AN INVESTIGATION INTO THE BIOLOGY AND MEDICINAL PROPERTIES OF *EUCOMIS* SPECIES.

by

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Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy
in
Department of Botany
University of Natal, Pietermaritzburg.

December 1999

This thesis is dedicated to my mother,

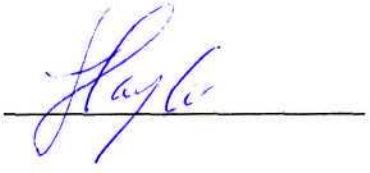
Diana Taylor,

an endless source of inspiration and encouragement.

DECLARATION

The experimental work described in this thesis was conducted in the Department of Botany, University of Natal Pietermaritzburg, from March 1996 to September 1999, under the supervision of Professor J. van Staden.

These studies are the result of my own investigations, except where the work of others is acknowledged, and have not been submitted in any other form to another University.



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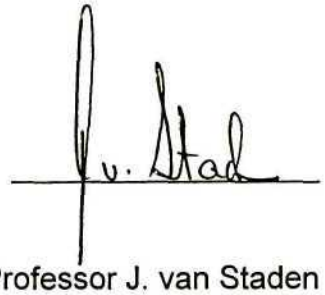

Professor J. van Staden
(SUPERVISOR)

December 1999

ACKNOWLEDGMENTS

I would like to extend my special thanks to Professor J. van Staden for supervising this work, and for his invaluable advice and encouragement throughout my academic career.

My sincere thanks go also to the members of my research committee, Dr A. K. Jäger and Dr J. F. Finnie, for their support and advice.

I have been most fortunate in that I have been able to draw on the technical and theoretical knowledge of a great many people in the Botany Department at the University of Natal. I would like to thank all those members of staff - academic, technical and especially research - who contributed so generously of their time and knowledge to assist my work. In particular, I would like to thank Dr Sibylle Zschocke for her invaluable advice and encouragement. I am also grateful to Dr Chris Viljoen for his assistance with the development of the molecular protocols and to Paul Hills and Nokwanda Makunga for their advice in this field. In addition, I would like to thank Professor Mulholland (University of Natal, Durban), Dr Boshoff (Mass Spectrometry Unit, Cape Technicon) and Mr Watson (University of Natal, Pietermaritzburg) for their prompt assistance in identifying the compounds described in this thesis.

I am most grateful to the Foundation for Research and Development for their generous financial support during my studies.

My thanks go to my friends, especially Sibylle, Cathy and Christina, as well as my adopted family in Pietermaritzburg, the Davies, for their, at times, heroic interest, and their support and encouragement during some very trying periods of research.

My special thanks go also to Barry, who has shown such faith in me, and on whose love and support I have relied.

Finally, I owe a special debt of thanks to my mother and brother, whose confidence in my abilities, and whose continued love and support have been an inspiration.

ABSTRACT

Eucomis (Family Hyacinthaceae) are deciduous geophytes with long, narrow leaves and erect, densely packed flower spikes. The bulbs are greatly valued in traditional medicine for the treatment a variety of ailments, and are thus heavily harvested for trade in South Africa's "muthi" markets. *Eucomis* species propagate relatively slowly from offsets and seed, and this, together with their over-utilization ethnopharmacologically, has led to their threatened status. This investigation focussed mainly on the study of the anti-inflammatory activity of plant extracts prepared from the leaves, bulbs and roots, and the development of suitable tissue culture protocols for the bulk propagation of the species under study.

Common underlying symptoms in the majority of ailments treated with traditional remedies prepared from *Eucomis* species are pain and inflammation. Prostaglandins are the primary mediators of the body's response to pain and inflammation, and are formed from essential fatty acids found in cell membranes. This reaction is catalysed by cyclooxygenase, a membrane-associated enzyme occurring in two isoforms, COX-1 and COX-2. Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting the activity of COX. The use of commercially available COX-1 inhibitors is associated with side-effects, including gastric and renal damage. Selective COX-2 inhibitors do not have these undesired effects, and are thus potentially very valuable to the pharmaceutical industry.

The relative inhibitory effects of different extracts of *Eucomis* species on the activities of purified cyclooxygenase enzyme preparations (COX-1 in sheep seminal vesicles, COX-2 in sheep placenta) were assessed. The COX-1 assay was used to screen extracts from 10 species of *Eucomis* and one hybrid species at a concentration of 250 μg mℓ^{-1} in the assay. High levels of anti-inflammatory activity were exhibited by the ethanolic extracts prepared from the dried leaves, bulbs and roots. Aqueous extracts (screened at 500 μg mℓ^{-1}) showed much lower levels of activity. In general, the highest levels of anti-inflammatory activity were observed for the ethanol bulb and root extracts. Comparison of the activity of the bulb extracts from bulbs harvested in summer and winter revealed very little difference in COX-1 inhibitory activity.

Eucomis extracts were separated using thin layer chromatography. The plates were developed in a solvent system of benzene : 1,4-dioxan : acetic acid, 90:25:4 and stained with anisaldehyde-sulphuric acid. The TLC fingerprints prepared from these extracts showed different chemical profiles for the leaf, bulb and root extracts, but many similarities between the different species. The position of the active R_f fractions was determined and correlated with the TLC-fingerprints.

The most widely utilized species medicinally, *E. autumnalis* subspecies *autumnalis,* was chosen for further investigation. The fluctuation of anti-inflammatory activity with season and physiological age was determined. Young plants were found to have high levels of COX-1 inhibitory activity, particularly in the leaves. As the plant matured, higher levels of activity were associated with the bulb and root extracts. The anti-inflammatory activity of the leaf, bulb and root extracts varied slightly throughout the year, with the highest levels detected towards the end of the growing season, shortly before the onset of dormancy.

This study of *E. autumnalis autumnalis* was extended to investigate the effects of environmental conditions on the levels of COX-1 inhibitory activity. The extent to which high temperature and light intensity, fertilization of the plants in summer with Kelpak preparations, and cold storage of the dry bulbs during winter, affected the levels of active compounds accumulated, was determined. Kelpak application decreased the anti-inflammatory activity of the leaf, bulb and root extracts, while high temperature / high light intensity had no significant effect on the COX-1 inhibitory activity of the leaf or bulb extracts. The root extract did show a significant increase in anti-inflammatory activity. Bulbs that were removed from the soil and stored at 10°C exhibited significantly higher COX-1 inhibitory activity than the control bulbs maintained in the soil. Higher COX-1 inhibition was observed in the leaf extracts from these plants when harvested half-way through the growing season. No significant difference was observed at this stage between the bulb and root extracts from the different treatments.

Bioassay-guided fractionation (using the COX-1 assay) was used to isolate the active principle(s) in the bulb extract. The bulb material was subjected to serial extraction using a Soxhlet apparatus. The ethyl acetate fraction showed the highest levels of

COX-1 inhibition, and this was further fractionated using a Sephadex LH-20 column and a solvent system of cyclohexane : dichloromethane : methanol (7:4:1). The most active fraction from this separation was then purified using semi-preparative TLC and HPLC. The primary compound eluting in this fraction had an IC_{50} value of 14.4 μg mℓ^{-1} in the COX-1 assay, and 30.5 μg mℓ^{-1} in the COX-2 assay. This compound was tentatively characterized as a phenol ring attached to a conjugated hydrocarbon chain (with a molecular weight of 390), and was a potent COX-1 inhibitor. The COX-2 / COX-1 inhibitory ratio was calculated to be 2.1.

A second, highly active compound, with IC_{50} values of 25.7 μg mℓ^{-1} and 21.8 μg mℓ^{-1} in the COX-1 and COX-2 assays respectively, crystalized from one of the Sephadex LH-20 column fractions. This compound was identified as a spirostane-type triterpenoid, eucosterol, previously isolated from *Eucomis* species but not specifically linked to the pharmacological activity of the extracts. This compound showed COX-2 / COX-1 inhibitory ratio of 0.8, indicating that it was a selective COX-2 inhibitor.

Two further compounds were identified from this extract, after crystallization from different fractions obtained from Sephadex LH-20 chromatography. These were both homoisoflavanones, 5,7-dihydroxy-6-methoxy-3-(4-methoxy benzyl)-chroman-4-one, and 5,7-dihydroxy-3-(4-methoxy benzyl)-chroman-4-one [eucomin], the latter having been isolated previously. The first compound exhibited very low levels of both COX-1 and COX-2 inhibition, and the second compound (eucomin) exhibited high COX-1, but low COX-2 inhibitory activity.

The *in vitro* propagation of the genus *Eucomis* was undertaken primarily to provide a source of material for experimentation, and also to optimize this technique for the bulk production of plants for commercial and conservation purposes. Multiple shoot production was initiated from leaf explants, in all species studied. A Murashige and Skoog (MS) medium, supplemented with 100 mg ℓ^{-1} myo-inositol, 20 g ℓ^{-1} sucrose, and solidified with 2 g ℓ^{-1} Gelrite® was used. The optimal hormone combination for shoot initiation in the majority of species was determined to be 1 mg ℓ^{-1} NAA and 1 mg ℓ^{-1} BA. Optimal root initiation was demonstrated on media supplemented with 1 mg ℓ^{-1} IAA, IBA or NAA, depending on species. A continuous culture system using this protocol produced 25-30 plantlets per culture bottle, with 10-25 specimens per bottle

available for acclimatization. To maximize plantlet survival, different support media used during the acclimatization process were necessary. Certain species responded best on a vermiculite medium, while perlite (which holds less water) was necessary for the optimal survival rate of other species. Acclimatized plantlets were repotted in a sand : soil mix (1:1).

Further experimental work aimed to determine the factors affecting the accumulation of anti-inflammatory compounds in *in vitro* plantlets. Extracts prepared from *in vitro* plantlets showed high levels of COX-1 and COX-2 inhibitory activity, with a COX-2/COX-1 ratio of 1.1. High levels of sucrose (40 g ℓ^{-1}) significantly increased the number of shoots initiated, but had no effect on the anti-inflammatory activity. Low levels of sucrose (10 g ℓ^{-1}) led to a significant decrease in COX-1 inhibition. Changing the levels of nitrogen in the medium (but not the ratio of nitrate to ammonium ions) had no significant effect on the COX-1 inhibitory activity of the extracts.

Callus was initiated from leaf explants and experiments were conducted to maximize callus proliferation. Optimal callus growth occurred on an MS medium supplemented with 100 mg ℓ^{-1} myo-inositol, 30 g ℓ^{-1} sucrose, 2 g ℓ^{-1} Gelrite® , and a hormone combination of 10 mg ℓ^{-1} 2,4-D and 2 mg ℓ^{-1} kinetin. Callus cultures maintained in the dark grew best. Callus extracts tested in the COX assays (250 μg mℓ^{-1}) showed a higher level of COX-2 inhibition (69%) than COX-1 inhibition (46%).

Lastly, the conclusive identification of the species under study was attempted, using DNA fingerprinting. Protocols were developed for the extraction of DNA from the leaves of *Eucomis* plants, and the optimization of the AP-PCR technique. Random sequence (10-base) oligonucleotide primers were screened, each primer used singly. Primers were selected on the basis that more than five distinct bands were detected. Differences were detected in the amplification products visualized using non-denaturing agarose gel electrophoresis stained with ethidium bromide. This work provides the basis for further studies into the phylogenetic relationships between the various species (and hybrids) of *Eucomis*.

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TAYLOR, J. L. S. AND VAN STADEN, J. 2000. Anti-inflammatory activity in *Eucomis* L'Herit. species. *Journal of Ethnopharmacology* (Submitted).

TAYLOR, J. L. S. AND VAN STADEN, J. 2000. *In vitro* propagation of *Eucomis* L'Herit. species - plants with medicinal and horticultural potential. *Plant Growth Regulation*. (Submitted).

TAYLOR, J. L. S. AND VAN STADEN, J. 2000. The effect of nitrogen and sucrose levels on the growth of *Eucomis autumnalis* (Mill.) Chitt. plantlets *in vitro*, and on the subsequent levels of anti-inflammatory activity in the extracts prepared from the *in vitro* plantlets. *Plant Growth Regulation*. (Submitted).

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PAPERS IN PREPARATION

TAYLOR, J. L. S. AND VAN STADEN, J. Isolation and identification of active principles from *E. autumnalis autumnalis* (Mill.) Chitt.

TAYLOR, J. L. S. AND VAN STADEN, J. A pharmacological study of *E. autumnalis autumnalis* (Mill.) Chitt. with respect to COX-1 and COX-2 activity.

TAYLOR, J. L. S. AND VAN STADEN, J. The effect of cold storage during winter on the anti-inflammatory activity of *Eucomis autumnalis autumnalis* (Mill.) Chitt.

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1997

International Symposium on Biotechnology of Tropical and Subtropical Species, Brisbane (Australia):-

Poster: *In vitro* propagation of *Eucomis* species. (J. L. S. Taylor and J. van Staden).

Centre for Indigenous Plant Research, Durban (South Africa):-

Paper: Tissue culture of *Eucomis* species - an indigenous plant with medicinal and horticultural potential. (J. L. S. Taylor and J. van Staden).

1998

24th Annual Congress of SAAB (South African Association of Botanists), Cape Town (South Africa):-

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Paper: Variation in anti-inflammatory levels in *Eucomis* with respect to species, season and tissue culture. (J. L. S. Taylor and J. van Staden).

IX International Congress on Plant Tissue and Cell Culture, Jerusalem (Israel):-

Poster: Micropropagation of *Eucomis* species: a plant with horticultural and medicinal potential. (J. L. S. Taylor and J. van Staden).

1999

25th Annual Congress of SAAB, Umtata (South Africa):-

Poster: Anti-inflammatory activity in *Eucomis* species. (J. L. S. Taylor and J. van Staden).

5th Joint Meeting of the ASP, AFERP, GA and PSE, Amsterdam (The Netherlands):-

Poster: Investigation of the anti-inflammatory principle in *Eucomis autumnalis*. (J. L. S. Taylor and J. van Staden).

XVI International Botanical Conference, St Louis (U.S.A):-

Poster: COX-1 inhibitory activity in extracts of *Eucomis* species. (J. L. S. Taylor and J. van Staden).

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AP-PCR	arbitrarily primed-polymerase chain reaction
bp	base pairs
BA	benzyl adenine
COX	cyclooxygenase
CTAB	cetyltrimethylammonium bromide
dd H_2O	de-ionised-distilled (HPLC grade) water
2,4 D	2,4-diclorophenoxyacetic acid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediaminetetracetic acid (chelating agent)
EPA	eicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroxyperoxyeicosatetraenoic acid
HPLC	high peformance liquid chromatography
IAA	indole-3-acetic acid
IBA	indole-butyric acid
K	kinetin
LT	leucotriene
M	molar
MS	Murashige and Skoog medium (1962)
MW	molecular weight
NAA	α-naphthaleneacetic acid
NMR	nuclear magnetic resonance
NSAIDs	Non-steroidal anti-inflammatory drugs
Or	origin
OPA	OPERON Technologies A series primers
PCR	polymerase chain reaction
PG	prostaglandin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2

PGF_2	prostaglandin F_2
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2
RAPD	random amplified polymorphic DNA
R_f	mobility relative to front
RFLPs	restriction fragment length polymorphisms
RNA	ribonucleic acid
SF	solvent front
Tris	2-amino-2(hydoxymethyl)-1,3-propanediol (buffer)
TAE	Tris-acetic acid-EDTA
TLC	thin layer chromatography
TX	thromboxane
UV	ultraviolet
V	volt
VNTR	variable number of tandem repeats

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Plants have been selected and used empirically as drugs for centuries, initially as traditional preparations then as pure active principles (KAMIL, 1993), with this knowledge and accumulated practice passing from generation to generation (COUZINIER AND MAMATAS, 1986; LEWIS, 1992). Natural products and their derivatives represent more than 50% of all the drugs in clinical use today with 25% originating from higher plants (BALANDRIN *et al.*, 1993). About 90% of the 119 plant-derived pharmaceutical products currently marketed are obtained by extraction from only about 90 species of plants (FARNSWORTH, 1994). Drugs such as digitalis, atropine, and morphine originate from plants, but with the emphasis placed on the isolation of single active ingredients, many other therapeutic agents could have been overlooked. In addition, from the approximately 500 000 species of higher plants, there remains an abundance of plants that have yet to be examined for their pharmacological activity, and their vast potential as sources of new pharmaceutical products has been largely underestimated by the pharmaceutical industry (HAMBURGER AND HOSTETTMANN, 1991; ADDAE-MENSAH, 1992; FARNSWORTH, 1994). The ultimate goal of therapeutic phytochemistry is the discovery of new active principles and their improvement for medical application (COUZINIER AND MAMATAS, 1986).

A drug is defined as a chemically identified substance, either derived from plants or animals, or produced by synthesis. With current advances in biotechnology, synthesis is being undertaken by genetically engineered micro-organisms (COUZINIER AND MAMATAS, 1986). The investigation of traditional medicines and phytotherapy is, however, once again receiving scientific attention, the aim being to develop effective drugs that are non-toxic and inexpensive, the latter being most important to

developing countries, particularly those in Africa (COUZINIER AND MAMATAS, 1986).

The definition of phytomedicines includes crude vegetable drugs (herbs) and the galenical preparations (extracts, fluid extracts, tinctures etc.) derived from them (TYLER, 1993). Laws and regulations governing the sale of phytomedicines (herbal remedies) are less strict in several European countries (e.g. Germany) than in the United States, and the development of medicines prepared from long-known plant drugs is supported by the European pharmaceutical industry and the medical community (TYLER, 1993). The use of phytomedicines is becoming more scientifically based, with increasing emphasis placed on proven product safety and efficacy.

Medicinal plant drugs can be placed into two broad categories. Firstly, they are included in complex mixtures containing a wide variety of compounds (e.g. infusions, essential oils, tinctures or extracts), and secondly they are used as pure, chemically defined active principles (HAMBURGER AND HOSTETTMANN, 1991). Pure compounds are used when the activity is strong and specific, and /or has a small therapeutic index (requiring accurate and reproducible dosage). Where plants show weaker and less specific pharmacological activity, or if the activity has not been fully categorized, the use of general plant extracts is appropriate (HAMBURGER AND HOSTETTMANN, 1991).

A joint report released by the United Nations Conference on Trade and Development (UNCTAD) and the General Agreement on Tariffs and Trade (GATT) in 1974 indicated that 33% of drugs produced in developed countries came from higher plants, with 25% of these originating from the tropical rain forests of Africa, Asia and South America, and having an annual retail value worldwide of between 20 billion to +40 billion US$, out of a total estimated worldwide drug market of more than 150 billion US$ (ADDAE-MENSAH, 1992). A further 27% of drugs originate from microbes and lower plants, resulting in an estimated +80% of current medicines derived either directly or indirectly from plant sources. This resource, however, represents only 15% of the estimated 500 000 plant species in the world (ADDAE-MENSAH, 1992). Increased demand for novel phytochemical drugs has coincided with the accelerated

destruction of tropical rain forests resulting in an estimated loss of 20 000 species over and above the normal rates of extinction (LEWIS, 1992).

Until the late 1980's there was relatively little attention shown by the developed world in indigenous traditional knowledge, and minimal assistance was provided to under-developed countries for the preservation, collection and systemization of this knowledge. Recently this attitude has undergone a substantial change, and the current interest in natural products has led to an increasing respect for the indigenous peoples and their knowledge (CORDELL, 1995).

The computerization of global literature on the pharmacological effects of extracts from living organisms, including plants, marine organisms, fungi and animals, as well as ethnomedical information on the uses of plants, and secondary metabolite occurrence and biological effect, began in 1975 and has been developed into an ongoing database known as NAPRALERT (NAtural PRoducts ALERT) (FARNSWORTH, 1994). This is a relational database and has systematic searches dating back to 1900. These include natural products used in the areas of cancer treatment, fertility regulation, diabetes, malaria and other parasitic diseases, viral diseases, sugar substitutes, molluscicides and anti-HIV agents. The aim of this database is to provide for the selection of plants with a high degree of probability that they would contain novel bioactive compounds if investigated scientifically. By combining the analysis of ethnomedical information and published scientific studies on plant extracts (ethnopharmacology), the number of plants that need to be screened for drug discovery should be reduced, leading to a greater success rate than random selection and mass bioscreening (FARNSWORTH, 1994). A similar database, the National Medicinal Plants Database (Medbase) is being established in South Africa, primarily to facilitate responsible land use, and the conservation and appropriate exploitation of medicinal taxa (CROUCH AND ARNOLD, 1997).

1.2 TRADITIONAL MEDICINE

A large proportion of the population of developing countries uses traditional medicines either due to the high cost of western pharmaceuticals and health care, or because the traditional medicines are more acceptable from a cultural and spiritual perspective (CUNNINGHAM, 1988a). In traditional African medicine many food plants are used for therapeutic purposes and in contrast to orthodox western medical practice, medicines are not viewed as "necessary poisons". In western medicine, drugs are, in essence, poisons which in low doses can cure diseases (IWU, 1993). African traditional medicine seeks to understand the underlying cause of the illness. The treatments are prescribed in relation to the perceived cause of the complaint and symptoms of the patient (VAN WYK *et al.*, 1997), and cover a wide area of medicine and pharmacology, including pharmacognosy (KOKWARO, 1995).The study of African medicinal plants has not in the past been taken as seriously, or documented as fully, as Indian and Chinese treatments. Over 5 000 plants are known to be used for medicinal purposes in Africa, but only a few have been described or studied. This documentation is becoming increasingly important due to the rapid loss of natural habitats, with many endemic medicinal plants becoming extinct before they can be investigated (IWU, 1993).

Of those African medicinal plants investigated for their chemical components, some have been found to contain compounds with interesting biological activity which have proved therapeutically active in controlled clinical evaluations (IWU, 1993). Research is, however, expensive and the success rate in drug development is low, about 1:10 000 (ADDAE-MENSAH, 1992). The major problem in these investigations lies in the fact that a variety of plants may be used in a single traditional medical preparation, and in the possibility of synergistic effects resulting from the interaction of the natural compounds. This can result in a loss of activity as the product is purified, and the compounds acting synergistically are lost (COUZINIER AND MAMATAS, 1986).

Drug development from ethnobotanical information has focussed either on the identification of single plant species with biologically active compounds, or the

characterization and standardization of traditional recipes for reformulation as commercial medicines. The first method has been very successful, while the second has been largely neglected despite the obvious advantages inherent in the optimization of these mixed remedies as formulated dosage forms (IWU, 1994).

LE GRAND AND WONDERGEM (1989) proposed two major reasons to promote herbal medicine. Firstly, traditional medicine is viewed as a vital means of improving health for the majority of the population in many developing countries. The World Health Organization's (WHO) resolution on a strategy for "Health for All" recognizes that health is a fundamental right (PATEL, 1983). The health needs of the entire population of the world cannot presently be met by modern health care, leading health workers to focus on traditional medicine as a way to strengthen primary health care (PHC). This resulted in the formation of a policy of promoting the integration of traditional medicine into national health care systems by the World Health Organization in 1978 (LE GRAND AND WONDERGEM, 1989). WHO has promoted the official use of traditional medicines, particularly in developing countries, by encouraging the incorporation of its useful elements (especially with respect to human resources) into national health care systems (SINDIGA, 1995b). This is important since in these countries western biomedical systems cannot successfully combat current morbidity and mortality rates which are especially high in children below the age of five years (SINDIGA, 1995b).

Secondly, the promotion of herbal medicine aims to encourage more rational drug use. Today, many modern drugs are available without adequate information on their use, resulting in over-consumption and drug abuse, even in the remote villages of developing countries. Synthetic drugs in general, have very high pharmodynamic effects, but this activity is accompanied by strong, possibly dangerous side effects. Medicinal plants (with a few exceptions), in contrast, do not have similarly high therapeutic potencies and neither do they show intense or serious side effects (NARANJO, 1995). The continued use of potent drugs is also associated with a gradual decrease in the body's resistence mechanisms as well as harmful side-effects. Herbal or plant preparations, have fewer harmful side effects, making them particularly suited for the treatment of less serious diseases, supportive treatments of chronic diseases, long-term treatment, and possibly for prophylactic medication

(GOVIL *et al.*, 1993). Traditional remedies and herbal drugs offer a cheaper, and relatively safer alternative in these circumstances (LE GRAND AND WONDERGEM, 1989; NARANJO, 1995).

Herbal medicines in general, depend on energizing and stimulating many systems within the body, in opposition to the modern medical approach which has, until recently, underplayed importance of the body's own immune system in the prevention of chronic diseases such as cancer (MOWREY, 1986). These herbal remedies stimulate blood and lymph flow, and phagocytic activity in the white blood cells or leucocytes, often in an attempt to prevent e.g. cancer, not cure it (MOWREY, 1986).

Traditional medicine is further advocated for its perceived accessibility, availability, acceptability and dependability to rural populations (SINDIGA, 1995b). In this respect it is an integral part of the particular culture and is thus both socially acceptable and effective in curing specific cultural health problems. It is holistic in its approach, and for many ailments it can be efficacious, at an affordable fee. Lastly these systems have wide spacial coverage, with each community supporting its own healers (SINDIGA, 1995b).

Modern and traditional health care often exist side by side but seldom co-operate, despite the important contribution that herbal medicine makes to primary health care. This is mostly due to the view that it has no scientific basis (ADDAE-MENSAH, 1992). There is a lack of standardization with respect to raw materials, methods of production and in quality control of the finished product (ANAND AND NITYANAND, 1984). The problems with traditional medicine, as described by WHO (1978) amongst others, are that traditional medicine does not keep pace with scientific and technological advancement, and its methods, techniques, medicines and training are often kept secret. The rational use of traditional medicine is also not well defined, and often relies on ritual, mysticism and intangible forces such as witchcraft, with some aspects based on spiritual and moral principles which are difficult to rationalize. While these may be valid psychologically, they cannot be rationalized scientifically (ADDAE-MENSAH, 1992). Other major objections expressed towards herbal practitioners are that the herbalist is not competent to diagnose, and that chronic illnesses are not diagnosed in time (ADDAE-MENSAH, 1992).

Toxicity of herbal medicines

Compared to synthetic drug treatments, the amount of information about the relative safety of herbal remedies is limited (ERNST, 1998). Little documentation exists on the long term toxicity of herbs. There is a lack of use of standard / measured doses, and the large volumes of the doses used are difficult to manage (ADDAE-MENSAH, 1992). While natural products do lack defined dose and potency data, they also benefit from containing many specific molecular principles in their natural state, which possess a variety of influences on human physiology, as opposed to purified synthetic drugs which are based on just a single molecular substance derived from the natural product (BLAND, 1986).

Examples of allergic reactions, toxic reactions, adverse effects related to pharmacological action, possible mutagenic effects, drug interactions, and mistaken plant identity have been reported (ERNST, 1998). In some cases the side-effects from the consumption of a combination of herbal medicines is not known. Other problems include an alleged low quality of care because of the lack of regulatory mechanisms, including control and licensing, and the lack of written registers or patient records. This causes difficulty in evaluation (especially in African systems). There is also a loss of knowledge (since the system is based on oral tradition), and errors are introduced through the large variety of herbs and other types of pharmacopoeia used (SINDIGA, 1995b). Other complaints are that the preparations are generally unhygienic and poorly packaged (ADDAE-MENSAH, 1992).

The use of herbal preparations can lead to hypersensitivity reactions ranging from transient dermatitis to anaphylactic shock (ERNST, 1998). Many widely used medicinal plants have been implicated as possible causes of long-term disease manifestations such as liver and kidney diseases. The widespread use of *Senecio*, *Crotalaria* and *Cynoglossum* have been implicated in the occurrence of liver lesions and tumours, lung and kidney diseases in certain areas of Ethiopia (ADDAE-MENSAH, 1992). The use of herbal enemas is considered the primary cause of some of the poisonings as well as cases of liver damage recorded in hospitals (HUTCHINGS, 1989a).

The preparative methods used in ethnomedicine often reduce toxicity, thus allowing the exploitation of potentially dangerous plants for their therapeutic value (ETKIN, 1986). Mucilages and gums have been found to slow the absorption of some active substances (ANTON *et al.*, 1986). Aqueous decoctions of *Aconitum* species (used in Oriental medicine) reduce the alkaloid content ±9-fold (by the hydrolysis and physical loss of alkaloids during aqueous extraction), and toxicity almost 100-fold. Since the reduction in alkaloid content is not on a par with loss of toxicity, other factors have been suggested to be important in preventing the remaining alkaloids from exerting any toxic effect (ETKEN, 1986).

The use of plant-based medications has become extremely popular in the United States and Europe, with the botanical industry in the US earning $ 1.5 billion per annum and the European market nearly three times as much (ERNST, 1998). Where herbal remedies are sold commercially, many of the reported cases of poisoning have been caused by substitution, or contamination, of the declared ingredients with a more toxic plant, a poisonous metal or a potent non-herbal drug (DE SMET, 1992; ERNST, 1998). This places the cause of such incidents in the realm of poor quality control rather than on the pharmacological activity of the particular herbal ingredients (DE SMET, 1992). The vast majority of herbal products are unlicensed and there is no requirement to demonstrate the efficacy, safety or quality of the product (ERNST, 1998).

Other contaminants in herbal preparations include micro-organisms and microbial toxins, pesticides and fumigation agents and synthetic and animal drug substances. In addition, the inappropriate use of the preparations by uneducated users is a factor leading to toxicity. In this respect, herbal medicinal products need to be accompanied by accurate and comprehensive consumer information on use and safety (DE SMET, 1992).

A history of traditional use is also not a reliable guarantee of safety. Delayed effects (e.g. mutagenicity), rare adverse effects, and adverse effects arising from long-term use may not be detected by traditional practitioners (ERNST, 1998).

Different medical systems utilize their own drugs and other pharmacopoeia. A *materia medica* refers to a compilation of monographs of medicines for different diseases, where each monograph represents a given medication, specifying the name and describing the raw materials and method used in preparation of the medicine, as well as its use, manner of administration, dose and other relevant data such as contraindications and storage requirements (SINDIGA *et al.*, 1995). Where possible, the constituents held responsible for the pharmaceutical activity of the herbal drug should be monitored. In cases where the active principle(s) are uncertain or unknown, quality control can be based on chemical markers (constituents which are considered characteristic for the herbal ingredient). The usefulness of such chemical markers has been recognized by the Committee for Proprietary Medicinal Products of the European Community (DE SMET, 1992).

Modern doctors know very little about the remedies people use traditionally, or how illness and healing are perceived. This can have serious repercussions in the use of prescribed medicines. The promotion of herbal drugs should stem from a primary health care approach that is comprehensive rather than selective, and includes concepts such as self reliance, equity and community participation. Studies in Ghana and Thailand demonstrate that modern health care workers in rural areas become involved in only a minority of illnesses, since most ailments are self treated. In both countries the wide availability and easy access to pharmaceuticals has led to over-consumption and abuse of drugs in self care (LE GRAND AND WONDERGEM, 1989). Various studies have indicated that rural people use modern drugs in a traditional way, and are not aware of the possible dangers and side effects of improper drug use. A better understanding of traditional medical concepts and healing practices is needed, as are ways to strengthen traditional medical practices (LE GRAND AND WONDERGEM, 1989).

Ethnopharmacology is defined as the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man (HOLMSTEDT AND BRUHN, 1995). The aim of this field of study is the validation of traditional preparations either through the isolation of active substances, or through pharmacological findings on indigenous drug preparations. Where biologically active principles are found, findings should be interpreted in light of traditional use

(HOLMSTEDT AND BRUHN, 1995). This should include an analysis and chemical and pharmacological evaluation of original drug preparations (e.g. water infusions) in order to establish dose-effect relationships for the quantitative use of the remedy. Although ethnopharmacological studies can contribute greatly to modern medicine, and can lead to many novel useful drugs, the modern and traditional uses may be entirely different (HOLMSTEDT AND BRUHN, 1995).

The evaluation and authentication of traditional remedies can contribute towards the formulation of an integrated health care system which combines both local and western practices (COTTON, 1996). This involves the documentation and testing of the efficacy and toxicity of medicinal plant extracts, and the identification of the active principles. This would enable local knowledge of medicinal plants to be compiled and conserved, and recommendations for usage to be based on an empirical understanding of the activity and toxicity.

1.3 TRADITIONAL MEDICINE IN SOUTH AFRICA

There is still a lack of detailed documentation on the use of medicinal plants in South Africa. This is becoming an urgent issue due to the fragility of oral-tradition knowledge and the rapid pace of urbanization and acculturation in this country (VAN WYK *et al.*, 1997). The informal (oral) traditional medical systems of the Khoi-San, the Nguni and the Sotho-speaking peoples of South Africa have not yet been systemized (VAN WYK *et al.*, 1997). Formal and informal (oral-tradition) systems of medicine exist together in South Africa, the first dating back only 300 years with the influx of European settlers and the latter possibly to palaeolithic times (VAN WYK *et al.*, 1997).

It is estimated that more than 80% of the world's population utilize plants as their primary source of medicinal agents (CORDELL, 1995) and that moreover, traditional medicine is still the only health resource available to about 60% of the world population (LE GRAND AND WONDERGEM, 1989). Estimates by WHO (1978) state that between 60 and 90% of Africa's population rely on medicinal plants to totally or partially meet their health care needs (ADDAE-MENSAH, 1992; KAMIL, 1993;

SINDIGA, 1995b). This is true also of South Africa where up to 60% of the population consult one of an estimated 200 000 traditional healers (VAN WYK *et al.*, 1997), especially in rural areas where traditional healers are more numerous and accessible than western doctors (CUNNINGHAM, 1988b). In KwaZulu-Natal, approximately 80% of the population seek medical advice from traditional healers, over or in addition to, western medicine.

A large part of the common, daily utilized medicines in South Africa are still derived from plants, and large volumes of plants or their extracts are sold in informal and commercial sectors of the economy. There is a growing interest in natural and traditional medicines as a source of new commercial products (VAN WYK *et al.*, 1997). The disadvantages evident in the South African context are, however, the over-exploitation of wild populations of certain popular species, with a rapid increase in the price of these plants as they become rare (CUNNINGHAM, 1988b).

In South Africa, and Africa as a whole, the evaluation and recognition of traditional medicine aims to improve its efficacy, safety, availability and wider application at low cost. This is coupled to a need to define the role of traditional medical practitioners in the community in order to formulate effective policies describing their role in PHC (SINDIGA, 1995a). The wide gap that exists between modern and traditional medical systems in South Africa is being addressed by attempts to formalize the practice of traditional medicine through the establishment of Healer's associations. The development of traditional medicine requires intervention to foster such professionalism and regulation (SINDIGA *et al.*, 1995). This includes proposals for the introduction of warehouses for the correct storage of herbal medicines marketed in the cities (VAN WYK *et al.,* 1997).

Despite firm foundations in the past, traditional medicine is not a static system, but is dynamic and adaptive (IWU, 1993; VAN WYK *et al.,* 1997) and although it reflects the value and perceptions of the people, it is under pressure by the introduction of western culture. The introduction of modern medicines and practices (including standardization of doses) as well as other socio-economic development processes (such as medical records and access to medical aid schemes) can improve the application of traditional healing. Although LE GRAND AND WONDERGEM (1989)

propose that many users could benefit from the dissemination of existing knowledge, this has become a controversial subject. Intellectual property rights and the exploitation of developing countries' natural resources have become the focus of ethnobotany in the past few years.

The South African flora consists of over 30 000 species of higher plants, with a biodiversity in the Cape comparable to the tropical rainforests in terms of species richness. Nearly 3 000 of these species are used as medicines with approximately 350 species forming the most commonly traded and used medicinal plants (VAN WYK *et al.,* 1997).

Wild medicinal plant resources are increasingly under threat from habitat destruction caused by agricultural, forestry, industrial and urban/housing encroachment. Professional herb gatherers and traditional healers thus have an exaggerated impact on the remaining wild stock. In the past, plants were collected and stored according to tradition, in this way preventing over-harvesting. Unemployment has brought non-specialist gatherers into commercial trading. The urban demand for medicinal plants generates a local and interprovincial trade supplied primarily by gatherers from rural areas who supply urban herbalists, diviners and herb traders (CUNNINGHAM, 1988a). Today, the urbanized healers purchase their ingredients from street markets and stores giving an economic incentive for the destructive harvesting of vulnerable medicinal plants (VAN WYK *et al.,* 1997). This problem is exacerbated by the rapid growth of the urban black population creating an increasing demand for traditional medicines collected from the remaining small resource base (CUNNINGHAM, 1988a). This in turn leads to commercial harvesting of medicinal plants vulnerable to over-exploitation, primarily due to the negative impact of large scale root and bark collection (CUNNINGHAM, 1988a).

1.4 CONSERVATION OF MEDICINAL PLANTS

The International Union for Conservation of Nature and Natural Resources (IUCN) and World Wide Fund for Nature (WWF) estimate that if present trends continue, 60 000 higher plant species could become extinct, or near extinct, by the middle of the next century primarily due to continuing habitat destruction (ETKEN, 1998). This is most apparent in areas experiencing rapid socio-economic transition, and occurs when western technology is suddenly introduced to traditional societies, with global market forces encouraging greater homogeneity of both the environment and of the products, including plants (ETKEN, 1998). Medicinal plants are under threat from population pressure, destruction by indiscriminate harvesting and foreign export (SINDIGA, 1995a). Traditional medicines require specific protection, enhancement and development, including the legal recognition of this medical system (SINDIGA, 1995a).

Ethnobotany is defined as the study of contextualized plant use i.e. the study of plant - human interrelationships embedded in dynamic ecosystems of natural and social components (ALCORN, 1995). This involves the study of the dynamic system of which plant use and plant management are a part (ALCORN, 1995). Conservationists argue in favour of the incorporation of traditional knowledge into the management of protected areas, since most habitats have been influenced significantly by humans in the course of evolution (COTTON, 1996). In addition, the maintenance of protected areas is closely linked to rural development (MARTIN, 1994). International conservation agencies, including the World Wide Fund for Nature, support the work of ethnobotanists in developing countries aiming to advance sustainable plant use and the application of this principle to conservation and community development (MARTIN, 1994).

The conservation implications of tropical hardwood logging have been the focus of global attention for over 10 years, while the implications of harvesting minor forest products has been considered only recently (COTTON, 1996). The agricultural and pharmaceutical industries accept that maintaining biodiversity may prove crucial in providing future genetic and biochemical resources of appreciable commercial value.

This is, however, not a consideration for local people who receive no financial compensation for conserving these resources in order to realize a potential future value, when the same resources can provide an immediate, if short-term source of income (COTTON, 1996).

In South Africa, more than 20 000 tons of plant material, are harvested, processed and sold annually as traditional medicine. Of this, approximately 14% comprises bulb material (MANDER, 1997) indicating the destructive nature of this type of harvesting. Bulbs are usually sold whole, sliced or chopped for preparation predominantly as decoctions used as emetics and enemas (WATT AND BREYER-BRANDWIJK, 1962; HUTCHINGS, 1989b; MANDER *et al.,* 1995; VAN WYK *et al.,* 1997). Medicinal plants in KwaZulu-Natal alone, support traditional health services with a value of more than two billion rands annually (CROUCH AND ARNOLD, 1997).

More than 500 plant species are traded as medicinal plants on the Witwatersrand alone. These are mostly bark and roots, although stems, leaves, whole plants and bulbs are also sold. Bulbs are the fleshy underground storage structures composed of successive layers of fleshy leaf bases or scales, and may be mistaken for fleshy stems (where the bulb scales are not too swollen). They are very popular for medicinal use (VAN WYK *et al.,* 1997). The active compounds in leaves, roots or bark can often differ considerably in nature and concentration, with one plant part harmless and another toxic. For this reason, whole plants are rarely used in medical preparations (VAN WYK *et al.,* 1997).

There has been a new awareness in many under-developed countries that biodiversity is valuable and that a country has the right to restrict access, and demand compensation for any access granted to these resources. In the long term, the conservation of genetic resources and the continued, controlled access to these resources (including terrestrial and marine species) are critical for the future development of natural product chemistry (CORDELL, 1995). The conservation of ecologically important habitats, vigorous cultivation, collection of data and compilation of national and regional pharmacopoeias of medicinal plants is necessary. This should be accompanied by modern scientific investigations of the active principles from traditional medicinal plants, with the aim of future development of modern drugs

(KOKWARO, 1995).

In the past, local practices facilitated the management of wild plants, either through the indirect methods of taboos on collection and other social or religious restrictions, or directly through the protection of plants during reproductive phases or sustainable harvesting (COTTON, 1996). Traditional methods of collection can be interpreted as prevention of over-harvesting. Strips of bark are removed from opposite sides of a trunk preventing ring barking, and some plants are only collected in winter leaving the summer season for the plant to reproduce undisturbed (VAN WYK *et al.*, 1997).

Preventing the wholesale destruction of vulnerable medicinal plant species through over-exploitation by pharmaceutical companies can be accomplished by a number of methods. Firstly, a better source of supply (e.g. a different species or cultivar), a different plant part, or cultivation can be investigated. Secondly, semi-synthesis from a more abundant precursor can be undertaken. Thirdly, total synthesis can be attempted, and lastly, tissue culture production of the compound or a closely related compound can be tried (CORDELL, 1995).

Programmes for the preservation of medicinal plants on a world-wide scale have been initiated by several organizations including the Food and Agriculture Organization (FAO), the Society of European Plant Breeders (EUCARPIA) and member states of the Council for Mutual Economic Aid (COMECON) while other countries (e.g. Germany, France, Hungary and U.S.A.) use existing environmental protection programmes to regulate the trade of medicinal plants gathered from the wild (MÀTHÉ, 1988).

Species that are designated for conservation in developing countries are usually identified by outsiders, culturally and politically detached from the threatened environment. The preservation of species is thus predominantly dictated by economics, and the significance of the taxa are not properly assessed in the cultural and ecological context of their use (ETKEN, 1998). Global efforts to sustain biodiversity would be better served by examining the variety of species used by local populations, especially wild plants, with consideration of the full scale of their uses. Local communities are removed from the global markets. They are thus places where

traditional cultures persist, and form repositories of knowledge of biological diversity through an intimate knowledge of native ecology and long experience of usage of the species in question (ETKEN, 1998).

The commercial propagation and inclusion of medicinal plants as part of an annual crop by subsistence farmers has been proposed in South Africa (BYE AND DUTTON, 1991). The high local demand for medicinal plants, as well as the simultaneous decline in availability has increased the price of traditional medicines and plants dramatically in the last 10 years (CUNNINGHAM, 1988a). A commercial venture for the growth of medicinal plants as a crop serves as a reliable source of the raw materials. This can, however, only succeed if large numbers of medicinal plants can be supplied at a lower price than that offered by the gatherers. At the present values, few species merit prices that would make cultivation profitable (CUNNINGHAM, 1988b).

1.5 TISSUE CULTURE

Natural habitats of wild plants are becoming increasingly endangered and, in the case of some endemics, are starting to disappear completely. This, coupled with the political instability of some 3rd World countries, and associated environmental destruction, can make it difficult to acquire certain plant-derived chemicals (BALANDRIN AND KLOCKE, 1988). The development of alternative sources of important natural plant products is thus desirable.

Plant tissue culture methods are widely used in the bulk propagation of plants. These techniques have been successfully applied to dicotyledons, monocotyledons and gymnosperms. Significant advances in the fields of haploid breeding; clonal propagation; mutant cultures; pathogen-free plants; secondary compound production; cryopreservation and genetic engineering have been made (DODDS AND ROBERTS, 1985).

Micropropagation is conducted under controlled, micro-organism-free conditions which enables rapid multiplication, irrespective of season or external environment, resulting in year-round availability. In addition, large-scale culture enables the exploitation of the biosynthetic potential of cells for the production of secondary metabolites, including pharmaceutical products (BAJAJ *et al.*, 1988).

Micropropagation

The establishment of a successful micropropagation protocol depends on the optimization of three stages, namely the establishment of an aseptic culture, initiation and multiplication of plantlets, and acclimatization of plantlets for re-establishment in the soil (MURASHIGE, 1974). This system has been modified by DEBERGH AND MAENE (1981) to include two additional steps, the first a pre-culture step to ensure healthy and uniform stock material, and the second a maturation step included before root initiation and acclimatization. This gives a total of five stages that are generally accepted today as summarizing a micropropagation protocol (RAYNS, 1993).

The initial preculture step (Stage 0) ensures that the mother plant material is healthy and disease-free. Stock plants are maintained in controlled environments and can be treated for surface and systematic microbial contaminants (RAZDAN, 1993). Effective control of pests and diseases results in vigorous stock plants, and consequently explants with a high regenerative potential (RAYNS, 1993). The choice of material for explants is important for the successful regeneration of plants. The regenerative potential of senescent tissues is very low, and while young plant parts tend to regenerate easily, this potential diminishes with each season of maturation. The season in which the explants are harvested can also effect the regenerative capacity of the tissue (RAZDAN, 1993), as can other factors related to the growth environment of the stock plants, such as light intensity, temperature and fertilizer application (RAYNS, 1993).

The first tissue culture stage (Stage 1) relies on the development of a suitable decontamination procedure enabling a large enough proportion of explants to survive free from fungal or bacterial infection. This is followed by the initiation and multiplication of buds from the sterile explants (Stage 2) which is most commonly accomplished via adventitious organogenesis, either of shoots or asexual embryos

(MURASHIGE, 1974). This is preferably achieved without the intermediate stage of callus formation, which can often lead to unwanted somatic variation. The initial plantlet-regeneration ability of callus may also decline over time (RAZDAN, 1993). The production of genetic aberrations is, however, still common with adventitious organogenesis. Axillary shoot multiplication results in virtually no genetically deviant plants, but is a much slower method of multiplication (MURASHIGE, 1974). A second step (Stage 3) is the elongation of these buds to shoots and the preparation of uniform shoots (DEBERGH AND MAENE, 1981). An optional third step (Stage 4) is the initiation of roots, which often requires a second media formulation.

The final step (Stage 5) entails acclimatizing plantlets grown in highly humid, sterile conditions with controlled temperature and light regimes, to external environmental conditions. Plantlets are transferred to suitable support media in misthouse conditions to allow the further development of roots and stomata, and protective structures such as cuticles. These plantlets usually show structural and physiological abnormalities manifest in abnormal leaf morphology and anatomy, poor photosynthetic efficiency, decreased epicuticular wax and malfunctioning stomata (RAZDAN, 1993). Large scale micropropagation is only successful if the high numbers of *in vitro* plants can be successfully transferred to the soil with a high survival rate.

Media requirements

Typically the basic nutrient medium, developed by MURASHIGE AND SKOOG (1962), is used. This combines inorganic salts (macro- and micronutrients), and vitamins (organic supplements), with a carbohydrate source and a gelling agent. This is modified by the addition of various plant hormones, generally cytokinins and auxins, to stimulate a growth response from the explant. The variation of these parameters, as well as the physical environment (light intensity, light regime and temperature) can induce morphogenic responses from plant tissue.

The concept of the hormonal control of organ formation was proposed by Skoog and Miller in 1957 (RAZDAN, 1993). In general, the addition of high concentrations of auxin promotes rooting whereas a higher proportion of cytokinin leads to bud or shoot initiation. Organogenesis is not determined solely by hormone ratios, but also depends on the source of plant tissue, environmental factors, composition of media,

polarity, growth substances and other factors (RAZDAN, 1993).

Direct morphogenesis

The differentiation of plants from *in vitro* cells through shoot-root formation or somatic embryogenesis is regarded as the fastest method of clonal multiplication (RAZDAN, 1993). Both axillary and apical shoots contain meristems that may be active or quiescent depending on the physiological state of the mother plant. *In vitro*, these can be induced to regenerate into shoots. The addition of suitable concentrations of cytokinin can induce axillary shoots to develop and proliferate into secondary and tertiary shoots. Division of these clumps during subculture will encourage further multiplication. Adventitious shoots are stem and leaf structures (including bulbs, corms, tubers) that arise naturally on plant tissues located in sites other than at normal leaf axil regions (RAZDAN, 1993). Adventitious shoot development can be induced *in vitro* from both leaf and bulb scale explants. Meristematic regions at the base of leaves and scales where they join the basal plate can be induced to regenerate multiple shoots. Adventitious shoots may arise from single epidermal cells resulting in little genetic aberration (RAZDAN, 1993).

Culture conditions

Despite the presence of chlorophyll in the regenerated shoots, these exist as heterotrophs and rely on the carbohydrate in the medium for growth. Light quality and photoperiod are, however, still important and can play a role in inducing morphogenesis. For most cultures, a diurnal illumination of 16 h light, 8 h dark, promotes shoot multiplication. A constant temperature level set at 25°C is optimal for the growth of most species that have been placed in culture.

Cell cultures

Liquid suspension cultures provide an opportunity to investigate the properties and potentialities of plant cells (RAZDAN, 1993). Interest has focussed on the potential of plant cell cultures as an alternative to traditional agriculture for the industrial production of secondary plant products (BALANDRIN AND KLOCKE, 1988). Using cell culture, the manipulation of chemical pathways has been achieved by biotransformation to produce natural products (BAJAJ *et al.*, 1988). Also, by utilizing the meristematic tissue, virus-free and disease-free propagules can be produced, and

germplasm can be maintained, leading to the formation of a Tissue Bank for medicinal plants (DATTA, 1993).

The use of cell suspension systems for the large scale culturing of plant cells from which specific secondary metabolites can be extracted, would enable the continuous, reliable production of natural products. These compounds would also be easier to purify due to the simpler nature of the extracts and the absence of large amounts of pigments, thus reducing processing costs (BALANDRIN AND KLOCKE, 1988).

Tissue culture techniques provide the basis for current plant genetic engineering and elite clone development, and the importance of these techniques should not be underestimated.

1.6 GENETIC ANALYSIS OF SPECIES VARIATION USING MOLECULAR MARKERS

The use of markers to determine patterns of inheritance underlies the study of genetics (WILLIAMS *et al.*, 1993). Although phenotypic features such as morphological characteristics can be used to distinguish closely related individuals, these can be influenced by the developmental stage of the plant, the heritability of the trait, and factors including environmental conditions, multi-genic and quantitative inheritance, and partial and complete dominance which influence the expression of a genetic trait (TINGEY AND DEL TUFO, 1993). The complexity of gene expression involved in phenotypic features limits the use of these external characters in distinguishing closely related hybrids (NEWBURY AND FORD-LLOYD, 1993).

Many DNA markers are used in genetic mapping, genetic diagnostics, molecular taxonomy and evolutionary studies (WILLIAMS *et al.*, 1993). The use of DNA-based markers allows direct comparison between the genetic material of two plants, avoiding interference on gene expression by environmental and other factors (NEWBURY AND FORD-LLOYD, 1993). DNA markers enable the detection of allelic differences in DNA sequences. These differences are termed DNA polymorphisms,

and are detected as changes in the size of restriction fragments (RFLP) or by amplification of the DNA by polymerase chain reaction (PCR) techniques (WILLIAMS *et al.*, 1993). Molecular ecology is a relatively new field in which polymerase chain reaction (PCR) techniques have had an important impact on the integration of genetic data with field observations on gene flow and diversity (WHITE, 1996).

Before the development of random amplified polymorphic DNA markers (RAPD markers), the most commonly used DNA markers were restriction fragment length polymorphisms (RFLPs). These assays detect DNA polymorphisms through restriction endonuclease digestions coupled with detection by DNA blot hybridization (TINGEY AND DEL TUFO, 1993), a process that is laborious and incompatible with applications requiring high throughput (WILLIAMS *et al.*, 1993). The related technique of VNTR (variable number of tandem repeats) also uses hybridization technology and identifies repeated DNA regions of differing lengths resulting from variable numbers of serial repeats of a core DNA sequence (NEWBURY AND FORD-LLOYD, 1993). PCR-sequencing analyzes variation by isolating base sequences of known genes and differences at the level of single base substitutions can be identified. A polymerase chain reaction is used to amplify the DNA between 20-base specific primers, which is then applied to electrophoretic gels for visualization (NEWBURY AND FORD-LLOYD, 1993). This reaction enables the amplification of regions of DNA from very small samples of DNA that are not necessarily completely intact (WHITE, 1996)

Genetic analysis using RAPD markers

This method is based on the PCR reaction (NEWBURY AND FORD-LLOYD, 1993).The use of RAPD genetic markers does not necessitate a knowledge of target DNA sequence, these are generated by amplification of random DNA segments with single primers of arbitrary nucleotide sequences (WILLIAMS *et al.*, 1993). In this reaction, a single species of primer of arbitrary nucleotide sequence binds to the genomic DNA at two different sites on opposite strands of the DNA template. Where these primer sites are within amplifiable distances, a discrete DNA product is produced through thermocyclic amplification (TINGEY AND DEL TUFO, 1993). The DNA copying enzyme *Taq* polymerase (originally isolated from *Thermus aquaticus*) works optimally at 72°C, and can thus be used to make copy strands of DNA under elevated temperature conditions, thereby ensuring that only specific regions of the

genome are replicated *in vitro*. The use of *Taq* polymerase in PCR reactions has improved the yield and sensitivity, generated more specific and longer products and facilitated automation (SAIKI *et al.*, 1988; GUYER AND KOSHLAND, 1989).

Once a DNA sequence has been copied, the copy can be copied resulting in an exponential increase in the number of copies (NEWBURY AND FORD-LLOYD, 1993). Short primers are used to ensure that amplification will result. Current PCR-technology does not allow for amplification of sequences longer than about 4 000 bases, so DNA sequences will only be amplified if two copies of the single primer used, hybridise to opposite strands of a piece of DNA, and they are separated by less than 4 000 bases (NEWBURY AND FORD-LLOYD, 1993). The presence of each amplification product identifies complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotide primer at each end of the amplified product (TINGEY AND DEL TUFO, 1993).

Advantages of RAPDs

In comparison to techniques such as RFLP analysis, RAPDs have proved technically simpler with far fewer steps, giving results in a much shorter time, making the assay more amenable to automation (TINGEY AND DEL TUFO, 1993). The costs of the two techniques are, however, similar. In addition, the amount of DNA required for the RAPD reaction is very low (since the analysis depends on amplification) necessitating only small amounts of starting material. Fluorescence is used as a visualization tool rather than radioactivity (TINGEY AND DEL TUFO, 1993). The major advantage of this technique is, however, that there is no requirement for sequence information (TINGEY AND DEL TUFO, 1993; WILLIAMS *et al.*, 1993).

The mean frequency with which variation is detected is higher per primer (RAPD method) than it is per probe (using RFLP methods). This is partly as a consequence of the typical production of more scorable bands by the use of a single primer, than with a single probe. RFLP analysis has a more limited use since the degree of homology between a probe derived from one species and the same locus in a different species may be insufficient to allow hybridization, resulting in no marker bands produced. In contrast, the same RAPD primers can be used for the analysis variance between taxonomically different plant groups. This is due to the fact that

usable bands are obtained from different species even though it is not necessarily the same loci that are yielding the amplifiable fragments (NEWBURY AND FORD-LLOYD, 1993).

Disadvantages of RAPDs

The dependence of this technique on the amplification of initially very small amounts of DNA sequence by PCR resulting in susceptibility to contamination of the reaction mixture by foreign DNA. Contamination can be as a result of the presence of fungal DNA sequences, cross-contamination during the extraction of the DNA from individual plants, or by the presence of amplified DNA sequences from previous PCR reactions. Usually, contaminating, potentially amplifiable sequences are present in much lower concentrations than the plant DNA sequences and may not amplify to sufficient levels to allow visualization in the gel (NEWBURY AND FORD-LLOYD, 1993).

Another disadvantage of RAPD analysis is that the information inherent in the banding patterns produced by RAPDs is less than in those produced by RFLPs. Unlike the bands produced by RAPD analyses, the bands on autoradiographs from RFLP analyses are known to have closely related sequences (or they would not hybridise to the probe). In RAPD analysis, there is a chance that two amplified fragments, from different individuals, which have the same size (as monitored by gel electrophoresis), may not actually have the same sequence. This is a problem since it is generally assumed that co-migrating DNA fragments are identical and thus allelic. Discrimination between homozygous and heterozygous individuals is thus not possible since both would produce bands of the same size, and the cycles of DNA copying that occur mask any differences in the quantity of target sequence produced, making detection by differences in band intensity unreliable (NEWBURY AND FORD-LLOYD, 1993).

DNA fingerprinting

DNA fingerprinting is used to identify varieties within species, to determine parentage within breeding material and to identify, characterize and classify clonal material (NEWBURY AND FORD-LLOYD, 1993). Phylogenetic relationships, especially those at the intra-specific level can be examined using RAPDs. The discrimination of closely-related individuals is based on the number and position of RAPD bands that

are observed (TINGEY AND DEL TUFO, 1993).

The question underlying this analysis is whether RAPD bands of equal molecular weight that are shared by individuals are homologous characters (i.e. inherited from a common ancestor), or homoplastic characters (i.e. arise independently within a population). It is probable that closely-related individuals co-inherited shared characters from a common ancestor, rather than acquiring the same character independently (TINGEY AND DEL TUFO, 1993).

RAPD markers can further be used in the determination of genetic stability of long term micropropagated plantlets that may exhibit somaclonal variation. This variation is often heritable and is thus undesirable in clonal propagation. Identifying variation early in the life of micropropagated plants is particularly important for slow growing economically important forest trees (GOTO *et al.*, 1998), but can be equally important in the horticultural and pharmacological industries. Micropropagation systems can be analysed for risk in terms of culture time and explant source using these techniques and different forms of regeneration (direct morphogenesis, callus or protoplast) tested for genetic variation (GOTO *et al.*, 1998). Further applications of this technique lie in the screening of genetic resources maintained in gene banks for genetic diversity. This can equally be applied to studies of biodiversity in natural habitats to enable the selection of high priority areas for conservation and aid in the development of planning conservation strategies (NEWBURY AND FORD-LLOYD, 1993).

1.7 THE GENUS *EUCOMIS*

The family Liliaceae *sensu lato* is one of the largest families of flowering plants including 250 genera and 3 700 species and consists mainly of herbs with a variety of storage organs such as bulbs, corms , tubers and tuberous roots (PERRY, 1985). This large family was restructured into separate smaller families belonging to one of the closely related orders Liliales and Asparagales.

Eucomis is a small genus of indigenous bulbs widely distributed in South Africa (PIENAAR, 1984). Petaloid monocotyledons comprise 64% of monocotyledons in South Africa, including the horticulturally important families: Iridaceae, Amaryllidaceae, Liliaceae, Orchidaceae and Hyacinthaceae. The genus *Eucomis* originally fell within the family Liliaceae, but under the restructured classification (DAHLGREN *et al.,* 1985; COMPTON, 1990) is placed in:

Superorder : Liliiflorae
Order : Asparagales
Family : Hyacinthaceae Batsch (1802)

The family Hyacinthaceae includes geophytes with a bulb as the underground storage organ. The leaves are basal and the inflorescence is a simple raceme. Perianth segments are free or united at the base and the fruit is a capsule (PERRY, 1985).

The name *Eucomis* is derived from the Greek *eukomes* meaning "beautiful headed", in reference to the characteristic tuft of leaf-like bracts at the crown of the flower spike (BRYAN, 1989). Common names for the genus *Eucomis* include Ananas Plant, King's Flower, Pineapple Flower (*E. bicolor*), and Pineapple Lily (*E. undulata*) (English); Schopflilie, Ananasblume (German) (DE HERTOGH AND LE NARD, 1993). The flower spikes resemble a pineapple, hence its common name (PIENAAR, 1984). The storage organ is a large, tunicated bulb, ovoid or globose in shape (BRYAN, 1989; DE HERTOGH AND LE NARD, 1993) with hard cortices (HUTCHINGS *et al.*, 1996). The roots are branched and contractile with root hairs (KAWA AND DE HERTOGH, 1992). The bulb produces a rosette of smooth, often shiny, leaves whose shape varies from narrow to broadly lance- or strap-like (DE HERTOGH AND LE NARD, 1993). The

leaves are green, basal and arching, curving back to the ground. The flowering stem varies from 30 cm to more than 100 cm in height, depending on the species.

Eucomis flowers in early summer, and may be sweetly or unpleasantly scented (DU PLESSIS AND DUNCAN, 1989). The scape bears an inflorescence which is an erect, densely packed spike or raceme, of predominantly green flowers forming a cylinder, on stout strong stems with a tuft of hairs above the flowers. The flowers are white, pale-green, or yellowish-green, with or without margins varying from pale to dark purple (DE HERTOGH AND LE NARD, 1993). The stamens are quite stubby and spaced so that they are quite prominent in the centre of the flowers, the perianth segments are almost equal. The swollen green fruits of *E. autumnalis* are almost triangular (BRYAN, 1989) and ripen to a capsule of hard, round black or brown seeds (DE HERTOGH AND LE NARD, 1993).

The genus *Eucomis* comprises about thirteen species (HUTCHINGS *et al.,* 1996), mostly native to South Africa, with two species extending into lower central (tropical) Africa (COMPTON, 1990; DE HERTOGH AND LE NARD, 1993). Most species occur in the eastern and north-eastern regions of South Africa, with one species, *E. regia*, occurring in the Western and South-Western Cape. *E. zambesiaca*, from Malawi, has the most northerly distribution (DU PLESSIS AND DUNCAN, 1989). The natural habitat comprises damp grasslands from the coast to the Drakensberg. These populations are vulnerable as they are currently common only in remote areas at high altitudes (MANDER *et al.,* 1995). *E. bicolor* is largely found at the base of damp cliffs in the Drakensberg and on moist inclines from 1 800 m to 2 600 m, while the distribution of *E. humilis* is mostly between 2 400 m to 2 900 m. *E. autumnalis* (syn *undulata*) is found in damp hollows in grasslands from 2 100 m to 2 400 m (TRAUSELD, 1969). *Eucomis* species are deciduous geophytes and are summer-growing and dormant in winter, with the exception of *E. regia,* which is winter-growing and dies down in early summer (DU PLESSIS AND DUNCAN, 1989) .

Eucomis species require full sun or partial shade and a rich, well-drained soil (BRYAN, 1989; DU PLESSIS AND DUNCAN, 1989). The summer-growing species are frost-tolerant in the Southern Hemisphere and in mild parts of the Northern Hemisphere. The genus is seldom troubled by serious pests or diseases (DU

PLESSIS AND DUNCAN, 1989). They are well suited as container plants, placed in rockeries and as herbaceous border plants (BRYAN, 1989). The flowers are long lasting and are attractive even when in fruit.

Eucomis can be used for cut flower production (DU PLESSIS AND DUNCAN, 1989) with specimens lasting for several weeks (BRYAN, 1989). *Eucomis* can be forced as potted plants, but no specific research has been conducted on the time of flower initiation, and the influence of external and other factors (DE HERTOGH AND LE NARD, 1993). A problem encountered in cut flower production is the propensity of the flower stems to bend. In limited studies conducted at North Carolina State University, it was concluded that clonal selections are a prerequisite for large scale use of *Eucomis* as forced plants (DE HERTOGH AND LE NARD, 1993).

The plants are senescent in autumn. Propagation is by offsets in autumn, after the leaves have started to die back. Offsets develop rather slowly, and propagation is easier by seed sown in spring. Young bulbs can be planted out at the beginning of their third season. Bulbs are planted in spring and flower in mid-summer. The minimum size for a flowering bulb is about 12 cm in circumference. Flowering can be expected in the third to fourth year if the plants are well-grown. (BRYAN, 1989; DU PLESSIS AND DUNCAN, 1989). *Eucomis* bulbs are planted with their tops at or just below ground level (DU PLESSIS AND DUNCAN,1989). Successful micropropagation has also been reported (DE LANGE *et al.*, 1989; DE HERTOGH AND LE NARD, 1993; AULT, 1995; McCARTAN AND VAN STADEN, 1995; McCARTAN *et al.*, 1999).

The breeding and commercial exploitation of South African petaloid monocotyledons in general, was dominated by Europe until the 1960's and 70's (STIRTON, 1980). The species of *Eucomis* under cultivation in The Netherlands include *E. bicolor* (green flowers with lilac margin, height about 75 cm) *E. bicolor* cv. var.'Alba', *E. comosa-punctata* (greenish white flowers with purple pistil, leaves spotted crimson, height about 60 cm) and *E. pole-evansii* (Green flower, height 1 m or more). Bulb production is very low (DE HERTOGH *et al.,* 1990) with only about two hectares being grown in The Netherlands. There is an absence of statistics for other countries. No specific breeding work is reported (DE HERTOGH AND LE NARD, 1993).

