gonorrhea, headaceh, lassitude, leprosy, mouth infection, peptic ulcers, phthisis, ringworm, stomach ache, toothache, wounds

APPENDIX 2: NMR Spectra

A2-1

1.17   3.21   2.29

11.08   4.16   4.37   9.77   4.13   14.00   11.45

7   6   5   4   3   2   1   ppm

A2-3

F2
(ppm)

SaCl3 in CDCl3
1H/13C ghsqc

Pulse Sequence: ghsqc_da

F2
(ppm)

1.5

2.0

2.5

3.0

3.5

4.0

4.5

5.0

5.5

6.0

6.5

7.0

150 140 130 120 110 100 90 80 70 60 50 40 30 20 10

F1 (ppm)

A2-7