Eucomis species:

The genus was originally described by BAKER (1897).

Eucomis autumnalis (Mill.) Chitt. **Subsp. *autumnalis*** (syn *E. clavata, E. undulata, E. robusta*) is native to the eastern Cape Province, Transvaal, Natal, and tropical South Africa. The bulb is large (8-10 cm) and ovoid in shape, bearing linear, lanceolate or ovate leaves with a distinct midrib, and a margin with a strongly undulate / wavy edge. The bulb produces 15-20 leaves, which are 5-7 cm wide and 60 cm long; extending a little way from the stem then falling back to lie along the ground.

E. autumnalis flowers in late summer. The flower spike reaches 45 cm in height, the top 30 cm of which is covered with flowers, each 1 cm in diameter, green (no purple colouration), fading to lighter yellow-green as it matures. The head is topped by a coma of green lanceolate bracts (pendulous with minutely undulate margins). The pedicels are short (0.3-0.9 cm) bearing flowers with white, yellowish green or green perianths (1.5-2.5 cm diameter). The filaments are green, fused to base of perianth forming a cup 0.3-0.4 cm high. The ovary has 3 lateral indentations with a membranous pericarp.

The subspecies ***clavata*** (Bak.) Reyneke (high altitude) is similar to the subspecies *autumnalis*, but it has larger flowers and a much wider distribution, occurring in Natal, Lesotho, eastern Orange Free State, Transvaal and Botswana. The subspecies is distinguished by wide, ovate / ovate-lanceolate leaves (6-13 cm wide) which usually lie flat on the ground. The peduncle is squat / stout (30 cm high) bearing whitish flowers. The flower stem is narrow as it emerges from the bulb and thickens into a club shape. This subspecies is further distinguished from *E. autumnalis autumnalis* by a seed capsule with a hard, double-layered pericarp (as opposed to the thin, sometimes inflated capsule of *E. autumnalis autumnalis*).

The subspecies ***amaryllidifolia*** (Bak.) Reyn. has narrower, linear-lanceolate leaves (13-30 cm x 1.5-4 cm). The inflorescence bears green or white flowers, which are densely packed. The perianth is short, with segments 0.6-1.0 cm in length. The seed capsule is small and papery (and is sometimes inflated) (REYNEKE, 1980).

Eucomis bicolor (Bak.) is native to Natal, Lesotho and Orange Free State. The species has a large bulb, bearing leaves that are 50 x 10 cm in length with a slightly wavy margin (undulate-edged). The leaves are broad, and dark green with their base clasping the flower spike. Leaves are often purple spotted, occasionally tinged with purple. The plants prefer partial shade, and need moisture throughout summer but can be allowed to dry out in autumn (BRYAN, 1989).

The peduncle is 60 (20-30) cm high, cylindric and often purple spotted or splotched dark brown (especially at the base). The racemes are 30 cm high, but not quite so densely packed as other species, bearing green or cream-coloured flowers with distinctive purple edges. The flower colour varies in the wild from pale green to purple, mostly with a dark purple centre (TRAUSELD, 1969). The coma sometimes has purple colouration on the bracts. The pedicels are pendulous, 2 cm long (1.5cm lobes; 2-3 cm in diameter). This species flowers in late summer and is unpleasantly scented.

Eucomis bicolor* var *"Alba" is white-flowered, with long pedicels that distinguish it from *E. autumnalis*, and its height and prominent coma differentiates it from *E. pole-evansii*. The leaves have no purple markings (BRYAN, 1989).

E. comosa* subsp. *comosa (syn *E. pallidiflora, E. punctata*) This species is native to the eastern Cape, Transkei and Natal, is one of the most distinctive of the genus and the one most commonly grown. The leaves are spotted purple at the base, and are 45-50 cm long.

The flower stem reaches 60 cm in height, and is half covered with sweetly scented flowers that are greenish-yellow in colour, spotted slightly with purple. The flowers have violet-purple ovaries. The petals curve back slightly, throwing the stamens well out in front and displaying the colour of the ovaries. The flower colour sometimes varies, being tinged with pink; a trait that has been selected for commercial production in New Zealand.

Eucomis comosa-punctata (Houtt.)Wehr. has a large, globose, often purple bulb. The leaves are oblong-lanceolate (30-80 x 3-10 cm) with smooth margins with a few gentle undulations. The leaves are green, often purple spotted, occasionally tinged with purple.

The peduncle is 40-60 cm high and often purple spotted or striped. The many

flowers are sweet-scented and have a coma of erect, often purple spotted or tinged bracts. The pedicels are erect (ascending pedicels) and the perianth is pale green, pink or purple (greenish white). The filaments are green, with a purple pistil and purple, conspicuous ovary. The pericarp has a characteristic purple hue.

Eucomis comosa-punctata* var *striata (Willd.) is characterized by leaves with purple spotting that develops into stripes (BRYAN, 1989)

Eucomis humilis (Baker) is a dwarf species with an ovoid bulb (3-5 cm in diameter). The size, erect leaves, short, rather slender inflorescence and swollen seed capsules distinguish this species. The leaves are small 6-35 x 6-10 cm, with smooth margins (no undulations). The leaf bases are occasionally attractively spotted and blotched underneath.

The peduncle is 20-30 cm high. The short dense flowered racemes are topped by a coma of short bracts. The flowers have somewhat pendulous pedicels, 2-4 mm long and a perianth that is greenish / purplish / wholly green. The filaments are purple with yellow anthers, and the ovary and capsule are large (fat), and dark, reddish purple in colour. The pericarp is a swollen seed capsule (BRYAN, 1989).

Eucomis pole-evansii (N.E.Brown) **= *Eucomis pallidiflora*** (Bak.) is native to the Transvaal and Swaziland. This is the largest species, reaching up to 2 m in height. There is no purple colouration The bulb is large and globose, bearing leaves that are 50-60 x 10-20 cm in length with minute serrations along the margin.

The peduncle can be 50-180 cm high supporting an inflorescence with stout, densely flowered racemes. The top 65 cm is covered with wide-opening green flowers. The thickness of the cylinder formed by the flowers can be as much as 20-25 cm. The coma consists of up to 30 oblong-lanceolate bracts with minutely ciliated margins. The pedicels are 1.5-2 cm long, and the perianth segments are white, greenish-yellow or green. The filaments and ovary are green (BRYAN, 1989).

E. regia (syn ***E. nana***) Native to western Karoo, in the Cape Province of South Africa; This is a dwarf species, with the peduncle only 30 cm in height, half covered with green flowers. The leaves are 60 cm long and 7-10 cm wide (BRYAN, 1989).


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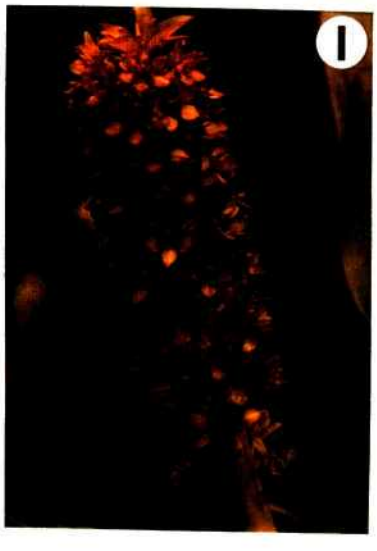
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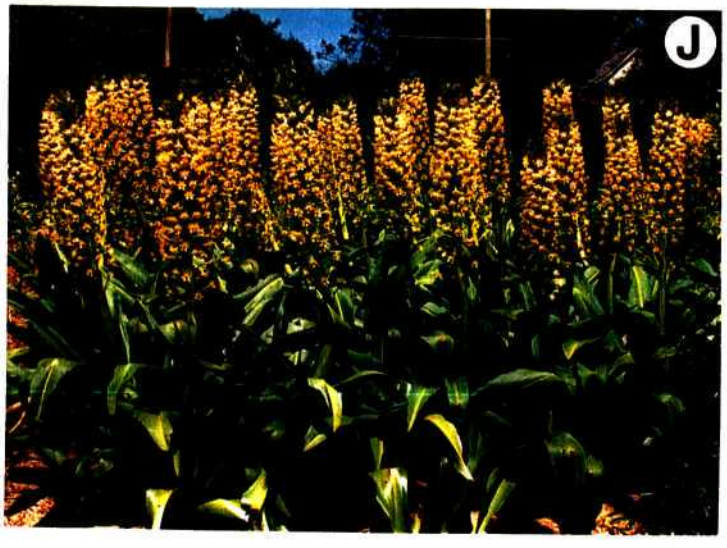
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Eucomis schjiffii (Reyn.) is the smallest, shortest species, only 4-10 cm high. The bulb is small (2-4 cm diameter) and globular. The 3-4 leaves are ovate, often glaucous and sometimes purple tinged or spotted at base. The margin is smooth, or minutely indented. The leaves are prostrate, 5-10 x 3-7 cm.

The peduncle is only 4-10 cm high, sometimes purple spotted. There is a coma of 10-15 ovate bracts, sometimes with purple margins. The flowers have no, or very short, pedicels and a dark reddish purple perianth. The filaments are purple and the ovary may be green, and often purple tinted. The pericarp capsule is inflated, and the seeds are pale brown. This species is characteristically squat, with glaucous, wide, flat leaves and short reddish inflorescences appearing in late summer (BRYAN, 1989).

Eucomis vandermerwei (Verdoorn.) is 15-25 cm high with a small bulb (3-6 cm diameter) and 3-4 leaves that are semi erect. The leaves are 40-60 x 15 cm with an undulate margin, and the lower surface may be spotted purple at the base.

The peduncle is 20-30 cm high, purple spotted with a fairly loose raceme. There is a coma of up to 11 bracts, with purple spots on the lower surface. The pedicels are 2-4 cm long and the perianth is green/greenish white, or may be purple-tinged, or with a purplish edge to segments. The filaments are green, the anthers mauve, and the ovary is green, and purple-tinged. The pericarp capsule is not inflated, but is 3-angled with a transparent pericarp. The flowers have an unpleasant smell (BRYAN, 1989).

Eucomis zambesiaca (Bak.) Native to E. Tropical Africa. The species is much like *E. comosa* var. *comosa*, and is distinguished from *E. autumnalis* by the absence of an easily recognizable cup of anthers or seed with thin pericarp. The plant is 15-25 cm high with a small (3-5 cm), ovoid, bulb attached to a swollen basal plate. The leaves are green, 30 x 5 cm with a undulate, minutely indented margin.

The peduncle is green, narrow, and 20-30 cm high. The raceme is densely packed. The flowers have a coma of short, ovate, lanceolate bracts and pedicels 2-5 mm long. The perianth is pale green, and white after anthesis with pale green filaments and white anthers. The ovary is green and obtusely 3-angled. The pericarp is a swollen seed capsule (BRYAN, 1989).

PLATE 1.1: Eucomis species (A) Inflorescence of *E. autumnalis autumnalis*; (*B) E. autumnalis autumnalis*; (C) *E. autumnalis clavata*; (D) *E. bicolor*; (E) *E. humilis*; (F) *E. zambesiaca*; (G) *E. comosa-punctata striata*; (H) *Eucomis* hybrid; (I) Mature inflorescence showing the characteristic inflated capsules; (J) *E. pole-evansii*.

Medicinal properties of *Eucomis*

Estimates of the trade in *Eucomis* species in KwaZulu-Natal by CUNNINGHAM (1988a) place the number of standard 50 kg bags sold annually as more than 581 for *E. autumnalis*, and ±224 for *E. bicolor* and other species. Further analysis of the bulbs showed that on average they were 47-62 mm in diameter. The bags contained between 324-432 bulbs, with each bag containing a bulb fresh mass of 35-39 kg. This implies that the age of the plants harvested is young, probably 1-2 years (based on bulb diameter), which has further implications for the scarcity of the species in its natural habitat. This is confirmed by the perceptions of the herb traders and herbalists themselves (both urban and rural), who list *Eucomis* species among the 15 scarcest medicinal plant species traded (CUNNINGHAM, 1988a). Over-exploitation is cited by traders as the major cause for the scarcity of medicinal plants (CUNNINGHAM, 1988a), and efforts are being made to rationalize conservation policies regarding plant collection for trade. Methods are needed that will prevent this current over-exploitation of indigenous flora and fauna for medicinal purposes without inhibiting cultural practices (BYE AND DUTTON, 1991).

E. autumnalis autumnalis (syn *E. undulata*) Ait. is a prized African medicinal plant, with both the leaves and bulbs having reported usage. Common names associated with the species include :

English : Eucomis

Afrikaans : Wilde pynappel, krulkop, (ROBERTS,1990) gifbol, pineapple flower, Pineapple plant (HUTCHINGS *et al.*, 1996)

Pedi : Maphuma-difala

Southern Sotho : Kxapumpu

Tswana : Mothuba-difala

Zulu : *Umakhunda* (ROBERTS,1990) *ukhokho, umakhandakantsele, umbola, umathunga* (HUTCHINGS *et al.*, 1996; MANDER *et al.*, 1995).

E. autumnalis autumnalis bulbs rank fifth on the list of ten most commonly stocked plants by traders. In general, plants harvested for the roots, bulbs and tubers are perceived by traders to be scarce, although this may be biased by the seasonal nature of the plants. They can only be identified and harvested in spring and summer when they are in flower (WILLIAMS, 1996).

Eucomis bulbs are identified as medium sized bulbs 50 -100 mm in diameter, with conspicuous horizontal stripes at the base and papery bulb scales which are brown to black in colour. When sliced, the yellow-white flesh is visible which turns brown on exposure to the air (MANDER *et al.*, 1995). The bulbs of *Eucomis* species are used in Zulu, Tswana, Sotho and Xhosa medicine, in decoctions prepared from shaved bulbs and roots boiled in water or milk, and used as ingredients in infusions. They are primarily used in the treatment of pain and fever, although the bulbs of *E. autumnalis* are regarded as toxic (HUTCHINGS, 1989a). In Zulu culture, *Eucomis* is used largely as an enema (*umuthi wokuchata*) and it serves to relieve biliousness (*ukuba nenyongo*), and to enhance sexual prowess (*umqundu wenkunzi*) (MANDER *et al.*, 1995). Other uses include decoctions used as protective charms (WATT AND BREYER-BRANDWIJK, 1962) and to treat kidney and bladder ailments, nausea and coughs (HUTCHINGS *et al.*, 1996; ROBERTS,1990). The bulbs are easily confused with *Cyrtanthus obliquus* and both are used in preparations for 'blood purifying' (*imbhiza*) (HUTCHINGS *et al.,* 1996). Further uses of the bulbs are listed in TABLE 1.1.

The long strap-like leaves are used as a poultice on suppurating sores and boils, and are wound round the wrists to bring down fever. The juice of the stems soothes sores and rashes, and the leaves may be added, with the bulbs, to *Alfalfa* or *Zea mays* leaves and given to cattle to treat gall sickness and other diseases (ROBERTS, 1990).

Eucomis has been suspected of causing human poisoning and is known to have produced death in sheep (WATT AND BREYER-BRANDWIJK, 1962). Symptoms of poisoning include abdominal pain, diarrhoea and renal failure (HUTCHINGS *et al.*, 1996). The bulb contains a dangerous haemolytic poison (MANDER *et al.*, 1995).

Phytochemical characters of the order Asparagales include the occurrence of steroidal saponins and chelidonic acid (DAHLGREN *et al.*, 1985). The family Hyacinthaceae comprises primarily glabrous, scapose perennial herbs with bulbs and contractile roots. Many of these plants have crystal raphides contained in mucilage cells or canals. The bulbs contain fructans and starch.

Eucomis plants have a sharp, bitter taste but no cardiac glycosidal effects have been observed in whole plants or bulbs (WATT AND BREYER-BRANDWIJK, 1962). This genus has been reported to contain saponins (WATT AND BREYER-BRANDWIJK, 1962) and eucomic acid (HELLER AND TAMM, 1974). The bulbs contain the spirocyclic nortriterpene, eucosterol (SIDWELL *et al.*, 1975; ZIEGLER AND TAMM, 1976; GLASBY, 1991), substituted dibenzo-α-pyrones such as autumnariol and autumnariniol (SIDWELL *et al.*, 1971; GLASBY, 1991) and homoisoflavones (BÖHLER AND TAMM, 1967; SIDWELL AND TAMM, 1970; FARKUS *et al.* 1971). The bulb wax yielded 5,7-dihydroxy-8-methoxy-chroman-4-one (HELLER AND TAMM, 1978).

TABLE 1: Medicinal uses of *Eucomis* bulbs.

SPECIES	USES	REFERENCE
E. autumnalis (Mill) Chitt	Colic, flatulence, kidney and bladder complaints.	HUTCHINGS *et al.*, 1996; ROBERTS, 1990.
	Nausea, coughs (respiratory problems) = emetic.	HUTCHINGS *et al.*, 1996; ROBERTS, 1990, WATT AND BREYER-BRANDWIJK, 1962.
	Lumbago.	WATT AND BREYER-BRANDWIJK, 1962.
	Hangovers.	HUTCHINGS *et al.*, 1996; WATT AND BREYER-BRANDWIJK, 1962.
	Syphilis (venereal disease).	HUTCHINGS, 1989a; HUTCHINGS *et al.*, 1996; ROBERTS, 1990.
	Abdominal distension, stomach ache, topical pain killer.	IWU, 1993; ROBERTS, 1990.
	Emetics and enemas (for fevers).	CUNNINGHAM, 1988a; GERSTNER, 1941; HUTCHINGS, 1989a.
	Urinary diseases, facilitating childbirth (taken regularly in pregnancy).	BRYANT, 1966; GERSTNER, 1941; HUTCHINGS, 1989a.
	Domestic stock diseases.	WATT AND BREYER-BRANDWIJK, 1962.
E. autumnalis (Mill.) Chitt. subsp *clavata* (Bak) Reyneke [Syn *E. regia* Ait.; *E. robusta* (Bak); *E. undulata* sensu Letty & Trauseld, non Ait] *umathunga* (Zulu)	Emetics and enemas for fever, biliousness and lumbago, respiratory ailments. Coughs, produce sleepiness Unspecified parts used for syphilis and blood disorders; venereal disease; to prevent premature childbirth, diarrhoea.	GERSTNER, 1941; WATT AND BREYER-BRANDWIJK, 1962. WATT AND BREYER-BRANDWIJK, 1962; HUTCHINGS *et al.*, 1996.

SPECIES	USES	REFERENCE
E. bicolor Bak	Colic, purgative. Purgative (*imbola*).	WATT AND BREYER-BRANDWIJK, 1962; BATTEN AND BOKELMANN, 1966; HUTCHINGS *et al.*, 1996. CUNNINGHAM, 1988a
E. comosa (Houtt) Wehrh var *comosa* [syn *E.pallidiflora* Bak. *E. punctata* L'Herit] *Ubuhlungu-becanti* (Zulu)	Anti-rheumatic, enema, for teething infants (Xhosa).	GERSTNER, 1939; WATT AND BREYER-BRANDWIJK, 1962; BATTEN AND BOKELMANN, 1966; HUTCHINGS, 1989a; HUTCHINGS *et al.*, 1996.
E. pole-evansii NE Brown	Mental diseases.	WATT AND BREYER-BRANDWIJK, 1962.
E. regia (L.) L'Herit	Venereal diseases, lumbago, diarrhoea, respiratory conditions especially coughs, biliousness and to prevent premature childbirth.	WATT AND BREYER-BRANDWIJK, 1962.

1.8 THE PHARMACOLOGICAL INVESTIGATION OF PLANTS

Pharmacognosy is defined as the botanical, chemical and biological study of drugs of therapeutic value, or the study of the starting materials and substances intended for therapeutics, of biological origin (i.e. obtained from plants; animals; or by fermentation from microorganisms) (BRUNETON, 1995).

The critical and systematic study of both the chemistry and biological properties of natural products developed at the start of the 19th century (KINGHORN AND BALANDRIN, 1993). This had a profound influence on the development of modern synthetic organic chemistry. Spectroscopic and X-ray crystallographic techniques allow the rapid structural determination of novel plant constituents and the high-throughput automated bioassays now available enable the production of detailed biological profiles using a few milligrams of a natural product. New chemical and biological technologies can facilitate the isolation and characterization of receptors, thus facilitating the application of drug design principles to potential natural product drugs (KINGHORN AND BALANDRIN, 1993).

Recent successes in the field of plant-derived compounds include the anti-cancer agents taxol and camptothecin, the Chinese anti-malarial drug, artemisinin, and the East Indian Ayurvedic drug, forskolin (BALANDRIN *et al.*, 1993). This illustrates the potential value and relevance of plant-derived secondary metabolites as viable compounds for modern drug development (BALANDRIN *et al.*, 1993).

Taxol is a plant-derived drug isolated from the Pacific yew. It was accorded final approval by the United States Food and Drug Administration (FDA) for the treatment of refractory ovarian cancer in 1993, the first naturally occurring plant-derived drug product (apart from semisynthetic natural products) to gain approval in more than twenty-five years (KINGHORN AND BALANDRIN, 1993). The approval of taxol has attracted widespread interest, both in terms of its therapeutic potential, and because of the necessity to preserve the natural stands of the Pacific yew while ensuring an adequate supply of the drug (KINGHORN AND BALANDRIN, 1993).

Preparation and storage of traditional medicine

Methods of preparation common in traditional medicine include boiling (roots and bark), soaking in cold water (crushed leaves or small herbaceous plants), burning of dried leaves or herbs (use of ash), chewing and heating or roasting (succulent leaves for poultices). The material is usually crushed or pounded prior to these processes, and in some cases the crushed material is applied directly to a wound (usually after being mixed with some type of oil). This represents an attempt on the part of the practitioner to extract the active principle in the plant for administration to the patient (KOKWARO, 1995).

The extraction processes used by traditional healers to obtain plant chemicals not exuded naturally from the plant (e.g. gum) vary according to the nature of the substance. Water soluble substances such as isoflavonoids and glycosidic compounds may be readily obtained through grinding tissues in water, others may require additional treatments such as heating or fermentation (COTTON, 1996). Experimentally, aqueous extracts are used to test plant extracts commonly used in the form of an infusion, while a chloroform extract is better for drugs administered in powdered form with oil, butter or milk, and a water-alcohol extract is suitable for drugs which are ground in solvents such as palm wine or home-made beer (CAVÉ, 1986; KOKWARO, 1995).

In traditional Zulu medicine, most plant remedies administered for inflammatory complaints such as headaches, are ground powders (snuff) inhaled through the nasal cavity. In this way digestion and metabolism of the compounds is avoided, and the active substances can dissolve in the watery mucosa and be absorbed directly into the circulatory system (JÄGER *et al.*, 1996). Aqueous extracts of the powdered extracts simulate this administration. This non-oral administration of the extracts can, however, present problems of solubilization, especially for lipophilic extracts (CAVÉ, 1986).

The treatment of plant material during storage, and after processing, can affect the chemical composition of the subsequent medical preparation. In modern phytomedicine, the stability of the active compounds is ensured, and packing materials are chosen that will not degenerate and contaminate the medicine. In

contrast, traditional medicines are routinely left exposed to sun, wind, dust, and human and animal contact, as is seen in the street markets (VAN WYK *et al.*, 1997).

Some medicinal plants or plant parts are only used fresh, while some may be stored in a dried state (cut into slices or dried whole in the sun or shade). The dried material is usually stored whole (or sliced) or may be ground to a powder. Containers include brown paper bags, newspaper, glass jars or tin cans (VAN WYK *et al.*, 1997). Storage of plant material dry and in the dark favours the preservation of active compounds. Some active substances (e.g. alkaloids) in plant material that is kept dry and with the cells intact can remain active for years (HOLMSTEDT, 1995).

Regardless of these methods, most experimental chemical work is done after air-drying the plant material. The chemistry of these dried plant parts is very different from that of fresh material, or that of a mixture of several different plants (usually prepared fresh) comprising a medical preparation. Volatile compounds, for instance, are easily lost through air-drying. Analysis of individual plants also neglects the reactions that may take place when chemicals from different plants are mixed together (PRANCE, 1995).

Screening for anti-inflammatory drugs

Since 90% of drugs now come from natural sources, pharmaceutical companies are renewing their interest in screening plants. Emphasis is, however, still placed on the partial or complete synthesis of active compounds in order to meet the potential market demands (FARNSWORTH, 1994). The example of taxol shows that while enough taxol can be obtained from the renewable resource of the needles of *Taxus* species, there are steps in place to prepare it by partial synthesis and possibly by tissue culture (FARNSWORTH, 1994).

There is a distinct difference in the types of illness predominating in western cultures and indigenous, third world populations. Cardiovascular disease, neoplasms, microbial infections and nervous system illnesses predominate in western societies, while diarrhoea, inflammation and complications associated with maternity concern indigenous healers (COX,1994). Indigenous peoples do not have the lifestyle or predicted lifespan associated with cardiovascular disease or cancer. These diseases

are also difficult to diagnose, as opposed to inflammatory, dermatological and gastrointestinal ailments which are more easily detected. In addition, the drugs active against ailments such as cancer, cardiovascular and central nervous system illnesses, have very narrow dosage windows and can be highly toxic in low doses. These, however, are the diseases that western medicine is pressured to treat, with as much as 72% of research funding devoted to these drugs (COX, 1994).

Thus, while the ethnobotanical approach to drug discovery has great potential for discovering potent new compounds, there are limitations on the type of drug likely to be discovered (COX,1994). Evaluations of the relative efficacy of ethno-directed sampling in relation to specific drug types, predict that properly designed ethnobotanical surveys could prove more successful in identifying drugs from specific plants used in the treatment of gastrointestinal, inflammatory and dermatological complaints, than for example of cancer (BALICK, 1994; COTTON, 1996). Anti-inflammatory drugs constitute only 4% of the US pharmacopoeia, while on average 12% of plants used in traditional medicine are used to treat inflammatory diseases. Compared to Western drug use pattern (based on information from US pharmacopoeia) there is a striking distinction between disorders receiving the greater attention in traditional versus orthodox medicine (COTTON, 1996). COX (1994) suggests that the differences in drug use patterns may reflect a number of factors including perceived severity of particular types of diseases, diagnostic ease of ailments, toxicity of specific treatments and prevailing economic incentives. Future ethno-directed sampling is more likely to succeed for drug categories which are more pertinent to indigenous medicine than those that reflect Western funding concerns (COTTON, 1996). There is, however, limited information detailing the chemical composition and full medicinal potential of 99.5 % of the plant kingdom, leaving great potential for the screening of randomly selected extracts in high-input screening assays (BALICK, 1994). Species selected by chemotaxonamy, where plants with a reported class of natural compound e.g. indole alkaloids or sesquiterpene lactones, and screened in specific assays can also prove successful (FARNSWORTH, 1994).

Compounds with potential anti-inflammatory activity need to be tested against animal or biochemical models of inflammation. There is no definitive model which covers all aspects of inflammation (LEWIS, 1989). Recent advances in biological test

methodology has removed reliance on *in vivo* assay procedures in the investigation of biological activity. Plant extracts and their purified compounds can now be applied to screening protocols to obtain a large amount of biological information. These tests can utilize for example bioengineered or synthetic enzymes, substrates, inhibitors and receptors and lend themselves to computerized automation (KINGHORN, 1992). These factors, coupled to the refinements in NMR and mass spectrophotometry techniques enable the detailed structural characterization of only a few milligrams of a natural product. Since the active principle(s) in crude extracts are usually present in low concentrations, the test system needs to be sensitive enough to detect their presence reliably, while maintaining a high selectivity to restrict the number of false positives, particularly from metabolites such as tannins (HAMBURGER AND HOSTETTMANN, 1991).

Enzyme inhibition assays, used to measure the inhibitory effect of plant extracts on specific enzymes involved in the expression of a disease or ailment, have been included as an integral tool in the search for new drugs. They can be utilized to investigate the biochemical mechanisms of the action of drugs, and have also been applied in the primary screening of plant extracts. These are highly specific, mechanism based tests, consequently any drug acting by a mechanism unrelated to the assay will be bypassed (FARNSWORTH, 1993). Enzyme assays are especially useful in evaluating the effects of traditional remedies for inflammation and pain related to the production of prostaglandins.

1.9 PROSTAGLANDIN SYNTHESIS

Inflammatory and related immune responses are normal defence mechanisms essential to health. These responses are, however, potentially harmful and play major roles in diseases such as rheumatoid arthritis and asthma (HINMAN, 1973). Inflammation is a complex process with many different mediators, including prostaglandins (CAMPBELL, 1990; TUNÓN *et al.*, 1990). Modulation of the release of such mediators can affect the result of the inflammation process. The inflammatory response involves a complex array of enzyme activation, mediator release, extravasation of fluid (oedema), cell migration, tissue breakdown and repair (VANE

AND BOTTING, 1995). The synthesis of prostaglandins has been implicated in the complex processes of inflammation and are responsible for pain. Prostaglandins (and eicosanoids) are not stored in tissues for later release, but are biosynthesized within seconds of the activation of the appropriate enzymes and are rapidly metabolized (SMITH, 1990).

The prostaglandins and related eicosanoids (thromboxanes and leucotrienes) are a family of pharmacologically active, acidic lipids produced by cell membranes (SMITH, 1990) and widely distributed in mammalian tissue and body fluids (Figure 1.2). They are among the most potent substances known, with concentrations as low as 0.01 mg $m\ell^{-1}$ active in *in vitro* systems and 10 ng $m\ell^{-1}$ active in *in vivo* systems, and are very versatile, exerting a variety of effects in different systems (WEEKS, 1973). They are thought to be involved in both physiological and pathological processes including reproduction, blood pressure regulation, broncho-constriction, inflammation, nerve transmission, gastric secretion, urine formation and platelet aggregation (SMITH, 1990).

Almost all the cells in the body contain both the appropriate fatty acid precursors (in the form of esters in their membrane phospholipids), and the enzymes involved in the oxygenative transformation of these fatty acids after release from the ester linkage (SMITH, 1990), and are thus capable of producing prostaglandins and eicosanoids. Eicosanoid precursors (usually arachidonic acid) are transformed into oxygenated products by one of two pathways in the body, catalysed either by prostaglandin endoperoxide synthetase or the lipooxygenases (SMITH, 1990).

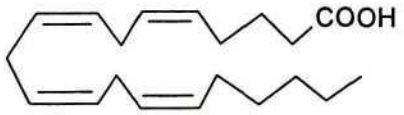
Unlike prostaglandin endoperoxide synthetase, which is present in the membranes of almost all cells, the lipooxygenases appear to be cytoplasmic and are almost entirely restricted to circulating or resident white blood cells (SMITH, 1990). The effect of prostaglandins, thromboxanes and leukotrienes on smooth muscle and other cells is produced through the interaction of these molecules with membrane-bound receptors, specific for eicosanoids. These receptors can differentiate between the different eicosanoids (SMITH, 1990). Leukotrienes are powerful chemotactic factors, attracting more phagocytes to the sites of inflammation (LEWIS, 1989).

Prostaglandins are formed naturally by the enzyme-catalysed oxidation and cyclization of certain 20-carbon polyunsaturated fatty acids (TAUBER *et al.*, 1973). Human calls and tissues contain almost exclusively arachidonic acid (5,8,11,14-eicosatetraenoic acid) esterified to phospholipids, with the exception of seminal vesicles which contain equal amounts of 8,11,14-eicosatrienoic acid and arachidonic acid, and lessor amounts of 5,8,11,14,17-eicosapentaenoic acid (EPA) (SMITH, 1990). These precursors are derived from dietary linoleic acid, an essential fatty acid that cannot be biosynthesized by humans, and are ingested as constituents of meat, with the exception of EPA which is assimilated by the ingestion of cold-water fish (SMITH, 1990).

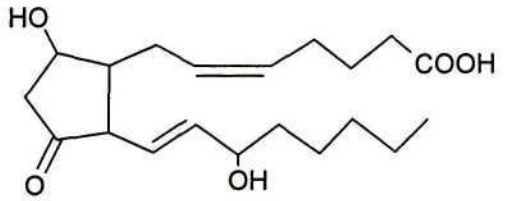
All prostaglandins (PGs) are analogs of a hypothetical compound, prostanoic acid. This is a 20-carbon fatty acid with a cyclopentane ring. Prostaglandins are classified according to the substituents attached to this ring (Classes A-I). The number subscript indicates the number of double bonds in the side chains (SMITH, 1990). The Prostaglandins G and H are intermediate cyclic endoperoxide derivatives formed from arachidonic acid (Figure 1.1 A) occurring during prostaglandin synthesis (Figure 1.2), while the activated cells release prostaglandins D, E, F and I (Figure 1.1 B-E).

The fact that commercial anti-inflammatory drugs, NSAIDs (non-steroidal anti-inflammatory drugs), inhibit prostaglandin formation but not leukotriene formation implies that it is the prostaglandins that contribute to the symptoms of inflammation. This suggests that the contribution of leukotrienes to the mediation of inflammatory responses is of lesser importance, although the possibility of these drugs (NSAIDs) acting at the levels of phospholipase, thus inhibiting both prostaglandin and leukotriene production, cannot be disregarded (SMITH, 1990).

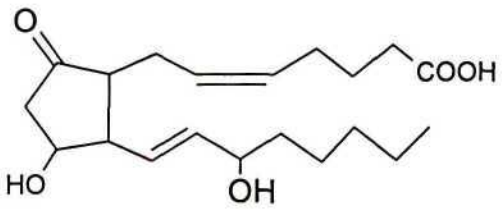
PGE_1, PGE_2 and PGI_2 inhibit both the output and volume of gastric acid. They stimulate the secretion of bicarbonate and mucin from the stomach and thus play a major role in the protection of the mucosa from the ulcerogenic effects of gastric acid (SMITH, 1990). In addition, these prostaglandins are potent dilators of almost all blood vessels. PGE_2 is synthesized in the kidney medulla and regulates blood flow to the inner and outer cortex.



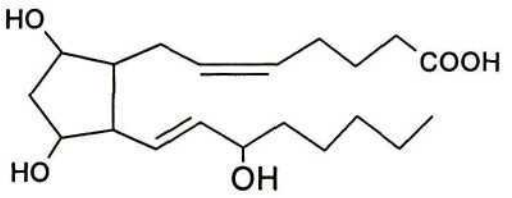
(A) Arachidonic acid



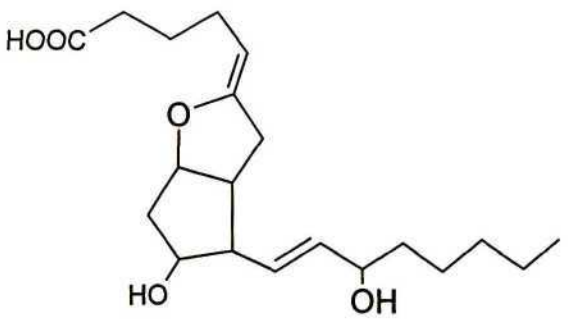
(B) PGD_2



(C) PGE_2



(D) $PGF_{2\alpha}$



(E) PGI_2 (Prostacyclin)

Figure 1.1: Chemical structure of (A) arachidonic acid; (B) PGD_2; (C) PGE_2; (D) $PGF_{2\alpha}$ and (E) PGI_2 (From SMITH, 1990; WAGNER AND JURCIC, 1991).

The E-prostaglandins differ from the F-prostaglandins in the presence of an oxygen at C_9, while the latter have an α-hydroxyl (Figure 1.1 C and D). Prostaglandin-E_2 is the predominant eicosanoid detected in inflammatory conditions (VANE AND BOTTING, 1995). The E-type prostaglandins are thus inflammatory, and cause pain, erythema (redness) and hyperalgesia (increased tenderness), with effects lasting up to ten hours. These compounds are produced locally in response to stimuli including heat, foreign particles and bacteria (SMITH, 1990). Prostaglandin-E_2 is a potent dilator of vascular smooth muscle which results in the vasodilation and erythema characterizing acute inflammation. This compound acts synergistically with other mediators in the production of inflammatory pain, and is a potent pyretic agent contributing to fever (VANE AND BOTTING, 1995). E-type prostaglandins specifically sensitize nerve endings and increase local blood flow resulting in enhanced pain and increased vascular permeability (SMITH, 1990).

Various other forms exist, differing in the double bonds or hydroxyls, and varying in biological activity. $PGF_2\alpha$ (Figure 1.1 D) is implicated as an agent which stimulates asthma with varying sensitivity and PGE_2 (Figure 1.1 C) usually causes a bronchodilator action to relieve asthma by stimulating the release in cyclic AMP concentrations (HINMAN, 1973). The bronchial smooth muscle of many species is sensitive to prostaglandins and can either relax or contract, depending on the type of prostaglandin (TOWNLEY AND ADOLPHSON, 1973). Other products of the COX-catalysed pathways, detected in inflammatory injuries, include $PGF_2\alpha$, PGD_2, prostacyclin (detected as 6-oxo-$PGF_1\alpha$) and TXA_2 (detected as TXB_2) (Figure 1.2). These compounds are usually present at less than a quarter of the concentration of PGE_2 (VANE AND BOTTING, 1995).

The primary enzyme responsible for prostaglandin synthesis is cyclooxygenase (WALLACE AND CHIN, 1997). This prostaglandin synthetase enzyme complex (cyclooxygenase) is ubiquitous, with the activity varying greatly with tissue type (WEEKS, 1973). This enzyme exhibits both cyclooxygenase and peroxidase activity (SMITH, 1990). The cyclooxygenase activity oxidizes arachidonic acid to endohydroperoxide PGG_2, and the peroxidase activity reduces hydroperoxide PGG_2 to the corresponding secondary alcohol PGH_2. These two sub-units are connected by a prosthetic heme (MANTRI AND WITIAK, 1994). The peroxidase activity prevents

the complete and irreversible inactivation of the cyclooxygenase enzyme by removing the hydroperoxide product from the active site. This is achieved by the reduction of the hydroperoxide functionality. The enzyme is thus able to provide sufficient endoperoxide intermediates for cellular prostaglandin, thromboxane and prostacyclin biosynthesis (MARKEY *et al.*, 1987).

Prostaglandins are not stored but are rapidly synthesized in response to various neural, chemical and mechanical stimuli including electrical nerve stimulation, exposure to venoms and chemical histamine releasers, tissue injury by ischemia, trauma, burn and osmotic shock and immediate type hypersensitivity reactions. In addition, prostaglandin synthesis is stimulated by substances such as growth factors and tumour promoters (TAKETO, 1998a). A common feature to this variety of stimuli may be an alteration in membrane function or activation of phospholipases (TAKETO, 1998a) resulting in the release of the fatty acid precursors (TAUBER *et al.*, 1973). The initiation of biosynthesis of prostaglandins and related eicosanoids is dependent on the release of these fatty acid precursors by enzymatic hydrolysis of the phospholipids. This is catalysed by phospholipase A_2 (Figure 1.2). The activity of this enzyme is enhanced by an increase in intracellular calcium ions which occurs during cellular stimulation (SMITH, 1990).

Various anti-oxidants can inhibit the fatty acid oxygenase of sheep vesicular gland, a component of the prostaglandin biosynthetic pathway. Certain dietary anti-oxidants (e.g. flavonoids) might provide an inhibition of prostaglandin production similar to that seen with non-steroidal anti-inflammatory drugs but more transient than the irreversible effect of aspirin since they inhibit the oxygenase by a reversible mechanism (VANDERHOEK AND LANDS, 1973).

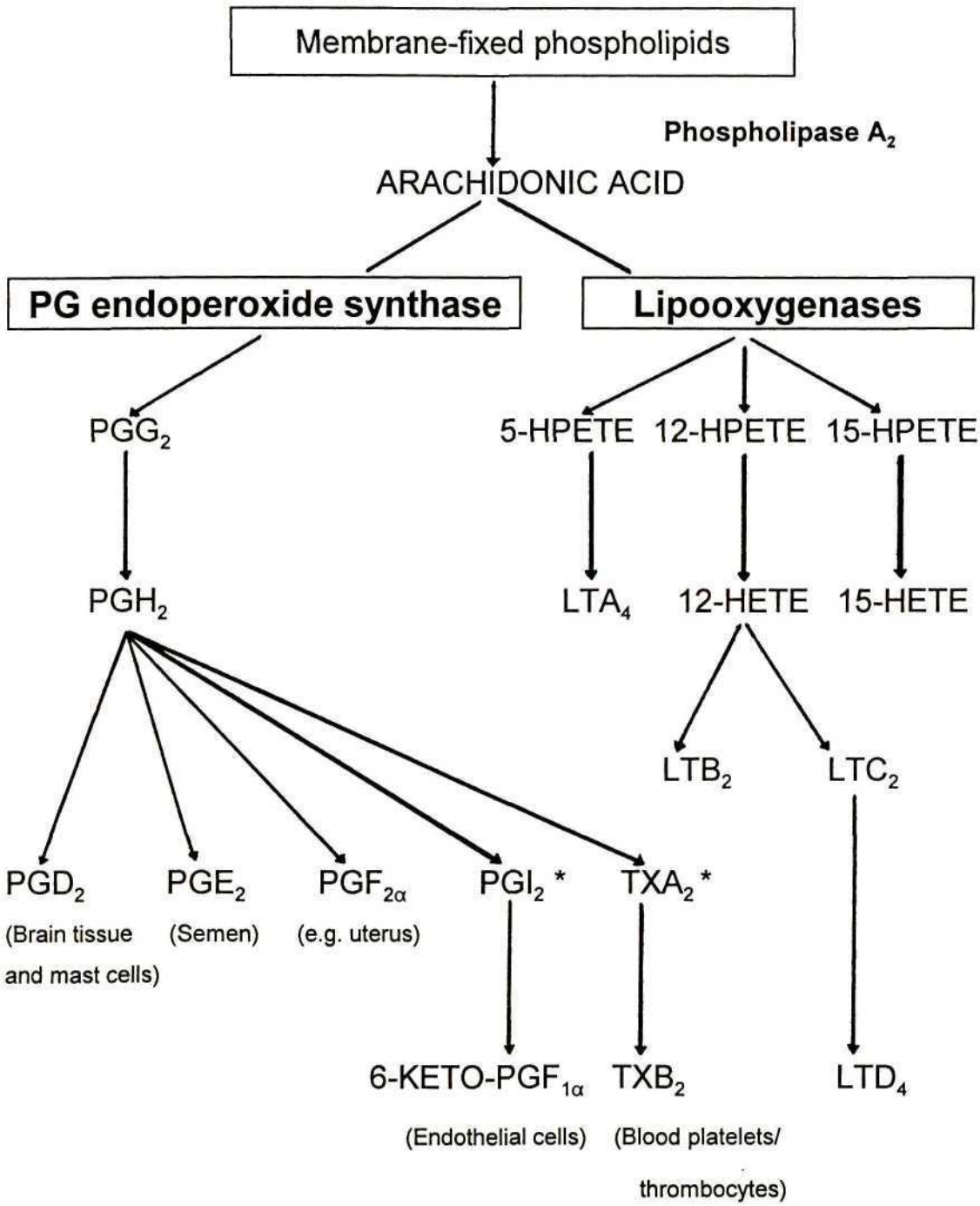


FIGURE 1.2: A schematic representation of the biosynthesis of prostaglandins and related eicosanoids. [KEY: PG = prostaglandin; TX = thromboxane; HPETE = hydroperoxyeicosatetraenoic acid; HETE = hydroxyeicosatetraenoic acid; LT = leukotriene]. * indicates unstable compounds that break down non-enzymatically. (Adapted from SMITH, 1990).

Cyclooxygenase enzymes: COX-1 and COX-2

The membrane-associated enzyme, cyclooxygenase (COX, prostaglandin-endoperoxide synthase, EC 1.14.99.1), occurs in two isoforms (VANE AND BOTTING, 1995), one located in the endoplasmic reticulum and the other in the nuclear envelope (GOETZL *et al.*, 1995). Both are of molecular weight ≈70 kDa, and have similar Km and Vmax values for their reaction with arachidonic acid (VANE AND BOTTING, 1995). Researchers have found clear pharmacological differences between the two enzymes (MITCHELL *et al.*, 1994). The existence of multiple forms of cyclooxygenase was first proposed in 1972, based on the selective inhibition of prostaglandin synthesis in different tissue types. This pharmacological data was substantiated by the molecular characterization of two distinct isoforms of cyclooxygenase (WALLACE AND CHIN, 1997).

This enzyme catalyses the first two steps (Figure 1.3) in the biosynthesis of prostanoids from arachidonic acid - the formation of prostaglandin G_2 (cyclooxygenase) and its subsequent reduction (peroxidase) to prostaglandin H_2 (QUELLET AND PERCIVAL, 1995). Both PGG_2 and PGH_2 are unstable intermediates (SMITH, 1990). Prostaglandin H_2 is then metabolized into different prostaglandins (PGs), prostacyclin and thromboxanes by other enzymes (MITCHELL *et al.*, 1994). The formation of the different end-products (PGD, PGE, PGF, PGI and thromboxanes) is dependant on the presence of the appropriate isomerase or synthase within the cell (SMITH, 1990). The release of prostacyclin by the endothelium is antithrombogenic, and by the gastric mucosa is cytoprotective (MITCHELL *et al.*, 1994).The prostanoids produced by COX-1 are believed to be involved in the maintenance of regular cellular physiology whereas those produced by COX-2 are involved in inflammatory processes (GOETZL *et al.*, 1995; QUELLET AND PERCIVAL, 1995).

The constitutively expressed COX-1 is present in cells under physiological conditions and when activated, induces the production of prostacyclin (MITCHELL *et al.*, 1994; FESSLER, 1996; BAUMGÄRTNER, 1997). The enzyme was first purified from sheep vesicular glands (MITCHELL *et al.*, 1994). COX-1 is found constitutively expressed in tissue such as the stomach, kidney and platelets. This enzyme is responsible for the production of prostanoids that maintain mucosal blood flow, promote mucous

secretion, inhibit neutrophil adherence, maintain renal blood flow and promote platelet aggregation (WALLACE AND CHIN, 1997). Suppression of COX-1 results in adverse side-effects including a reduction in mucosal blood flow and mucous secretion, delay in the healing of ulcers and a reduction in renal blood flow (WALLACE AND CHIN, 1997). Concentrations of this enzyme remain largely stable, with small increases (2-4 fold) occurring in response to hormones or growth factors (VANE AND BOTTING, 1995).

In contrast to COX-1, COX-2 is effectively absent in healthy tissue and is induced in migratory and other cells by proinflammatory agents, such as cytokines, mitogens and endotoxins presumably in pathalogical conditions such as inflammation (MITCHELL *et al.*, 1994). COX-2 is encoded by a different gene from the constitutive enzyme (COX-1), and the amino acid sequence of its cDNA shows a 60% homology with the sequence of the COX-1 enzyme (MANTRI AND WITIAK, 1994; VANE AND BOTTING, 1995). The COX-2 gene belongs to a family of primary response genes, induced during inflammation and cell growth (VANE AND BOTTING, 1995). Both enzymes have similar active sites, although the active site of the COX-2 enzyme is larger than that of COX-1 and can accept a wider range of structures as substrates. With the onset of inflammation or tissue damage, levels of COX-2 increase substantially (VANE AND BOTTING, 1995). Prostanoids produced by COX-2 promote oedema, fever and pain (WALLACE AND CHIN, 1997).

Selective COX-2 inhibitors have further been investigated with respect to their possible role in the treatment of human cancers. It is still necessary to determine, however, whether COX-2 is induced and plays a key role in various cancer and/or precancerous conditions. The establishment of such an association can lead to the use of these drugs in chemotherapy (TAKETO, 1998b).

The rate-limiting step in arachidonate metabolism is mediated by cyclooxygenase (TAKETO, 1998b). Nonsteroidal anti-inflammatory drugs (NSAIDs) are a large, chemically diverse group of drugs that act by inhibiting the activity of COX (VANE AND BOTTING, 1995). This explains both their effectiveness (in the inhibition of COX-2) and their side effects, leading to gastric and renal damage (in the inhibition of COX-1, which produces protective substances for the stomach and kidney)

(MITCHELL *et al.*, 1994).

Research has, for the past fifty years focussed on the development of non-steroidal anti-inflammatory drugs that show reduced toxicity in the gastrointestinal tract and kidney, manifest in gastrointestinal bleeding and perforation. This is complicated by the mechanistic link existing between the beneficial and adverse effects of NSAIDs, both of which relate to the ability of the drug to inhibit prostaglandin synthesis (WALLACE AND CHIN, 1997).

Two NSAIDs (nabumetone and etodolac) with moderate selectivity (5-10 times) towards COX-2 have been developed and are commercially available. These drugs appear to have reduced gastrointestinal toxicity in comparison to other NSAIDs (WALLACE AND CHIN, 1997). These drugs retain the ability to suppress COX-1, a factor which is likely to contribute to their anti-inflammatory and analgesic effects. NSAIDs block the cyclooxygenase activity of COX-1, but not the peroxidase activity (TAKETO, 1998a). Highly selective COX-2 inhibitors (more than 100-fold selectivity) caused less gastrointestinal damage than standard NSAID's in *in vivo* experiments. The effectiveness of these compounds in the reduction of pain and inflammation has not, however, been proven for doses that only affect COX-2 activity (WALLACE AND CHIN, 1997).

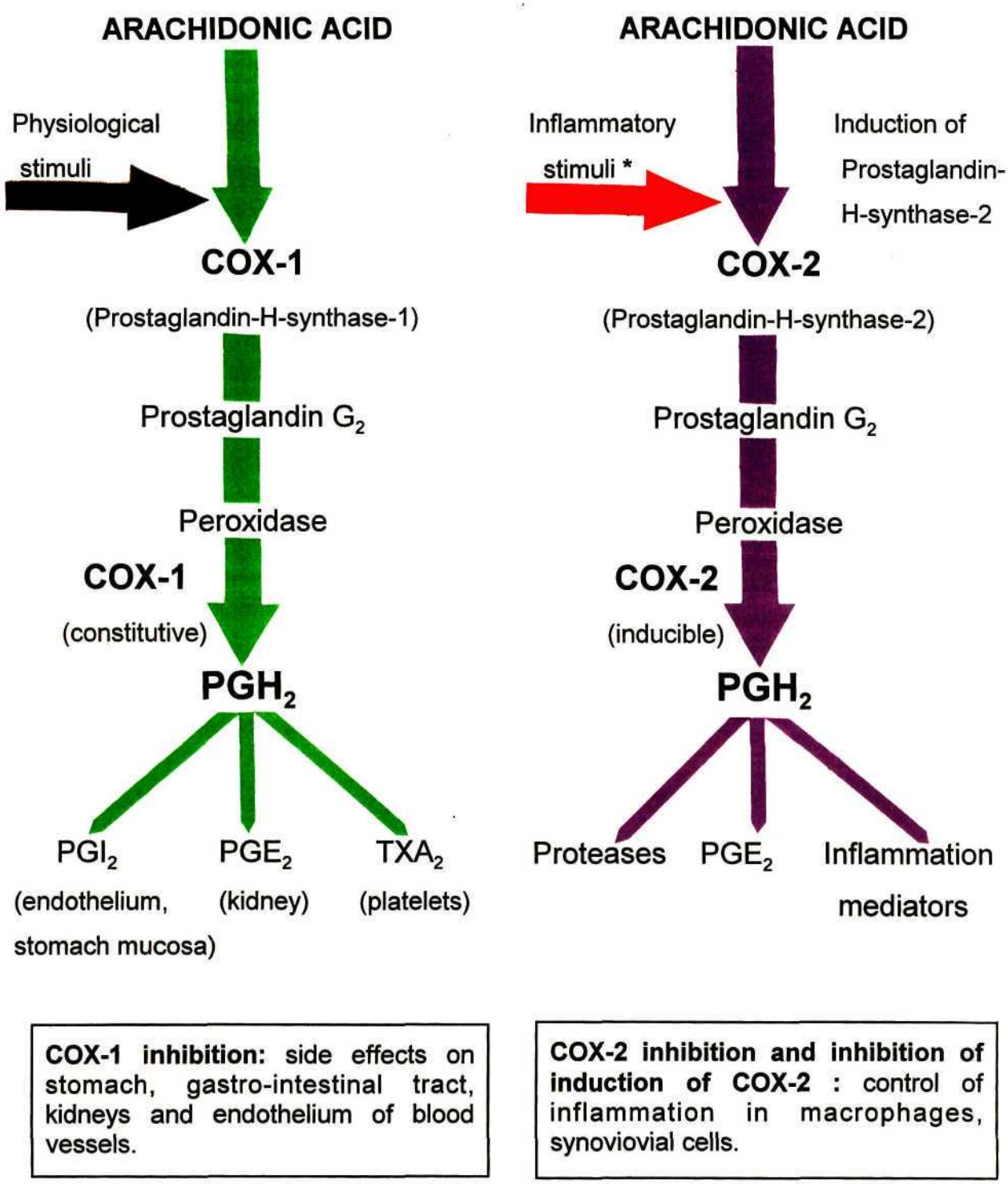


FIGURE 1.3: A diagrammatic representation of a comparison between cyclooxygenase-1 and cyclooxygenase-2 catalysed prostaglandin synthesis (* e.g. cytokines and growth factors).(Adapted from VANE AND BOTTING, 1995; FESSLER, 1996; BAUMGÄRTNER, 1997; TAKETO, 1998a).

Inhibition of cyclooxygenase

Aspirin-like drugs share a common mechanism in that they block prostaglandin synthesis which is related to anti-inflammatory activity (WILLOUGHBY *et al.*, 1973). This group of drugs is referred to as the non-steroidal anti-inflammatory drugs to distinguish them from the glucocorticoids, which represent the alternative group of agents used in the treatment of inflammation (VANE *et al.*, 1990). Despite the diverse chemical structures of drugs belonging to the NSAIDs, they all exhibit similar therapeutic properties. These include the alleviation of swelling, erythema and pain of inflammation, the reduction of a general fever, and the relief from headache pain (TAKETO, 1998a). All NSAIDs inhibit prostaglandin biosynthesis, with both enzymes (COX-1 and COX-2) being the major pharmacological targets of these drugs (TAKETO, 1998a). These NSAIDs tend to be large, hydrophobic molecules, analogous to the substrate arachidonic acid (MARKEY *et al.*, 1987). Compounds inactive against prostaglandin synthase (< 10 % inhibition at 100 μg mℓ^{-1}) include morphine, atropine and phenoxynenzamine (VANE *et al.*, 1990). The two strongest inhibitors of COX-1 in all assay systems were aspirin and indomethacin, the two NSAIDs that cause the most gastric damage. Although indomethacin is more potent than aspirin as an inhibitor of COX activity in a variety of test systems, and as an anti-inflammatory drug, it is also more potent than aspirin as an ulcerogenic agent. What is more important than the relative potencies of NSAID's against each other on COX, is the ratio of activities on COX-1 and COX-2. Thus, assuming COX-2 is relevant to inflammation, an anti-inflammatory dose of indomethacin or aspirin will suppress COX-1 activity in the stomach and kidney with a 50-fold greater potency than it will COX-2 activity (MITCHELL *et al.*, 1994).

The different NSAIDs currently on the market function by different mechanisms. Two classes of NSAIDs exist in terms of the kinetic mechanism by which they inhibit cyclooxygenase, although both appear to act competitively with the substrate arachidonic acid. The first type of NSAID acts in a time-independent manner, consistent with the formation of a reversible enzyme-inhibitor complex. The second exhibits time-dependent action, involving a two-step inhibitory mechanism. These NSAIDs are, in general, more potent, since the second, time-dependent step involves the essentially irreversible formation of a non-covalent enzyme-inhibitor complex (QUELLET AND PERCIVAL, 1995).

The active site on the COX enzyme is a long, hydrophobic channel (VANE AND BOTTING, 1995). The active site of COX-2 is thought to be slightly larger than that of COX-1. This is suggested by the broader fatty acid substrate specificity of COX-2 and the lower relative affinities of NSAIDs for COX-2 (TAKETO, 1998). The cyclooxygenase subunit of the enzyme serves as the binding site for both the arachidonic acid substrate and for competitive and non-competitive NSAIDs, while the peroxidase binding site accommodates secondary alkyl hydroperoxides (MANTRI AND WITIAK, 1994).

Aspirin irreversibly inhibits COX-1 by acetylation of a single serine residue on the enzyme (excluding access for arachidonic acid) (VANE *et al.*, 1990) but has no effect on peroxide activity (MANTRI AND WITIAK, 1994), while indomethacin acts by binding to the active site of the enzyme, and subsequently rendering it inactive, possibly by producing a conformational change in an essential protein radical (MITCHELL *et al.*, 1994). Ibuprofen inhibits the COX enzyme by substrate competition with arachidonic acid. This drug induces fewer side effects than the previous two due to its selective COX-2 inhibition, which has been demonstrated in intact cell tests (MITCHELL *et al.*, 1994). The mechanism of action of the inhibitor salicylate in intact cells is aided by its suppression of the induction of COX. Acetaminophen is a weak anti-inflammatory agent (less potent inhibitor of both COX-1 and COX-2) but has stronger analgesic and antipyretic action. A third isoform of COX (COX-3) in the endothelial cells of the brain is theorized to explain the effectiveness of this action (MITCHELL *et al.*, 1994). There is a group of NSAIDs with a COX-2/COX-1 ratio (IC_{50}) of one (equipotent) in intact cells. These inhibitors (e.g. diclofenac, naproxen) are less ulcerogenic than aspirin or indomethacin at anti-inflammatory doses, showing the advantages of strong COX-2 inhibition (MITCHELL *et al.*, 1994).

Published ratios of COX-2 to COX-1 inhibitory activity can vary according to whether the test was conducted using pure enzymes, cell homogenates, intact cells or with the type of cells used (VANE AND BOTTING, 1995). Some inhibitors have shown higher activity in intact cells than in purified enzyme preparations, suggesting that the latter may not always be indicative of therapeutic action (MITCHELL *et al.*, 1994). Penetration of the cell membrane is an important aspect of the activity of NSAIDs

(VANE AND BOTTING, 1995). The inhibition of purified enzymes as a screening method for anti-inflammatory activity is still a logical starting point, but results using intact cell preparations may correlate better with biological activity in animals and humans (MITCHELL *et al.*, 1994).

The inhibition of COX may occur at several different levels in the cascade of events leading to the induction of enzyme activity. Inflammatory mediators have been found to increase the expression of phospholipase A_2, leading to increased prostaglandin production through increased arachidonic acid release. Compounds such as the corticosteroids inhibit the activity of phospholipase A_2, and thus inhibit the formation of prostaglandins, thromboxane and leukotrienes (VANE AND BOTTING, 1995).The action of proflammatory mitogens can be blocked with receptor antagonists or antibodies (MITCHELL *et al.*, 1994). Alternatively, once the cell is activated, the synthesis of COX-2 can be blocked by agents such as glucocorticosteroids or salicylates (MITCHELL *et al.*, 1994). Lastly, once COX-2 has been synthesized, selective COX-2 inhibitors can inhibit the production of pro-inflammatory prostanoids without affecting prostacyclin production by the endothelium. The identification of selective inhibitors of COX-1 and COX-2 is important firstly in order to investigate these steps, and may also lead to advances in the treatment of inflammation. As new inhibitors of COX-2 are discovered, the use of aspirin will decrease in the treatment of inflammation, but extend as an inhibitor of COX-1 in platelets for the prevention of thrombosis (MITCHELL *et al.*, 1994).

Most commercial NSAIDs show a much higher inhibition of COX-1 than COX-2. Piroxicam is 250 times more active on COX-1 than on COX-2, while aspirin is 166 times more active and indomethacin is 60 times more active against COX-1. Meloxicam and a drug classified as BF389 are potent new NSAIDs with COX-2 to COX-1 activity ratios of 0.8 and 0.2 respectively. These drugs show minimal damage to the gastrointestinal tract (VANE AND BOTTING, 1995). The pharmaceutical industry is currently searching for selective inhibitors of COX-2, which will in theory have fewer side effects than drugs in current usage. New NSAIDs are being developed which have +1000 fold selectivity for COX-2 over COX-1 (VANE AND BOTTING, 1995).

Prostaglandins are, however, not the only mediators involved in chronic inflammation, and even with the development of highly selective COX-2 inhibitors, these drugs may not be as potent as the steroids in the relief of inflammation, especially that associated with arthritis (VANE AND BOTTING, 1995).

Assays for the detection of cyclooxygenase inhibition

The development of screening methods of plant extracts is orientated towards the major and fundamental pathophysiological processes involved in inflammatory and allergic reactions. Four major pathophysiological areas are recognized as possible sites for drug intervention. These include arachidonic acid metabolism; phagocytic and other cell functions involved in inflammatory processes; complement factors and autoimmune processes (WAGNER AND JURCIC, 1991). Initial assay methods to confirm pharmacological activity should be fairly broad in order to take into account several modes of action. This is true of the anti-inflammatory response where the mechanisms of action are diverse, and may include mechanisms of action not fully elucidated. This research has the potential to discover new types of compounds with novel mechanisms of action that extend current therapy methods (CAVÉ, 1986).

The cyclooxygenase pathway in arachidonic acid metabolism leads to the formation of the stable prostaglandins PGE_2, PGD_2, $PGF_{2\alpha}$, prostaglandin PGI_2 and thromboxane A_2. Vasodilation, increased blood vessel permeability, allergic reactions, chemotaxis of granulocytes, cartilage- and joint destruction, and increased or suppressed proliferation of B- and T-lymphocytes are totally or partially caused by these pro-inflammatory mediators (WAGNER AND JURCIC, 1991).

Prostaglandin synthesis-inhibition can be evaluated using an *in vitro* assay originally described by WHITE AND GLASSMAN (1974). This assay has been used extensively in the evaluation of medicinal plant extracts and plant-derived compounds. This coupled to the bioassay-directed fractionation of plant extracts has led to the characterization of several new inhibitors of COX-1 catalysed prostaglandin synthesis (NOREEN *et al.*, 1998).

Sources of the cyclooxygenase enzyme

Cyclooxygenase (as whole cell systems or homogenates) can be obtained from sheep seminal vesicle microsomes (SSVM), porcine seminal vesicle microsomes (PSVM), human thrombocytes (platelets) (HP), polymorphonuclear leucocytes (PMNL) and rabbit or rat kidney medulla (RRM, RtRM) (WAGNER AND JURCIC, 1991). Of these, the SSVM and HP are the preparations used most frequently. The SSVM preparation metabolizes arachidonic acid to PGE_2, PGD_2, $PGF_{2\alpha}$, and 6-oxo-$PGF_{1\alpha}$, and to a smaller extent to HHT (12 hydroxy-5-*cis*, 8-*trans*, 10-*trans*-heptadecatrienoic acid), 11-HETE and 13-HETE (hydroxy eicosatetraenoic acid). The HP are a whole cell system which provides information on the properties of an inhibitor under physiological cell conditions. Tests with this cell system indicate simultaneously to what extent an inhibitor possesses membrane permeability. The metabolites produced are predominantly 12 HETE, HHT and TXB_2 (WAGNER AND JURCIC, 1991).

Cyclooxygenase assay technique

The cyclooxygenase bioassay (COX-1) used in this thesis was developed by WHITE AND GLASSMAN (1974) and modified by JÄGER *et al.* (1996). The bioassay can be used to measure the effectiveness of extracts from plant material at inhibiting the conversion of the arachidonic acid precursor to prostaglandins. The assay entails a simple method for the incubation of the radiolabelled substrate (^{14}C-arachidonic acid) with the prostaglandin synthetase and the potential inhibitor. This mixture is then applied to small disposable silica gel columns to separate the unreacted ^{14}C-arachidonic acid from the prostaglandins synthesized in the test. The ^{14}C-prostaglandins can then be quantified using a scintillation counter, and the degree of inhibition of the enzyme calculated.

E. autumnalis autumnalis extracts have been tested previously for anti-inflammatory activity in this bioassay. Aqueous and ethanolic bulb extracts were shown to exhibit 73% and 90% inhibition respectively. This was high compared to the 66% inhibition shown by the standard used (commercial indomethacin 5μM) (JÄGER *et al.*,1996).

Systematic screenings performed in various labs have shown that promising non-steroidal inhibitors of cyclooxygenase can be found in similar classes of compounds

of plant origin, the most potent inhibitors being found in the classes of flavonoids, phenolic phenylpropanoids, diarylheptanoids, phenylalkan-on-ols, depsides, naphthoquinones, tannins, simple phenols, alkylamides and thiosulphinates (WAGNER AND JURCIC, 1991).

1.10 PROBLEMS ASSOCIATED WITH SCREENING PROCEDURES

The most common problem encountered in the detection of pharmacological activity is that even extracts from single plants are a mixture of several compounds which can be subject to variation in concentration or composition according to ecological changes (FARNSWORTH, 1993). Traditional remedies are seldom comprised of a single plant extract, and in many cases the therapeutic benefits are attributed to the consumption of plant mixtures in which whole plants or plant parts are prepared and/or consumed in combination or in sequence (ETKEN, 1986). This complicates the pharmacological investigation of such preparations, since determining which of the many constituents of a single plant is active requires much work, and this is compounded by the presence of constituents from several plants (ETKEN, 1986). This is further complicated by the possibility that a wide range of structurally diverse compounds contribute to the overall pharmacological activity of a plant extract, or that synergistic effects exist between the active principles. This is found particularly where medicinal plants show less specific activity (HAMBURGER AND HOSTETTMANN, 1991). Some plants may be added to reduce the toxicity of the more therapeutically effective plants. The effects of drug interactions in medicinal preparations is largely unknown since a therapeutic profile on each constituent would be required. Some pharmacological studies have been initiated on multi-drug preparations, revealing significant interactions (FARNSWORTH, 1993).

Even where a purified extract or synthetic analogue is identified, there is the possibility that some of the apparently pharmacologically-inert constituents could have important effects with respect to the administration of the drug. The administration of Digitalis in western medicine requires temporary interruptions to avoid dangerous accumulations of this drug. In contrast, such precautions are unnecessary in the ethnomedical use of the whole *Digitalis* plant, suggesting an interaction of the active

principle with other constituents in the plant (ETKEN, 1986). Most indigenous pharmacopoeias utilize species in composite preparations. This could be as mechanical and/or symbolic adjuvants, vehicles or facilitators of other constituents to enhance the bioavailability of active principles in other plant medicines (ETKEN, 1986).

Existing assays can not necessarily reliably predict clinical efficacy, and suitable pharmacological models have not yet been developed for many common diseases with unknown, or multifactorial origins (HAMBURGER AND HOSTETTMANN, 1991).

The lack of a positive result in a screening assay does not always mean the absence of bioactive constituents. The active principle(s) may be present in insufficient quantities in the crude extracts to show activity in the dose levels employed. Lack of activity can thus only be proven by using large doses. Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the active principles during the assay (FARNSWORTH, 1993). Fractionation of the extracts before screening by partitioning or serial extraction, can in some cases overcome this problem, although this multiplies the number of samples to be tested. *In vitro* screening methods have a further inherent problem in that some compounds showing good activity in an *in vitro* assay may be metabolized *in vivo* into inactive metabolites. Conversely, some extracts show only *in vivo* activity due to the metabolism of inactive compounds into active forms (FARNSWORTH, 1993).

Once an extract has shown promising activity in the screening tests, fractionation and purification procedures are conducted in order to isolate the active principle. Firstly, the procedures employed must be effective and appropriate to isolate and separate the active compounds. Even using bioassay-guided fractionation, there is a real possibility of loss of activity. This can be caused by the compound in question being labile (sensitive to temperature, light, acidity, basicity or the extracting solvent) and consequently is progressively degraded during fractionation (FARNSWORTH, 1993). Alternatively, some compounds are inactive *in situ* but act synergistically with other constituents of the extract. Separation into different fractions during purification will thus result in a decrease or total loss in activity in all the fractions.

The activity of some compounds is dose-dependent, causing different or antagonistic activity with the increase in concentration brought about by the fractionation procedures. This can be overcome by adjusting the dose levels to be assayed at progressive stages (FARNSWORTH, 1993). In addition, minor compounds in the crude extracts may become concentrated in later fractions and exhibit unexpected biological activity.

The duplication of pharmacological results is important. Failure to do so could result from a variation in the concentration of active compounds due to environmental or genetic factors (FARNSWORTH, 1993). These include the season, area of collection, physiological age of the plant, and physiological state (e.g. flowering). Genetic variation (genotype) usually causes qualitative differences in different samples, while the environmental factors usually affect quantitative results. Another cause is the failure to collect the same specimen on separate occasions, which emphasizes the importance of voucher specimens. Initial thin layer chromatogram (TLC) profiles or fingerprints of active extracts as well as the subsequent active fractions, should be performed and filed before the active principle(s) are isolated and identified to enable comparison between samples (FARNSWORTH, 1993).

In many cases, however, the tests used to screen herbal remedies are inadequate, largely due to the complex nature of these preparations. Pharmacological tests need to be tailored to fully evaluate herbal remedies (FARNSWORTH, 1993).

1.11 DRUG DEVELOPMENT

Modern chemical methods have led to a dramatic increase in the number of natural or synthetic molecules available for pharmacological research. There is, however, a large gap between the detection of a specific activity and the development of a drug, or even a biochemical tool of interest for research purposes (RASOLONJANAHARY *et al.,* 1986).

The aims of research in the field of drug development are the identification of the active principles in medicinal plants, and investigation of the extracts to ensure that

they are safe, effective and of constant activity; the isolation of the active principles, and determination of their structure in order that they may be synthesized, structurally modified or simply extracted more efficiently (CAVÉ, 1986).

A defined active principle can be obtained by total synthesis (which may be very difficult due to the complexity of some natural compounds); by partial synthesis (using precursors from the plant that are easy to extract in sufficient quantities); by extraction (a common process which may be improved by selection of the most productive plants); by improvement of the original culture technique or methods of harvesting; by genetic manipulation; by tissue culture to reproduce a particularly productive clone of a plantlet; and finally by fermenter culture of plant cells (not always profitable) which is being used to investigate intermediate metabolism as a first step to plant genetic engineering (COUZINIER AND MAMATAS, 1986).

Any system producing plant-derived natural products must meet certain criteria. It must be economic, sustainable, reliable with minimal impact on the environment (McCHESNEY, 1993). This entails the discovery and development of a superior source of the natural product with a consistently high yield of the active principle, an effective production and harvesting system, the appropriate technology for the processing and storage of plant biomass, and an economic and efficient extraction and purification system which minimizes waste-product generation (McCHESNEY, 1993). The lack of a stable and reliable supply of plant-derived drugs at a predictable cost will significantly restrict the potential clinical utilization of the active constituent(s).

Under these conditions, wild populations of medicinal plants may not be a reliable source of a drug, and their harvesting may be counter-productive to the development of a reliable, cost-effective and long-term production system of a clinically utilized drug (McCHESNEY, 1993). Wild populations are naturally under threat from forest fires, annual climatic variations, natural phenotypic variation and presence of chemotypes in wild populations. In addition, there is increasing pressure to protect and regulate the harvesting of wild plants. Other considerations include the high cost of collection of scattered plants, and transportation to processing facilities, and the lack of assured accessibility to populations which occur on public and privately held

lands (McCHESNEY, 1993). Reliance on the harvesting of wild plants for production may thus lead to uncontrollable interruptions of drug supply.

Wild harvesting also risks the destruction of germplasm reserves essential for the future cultivation of the plant for drug production. These reserves include genes for disease and pest resistance, hardiness, and tolerance to full sunlight, drought and flooding as well as genes for high growth rates and high levels of drug production. The preservation of wild gene pools can be critical to the development of long-term, cost-effective supplies, whether produced by cultivated plants, tissue culture or genetically modified micro-organisms (McCHESNEY, 1993).

An alternative may be the utilization of cultivated plant sources. This may be accomplished either by the cultivation of the currently recognized source of the drug, or by evaluating and selecting currently cultivated varieties for drug or drug precursor content (McCHESNEY, 1993). The former method encompasses the problems and uncertainties common to the introduction of any new plant into cultivation. Only about 3 000 species of the estimated 500 000 plant species known to occur in the world have successfully been cultivated (McCHESNEY, 1993). With the cultivation of medicinal plants there is the added necessity of establishing reliable drug production, a process that may take several years (McCHESNEY, 1993). An advantage of the second method is the known genetic origin and uniformity of the cultivated plant variety, which enables greater flexibility in terms of response to demand. Cultivation provides high plant densities in defined locations which will significantly reduce collection and transportation costs. This system can be placed into production for drugs faster and more reliably than any other production system (McCHESNEY, 1993).

In terms of estimating the production of drugs from cultivated plants, McCHESNEY (1993) gives the following example. To produce 20 kg of a drug at an isolated yield of 0.03% w/w of the weight of the biomass, it would be necessary to collect and process approximately 70 000 kg of biomass. Assuming 10 kg of dry biomass per plant is produced, the biomass from 7 000 000 plants would have to be harvested. With 4 500 plants grown per acre, less than 1 600 acres would provide all the biomass required to meet the clinical need. This scale of production is easily accomplished in a

relatively short period of time, and can potentially exceed one hundred times this amount (McCHESNEY, 1993).

A potential drug will always require *in vivo* testing to provide more in-depth information. This involves considerable time, skill, effort and expense and exposes the animal to risk. This is, however, a crucial stage that cannot be entirely replaced by computerized modelling. *In vivo* testing (intact animals), which precedes human clinical trials, is ultimately used to determine the potency, selectivity, oral activity and toxicity of a drug under laboratory conditions (BURGER, 1982).

Other areas requiring study include pharmacokinetics and biotransformation. Pharmacokinetics involves the study of the differences in activity in the body caused by absorbtion, distribution, blood levels, metabolism and excretion. Oral administration is mandatory for most therapeutic preparations, especially for prolonged drug use. The active compounds are therefore exposed to salivary, gastric and intestinal digestive enzymes, as well as to gastric acid, before absorbtion can occur across the stomach or intestinal wall (usually into the portal circulation). The drug is transported to and metabolized by the liver or kidneys, resulting in only a small percentage of the originally administered dose remaining available for action at its receptors (BURGER, 1982).

Biotransformation is the study of the metabolism of drugs by organs such as the liver and kidneys and the resultant metabolites, and is conducted in conjunction with screening and drug development. Few compounds are excreted unchanged. Mostly metabolism is associated with a reduction in activity but a minority of drugs are bioactivated (i.e. the metabolites are more active, or more toxic than the parent drug). The primary metabolic pathways of a foreign compound in the body are oxidation, reduction, methylation, dealkylation and some other more minor reactions. Secondary metabolism (conjugation) solubilizes and detoxifies compounds as a prerequisite to excretion. Hydroxylated compounds are conjugated as esters of sulfuric acid, amines, or acids as amides of metabolic amino acids, as glucuronides etc. (BURGER, 1982).

1.12 AIMS AND OBJECTIVES

The aim of this study was firstly to extend the screening of the *Eucomis* species for prostaglandin-synthesis (COX-1) inhibitors. The results obtained by JÄGER *et al.* (1996) showed high levels of COX-1 inhibitory activity in the bulbs of *E. autumnalis*. This investigation aimed to conduct a comparison of the COX-1 inhibitory activity in extracts obtained from different *Eucomis* species, not all of which have reported usage in traditional medicine. The COX-1 inhibitory activity of the different plant parts was also investigated in order to determine whether a renewable plant part (e.g. the leaves) could be substituted for the bulbs and roots, in terms of the sustainable utilization of the plant.

Further tests focussed on determining whether these levels of pharmacologically active compounds changed with season, or with the physiological age of the plant. This data can be used to ascertain the pharmacological status of the plant at harvest, and can thus indicate the optimal harvest period.

This initial screening test was followed by the development of a bioassay-guided isolation and purification procedure using chromatography techniques, with sequential testing of fractions with the COX-1 assay, to identify the fractions containing the active compounds. Finally, the compounds in sufficiently pure samples were characterized and identified using HPLC, NMR and MS analytical techniques.

A thin layer chromatography (TLC) solvent system was developed to give a clear chemical profile of the plant extracts. These TLC fingerprints were compared for the extracts prepared from different species, and for the extracts prepared from summer and winter harvests.

The bulb extracts were also tested for COX-2 activity. This study was extended to investigate the COX-2 inhibitory activity of leaf, bulb and root extracts of *E. autumnalis autumnalis*. Further COX-2 tests were conducted on the purified active fractions from the COX-1 bioassay-guided fractionation.

A set of experiments was designed to investigate the effect of selected environmental factors on the accumulation of the active principle(s) in *E. autumnalis autumnalis*. Specifically, the effect of fertilization, light intensity and cold storage of bulbs (during winter) was determined.

A tissue culture protocol was established for the rapid and economical bulk propagation of the species under study. The COX assays were further utilized to screen for the active compounds in plants grown *in vitro*. Further experimentation involving factors affecting the accumulation of the active compounds in *in vitro* plantlets was conducted.

The success of this study led to attempts to establish callus cultures from the cultured material. This callus was also screened for active compounds, but the limited success in terms of the proliferation of the callus restricted further experimental work.

The last study was included to establish DNA fingerprints for the various species. A conclusive identification system is necessary, in part due to the similar vegetative appearance of many of the species, and due to the high frequency of hybridization shown by the genus.

CHAPTER 2

EXTRACTION AND SCREENING

2.1 INTRODUCTION

The sciences of ethnobotany and ethnomedicine are developing rapidly due to the recent improvements in screening techniques, including *in vitro* bioassays. In the past, analyses of plants have not successfully isolated the active components because the assays lacked sufficient sensitivity. Current tests could prove successful where negative results were obtained 20 years ago (PRANCE, 1994). With the development of more selective *in vitro* assays, the quantities of plant material that need to be collected and stored has dropped from 5 - 10 kg to 50 - 500 g. This obviously has important ecological implications. In addition, conservation trends have been extended to the collection of different plant parts, with greater emphasis placed on sustainable harvest - bark is not collected if this would endanger the survival of the tree, and limited amounts of root systems are harvested (CORDELL, 1995).

Screening assays for biologically active compounds

Practically, the screening of large numbers of plant extracts from a wide range of geographical locations makes the use of dried plant material a standard procedure, even though some constituents may be lost or degraded in the drying process (O'NEILL AND LEWIS, 1993). Plants are usually crudely chopped to facilitate and speed drying. The majority of high through-put screening programmes allow any plant part, or combination of plant parts to be tested, providing sufficient quantity of material is available (O'NEILL AND LEWIS, 1993).

Many plants concentrate certain secondary metabolites in specific organs, and in terms of anticipated chemical diversity several samples from the same phenotype are often screened. It is important also to consider seasonal variation, and a record of the harvest date enables any seasonal variation to be catered for. This can be important

important should re-collection become necessary (O'NEILL AND LEWIS, 1993).

Selective extraction procedures are only employed where specific types of chemicals are required. High through-put screening, however, requires that the chances of detecting any type of biologically active molecule present in the plant extract be maximized, preferably while restricting the presence of non-selective interfering agents. This is achieved by screening a variety of solvent extracts from each plant sample, or one extract from an increased number of plant samples. The route chosen should be based on the potential sample supply, capacity, range and selectivity of the screens, as well as the potential for automation (O'NEILL AND LEWIS, 1993).

Advances in the fields of cell biology and molecular pharmacology have increased the importance of mechanism-based bioassays. The advantages of these assays lie in their selectivity and sensitivity, as well as good reproducibility and high sample throughput. Their major disadvantages lie firstly in their inability to detect active compounds with unknown mechanisms of action, and secondly in the occurrence of non-specific interactions, including enzyme inhibition by tannins, which lead to false positives.

False positives in screening assays

In the case of higher plant extracts, the majority of false positives can be attributed to the presence of polyphenols, detergents such as saponins, certain pigments or fatty acids (O'NEILL AND LEWIS, 1993). Phenols affect highly purified enzyme-based targets, while saponins primarily disrupt membranes in cellular targets or dislodge substrates adsorbed onto assay wells. Pigments tend to interfere with read-outs in colorimetric or quenched assays. Fatty acids show activity through a variety of mechanisms (O'NEILL AND LEWIS, 1993). The removal of these undesirable compounds from the plant extract is preferable before primary screening, but it is usually easier to run the crude extracts through the primary screening assay, and to introduce measures to discriminate between false and true positive results at a later stage in the process (O'NEILL AND LEWIS, 1993).

The cyclooxygenase assay

The cyclooxygenase assay is an example of a mechanism-based assay that uses subcellular structures (i.e. enzymes) to detect inhibitors of inflammation (HAMBURGER AND HOSTETTMANN, 1991). This bioassay tests for the presence of non-steroidal anti-inflammatory compounds by measuring the degree of inhibition of the cyclooxygenase enzyme, which is active in prostaglandin synthesis. The constituents of a plant extract may have an inhibitory effect in the assay by denaturing (or otherwise destroying) the enzyme, or by acting on the prosthetic group thus inactivating the enzyme. Since tannins and phenols have such effects in *in vitro* tests, these have to be negated as the cause of the inhibition (TUNÓN *et al.*, 1995).

The cyclooxygenase enzyme exists in two isoforms, COX-1 which is constitutive and COX-2 which is transcriptionally induced by inflammatory stimuli (VANE AND BOTTING, 1996). Nearly all of the non-steroidal anti-inflammatory drugs (NSAIDs) presently on the market, such as aspirin, ibuprofen and indomethacin, are COX-1 inhibitors. COX-1 inhibition is at present thought to be associated with the side-effects of NSAIDs, whereas the specific inhibition of COX-2 seems to be without side-effects. COX-2 selective inhibitors, defined by a COX-2/COX-1 inhibition ratio of less than zero, are thus the preferential type of inhibitor to be discovered.

Very few compounds isolated from plants (or other natural sources) have exhibited selective COX-2 inhibitory activity. Among those compounds showing such activity are manoalide and scalaradial (sesterterpenes of marine origin), akendo 3 (diterpene derivative) and resveratrol (a *trans*-stilbene) (NOREEN *et al.*, 1998). The use of both the COX-1 assay, originally developed by WHITE AND GLASSMAN (1974) and the COX-2 assay (NOREEN *et al.*, 1998) in conjunction to test plant extracts is thus an important research area.

Based on the reported usage of *Eucomis* extracts in African traditional medicine, extracts of this plant were tested for potential anti-inflammatory activity using COX-1 and COX-2 enzyme assays. Levels of COX-1 inhibitory activity were determined for leaf, bulb and root extracts (all of which have reported ethnopharmacological usage) from adult *Eucomis* specimens. This study compared levels of activity across species and in summer and winter. A further study was included to contrast anti-inflammatory

activity across age groups as well as throughout the year. The latter investigation was restricted to one species, *E. autumnalis autumnalis*. This species was chosen specifically due to its extensive use by traditional healers. COX-2 inhibitory activity was investigated for the bulb extracts from adult specimens, as well as for the leaf, bulb and root extracts from *E. autumnalis autumnalis*.

2.2 MATERIALS AND METHODS

2.2.1 COLLECTION

Plant material was collected from a total of eleven species / subspecies of *Eucomis*. These were housed in shadehouses in the University of Natal, Pietermaritzburg Botany Department Gardens. Herbarium voucher specimens were prepared and submitted to the University of Natal (NU) Herbarium. The species under study included the three subspecies of *E. autumnalis* (*E. autumnalis* subsp *autumnalis* Taylor01; *E. autumnalis* subsp *amaryllidifolia* Taylor02; *E. autumnalis* subsp *clavata* Taylor03); *E. bicolor* Taylor04; *E. comosa-comosa* Taylor05; *E. humilis* Taylor06; *E. pole-evansii* Taylor07; *E. comosa-punctata* Taylor08; *E. comosa-punctata* var *striata* Taylor09; *E. zambesiaca* Taylor10) and a garden hybrid species (*Eucomis* Taylor11).

It is important to use material free of viral, bacterial or fungal infection since products synthesized by the microorganisms may be detected in the extract. Diseased plants may also have altered metabolism which could result in the accumulation of large amounts of unexpected products (HARBORNE, 1998). Plant specimens were checked regularly for infestation, and only healthy plants were harvested.

Specimens were harvested in summer (January) and winter (July) to detect seasonal variation.

2.2.2 EXTRACTION

Harvested plant material was divided into leaves, bulbs and roots. This material was cut up, placed in brown paper bags, and dried at 50°C for three days. These were stored at room temperature in the dark until extracted. The dried material was finely ground, and extracted in a sonication bath using ethanol or water as the extracting solvent. Grinding facilitates the penetration of the solvent to the cellular structures of the plant tissues, aiding the extraction of secondary metabolites and increasing the yields of extraction. Generally, the smaller the particle size of the plant material, the more efficient the extraction (SILVA *et al.*, 1998). The extracts were filtered through Whatman No. 1 filter paper and dried under vacuum at 35°C. The residue was resuspended in ethanol (10 mg $m\ell^{-1}$) or in water (2.5 mg $m\ell^{-1}$).

2.2.3 ENZYME PREPARATION:

***COX-1*:**

The enzyme was prepared on ice (4°C). Sheep seminal vesicles were homogenized in potassium phosphate buffer with 1mM EDTA. This homogenate was centrifuged and the cell debris discarded. The microsomes were isolated by centrifugation of the supernatant at 100 000 g for 1h. The microsomal pellet was resuspended in 0.1 M K-Pi (pH 7.4) and the enzyme concentration determined by a protein assay (Appendix I). Aliquots (10$\mu\ell$) were stored at -70°C.

Standardization:

The enzyme preparation was tested at various concentrations (Appendix I) using solvent blanks. A dilution giving a dpm count ±⅓ of the total of the ^{14}C-arachidonic acid stock was chosen (ie 6 000 - 8 000 dpm). This concentration was then tested for a range of incubation periods (Appendix I). Standard curves were prepared for indomethacin using 3 separate tests (Appendix I). A stock solution of 5μM indomethacin was used as a positive control in each subsequent assay.

COX-2:

Purified COX-2 enzyme, isolated from sheep placental cotyledons was purchased from Cayman Chemicals. Aliquots (10 $\mu\ell$) containing 3 units were prepared. A stock solution of 200 μM indomethacin was used as a positive control in each assay.

2.2.4 ASSAY: COX-1

The assay was performed as described by WHITE AND GLASSMAN (1974), with slight modifications by JÄGER *et al.* (1996). Ten microlitres of the standardized enzyme preparation, and 50 $\mu\ell$ of co-factor solution (per sample) were preincubated for 15 min on ice. This solution (60 $\mu\ell$) was added to the test solution (plant extract at 2.5 $\mu\ell$ of ethanol extract + 17.5 $\mu\ell$ water; or 20 $\mu\ell$ of water extract) and preincubated for 5 min at room temperature. ^{14}C-Arachidonic acid (20 $\mu\ell$) was added to this enzyme-extract mixture and incubated for exactly 8 min in a water bath at 37°C. The reaction was terminated with 10$\mu\ell$ 2 N HCℓ.

2.2.5 ASSAY: COX-2

The COX-2 assay follows the same protocol as the COX-1 assay, thus allowing a comparison of the activities of the extracts on the two different enzymes. The protocol described by NOREEN *et al.* (1998) was followed, with minor modifications. The enzyme (3 units) was activated with 50 $\mu\ell$ co-factor solution on ice (5 min). The enzyme solution (60 $\mu\ell$) and sample (2.5 $\mu\ell$ ethanolic extract + 17.5 $\mu\ell$ water) were preincubated for 5 min at room temperature. The reaction was initiated with the addition of 20 $\mu\ell$ ^{14}C-arachidonic acid and the solutions were incubated for exactly 10 min in a water bath at 37°C. The reaction was terminated with 10$\mu\ell$ 2 N HCℓ.

2.2.6 CONTROLS

A sample resuspended at 10 mg mℓ^{-1} solvent is tested in the assay at a concentration of 250 μg mℓ^{-1} test solution. In each test four controls were run (2.5 $\mu\ell$ ethanol + 17.5 $\mu\ell$ water). Two were backgrounds in which the enzyme was inactivated with HCℓ before the addition of ^{14}C-arachidonic acid and which were kept on ice, and two were solvent blanks. Indomethacin was tested as a positive control.

2.2.7 SOLUTIONS

Co-factor solution (COX-1): 0.003g L-adrenalin/*l*-epinephrine and 0.003g reduced glutathione in 10mℓ 0.1 M Tris buffer, pH 8.2.

Co-factor solution (COX-2): 0.006g L-adrenalin/*l*-epinephrine (0.9 mM), 0.003g reduced glutathione (0.49 mM) and 1 μM hematin in 10mℓ 0.1 M Tris buffer, pH 8.0.

^{14}C-arachidonic acid: 16 Ci/mole, 3 mM.

Prostaglandin carrier solution: 0.2 mg mℓ$^{-1}$ of unlabeled prostaglandins (PGE_2:PGF_2 in the ratio 1:1).

Eluent 1: hexane:1,4-dioxan:acetic acid (350:150:1 v/v/v).

Eluent 2: ethyl acetate:methanol (85:15 v/v).

Standards

Indomethacin	5 μM for COX-1 and 200 μM for COX-2
Hematin (COX-2)	1.26 mg + 96.9 mg TRIS in 10 mℓ water (1 μM in assay)
Nimesulide (COX-2)	24.46 mg + 10 mℓ ethanol (20 $\mu\ell$ batches) (200 μM)

2.2.8 SILICA COLUMNS

Unlabelled prostaglandin carrier solution (4 $\mu\ell$ per sample) was added to the reaction mixture, and ^{14}C-prostaglandins synthesized in the assay were separated from unmetabolized arachidonic acid by column chromatography using silica columns. Silica gel, (Kieselgel 60, Korngroesse 0.063-0.2 mm, 70-230 mesh ASTM) in eluent 1, was packed to a height of 3 cm in Pasteur pipettes stoppered with glass wool. The assay mixture was applied to the column with 1 mℓ eluent 1. This was followed by an additional 4 mℓ eluent 1 to elute the unreacted arachidonic acid, which was then discarded. The prostaglandins were then eluted into scintillation vials using 3 mℓ eluent 2. Scintillation fluid (4 mℓ) was added and the radioactivity was counted after 1 h in the dark, using a Beckman LS3801 scintillation counter.

2.2.9 CALCULATION OF INHIBITION

The percentage inhibition of the extracts was obtained by measuring the amount of radioactivity in the solutions relative to that of the solvent blank. Inhibition refers to the reduction of PGE_2 formation with reference to an untreated sample (solvent blank). All samples were tested in triplicate. Statistical analyses were based on the results obtained from 3 different samples. One-way ANOVA and Tukey HSD tests were performed using Minitab Xtra version 10.52.

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

2.2.10 SAPONIN DETERMINATION

The 10th Edition of the French Pharmacopoeia gives a protocol for the detection of saponins based on their foam value i.e. "the dilution of a decoction of the drug which in the presented conditions produced a lasting foam" (BRUNETON, 1995). In practice this value is determined by the prolonged boiling (30 min) of 1 g of drug in 100 mℓ water. A series of calibration tubes containing increasing dilutions of this decoction are agitated: the foam value is the drug dilution in the tube giving 1 cm of foam after 15 min at rest (BRUNETON, 1995). Based on this protocol, a crude test for the presence of saponins was conducted simply by vigorously shaking the water extracts.

The detection and quantification of saponins based on their haemolytic ability is facilitated by the use of blood agar plates or by changes in absorbance of erythrocyte suspensions. Blood agar plates (B17:9) were obtained from the Allerton Vetinary Laboratory in Pietermaritzburg. Five wells (8 mm in diameter) were made per plate. A distilled water control was used for the central well and 50 $\mu\ell$ of extract (10 mg $m\ell^{-1}$) was added to the other wells. Both leaf and bulb extracts of *E. autumnalis autumnalis* were tested. The saponin activity of the extracts was calculated by measuring the diameter of the haemolytic zone of the extract relative to that of the distilled water control. This was expressed as a percentage value.

2.3 RESULTS

2.3.1 COX-1 INHIBITION

A dilution curve was prepared for the commercial NSAID indomethacin used as a standard (Appendix I). The IC_{50} value was calculated to be 3.1 μM.

Anti-inflammatory activity of bulb extracts

Figure 2.1 represents the percentage inhibition for the ethanolic and aqueous bulb extracts from all 11 specimens. Four levels of activity were defined by TUNÓN *et al.* (1995) in their investigation of Swedish medicinal plants. Using a test concentration of 200 μg mℓ^{-1},activity below 20% was considered insignificant, 20-40% low, 40-70% moderate, and 70-100% high. Accordingly, in this study, using a screening concentration of 250 μg mℓ^{-1} in the assay, a minimum activity level of 40% was chosen, with activity above 70% considered significant. The levels of COX-1 inhibitory activity can thus be considered moderate for all the species, and especially high for *E. bicolor, E. humilis* and the hybrid species. The aqueous extracts were tested at a higher concentration in the assay (500 μg mℓ^{-1}). There was no consistent correlation between the anti-inflammatory activity of the aqueous and ethanolic extracts from a particular species (Figure 2.1). Three species (*E. bicolor*, *E. humilis* and the hybrid species) showed significantly higher levels of anti-inflammatory activity for the ethanol extract than for the aqueous extract. Since the activity was invariably higher in the ethanolic extracts, and considering the relative difficulty entailed in the preparation of aqueous extracts (due to the high levels of saponins), it was decided to restrict the remaining screening procedures to ethanolic extracts.

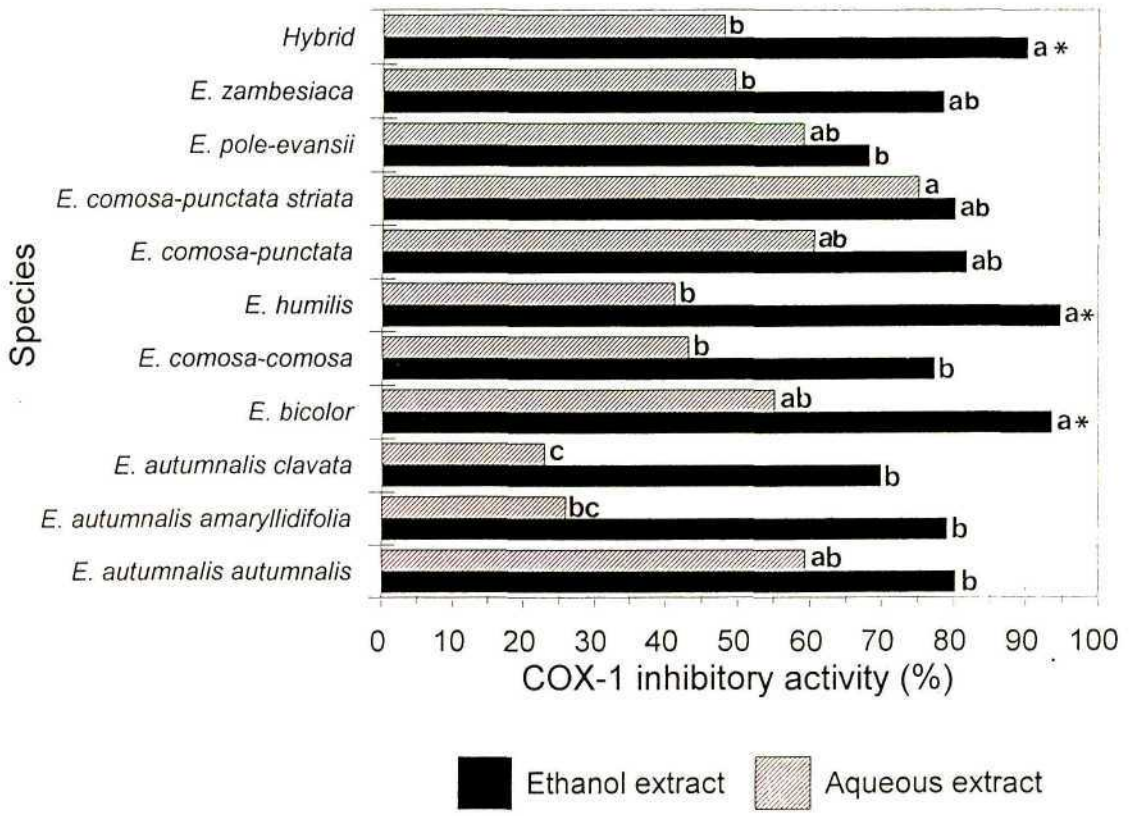


FIGURE 2.1: The % inhibition of the COX-1 enzyme by aqueous and ethanolic extracts of the bulbs of various *Eucomis* species. Screening concentration for crude extracts was 250 μg mℓ^{-1} for ethanol extracts, and 500 μg mℓ^{-1} for aqueous extracts. Bars bearing different letters are significantly different, $P \leq 0.05$. Species marked with an asterisk (*) show significant differences between the activity of the aqueous and ethanol extracts of that particular species.

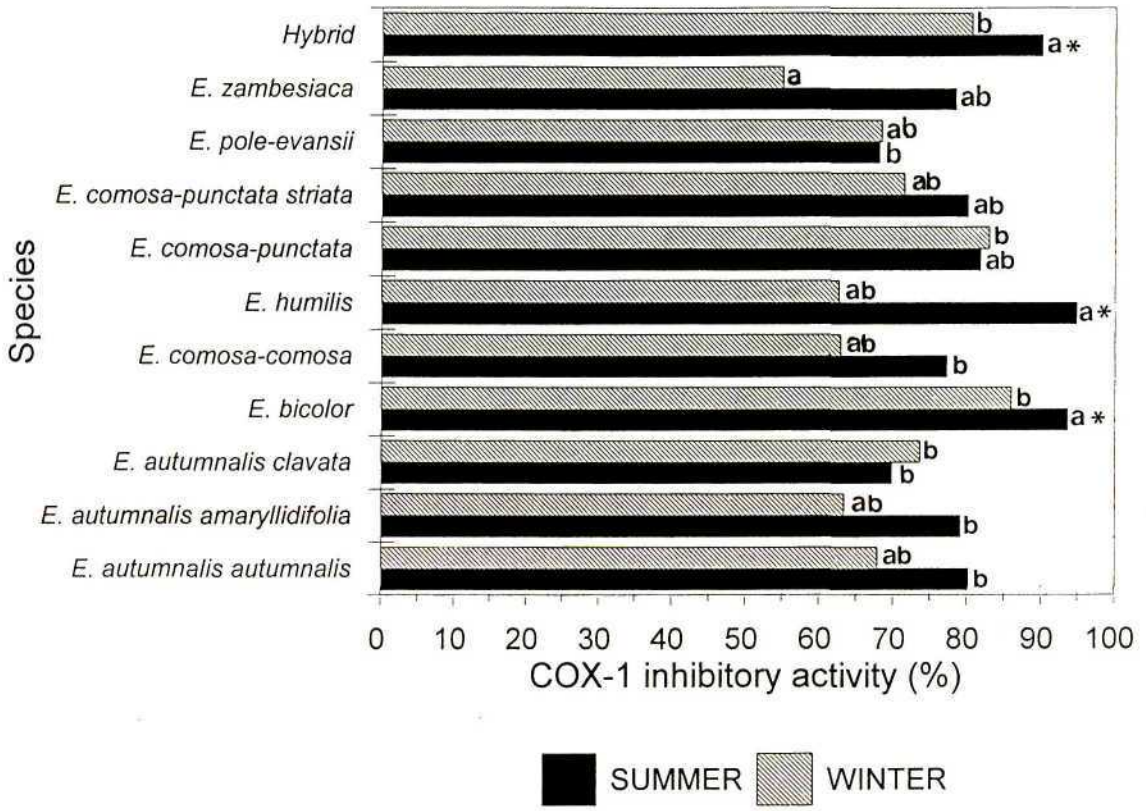


FIGURE 2.2: The % inhibition of the COX-1 enzyme by ethanolic extracts of the bulbs of various *Eucomis* species harvested in summer and winter. Screening concentration for crude extracts was 250 μg mℓ^{-1}. Bars bearing different letters are significantly different, $P \leq 0.05$. Species marked with an asterisk (*) show significant differences between activity in summer and winter.

Comparison of summer / winter data for bulb extracts (ethanol)

Very little difference can be observed in the levels of active compounds present in the bulb extracts made in summer and winter (Figure 2.2). In most of the species, the summer values are higher than those in winter, but this difference was only significant for four species (*E. autumnalis autumnalis*; *E. bicolor*; *E. humilis* and the hybrid).

Anti-inflammatory activity of extracts prepared from different plant parts

The different species were divided into leaf, bulb and root material and these extracts were assayed (at 250 μg mℓ^{-1}) to determine whether the different plant parts vary significantly in levels of anti-inflammatory activity (Table 2.1).

TABLE 2.1: Anti-inflammatory activity of extracts prepared from the different plant parts of various *Eucomis* species (Screening concentration 250 μg mℓ^{-1}).

Species	% Inhibition of COX-1*		
	Leaf	Bulb	Root
E. autumnalis subsp *autumnalis*	78 ± 5.3 b	79 ± 5.5 ab	71 ± 6.4 ab
E. autumnalis subsp *amaryllidifolia*	62 ± 8.8 a #	79 ± 4.6 ab	89 ± 3.2 c
E. autumnalis subsp *clavata*	66 ± 7.7 a	70 ± 6.7 a	61 ± 7.8 a
E. bicolor	96 ± 4.4 c	94 ± 2.6 c	84 ± 8.4 bc
E. comosa-comosa	83 ± 8.8 bc	77 ± 9.2 ab	80 ± 8.9 bc
E. humilis	64 ± 6.3 a #	95 ± 3.3 c	88 ± 6.4 c
E. comosa-punctata (striata)	62 ± 7.5 a #	82 ± 4.5 b #	59 ± 8.9 a
E. comosa-punctata	91 ± 2.5 bc#	80 ± 1.7 ab	73 ± 2.5 ab
E. pole-evansii	89 ± 2.9 bc #	68 ± 9.1 a	65 ± 4.4 ab
E. zambesiaca	67 ± 7.1 a	78 ± 6.4 ab	67 ± 2.9 ab
Hybrid	91 ± 3.8 bc	90 ± 1.0 bc	75 ± 3.8 b #

* Different letters represent significant differences within groups, $P \leq 0.05$.

represents a significant difference between plant parts of a species.

In general, the highest activity was observed in the bulbs and roots of the plants, but some species (especially the larger forms of *E. bicolor*, *E. pole-evansii* and the hybrid species) yielded higher levels of activity in the leaf extracts.

The COX-1 inhibitory activity of the leaf, bulb and root extracts was analysed for each species to determine whether significantly higher levels of anti-inflammatory activity are exhibited by particular plant parts, or whether high activity is typical of a species. The activity of the extracts from most species did not differ significantly with plant part.

E. autumnalis amaryllidifolia and *E. humilis* showed lower levels of anti-inflammatory activity in the leaves relative to the bulb and roots, while the leaf extracts of *E. comosa-punctata* and *E. pole-evansii* had significantly higher activity than the bulb and root extracts. The bulb extract from *E. comosa-punctata (striata)* showed significantly higher activity than the leaf and root extracts.

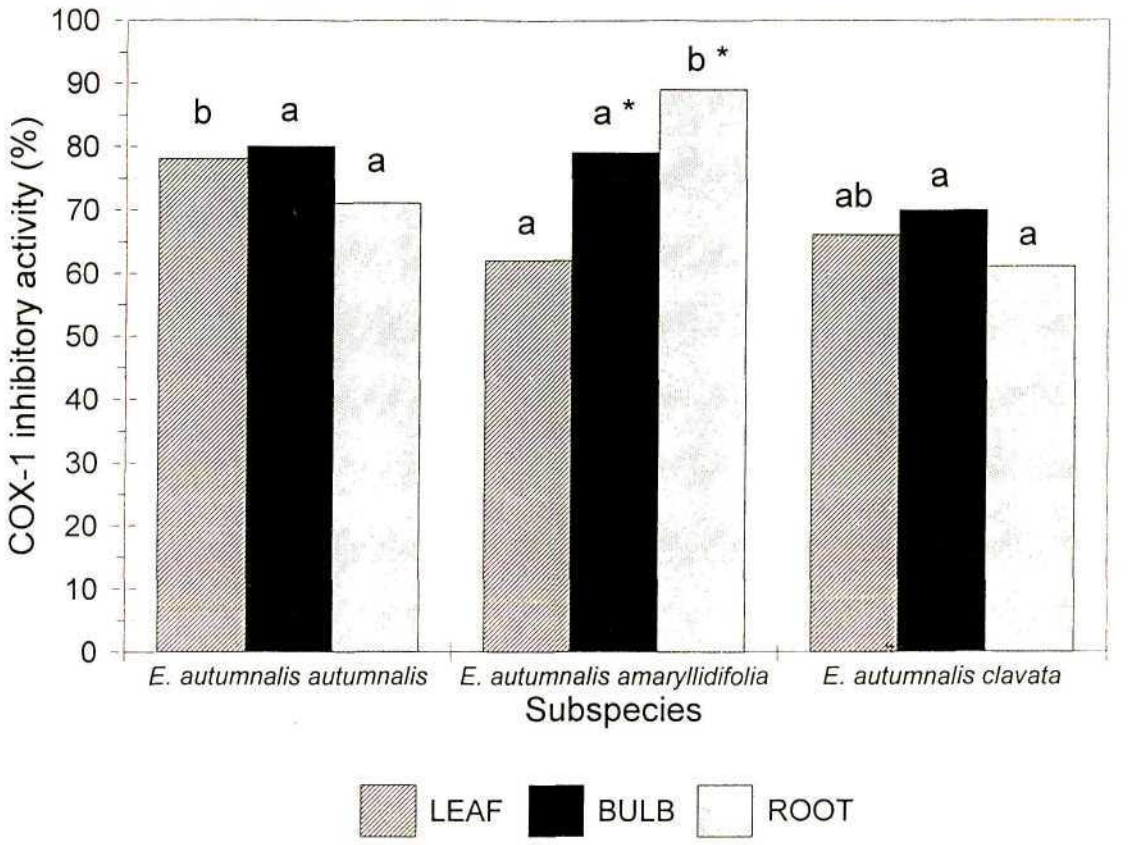


FIGURE 2.3: The % inhibition of the COX-1 enzyme by ethanolic extracts of the leaves, bulbs and roots of the 3 subspecies of *E. autumnalis.* Screening concentration of extracts was 250 μg mℓ^{-1}. Bars bearing different letters are significantly different, $P \leq 0.05$. Bars marked with an asterisk (*) indicate significant differences between the activity of the different plant parts of a particular subspecies.

2.3.2 INVESTIGATION OF *E. autumnalis autumnalis*

COX-1 inhibitory activity of leaf / bulb / root extracts

Figure 2.3 depicts the percentage inhibition of cyclooxygenase by ethanolic extracts from the leaves, bulb and roots of the three subspecies of *E. autumnalis*. All three subspecies exhibited similarly high levels of activity for leaf, bulb and root extracts. The subspecies *amaryllidifolia* differs from the other two subspecies in that the root extract exhibited relatively high levels of activity, and the leaf extract significantly lower activity than the bulb and root extracts.

Juvenile vs adult leaf / bulb / root extracts

Specimens of *E. autumnalis autumnalis* representing different physiological ages were harvested together. ***Juvenile*** material was harvested from tissue cultured specimens after acclimatization in a misthouse. Due to the limited amount of material in plants this immature, the extract was prepared from entire specimens. These plantlets consisted predominantly of leaf material with poorly developed bulblets and practically no root system. ***Young*** material was obtained from specimens that had been maintained for a period of one year in a greenhouse and the ***Adult*** specimen had reached flowering size (minimum 3 years old). Very high levels of COX-1 activity were associated with the juvenile state of the plant. As the plant aged, the extracts showed relatively less activity per gram dry mass, and the high levels of activity exhibited by the juvenile leaves decreased to levels comparable to the bulb and root extracts (Figure 2.4). The activity of the juvenile extract and young leaf extract was significantly higher than that of the mature leaf extract. In addition, the young plant showed relatively higher levels of anti-inflammatory activity in the leaf extract than in the bulb and root extracts, while the activity in the mature plant parts did not differ notably (Figure 2.4). The difference in activity of extracts from various plant parts for both the young and adult plants was not, however, statistically significant.

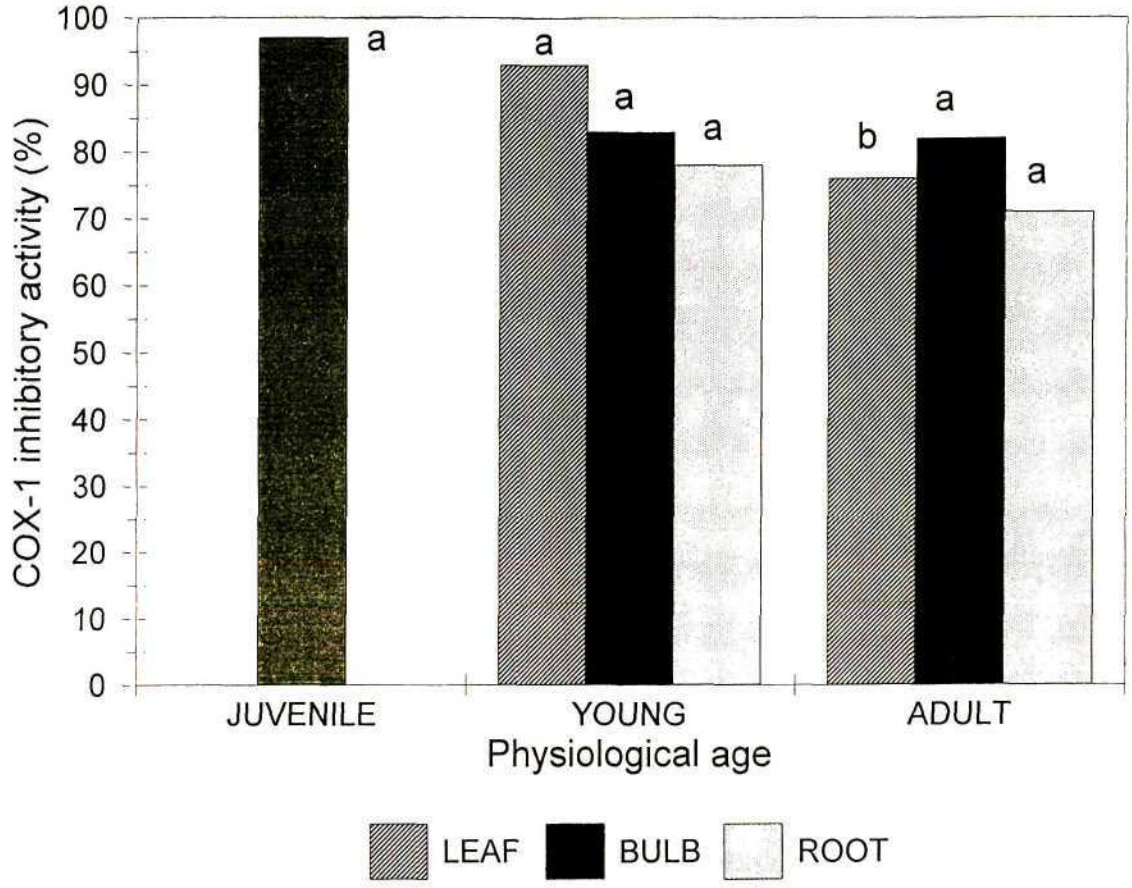


FIGURE 2.4: The variation with physiological age of the % inhibition of the COX-1 enzyme by extracts (ethanol) of *E. autumnalis autumnalis* leaves, bulbs and roots. Screening concentration of extracts was 250 μg mℓ^{-1}. Bars bearing different letters are significantly different, $P \leq 0.05$.

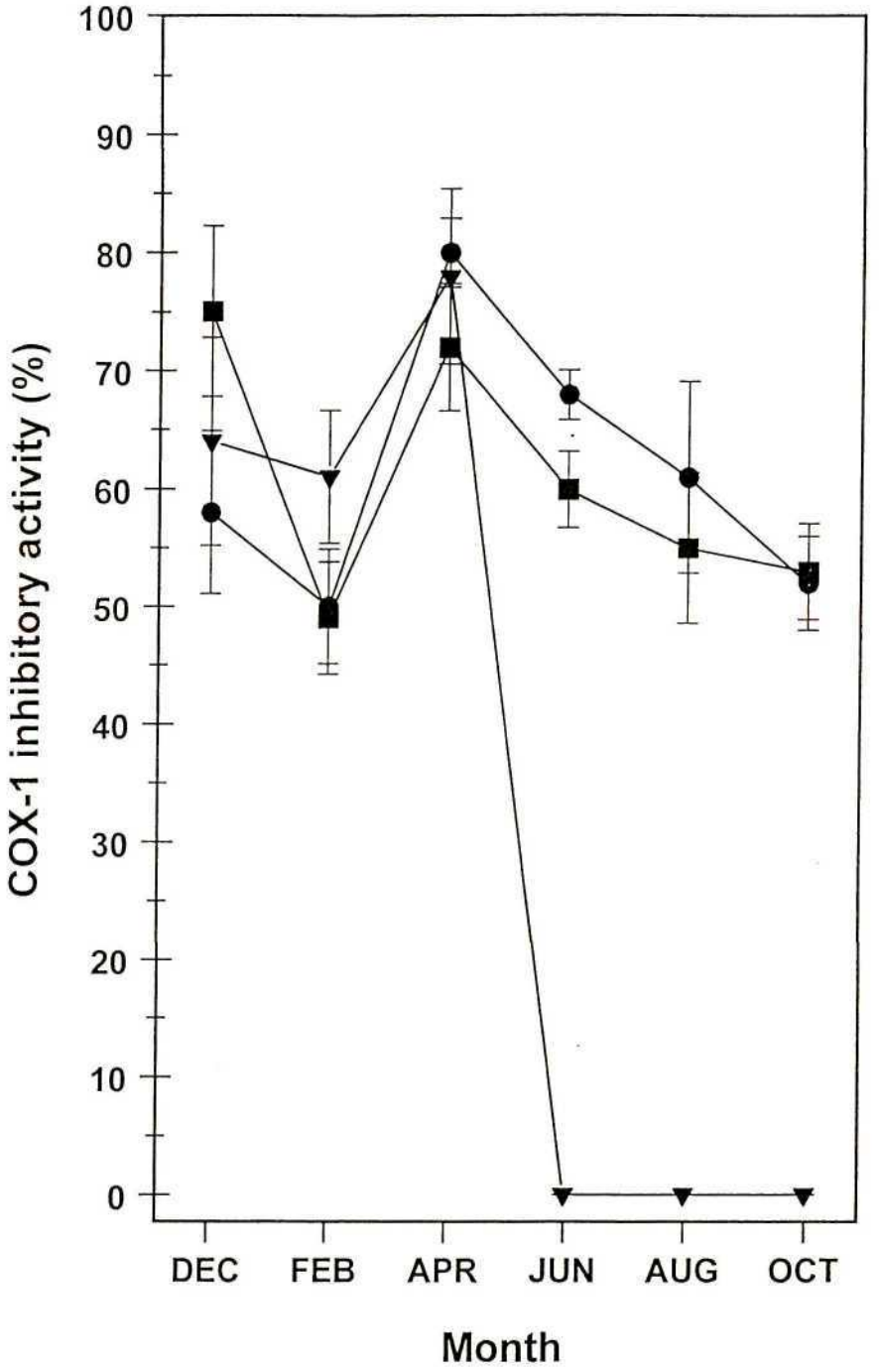


FIGURE 2.5: Seasonal variation of the % inhibition of the COX-1 enzyme by ethanolic extracts of *E. autumnalis autumnalis* leaves(▼), bulbs (●) and roots (■). Screening concentration of extracts was 250 μg mℓ^{-1}. Bars indicate standard deviation from the mean.

Seasonal (two-monthly) harvests of leaf / bulb / root material

A further investigation was conducted to observe seasonal changes in the levels of active compounds in more detail (at two-monthly intervals). Again, little variation was evident (Figure 2.5). The leaf extracts showed no significant difference in anti-inflammatory activity. Since *Eucomis* plants are dormant in winter there was no data for the leaves for the months June through October. The highest levels of activity were observed in summer (April) towards the end of the growing season. The bulb extract for April showed significantly higher activity for this month than any other.

Storage of dried material

Extracts from material harvested and dried in 1996, but not extracted, as well as ethanolic extracts made in 1996 were tested in the COX-1 assay, and compared to fresh extracts made in 1999. No significant difference was observed in the levels of activity detected in the assay (Table 2.2). These extracts were stored in the dark since ultraviolet radiation may produce chemical reactions that give rise to compound artifacts (SILVA *et al.*, 1998).

TABLE 2.2: Anti-inflammatory activity of *E. autumnalis autumnalis* extracts prepared fresh and after storage as dried, ground material and as an ethanol extract.

Extract	COX-1 inhibitory activity (%)		
	1996	1996 (dry)	1999
LEAF	79 ± 4.1	75 ± 6.2	78 ± 5.3
BULB	78 ± 5.9	80 ± 6.8	79 ± 5.5
ROOT	67 ± 3.2	68 ± 4.2	71 ± 6.4

There was no significant difference ($P \leq 0.05$) in the activity of extracts stored over the period of this study. Plant material stored dry (ground) and in the form of a crude ethanolic extract showed similar activity to the freshly prepared extracts from similarly aged specimens.

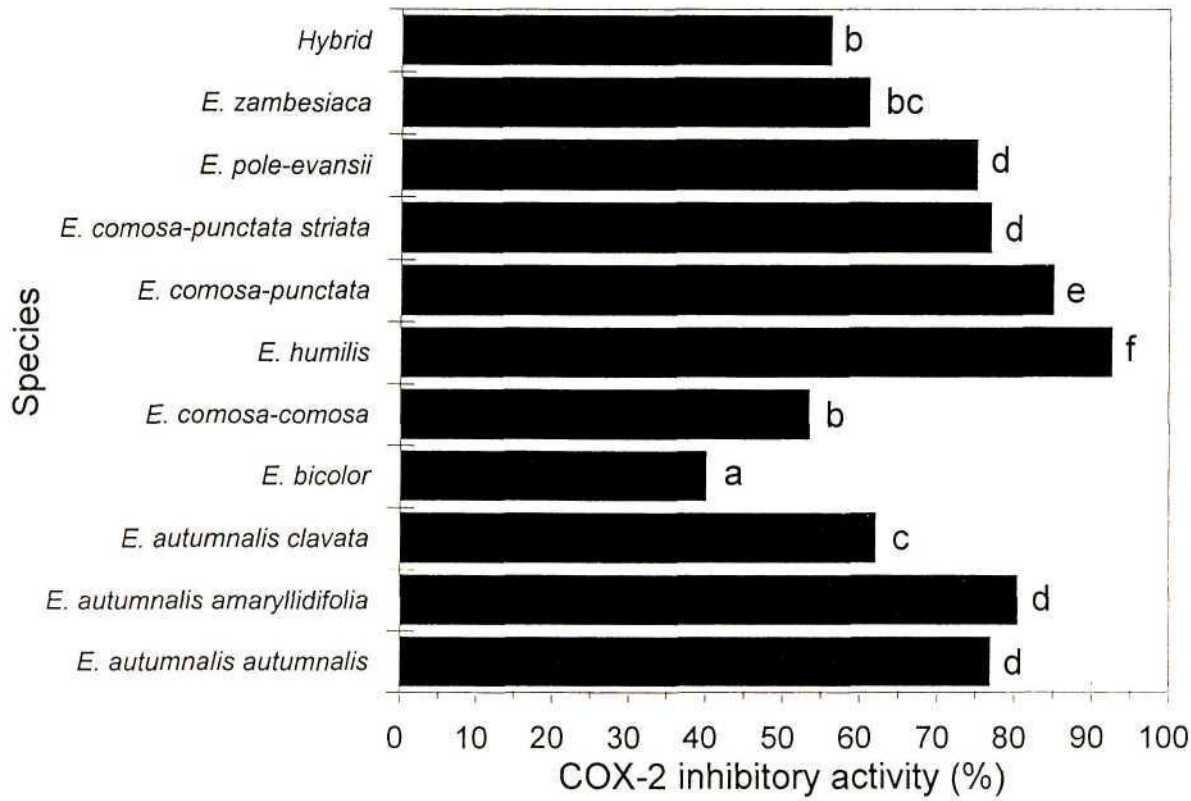


FIGURE 2.6: The % inhibition of the COX-2 enzyme by extracts (ethanol) of the bulbs of various *Eucomis* species. Screening concentration for crude extracts was 250 μg mℓ^{-1}. Bars bearing different letters are significantly different, $P \leq 0.05$.

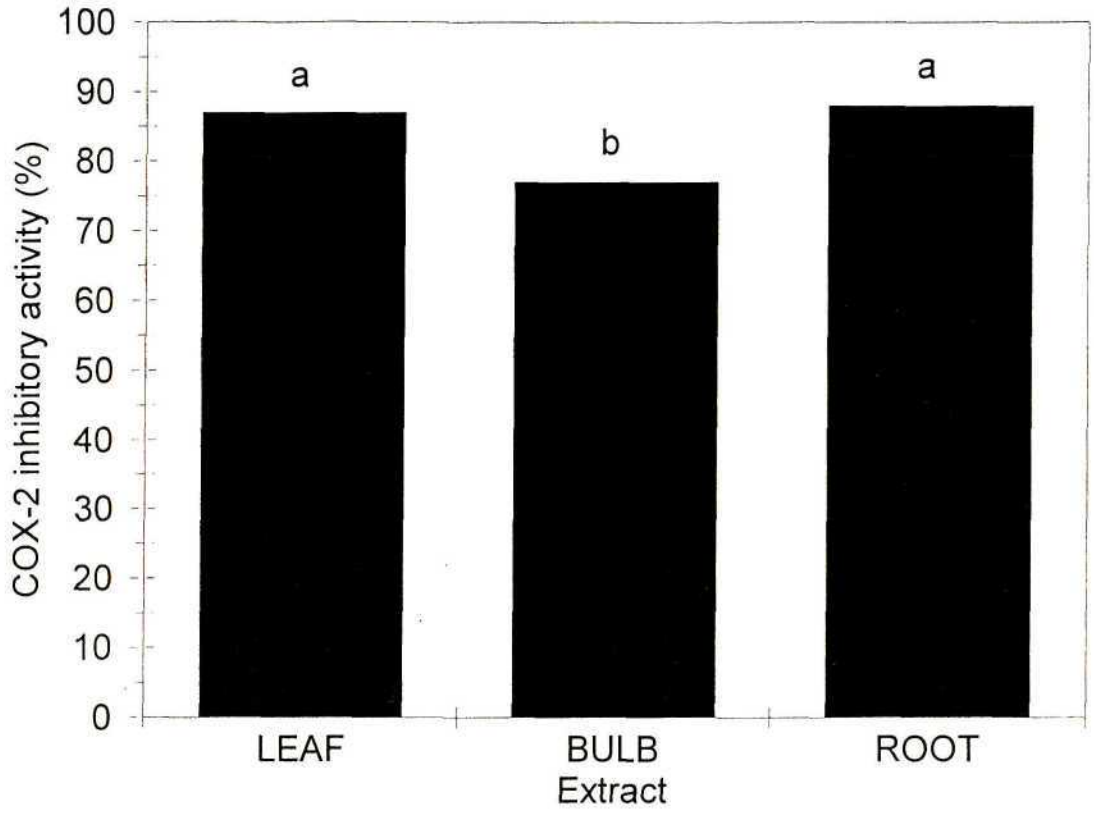


FIGURE 2.7: The % inhibition of the COX-2 enzyme by extracts (ethanol) of the leaves, bulbs and roots of *E. autumnalis autumnalis*. Screening concentration of extracts was 250 μg mℓ^{-1}. Bars bearing different letters are significantly different, $P \leq 0.05$.

2.3.3 COX-2 INHIBITION

A dilution curve was prepared using the commercial NSAID indomethacin (Appendix I). The IC_{50} value was calculated to be 188 μM.

Comparison of bulb extracts(ethanol) from different Eucomis species

High levels of COX-2 inhibitory activity were exhibited by ethanol extracts from the bulbs of *E. humilis, E. comosa-punctata* and *E. autumnalis amaryllidifolia, E. autumnalis autumnalis, E. comosa-punctata (striata)* and *E. pole-evansii* (Figure 2.6). High levels of COX-2 inhibitory activity were not, however, consistently correlated with high levels of COX-1 inhibitory activity (Figure 2.1).

E. autumnalis autumnalis leaf / bulb / root extracts

Unlike COX-1 inhibitory activity, which in *E. autumnalis autumnalis* did not differ significantly with plant part (Figure 2.3), COX-2 inhibitory activity was highest in the leaf and root extracts (Figure 2.7). The COX-2 inhibitory activity of the bulb extract, although still considered high, was significantly lower than the leaf and root extract activity.

Comparison of COX-1 and COX-2 inhibitory activity

A dilution series was prepared from the crude ethanol extracts and tested in both assays (Figure 2.8). The COX-1 inhibitory activity was similar to the COX-2 inhibitory activity for the leaf, bulb and root extracts. The IC_{50} values for each extract were calculated by regression analysis of the log concentrations and are presented in Table 2.3.

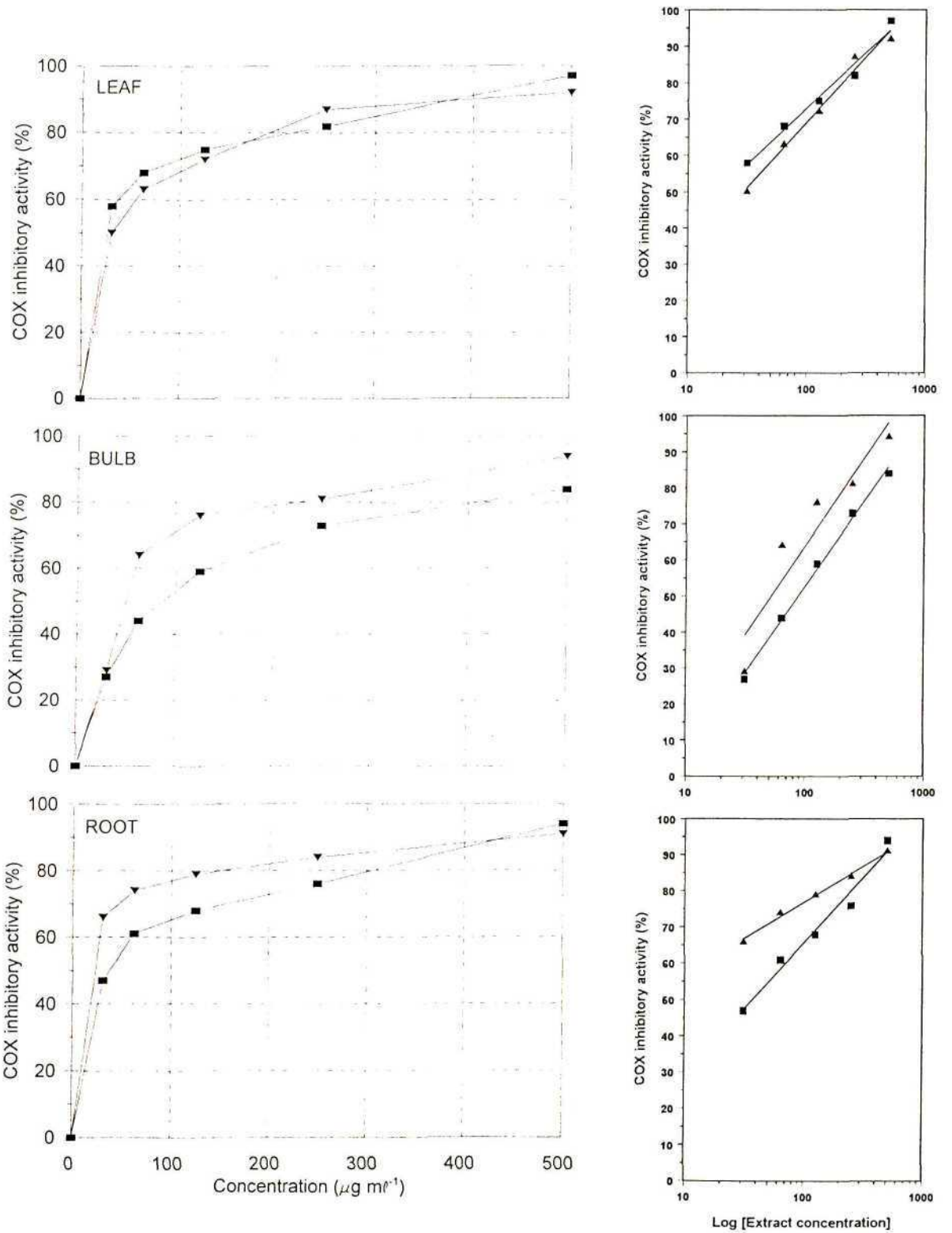


FIGURE 2.8: Dilution curves (Left) and regression analyses (Right) of the crude extracts (ethanol) prepared from the leaf; bulb and roots of *E. autumnalis autumnalis*, tested in the COX-1 (■) and COX-2 (▼) assays.

TABLE 2.3: IC_{50} values for COX inhibition by the crude ethanol extracts from the leaves, bulbs and roots of *E. autumnalis autumnalis.*

EXTRACT	IC_{50} VALUES (μg mℓ^{-1})		COX-2/COX-1 RATIO
	COX-1	COX-2	
Leaf	15	29	1.9
Bulb	72	55	0.8
Root	27	18	0.7
Indomethacin (μM)	3.1	188	61

The IC_{50} value obtained for the indomethacin standard correlates well with values recorded in the literature. A ratio of COX-2/COX-1 activity of 60 is given for indomethacin while the ratio for aspirin is given as 166 and for ibuprofen, 15 (VANE AND BOTTING, 1995; TAKETO, 1998a).

2.3.4 OTHER TEST RESULTS

Saponins

A simple test for saponins was conducted by vigorously shaking the water extracts. The resulting froth consisted of a high volume (4 cm) of large but stable bubbles, lasting more than 4 hours. Ethanol extracts from both the leaves and bulbs showed the presence of saponins, using a haemolytic test (Table 2.4). Since the extracts tested were from a serial extraction, it is to be expected that the water extract would show less activity than the ethanolic fraction.

TABLE 2.4: Measurement of saponins in *E. autumnalis autumnalis* extracts, based on haemolytic activity, expressed as a percentage of the water control.

	Degree of Saponin activity (% of water control)			
EXTRACT	Hexane (20 mg mℓ^{-1})	Ethyl acetate (20 mg mℓ^{-1})	Ethanol (20 mg mℓ^{-1})	Water (2.5 mg mℓ^{-1})
LEAF	0	112	140	109
BULB	106	106	155	131

2.4 DISCUSSION

The addition of cofactors to the enzyme preparation is necessary for the efficient conversion of arachidonic acid to the prostaglandin products by COX-1 and COX-2 (NOREEN *et al.*, 1998). Reduced glutathione stimulates the biosynthesis of prostaglandin E_2 from arachidonic acid, in sheep vesicular gland microsomes (RAZ *et al.*, 1976). The purified protein, in the presence of excess reducing substrate, converts arachidonic acid to PGE_2. In the absence of reducing substrates, prostaglandin synthase rapidly undergoes inactivation, by low levels of hydroperoxide. This inactivation during enzymatic turnover is related to the peroxidase activity of the enzyme (MARKEY *et al.*, 1987). The formation of PGE_2 (as well as $PGF_{2\alpha}$ and PGD_2) increases in the presence of l-epinephrine (NOREEN *et al.*, 1998). Since PGE_2 is an important mediator of inflammation, high yields of this prostaglandin in the control reaction (solvent blank) increase the efficiency of the assay. The inclusion of a pre-incubation step of the extract and enzyme (ZSHOCKE pers comm), before the addition of the substrate arachidonic acid is necessary to detect the presence of time-dependent inhibitors (which include indomethacin) of both COX-1 and COX-2 (QUELLET AND PERCIVAL, 1995).

2.4.1 ANTI-INFLAMMATORY ACTIVITY OF *EUCOMIS* EXTRACTS

Non-steroidal anti-inflammatory compounds can relieve the pain and inflammation associated with elevated levels of prostaglandins in the body. Inhibition of prostaglandin action may lead to the relief of headache pain (RANG AND DALE, 1987). Similar compounds are proposed to be the agents responsible for the action of traditional herbal remedies associated with the reduction of pain, fever and inflammation. Primarily the bulbs and in some instances the leaves and roots, of *Eucomis* species are widely utilized for this purpose.

E. autumnalis autumnalis is regarded as very popular as an emetic and enema for feverish conditions, but very dangerous since it contains a haemolytic toxin (CUNNINGHAM, 1988a). The active principle(s) are proposed to be anti-inflammatory compounds, hence the screening procedure conducted with the COX-1 assay. Initial screening procedures in this study have shown the presence of COX-1 inhibitors

associated with extracts from *Eucomis* plant material, which corroborated previous results obtained by JÄGER *et al.* (1996). The anti-inflammatory activity of the bulb extracts (ethanol) from all eleven species can be considered high (ie. 70-100 % inhibition). In addition, nine of the eleven corresponding water extracts show moderate anti-inflammatory activity (ie. 40-70 % inhibition) in the test used. This is a highly significant result in terms of the pharmacological evaluation of the genus, and provides sufficient evidence both to validate the use of this plant in traditional medicine, and to warrant further investigation into the nature of the active principle(s). The results from the leaf, bulb and root extracts are also significant in that they validate their individual use in traditional medicinal remedies. Another interesting point is that the bulb extracts of the three subspecies of *E. autumnalis* do not differ significantly in anti-inflammatory activity (Figure 2.3). The bulbs of the subspecies *autumnalis* and *clavata* have documented uses (Table 1.1) and considering the similar morphology of these plants, the bulbs of the three subspecies are conceivably used interchangeably.

Water and ethanol are the solvents commonly available to healers, with an aqueous medium most commonly used in the administration of *Eucomis*. Both these solvents were tested initially, but after the preliminary results, the ethanol extracts were chosen to compare the activity of the extracts. Aqueous fractions are highly complex, and include the majority of primary metabolites from the plant material (LEWIS, 1989). Ethanol dissolves the more lipophilic compounds in the extracts ensuring that more potential inhibitors are included in the extract. It should also be noted that extracts showing low activity in the assay do not necessarily lack anti-inflammatory activity - they may contain active compounds that act at other sites in the processes of pain and inflammation (McGAW *et al.*, 1997)

Although traditional healers use predominantly boiling, or hot water extracts (up to 90%), in many cases the active compounds are found to be completely insoluble in water. The most probable reason for the efficacy of these preparations is that there are saponins in the extract, or other materials that affect surface tension and solubilize, or bring into aqueous phase, water-insoluble compounds (BALICK, 1994). In terms of the scientific investigation of these plants, two extracts are thus required, which in commercial terms doubles the cost. The same situation arises every time the

extract is partitioned. For drug companies this becomes financially indefensible and they adopt a hit and miss policy (BALICK, 1994).

With respect to both aqueous and ethanolic extracts, compounds that have little or no intrinsic activity can exhibit a potentiating action, resulting in the extract having higher activity than the purified active compound. Polar compounds included in the aqueous extracts may play a role in lowering toxicity and / or enhancing activity (FARNSWORTH, 1993). This may be relevant for extracts of *Eucomis* species since the bulbs are reported to have toxic effects (HUTCHINGS *et al.,* 1996; WATT AND BREYER-BRANDWIJK, 1962). Extraction with an ethanolic solvent may also help to reduce the number of micro-organisms due to direct contact with alcohol, while extraction with boiling water (the most common method of preparing medicinal teas) can serve a similar decontamination purpose (DE SMET, 1992).

Variation in anti-inflammatory activity within species

Variation in morphological and chemical traits is common within populations, possibly as an adaptation for survival and growth within changing ecosystems. This can result in differences in type and quantity of the natural products produced by the plants. Differences in natural product accumulation have been linked to age. MÁTHÉ (1988) found that differences in total glycoside accumulation within single plant populations of *Adonis vernalis* L. were primarily as a result of differences in plant age, not heterogenicity. The frequency distribution for total glycoside content of plants within the same age group fitted a normal distribution curve. This indicates that the trait is genetically based. In genetically based studies, researchers have found plants (e.g. *Papaver bracreatum*) showing discrete morphological and chemical properties within populations, indicating different chemical taxa within the species (MÁTHÉ, 1988).

Results obtained from these investigations showed that while there was little seasonal variation (Figure 2.2) within the activity of the bulb extracts, high levels of COX-1 inhibitory activity were linked to juvenility in *E. autumnalis autumnalis.* This was also associated with a different distribution of activity within the plant parts (Figure 2.4) Juvenile plants (whole plant extract) consisted mainly of leaf material. This extract, and that made from the young leaves showed higher levels of activity than those prepared from the adult plant. In the older plant the activity of the leaf extracts

decreased, and no significant difference could be detected in the COX-1 inhibitory activity of the different plant parts. The only significant change in COX-1 inhibitory activity on a seasonal basis, was detected for the bulb extract in April (Figure 2.5). This is just before the leaves begin to die back in preparation for winter dormancy and could represent a redistribution of the active compound(s) in readiness for this physiological change. These results are important in terms of the choice of the season and age of plants for harvest. In addition, these results present further implications for the standardization of traditional herbal remedies. The fact that the anti-inflammatory activity of the plant extracts has been shown to vary, makes standardization of such preparations very difficult.

A further consideration in the screening of traditional medicinal plants for pharmacological activity is the possibility of variation due to ecological change over centuries (TUNÓN *et al.*, 1995). Such pressures on the system of traditional phytomedicine account for its dynamic nature. This problem is not, however, relevant in this case where the genus investigated is currently used by traditional healers.

2.4.2 COX-2 INHIBITION

Aspirin, indomethacin and ibuprofen have been found to be more potent inhibitors of COX-1 than COX-2 in several model test systems. The relative potencies of aspirin and indomethacin vary slightly between models although the IC_{50} values are different (MITCHELL *et al.*, 1994). Very few compounds of natural origin have been shown to inhibit COX-2 (NOREEN *et al.,* 1998). Investigation of plant extracts in terms of COX-2 inhibition is thus of primary importance, considering the potential value of such compounds.

The levels of activity shown by the bulb extracts of *Eucomis* species in the COX-2 assay were not as high as those shown by the leaves and roots. This was, however, a crude extract and could have included compounds acting in opposition to these active compounds. Extraction using solvents of different polarities (conducted in CHAPTER 3) showed slightly different results. The ratio of COX-2/COX-1 inhibitory activities, for the bulb and root extracts in particular, were low indicating the selective COX-2 inhibitory activity of the extracts. This selective COX-2 inhibition further validates the

use of these particular plant parts in traditional medicine. High COX-2 inhibitory activity makes these extracts valuable medicinal preparations and emphasizes the potential traditional remedies show for the discovery of new drugs. These preparations have thus been utilized for centuries as drugs with a pharmacological value that current medical practitioners only recently have fully appreciated.

Sustainable use

The high levels of activity exhibited by the leaf extracts suggest that this may be a viable alternative to the use of the bulbs. While the COX-1 inhibitory activity in the leaves does decrease slightly with age (Figure 2.4), in the mature plant there is no significant difference between the COX-1 inhibitory activity of the leaves, bulbs and roots (Figure 2.3). The COX-2 inhibitory activity of the crude leaf extracts, however, is significantly higher than that of the bulbs (Figure 2.7). This is an important finding in terms of the conservation status of this species. If the traditional use of this plant could shift to utilize the leaves, a renewable resource, the destructive harvesting of *Eucomis* species for the bulb material would not be necessary, and cultivation of the species would be encouraged. These results show that this is a viable option - one that merits further investigation and development.

2.4.3 OTHER TEST RESULTS

Storage of dried material

Testing the COX-1 inhibitory activity of both the dried, ground plant material, and the plant extracts (ethanol) over a number of years resulted in no significant difference in inhibitory activity. This implies that the active compound(s) are stable, both in the dried plant, and in solution. Plant material stored carefully (paper bags kept in cool, dry, dark environments) will thus maintain these pharmacological properties for periods in excess of three years. The implications of this finding are significant in terms of the pharmacological screening and isolation of active compounds (over an extended time period), and for the traditional healers and collectors.

Anti-bacterial activity

Aqueous and methanol extracts of *E. autumnalis autumnalis* leaves and bulbs have been previously tested for anti-bacterial activity using the agar diffusion and dilution methods (RABE AND VAN STADEN, 1997). Bacteria tested were *Staphylococcus aureus, Staphylococcus epidermis, Bacillus subtilis, Klebsiella pneumoniae* and *Escherichia coli.* Only the methanol bulb extract showed slight activity against *Bacillus subtilis*, a gram positive bacteria. The poor anti-bacterial activity shown in these results did not warrant further investigation in this field.

Saponins

The saponins are a group of glycosides, ubiquitous in plants and characterized by surface active properties - they dissolve in water to form foamy solutions. Steroidal saponins are almost exclusively present in Monocotyledonous angiosperms. Most saponins have haemolytic properties and are toxic for cold-blooded animals especially fish (BRUNETON, 1995).

The detection and quantification of saponins is based on their ability to rupture erythrocytes. In general, the change in absorbance of the supernatant of an erythrocyte suspension is measured after haemolysis by a saponin or saponin-containing drug (BRUNETON, 1995). Alternatively, the zone of haemolytic activity can be measured using blood-agar plates. Using the second method, *E. autumnalis autumnalis* extracts were found to contain significant levels of saponins (Table 2.4). Higher levels of activity were detected in the ethanolic extracts of the serial extraction. Since saponins are soluble in water they can be extracted with this solvent (generally at the boiling point). An aqueous medium, however, favours the hydrolysis of the bidesmosides and it is often better to use alcohols (methanol) or hydroalcoholic solutions after partial lipid removal. Although saponins often occur in substantial quantities, they are found as complex mixtures, and their high polarity, relative fragility and the minute structural differences between high molecular weight constituents complicates their extraction (BRUNETON, 1995).

The haemolytic properties of saponins are generally attributed to their interaction with the sterols of the erythrocyte membranes, which induces an increase in permeability and a loss of haemoglobin. Taken orally by warm-blooded species, saponins are most

often only weakly toxic, probably because they are not absorbed much, but different effects are seen when administered by methods other than via the alimentary canal. Several drugs owe their anti-inflammatory properties to saponins. Many saponin containing drugs are traditionally used for their anti-tussive (cough) and expectorant properties (BRUNETON, 1995).

Saponins are also known to have an irritant effect on mucosa, which makes them effective emetics. Mucilaginous exudates from the bulbs of some Amaryllidifolia species are used for wound healing and rashes, as well as in purging medicine. Mucilage applied externally would promote healing by forming a barrier to further irritation. Taken internally, it has a laxative effect which is attributed to its property of swelling in water (HUTCHINGS, 1989b). These uses have been reported for preparations of *E. autumnalis autumnalis* and *E. autumnalis clavata* (WATT AND BREYER-BRANDWIJK, 1962; HUTCHINGS *et al.*, 1996).

2.4.4 POSSIBLE CONTAMINANTS OF THE COX ASSAY

The use of polar extracts in screening assays, introduces the possibility that the extract contains polyphenolic compounds that are able to interfere in the COX assay, giving false positive results. These include compounds such as tannins (bark) and chlorophyll degradation products (leaf) which contaminate the assay by unspecific binding to the enzyme complex.

Polypenols (tannins)

These are ubiquitous in plant extracts and show activity in a large number of assays. In general, their nonspecific activity is attributed to their ability to complex metal ions, scavenge radicals and reduce active oxygen species, and form tight complexes with a wide variety of proteins and polysaccharides. This will result in false positives in most assays involving purified protein by forming a precipitate with the protein (VAN MIDDLESWORTH AND CANNELL, 1998). Since polyphenolics (tannins) bind to protein, if they are absorbed in the body they bind to serum and are therefore not bioavailable. This may have an effect in treating diarrhoeal diseases, since polyphenols are not well absorbed and are very astringent (BALICK, 1994) Tannins thus need to be disqualified as the source of the extract's inhibitory activity in the COX-assays.

2.5 CONCLUSION

The screening of plant extracts is motivated by the potential discovery of new drugs that could be developed for use later in clinical medicine. This procedure has the advantage of uncovering unexpected chemical structural types as active principles under test conditions. These can then serve as starting points for further elaboration into suitable drugs (BURGER, 1982). The screening of *Eucomis* extracts for COX inhibitors has revealed the great potential that this genus shows for the discovery of COX-1 and more importantly, selective COX-2 inhibitors. In addition, this screening procedure has validated the extensive use of this plant in African traditional medicine.

The concentration of the constituents assumed to be responsible for the activity varies depending on the stage of growth and the ecological conditions under which the plants were grown (ANTON *et al.,* 1986). This is true of *Eucomis* specimens, as is evident from the results presented for the variation of COX-1 activity with age and for the seasonal variation seen in *E. autumnalis autumnalis* extracts. Further experiments involving this premise were conducted (CHAPTER 9). The implications of this finding are significant in terms of the current emphasis on the standardization of traditional remedies with the aim of improving their safety and efficacy. This would make these remedies acceptable for endorsement by both medical and government associations.

The positive results from these preliminary screening assays formed the basis for the development of an isolation procedure, designed to identify the major active compound(s) in these *Eucomis* extracts. The attention generally given to the most abundant substances with the largest molecules detracts from the low-molecular-weight molecules which are present in noteworthy proportions in many extracts and which may be the true active principles. While the isolation of new natural compounds is an important field, the isolation of the pharmacologically active compounds is of greater importance and interest. This can only be accomplished effectively by the bioassay-guided fractionation of the complex mixtures of natural substances present in plant extracts. This entails the use of specific bioassays, with the utilization of appropriate controls. This method is still inherently impaired in that with the isolation of the primary active compound, other potentially important, novel active principles

may be overlooked. Due to these limitations of the assays used, the bioassay-guided fractionation, as described in CHAPTERS 3 and 4, concentrated on the primary active principle detected in extracts of *E. autumnalis autumnalis*.

CHAPTER 3

ISOLATION OF THE ACTIVE PRINCIPLE IN *E. AUTUMNALIS AUTUMNALIS*

3.1 INTRODUCTION

The selection, collection, extraction and biological evaluation of medicinal plants no longer represent obstructions in the development of new drugs. This is now represented by bioactivity-directed fractionation (CORDELL, 1995). A screening procedure may identify a few (10-20) or several hundred samples showing activity. The isolation and elucidation of biologically active principles is a labour intensive procedure, usually assigned to a chemist. A limited number of samples can be worked on at once: up to 10, providing the activity is concentrated in limited fractions, or 6 if the activity is spread through several polarity / chemical groups. Secondary selection criteria are needed to identify samples for further work. In species-screening protocols this can be done using chemotaxonomic or ethnomedical information to eliminate species that have been well studied previously, or are likely to yield known active metabolites. This process is driven by the search for novel compounds in order to establish patents (CORDELL, 1995). In this study, the secondary criteria were primarily the abundance and type of material available.

The large scale isolation of biologically active plant constituents can only be achieved through the large-scale re-collection of the plant of origin. This does not, however, always result in the detection of the original biological activity, frequently due to biological variation in the plant material (KINGHORN, 1992). This problem was discussed in more detail in CHAPTER 2.

The current bioassay-guided methods for separating and identifying active compounds originated in the 1980s with experimentation on whole plant extracts, crude products and chromatographic fractions, using the standard biological tests for

pure compounds (LOZOYA, 1994). Results showed that the extracts and crude fractions performed well in *in vitro* assays, with the utilization of the appropriate controls. This system requires interaction between chemists and pharmacologists - with extracts and fractions being tested for biological activity. Once dose dependent effects of the extracts have been demonstrated (Figure 2.8), fractionation is attempted. After chromatography, the fractions are retested and the one showing increased biological activity with respect to the original extract is fractionated further. This is repeated until the pure active compounds are isolated. This approach frequently results in the isolation of several active compounds from the same extract. These compounds have often been associated with the polar, aqueous fraction (from which they are difficult to separate) (LOZOYA, 1994).

Ideally, pharmacological tests on purified fractions should show 100% of the original activity in one fraction, or a distribution among two or more fractions. In the latter case different separation methods should be tried. If any activity has been lost, it is necessary to identify the step where this occurred. Loss of activity may be caused by degradation of products, or the removal, during purification steps, of a compound that could have initially increased the effectiveness of the active principle. This cannot, however, entirely explain loss of activity (CAVÉ, 1986).

The size of a dose, relative to the weight of the original dried material, is vital information regardless of the purity of the extract in question. This allows the correct interpretation of the results in order to draw valid conclusions on which to base subsequent stages of work. Fractionated extracts are expressed as a percentage weight of the weight of the original plant material in order to calculate the yield of each extraction (CAVÉ, 1986).

Column chromatography -Sephadex LH 20

The chromatographic separation of labile natural products can be efficiently conducted using fairly inert polymers of carbohydrates. Polysaccharides can be crosslinked to produce three dimensional networks which can be formed into beads and packed into columns. Sephadex® is a water insoluble polymer formed by crosslinking the water soluble dextran to epichlorohydrin, with glycerin-ether bonds as crosslinkers. The gel is hydrophilic, and swells in water separating compounds based

on their molecular size. The predominant mode of separation is gel filtration (size exclusion), with additional adsorbtion mechanisms such as hydrogen bonding, resulting in good fractionations (SALITURO AND DUFRESNE, 1998).

Sephadex LH-20 is a hydroxypropylated form of Sephadex G-25, which adds lipophilicity to the gel while retaining its hydrophilicity (SALITURO AND DUFRESNE, 1998). The gel swells in polar solvents (e.g. water, methanol) to about four times its dry volume. This gel is suited for the fractionation of organic-soluble natural products due to the added lipophilicity that allows the gel to swell adequately in organic solvents. The useful fractionation range is for compounds of molecular weight 100-4000 (SALITURO AND DUFRESNE, 1998) .

The best separation results are obtained when a mixture of polar and non-polar solvents are used. In a solvent mixture, the more polar of the solvents is taken up by the gel preferentially, resulting in a two-phase system with stationary and mobile phases of different compositions. Chromatographic separations thus take place by a partition mechanism. As an added advantage, it was found that phenolic and heteroaromatic compounds have unusual affinity for this type of gel. These compounds are thus generally more strongly retained than predicted based on their size, especially when lower alcohols are used as eluting solvents (SALITURO AND DUFRESNE, 1998).

Sephadex gels are stable in all solvents except for strong acids, which would hydrolyse the glycosidic linkages. In addition, since these polysaccharide materials are fairly inert, they rarely irreversibly adsorb compounds, consequently biological activity is almost always fully recovered. The same column can also be used for several experiments without the need for regeneration (SALITURO AND DUFRESNE, 1998).

Flavonoids and homoisoflavonoids

Flavonoids cover a large group of naturally occurring phenolic compounds in which two benzene rings are linked by a propane bridge (C6-C3-C6). Many possess strong anti-inflammatory activity (KAMIL, 1993). The flavonoids occur in the free state and as glycosides. The flavones and related compounds are usually yellow and are widely

distributed in higher plants. They occur primarily in young tissues and are found in the cell sap. The glycosides are generally soluble in water and alcohols, but not in organic solvents.

Chemical constituents previously isolated from *Eucomis* include the homoisoflavones punctatin, autumnalin and eucomin (SIDWELL AND TAMM, 1970; FARKAS *et al.,* 1971). These have not, however, been linked to the specific activity of *Eucomis* extracts using bioassays, or by bioassay-guided fractionation. Other compounds isolated include two di-benzo-α-pyrones, autumnariol and autumnariniol, the spirocyclic nortriterpene, eucosterol and choladienoic acid (SIDWELL *et al.* 1971; SIDWELL *et al.* 1975; ZIEGLER AND TAMM, 1976; GLASBY, 1991).

Many medicinal plants contain flavonoids - they have been detected in over 100 herbal preparations on the European market (ERNST, 1998). Flavonoids have been associated both with beneficial effects such as antioxidative activity and reduction in vascular permeability, and adverse reactions including haemolytic anaemia, chronic diarrhoea, severe nephropathy and colitis (ERNST, 1998). The inhibition of platelet aggregation is associated with their ability to interfere with the development of vascular diseases. In addition, flavonoids inhibit prostaglandin metabolism and the synthesis of mediators of inflammation, protect endothelial cells and modify blood rheological parameters (ANTON *et al.*, 1987). Flavonoids show very low toxicity, and are synthesized in a wide variety of chemical structures (ANTON *et al.*, 1987).

Saponins

Haemolytic sapogenins and organic acids have been found in the bulbs of several *Eucomis* species (WATT AND BREYER-BRANDWIJK, 1962), with decoctions from the bulb and roots of an unidentified species reputed to produce rashes.

The saponins are a group of glycosidic compounds with a terpenoid aglycone (sapogenin), widely distributed in higher plants, especially dicotyledons (HILLER, 1987). Saponins include three major structural classes: the steroids, spirostanols and triterpenoid gycosides, the last being the main group (HILLER, 1987).

Saponin based drugs are primarily triterpene glycosides, but some may also, or only, contain steroid saponins. The structure of the glycoside is primarily responsible for the surface activity of these compounds. The number and arrangement of the functional groups affect the haemolytic activity (HILLER, 1987).

The triterpene glycosides may contain sugar residues linked via the OH group at C-3-OH of the aglycone (monodesmosidic saponins), or more rarely, linked via two OH groups or a single OH group and a carboxyl group of the aglycone moiety (bidesmosidic saponins). The carbohydrate moiety usually contains one to six monosaccharide units, commonly glucose, galactose, rhammose, arabinose, fructose, xylose, glucuronic and galacturonic acids (WAGNER AND BLADT, 1996).

The presence of carbonyl groups in the algycone and/or sugar moieties give these saponins their acidic properties. Triterpene saponins possess an oleanane or, more rarely, an ursane or dammarane ring system. Sapogenins may also contain other oxygen-containing groups (e.g. -OH , $-CH_2OH$ or -CHO). Most triterpene saponins possess varying degrees of haemolytic activity, depending on the pattern of substitution (WAGNER AND BLADT, 1996).

The sapogenins of the steroid saponins are generally spirostanols. Furostanol derivatives are usually converted into spirostanols during isolation procedures. These sapogenins do not contain carboxyl groups and contain fewer sugar units than the triterpene saponins. In contrast to monodesmosides, the bidesmosidic furostanol glycosides exert no haemolytic activity (WAGNER AND BLADT, 1996).

3.2 MATERIALS AND METHODS

All solvents used were re-distilled using a rotary evaporator.

3.2.1 TLC SYSTEM

Various TLC solvent systems were tested on the crude ethanolic leaf and bulb extracts. Benzene : 1,4 dioxan : acetic acid (90 : 25 : 4) was found to give the best separation for both extracts and was subsequently used for the preparation of TLC "fingerprints" (CHAPTER 5). This solvent system was used in all further sample separations.

The extracts (0.5 mg) were streaked on the TLC plates in 0.5 cm bands. TLC plates (10 cm) were developed over 8 cm. The solvent front was marked and the plates were allowed to dry before examination under UV_{254nm} and UV_{366nm}. The plates were then stained with anisaldehyde-sulphuric acid reagent (0.5 mℓ anisaldehyde, 10 mℓ sulphuric acid, 85 mℓ methanol and 5 mℓ concentrated sulphuric acid mixed in this order). The stain was poured over the plates, which were then heated for 10 min at 110°C. Photographs were taken of the plates using UV and standard fluorescent lighting.

3.2.2 SERIAL EXTRACTION

Serial extractions were performed on the leaf, bulb and root material using a Soxhlet apparatus. Dried ground material (10 g) was placed in a thimble and covered with glass wool. The solvent (150 mℓ) was heated to boiling and maintained for 2½ h. This was allowed to cool and drain, before being filtered through Whatman No. 1 filter paper and dried under vacuum at 35°C. The residue was weighed and resuspended at 10 mg $m\ell^{-1}$. This procedure was repeated for the next solvent using the same material and thimble. The solvents used were hexane, followed by ethyl acetate, ethanol and water.

The extracts were then tested in the COX-1 and COX-2 assays at a concentration of 250 μg $m\ell^{-1}$ (Figure 3.1 - 3.3). A dilution series was prepared for the bulb extracts (all

three fractions) for testing in the COX-1 and COX-2 assays (Figure 3.4 A & B).

IC_{50} values were calculated by regression analysis of the results for four different concentrations of the sample for the bulb extract (ethyl acetate fraction). The IC_{50} value for indomethacin was determined to be 3.1μM for the COX-1 assay and 188 μM for the COX-2 assay (Appendix I).

3.2.3 CHLOROPHYLL SEPARATION AND ASSAY

Chlorophyll has been found to exhibit activity in the COX-1 assay, which necessitated the introduction of a further purification step before the activity of the leaf extracts could be verified. A Sephadex® (LH-20) column (25 mm x 520 mm) was prepared using a solvent system of cyclohexane : dichloromethane : methanol (7 : 4 : 1), and maintained in a coldroom at 10°C. Dried leaf material (5 g) was extracted in 100 mℓ ethanol giving a residue of 779 mg. The residue (525 mg) was resuspended in 2 mℓ of the solvent system and applied to the column. This was followed by a 1 mℓ rinse and this was allowed to run into the column before the eluent was added. The remaining residue (254 mg) was resuspended at 10 mg $m\ell^{-1}$ and assayed in the COX-1 assay. Fractions (60) were collected at 20 min intervals (± 15 mℓ). These were dried down and combined into 14 fractions according to their TLC profiles, before being resuspended at 10 mg $m\ell^{-1}$. This gives a test concentration of 250 μg $m\ell^{-1}$ in the assay.

3.3.4 BULK EXTRACTION

Dried, ground bulb material (500 g) was left in hexane (500 mℓ) for 24 h. This solution was mixed using an UltraThurax for 2 periods of 5 min. This was allowed to stand for 45 min before the hexane was decanted. Fresh solvent was added (500 mℓ) and the procedure was repeated until the solvent was clear (three times in total). TLC plates were used to check the extract. The decanted solvents were filtered through Whatman No. 1 filter paper and the combined extract was dried under vacuum at 35°C. The bulb material was allowed to dry for 48 h. This material was divided into 40 g portions and placed in thimbles for extraction using a Soxhlet apparatus. These were extracted for 4 h using ethyl acetate (200 mℓ). The solvent was replaced and the

material extracted for a further 4 h (until the solvent was clear). The extracts were combined, filtered and dried down. TLC plates were run to check the profile against the earlier serial extraction. The Soxhlet extraction was repeated using ethanol and water.

3.2.5 COLUMN CHROMATOGRAPHY - BULK EXTRACT (BULB)

The bulk ethyl acetate extract was chosen to attempt the isolation of the active principle. A portion of the residue (431 mg) was resuspended in the eluent (3 mℓs of cyclohexane : dichloromethane : methanol, 7:3:1) and applied to a Sephadex LH-20 column (25 mm x 520 mm) at 10°C. Fractions (580) were collected at 15 min intervals (±10 mℓ). These were dried down and run on TLC plates. Fractions with similar profiles were combined (69 sample fractions) and assayed in the COX-1 test at a concentration of 100 μg mℓ^{-1}. The fractions with the highest activity were chosen for further purification.

3.3 RESULTS

3.3.1 SERIAL EXTRACTION

Extracts were prepared (10 mg $m\ell^{-1}$) from the hexane, ethyl acetate and ethanol fractions obtained through the serial extractions of the leaves, bulbs and roots of *E. autumnalis autumnalis*. These extracts were tested for activity in COX-1 and -2 assays (Figures 3.1 to 3.3). The aqueous fractions showed insignificant activity in the assays (and did not separate on TLC), and were thus not investigated further. It is evident from these graphs that the COX-1 inhibitory activity of the non-polar fractions was higher than that of COX-2 in the leaves and bulbs, while the roots showed higher COX-2 activity for all three fractions. Root material from *Eucomis* bulbs is, however, limited and the bulk extraction for the isolation of active principles is not feasible. Compounds from the leaf extract showing COX-2 inhibitory activity were non-polar in nature since they were all extracted by the non-polar hexane and ethyl acetate solvents, with the polar ethanol solvent showing no activity in the COX-2 assay. The bulb and root extracts, however, showed high COX-2 activity in all three fractions, suggesting that more than one type of anti-inflammatory compound (showing activity in the COX-2 assay) was present in these plants.

Figure 3.4 represents dilution curves of these fractions. Regression analysis was performed on the ethyl acetate fraction to enable calculation of the IC_{50} values (Table 3.1). The extracts showed high levels of both COX-1 and COX-2 inhibitory activity, but the COX-1 assay was chosen for further bioassay-guided fractionation due to both the relative expense and availability of the enzymes.

TABLE 3.1: IC_{50} values for COX inhibition by the ethyl acetate fraction obtained from *E. autumnalis autumnalis* bulbs.

	IC_{50} VALUES ($\mu g\ m\ell^{-1}$)		COX-2/COX-1 RATIO
	COX-1	COX-2	
E. autumnalis autumnalis	22	45	2.0
Indomethacin	3.1	188	61

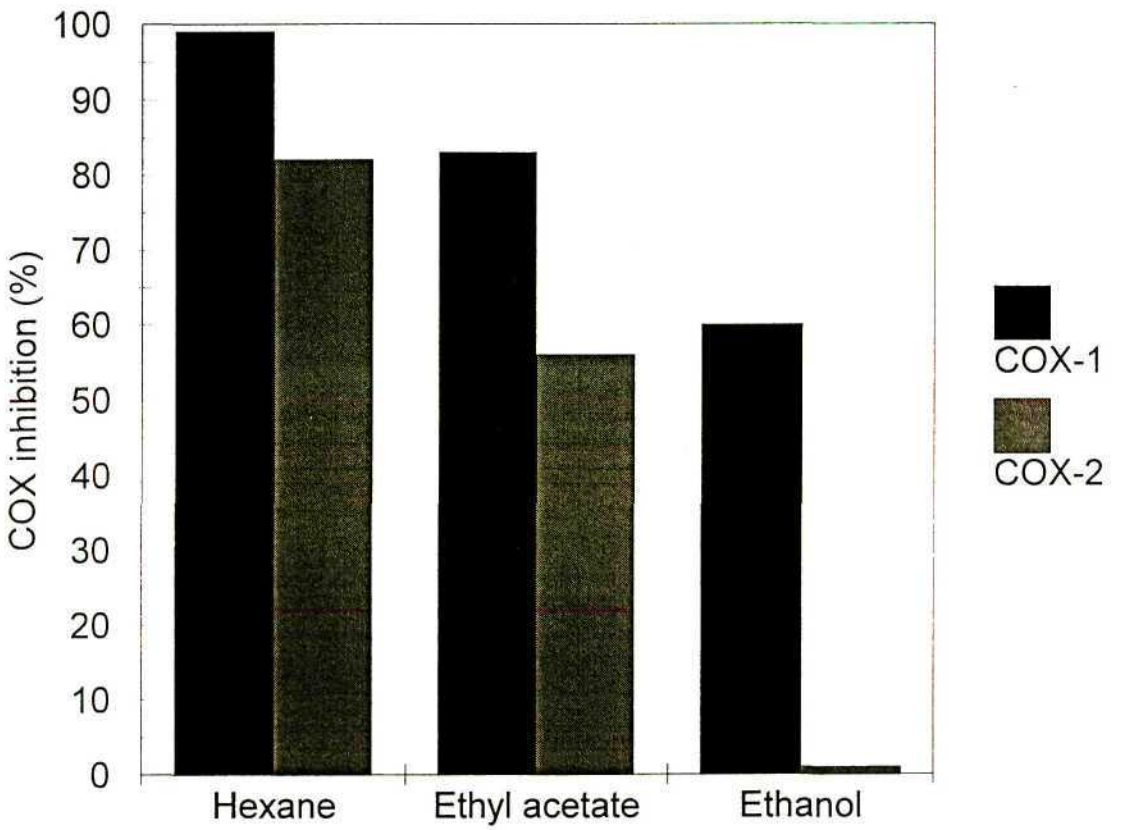


FIGURE 3.1: The relative inhibition of COX-1 and COX-2 enzymes by the hexane, ethyl acetate and ethanol fractions from the serial extraction of *E. autumnalis autumnalis* leaves. Screening concentration of extracts was 250 μg mℓ^{-1}.

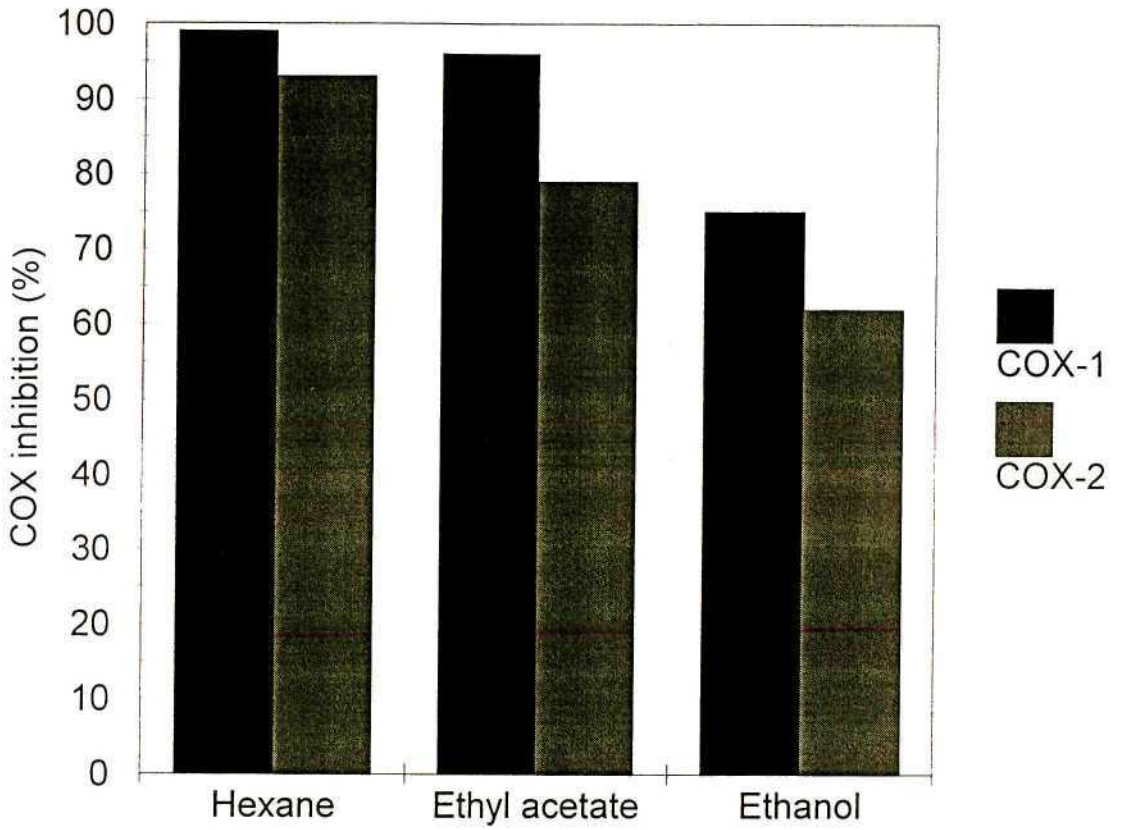


FIGURE 3.2: The relative inhibition of COX-1 and COX-2 enzymes by the hexane, ethyl acetate and ethanol fractions from the serial extraction of *E. autumnalis autumnalis* bulbs. Screening concentration of extracts was 250 μg mℓ^{-1}.

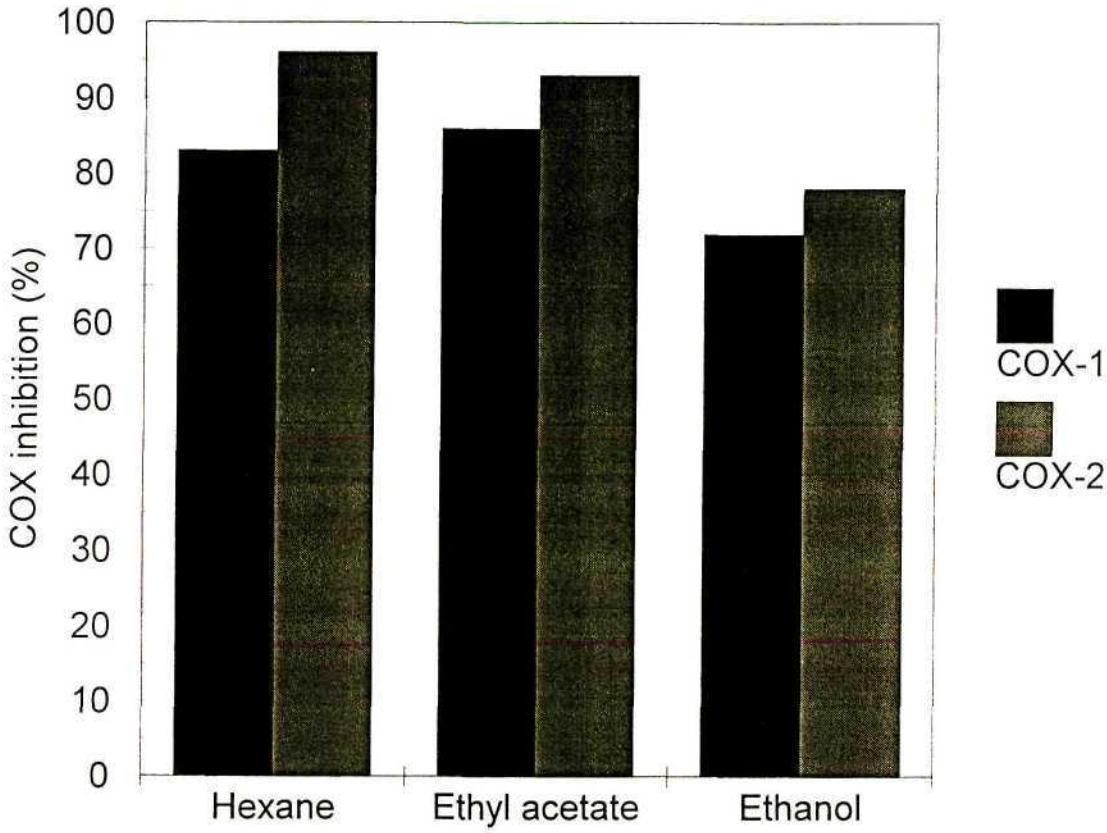


FIGURE 3.3: The relative inhibition of COX-1 and COX-2 enzymes by the hexane, ethyl acetate and ethanol fractions from the serial extraction of *E. autumnalis autumnalis* roots. Screening concentration of extracts was 250 μg mℓ^{-1}.

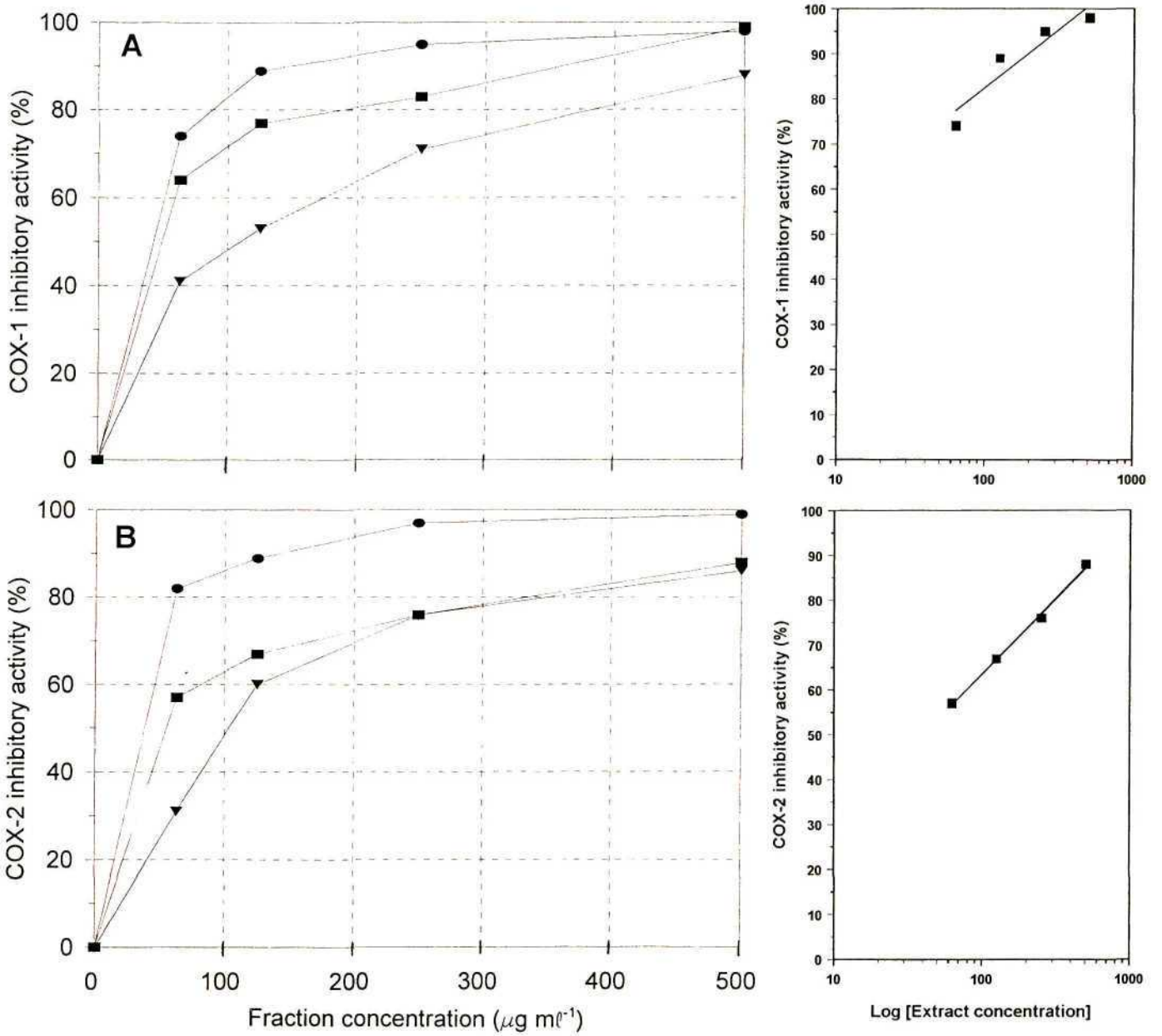


FIGURE 3.4: The relative inhibition of (A) COX-1 and (B) COX-2 enzymes by dilutions of the hexane (●), ethyl acetate (■) and ethanol (▼) fractions obtained from the serial extraction of *E. autumnalis autumnalis* bulbs (Left). Regression analyses of the ethyl acetate fraction (Right).

3.3.2 CHLOROPHYLL SEPARATION AND ASSAY

The combined fractions collected from the Sephadex LH-20 column separation of the ethanolic leaf extract were run on a TLC plate (Plate 3.2). It is evident that Fractions 2-5 and 7-10 exhibited the red luminescence under UV light (366nm) associated with chlorophyll molecules. These fractions were dried and resuspended at 10 mg $m\ell^{-1}$ before testing in the COX-1 assay (Figure 3.5). Fractions 5, 13 and 14 showed greater activity than the original extract (W).

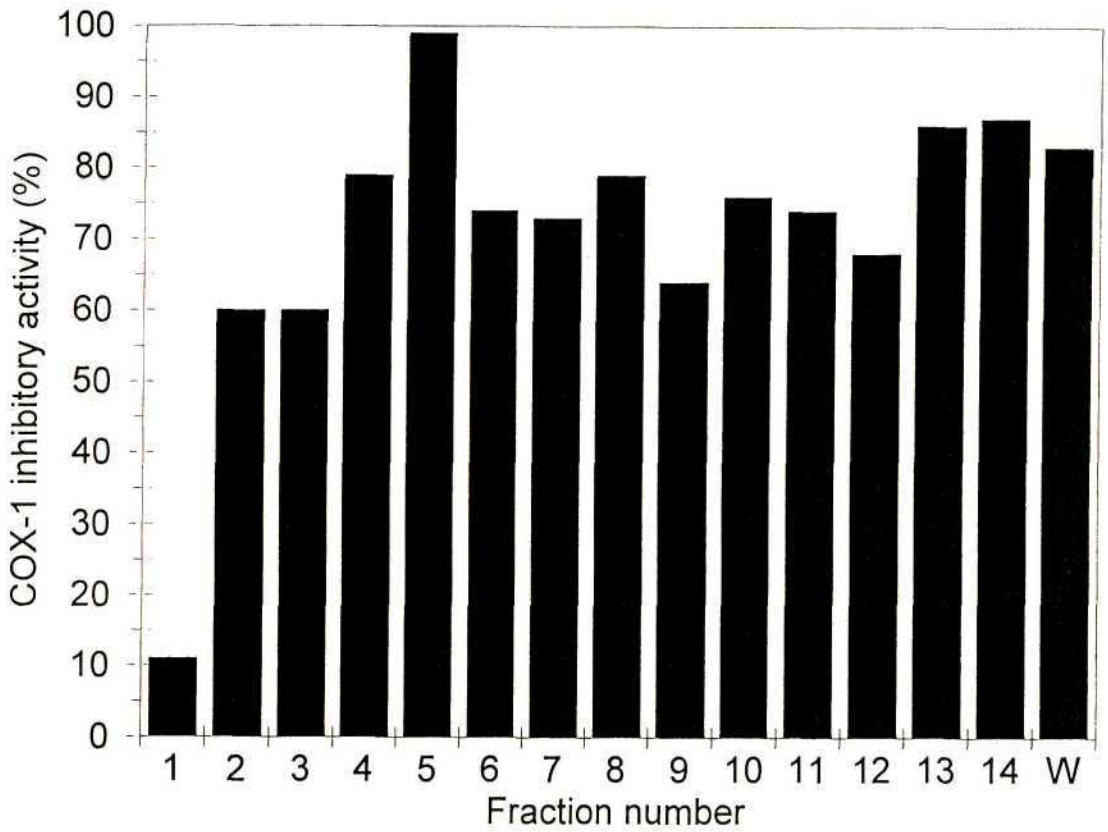


FIGURE 3.5: The % inhibition of the COX-1 enzyme by different fractions obtained from the Sephadex LH-20 separation of the ethanol extract of leaves of *E. autumnalis autumnalis* (solvent system of cyclohexane : dichloromethane : methanol (7:4:1)). Screening concentration for extracts was 250 μg $m\ell^{-1}$.

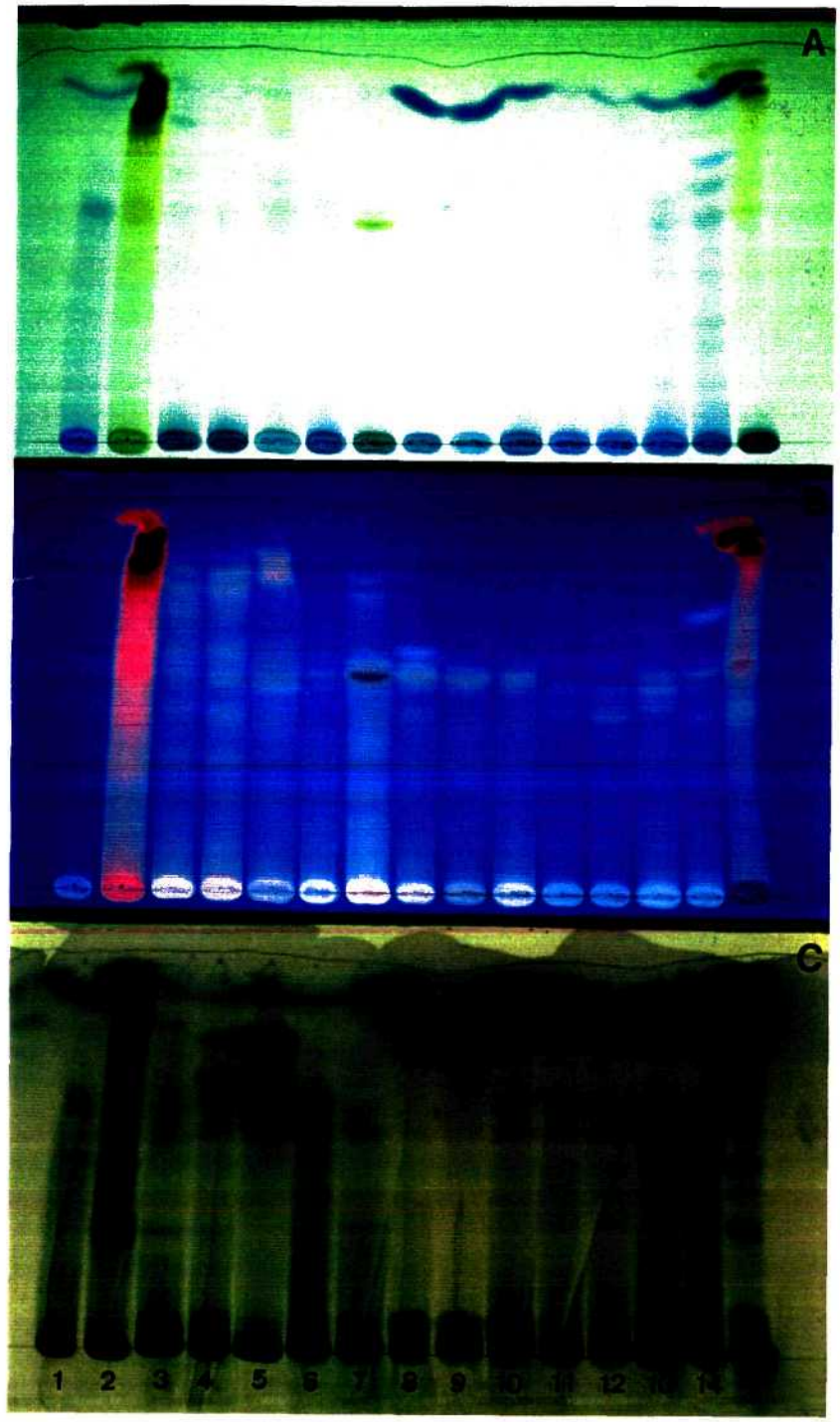


PLATE 3.1: TLC profiles of fractions obtained from the separation (Sephadex LH-20 column, solvent system of cyclohexane : dichloromethane : methanol (7:4:1)) of the ethanol extract of the leaves of *E. autumnalis autumnalis*. Viewed under (A) $UV_{254\ nm}$; (B) $UV_{366\ nm}$; and (C) after staining with anisaldehyde. TLC solvent system used was benzene : 1,4-dioxan : acetic acid (90 : 25 : 4). [W = whole extract].

3.3.3 BULK EXTRACTION

The hexane extract was yellow in colour, and dried down to a yellow, oily residue that solidified on cooling (3.107 g) indicating the presence of lipids. This melted over a low heat, and 50 mℓ hexane was added to the residue for storage in the cold room.

The ethyl acetate residue measured 5.011 g (oil), representing 1.02 % of the original extract of 500 g. This was an orange-yellow extract which dried down to an orange-brown oily residue. On standing, this separated into two layers, a lower orange solution with a thin film of a dark orange-brown oil on top. This oil was tested against the solution using TLC and very similar profiles were obtained (Figure 3.6). Before removing a proportion of this fraction to apply to the Sephadex LH-20 column, the extract was shaken to mix the two layers.

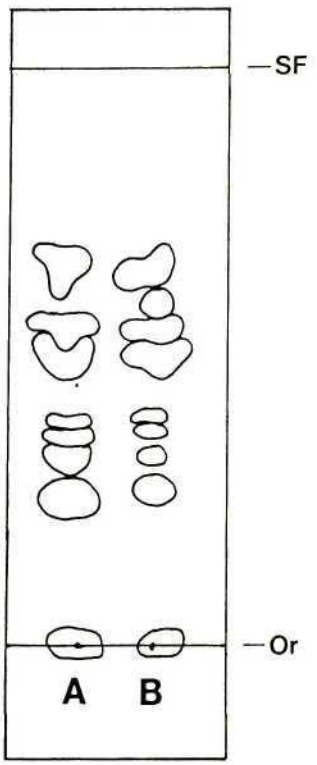


Figure 3.6: Diagrammatic representation of the separation of the (A) oil, and (B) solution comprising the ethyl acetate fraction. Solvent system used was benzene:1,4-dioxan:acetic acid (90:25:4).

The ethanol fraction was orange in colour and dried down to give an orange-white precipitate and an orange-brown sticky toffee-like residue which could not be dried to weigh.

3.3.4 COLUMN CHROMATOGRAPHY - BULK EXTRACT (BULB)

The ethyl acetate fraction was chosen to conduct bioassay-guided fractionation. High levels of activity were exhibited by this fraction, and the polarity of this solvent is such that it extracts a wide range of natural compounds. The combined fractions from the Sephadex LH-20 column separation of the extract were dried and resuspended at 4 mg $m\ell^{-1}$ for screening in the COX-1 assay (Figure 3.7). The fractions were separated using TLC and the results (Plate 3.2 and 3.3) showed the presence of a wide variety of different compounds in this extract. High levels of inhibitory activity were obtained for Fractions 4-6, with several other fractions showing high anti-inflammatory activity (Fractions 18; 19; 23; 31 and 51). This suggested the presence of more than one compound active in this assay. The activity of the whole extract (originally applied to the column) is represented on each graph by 'W'. Fraction 4 was chosen for further isolation (CHAPTER 4).

3.3.5 CRYSTALLIZATION

The evaporation of the elution solvents resulted in crystal formation from Fractions 6 (colourless / white crystals), 15 and 23 (yellow crystals). As the proportion of dichloromethane / methanol decreased, the crystals dropped out of solution, indicating their insolubility in cyclohexane. The crystals were washed in cold hexane and stored. The fractions from which the crystals were obtained were tested in the COX-1 assay (Figure 3.6), as were the crystals (Table 3.2).

TABLE 3.2: Anti-inflammatory activity of the crystals obtained from the Sephadex LH-20 fractionation of the ethyl acetate bulb extract using a solvent system of cyclohexane:dichloromethane:methanol (7:3:1).

Fraction (100 μg $m\ell^{-1}$)	COX inhibition (%)	
	COX-1	COX-2
Fraction 6	76	86
Fraction 15	35	19
Fraction 23	73	21

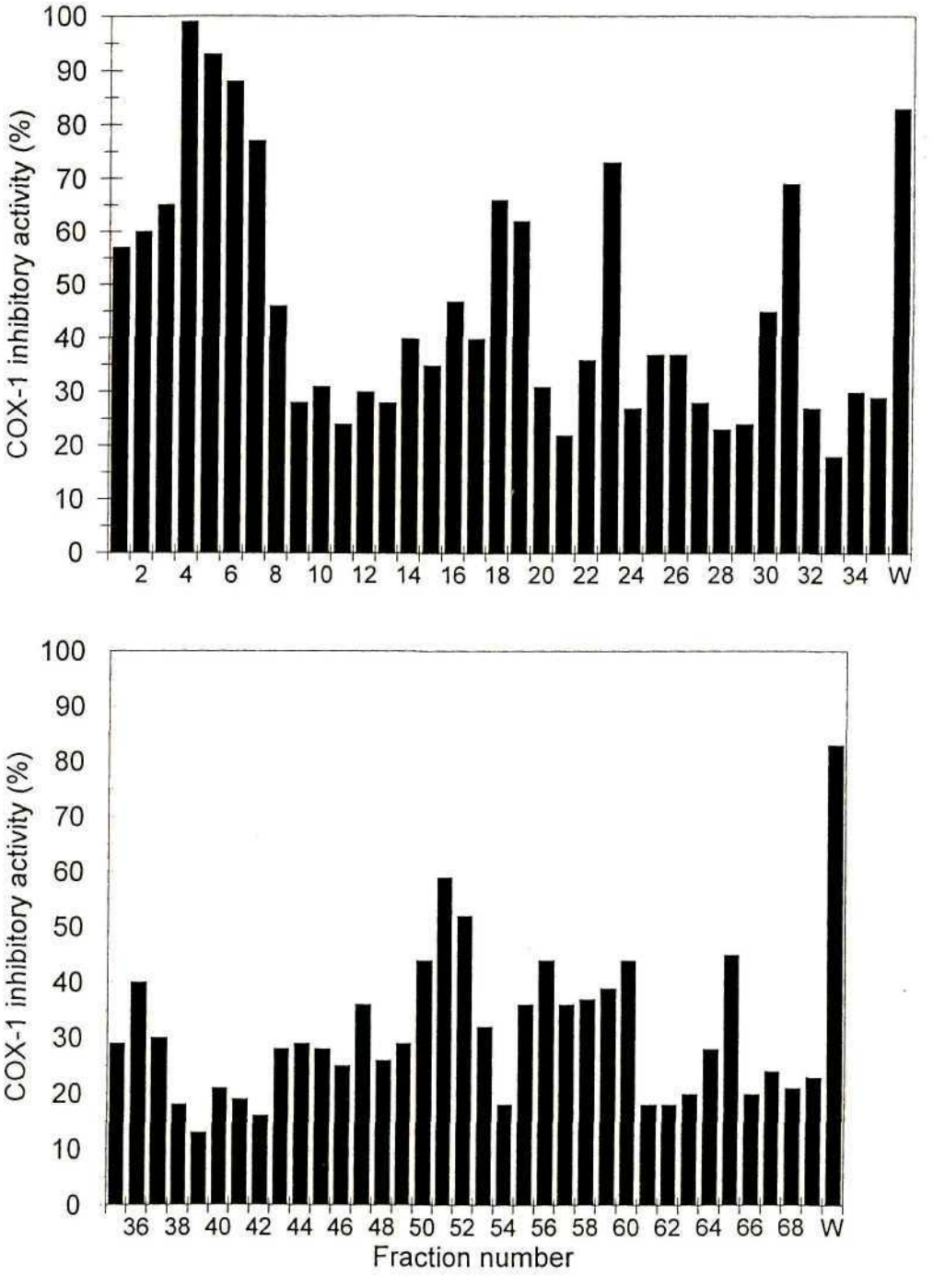
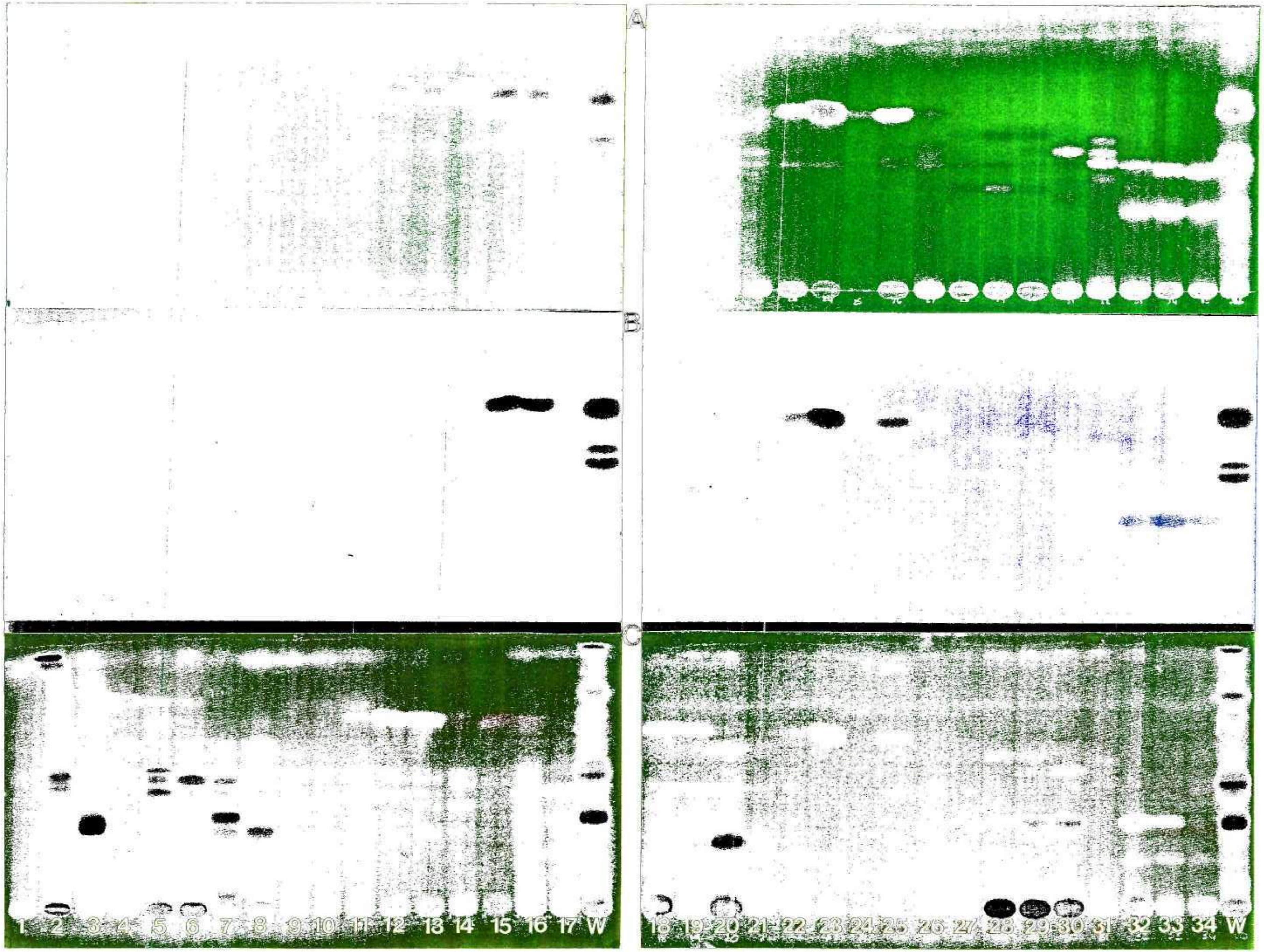
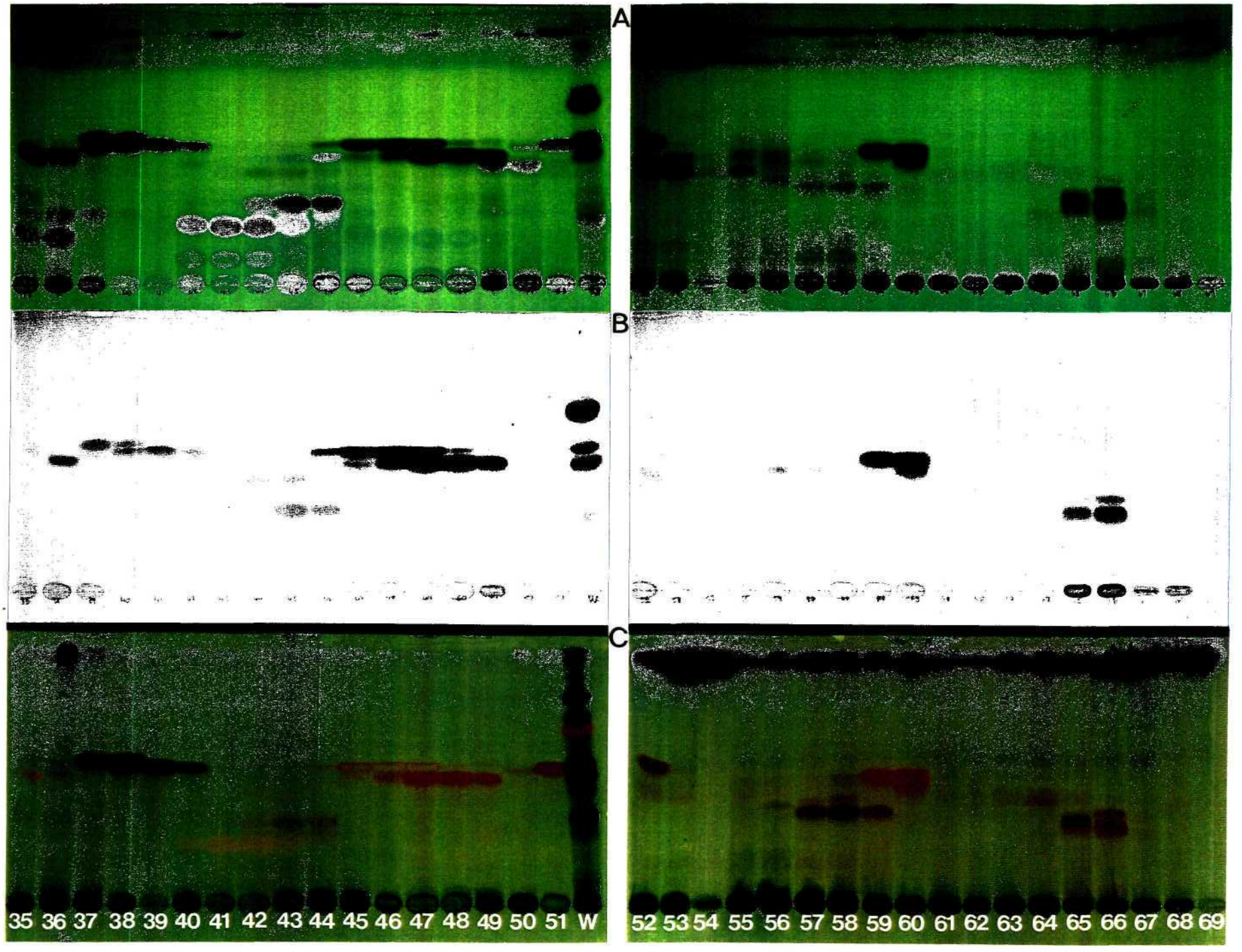


FIGURE 3.7: The % inhibition of the COX-1 enzyme by the different fractions obtained by the Sephadex LH-20 separation of the ethyl acetate extract of the bulbs of *E. autumnalis autumnalis*. (Solvent system of cyclohexane : dichloromethane : methanol (7:4:1)). Screening concentration was 100 μg mℓ^{-1}.


A
B
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 W
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 W


A
B
C
35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 W
52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69

Fraction 6 showed high activity in both the COX-1 and COX-2 assays, while Fraction 23 only showed activity in the COX-1 assay. The crystals isolated from Fraction 15 did not show significant activity in either assay. These crystals were sent for identification using NMR and mass spectrometry (CHAPTER 4).

3.4 DISCUSSION

It is important from both a financial and time economic perspective, at every stage of the isolation process to have two fractions available to test for comparative activity, one from the previous fraction in the purification sequence and the second the most recently purified fraction. This enables the rapid evaluation of the separation steps conducted and directs further steps to the most active principle in the sample.

3.4.1 SERIAL EXTRACTION

The COX test results from the serial extraction of the different plant parts, analysed with the TLC fingerprints enabled a clear comparison of the chemical constituents of the respective plant organs. These plants show high levels of activity in both the COX-1 and COX-2 assays, which validates their role as anti-inflammatory agents in traditional medicine. The combination of activity against both isoforms of the cyclooxygenase enzyme increases their efficiency. While serial extraction is a classical method for the extraction of organic compounds from plant material, this procedure does have drawbacks in that the complete separation of the compounds is rarely achieved and the same compounds may be recovered in varying proportions in several fractions (HARBORNE, 1998). This resulted in three of the fractions showing high levels of COX inhibition (Figure 3.1-3.3).

3.4.2 CHLOROPHYLL SEPARATION AND ASSAY

Photosynthetic pigments (chlorophylls and carotenoids), are isoprenoid plant lipids, or prenyl lipids. This group includes the sterols, prenylquinones and prenols (LICHTENTHALER, 1987). These fat-soluble compounds are extracted from plant tissues with organic solvents, especially solvents such as methanol and ethanol which can take up water. The crude leaf extracts thus contained high levels of these pigments, which are known to create false positives in the COX assay.

The results from this test show that although some of the activity associated with the leaf extracts was due to the presence of chlorophyll, once this had been separated from the extract, significant levels of COX-1 inhibitory activity were detected. Leaves are reportedly applied as poultices and are used to wind around the wrists to lower fever (ROBERTS, 1990). The presence of compounds in the leaves, shown to be active against both COX-1 and COX-2 enzymes, explains this usage. This has further implications for the sustainable use of this plant from a medicinal and conservation standpoint.

3.4.3 BULK EXTRACTION AND SEPARATION

Separation using Sephadex LH-20 columns is highly efficient in the purification of natural products. The advantages lie in the inert nature of the stationary phase, which unlike silica gel, does not have the disadvantage of irreversibly binding many types of compounds. The column gives a clear and effective separation of relatively large volumes of extracts. A further advantage is that it can be used for non-aqueous systems and that it utilizes both size exclusion chromatography (gel filtration) and adsorbtion in the separation of molecules (CANNEL,1998).

The use of this system in the fractionation of the ethyl acetate bulb fraction was highly efficient, from both a time and economic viewpoint. The small size of the fractions collected gave a good division of the compounds in the extract, and the use of disposable TLC plates enabled fractions containing similar compounds to be combined. The inert nature of the Sephadex LH-20 column meant that irreversible binding of compounds to the stationary phase (as is found with silica columns) was minimized, and a high degree of compound recovery was obtained.

Testing these fractions with the COX-1 assay facilitated the identification of those with high anti-inflammatory activity. The 8-10 fractions thus identified could then be worked on further. Fraction 4 (representing 5.89 % of the fractionated extract and a yield of 0.0051 % of the original plant material) exhibited the highest COX-1 inhibitory activity and was selected for further purification studies. This represents, however, just one of the active compounds in this plant. Further studies are necessary to identify the remaining anti-inflammatory compounds in *E. autumnalis autumnalis*.

3.5 CONCLUSION

Using this system, several fractions were detected that showed high levels of COX-1 inhibitory activity. The TLC separation of these fractions showed their relative impurity, with the presence of several different lipophilic compounds eluting together. This necessitated further purification steps before identification could be attempted (CHAPTER 4).

The TLC profiles presented indicate the highly complex nature of these purified fractions. This, together with the detection of activity in several of the Sephadex LH-20 fractions, suggests the presence of several compounds exhibiting anti-inflammatory activity in these assays. This highlights the complexity of traditional medicinal remedies, compared to western drugs, which primarily consist of only one or two active constituents.

CHAPTER 4

IDENTIFICATION OF THE PRIMARY ACTIVE PRINCIPLE IN *E. AUTUMNALIS AUTUMNALIS*

4.1 INTRODUCTION

The preparation used as a medicine by local peoples is usually not identical to the sample tested in the laboratory. Most effective brews are mixtures of green plants prepared in the field. Laboratory work is done on individual plants that are collected, dried and chemically analysed. The traditional medicine is in essence a complex chemical mixture - chemical reactions occur within a herbal tea brewed as medicine - which is very different to the single compounds isolated from a dried plant (PRANCE, 1994).

Historically, ethnobotanical studies have led to the discovery of drugs utilized in three different forms. Firstly, drugs can be generated from unmodified natural products where ethnomedical use alluded to clinical efficacy (e.g. Digitalis). Alternatively, drugs have been developed from unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use (e.g. Vincristine). Lastly, modified natural or synthetic substances based on a natural product used in traditional medicine (e.g. Aspirin) have been produced (COX, 1994). This investigation followed the first route, with the use of bioassay-guided fractionation to direct the purification of the primary active compounds in the extracts.

Preparative Thin Layer Chromatography

After column chromatography, fractions may be further separated using preparative TLC on glass-backed TLC plates. Preparative TLC utilizes thick (up to 1 mm) instead of thin (0.10 - 0.25 mm) layered adsorbent (HARBORNE, 1988). The separation of compounds using silica TLC plates does carry the danger that the compounds may be irreversibly bound to the silica. The compounds must thus be exposed to the silica

for as short a time as possible. The separated compounds are recovered by scraping off the silica at appropriate places on the developed plate (HARBORNE, 1988). Compounds may be desorbed from the adsorbent (silica) using Pasteur pipettes fitted with a plug of glass wool and packed with the compound-rich sorbent. This mini-column can be eluted with solvent to recover the pure compound, using a solvent that is slightly more polar than is normally required to dissolve the sample. This ensures maximum recovery from the sorbent and minimizes the possibility of the product remaining strongly bound to the solid phase (GIBBONS AND GRAY, 1998).

High Performance Liquid Chromatography

Another purification technique widely utilized is that of high performance liquid chromatography (HPLC). This is a highly efficient separation method using the same principles as the column chromatography used in CHAPTER 3. This chromatography technique utilizes a stationary phase bonded to a porous polymer held in a narrow bore stainless steel column, and a mobile phase which is forced through the column under high pressure. The mobile phase is a miscible solvent mixture, either in constant (isocratic separation), or changing proportions (gradient elution) (HARBORNE, 1988).

A detector, usually UV, is used to monitor compounds as they elute off the column. Compounds have characteristic absorbtion coefficients, and this absorbance may be different for different compounds. Changes in UV absorbance can thus be used to monitor chromatographic separations. The monitoring of unknown compounds by UV detection is best conducted using short wavelengths near the "end absorbance", typically 200 and 220 nm (CANNELL, 1998). Almost all organic compounds will exhibit some absorbance in this range. Shorter wavelengths tend to cause interference by absorbtion by the mobile phase solvents.

Every compound has a characteristic UV spectrum, and a chromatogram can thus provide useful information about the compounds in the mixture (CANNELL, 1998). HPLC is mainly used in the separation of classes of compounds which are non-volatile (e.g. higher terpenoids, phenolics, alkaloids, lipids and sugars). The technique is most effective for compounds that can be detected in the UV or visible regions of the spectrum (HARBORNE, 1988).

Mass Spectrometry

Mass spectrometry (MS) is used to provide an accurate molecular weight of a compound. This technique requires microgram quantities of a sample, and yields a complex fragmentation pattern which is often characteristic of a particular compound. Trace amounts of an organic compound are degraded (ionised), and the fragmentation pattern based on the fragment mass is recorded. The positive ions generated are accelerated in a magnetic field, which disperses the ions, and allows the measurement of the relative abundance of ions of a given mass-to-charge ratio. This results in a mass spectral graph of ion abundance versus mass. The accuracy of this technique enables the exact molecular formula to be determined (HARBORNE, 1988).

Nuclear Magnetic Resonance

Proton NMR spectroscopy allows the structural determination of organic compounds based on the measurement of the magnetic moments of the hydrogen atoms. This enables the identification of different functional groups, but cannot provide direct information on the carbon skeleton (which is obtained using ^{13}C-NMR). The advantage of this technique (over MS) is that the sample is recovered unchanged (HARBORNE, 1988). The combination of the ^{13}C-NMR and ^{1}H-NMR techniques result in a powerful means for the structural elucidation of new terpenoids, alkaloids and flavonoids. The application of NMR spectroscopy to the structural determination of natural plant products advanced considerably in the 1980's. This has resulted in the production of high resolution spectra using more powerful instruments, and the development of additional procedures such as NOE, COSY, NOESY, INEPT and DEPT (HARBORNE, 1988). The two-dimensional spectra provide additional information to complement the basic one-dimensional ^{13}C-NMR and ^{1}H-NMR spectra.

4.2 MATERIALS AND METHODS

4.2.1 TLC PURIFICATION

Fraction 4 (25.4 mg), obtained from the Sephadex LH-20 separation of the ethyl acetate bulb extract (CHAPTER 3) was resuspended in 1 mℓ ethanol and streaked on two silica gel 60 F_{254} plates (20 x 20 cm). An additional small plate (5 x 20 cm) was run to stain with anisaldehyde. These were developed using the solvent system described in CHAPTER 3 (Plate 4.1). The two plates were divided into 11 R_f fractions according to the profile developed on the sample plate. The compounds were eluted from the silica using disposable Pasteur pipette columns stoppered with glass wool and packed with celite, using dichloromethane as the elution solvent. The celite facilitates the removal of silica from the sample. The fractions were dried and resuspended in 1 mg $m\ell^{-1}$ ethanol before testing in the COX-1 assay (Table 4.1).

4.2.2 HPLC SEPARATION

The three TLC fractions showing activity in the COX-1 assay were dried down and resuspended at 4 mg $m\ell^{-1}$. High Performance Liquid Chromatography separations were conducted using a Varian model 5000 Liquid Chromatograph linked to a Spectrasystem UV3000 HR detector (deuterium lamp). Separations were initially performed using an analytical Millipore NovaPak C_{18} column. Acetonitrile and water were used as the mobile phase and an optimal elution program was established to give clear separation of the peaks. This was determined to be an initial 70/30 mixture of acetonitrile / water grading to 100% acetonitrile over 30 min, with a flow rate of 1 mℓ min^{-1}. The detector was run for 40 min. This program was run for fractions 2, 3 and 4 with an injection volume of 10 $\mu\ell$.

Fraction 3 (8.8 mg) exhibited high COX-1 activity, with the highest residue mass, and gave the cleanest HPLC profile. This was thus chosen for semi-preparative HPLC separation to isolate the active peak. A semi-preparative Phenomenex Hypersil S C_{18} 250x10 mm, 5 micron column was used, with the same elution program and a flow rate of 2.5 mℓ min^{-1}. The remaining sample was injected (4 x 100 $\mu\ell$) and the different peaks were collected, resuspended in 1mℓ ethanol and tested for COX-1 activity. The

active peak was dried, weighed and sent for analysis. Fractions 2 and 4 were subjected to the same elution programme, and the UV 200 nm traces were compared.

4.2.3 MASS SPECTROMETRY AND NMR ANALYSIS

One-dimensional NMR spectra (^{1}H, ^{13}C and DEPT), and two-dimensional NMR spectra (NOSEY, COSY and HMBC) were performed by Professor Mulholland in the Natural Products Research Group of the Organic Chemistry Department (University of Natal Durban) and Mr Martin Watson in the School of Chemistry and Physical Sciences (University of Natal Pietermaritzburg). Four samples were sent for NMR analysis: the active peak collected from the HPLC separation, and three sets of crystals collected from the Sephadex LH-20 fractions (Fraction 6; 15 and 23). The primary active compound was further subjected to mass spectrometry by Dr Boshoff in the Mass Spectrometry Unit of the Cape Technicon.

A summary of the extraction and isolation procedures is represented in Figure 4.1.

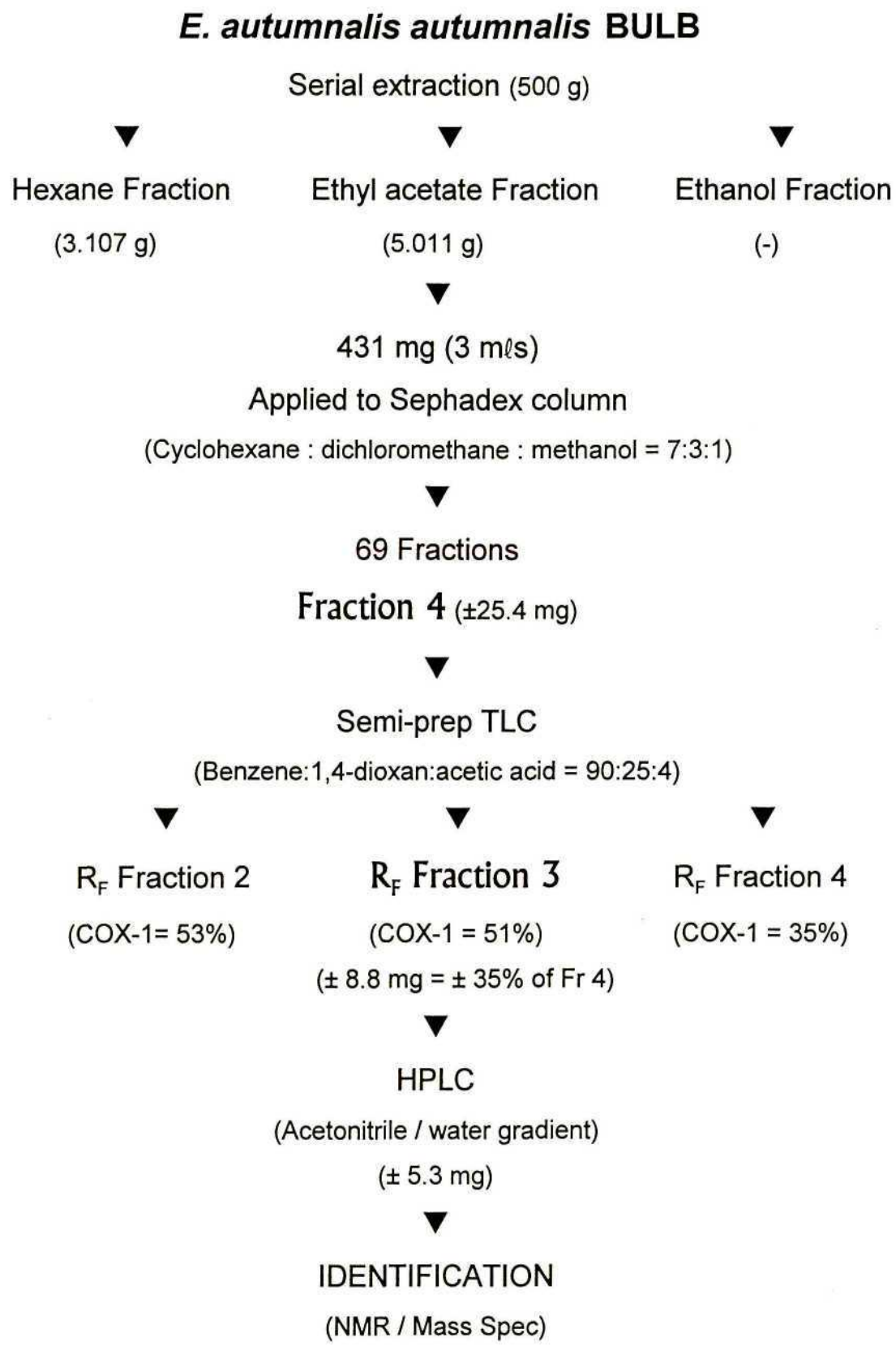


FIGURE 4.1: Flow chart representing the procedures followed in the isolation of the active principle from *E. autumnalis autumnalis* bulbs.

4.2.4 ANTI-INFLAMMATORY ACTIVITY

Dilution series were prepared from the active fraction obtained from the HPLC separation and the crystals obtained from Fraction 6 of the Sephadex LH-20 fractionation (CHAPTER 3). These samples were tested in the COX-1 and COX-2 assays. IC_{50} values were then calculated from regression analysis of these curves and the COX-2/COX-1 ratio was determined.

4.2.5 TOXICITY STUDY

Both the active compound and the crystals obtained from Fraction 6 were tested for their ability to initiate DNA damage. This test is utilized in the search for anti-cancer agents, since active compounds would be able to initiate DNA damage within tumour cells (WHITE *et al*, 1986). The procedure described by WHITE *et al.* (1986) was followed using concentrations of 10 and 100 μg $m\ell^{-1}$. The two compounds tested in this assay were found to be inactive (T. RABE pers comm.).

4.3 RESULTS

4.3.1 SEMI-PREPARATIVE TLC

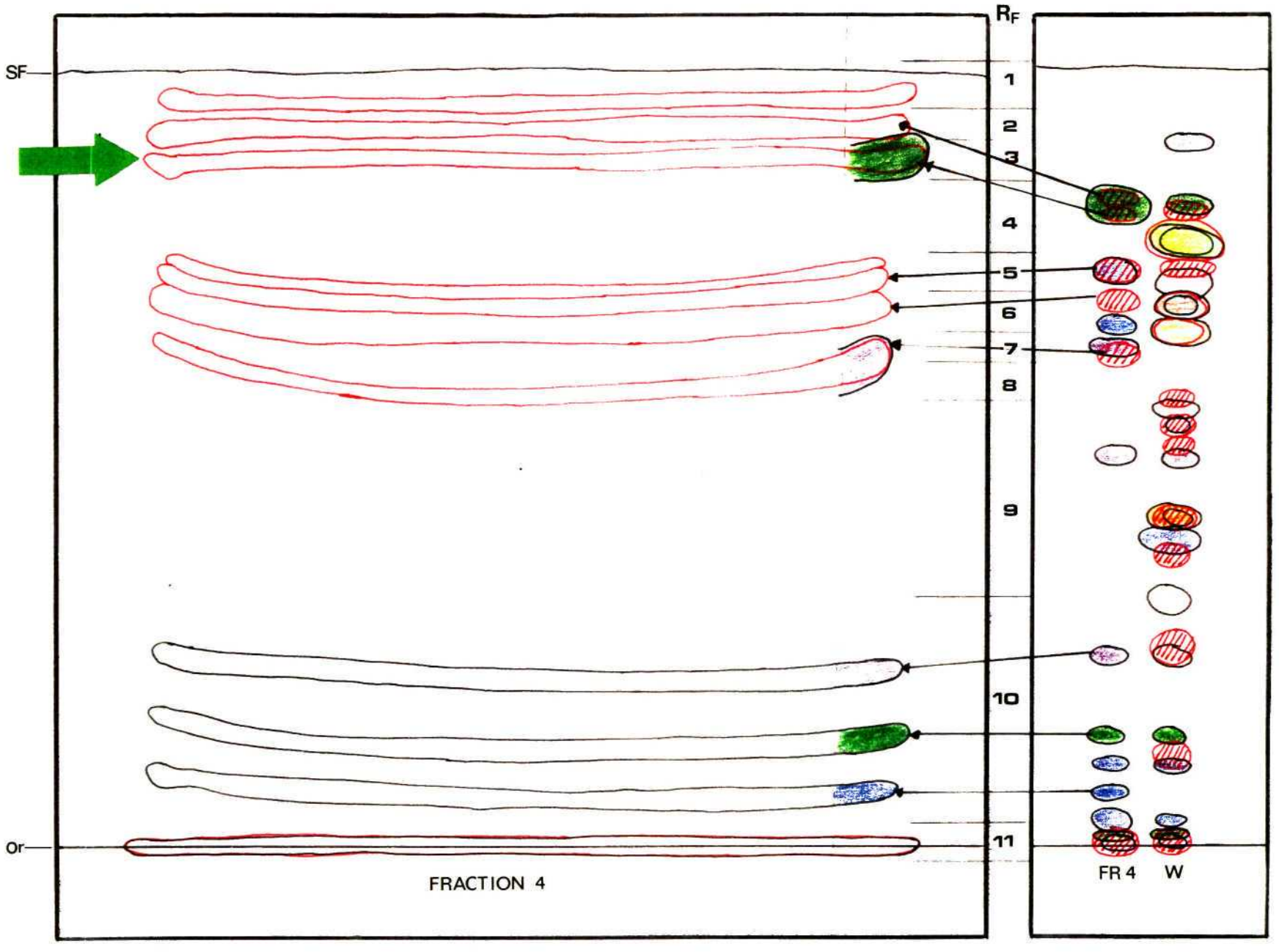
The TLC plates were viewed under UV light (Plate 4.1). Spots that luminesce under UV light are marked in red. The plates were divided into R_f fractions as marked on Plate 4.1. The COX-1 inhibitory activity of these fractions is given in Table 4.1. Extracts were diluted to a test concentration of 25 μg $m\ell^{-1}$. This was compared to the activity of the whole fraction (FR 4) at 25 μg $m\ell^{-1}$, and the activity of the original ethyl acetate extract (W) at 100 μg $m\ell^{-1}$.

Table 4.1: The residue masses obtained from the dried TLC fractions (eluted with dichloromethane), correlated with COX-1 inhibitory activity.

RF fraction	Residue (mg)	COX-1 inhibition (%)
1	0.6	0
2	**1.5**	**54**
3	**8.8**	**51**
4	**3.2**	**35**
5	0.2	12
6	0.7	6
7	0.7	5
8	2.8	0
9	1.2	3
10	1.1	5
11	4.6	0
FR 4	-	96
W	-	60

Relatively high activity was obtained for Fractions 2, 3 and 4. Fraction 3 was most abundant in terms of residue mass, and this fraction was fractionated further.

PLATE 4.1: Semi-preparative TLC separation of Fraction 4, obtained from the fractionation of the ethyl acetate extract of the bulbs of *E. autumnalis autumnalis* on a Sephadex LH-20 column (cyclohexane : dichloromethane : methanol, 7:4:1). TLC solvent system used was benzene : 1,4-dioxan : acetic acid (90 : 25 : 4). Spots that luminesce under UV light are marked in red. Mini-plate (Right) shows the separation of Fraction 4 compared to the original ethyl acetate extract (W). Colours indicate different bands obtained after staining with anisaldehyde-sulphuric acid.


SF
Or
R_F
1
2
3
4
5
6
7
8
9
10
11
FR 4
W
FRACTION 4

4.3.2 HPLC SEPARATION

The HPLC profiles for TLC fractions 2, 3 and 4 are represented in Figures 4.2 - 4.4. The different peaks obtained through the HPLC separation of Fraction 3, and the corresponding activity obtained using the COX-1 assay is represented in Table 4.2. The highest COX-1 inhibitory activity was obtained for Fraction 6. This active peak (sent for identification) is indicated by the arrow in Figure 4.3. This fraction represents 2.04 % of the fractionated extract (431 mg).

TABLE 4.2: COX-1 inhibitory activity of the HPLC fractions collected (Sample concentration was 25 μg mℓ^{-1} in assay).

FRACTION	ACTIVITY (%)
Fraction 1 (T_0 - $T_{10.11}$)	10
Fraction 2 ($T_{10.11}$ - $T_{13.52}$)	20
Fraction 3 ($T_{13.52}$ - $T_{16.78}$)	19
Fraction 4 ($T_{16.78}$ - $T_{22.04}$)	4
Fraction 5 ($T_{22.04}$ - $T_{24.91}$)	35
Fraction 6 ($T_{24.91}$ - $T_{30.40}$)	64
Fraction 7 ($T_{30.40}$ - $T_{40.00}$)	4
Fraction 8 (Blank run)	7
Whole sample	50

4.3.3 MASS SPECTROMETRY AND NMR ANALYSIS

Crystals

The coloured (yellow) crystals were identified as homoisoflavanones. These samples were isolated from Fractions 15 and 23 of the Sephadex LH-20 separation, and their structures are given in Figure 4.5 A and B respectively. The structure depicted in Figure 4.5 A is 5,7-dihydroxy-6-methoxy-3-(4-methoxy benzyl)-chroman-4-one [Compound A], and in Figure 4.5 B is that of Eucomin (5,7-dihydroxy-3-(4-methoxy benzyl)-chroman-4-one) [Compound B], a compound previously isolated from *Eucomis* species (SIDWELL AND TAMM, 1970; FARKAS *et al.*, 1971).

Although very similar in structure, these compounds did not show similar levels of anti-inflammatory activity.

Results from the COX-1 assay show high inhibitory activity associated with Fraction 23 (Compound B), but insignificant inhibition associated with Fraction 15 (Compound A). Neither fraction showed significant COX-2 inhibition (Table 4.2).

The crystals obtained from Fraction 6 were colourless-white and exhibited significantly high levels of COX-1 and COX-2 inhibitory activity (Table 4.2). This compound [Compound C] was identified as eucosterol, a compound previously isolated from *Eucomis* species (SIDWELL *et al.*, 1975; ZIEGLER AND TAMM, 1976, GLASBY, 1991). The structure of this compound, a spirostane-type triterpenoid with the molecular formula $C_{29}H_{44}O_5$, is given in Figure 4.5 C.

Active principle from E. autumnalis autumnalis

Using bioassay-guided fractionation and focussing on the most active fraction from each procedure, one of the most active compounds in the bulb extract was determined to be a polar compound with a molecular weight of 390. This most probably represents a phenol group attached to a conjugated hydrocarbon chain [Compound D]. MS spectra revealed no molecular ion peak, and indicated a single cleavage at the phenol group, with no fragmentation pattern (fairly intact molecule). Further work is being done to fully identify and characterize this molecule (BOSHOFF, pers comm.).

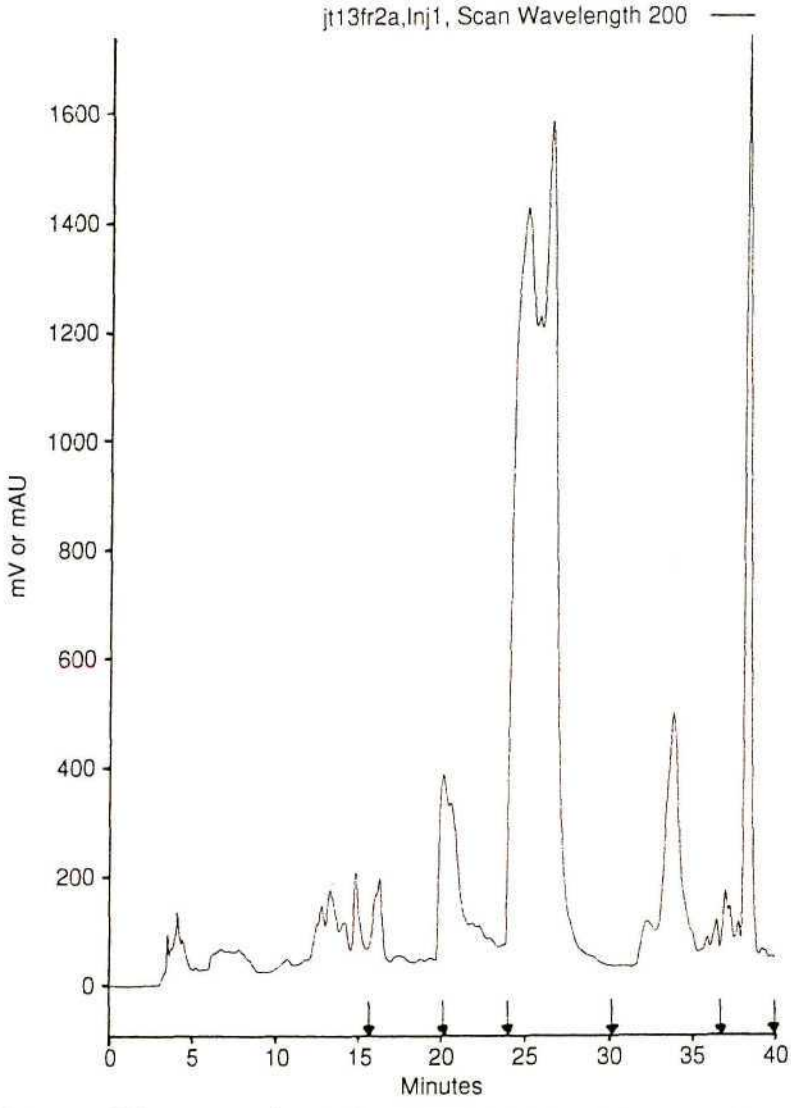


Figure 4.2: HPLC trace of the separation of Fraction 2 (obtained from semi-preparative TLC). Gradient elution of 70/30 acetonitrile/water changing to 100/0 acetonitrile/water over 30 min. Detection at 200 nm.

Table 4.3: The retention times of fractions collected from the HPLC separation of Fraction 2 (semi-prep TLC).

Fraction	Retention time
Fraction 1	T00.00 - T16.21
Fraction 2	T16.21 - T20.21
Fraction 3	T20.21 - T24.16
Fraction 4	T24.16 - T30.38
Fraction 5	T30.38 - T37.42
Fraction 6	T37.42 - T40.00
Fraction 7	T40.00 - T62.00

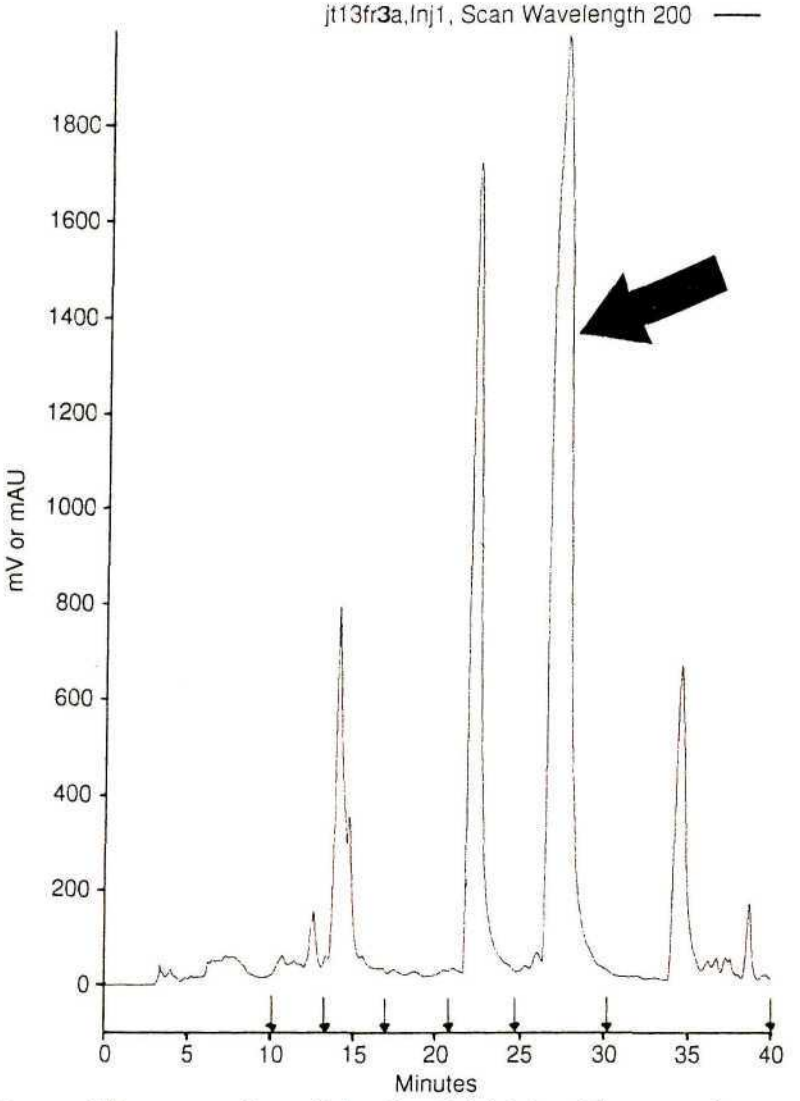


Figure 4.3: HPLC trace of the separation of Fraction 3 (obtained from semi-preparative TLC). Gradient elution of 70/30 acetonitrile/water changing to 100/0 acetonitrile/water over 30 min. Detection at 200 nm. Peak showing COX-1 inhibitory activity indicated by arrow.

Table 4.4: The retention times of fractions collected from the HPLC separation of Fraction 3 (semi-prep TLC).

Fraction	Retention time
Fraction 1	T00.00 - T10.11
Fraction 2	T10.11 - T13.52
Fraction 3	T13.52 - T16.78
Fraction 4	T16.78 - T22.04
Fraction 5	T22.04 - T24.91
Fraction 6	T24.91 - T30.40
Fraction 7	T30.40 - T37.28
Fraction 8	T37.28 - T40.00
Fraction 9	T40.00 - T50.00

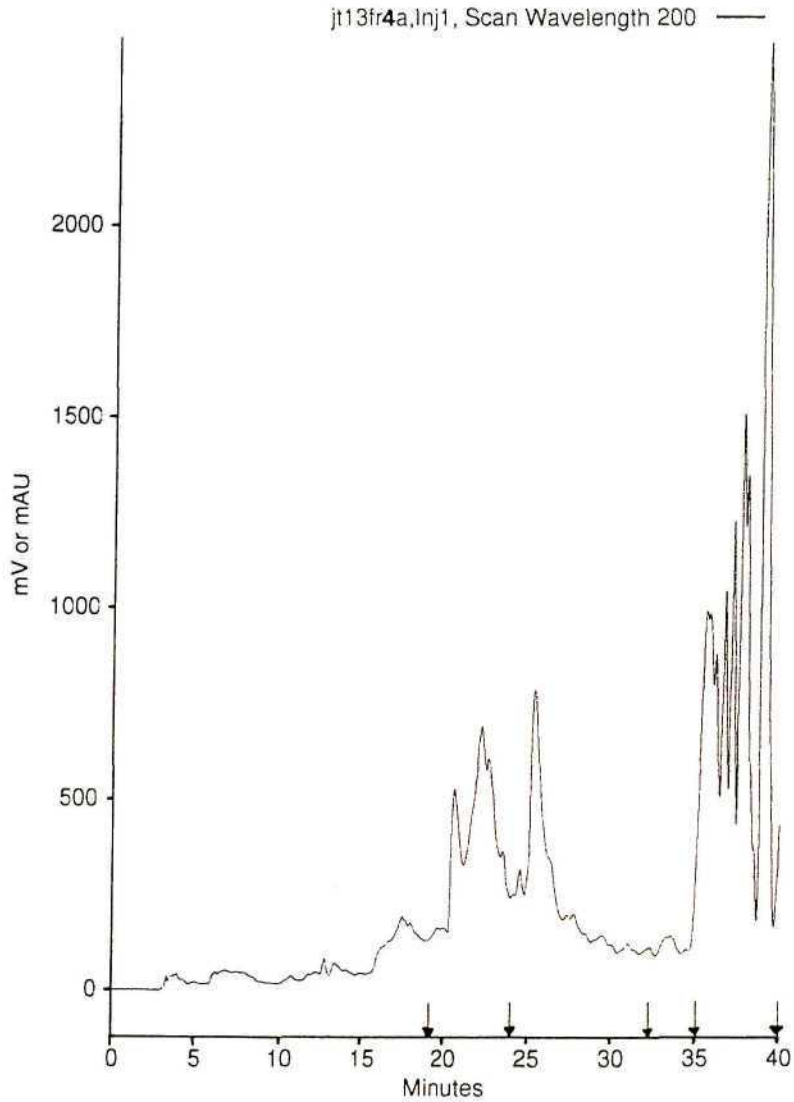


Figure 4.4: HPLC trace of the separation of Fraction 4 (obtained from semi-preparative TLC). Gradient elution of 70/30 acetonitrile/water changing to 100/0 acetonitrile/water over 30 min. Detection at 200 nm.

Table 4.5: The retention times of fractions collected from the HPLC separation of Fraction 4 (semi-prep TLC).

Fraction	Retention time
Fraction 1	T00.00 - T19.42
Fraction 2	T19.42 - T24.62
Fraction 3	T24.62 - T33.05
Fraction 4	T33.05 - T35.28
Fraction 5	T35.28 - T40.00
Fraction 6	T40.00 - T50.00

4.3.4 DETERMINATION OF RELATIVE COX-1 AND COX-2 INHIBITORY ACTIVITY

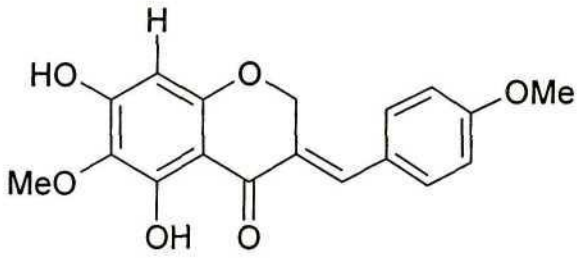
Portions of the primary active principle (Compound D), and of Compound C (crystals), were retained for testing in the COX-1 and COX-2 assays. Dilution series were prepared (Figure 4.6 and 4.7) and tested in the assays. The IC_{50} values and COX-2 / COX-1 ratios (Table 4.6) were calculated with the aid of regression analysis (using log values for the concentrations).

Table 4.6: The IC_{50} values for COX inhibition by the active compounds isolated from the bulbs of *E. autumnalis autumnalis*.

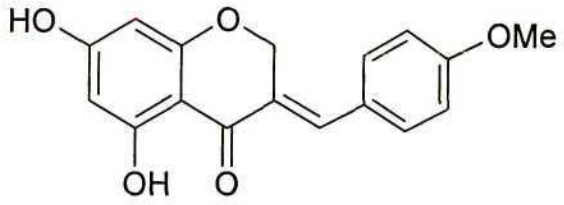
	IC_{50} VALUES ($\mu g\ m\ell^{-1}$)		COX-2/COX-1 RATIO
	COX-1	COX-2	
Compound D	14.4	30.5	2.1
Compound C	25.7	21.8	0.8
Indomethacin (μM)	3.1	188	61

A low ratio indicates that the compound is a selective COX-2 inhibitor. The results in this table indicate that while the active compound (D) isolated by bioassay-guided fractionation emerged as the most potent COX-1 inhibitor, the crystals obtained from Fraction 6 (Compound C) were more effective anti-inflammatory agents since the COX-2/COX-1 ratio was less than zero. Compound C (eucosterol) can thus be regarded as a selective COX-2 inhibitor, while Compound D favours COX-1 inhibition.

(A)



(B)



(C)

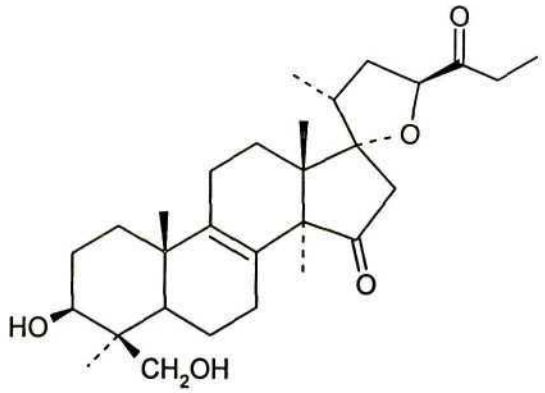


FIGURE 4.5: Molecular structures of the compounds isolated from the ethyl acetate bulb extract of *E. autumnalis autumnalis*, separated on a Sephadex LH-20 column using cyclohexane : dichloromethane : methanol (7:4:1).
(A) 5,7-dihydroxy-6-methoxy-3-(4-methoxy benzyl)-chroman-4-one (isolated from Fraction 15); (B) 5,7-dihydroxy-3-(4-methoxy benzyl)-chroman-4-one [eucomin] (isolated from Fraction 23); (C) eucosterol (isolated from Fraction 6).

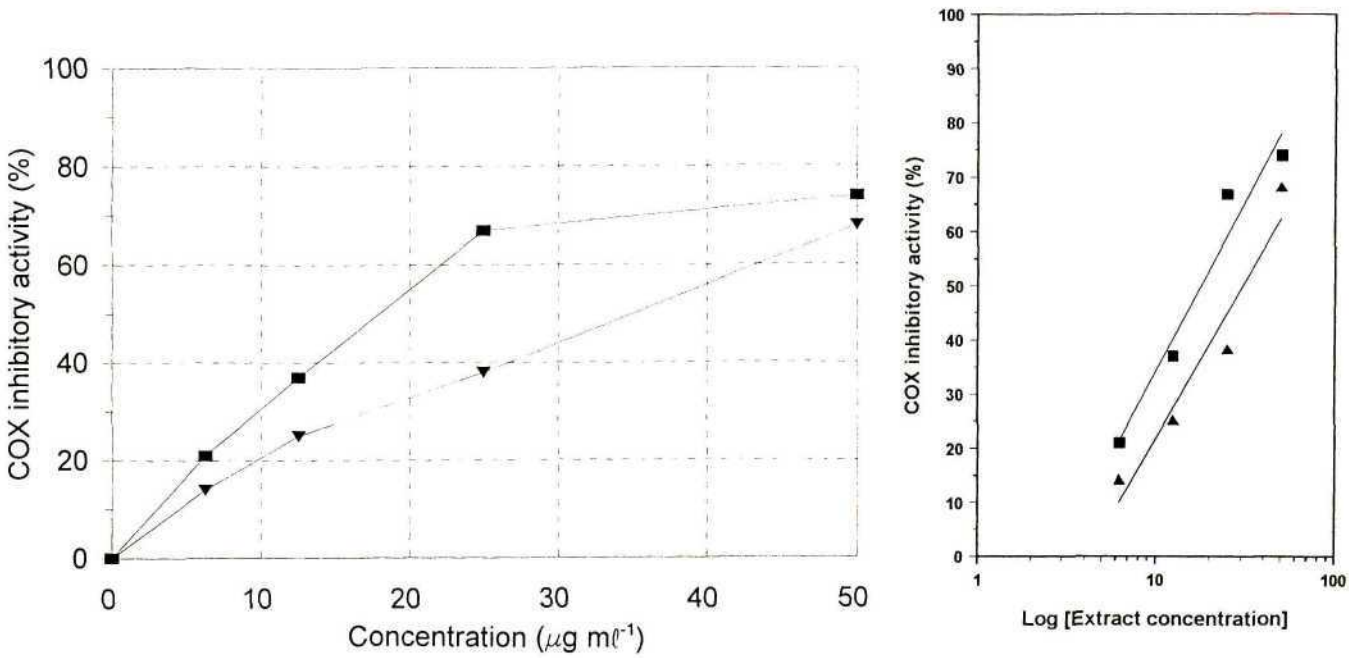


FIGURE 4.6: Dilution curves (Left) and regression analysis (Right) of Compound D (after HPLC separation) tested in the COX-1 assay (■) and the COX-2 assay (▼).

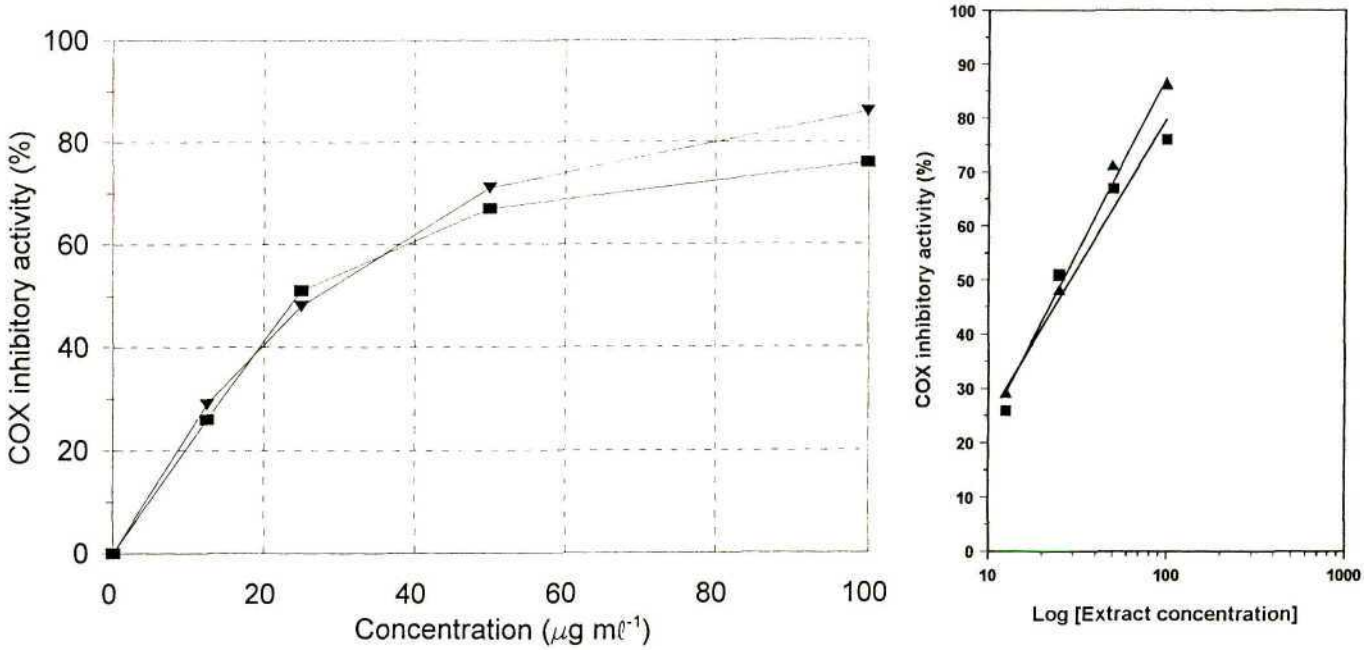


FIGURE 4.7: Dilution curves (Left) and regression analysis (Right) of Compound C (crystals from Fraction 6 of the Sephadex LH-20 column) tested in the COX-1 assay (■) and the COX-2 assay (▼).

4.4 DISCUSSION

4.4.1 TLC PURIFICATION

Silica gel is described as a "reactive" sorbent, and some natural products may be unstable on this phase (GIBBONS AND GRAY, 1998). The period between development of the TLC plate and elution of the compounds from the silica should thus be minimized to prevent irreversible binding of the compounds to the sorbent. The area of silica stained with anisaldehyde-sulphuric acid reagent was separated from the rest of the plate by a scored line to minimize migration of the reagent (GIBBONS AND GRAY, 1998). This silica, contaminated by the stain-reagent, was not eluted. The TLC solvent system used in the separation was, however, not totally efficient, as indicated by the presence of the active compound in three of the R_f fractions collected. HPLC was necessary to more effectively separate the active compound from contaminating substances.

4.4.2 HPLC SEPARATION

A compound will always have the same retention time under a given set of conditions. The identification of a peak or compound based on retention time is, however, not conclusive, as other compounds may have the same, or indistinguishably similar, retention times. On a chromatogram with sharp peaks, the relationship between peak height and concentration is closely related to that between peak area and concentration. Peak height can thus be used to approximate the proportion of natural product recovered at each stage of the purification (CANNELL, 1998). The HPLC separation represented in Figure 4.3 shows a high proportion of the active compound, as well as the presence of other contaminating substances. The active peak is also represented in Figures 4.2 and 4.4, explaining the activity of these fractions before HPLC separation. Fraction 2 (which showed the highest activity in the COX-1 assay) shows a high proportion of the active compound, but is contaminated by the presence of other compounds with similar elution times.

The use of gradients in HPLC separations is advantageous in that they allow for the separation of components with widely different properties in a single separation. This method also often results in better separations of closely eluting solutes, since the

gradient sharpens and focuses the chromatographic bands (CANNELL, 1998). Isocratic elutions (a single, unchanging solvent system) are suitable for mixtures where all the components are eluted from the column within a reasonable time. Where this does not occur, a system in which the mobile phase composition changes during the separation can be used (gradient elution). The use of two solvents, the proportions of which change during a run, enables the separation of compounds with widely different retention times. The mobile phase changes may occur either at a number of set intervals (step gradient) or continuously throughout the run (continuous gradient) (CANNELL, 1998). The continuous gradient developed and used in this separation gave a relatively sharp peak for the active compound (Figure 4.3), well separated from any contaminants. This fraction (HPLC Fraction 6) was considered pure enough to send for further analysis using NMR and mass spectrometry.

4.4.3 MASS SPECTROMETRY AND NMR ANALYSIS

Mass spectrometry (MS) requires only microgram amounts of material and can provide an accurate molecular weight of natural compounds, and give its molecular formula. MS yields complex fragmentation patterns that are often characteristic of particular compounds. Proton nuclear magnetic resonance spectrometry (NMR) facilitates the structural determination of a compound by identifying the nature and position of the hydrogen atoms attached in different functional groups (e.g. $-CH_3$, -CHO) (HARBORNE, 1998). Used together, these techniques can accurately characterize and identify the molecular structure of a compound. These specialized techniques were utilized to yield the structural data on Compounds A to D.

Flavonoids and homoisoflavanones

Flavonoids, alkaloids, quinones and terpenoids are stable over time, and have successfully been isolated from dry herbarium specimens several years old (HARBORNE, 1998). Flavonoids are widely distributed in plants, concentrated in aerial plant parts. They act as inhibitors of a wide variety of mammalian enzyme systems and have been attributed with diverse pharmacological activity (ANTON *et al.*, 1986; MIDDLETON, 1988). Flavonoids are low molecular weight compounds, and the phenyl benzo-γ-pyrones (phenyl chromones) are present in most vascular plants (LEWIS, 1989). Selected flavonoids are thought to affect immunological functions and exert protective effects against cell damage produced by lipid peroxidation stimulated

by toxins. Other effects include modulation of the activity of enzymes such as phospholipase A_2 (Refer to Figure 1.2), phospholipase C, the lipooxygenases and cyclooxygenases (Refer to Figure 1.2 and 1.3), and the protein kinases (MIDDLETON, 1988). This action may account for the anti-allergic and anti-inflammatory activity of some flavonoids. In addition, certain flavonoids are thought to inhibit tumour promoter activity (potential anti-cancer agents), while others possess potent anti-viral activity (MIDDLETON, 1988).

Flavonoids are known to potentiate the action of prostacyclin PGI_2. This is an anti-aggregating agent secreted by the vascular endothelium. It is thought that flavonoids act through a variety of mechanisms, for example on membrane ATPases, or on prostaglandin synthetase. They are thought to be moderators of metabolic pathways in the body. Natural flavonoids are, however, metabolized in the body, making their effect transient at best (ANTON *et al.*, 1986). In *in vitro* tests this situation differs, and activity on an isolated enzyme cannot necessarily be projected on more complex cell constituents (ANTON *et al.*, 1986). Although flavonoids have been linked to anti-allergic, anti-inflammatory and anti-asthmatic activity, their pharmacological activity has to be considered in terms of their bioavailability, which is probably limited (ANTON *et al.*, 1986).

Homoisoflavanones are 3-benzyl-4-chromanones, and have been isolated from the bulbs of some species of the Liliaceae family (the genera *Eucomis*, *Scilla* and *Muscari*) (DELLA LOGGIA *et al.*, 1989). They are structurally related to the flavonoids and could share some of their biological activities. Structurally, they differ from the isoflavonoids by the presence of an extra carbon atom (DEWICK, 1975). Homoisoflavanones have been shown to possess anti-inflammatory, anti-allergic, anti-histaminic and angioprotective activity (AMSCHLER *et al.*, 1996). In addition, these compounds, in common with flavonoids, flavanones and isoflavanones, show potent phosphodiesterase (PDE) inhibitor activity (AMSCHLER *et al.*, 1996).

Homoisoflavanones (eucomin and derivatives) have been found in the waxy material between the bulb scales of *E. bicolor* (HELLER *et al.*, 1976). The eucomin content of *E. bicolor* reaches a maximum in the dormant bulb, and decreases as growth progresses and the flower spike is produced. Levels of eucomin increase (in the bulbs

and roots) during the fruiting period (DEWICK, 1975). The results presented in this study linked eucomin to high COX-1 inhibitory activity (73%), but low COX-2 inhibition (21%) (Table 3.2). The seasonal variation in COX-1 activity, seen in CHAPTER 2, can be partially explained by the changes in eucomin content. It is probable that the other active compounds show similar changes in concentration.

Investigating the differences in structure between the two homoisofavanones isolated in this study, one compound exhibiting high COX-1 inhibitory activity, and the other showing insignificant activity, can elucidate structure-activity relationships. These compounds differ only in the presence of an additional methoxy group, which is associated with the loss of activity observed in the first homoisoflavanone: 5,7-dihydroxy-6-methoxy-3-(4-methoxy benzyl)-chroman-4-one (Figure 4.5 A).

Triterpenoids

The triterpenoids are a group of compounds, widely distributed in plants, with a basic structure comprising six isoprene units. These are colourless crystalline compounds with high melting points and show wide structural diversity. Many of the triterpenoids are optically active and the functional groups include alcohols, aldehydes and acids (LEWIS, 1989). Some compounds belonging to the triterpene saponins are characterized by low haemolytic activity, and low toxicity, and are regarded as practically harmless (SOKOLOV, 1986). Triterpenoid derivatives inhibit tumour promotion, inflammation and lipid peroxidation. The structural requirements for activity have been linked to the presence of a carboxylic group and alcohol group at specific sites, rather than the basic carbon skeleton of these molecules (DEL CARMON RECIO *et al.*, 1995).

The crystals [Compound C] obtained from Fraction 6 (Sephadex LH-20 column), were identified as eucosterol (Figure 4.5 C), a compound previously isolated from *Eucomis* species (SIDWELL *et al.*, 1975; ZIEGLER AND TAMM, 1976; GLASBY, 1991), but not specifically linked to the pharmacological activity of the extracts. In this study, eucosterol ($C_{29}H_{44}O_5$) was found to exhibit significantly high levels of both COX-1 and COX-2 inhibitory activity, and was determined to be a selective COX-2 inhibitor (with a COX-2/COX-1 ration of 0.8).

Active principle from E. autumnalis autumnalis

An important consideration in the isolation of pharmaceutically active compounds is the possibility of damage to the compound during the isolation and characterization procedure. This can often result in a loss of activity in the final stages of the procedure (HARBORNE, 1998). The isolated compounds sent for analysis were thus re-tested in the assay and were found to still exhibit activity.

The use of bioassay-guided fractionation employing the COX-1 assay as a screening test, resulted in the isolation of a highly active COX-1 inhibitor (Compound D) in the extract. This compound, tentatively identified as a phenol ring with an attached conjugated hydrocarbon chain, shares some structural similarities with acetyl salicylic acid (aspirin), viz the phenol group, and may have a similar mode of action as a COX inhibitor. It is, however, evident that this was not the only anti-inflammatory compound in this extract responsible for the anti-inflammatory activity.

These results certainly support the use of this plant in traditional medicine. While the COX-2 selectivity of the crystals are an important finding, the current emphasis in pharmaceutical research is for compounds showing over 1000-fold selectivity for COX-2 over COX-1 (VANE AND BOTTING, 1995). The important factor, as defined by the pharmaceutical industry, is weak COX-1 inhibitory activity which significantly reduces the side-effects of such drugs. Although this compound showed similar COX-1 and COX-2 IC_{50} values, it might still have potential as a drug. Researchers have found discrepancies between results obtained using purified enzymes and whole cells. Investigations have found that while estimates of COX-1 inhibitor potency compare well between the two systems, most compounds analysed were consistently more potent against human COX-2 in whole cell tests (TAKETO, 1998a). This suggests an extremely high sensitivity to certain NSAIDs. Another possibility proposed to explain this discrepancy was that it resulted from conditions affecting the availability of arachidonic acid, and from the allosteric regulation of COX-1 by both arachidonic acid and the inhibitors (TAKETO, 1998a).

With the identification of activity in screening procedures, attention is concentrated on the most active sample for isolation and characterization. In instances without any ethnomedical data, these compounds often fall short of expected therapeutic value in

clinical and toxicity trials since the margin between efficacy and toxicity is too narrow (LEWIS AND ELVIN-LEWIS, 1994). Linking ethnomedical information to this method increases the chances of isolating potentially effective drugs.

Many large pharmaceutical companies have as their primary objective in the use of random screening of plant products, the acquisition of information on structure-activity relationships (FARNSWORTH, 1994). Secondary compounds from plants often have unusual and highly complex structures, difficult to synthesize in a laboratory. These structures are used to complement chemical libraries and gain information on new structure-activity relationships. This data can be used by medicinal chemists in substructure investigations to determine which structural elements are important and to perform exploratory modifications in an attempt to develop structurally related products that are more easily accessible (FARNSWORTH, 1994). Research has focussed on the discovery of unique structures and how their use can be improved by molecular modification. These molecules can be patented and are easily distinguishable from phytochemicals (LEWIS AND ELVIN-LEWIS, 1994).

Alternatively, the discovery of new active structures can serve as departure points for synthetic chemists. Reserpine, cocaine, the coumarins, quinine all served as models from which whole groups of synthetic drugs were created (ANTON *et al.*, 1986). Many of the natural products responsible for medicinal activity from natural drugs have been isolated and a large number are now available through synthesis, others have served as templates for the synthesis of derivative drugs with greater effectiveness, or special properties (NIGG, 1992).

4.5 CONCLUSION

Often medicinal plants contain more than one bioreactive component which can act additively or synergistically to effect the full potential of the remedy, or a mixture of medicinal plants could act in this way. Extracts of *E. autumnalis autumnalis* bulbs yielded two highly active compounds which were isolated and identified. Furthermore, there exists the possibility that other bioactive compounds are present in these complex extracts.

Most natural products, once purified from mixtures, do not make good pharmaceuticals. They may not formulate well, may not be bioavailable, have the appropriate stability or have toxic side-effects as single agents (CRAGG *et al.*, 1994). Research on the pharmacology of the derivatives of plant derived drugs is essential in the development of analogues which can more safely be administered to patients, but this in effect, can circumvent patent rights, another highly relevant consideration in the pharmaceutical industry.

The results presented here have shown the presence of several highly active anti-inflammatory compounds. COX-1 inhibitory activity was linked to eucomin, a compound previously isolated but not tested specifically for anti-inflammatory activity. An additional homoisoflavanone isolated in this study (5,7-dihydroxy-6-methoxy-3-(4-methoxy benzyl)-chroman-4-one), differing structurally from eucomin only in the presence of an extra methoxy group on the A-ring, showed insignificant COX-1 inhibitory activity. This result has implications for the determination of structure-activity relationships. In addition, high COX-1 inhibitory activity has been associated with several other compounds in the extract, including Compound D, the phenol-hydrocarbon chain. The anti-inflammatory effect of these extracts is, however, enhanced by the presence of compounds showing selective COX-2 inhibitory activity, specifically eucosterol (Compound C), the spirostane-triterpenoid. This is an important finding in terms of the search for new drugs of this type.

This type of research remains an important and integral part of drug discovery and development. This study specifically linked anti-inflammatory activity to particular compounds in *Eucomis* extracts. These compounds were then characterized and identified. This scientific approach, involving bioassay-guided fractionation, is advantageous from both a chemical and pharmaceutical perspective. Proving the efficacy of traditional medicinal preparations is the first step towards validating this medical system. The development of new, potent drugs from these plants is a bonus, more relevant to western society.

CHAPTER 5

TLC FINGERPRINTING

5.1 INTRODUCTION

Thin-layer chromatography is widely used for the rapid analysis of drugs and drug preparations. This analytical system can be used to efficiently demonstrate most of the characteristic constituents of a drug. In addition to this qualitative detection, TLC can also be employed to assess drug quality by providing semi-quantitative information on the major active constituents of a drug or drug preparation (WAGNER AND BLADT, 1996). Where the active principles of a group of drugs are very similar (e.g. drugs from the Solanaceae or saponin drugs), the differentiation and identification on the basis of the active principles is difficult or impossible. In such cases, other classes of compounds need to be used for the purpose of differentiation (WAGNER AND BLADT, 1996).

TLC also enables the qualitative and quantitative detection of known natural products or compounds (e.g. pharmaceuticals), and their metabolites or breakdown products (GIBBONS AND GRAY, 1998). This approach has been used to develop secondary metabolite "fingerprints" which can facilitate the classification of the same range of metabolites, and the highlighting of those with different or interesting profiles (VAN MIDDLESWORTH AND CANNELL, 1998).

The use of photographic plates can serve as a catalogue of spectra of phytochemical preparations. Where stains are used, these are generally chosen to give the most striking colours (WAGNER AND BLADT, 1996). A photographic drug atlas can be an aid to the routine identification and purity testing of drugs in control laboratories, and it can be used without previous pharmacognostic training. Plates of "standard" drugs can be used for comparison in the identification of an official drug or medical preparation. For drugs with unknown or incompletely known active principles, identification needs to be based on other non-active but easily detectable constituents

that can be regarded as "guide substances". An unknown sample of a commercial drug can thus be classified by comparison with the visual record in the TLC atlas (WAGNER AND BLADT, 1996).

Standardization of TLC plates

The reproducibility of these TLC separations is very important and can only be guaranteed if standardized adsorption layers are used. Silica gel is an efficient adsorbent for the TLC separation of most drug extracts. Commercially available TLC plates are generally used: Silica gel 60 F_{254} - precoated TLC plates from Merck (Germany).

Analytical TLC has also traditionally been used in the detection and monitoring of compounds throughout a separation process. Natural products may be detected by running analytical TLC plates of fractions obtained from other separation processes (e.g. column chromatography or HPLC).

Visualization of TLC plates

Once a natural product mixture has been separated on TLC, the first step in the detection and localization of compounds on the plates is viewing under UV light. The primary advantage of UV detection is that it is non-destructive, and the detection of compounds can be conducted at any stage in the separation process (GIBBONS AND GRAY, 1998).

Compounds that absorb light at either 254 nm (short wavelength = pale green) or 366 nm (long wavelength = pale purple) appear as dark spots on a light background. Long wavelength (366 nm) light is normally used for the detection of compounds that fluoresce under UV light (e.g. orange, yellow or red) as is the case for some chlorophylls. The major disadvantage of UV detection, however, is that all compounds do not absorb UV light at 254 or 366 nm, and those that do not will thus be invisible. Such compounds require the use of spray reagents to facilitate visualization (GIBBONS AND GRAY, 1998).

The most common chemical tests performed on TLC plates are the application of colorimetric stain reagents that give a visual indication of the presence of various

functional groups. Although easy to perform, these tests are not always specific in that they seldom detect compounds of the specific class only, and they often will not react with every compound of that class (VAN MIDDLESWORTH AND CANNELL, 1998).

5.2 MATERIALS AND METHODS

5.2.1 SAMPLE PREPARATION

Ethanolic extracts were prepared at a concentration of 10 mg $m\ell^{-1}$ and streaked on the plates in 25 $\mu\ell$ aliquots. All TLC separations were performed at room temperature i.e. 22-25°C.

Sharply resolved zones are obtained by restricting the quantity of material applied to the chromatogram. Larger sample volumes are, however, often necessary for the detection (by colour reaction) of substances that are present in low concentrations, which inevitably leads to the broadening and overlapping of zones. Compounds can be located on TLC plates through the calculation of R_f values. These values represent the ratio of the distance travelled by the compound (spot or band) to the distance travelled by the solvent front, and should be consistent for different TLC separations run under standard conditions.

5.2.2 TLC SEPARATION

The benzene : 1,4 dioxan : acetic acid (90 : 25 : 4) system described in CHAPTER 3 gave the best separation of both leaf and bulb extracts and was subsequently used for the preparation of TLC "fingerprints". The addition of a small amount of acid (e.g. 1% acetic acid) to the mobile phase maintains acidic groups in a non-ionised form, and thus reduces the "tails" formed from interaction between the acidic groups and silanols (GIBBONS AND GRAY, 1998).

The plant extracts (0.5 mg) were applied to TLC plates (Merck Silica gel 60 F_{254}) as 1 cm bands. Plates (10 cm) were developed over 8 cm. These were allowed to dry and viewed under $UV_{254\ nm}$ and $UV_{366\ nm}$ before staining with anisaldehyde-sulphuric

acid reagent (0.5 mℓ anisaldehyde, 10 mℓ sulphuric acid, 85 mℓ methanol and 5 mℓ concentrated sulphuric acid mixed in this order). The stain was poured over the plates, which were then heated for 10 min at 110°C. Photographs were taken of the plates using UV and standard fluorescent lighting.

5.2.3 LOCALIZATION OF ACTIVITY

A standard TLC plate (5 x 20 cm) was streaked with 50 $\mu\ell$ of the ethyl acetate bulb extract. After developing in the solvent system described above, this plate was divided into eight fractions and the compounds eluted from the silica with dichloromethane. The fractions were dried, and resuspended in 50 $\mu\ell$ ethanol before testing in the COX-1 assay. The results are presented in Figure 5.1.

5.2.4 TLC FINGERPRINTS

Leaf, bulb and root extracts from the eleven different *Eucomis* species were streaked on TLC plates. These plates were developed as described in 5.2.2. This facilitated the comparison of the extracts at both a species level, and at the level of different plant parts (Plates 5.1 and 5.2). Bulb extracts from plants harvested in winter were also fingerprinted to compare the TLC profile on a seasonal basis (Plate 5.1).

5.2.5 SERIAL EXTRACTION

The serial extraction (CHAPTER 3) of the different plant parts using solvents of increasing polarity was undertaken to further compare both the COX-1 inhibitory activity of the extracts, and their chemical constituents. The TLC fingerprints from the separation of these fractions are presented in Plate 5.3.

5.2.6 COMPARISON OF TLC FINGERPRINTS WITH PURIFIED SAMPLES

Lastly, TLC plates were run for the samples sent for identification (CHAPTER 4). These were compared to the ethyl acetate extract originally applied to the Sephadex LH-20 column, as well as to the crude ethanol extracts of the leaves, bulb and roots of *E. autumnalis autumnalis* (Plate 5.4).

5.3 RESULTS

5.3.1 CORRELATION OF COX-1 INHIBITORY ACTIVITY WITH R_f FRACTIONS

Anisaldehyde-sulphuric acid can be used for the detection of many compounds, especially terpenes, sugars, phenols and steroids (GIBBONS AND GRAY, 1998). Since apparently pure spots on a TLC plate can consist of several compounds with identical R_f values, different separation procedures should be employed, or more than one solvent system should be used for TLC separations (GIBBONS AND GRAY, 1998).

The R_f fraction containing the active principle (Figure 5.1), can be identified by the presence of spots that stain green-orange with anisaldehyde (R_f ~ 0.7). Sephadex LH-20 separations conducted in CHAPTER 3 showed the presence of compounds that stained red-orange and green with the anisaldehyde-sulphuric acid stain, eluting in the same R_f fraction (the 7th fraction represented in Figure 5.1). Several other R_f fractions show low to moderate activity (35-40% inhibition) indicating the presence of more than one active compound.

5.3.2 COMPARISON OF TLC FINGERPRINTS ACROSS SPECIES

It is evident from Plate 5.1 (A) that the ethanolic bulb extracts of the different *Eucomis* species exhibited similar chemical profiles. Clear bands were detected at R_f ~ 0.7, corresponding to the R_f fraction for which the highest COX-1 inhibitory activity was detected. Under UV_{366nm} these spots were dark blue (Plate 5.1). Differences were observed, on a quantitative level, by comparing the relative intensities of the spots. This was not, however, a suitable estimation of anti-inflammatory activity, since a higher intensity spot on the TLC plates did not necessarily correspond to higher COX-1 inhibitory activity, as determined in CHAPTER 2.

TLC fingerprints (UV_{366nm}) of the ethanolic extracts from the leaves (Plate 5.2 B) were characterized by the red fluorescence typical of chlorophyll and its degradation products. Dark blue spots (clearly visible on Plate 5.1 B) were notably absent on Plate 5.2 B. This indicates that while these bands mark the position of the active

compounds (for the bulb extracts), these compounds are not necessarily themselves active in the COX-1 assay. Another possibility is that it is different compounds in the leaves, roots and bulbs that are active in this assay. Without the chlorophyll, the leaves display a similar TLC profile to the roots. The profiles of both the leaf and root extracts differ significantly from that of the bulb extracts. These are complex profiles, which makes comparison more difficult.

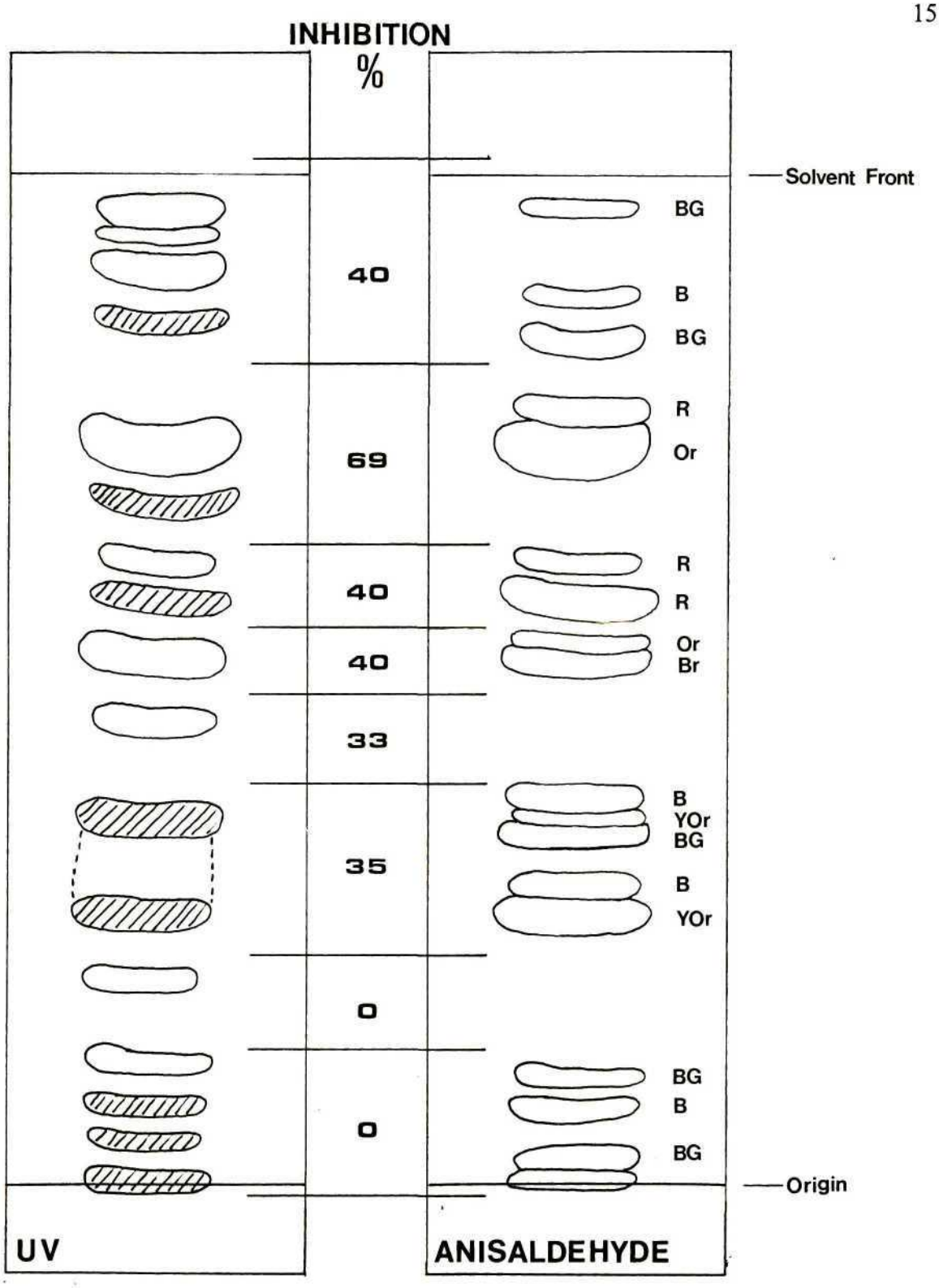


FIGURE 5.1: Diagrammatic representation of a TLC plate, streaked with 50 $\mu\ell$ bulb extract (ethyl acetate fraction), and developed in a solvent system of benzene : 1,4-dioxan : acetic acid (90 : 25 : 4). Viewed under UV light (left) and stained with anisaldehyde (right). Shaded spots luminesce under UV light. R_f fractions correlated with percentage COX-1 inhibitory activity.

[BG = blue-green; B = blue; R = red; Or = orange; Br = brown; YOr = yellow-orange and Bl = black].

PLATE 5.1: TLC separation of the ethanol extracts of the bulbs of *Eucomis* species, harvested in summer and winter. Viewed under (A) UV_{254nm}; (B) UV_{366nm}; and (C) after staining with anisaldehyde. Solvent system used was benzene : 1,4-dioxan : acetic acid (90 : 25 : 4).

[A = *E. autumnalis autumnalis*; B = *E. autumnalis amaryllidifolia*; C = *E. autumnalis clavata*; D = E. *bicolor*; E = *E. comosa-comosa*; F = *E. humilis*; G = *E. comosa-punctata - striata*; H = *E. comosa-punctata*; I = *E. pole-evansii*; J = *E. zambesiaca*; K = Hybrid].

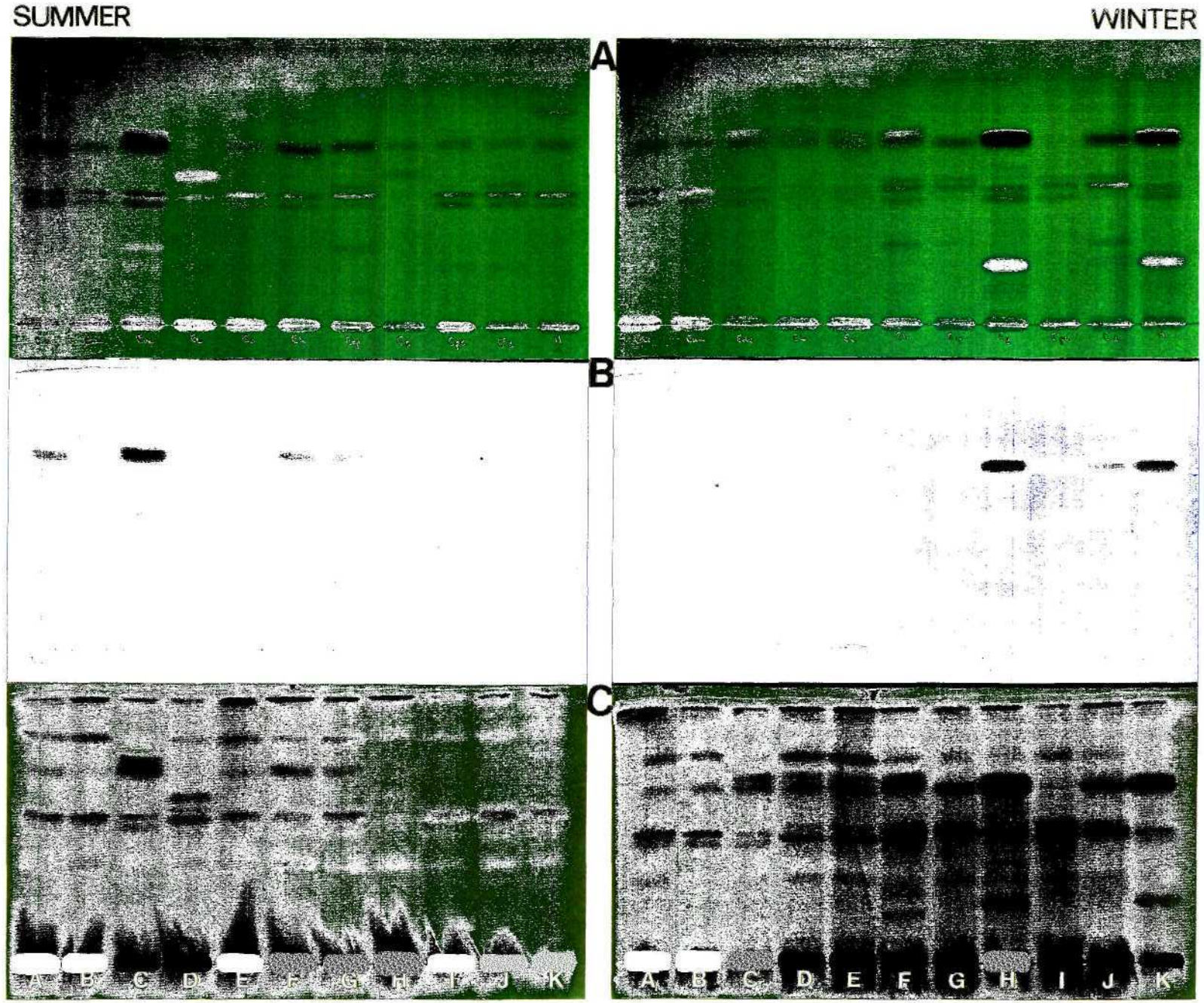
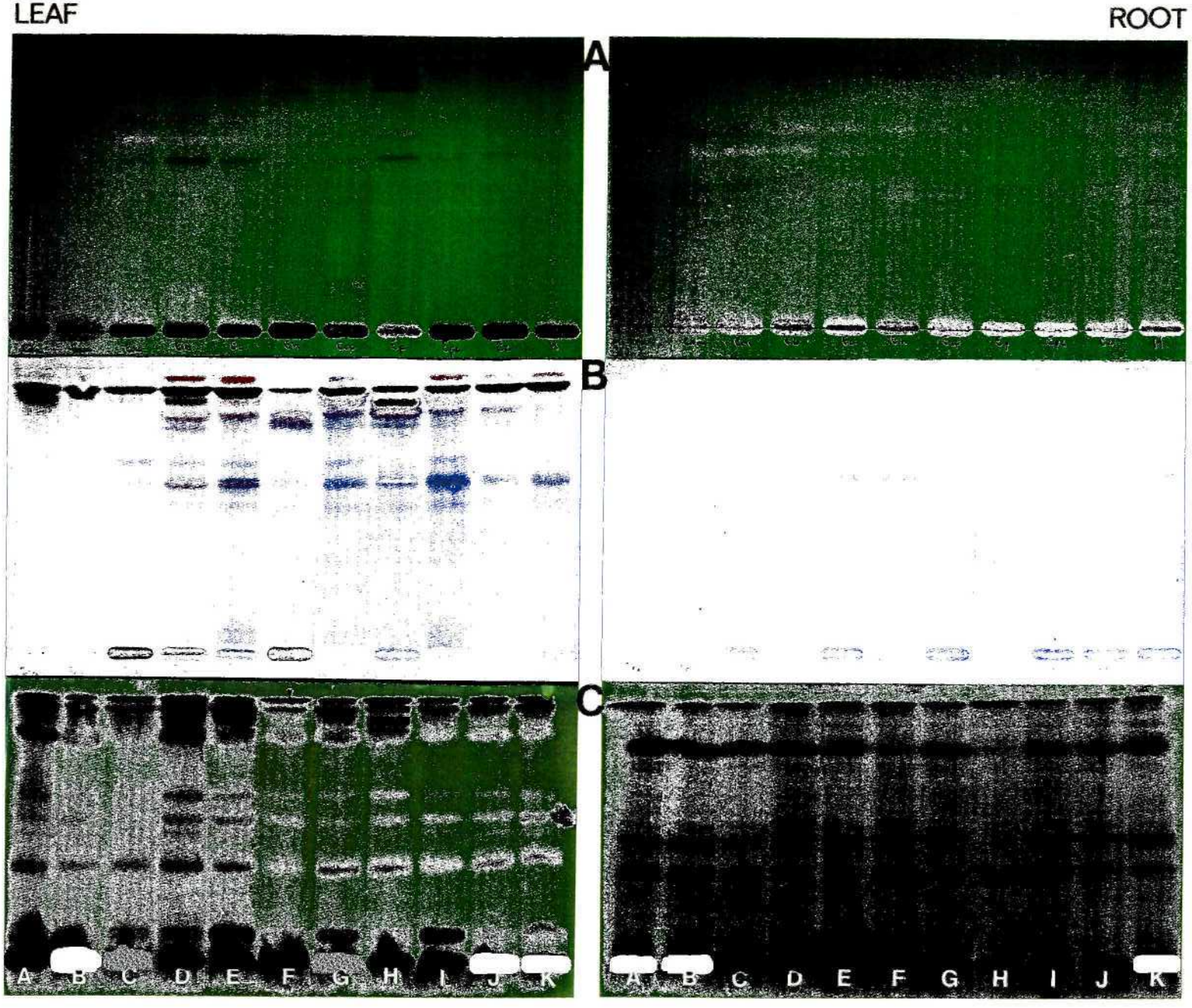

SUMMER
WINTER
A
B
C
A B C D E F G H I J K
A B C D E F G H I J K

PLATE 5.2: TLC separation of the ethanol extracts of the leaves and roots of *E. autumnalis autumnalis*, harvested in summer. Viewed under (A) $UV_{254\ nm}$; (B) $UV_{366\ nm}$; and (C) after staining with anisaldehyde. Solvent system used was benzene : 1,4-dioxan : acetic acid (90 : 25 : 4).

[A = *E. autumnalis autumnalis*; B = *E. autumnalis amaryllidifolia*; C = *E. autumnalis clavata*; D = *E. bicolor*; E = *E. comosa-comosa*; F = *E. humilis*; G = *E. comosa-punctata - striata*; H = *E. comosa-punctata*; I = *E. pole-evansii*; J = *E. zambesiaca*; K = Hybrid].


LEAF
ROOT
A
B
C
A B C D E F G H I J K
A B C D E F G H I J K

5.3.3 COMPARISON OF ETHANOLIC EXTRACTS FROM THE BULBS HARVESTED IN SUMMER AND WINTER

A comparison of the levels of COX-1 inhibitory activity in summer and winter (CHAPTER 2) showed few differences. The TLC profiles (Plate 5.1) also showed little difference between the TLC-fingerprints for the summer and winter extracts, corroborating this result. Additional compounds were visible for some species, notably at R_f ~ 0.2, under UV_{254nm} (Plate 5.1 A). The presence of these bands was not, however, correlated to any change in COX-1 inhibitory activity (CHAPTER 2).

5.3.4 COMPARISON OF TLC SEPARATIONS OF FRACTIONS OBTAINED FROM THE SERIAL EXTRACTION OF THE DIFFERENT PLANT PARTS.

The serial extraction of leaf, bulb and root material using hexane, ethyl acetate and ethanol gave different TLC fingerprints (Plate 5.3). Since ethanol is a relatively polar solvent, the TLC-fingerprints obtained earlier showed too many compounds to be useful in distinguishing minor differences between species and plant parts. The ethyl acetate fraction showed a clearer TLC-fingerprint and the presence and intensity of the spot indicating the position of the active compounds was easier to detect.

E. autumnalis autumnalis leaves

The leaf extracts (Plate 5.3 L) were clearly distinguished by the presence of chlorophyll, which, under $UV_{366\ nm}$ appeared red. There was no clear band detectable at R_f ~0.7, the region where the active compound(s) should be found (Figure 5.1). Neither the leaf nor root extract fingerprint showed the presence of the highly coloured bands visible in the bulb extract profile, after staining with anisaldehyde-sulphuric acid.

E. autumnalis autumnalis bulbs

The TLC fingerprints of the bulb extracts (Plate 5.3 B) are characterized by the presence of highly coloured bands, after staining with anisaldehyde-sulphuric acid. These plates clearly show red/orange-coloured spots in the region corresponding to the position of the active compound(s). These bands are equally clearly visible under UV light. The ethyl acetate fraction showed the highest occurrence of these compounds, as indicated by the intensity of the spots.

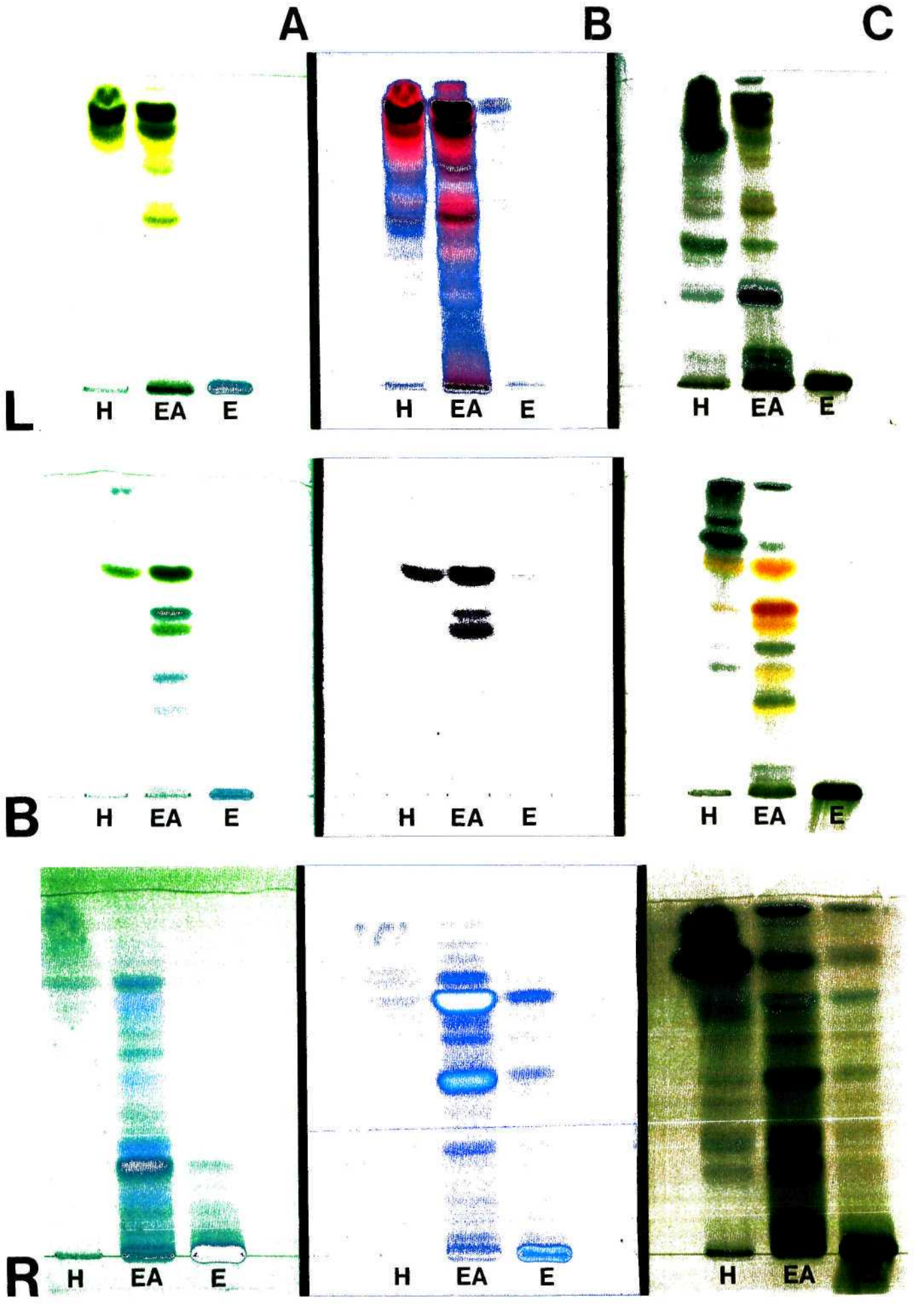
E. autumnalis autumnalis roots

This plate (Plate 5.3 R) shows luminous bands (especially for the ethyl acetate fraction) that were not detectable in the TLC separation of the crude ethanol extract. These compounds were probably present in too low a concentration in the crude extract, or were masked by other compounds. These bands also elute in the region characterized by high COX-1 inhibitory activity.

5.3.5 COMPARISON OF TLC FINGERPRINTS WITH PURIFIED SAMPLES

Plate 5.4 illustrates the TLC profiles of the purified samples (CHAPTER 4) compared to that of the whole extract (Plate 5.4 I and II). Plate 5.4 III represents the ethyl acetate extract fingerprint of the leaves (L), bulbs (B) and roots (R) of *E. autumnalis autumnalis*. This plate further facilitates the correlation of the bands with COX-1 inhibitory activity.

PLATE 5.3: TLC separation of the hexane (H), ethyl acetate (EA) and ethanol (E) fractions from the serial extraction of *E. autumnalis autumnalis* leaves (L), bulbs (B) and roots (R). Viewed under (A) $UV_{254\ nm}$; (B) $UV_{366\ nm}$; and (C) after staining with anisaldehyde. Solvent system used was benzene : 1,4-dioxan : acetic acid (90 : 25 : 4).


A
B
C
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EA
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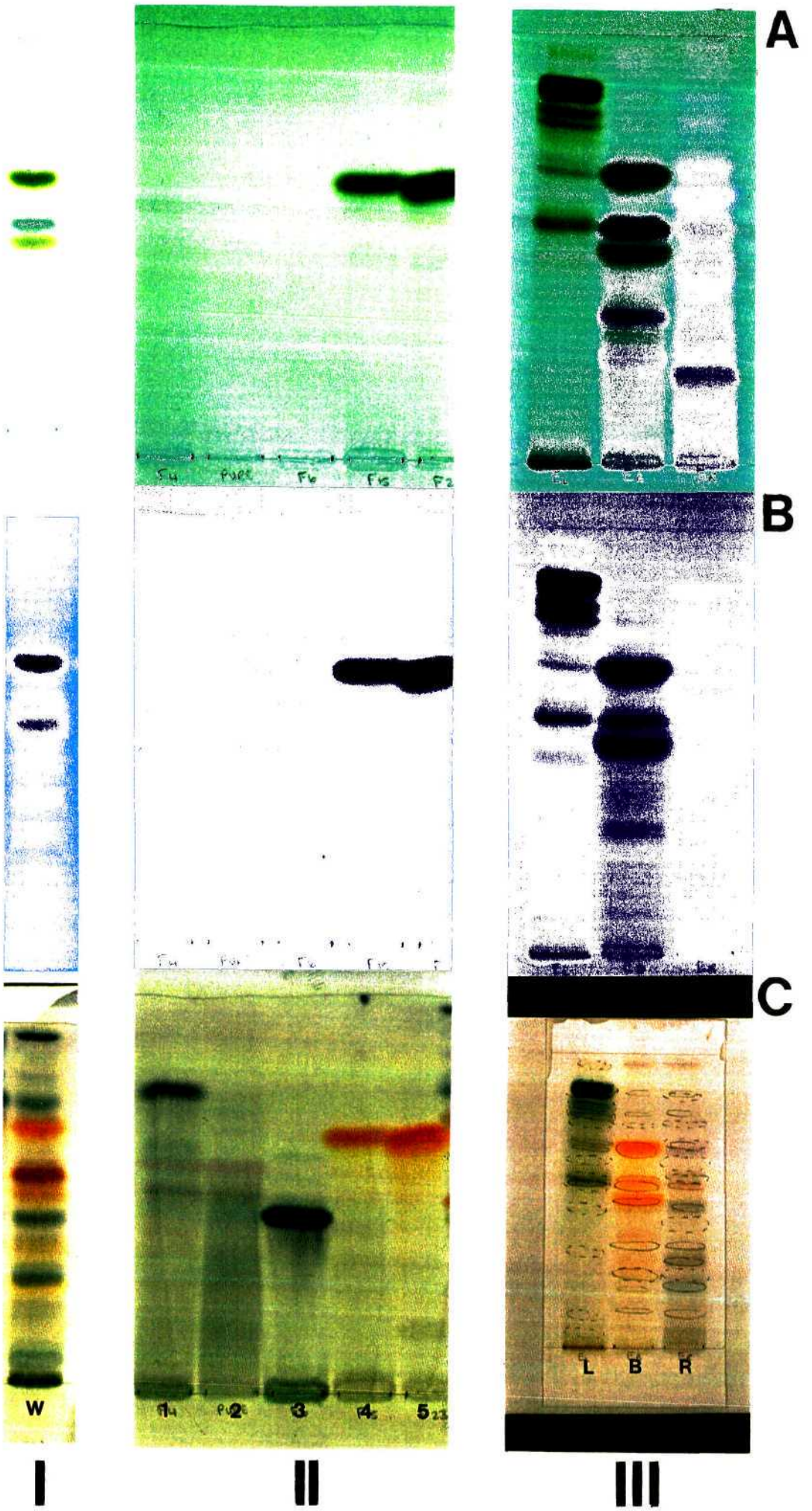
R
H
EA
E
H
EA
E
H
EA

PLATE 5.4: TLC separation of (I) the ethyl acetate fraction of the bulb extracts [W]; (II) Fraction 4 [1], Compound D (Fr 4) [2], Compound C (Fr 6) [3], Compound A [4] and Compound B [5]; (III) the crude ethyl acetate extracts of the leaves [L], bulbs [B] and roots [R] of *E. autumnalis autumnalis*. Viewed under (A) $UV_{254\ nm}$; (B) $UV_{366\ nm}$; and (C) after staining with anisaldehyde. Solvent system used was benzene : 1,4-dioxan : acetic acid (90 : 25 : 4).


A
B
C
W
L
B
R
I
II
III

5.4 DISCUSSION

The separation of a drug preparation by TLC provides a chromatographic drug fingerprint. It is therefore suitable for monitoring the identity and purity of drugs and for detecting adulterations and substitutions. With the aid of appropriate separation procedures, TLC can be used to analyse drug combinations and phytochemical preparations (WAGNER AND BLADT, 1996).

This system obviously works best for simple mixtures, where minor differences are immediately detectable, and the presence or absence of key compounds can be clearly discerned. The fingerprints prepared from the serial extraction of the plant parts (specifically the ethyl acetate fraction, pictured in Plate 5.3) showed distinct advantages over those prepared from the crude ethanol extracts (Plate 5.1 and 5.2), in terms of clarity and resolution of the bands.

The use of photographic plates of thin-layer separations has a distinct advantage over graphical representations. The colour and separation of the bands are displayed visually with an immediate clarity of representation that facilitates close comparison (WAGNER AND BLADT, 1996).

The leaves, bulbs and roots of *Eucomis* showed distinctly different fingerprints under UV_{254nm} and UV_{366nm} light as well as after the application of the anisaldehyde stain (Plates 5.1-5.2). The TLC chromatogram of the leaf extract was dominated by the presence of chlorophyll and its degradation products, which show red fluorescence under UV_{366nm}. The fingerprint obtained for the bulb extracts showed a strong band eluting at R_f 0.7, with a second band at R_f 0.55. These were visible at dark spots under UV light and turned a bright orange-red colour when treated with the anisaldehyde stain reagent. The chromatogram of the root extracts showed several fluorescence quenching zones under UV_{254nm}, predominantly in R_f's 0.7 and 0.5. These compounds exhibited bright blue fluorescence under UV_{366nm}. This would suggest that the major chemical constituents of the various plant parts differ considerably.

5.4.1 SAPONINS AND ISOFLAVONOIDS

The TLC detection of saponins using an anisaldehyde-sulphuric acid reagent is indicated by mainly blue, blue-violet and sometimes red or yellow brown zones (WAGNER AND BLADT, 1996). Inspection under $UV_{366\ nm}$ resulted in blue, violet and green fluorescent zones. The bulb, and the root extracts would thus seem to contain high levels of saponins, a conclusion substantiated both by the results obtained in CHAPTER 2 and by the literature. These were not, however, the active compounds in this extract. The purified samples (Fractions 15 and 23) showing dark blue fluorescence under UV_{366nm} were identified as isoflavonoids. The application of anisaldehyde-sulphuric acid to flavonoids results in highly coloured bands (LEWIS, 1989). The bright orange bands visible after staining with anisaldehyde-sulphuric acid are characteristic of these compounds.

5.4.2 ACTIVE COMPOUNDS

The active principle (Compound D) isolated by bioassay-guided fractionation (CHAPTER 3 and 4) was not visible under UV light, and was not highly coloured after staining with anisaldehyde (Plate 5.4 II [2]). The second active compound (Compound C) isolated from the extract was determined to be a spirostane-type triterpenoid (eucosterol). This compound showed no fluorescence under UV light, but was visible as a dark blue-black band after staining with anisaldehyde-sulphuric acid (Plate 5.4 II [3]). This indicates the weakness of the TLC fingerprints in identifying specific active compounds in crude extracts, particularly if the concentration of such compounds is very low. In a purified sample, there is a much greater possibility of successfully demonstrating the presence of such a compound.

5.5 CONCLUSION

Thin layer chromatography represents one of the fastest, cheapest and most effective methods of obtaining a characteristic analytical fingerprint of a plant extract (WAGNER AND BLADT, 1996). This technique can show significant differences, as well as similarities, between the major chemical constituents of different plant parts. Comparisons can also be made between species, and between extracts prepared in summer and winter.

The chemical assay of complex mixtures of traditional plants containing unknown substances is not feasible. Chemical fingerprints obtained from chromatographic separations of these preparations would, however, give a qualitative idea of their chemical composition, and would thus enable some form of standardization to be applied (ANAND AND NITYANAND, 1984). Once an active principle has been isolated and characterized under UV light, and after staining with a particular reagent (in this case anisaldehyde-sulphuric acid), its presence can be detected in more complex mixtures, provided it is present in sufficient amounts.

It is thus evident that while the development of TLC fingerprints for herbal medical preparations has been useful, its application to traditional medicine, which is characterized by highly complex mixtures of plants, presents many difficulties. This system can more profitably be applied to the identification of a medicinal plant in a taxonomic situation, and for the determination of chemotaxonomic relationships within species and genus classifications.

CHAPTER 6

TISSUE CULTURE OF *EUCOMIS* SPECIES

6.1 INTRODUCTION

The horticultural propagation of bulbous plants is almost exclusively vegetative in nature, largely due to the need to preserve the clonal nature and uniform character of the cultivar, many of which are sterile and are unable to set seed. In the wild, however, there is a much greater dependence on seed propagation, which naturally introduces variety into populations (REES, 1992). The seed of some bulbous plants has limited viability and is best planted fresh. In addition,many spring flowers have a cold requirement before germinating (REES, 1992). Commercially, propagation by seed is only preferred where there is large and reliable seed set and the plants are characterized by short juvenile periods. Plants produced from seed are generally virus free, unlike material propagated vegetatively (REES, 1992).

In general, petaloid monocotyledons are attractive plants, usually easily propagated by vegetative methods, but are especially vulnerable to over-collection and exploitation. In South Africa, this is exacerbated firstly by their restricted distribution, and secondly through their increasing displacement by encroaching agriculture and exotic plant invaders. There is, in addition, little known about the dispersal ecology and phenology of most petaloid monocotyledons in South Africa, and the impact of modern farming methods and fire on the dispersal, phenology and pollination biology is difficult to evaluate (STIRTON, 1980).

A major concern regarding micropropagated plants is the genetic stability of the cultured material, although all vegetatively propagated plants are, to some extent, subject to mutation (usually at a low rate depending on the method used). Adventitious shoot meristems are more prone to mutation than axillary shoot meristems because they are more frequently derived from single cells (REES, 1992).

Cultivation of medicinal plants

Plant species used for ethnomedical purposes have traditionally been collected and gathered from the wild as opposed to cultivated on a commercial scale (MÀTHÉ, 1988). This practice is ongoing, with large quantities of medicinally utilized species being removed from their native ecosystems all over the world. The majority of plants used for traditional medicinal purposes in South Africa are harvested from the wild and have not yet been fully analysed for their bioactive compounds.

The introduction of both aromatic and medicinal plants as cultivated crops is receiving more attention as the value of these crops to the food and pharmaceutical industries increases (MÀTHÉ, 1988). This is coupled to an awareness of the need to conserve plant genetic resources that are threatened by natural habitat destruction, making cultivation an attractive alternative. In South Africa, this is being addressed by conservation organizations and nurseries such as Silverglen (in KwaZulu-Natal), in an attempt to encourage small scale farming and create both employment and conservation opportunities.

The successful domestication of wild medicinal plant species would ensure a continuous supply of the plants and plant products on a commercial scale, which can be improved upon by standard farming practices. This would improve the quality and consistency of the raw crude plant extract through the harvest of the homogeneous plant populations necessary for modern production, thus simplifying standardization of the concentration of the active ingredients as well as processing and utilization (MÀTHÉ, 1988).

There is growing concern in South Africa for the conservation of rare and endangered plants and appreciation of the economic value of rare and endangered plants as renewable resources (STIRTON, 1980). All Liliaceae are listed as specially protected under South African law (MANDER, 1988a). The conservation status of *E. autumnalis* is described as declining by MANDER (1988a), meaning that the species was previously widespread but is likely to become vulnerable and to continue to decline if destruction of wild populations continues. Although legislation has been passed protecting some *Eucomis* species (MANDER *et al.*, 1995), indiscriminate collection by traders is endangering natural populations. Three species of *Eucomis* are utilized

depending on which is locally available. Since these are such slow-growing plants, the rapid and economic techniques of *in vitro* propagation have great potential for the alleviation of this pressure by providing alternative plant sources (McCARTAN AND VAN STADEN, 1995), thus facilitating the conservation of this valuable genus.

Although *Eucomis* plants can be propagated by seed and offsets, this is a relatively slow process. *Eucomis* plants require 3-4 years to mature, and in the larger species, require a minimum bulb diameter of 12 cm to flower. The obvious advantage of a continuous micropropagation system lies in the rapid and economic bulk production of plants, which furthermore can be initiated from limited or juvenile stock. In addition, the rapid induction of new lines and varieties or hybrids in the market place can be accomplished. *Eucomis* shows great potential for the development of attractive flower stalks by hybridization (Plate 1.1 H), which can then be easily bulked up by micropropagation methods, while retaining the clonal nature of the hybrid.

Horticultural value

Floriculture is emerging as a high value industry in many sub-Saharan African countries where it generates both employment and foreign revenue. The value of the flower industry in South Africa has increased substantially in the past ten years and currently has an estimated value in excess of R 426 million (NIEDERWIESER *et al.*, 1998). The development of new products and new varieties is an essential facet of this industry. In addition, the establishment of genebanks to facilitate hybrid crosses has the added advantage of preserving biological diversity. *Eucomis* species respond well as cut flowers. The flower stalks vary in size considerably, and the flowers are long-lasting and attractive, varying in colour from pale green and yellow to dark purple.

Flower production in South Africa is orientated towards the export market. As a sub-section of this market, the flower bulb industry facilitates the sale of dry bulbs and the forcing of potted plants under controlled conditions (NIEDERWIESER *et al.*, 1998). *Eucomis* species show great potential for contribution both to the cut flower industry and for the sale of dry bulbs for use in gardens and as pot plants.

Propagation of this genus has become important primarily due to its destructive harvesting in the wild, but the added incentive of the great horticultural potential several of the smaller species (e.g. *E. zambesiaca* and *E. humilis*) show as pot plants (Plate 1.1 E and F), and the larger species show as garden plants (Plate 1.1 H and J), make this a commercially important venture as well. The demand for flower bulbs in general, has increased substantially in the past ten years, with a simultaneous doubling in commercial value (NIEDERWIESER *et al.*, 1998). The aim was thus to establish a tissue culture protocol to enable the rapid propagation of these species. Species of *Eucomis* have been successfully micropropagated (DE LANGE *et al.*, 1989). The tissue culture of *E. autumnalis*; *E. comosa-comosa* and *E. zambesiaca* using twin-scales (AULT, 1995); of *E. pole-evansii* using leaf explants from seedlings germinated *in vitro* (McCARTAN AND VAN STADEN, 1995) and of *E. vandermerweii* (McCARTAN *et al.*, 1998) has been achieved. New protocols for all three subspecies of *E. autumnalis,* viz *autumnalis*, *amaryllidifolia* and *clavata* , *E. bicolor*, *E. comosa-punctata*, *E. humilis*, *E. comosa-punctata* var *striata* and a hybrid species were established. The tissue culture of *E. comosa-comosa*, *E. pole-evansii* and *E. zambesiaca* was also investigated using leaf explants.

6.2 MATERIALS AND METHODS

Stock plants were maintained in a sand : soil (1 : 1) mixture under 20% shade and watered once a week (Plate 6.1 F).

6.2.1 DECONTAMINATION

The suitability of both leaf and bulb explants for *in vitro* culture was investigated. Initially, a decontamination grid was investigated using 1.75% and 3% sodium hypochlorite (JIK®) for 10 and 20 min. In subsequent experiments the following decontamination regime was observed:

The plant material was washed to remove loose dirt. Leaves were placed in 70% ethanol for 5 min, followed by 10 min in 0.2% Benlate®. The material was then sterilized for 20 min in 1.75% sodium hypochlorite, before rinsing in sterile distilled water.

Slices of bulb scales were soaked in water for 30 min followed by a 1 min dip in 100% ethanol. The bulb scales were then placed in 0.2% Benlate® for 5 min, followed by 20 min in 3% sodium hypochlorite. The material was then rinsed in sterile distilled water.

Leaf or bulb explants (10 mm x 10 mm) were aseptically transferred to initiation media in culture tubes (24 mm x 100 mm). These were maintained in a growth room under a 16 h light / 8 h dark light regime (27 μmol m^{-2} s^{-1}) at 25±3°C.

6.2.2 INITIATION MEDIA

A modified Murashige and Skoog medium (1962) supplemented with 100 mg ℓ^{-1} myo-inositol, 20 g ℓ^{-1} sucrose and solidified with 2 g ℓ^{-1} Gelrite® was used (Appendix II). The pH of the medium was adjusted to 5.8 with 1 M NaOH before autoclaving at 121°C and 103 kPa for 20 min.

Adventitious shoot initiation

The hormones added to promote shoot initiation were NAA and BA in a factorial grid (Table 6.1). Successful shoot initiation was obtained for the combinations of NAA : BA of 1:1; 1:2 and 2:2 (mg ℓ^{-1}), and these were the experiments that were repeated. Results for the remaining hormone combinations are not presented.

Table 6.1: Replicate numbers for the hormone combinations used to test shoot initiation from leaf and bulb explants of *Eucomis* species.

	AUXIN CONCENTRATION (mg ℓ^{-1})			
CYTOKININ CONCENTRATION (mg ℓ^{-1})	0	1	2	5
	0	25	25	25
	1	50	50	25
	2	50	50	25
	5	25	25	25

Orientation

With respect to the leaf explants, an experiment was included to determine the effect of explant orientation on shoot initiation. Leaf explants from *E. autumnalis autumnalis* were positioned on the medium such that either their abaxial or adaxial surface was in contact with the gelrite. Differences in the number of shoots initiated were determined.

Root initiation

Shoots were excised and transferred to the same modified MS medium, supplemented with the auxins IAA, IBA or NAA at a concentration of 1 mg ℓ^{-1}, to accelerate root formation.

6.2.3 ACCLIMATIZATION

Rooted specimens were removed from culture and transferred to a misthouse with a bottom heat of 30°C to harden off. The plantlets were washed to remove all traces of sucrose and culture medium to discourage the growth of microorganisms. Two support media were investigated for this process, namely perlite and vermiculite. After 6 weeks the plants were ready to be transferred to pots containing a sand :soil mix of 1 : 1. These were placed in a greenhouse (light intensity 1990 μmol m^{-2} s^{-1}) and watered once a week. Mature plants (2 years) were repotted and relocated to 20% shadehouses (light intensity 1220 μmol m^{-2} s^{-1}).

6.2.4 BULK PROPAGATION

Sterile leaf material from the *in vitro* plantlets was used to reinitiate cultures in a continuous culture system. Explants (5 mm x 10 mm) were prepared from the leaf material and transferred to bottles (9 explants per culture bottle) for multiple shoot initiation. Plantlets were excised and transferred to rooting media to accelerate root formation and allow bulblet development.

6.2.5 ANALYSIS OF RESULTS

Twenty-five replicates were used per treatment and experiments were repeated at least twice. One-way ANOVA and Tukey HSD tests were performed using Minitab Xtra version 10.51.

6.3 RESULTS

6.3.1 DECONTAMINATION

Figure 6.1 represents the percentage survival for *E. autumnalis autumnalis* leaf and bulb explants placed in culture. The optimal sterilant concentration chosen was 1.75% sodium hypochlorite for 20 min for the leaf explants and 3% sodium hypochlorite for 10 min for the bulb explants. Using these decontamination regimes, the bulb explants for all 11 species showed on average, a significantly higher percentage contamination (55-65%), compared to the leaf explants (10-15%).

6.3.2 SHOOT INITIATION

Successful shoot initiation was achieved for both leaf and bulb explants with the younger leaf material responding best to the culture conditions (Plate 6.1 A and B). In addition, leaf explants produced more shoots per explant (up to 8), while bulb explants produced only 2-3 shoots. For this reason, subsequent experiments were conducted using leaf explants.

With respect to leaf explant orientation, the results presented in Figure 6.2 show that significantly higher numbers of adventitious shoots were initiated where the abaxial leaf surface was placed in contact with the medium.

Figure 6.3 shows the percentage shoot initiation from the leaves of the different species. The remaining hormone combinations (Table 6.1) did not show a significant morphological response and the data is thus not included. The majority of species showed optimal shoot initiation for a 1:1 (NAA:BA) medium. Where no significant difference in percentage shoot initiation between media was found, the number of shoots initiated per explant was used to choose an optimal initiation medium (Figure 6.3).

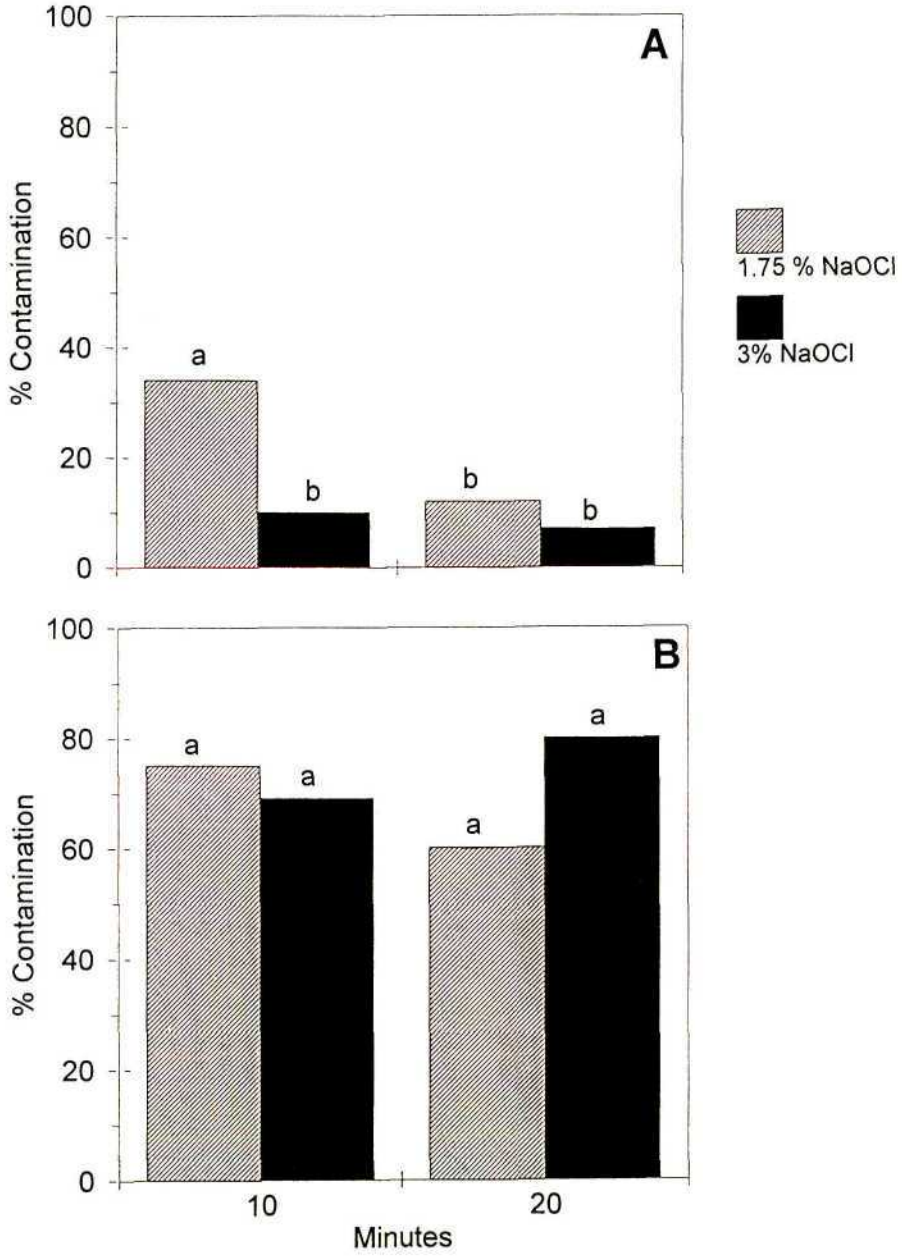


FIGURE 6.1: Percentage contamination of (A) leaf and (B) bulb scale explants sterilized for 10 and 20 min using 1.75 % and 3 % sodium hypochlorite. Bars bearing different letters are significantly different, $P \leq 0.05$.

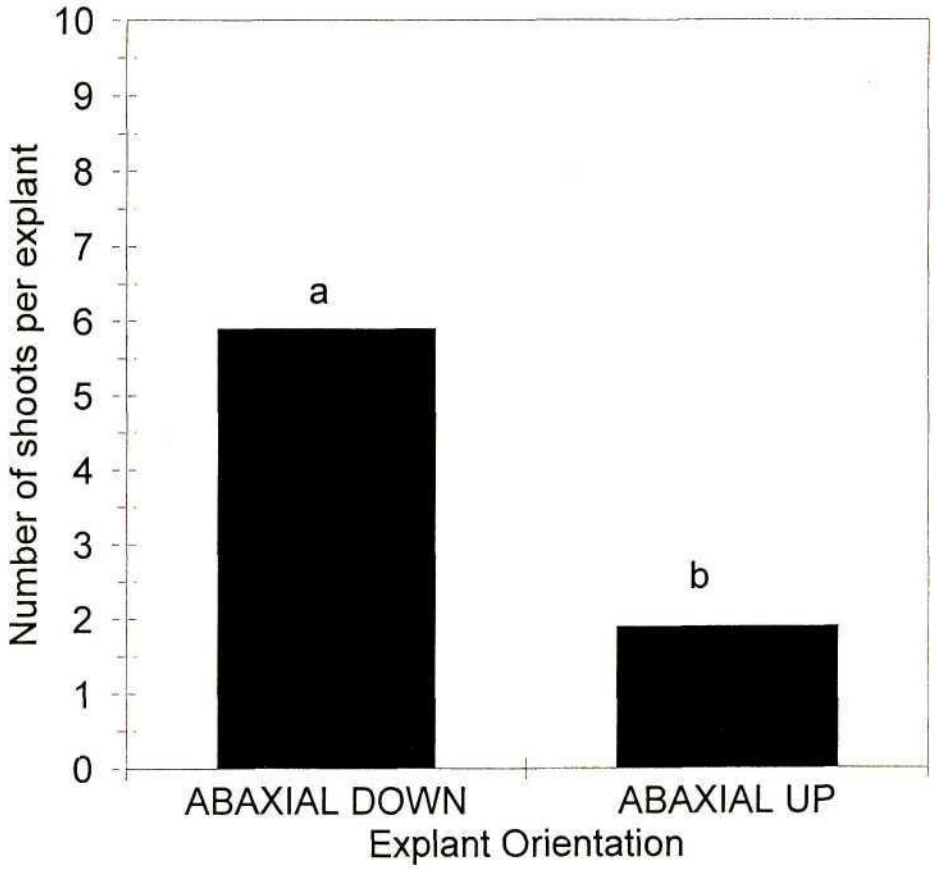


FIGURE 6.2: The effect of leaf explant orientation on shoot initiation for *E. autumnalis autumnalis*. Bars bearing different letters are significantly different, $P \leq 0.05$.

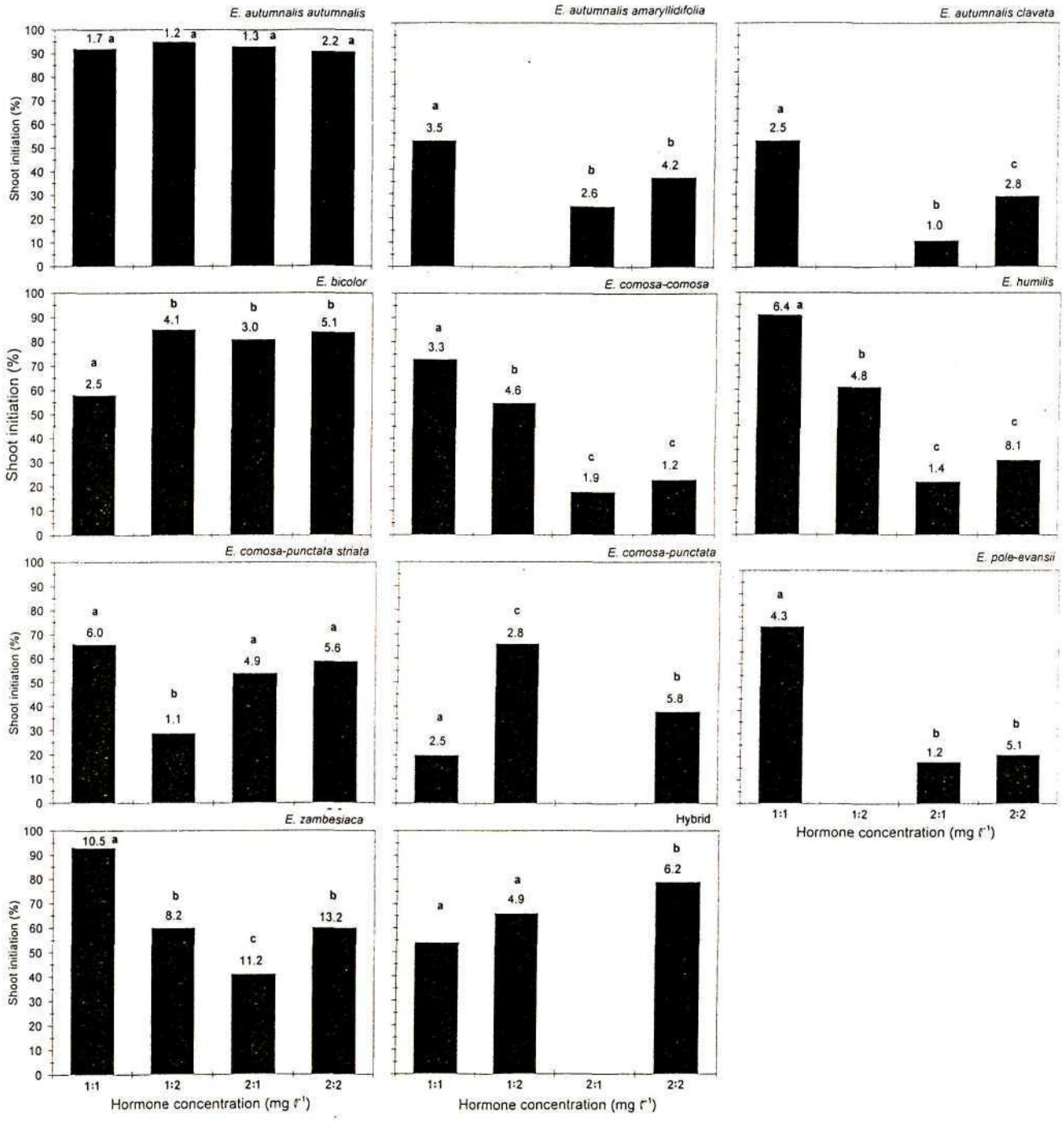


FIGURE 6.3: Adventitious shoot initiation in leaf explants from different *Eucomis* species for four initiation media supplemented with combinations of NAA and BA. Number annotations on graphs indicate average number of shoots per explant. Bars bearing different letters are significantly different, $P \leq 0.05$.

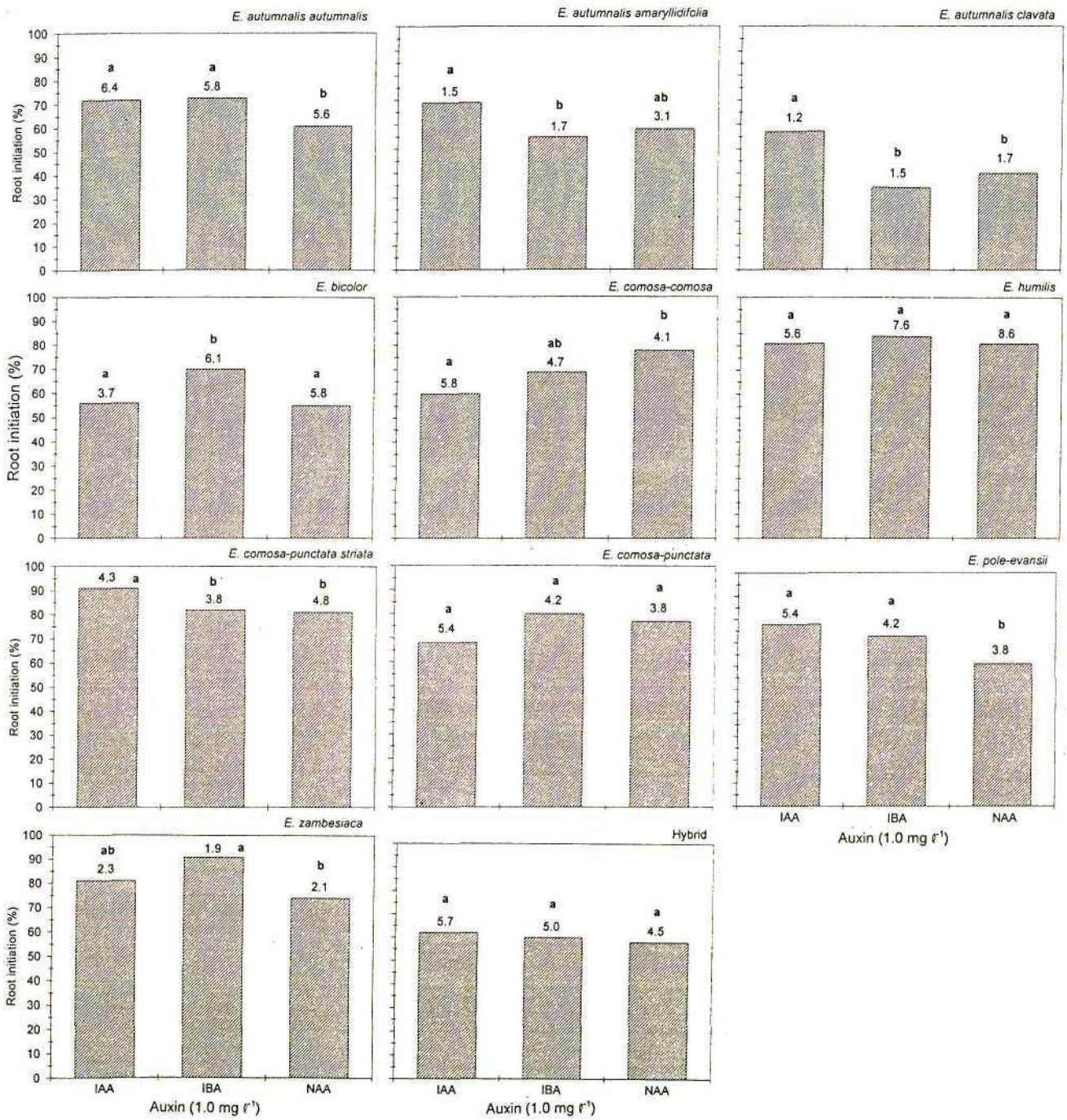


FIGURE 6.4: Adventitious root initiation for *in vitro* shoots of various *Eucomis* species subcultured onto media containing 1 mg ℓ^{-1} auxin. Number annotations on graphs indicate average number of roots per shoot. Bars bearing different letters are significantly different, $P \leq 0.05$.

6.3.3 ROOT INITIATION

Figure 6.4 shows the results for root initiation for the different species under study. Most species did not show a significant difference in percentage root initiation on the various rooting media. An optimal root initiation medium for each species was thus identified using the number of roots initiated.

6.3.4 ACCLIMATIZATION

The suitability of two different support media (perlite and vermiculite) was tested in the acclimatization of the different species (Figure 6.5). Vermiculite tends to hold more water than the perlite medium and was not optimal for all species. Specimens were kept in the misthouse for 6-8 weeks to allow adequate root formation (Plate 6.1 D).

6.3.5 BULK PROPAGATION

Once the optimal conditions for shoot and root formation had been established, the culture vessels were changed from tubes to bottles and a bulk propagation system was established. For several of the species, the optimal initiation media (Table 6.2) proved to differ slightly from those presented in Figures 6.2 & 6.4, possibly due to the age and physiological status of the *in vitro* grown material. Under this system each leaf explant initiates 3-8 shoots, giving an average of 25-30 plantlets per culture bottle (Plate 6.1 C).

Root formation can be accelerated by subculturing the plantlets onto a rooting medium, resulting in 10-25 specimens per bottle. These specimens can then be hardened off in the misthouse, while still retaining sterile leaf material to reinitiate shoots *in vitro*.

Table 6.2: Optimal initiation media for the continuous culture of *Eucomis* species.

SPECIES	Optimal initiation media (mg ℓ^{-1})	
	Shoot (NAA: BA)	Root
E. autumnalis subsp *autumnalis*	1:1	1 NAA
E. autumnalis subsp *amaryllidifolia*	1:1	1 NAA
E. autumnalis subsp *clavata*	1:1	1 NAA
E. bicolor	1:2	1 IBA
E. comosa-comosa	1:1	1 IBA
E. humilis	1:1	1 IBA
E. comosa-punctata striata	1:1	1 NAA
E. comosa-punctata	1:2	1 IBA
E. pole-evansii	1:1	1 IAA
E. zambesiaca	1:1	1 IAA
Hybrid	2:2	1 NAA

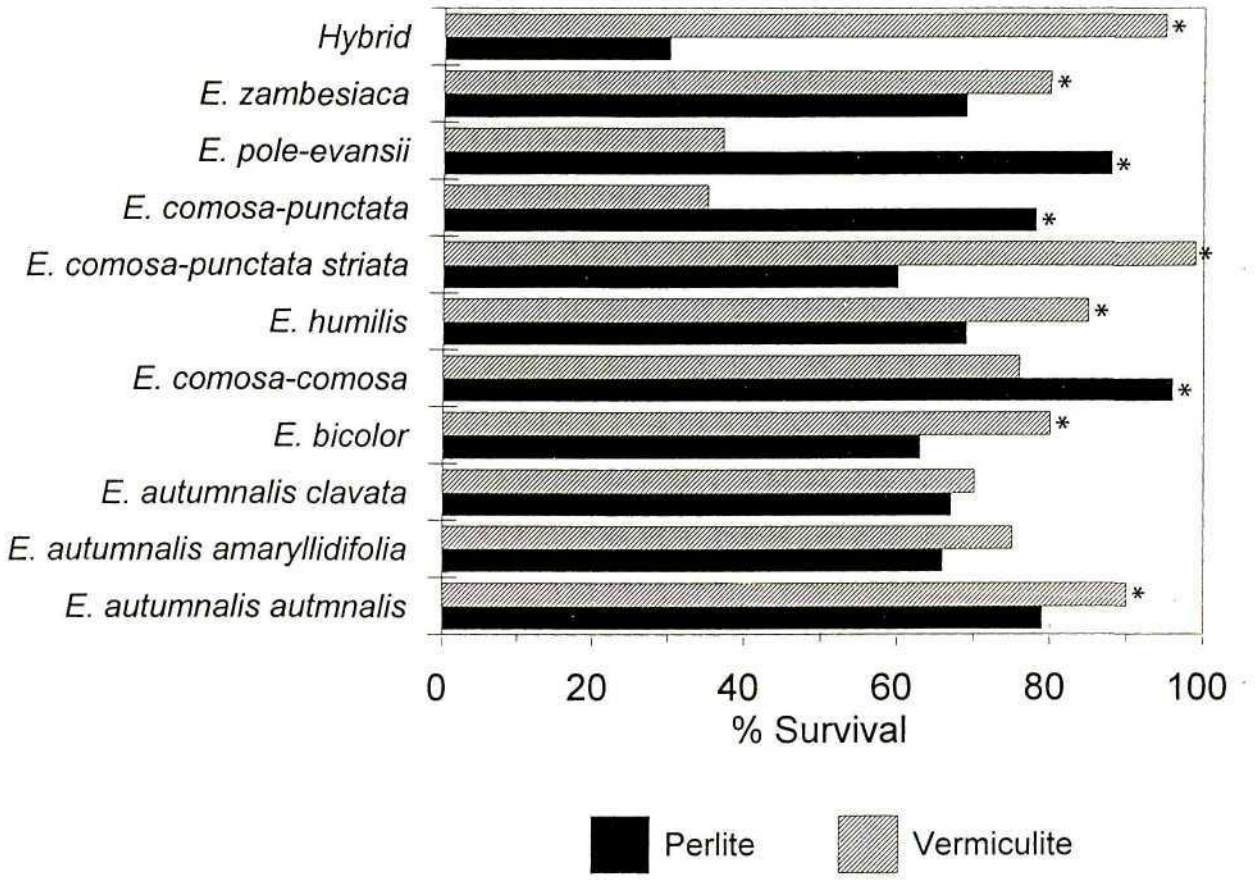
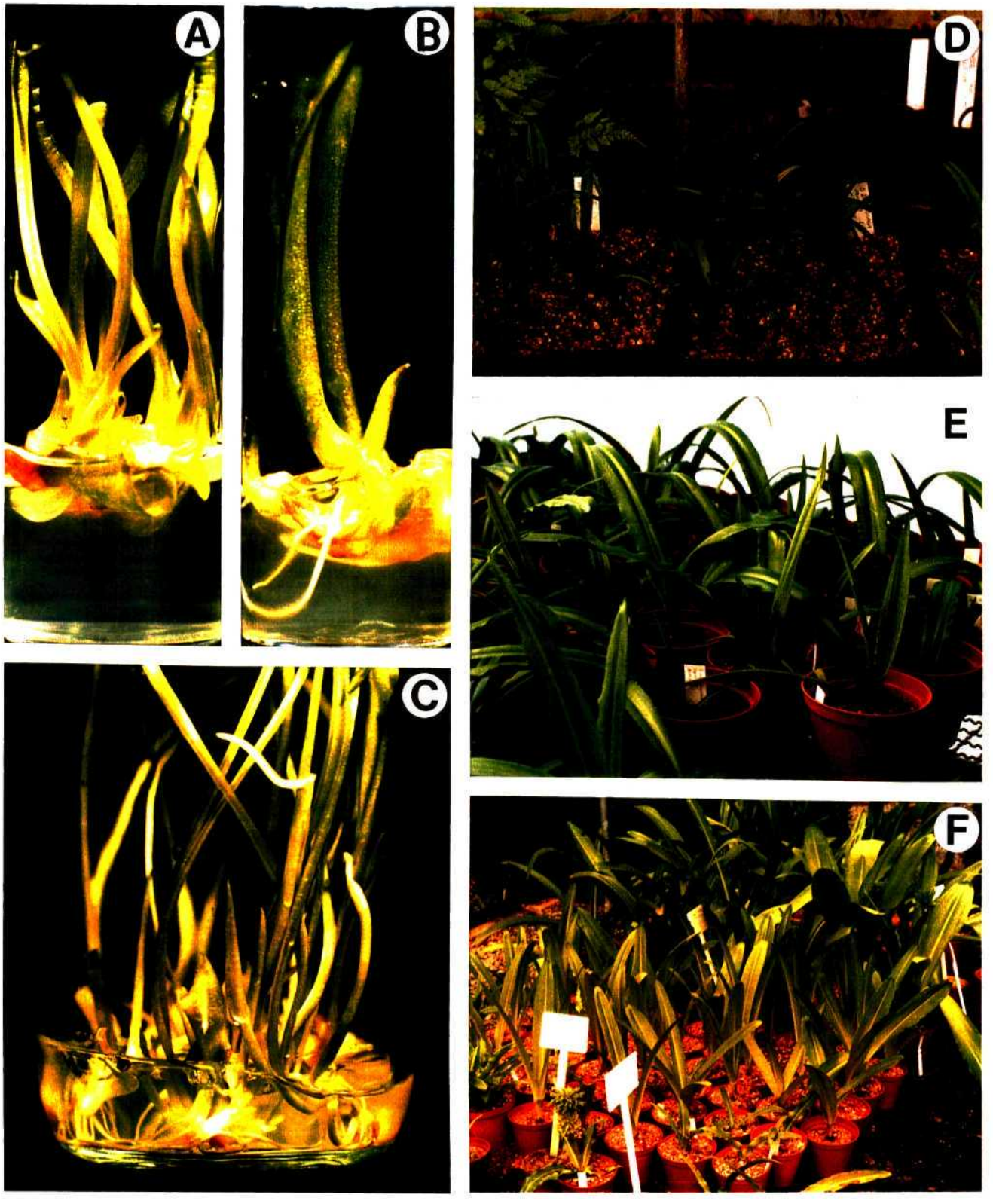


FIGURE 6.5: The effect of different acclimatization support media on plantlet survival for various *Eucomis* species. Bars bearing asterisks (*) indicate a significant difference in survival between the two media, $P \leq 0.05$.

PLATE 6.1: *In vitro* production of *E. autumnalis autumnalis*. Adventitious shoots produced from (A) a leaf explant; (B) a bulb scale explant; (C) Multiple shoot production; (D) Plantlets (± 6 weeks *ex vitro*) acclimatized in vermiculite (in a misthouse); (E) Plants (± 12 months) maintained in a greenhouse; (F) Stock plants (±24 months) maintained in a 20 % shadehouse.


A
B
C
D
E
F

6.4. DISCUSSION

6.4.1 DECONTAMINATION

In general, leaf material was easier to decontaminate and initiated higher numbers of adventitious shoots than the bulb explants. Decontamination is a common problem with bulb material since the open structure of the bulb scales allows entry of micro-organisms which are then difficult to eliminate with conventional sterilizing agents (HUSSEY, 1974). Leaf tissues (and other aerial plant parts) are, in comparison, almost entirely free of contamination. Previous protocols published for *Eucomis* species (AULT, 1995) focussed on the use of the bulb as the explant source, specifically concentrating on the use of twin-scales. Leaf material is a more efficient source of explant material than bulb slices, since harvesting is non-destructive. This is important where the plants are rare or endangered and only limited material is available for culture. McCARTAN AND VAN STADEN (1995) published a protocol utilizing *E. pole-evansii* seedlings germinated *in vitro*, as a source for leaf explants. Seeds are relatively easy to decontaminate without causing senescence. While this method has the advantage of a source of sterile material, seeds of endangered species are difficult to obtain. In addition, this method has none of the advantages presented by vegetative propagation. This is especially important for hybrid propagation. The disadvantage associated with the use of this particular type of explant lay in the seasonal availability of the leaves. Young leaves (spring) proved best for the initiation of adventitious shoots, thus limiting the period for the successful introduction of the plants into culture. In general, the use of leaf material as an explant source was, however, preferable due to its high decontamination success and its suitability in terms of clonal propagation.

Even healthy, symptomless stock plants can be systematically infected with bacteria and / or fungal pathogens (DEBERGH AND MAENE, 1981). There is a high probability for the spread of systemic pathogens in vegetatively propagated plants. The widespread use of systemic pesticides destroys the natural equilibrium of micro-flora coexisting with the plant. The pathogen is able to develop extensively since plants have lost their natural resistance to micro-organisms (DEBERGH AND MAENE, 1981).

Eucomis plantlets maintained for long periods in culture were susceptible to an internal pathogen. This usually could be eliminated by decreasing the periods between sub-cultures, but where the infection was prolific, the culture vessels had to be discarded and reinitiated from other cultured material, or where necessary, from stock plants. This problem highlighted a further advantage of the use of leaf material to supply explants, in that the same plant could be used to reinitiate the culture.

6.4.2 SHOOT INITIATION

Adventitious organogenesis is the most desirable form of multiplication since it enables substantially faster increases in plantlet numbers (DEBERGH AND MAENE, 1981). Although the various *Eucomis* species had differing requirements for optimal shoot initiation, an acceptable percentage shoot initiation was obtained for the majority of species using a 1:1 medium containing NAA and BA. This is an important factor in the commercial propagation of this plant since a single standard medium preparation would be far more economical for plantlet production, in terms of labour and materials. In all cases the young leaf material proved best for adventitious shoot initiation. Adventitious shoots were induced directly, without the intervening step of callus formation, and in many cases adventitious roots were initiated spontaneously. With respect to the bulb scales, shoot initiation is generally more difficult from isolated scale pieces and basal plate tissue than is the production of plantlets from twin-scales (HUSSEY, 1974). This accounts for the difference in the number of shoots initiated from the leaf and bulb explants. Although young, elongating inflorescence stems have proved the most consistently successful source of explants for Liliaceous species (HUSSEY, 1974), this was not the case for *Eucomis* species. These explants, placed on the initiation media described above tended to produce callus. Overall, the leaf material from *Eucomis* species was most suitable for multiple shoot production.

Explant orientation affected the regenerative potential of the leaf tissue (Figure 6.3), with fewer shoots initiated on explants placed with the adaxial surface placed in contact with the medium. Similar results were obtained for *Lilium longiflorum* bulblet production from bulb scales (LESHAM *et al.*, 1982). In both cases, shoot initiation predominated on the adaxial surface of the bulb scale or leaf explant, possibly due to polarity between the two surfaces. Where this surface was placed down, the few shoots that were formed grew into the medium.

The route of shoot multiplication (adventitious or axillary) can be influenced by the juvenility of the tissue. Cultures originally producing axillary shoots have been found to produce abundant adventitious shoots after several sub-cultures. The different response of juvenile tissue can result in the production of more off-type plants (DEBERGH AND MAENE, 1981). In cultures initiated from *Lilium* bulb scales, external bulb scales from older bulbs showed a reduced ability to initiate bulblets, with increased callus production. Higher levels of soluble nitrogen and lower levels of sugars were observed in the internal bulb scales relative to the outer ones (TAKAYAMA AND MISAWA, 1980). The hormones used in the initiation medium, however, remain the most important factors in the regulation of differentiation (TAKAYAMA AND MISAWA, 1980). Juvenile leaf tissue from *Eucomis* species, in addition to the relative ease of decontamination, showed a faster response time for shoot initiation, compared to older leaf material.

6.4.3 ROOT INITIATION

This step is the most labour intensive stage since it is necessary to handle single shoots. Root formation is usually not optimal using a single subculture. The exogenous auxins required for the initiation of roots tend to inhibit root elongation (DEBERGH AND MAENE, 1981), and a hormone-free medium may prove optimal for good root development. In addition, *in vitro* roots are often damaged during transplanting which can increase the chances of pathogen attack. Many tissue culture protocols thus eliminate this step, preferring to treat the *in vitro* plantlets as cuttings and to induce root formation during the acclimatization stage (DEBERGH AND MAENE, 1981). The use of a single root initiation medium (1 mg ℓ^{-1} IAA) for the propagation of the various *Eucomis* species would reduce costs. Results published by AULT (1995) showed that the addition of NAA to the rooting medium had no significant effect on the percentage rooting (of *E. autumnalis*, *E. comosa* and *E. zambesiaca*), relative to the control (0 mg ℓ^{-1} NAA). For most species in this study, root initiation could be eliminated as a micropropagation step by delaying the transfer of plantlets from the shoot initiation medium, and allowing sufficient time for the onset of root initiation. This would be the most advantageous protocol in terms of financial constraints.

6.4.4 ACCLIMATIZATION

In general, vermiculite (Plate 6.1 D) proved adequate for the acclimatization of the plantlets from culture. *E. comosa-comosa; E. comosa-punctata,* and *E. pole-evansii*, however, showed significantly better survival rates when placed in a perlite medium for acclimatization. In vermiculite, these plantlets tended to rot and die quickly. Perlite tends to hold less water than vermiculite and thus was more suitable for the less hardy plantlets. Plantlets removed from culture are susceptible to fungal rot due to the thin epicuticular wax deposits and poorly developed roots (RAYNS, 1993). It is difficult to induce a functioning root system *in vitro* (roots formed *in vitro* are often poorly connected to the plantlets vascular system, and rarely have functioning root hairs (GEORGE, 1993)), and these roots generally die during the acclimatization period and are replaced by new roots. This step is thus usually accompanied by a lag phase in growth (DEBERGH AND MAENE, 1981). This may entail an extended period of acclimatization in a misthouse. *Eucomis* plantlets required a minimum period of 6 weeks in misthouse conditions to fully acclimatize. Where these plantlets were left under these conditions for periods in excess of 18 weeks, their survival rate decreased. Plantlets with well developed root systems and bulbs were transplanted to a potting mix (sand / soil) and maintained in a greenhouse (Plate 6.1 E).

Eucomis plants transferred from misthouse conditions to the greenhouse, did not undergo normal winter dormancy for 1-2 seasons. Phenotypic alterations are often observed in plantlets produced *in vitro.* These are as a result of epigenetic changes occurring *in vitro* as a result of the culture procedure (RAYNS, 1993). Epigenetic changes involve selective gene expression and are stable, heritable at the cellular level, but are potentially reversible (DODDS AND ROBERTS, 1985).

6.4.5 BULK PROPAGATION

The efficient micropropagation of a species requires that plantlets can continuously be initiated from sterile explants. Shoots initiated from secondary bulb explants of *Ornithogalum umbellatum*, another Liliaceous species, were found to be genetically stable after five years in culture, providing the rapid and stable production of these plants for commercial use (NAYAK AND SEN, 1995). In the system described above, sterile leaf explants were excised from *in vitro* plantlets before these were acclimatized and prepared for replanting. Bulk propagation was conducted using

culture bottles as opposed to tubes. Container size (volume) can sometimes affect growth and morphogenesis, probably due to different concentrations of oxygen, carbon dioxide, ethylene and other volatiles in the vessel, as well as the ratio of explant to medium, explant to air and medium to air. Shoot proliferation is usually not optimal in small containers, and fresh weight increases, morphogenesis and the rate of axillary shoot multiplication are frequently affected by container size and shape (GEORGE, 1993). These factors were not, however, readily detectable for the propagation of *Eucomis* species in tubes and culture bottles.

Before the adoption of a protocol on a commercial scale, it is important to determine whether the adventitious bud system produces true-to-type plants, and the frequency of off-type plants should be determined (DEBERGH AND MAENE, 1981). Regular subculturing is a highly labour-intensive practice, and increases the possibility of contamination and genetic instability, as well as the mislabeling of similar species or clones (LOUW, 1995). The frequency of mutations and variations increases with the number of subcultures, necessitating the maintenance of stock plants that can be used to re-initiate cultures utilizing an efficient, reproducible decontamination and initiation protocol. Regular renewal of these plants should eliminate many of the problems associated with plants propagated through adventitious shoots (DEBERGH AND MAENE, 1981). This illustrates again, the importance of a stable explant source (such as leaf material), which is not destructively harvested for the provision of explants.

6.4.6 PROBLEMS ASSOCIATED WITH CONTINUOUS PROPAGATION

The appearance of bacterial contamination in cultures maintained for long periods *in vitro*, suggested the presence of an internal source of contamination. This was possible to eliminate with shorter periods between sub-cultures. The use of antibiotics was not considered to be cost-effective in this case, and has in other research laboratories not given a bactericidal effect in the concentration range that allowed the survival of the explant (DEBERGH AND MAENE, 1981). In light of the increased genetic variation associated with extended periods in culture, it would probably be beneficial to reinitiate these cultures periodically from stock plants. The clonal propagation of hybrid material either for commercial exploitation or breeding purposes depends on maintaining the integrity of the genotype (HUSSEY, 1974).

6.4.7 ECONOMIC CONSIDERATIONS

The most labour intensive step in the production of *in vitro* plantlets is sub-culture (GEORGE, 1993; RAYNS, 1993). This thus represents a major proportion of the cost of such plants. It was found that by leaving the plants in culture for extended periods they began to initiate *de novo* roots. These plantlets were able to survive acclimatization to the same degree as those with roots formed on specific rooting media. The elimination of this step would significantly reduce the production cost of *Eucomis* plants.

6.5 CONCLUSION

Micropropagation is a relatively new industry still undergoing rapid changes. The introduction of new methods has increased the range of products that can be offered at prices that are highly competitive with established propagation techniques, and in most cases with greatly improved product quality (RAYNS, 1993). These techniques are the basis of all other areas of plant biotechnology and are thus a critical research tool. With respect to the commercial uses of these techniques, however, the major restrictions are in terms of cost.

With such rapid rates of propagation, it is easy to achieve an initial high number of plants, but these techniques are too expensive to continue indefinitely without a fixed market. Depending on the facilities available, micropropagation could be accompanied by traditional vegetative methods such as chipping or twin scaling followed by chipping to maintain stock. This entails the optimization of all growing factors to maximize yield per plant for the main bulk of stock (REES, 1992).

Eucomis plants show great potential both ethnopharmacologically and horticulturally. They have no reported susceptibility to common pests and diseases. They grow well in full sun or partial shade, requiring a rich soil and are dormant in winter. The flowers are long lasting and attractive, both as cut flowers and garden specimens. Hybrid varieties are available, and these can be further capitalized upon by micropropagation. This can also aid the conservation of this heavily exploited genus

through the stocking of "muthi" gardens which are growing in popularity with traditional healers in southern Africa. The development of a simple, economical micropropagation protocol is thus of great value to the horticultural industry.

The protocol described above presents several advantages relevant to successful commercial propagation. The use of leaf explants to initiate cultures proved beneficial in terms of the renewable nature of the plant organ, and in the relative ease of decontamination. This explant source further produced a higher number of shoots per explant, increasing the efficiency of the protocol. The root initiation step (and thus the additional subculture onto a specific rooting medium) could be eliminated in the bulk production of the plants by extending the period on the initial (shoot initiation) medium. Transferal of the rooted plantlets to an optimal acclimatization medium, maximizes their survival during the transition period from culture vessel to greenhouse conditions. This further minimized losses common to this stage in the propagation of *in vitro* plantlets. This protocol thus facilitates the rapid and economical bulk production of *Eucomis* species.

CHAPTER 7

TISSUE CULTURE TREATMENTS

7.1 INTRODUCTION

The procurement, cultivation, regeneration and import/export of medicinal plants used in indigenous systems of medicine is of current scientific importance, as the pharmaceutical industry depends on these plants for raw materials (GOVIL, *et al.*, 1993). Over 10% of the world's flora is threatened with extinction within the next few years, particularly in tropical rainforests, and this together with the loss of folkloric information as traditional cultures change their way of life, are distinct threats to the development of plant-derived drugs. Conservation efforts are necessary to preserve endangered genetic resources for future generations to investigate more thoroughly than is possible at present (KINGHORN, 1992). Advances in tissue culture have the potential to overcome the problem of supply of promising plant-derived compounds.

Plants synthesize and accumulate a variety of primary and secondary metabolites, some of which are used by man as medicines. The leaves, roots, flowers and fruits consist of complex cellular tissues containing compartmentalized phytochemicals (TOWERS AND ELLIS, 1993). Secondary metabolites in plants fulfill a variety of roles, including the storage of energy, attraction of pollinators and other dispersal agents, or in the protection of plants against a range of environmental stress factors (COTTON, 1996).

The growth of plant organs, tissues and cells *in vitro*, introduced the possibility of the production of such compounds on a large scale. Cell, callus and organ cultures are capable of producing most types of secondary compounds including phenylpropanoids, polyketides, terpenoids, alkaloids and carbohydrates, and since they consist of populations of actively growing cells, they offer potential materials for biosynthetic studies (TOWERS AND ELLIS, 1993). The biosynthesis of phytochemicals in plants and in tissue culture is, however, often dependent on the

presence of differentiated tissues, or on the development of specialized cells (TOWERS AND ELLIS, 1993). In addition, these cultures usually only produce small quantities of the desired product (BURBIDGE, 1993). During culture, the concentrations of secondary metabolites may vary depending on the plant hormones added, and physical parameters such as the presence of light. Metabolites may in some cases be produced in culture that are absent in the plant from which the tissue was derived (HARBONE, 1998).

The manipulation of the culture environment and media can affect the rates of both cell growth and accumulation of secondary metabolites. Secondary metabolite biosynthesis is extremely complex (with respect to enzyme regulation, cellular and subcellular sites of synthesis and pathways) and hence poorly understood. In order to successfully increase yield, the biosynthetic pathways, physiology and localization of secondary metabolites in plants need to be understood. By clarifying the physiological and biochemical factors governing secondary metabolite biosynthesis and degradation, the ability to increase the amounts of secondary metabolites produced and accumulated by cultured plant cells can be enhanced (BALANDRIN AND KLOCKE, 1988).

These experiments are usually conducted using cell suspension cultures, in a manner similar to microbial fermentation techniques. Plant cells, however, differ from microbial cells primarily in that they function in complex tissues as opposed to single celled organisms, and often the desired secondary product is confined to particular groups of differentiated cells within the plant. Undifferentiated cells, such as those comprising cell cultures, cannot be manipulated to produce these compounds since they have reverted to an unspecialized state (BURBIDGE, 1993).

For this reason, the following experiments were conducted using entire plantlets *in vitro*, to investigate the effect of manipulation of the culture medium on the potential anti-inflammatory activity of extracts prepared from these plantlets.

Constituents of tissue culture media

Intact plants, tissues and organs take up nitrogen and show faster growth rates on media containing both nitrate and ammonium ions. Nitrogen, in tissue culture media, is thus supplied in the form of salts of both nitrate and ammonium ions (GEORGE, 1993; RAYNS AND FOWLER, 1993). In this respect, both the total nitrogen concentration in the medium, and the ratio of NO_3^- to NH_4^+ ions is important. MS medium contains a high ratio of nitrate to ammonium ions (66:34) and has a higher total nitrogen content than most other media (GEORGE, 1993). Experimentation has shown that changes to the nitrogen content of MS medium resulted in the improved growth or morphogenesis of a number of species. Both the ratio of ions and the total nitrogen content seem to be key factors in determining growth or morphogenesis, and require optimization for different species. In addition, this improved morphogenesis on media supplemented with high nitrogen levels may not be apparent unless the sucrose concentration is adequate. Concentrations of nitrogen higher than that prescribed in MS medium rarely proved beneficial to growth (GEORGE, 1993).

The culture of cells, tissues and organs requires the addition of a carbon source into the medium, since very few cultures are autotrophic. Sucrose is generally considered the best source of carbon for tissue cultures (GEORGE, 1993). Sucrose is usually hydrolysed partially or completely in the medium into the component monosaccharides glucose and fructose, which are taken up by the plant tissues partly through active transport,and partly through passive permeation (GEORGE, 1993). The optimal concentration of sucrose to induce morphogenesis or growth,differs with species. Sucrose levels in media are generally in the range 2-4% w/v, but increased growth on media containing up to 6% sucrose has been reported (GEORGE, 1993).

7.2 MATERIALS AND METHODS

7.2.1 COX-1 ASSAY

Plantlets grown *in vitro* (CHAPTER 6) from three species (*E. autumnalis autumnalis*; *E. bicolor* and *E. pole-evansii*) were harvested and dried at 50°C. Extracts were made using ethanol and water (CHAPTER 2) and these were tested for COX-1 and COX-2 inhibitory activity (CHAPTER 2). A dilution series was prepared for the *E. autumnalis autumnalis* extract (ethanol) and the IC_{50} value was calculated using regression analysis.

7.2.2 GROWTH MEDIA

A modified MS medium (1962), with 100 mg ℓ^{-1} myo-inositol and 2 g ℓ^{-1} Gelrite® as the solidifying agent was used (Appendix II). The optimal hormone combination for shoot initiation from CHAPTER 6 was chosen (i.e. 1 mg ℓ^{-1} NAA and 1 mg ℓ^{-1} BA). This was supplemented with different levels of sucrose or nitrogen. The pH of the medium was adjusted to 5.8 with 1 M NaOH before autoclaving at 121°C and 103 kPa for 20 min.

Sucrose concentration

The basic medium described above was supplemented with 10 g ℓ^{-1} (low), 20 g ℓ^{-1} (control) or 40 g ℓ^{-1} (high) sucrose.

Nitrogen concentration

For the high nitrogen treatment, double the volume of Stocks 1 and 2 of the modified MS medium (APPENDIX II) were used, while for the low nitrogen treatment, half the volume of Stocks 1 and 2 were added. The control treatment contained the normal volumes of Stocks 1 and 2. Each treatment contained 20 g ℓ^{-1} sucrose. The concentration of nitrogen in each medium was as indicated in Table 7.1.

Table 7.1: The concentration of nitrogen in the modified MS media.

Medium	Nitrogen concentration	
	g ℓ^{-1}	mM
Control	0.84	60.00
High nitrogen	1.68	119 .94
Low nitrogen	0.42	29.99

7.2.3 EXPERIMENTAL PROCEDURE

Leaf explants (5 mm x 10 mm) were excised from sterile leaf material of *E. autumnalis autumnalis* plantlets grown *in vitro* (CHAPTER 6). These explants (9) were transferred to culture bottles containing the different media described above, and placed in a growth room with a 16 / 8 h light / dark regime (light intensity of 27 μmol m^{-2} s^{-1} at 25±3°C). After 10 weeks the plantlets were harvested, the number of shoots counted and weighed. These were then dried and the dry mass determined. Ethanol extracts (10 mg $m\ell^{-1}$) were prepared and these were tested in the COX-1 assay (CHAPTER 2).

7.2.4 ANALYSIS OF RESULTS

The rate and number of shoots initiated on media containing high and low levels of nitrogen or sucrose was determined. In addition, the fresh and dry mass of cultured shoots from the different treatments, and the ratio of fresh mass : dry mass were determined. Dried material was also extracted and tested for COX-1 inhibitory activity (CHAPTER 2). The results were analysed using a One-way ANOVA and Tukey HSD test to identify significant differences.

7.3 RESULTS

7.3.1 SCREENING RESULTS FOR *IN VITRO* PLANTLETS

Figure 7.1 depicts the percentage inhibition of COX-1 by both aqueous and ethanol extracts from three species of *Eucomis* plantlets, harvested directly from the culture vessels. Ethanol extracts were screened at a concentration of 250 μg mℓ^{-1} and the aqueous extracts at 500 μg mℓ^{-1} in the assay. Significantly high levels of activity (above 70%) were detected for the ethanol extracts from all three species, with all three species exhibiting higher levels of activity than those of the corresponding bulb extracts from the adult plants (Figure 2.1). The aqueous extracts showed lower levels of activity than those detected in the bulb extracts from the respective adult plants (Figure 2.1). In each case, the level of activity detected in the aqueous extract was significantly lower than that of the ethanol extract. Analysis of variance across the species showed that the ethanol extract from *E. autumnalis autumnalis* showed significantly higher activity than the other two extracts, and the activity of the aqueous extract from this species was significantly higher than that of *E. bicolor*.

The results for the dilution series tested in both the COX-1 and COX-2 assays are presented in Figure 7.2. The IC_{50} values were calculated to be 117 μg mℓ^{-1} for COX-1 inhibition, and 124 μg mℓ^{-1} for COX-2 inhibition, giving a COX-2/COX-1 ratio of 1.10. These IC_{50} values were higher than those calculated for the crude ethanol extracts presented in Table 2.3. The COX-2/COX-1 ratio for the *in vitro* plantlets was, however, lower than that of the crude leaf extract (Ratio = 1.9), and is closer to an average of the three values given for the adult plant (Table 2.3).

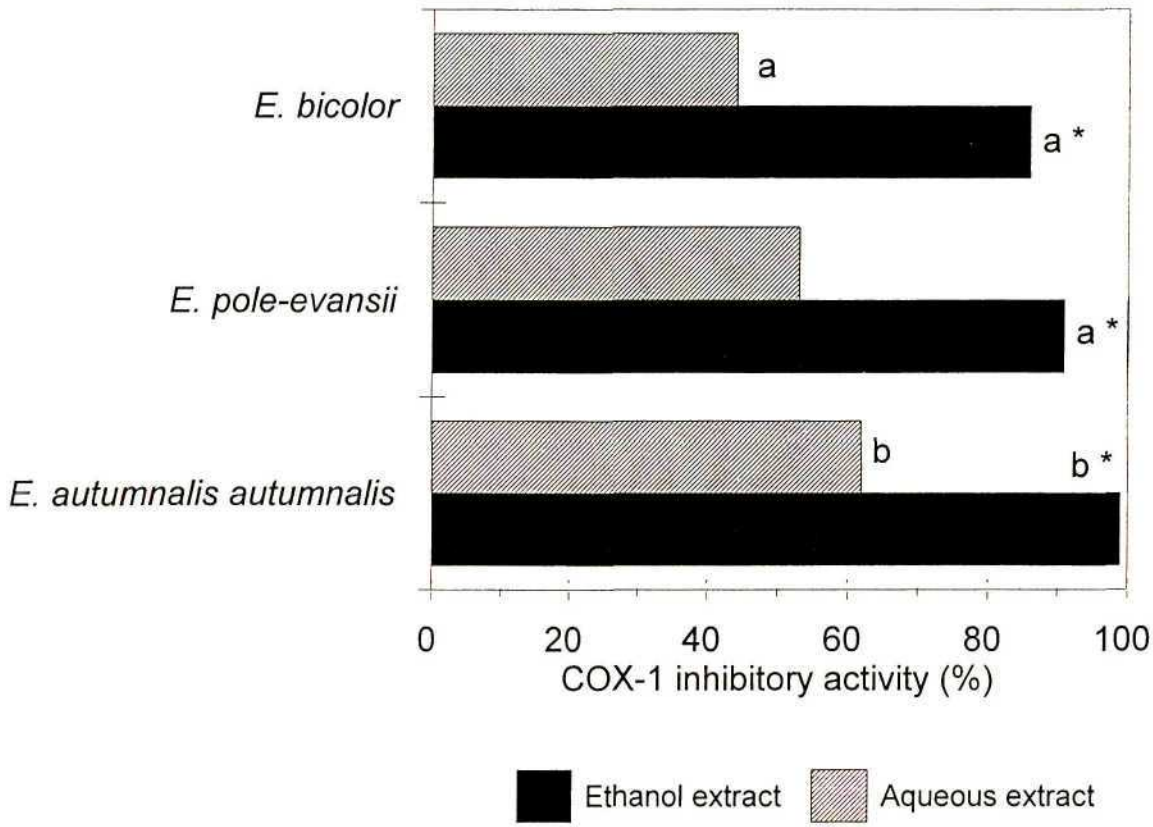


FIGURE 7.1: The % inhibition of the COX-1 enzyme by aqueous and ethanolic extracts from *E. autumnalis autumnalis, E. bicolor* and *E. pole-evansii.* Extracts were prepared from entire *in vitro* grown plantlets. Screening concentration for aqueous extracts was 500 μg mℓ^{-1} and for ethanol extracts was 250 μg mℓ^{-1}. Bars bearing different letters are significantly different, $P \leq 0.05$. An asterisk (*) indicates a significant difference between the aqueous and ethanol extract of a species.

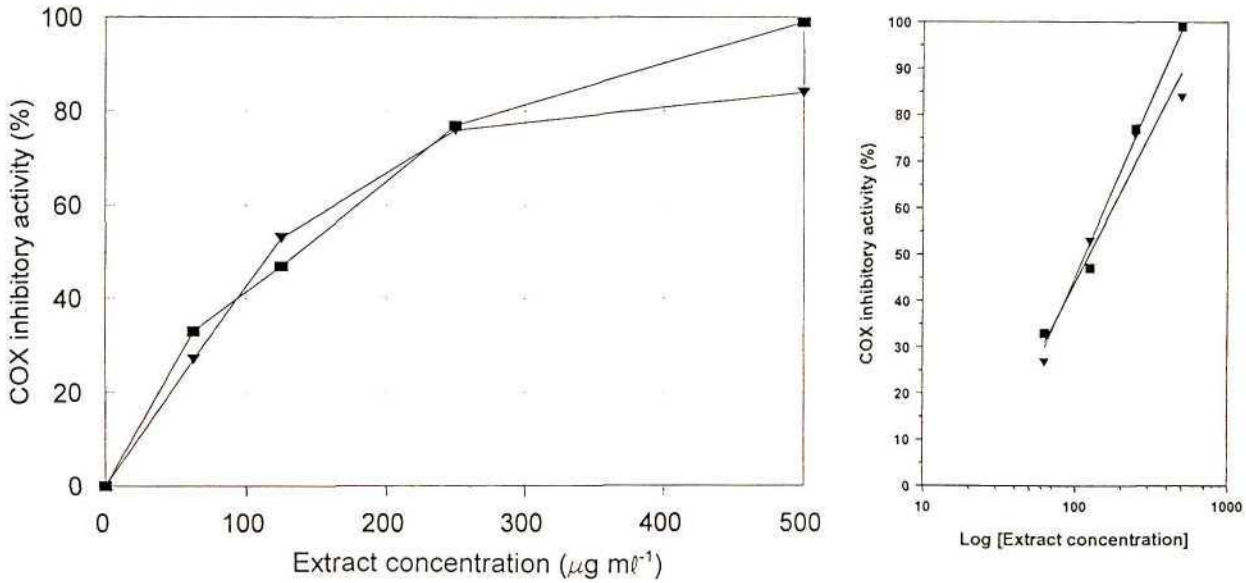


FIGURE 7.2: Dilution curves (Left) and regression analysis (Right) prepared from the crude ethanol extracts of *in vitro* grown *E. autumnalis autumnalis* plantlets, tested for inhibitory activity in the COX-1 (■) and COX-2 (▼) assays.

7.3.2 GROWTH DATA

Effect of sucrose

Changing the sucrose concentration in the initiation medium resulted in a significant change in the number of shoots produced per culture flask (Figure 7.3), as well as in the average fresh and dry mass of the plantlets (Figure 7.4). High sucrose levels increased the number of shoots to nearly double that produced in the control treatment. Lowering the sucrose level had less of an effect on shoot number, but made a significant difference to the fresh and dry mass. A high sucrose concentration in the medium led to an increase in the average mass of each plantlet, while low sucrose levels significantly decreased the mass of the individual plantlets. Analysis of the ratio of fresh to dry mass (Figure 7.5) showed that high sucrose levels significantly lowered this ratio with respect to the control, but a low sucrose level in the medium had no significant effect. This indicates that the increase in mass was due increased growth and that the plants grown with high sucrose levels contained less water than the other two treatments.

Effect of nitrogen

Decreasing the amount of nitrogen added to the initiation medium significantly lowered the number of shoots produced (Figure 7.6), but had little effect on the average fresh and dry mass (Figure 7.7). High levels of nitrogen also decreased shoot number, but to a much lesser degree, and led to a significant increase in average fresh and dry mass. Changing the nitrogen content of the medium (high and low nitrogen treatments) increased the average fresh mass of the individual plantlets produced. The ratio of fresh to dry mass for the high nitrogen treatment was significantly higher that of the low nitrogen treatment, indicating a higher water content. Neither treatment differed significantly from the control (Figure 7.8).

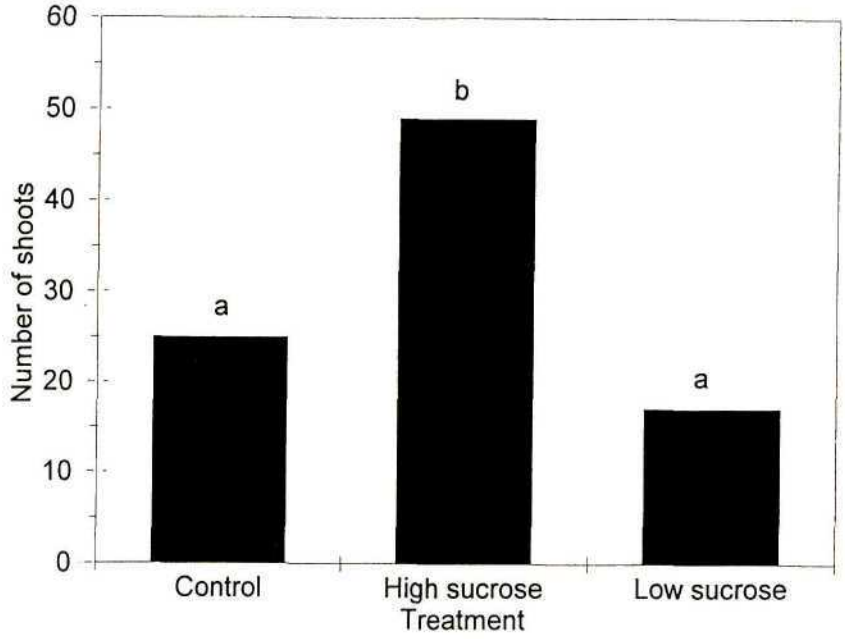


FIGURE 7.3: The number of shoots initiated per treatment for shoot initiation media supplemented with high (40 g ℓ^{-1}) and low (10 g ℓ^{-1}) levels of sucrose. The control contained 20 g ℓ^{-1} sucrose. (Bars bearing different letters are significantly different, $P \leq 0.05$).

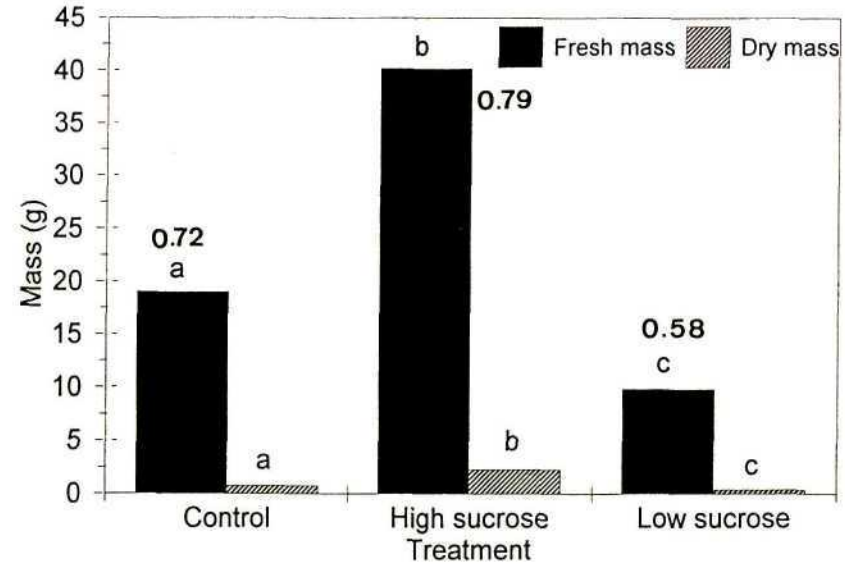


FIGURE 7.4: The average fresh and dry mass of shoots initiated (per culture bottle) on media supplemented with high (40 g ℓ^{-1}) and low (10 g ℓ^{-1}) levels of sucrose. The control contained 20 g ℓ^{-1} sucrose. (Bars bearing different letters are significantly different, $P \leq 0.05$). Annotations indicate average mass of individual plantlets (g).

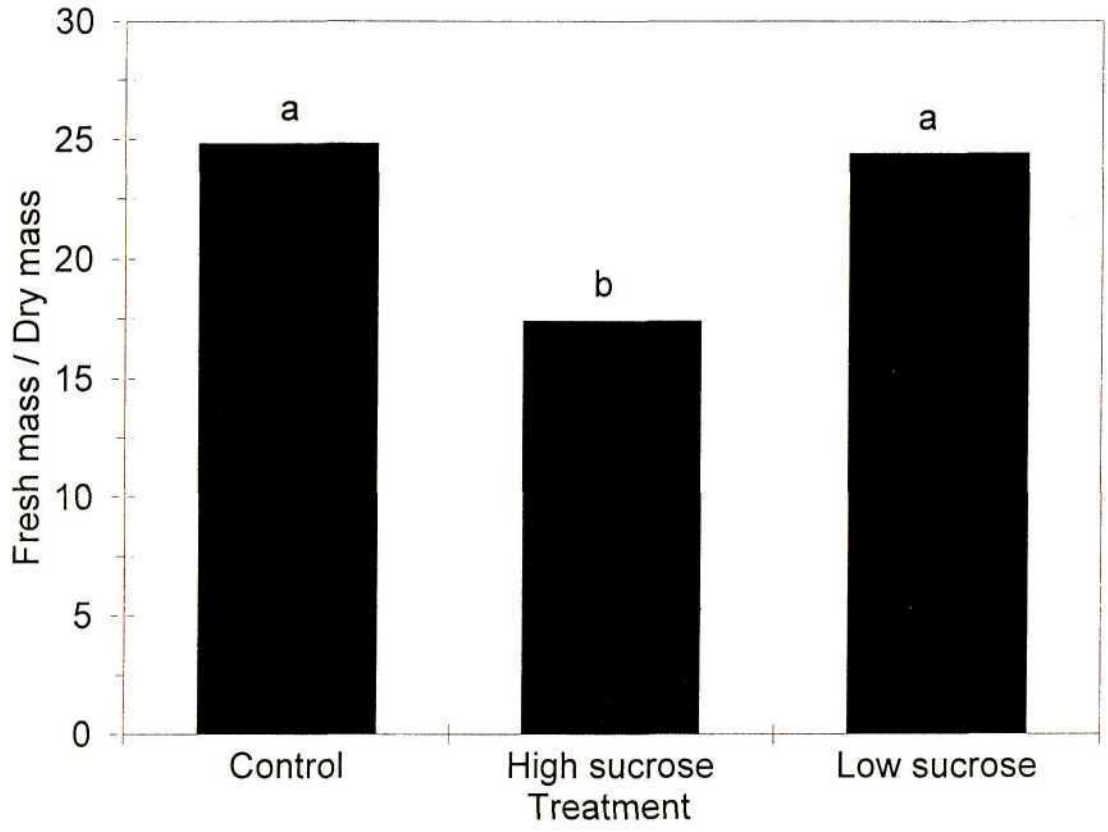


FIGURE 7.5: Variation in the ratio of fresh to dry mass of shoots initiated *in vitro* on media supplemented with high (40 g ℓ^{-1}) and low (10 g ℓ^{-1}) levels of sucrose. The control contained 20 g ℓ^{-1} sucrose. (Bars bearing different letters are significantly different, $P \leq 0.05$).

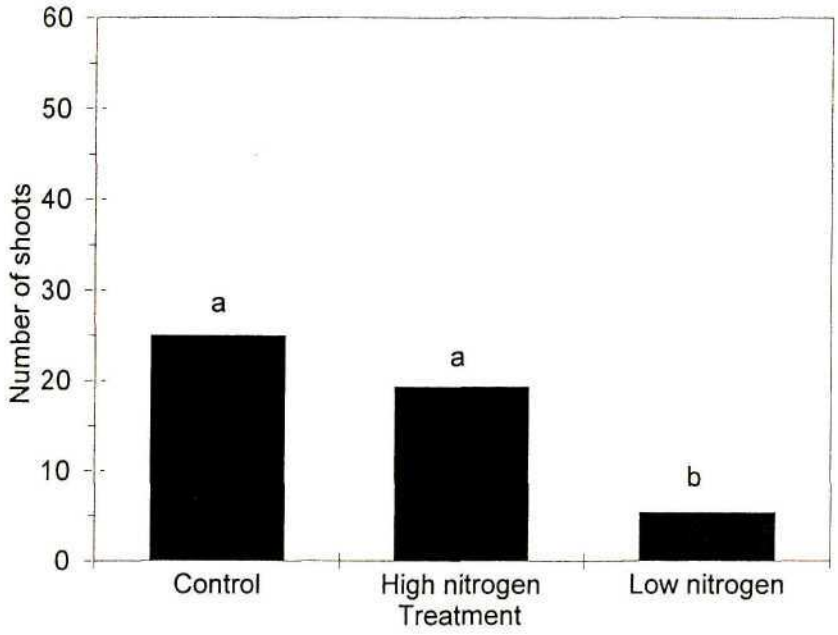


FIGURE 7.6: The number of shoots initiated per treatment for media supplemented with high (120 mM) and low (30 mM) levels of nitrogen. The control contained 60 mM nitrogen. (Bars bearing different letters are significantly different, $P \leq 0.05$).

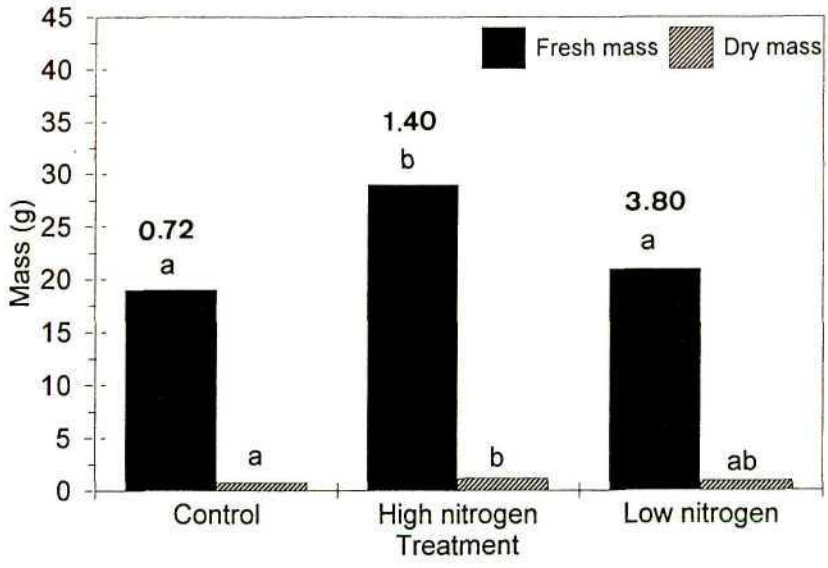


FIGURE 7.7: The average fresh and dry mass of shoots initiated per culture bottle for media supplemented with high (120 mM) and low (30 mM) levels of nitrogen. The control contained 60 mM nitrogen. (Bars bearing different letters are significantly different, $P \leq 0.05$). Annotations indicate average mass of individual plantlets (g).

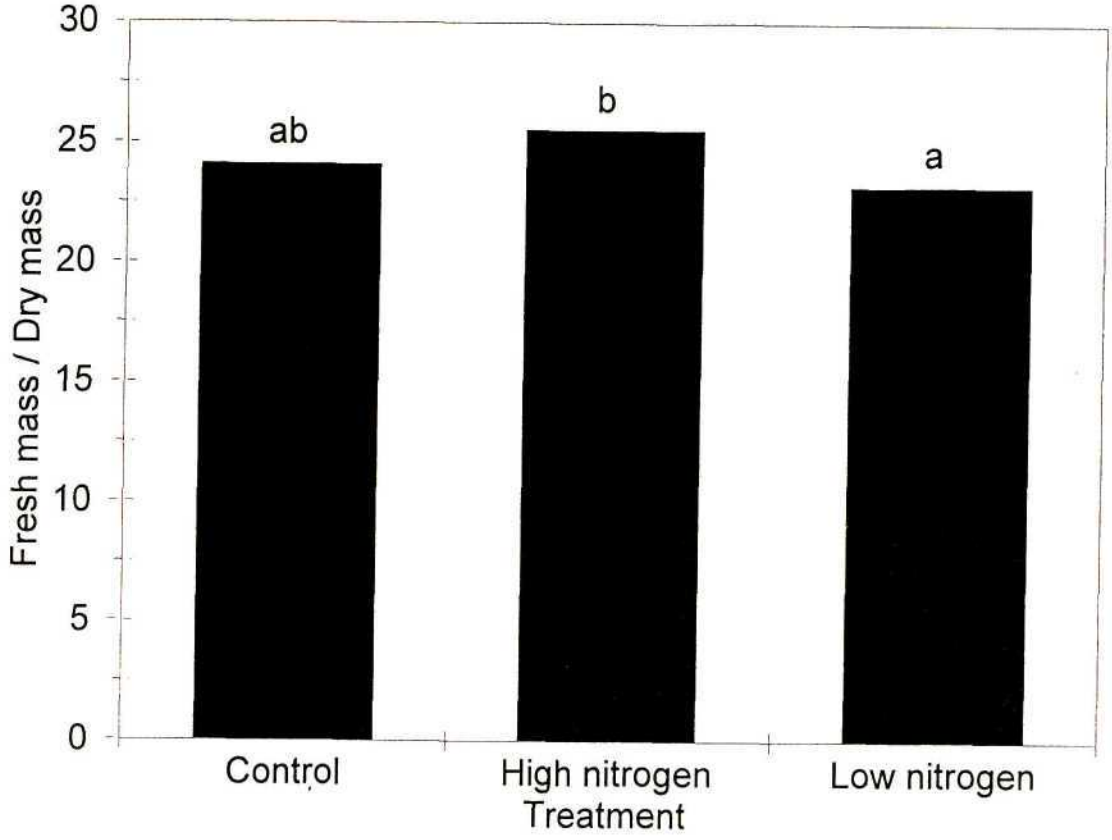


FIGURE 7.8: Variation in the ratio of fresh to dry mass of shoots initiated *in vitro* on media supplemented with high (120 mM) and low (30 mM) levels of nitrogen. The control contained 60 mM nitrogen. (Bars bearing different letters are significantly different, $P \leq 0.05$).

7.3.3 ANTI-INFLAMMATORY ACTIVITY OF EXTRACTS

Effect of sucrose

Although high levels of sucrose resulted in an increase in the fresh mass of the plantlets, this treatment had no significant effect on COX-1 inhibitory activity. In the plantlets grown on low levels of sucrose there was, however, a significant decrease in the COX-1 inhibitory activity of the extracts (Figure 7.9).

Effect of nitrogen

Changing the levels of nitrogen in the growth medium had no significant effect on COX-1 inhibitory activity (Figure 7.10).

7.3.4 ANALYSIS OF VARIANCE ACROSS ALL TREATMENTS

The pooled data from both experiments was subjected to a One-way ANOVA and Tukey's HSD test. High levels of sucrose and nitrogen significantly increased shoot number, fresh mass and dry mass relative to the control. Treatments containing low levels of sucrose and nitrogen did not differ from the control in terms of shoot number, but produced significantly lower results for fresh mass and dry mass. Analysis of variance showed that the fresh to dry mass ratios for high sucrose and nitrogen treatments were significantly lower than the control but that the low sucrose and nitrogen treatments had no effect on this ratio. In terms of COX-1 inhibitory activity, only the low sucrose treatment had a significant effect. This was evident as a decrease in the anti-inflammatory activity of the extracts.

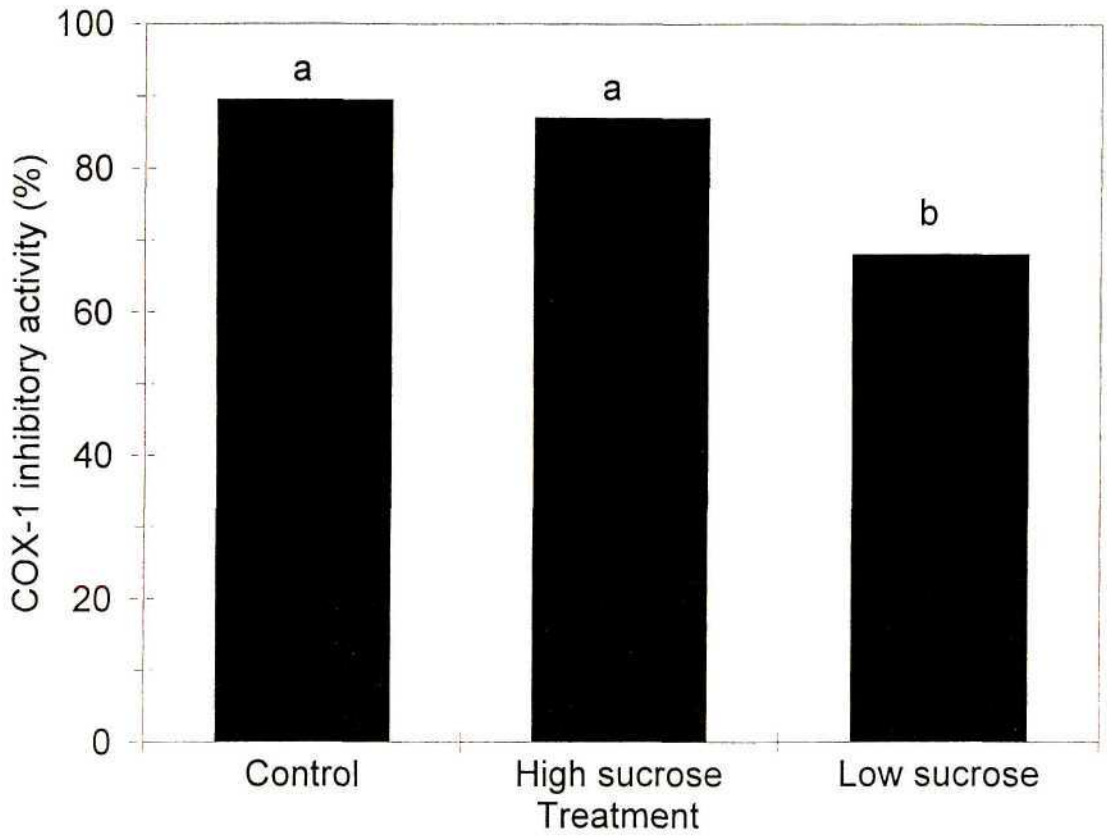


FIGURE 7.9: The % inhibition of the COX-1 enzyme by ethanolic extracts prepared from *in vitro* grown plantlets grown under different sucrose concentrations. High sucrose = 40 g ℓ^{-1}; Low sucrose = 10 g ℓ^{-1} and Control = 20 g ℓ^{-1} sucrose. Screening concentration for crude extracts = 250 μg mℓ^{-1}. (Bars bearing different letters are significantly different, $P \leq 0.05$).

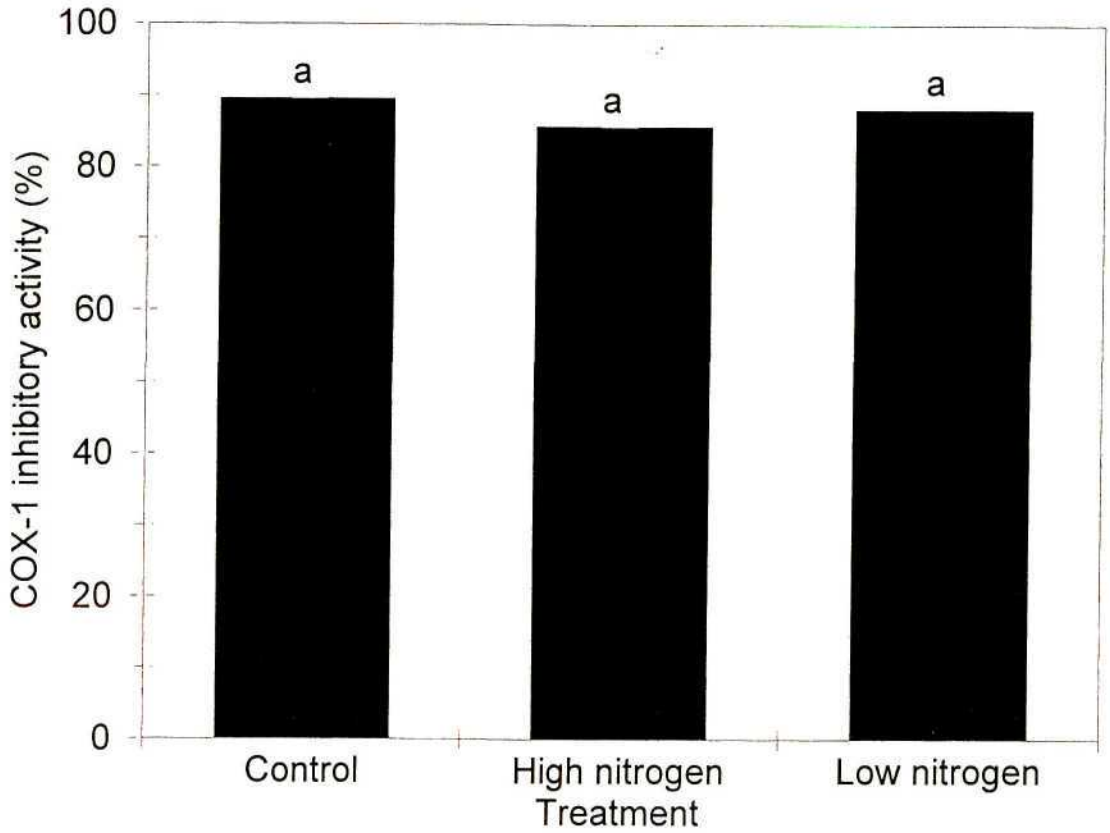


FIGURE 7.10: The % inhibition of the COX-1 enzyme by ethanolic extracts prepared from *in vitro* grown plantlets grown under different nitrogen concentrations. High nitrogen = 120 mM; Low nitrogen = 30 mM and Control = 60 mM nitrogen. Screening concentration for crude extracts = 250 μg mℓ^{-1}. (Bars bearing different letters are significantly different, $P \leq 0.05$).

7.4 DISCUSSION

7.4.1 SUCROSE

Sucrose (at a concentration of 30 g ℓ^{-1}) is the mostly commonly used carbohydrate source for the culture of geophytes. Relatively few studies have been conducted on the effect of altering sucrose (or carbohydrate) concentrations on the initiation and growth of bulbous plants *in vitro* (VAN RENSBERG AND VCELAR, 1989). Sucrose concentration significantly altered the number of buds formed per explant in different genotypes of *Lachenalia*. Omitting sucrose from the culture medium generally had a negative effect on shoot initiation, as did supra-optimal levels of sucrose. Optimal requirements for the initiation of buds and the subsequent growth of shoots *in vitro* differed, as did the requirements for different genotypes (VAN RENSBERG AND VCELAR, 1989). In other studies, increased sucrose concentrations decreased both the number and fresh mass of *Hyacinthus orientalis* shoots *in vitro* (BACH *et al.*, 1992). Shoot initiation in *E. autumnalis autumnalis* is promoted, to a significant extent, by high sucrose levels. Lowering the sucrose concentration had less of an effect, but did reduce shoot number.

Investigations into hyperhydricity showed that this can be reduced by altering the carbohydrate source, but the effect of this on leaf metabolism is unknown. Differences in fresh mass, but not dry mass accumulation, indicate that a large proportion of the mass difference between treatments is due to water uptake (LESHAM *et al.*, 1982). High sucrose levels in this study resulted in increased fresh and dry mass accumulation (Figure 7.4). The ratio of these masses showed a significant decrease relative to the control, indicating that this change is due to increased growth, rather than water accumulation. No significant difference was observed for the low sucrose treatment, indicating that this medium contained sufficient quantities of the carbohydrate source for growth. The effect of omitting the carbohydrate source from the medium was not investigated.

Increased sucrose concentrations promote hyperhydricity, and the accumulation of sugars in the leaves of the plantlets (ZIMMERMAN AND COBB, 1989). Organogenesis varies with the type and quantity of carbohydrate source. This is

proposed to be due to changes in the osmotic potential as well as in metabolism. In this respect, the hormonal and nutritional status of the explant has an effect on organogenesis (VAN RENSBERG AND VCELAR, 1989). Increasing sucrose concentration (up to 90 g ℓ^{-1}) inhibited scale leaf formation in *Lilium* bulb scales, with 30 g ℓ^{-1} proving optimal (TAKAYAMA AND MISAWA, 1980). In this study, high sucrose concentrations promoted shoot initiation (Figure 7.3). There was no noticeable increase in hyperhydricity.

The increase in shoot initiation had no impact on the size of the individual shoots, since the average mass per plantlet for this treatment was similar to that of the control. Low sucrose levels did not significantly reduce the number of shoots initiated, but these were significantly smaller than those of the control. The average fresh and dry mass of this treatment was significantly lower than the control, indicating a negative effect on shoot growth.

The increased growth observed on the medium containing high levels of sucrose did not impact significantly on the accumulation of anti-inflammatory compounds in the plantlets. The COX-1 inhibitory activity of these extracts was very similar to that of the control. Plantlets grown on media with low sucrose concentrations, however, showed significantly reduced levels of anti-inflammatory activity. This could indicate that sufficient carbohydrates were available for growth and development (primary metabolism), but that the pathways producing the anti-inflammatory compounds were not functioning optimally.

7.4.2 NITROGEN

Nitrogen in plant cells exits in the reduced form of ammonium ions (NH_4^+), for incorporation into amino acids (GEORGE, 1993; RAYNS AND FOWLER, 1993). There is thus an advantage in terms of energy conservation to supplying nitrogen in a form that does not require reduction before incorporation into biological compounds. Nitrogen is not supplied solely as ammonium ions since this would be toxic, and high concentrations result in hyperhydricity (RAYNS AND FOWLER, 1993).

A balance of ammonium and nitrate ions (NO_3^-) further acts as a pH buffering system in that the uptake of ammonium ions from the medium is accompanied by the release of H^+ ions resulting in acidification, while the uptake of nitrate ions results in the excretion of OH^- ions (RAYNS AND FOWLER, 1993). The form in which nitrogen is supplied markedly affects the growth and development of cultures (GEORGE, 1993; RAYNS AND FOWLER, 1993).

Associated with the increase in nitrate ions is an increase of the corresponding cation, potassium. This is the most important cellular cation as it plays a role in cellular homeostasis (GEORGE, 1993). Potassium is involved in the regulation of pH, osmosis as well as the action of some enzymes. Potassium is readily transported across cell membranes and thus regulates turgor (GEORGE, 1993).

Morphogenesis is effected by the total amount of nitrogen provided, and in most cases this must be supplied as both ammonium and nitrate ions. Shoot initiation has been found to occur in the absence of ammonium ions (e.g. strawberry). Addition of ammonium ions can cause phytotoxicity and/or hyperhydicity. Root growth is often depressed by ammonium and promoted by nitrate ions (GEORGE, 1993). Increasing the level of available nitrogen in the medium, without altering the ratio of ammonium to nitrate ions had no significant effect on the number of shoots initiated by leaf explants of *E. autumnalis autumnalis*. This medium did, however, result in increased growth, with a substantial increase in both the average fresh and dry mass. Low nitrogen levels significantly reduced shoot initiation, and root development. This was accompanied by a decrease in growth, since the average fresh and dry mass did not differ significantly from the control. This implies that the reduced number of plantlets were larger than the control plantlets in terms of leaf (and possibly bulblet) biomass (not root biomass).

Changing the nitrogen levels had no significant effect on the anti-inflammatory activity.The most important factors controlling secondary metabolite synthesis are reported to be hormones (DATTA, 1993). This was not, however, a parameter tested in this study.

7.5 CONCLUSION

In vitro grown plantlets of *Eucomis* species show high levels of anti-inflammatory activity, indicating that these compounds are produced *in vitro*. Extracts prepared from these plants show high COX-1 and COX-2 inhibitory activity, with a COX-2/COX-1 ratio comparable to those obtained from the intact plant organs (CHAPTER 2).

It is evident that optimal sucrose concentration differs with plant species, and that the effect of changing this level is not consistent. In *E. autumnalis autumnalis*, high levels of sucrose promote shoot initiation, but not growth of individual plants. Low sucrose levels impact negatively on shoot growth.

Altering the levels of nitrogen from those proposed by MURASHIGE AND SKOOG (1962) primarily had a negative effect on shoot initiation, but led to increased growth of individual plants, possibly due to the extra sucrose available for uptake.

While altering the levels of nitrogen and sucrose in the medium altered the primary metabolic systems involving growth and development, they had a less significant effect on secondary metabolism, with only low sucrose levels significantly altering the production of anti-inflammatory compounds in the plantlets. This last factor could also be linked to the sub-optimal functioning of the primary metabolic systems. The plantlets grown under these conditions were both less in number, and smaller. Of the different treatments, optimal sucrose levels are most important in terms of anti-inflammatory activity.

CHAPTER 8

CALLUS STUDIES

8.1 INTRODUCTION

Most plant-derived pharmacologically active compounds have complex structures, making chemical synthesis an economically uncompetitive option. The majority of the plants from which important pharmaceutics are isolated, are grown on large scale plantations. Factory-type production is an attractive option for the pharmaceutical industry since this would minimize general problems such as crop failure and transport costs. Often these medicinal plants have to be grown (for climatic reasons) in regions far away from the site of drug preparation (BERLIN, 1988).

Plant cells can be cultured successfully as undifferentiated or partially differentiated cell suspensions, callus, or completely differentiated organs, depending on nutrient supplies, hormonal levels, light regimes, temperature and access to oxygen and carbon dioxide (TOWERS AND ELLIS, 1993). The production of secondary metabolites in culture systems, on a large scale, involves an understanding of plant physiology, phytochemistry and chemical engineering. The desired phytochemicals may be confined within the cells or excreted into the medium or be distributed between both these compartments (TOWERS AND ELLIS, 1993).

The accumulation of high concentrations of secondary metabolites in higher plants tends to occur in specific cell types at specific developmental stages, often in response to environmental stresses. Tissue cultured cells from higher plants similarly accumulate large amounts of secondary compounds when subjected to certain chemical stresses or under certain culture conditions (BALANDRIN AND KLOCKE, 1988). This results in a system technically difficult to optimize and limited success has been obtained in this field.

Tissue culture systems aimed at the formation of medicinally important alkaloids continue to be established (CORDELL, 1995). The secondary metabolites of *Artemisia annua* tissue cultures have been studied for several years, but little success with respect to producing the desired levels of artemisinin has been achieved. Undifferentiated callus from these plants produces no artemisinin, while the differentiated shoots showed a terpenoid profile similar to that of the intact plant (CORDELL, 1995).

Potentially, valuable pharmaceutical intermediates and drugs can be produced on an industrial scale in +1000ℓ bioreactors. This has succeeded for the industrial production of antibiotics and other chemicals by continuous fermentation of microbial cultures (BALANDRIN AND KLOCKE, 1988). This system depends for success on both the physiological limitations of the plant cells, and on advancements in understanding of both the biological and engineering factors associated with tissue culture systems. These include the development of high yielding, genetically stable cell lines with shorter doubling times, and on the improvement of contamination-free culture methods and bioreactor design (BALANDRIN AND KLOCKE, 1988). More than 30 natural products have been biosynthesized and accumulated in cell culture at levels higher than in the respective whole plant. These successes, however, did not involve the production of any commercially important compounds (BALANDRIN AND KLOCKE, 1988).

Callus culture

Callus is defined as a coherent and amorphous tissue, formed by the disorganized multiplication of plant cells, and consisting of meristematic and unspecialized parenchyma cells (GEORGE, 1993). Callus does not conform to any predictable organizational pattern, but localized centres of meristematic activity are present, sometimes accompanied by a simple cambial region with zones of vascular differentiation (DODDS AND ROBERTS, 1985). Callus formation occurs in plants as a result of wounding, stress, and insect or microorganism attack, and is controlled by the endogenous hormones auxin and cytokinin (DODDS AND ROBERTS, 1985; GEORGE, 1993). This response is associated with increased metabolic activity, including polyphenols to strengthen cell walls and the synthesis of compounds to protect against pathogen attack (ALLAN, 1991).

The induction of callus *in vitro* requires a change in cell metabolism from a quiescent state to one of active cell division, which often necessitates the reversal of cell differentiation and specialization (GEORGE, 1993). Callus formation can be induced *in vitro* in plant tissues and organs not usually characterized by callus formation in response to injury (DODDS AND ROBERTS, 1985).

Callus growth differs widely in morphological appearance and anatomy. Some are heavily lignified and hard in texture, while others break apart easily into small fragments (friable). The appearance of callus cells varies from white to yellow, green or pigmented (DODDS AND ROBERTS, 1985). The extent and type of cellular differentiation also differs, with a callus consisting entirely of homogenous parenchyma cells rarely found. Tracheary elements, sieve elements, suberized cells, secretory cells and trichomes occur, and groups of dividing cells form 'meristemoids' or vascular nodules that may form the site of shoot apices, root primordia or incipient embryos (DODDS AND ROBERTS, 1985).

The maintenance of callus in both solid and suspension culture is regulated by a three phase cycle in growth (ALLAN, 1991). During the initial induction phase, metabolism is stimulated as the cells prepare for division. The duration of this phase depends on the physiological status of the explant, as well as the culture environment (DODDS AND ROBERTS, 1985). This is followed by active cell division involving the dedifferentiated cells. The biomass of the culture increases substantially during this phase due to cell division and cell enlargement (DODDS AND ROBERTS, 1985). This active growth phase is limited and is followed by a stationary phase, since the viability of the cells in culture decreases due to the exhaustion of nutrient factors or the accumulation of toxic substances in the medium. The third phase is characterized by cellular differentiation and the expression of certain metabolic pathways leading to the accumulation of secondary products (DODDS AND ROBERTS, 1985).

The timing and quantification of subculturing is important - the incubation period from culture initiation to the stationary phase is determined primarily by factors such as initial cell density, the duration of lag phase and the growth rate of cell line. These can be determined by regular measurements of the mass or cell density of callus cultures (DODDS AND ROBERTS, 1985).

The major disadvantage associated with the use of callus and suspension cultures is their genetic and karyological instability (TOWERS AND ELLIS, 1993). This can be minimized by frequent subcultures. Long subcultures intervals, where cells remain for a long time in a stationary phase, may lead to increases in ploidy level (TOWERS AND ELLIS, 1993). After subculture and mitotic stimulation, a mixoploid population is often obtained. This is referred to as endoreduplication (TOWERS AND ELLIS, 1993). Genetic instabilities may be displayed in different ways including reduced growth rates, and inconsistent or aborted biosynthesis of the desired chemical (TOWERS AND ELLIS, 1993).

Callus cultures exhibit heterogeneity both between cultures and within the callus itself (ALLAN, 1991). This is evident as differences in colour, structure, growth and metabolism. Visibly uniform cultures can consist of cells differing in ploidy and metabolic capacity (ALLAN, 1991). In addition, gradients (nutrients, gases, plant growth regulators) exist in the callus creating micro-environments that affect growth patterns. This necessitates random selection of callus during subculture to promote standardized results. In addition, callus derived from the same explant but cultured on different media should be considered separate cell lines. Although this heterogeneity suggests that no two calluses are identical, callus cultures do become less variable with time and repeated subculture enabling some consistency to be achieved (ALLAN, 1991). Some callus cultures have proved very stable genetically (REES, 1992).

Secondary metabolite production

In vitro cultures often only produce small quantities of the desired metabolite (SCRAGG, 1993). The biosynthesis of secondary metabolite production by tissue culture is often complicated by the biosynthesis of a metabolic block, which needs to be overcome by the control of several biological and environmental factors (DATTA, 1993). High growth rates of callus have been directly correlated to the high quantity of the production of secondary metabolites, but in other cases the media for rapid growth may be different from that of optimal secondary metabolite synthesis (DATTA, 1993). The establishment of a medium suitable for secondary metabolite synthesis depends on the incorporation of components which can differ with species, or genotype (DATTA, 1993).

Callus cultures need to exhibit both rapid growth, and the production of the required compound in high and reproducible amounts. Biochemical pathways are often non-linear and thus are inherently chaotic systems which may pose problems over long periods of culture (TOWERS AND ELLIS, 1993). Tissue culture systems lend themselves well to the use of elicitors to enhance the production of secondary metabolites. Plant cells subjected to environmental stresses often show enhanced production of some of the usual secondary metabolites produced in the plant tissues. This phenomena is known as elicitation and there are two major types of elicitors. Biotic elicitors are prepared from pathogenic bacteria and fungi, and abiotic elicitors include salts of heavy metals. The use of live pathogens has been discontinued and has been replaced by cell wall preparations, since live organisms would overrun the culture (TOWERS AND ELLIS, 1993). This system was not, however, investigated for the callus produced in this particular study.

Culture growth is proposed to be incompatible with secondary metabolism in some systems (SCRAGG, 1993). In these cases the formation of secondary metabolites is induced when the cultures have stopped growing and have entered the stationary phase. The optimization of such systems requires a two-phase system supporting growth then metabolite production. Two media formulations are required, the first to maximize biomass accumulation, and the second to divert cell activity from growth to secondary metabolite production. The second medium often contains reduced nitrogen or potassium to discourage growth (SCRAGG, 1993).

8.2 MATERIALS AND METHODS

8.2.1 INITIATION PROCEDURES

Callus induction was attempted from both *in vitro* leaf material and explants from young leaves of adult plants. The latter proved more successful for the induction of callus. The initiation medium consisted of a modified MS medium (Appendix II) supplemented with 100 mg ℓ^{-1} myo-inositol, 2 g ℓ^{-1} gelrite and 20 g ℓ^{-1} sucrose. A hormone grid was established using 2,4-D and kinetin (Table 8.1).

Subsequent subcultures of this callus required a high level of auxin combined with cytokinin to maintain the friable nature. High levels of 2,4-D promote genetic aberrations. Attempts were therefore made to reduce the concentration of 2,4-D, or to substitute IAA, but this resulted in callus that was morphogenic in nature. The auxins used were 2,4-D (10 mg ℓ^{-1}) or IAA (5 or 10 mg ℓ^{-1}) in combination with the cytokinin, kinetin, at a concentration of 1, 2 or 3 g ℓ^{-1}.

8.2.2 CULTURE CONDITIONS

Initial callus induction was conducted in a light/dark regime of 8h/16h (27μmol m^{-2} s^{-1} at 25±3°C). Callus was initiated from explants placed in tubes and this was subcultured onto media in tubes and in petri dishes. Callus from different replicates was combined before subculture, and experiments were conducted using callus from one treatment, in an attempt to standardize the results. In addition, cells at the centre of older callus cultures do not divide and may be dead (ALLAN, 1991). This necrotic callus was soft and brown in colour, and was separated from the living callus and discarded. Replicates of all experiments were conducted using both culture tubes and plastic petri dishes (7 cm diameter).

8.2.3 EFFECT OF SUCROSE CONCENTRATION

The effect of two concentrations of sucrose on the growth and type of callus proliferation were tested. The same (initiation) MS medium to which 2,4-D and kinetin (in a ratio of 10:2 or 10:3) was added, was supplemented with 20 g ℓ^{-1} or 30 g ℓ^{-1} sucrose. Callus (0.5 g) was subcultured onto this medium and allowed to proliferate for 8 weeks.

8.2.4 EFFECT OF LIGHT

A further experiment was initiated using media supplemented with 30 g ℓ^{-1} sucrose and 2,4-D and kinetin in a ratio of 10:2 and 10:3. Half these cultures were placed in the dark at 25±3°C, and half were maintained in continuous light (41 μmol m^{-2} s^{-1}). Callus (0.5 g) was subcultured onto this medium. After 8 weeks the callus was weighed and the increase in mass calculated.

8.2.5 SUSPENSION CULTURE

Attempts were made to maintain this callus in suspension culture using the same media but in liquid form. This did not, however, prove successful.

8.2.6 ANALYSIS OF RESULTS

The initial callus produced from each treatment was combined and randomly subcultured onto the different experimental media. After 8 weeks this was harvested and weighed to determine the increase in mass. Loose, friable (white) callus was separated from hard, organogenic callus and weighed. Soft, brown (necrotic) callus was discarded. The percentage soft, friable, meristematic callus was calculated and this was used in subsequent subcultures.

8.2.7 ANTI-INFLAMMATORY ACTIVITY

Callus was ground in liquid nitrogen and extracted for 30 min in 20 mℓ ethanol using a sonication bath. The extract was filtered and dried down and was resuspended in ethanol at a concentration of 20 mg $m\ell^{-1}$. This extract was tested for both COX-1 and COX-2 inhibitory activity.

8.3 RESULTS

8.3.1 CALLUS INITIATION

High concentrations of auxin (10 mg ℓ^{-1} 2,4-D) was required to initiate callus. This was a loosely packed, friable callus, termed primary callus as it was formed on the original explant (GEORGE, 1993). Secondary callus is initiated from primary callus. On subculturing the nature of the callus tended to change, becoming organogenic, and prone to root initiation (Plate 8.1 B). The callus had an extended lag phase following subculture, and the growth of the secondary callus in general was erratic, with very little change in mass observed for the first four weeks. Growth occurred in weeks 5-8.

TABLE 8.1: Hormone grid and percentage primary callus initiation from *E. autumnalis autumnalis* leaf explants.

% Callus Formation		2,4-D (mg ℓ^{-1})		
		1	5	10
K (mg ℓ^{-1})	0	-	-	0
	0.1	0	33	0
	2	14	17	62

A medium containing 2,4-D and kinetin (10:2) was the only successful hormone combination for callus induction.

8.3.2 HORMONE GRID

The production of secondary callus was attempted using 2,4-D or IAA as the auxin source and kinetin as the cytokinin. Figure 8.1 shows a significant increase in the average mass of callus produced on media containing IAA. The percentage of this callus that could be used in subsequent subcultures however, decreased significantly on media containing IAA. The notable increase in mass was largely due to the organogenic nature of the callus, which was hard and white in colour. Subsequent experiments utilized 2,4-D and kinetin as the hormones.

8.3.3 EFFECT OF SUCROSE CONCENTRATION

Increasing the sucrose concentration from 20 to 30 g ℓ^{-1} significantly increased callus mass (Figure 8.2). The percentage of this callus classified as white and friable (Plate 8.1 A) did not differ significantly with hormone concentration, but did increase significantly with increased sucrose concentration (Plate 8.1 A vs E).

8.3.4 EFFECT OF LIGHT

Cultures maintained in the dark showed a substantial increase in total callus mass (Figure 8.3), as well as in the percentage friable callus. This occurred for both hormone combinations tested (Plate 8.1 A, C, E vs B, D, F).

These results indicate that callus initiated from *E. autumnalis autumnalis* is best maintained in the dark, on a medium supplemented with 30 g ℓ^{-1} and a hormone combination of 2,4-D : kinetin (10:2).

8.3.5 ASSAY RESULTS.

This callus was tested for activity in the COX assays at a concentration of 500 μg mℓ^{-1}. The callus showed high COX-2 inhibitory activity (69 %), relative to COX-1 inhibitory activity (46 %). These test concentrations are, however, very high, double the normal screening concentration of 250 μg mℓ^{-1}.

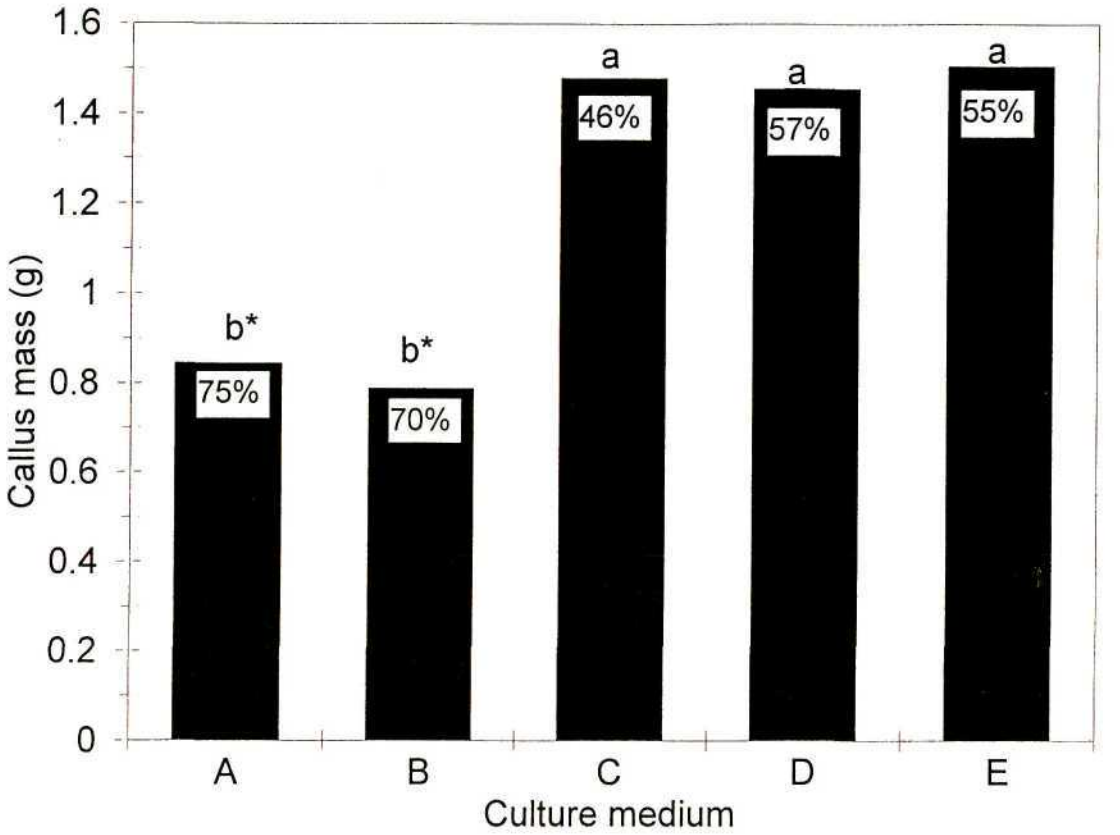


FIGURE 8.1: The effect of different hormone concentrations (mg ℓ^{-1}) on callus production. (A) 10:2 (2,4-D:kinetin); (B) 10:3 (2,4-D:kinetin); (C) 10:3 (IAA:kinetin); (D) 5:2 (IAA:kinetin) and (E) 5:1 (IAA:kinetin). Annotations indicate the percentage friable callus produced. (Bars bearing different letters are significantly different, $P \leq 0.05$).

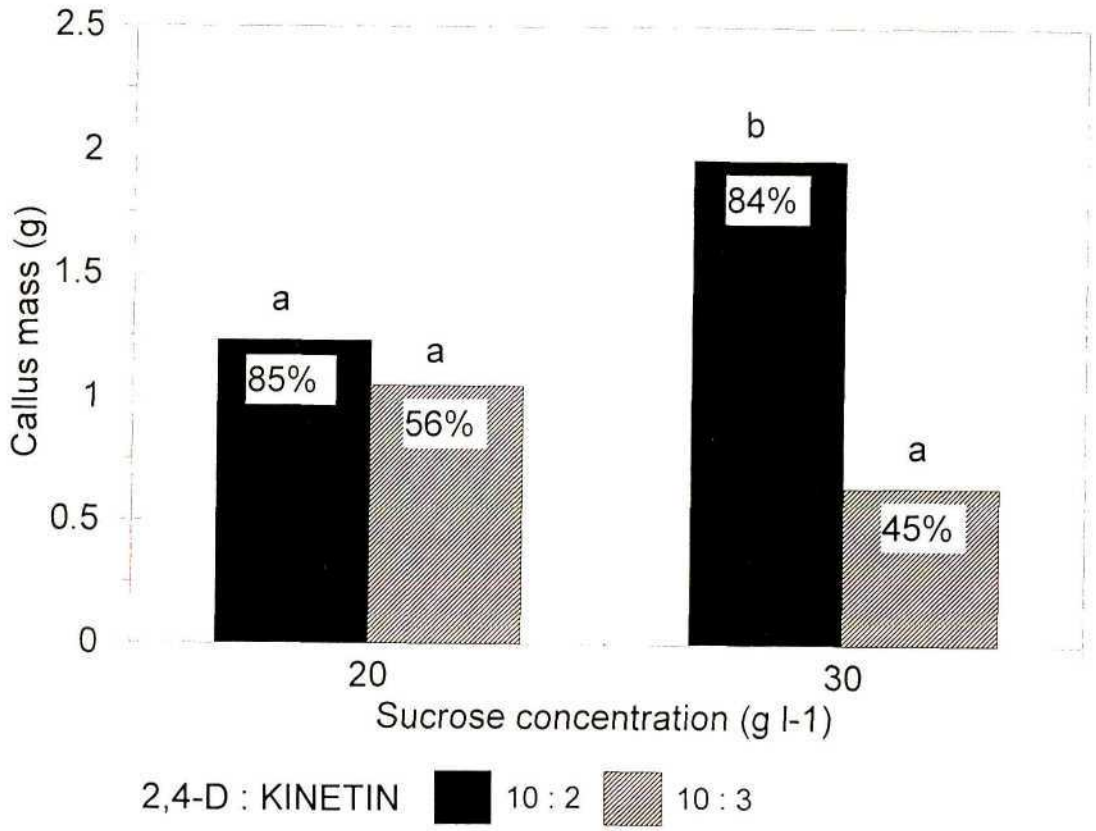


FIGURE 8.2: The effect of different sucrose concentrations (g ℓ^{-1}) on callus production. Hormone combinations (mg ℓ^{-1}) used were 10:2 (2,4-D:kinetin) and 10:3 (2,4-D:kinetin). Annotations indicate the percentage friable callus produced. (Bars bearing different letters are significantly different, $P \leq 0.05$).

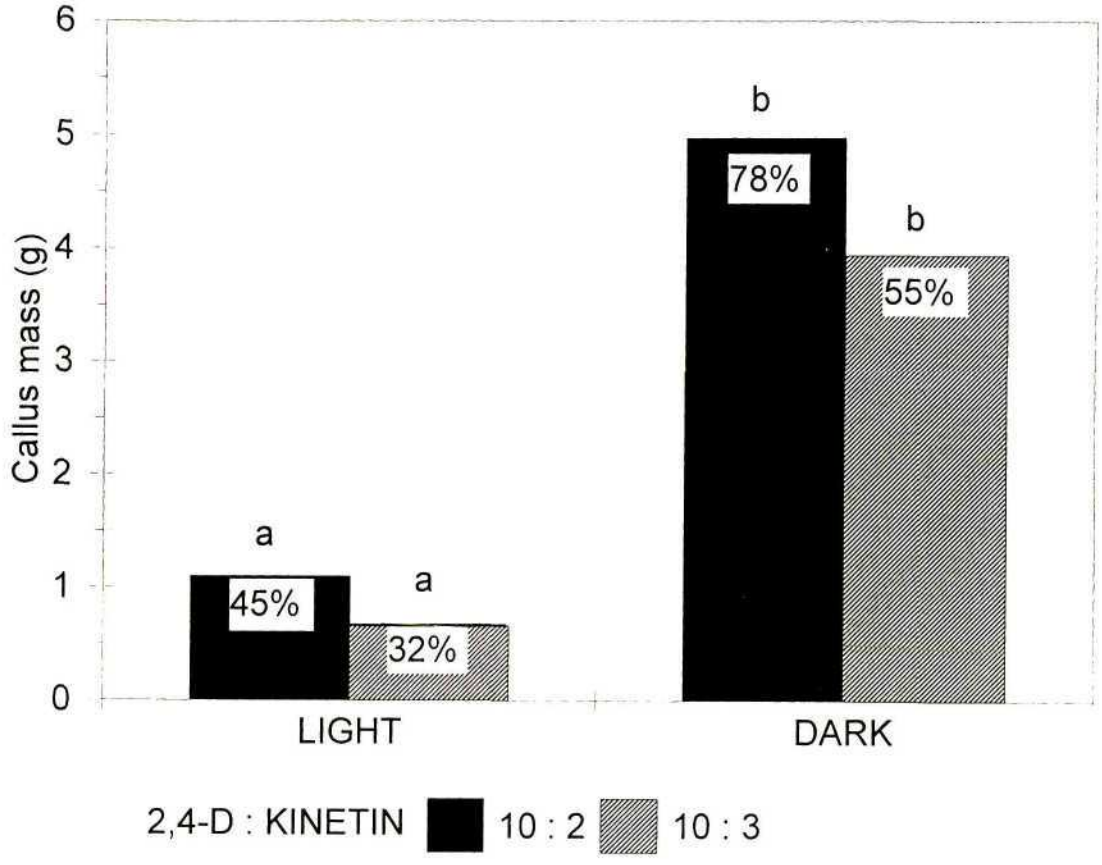
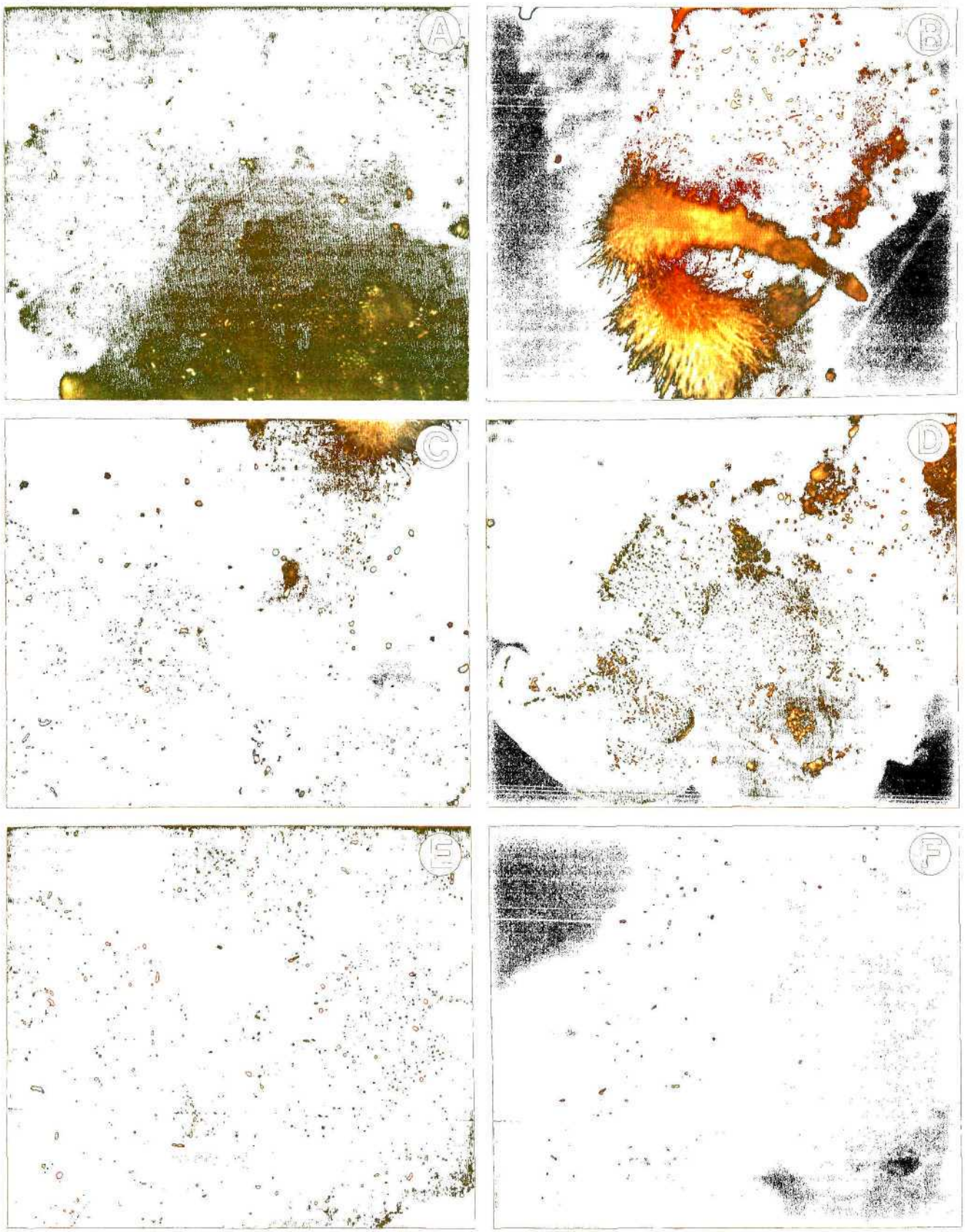


FIGURE 8.3: The effect of light and dark on callus production. Hormone combinations (mg ℓ^{-1}) used were 10:2 (2,4-D:kinetin) and 10:3 (2,4-D:kinetin). Annotations indicate the percentage friable callus produced. (Bars bearing different letters are significantly different, $P \leq 0.05$).

PLATE 8.1: Callus produced on MS media supplemented with 30 g ℓ^{-1} sucrose, 10 mg ℓ^{-1} 2,4-D and 2 mg ℓ^{-1} kinetin, maintained (A) in the dark and (B) in the light. Callus produced on MS media supplemented with 30 g ℓ^{-1} sucrose, 10 mg ℓ^{-1} 2,4-D and 3 mg ℓ^{-1} kinetin, maintained (C) in the dark and (D) in the light. Callus produced on MS media supplemented with 20 g ℓ^{-1} sucrose, 10 mg ℓ^{-1} 2,4-D and 2 mg ℓ^{-1} kinetin, maintained (E) in the dark and (F) in the light.

DARK

LIGHT



8.4 DISCUSSION

Plant cells are totipotent, and in theory all cells, and in some cases even the protoplasts of differentiated cells can be regenerated, forming entire plants (DATTA,1993). During the dedifferentiation of cells preceding cell division and callus formation, the storage products typically found in quiescent cells usually disappear. The new meristems formed produce undifferentiated parenchyma cells lacking the structural order characteristic of the organs and tissue from which the callus was derived (GEORGE, 1993).

8.4.1 OPTIMAL CONDITIONS FOR GROWTH OF CALLUS FROM *E. AUTUMNALIS AUTUMNALIS*

The optimal conditions for callus growth were a high level of sucrose (30 g ℓ^{-1}), and darkness. Darkness is essential for the initiation and growth of callus from some plants (GEORGE, 1993). In other species, light may be required for the initiation of callus, but once obtained this callus is subcultured and maintained in the dark (GEORGE, 1993). Although explants of *E. autumnalis autumnalis* did produce callus in the light, both the proliferation and type of callus produced after subculture was improved in the dark.

Auxin is usually required in the medium for the induction of callus from explants, the most common being 2,4-D (GEORGE, 1993). This hormone is, however, associated with genetic variability and it is preferable to transfer callus to media supplemented with NAA or IAA after subculture. The addition of a cytokinin is not always necessary for the induction of callus from monocotyledons, and a higher concentration of auxin (2.0-10.0 mg ℓ^{-1}) is typically needed (GEORGE, 1993). Leaf explants of *E. autumnalis autumnalis* responded best on media supplemented with a high concentration of auxin (2,4-D at 10 mg ℓ^{-1}) and kinetin at 2 mg ℓ^{-1}.

Although callus is an unorganised tissue, as growth proceeds, certain specialized cells may reappear. This differentiation may appear random, but may be associated with centres of morphogenesis which can give rise to organs such as roots, shoots or

embryos (GEORGE, 1993). Although biosynthesis of secondary products may naturally be repressed in, for example leaf tissue, as soon as these cells enter another state of differentiation, biosynthesis may resume (DATTA, 1993). The degree of secondary product synthesis in callus can thus not be predicted from levels of compounds detected in the intact plant tissues. Callus cells represent a unique physiological state, which might be transient in the intact plant, if it occurs at all (DATTA, 1993).

8.4.2 ANTI-INFLAMMATORY ACTIVITY OF CALLUS EXTRACTS

Callus produced from *E. autumnalis autumnalis* was found to exhibit relatively high COX-2 inhibitory activity, in comparison to COX-1 inhibitory activity. This represents different metabolic activity to both plantlet tissues *in vitro* (CHAPTER 7) and to tissues from the intact plant where COX-1 inhibitory activity is typically higher than, or very similar to COX-2 inhibitory activity (CHAPTER 2).

8.4.3 APPLICATIONS OF CALLUS CULTURE

The production of secondary metabolites in plant cell cultures is only economically viable where more than 1 g of compound per litre of cell culture can be achieved, and only for compounds with a market value exceeding $500 to $1000 per kg. In addition, the total market demand for the compounds of interest must be large enough to justify the large capital expenses needed for the development of tissue culture systems. With these economical constraints, commercial production utilizing plant cell and tissue culture techniques is limited to a few types of high-value plant-specific compounds (BALANDRIN AND KLOCKE, 1988). Another recent application of tissue culture preparations is as a source of enzymes employed in secondary metabolism . These enzymes have the potential to be integrated into the production of secondary metabolites either wholly, or as part of a chemo-enzymatic synthesis (CORDELL, 1995). This could become important in the future if certain resources become depleted, restricting the production of crucial natural products. This application relies on the isolation of the gene encoding the enzyme and the subsequent over-expression in an appropriate host system of its cDNA (CORDELL, 1995).

8.5 CONCLUSION

Although the initiation and production of callus from *E. autumnalis autumnalis* was not reliable and prolific, this system does have potential in the production of anti-inflammatory compounds. The compounds produced show high COX-2 activity, and further research in this area could prove rewarding. Further experimentation is needed in order to optimize callus growth, before the conditions suited to maximize the accumulation of the active compounds can be investigated. These compounds should then be compared to those in the intact plant, to determine whether the callus cells produce different compounds, or different proportions of the same compounds, resulting in the observed difference in activity of the respective extracts.

CHAPTER 9

THE EFFECT OF SELECTED ENVIRONMENTAL FACTORS ON GROWTH AND ANTI-INFLAMMATORY ACTIVITY

9.1 INTRODUCTION

The potency of medicinal plants is known to be affected by a number of factors. The biochemistry varies within individual plant species, and is influenced by the ecology, soil and climate (PRANCE, 1994; TUNÓN *et al.*, 1995). Plants have evolved in conjunction with specific, complex ecosystems. Changes to the environment can cause substantial modifications in plant growth and development, which in turn can often result in changes in the natural product content (MÀTHÉ, 1988). Plants are sensitive to local conditions and consequently do not consistently produce the same chemicals, and in the same quantities (PRANCE, 1994). Experienced gatherers and healers consider the particular locality, the correct season of collection, the best aspect on a slope, the type of soil and moisture, and the time of day when harvesting their plants (VAN WYK *et al.*, 1997).These factors can, however, be problematic when cultivating species of medicinal plants.

Domestication

The domestication and cultivation of medicinal plants from the wild is a complex process and involves more than a simple transfer of the plants from their natural habitats to cultivated fields in a new location. Conditions in a new, foreign environment alter the suitability of the plant for both cultivation and the value of the plant chemical products (MÀTHÉ, 1988). These are not, however, always economically detrimental in terms of lowered plant growth and yield. For example, *Vinca minor* L. (periwinkle), naturally found in shady environments, shows enhanced alkaloid yield when exposed to the higher light intensity in open cultivated field conditions. Similarly, *Convallaria majalis* L. (lily-of-the-valley), *Origanum majorana* L. (marjoram) and *Pelagonium graveolens* L'Her. Ex Ait. (Rose geranium) benefit from

the stresses applied by cultivation (MÀTHÉ, 1988).

The domestication of any species for cultivation in a new geographical location involves both a study of the natural populations as well as experimental cultivation trials. Medicinal plant cultivation differs from other crop farming in that the desired concentration of natural products needs to be maintained or enhanced, while sustaining optimal growth. For medicinal plants, field trials must be accompanied by the screening of the introduced plants for natural product content, and determinations of the composition of the plant with reference to biomass production during normal growth and development over the growing season (MÀTHÉ, 1988).

These studies thus need to be evaluated in terms of the economically viable production of the natural products with respect to growth and reproduction as well as product accumulation. Any field conditions that impact negatively on product accumulation need to be eliminated or adjusted. This may involve the manipulation of the field ecology to match the environmental conditions in the natural ecosystems to ensure growth and reproduction, or alternatively, manipulation of the plant through selection and breeding. Factors such as planting times, pest control, soil conditions and water supply need to be appraised to ensure vigorous plant growth with high product yield (MÀTHÉ, 1988).

Attention is focussing on the collection and preservation of wild plants, and the introduction of economically important species into cultivation. Approximately fifty species of medicinal and aromatic plants have been successfully introduced and maintained in large-scale cultivation in temperate zones (MÁTHÉ, 1988). Limitations in the availability of gathered medicinal plants, and a re-assessment of the role of large- versus small-scale production systems has brought new perspectives to the medicinal plant market. The introduction of such crops in less favourable agricultural regions can extend the agricultural and economic base of the area (MÁTHÉ, 1988).

Commercial production of *Eucomis* (for ornamental use)

The commercial production of *Eucomis* species in Europe is for their ornamental value. Bulb production is, however, low, with about 2 hectares grown in The Netherlands (no data available for other countries). Approximately 700 to 1 000 bulbs

are planted per acre (DE HERTOGH AND LE NARD, 1993).

Bulbs are generally planted at a depth of 8-10 cm in sandy, well-drained soil. A complete fertilizer (7 kg per acre of a 7-14-28 mixture) and calcium nitrate (at 94 kg per acre) are applied. Weeds are controlled by soil sterilization (for seedlings) or by a Chlorprofam® application before plant emergence (for bulbs) (DE HERTOGH AND LE NARD, 1993).

Bulb harvesting of commercial bulbs and planting stock takes place in November. After lifting, bulbs are stored between 13-20°C in a well ventilated room during winter (DE HERTOGH AND LE NARD, 1993). Bulbs have to be maintained during storage and transport in frost-free conditions (DE HERTOGH AND LE NARD, 1993).

The aim of this particular study was to ascertain the effect of fertilization, and light intensity, on the growth and biomass accumulation of *E. autumnalis autumnalis*, and to determine the effect of this on the accumulation of the active compounds. In addition, the effect storing the dormant bulbs under different conditions during the winter months was studied in terms of the effect on the activity of extracts prepared at the beginning of spring and half way through the growing season.

9.2 MATERIALS AND METHODS

9.2.1 EFFECT OF KELPAK APPLICATION

Three dilutions of a commercial Kelpak preparation were applied as a foliar spray (10 mℓ), to 10 specimens of *E. autumnalis autumnalis* maintained under uniform conditions in a greenhouse, for a period of six months. A water control was applied in the same manner to a further 10 plants. Treatment 1 = 0.5 mℓ Kelpak / 250 mℓ water, once every two weeks; Treatment 2 = 1.0 mℓ Kelpak / 250 mℓ water, once every two weeks; Treatment 3 = 1.0 mℓ Kelpak / 250 mℓ water, once every 4 weeks. The recommended dilution of Kelpak is 1 part per 500 applied as a foliar spray 3-5 times during the growing season, at 3-4 week intervals.

9.2.2 EFFECT OF LIGHT INTENSITY

Specimens of *E. autumnalis autumnalis* were maintained in low (1990 μmol m^{-2} s^{-1} and 15-35 °C) and high (2335 μmol m^{-2} s^{-1} and 12-40 °C) light intensity greenhouses (where direct sunlight = ± 3400 μmol m^{-2} s^{-1}). Two harvests were performed, one half way through, and one at the end of the growing season.

9.2.3 EFFECT OF WINTER STORAGE OF BULBS

Uniform specimens of *E. autumnalis autumnalis* were collected at the end of summer, once the foliage had died back. Eight specimens were stored dry in brown paper bags in the potting shed (18 ± 2 °C), eight were stored dry in a coldroom (10±1.5 °C) and eight were replaced into a soil / sand mix and returned to the greenhouse (15-24 °C). All specimens were dusted with Bexadust® to discourage fungal growth. One harvest was performed at the end of the winter season and one mid-way through the growing season, when the bulbs had developed leaves.

9.2.4 ANALYSIS OF RESULTS

Harvested specimens were divided into leaf, bulb and root material, weighed to determine fresh mass, and dried at 50°C for three days. Ethanol extracts were prepared and tested for inhibitory activity in the COX-1 assay. The results were analysed using a One-way ANOVA and Tukey HSD test.

9.3 RESULTS

9.3.1 KELPAK TREATMENT

Growth data

Fertilization with Kelpak had no significant effect on the fresh or dry mass accumulation of the leaves, bulbs or roots (Figure 9.1 A-C). A trend of increasing fresh mass accumulation was observed for the leaves treated with Kelpak, with Treatment 2 showing the highest fresh mass accumulation (Figure 9.1 A). There was a decrease in root dry mass for all three treatments, relative to the control (Figure 9.1 C). Differences in the ratio of fresh to dry mass accumulation did not prove significant for the leaves, bulbs or roots (Figure 9.2).

Anti-inflammatory activity

COX-1 inhibitory activity was significantly higher in the control plants than in the treated plants (Figure 9.3). Bulb extracts from plants treated with Kelpak showed the most noticeable reduction in levels of activity. The ratios of leaf : bulb : root extract activity were calculated for each treatment, and analysed statistically. There were, however, no significant differences in the ratios of activity for leaf : bulb : root extracts, calculated for each treatment.

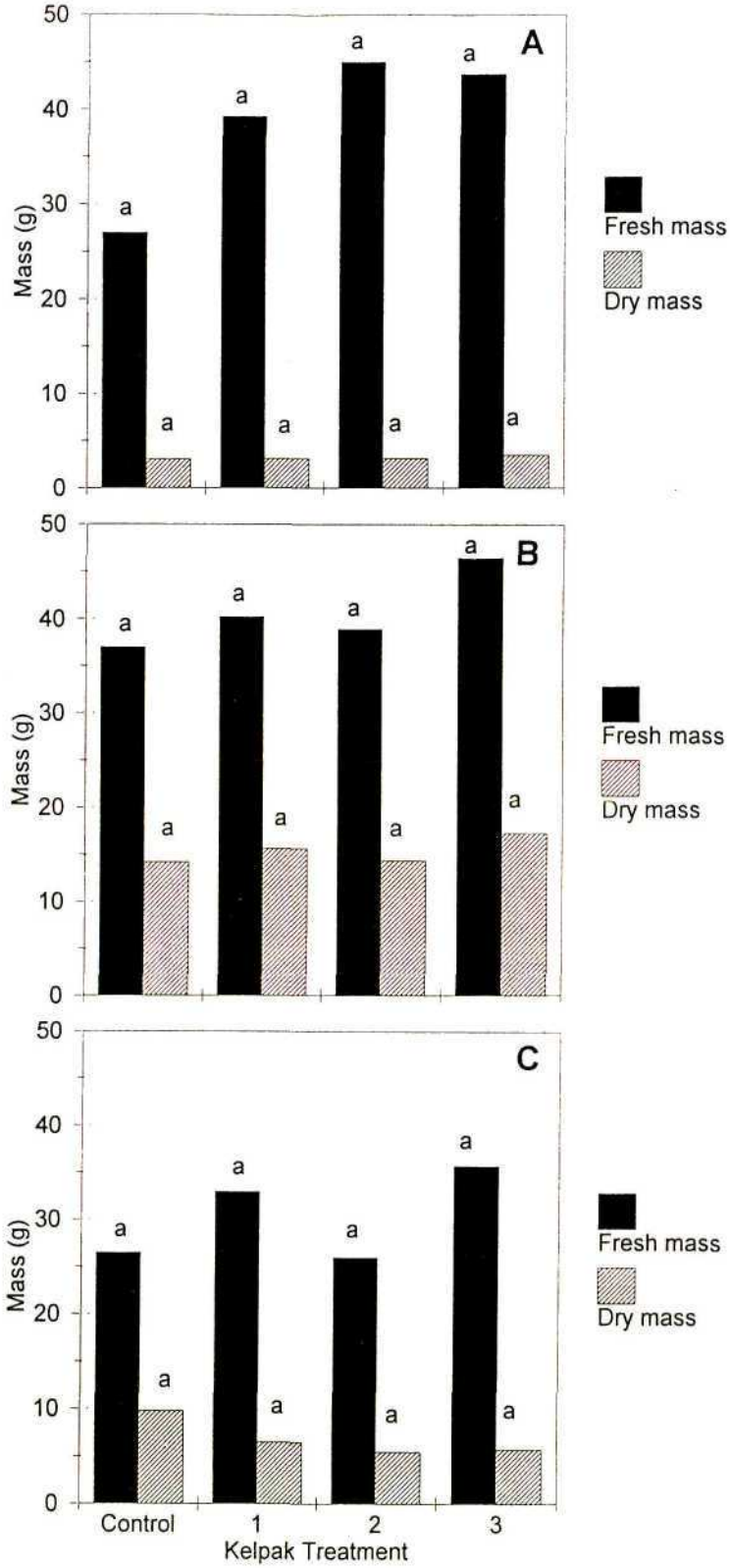


FIGURE 9.1: Variation in fresh and dry mass of *E. autumnalis autumnalis* plants treated with different Kelpak concentrations. (A) Variation in leaf growth; (B) Variation in bulb growth and (C) Variation in root growth. (Bars bearing different letters are significantly different, $P \leq 0.05$).

Control = distilled water; 1 = 0.5 mℓ Kelpak / 250 mℓ water, once every two weeks; 2 = 1.0 mℓ Kelpak / 250 mℓ water, once every two weeks; 3 = 1.0 mℓ Kelpak / 250 mℓ water, once every four weeks.

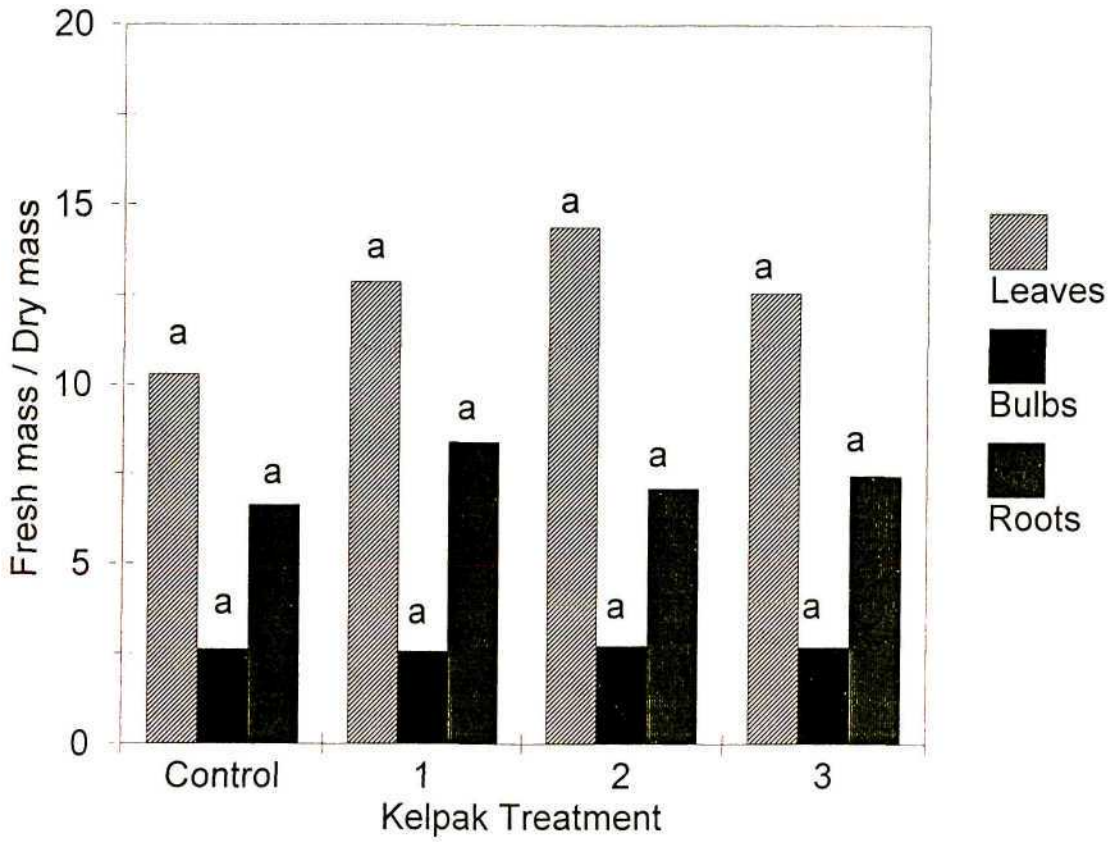


FIGURE 9.2: Variation in the ratio of fresh mass to dry mass of *E. autumnalis autumnalis* plants treated with different Kelpak concentrations. (Bars bearing different letters are significantly different, $P \leq 0.05$).

Control = distilled water; 1 = 0.5 mℓ Kelpak / 250 mℓ water, once every two weeks; 2 = 1.0 mℓ Kelpak / 250 mℓ water, once every two weeks; 3 = 1.0 mℓ Kelpak / 250 mℓ water, once every four weeks.

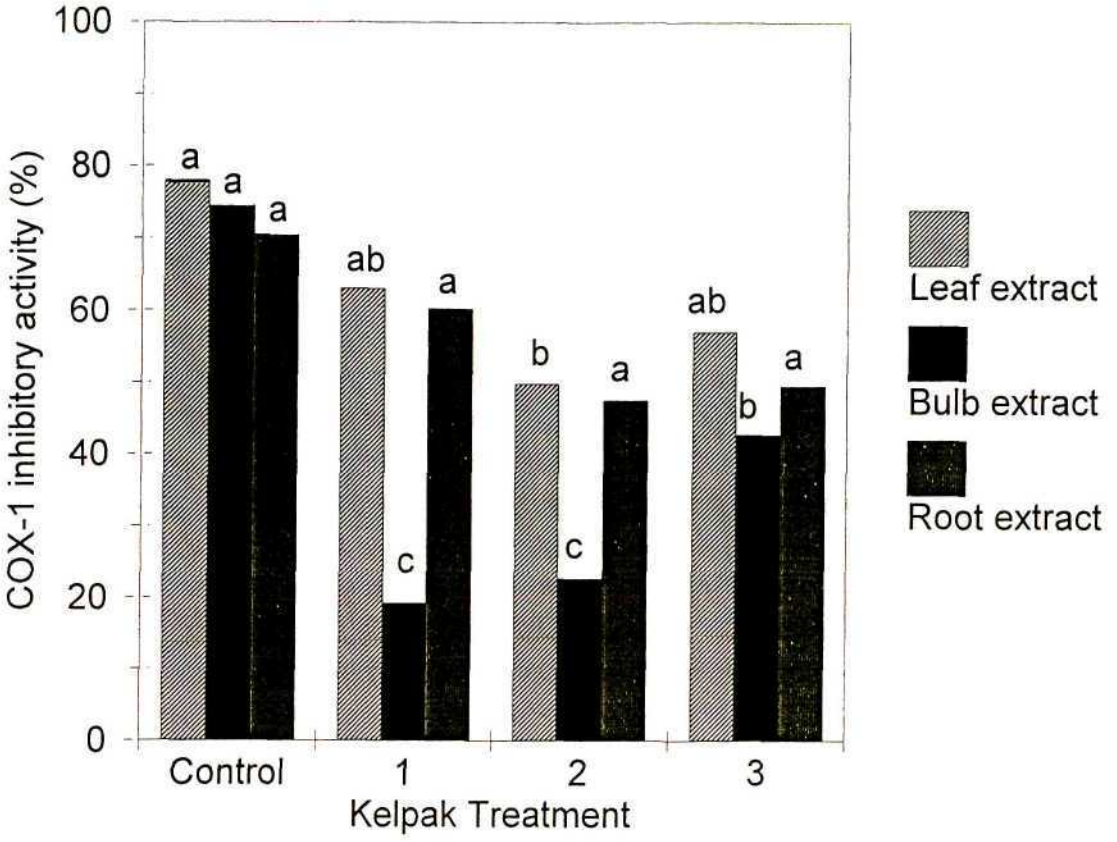


FIGURE 9.3: The % inhibition of COX-1 by ethanolic extracts of leaves, bulbs and roots of *E. autumnalis autumnalis* plants treated with different Kelpak concentrations. (Bars bearing different letters are significantly different, $P \leq 0.05$).

Control = distilled water; 1 = 0.5 mℓ Kelpak / 250 mℓ water, once every two weeks; 2 = 1.0 mℓ Kelpak / 250 mℓ water, once every two weeks; 3 = 1.0 mℓ Kelpak / 250 mℓ water, once every four weeks.

9.3.2 LIGHT INTENSITY EXPERIMENT

Growth data

No significant differences between the control and treated plants were observed in terms of fresh mass or dry mass accumulation for the leaves or roots at Harvest 1 or 2 (Figure 9.4 A and C). The control bulbs showed a significant increase in fresh and dry mass between Harvest 1 and 2, which was not reflected in a comparison between treated plants at Harvest 1 and 2 (Figure 9.4 B). The control plants showed a higher rate of bulb growth compared to the treated plants at Harvest 2 (Figure 9.4 B). The ratio of leaf fresh mass to dry mass did not differ significantly from the control at Harvest 1 or 2 (Figure 9.5). At the end of the experiment, the ratio for the control leaves had decreased significantly relative to the first harvest. The fresh to dry mass ratio for the control and treated bulbs did not change significantly over the growing season. The control ratio for the bulbs differed significantly from that of the treated plants at Harvest 1 and at Harvest 2 (Figure 9.5).

Anti-inflammatory activity

The average levels of anti-inflammatory activity detected in the leaf and bulb extracts did not differ significantly, either from the control (at both harvests) or between harvests (Figure 9.6). The activity of the root extracts at the end of the growing season was significantly higher than that of the controls, and that of the initial harvest.

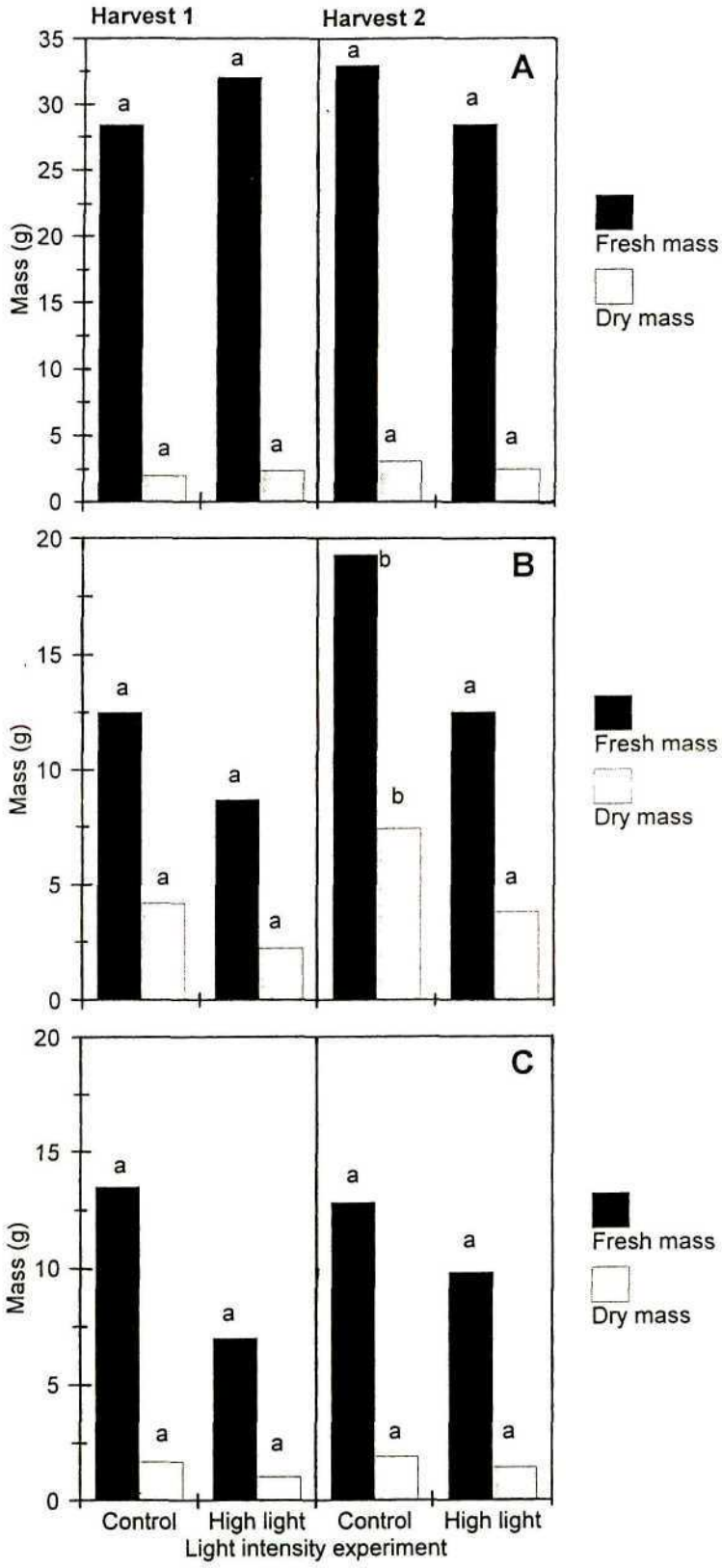


FIGURE 9.4: Variation in fresh and dry mass of *E. autumnalis autumnalis* plants maintained under low (Control) and high light intensity. (A) Variation in leaf growth; (B) Variation in bulb growth; and (C) Variation in root growth. (Bars bearing different letters are significantly different, $P \leq 0.05$).

Control = 1990 μmol $m^{-2}s^{-1}$; High light = 2335 μmol $m^{-2}s^{-1}$.

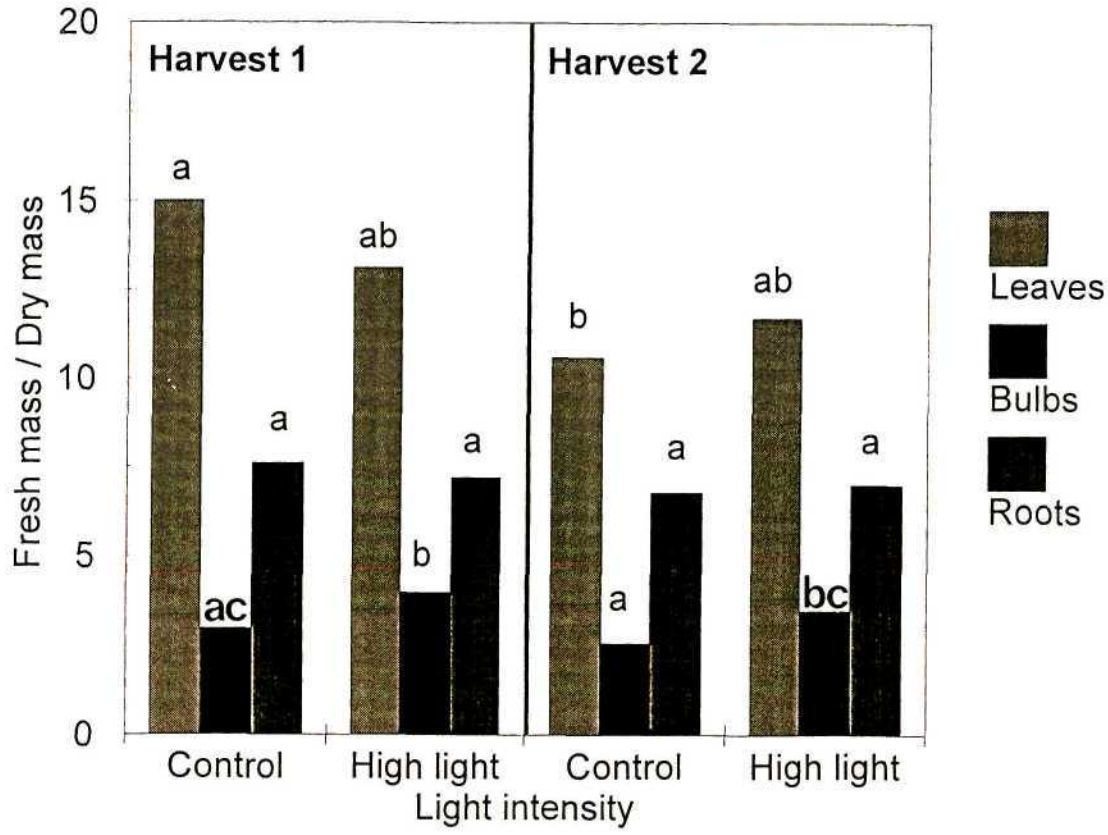


FIGURE 9.5: Variation in the ratio of fresh mass to dry mass of *E. autumnalis autumnalis* plants maintained under low (Control) and high light intensity. (Bars bearing different letters are significantly different, $P \leq 0.05$).

Control = 1990 μmol $m^{-2}s^{-1}$; High light = 2335 μmol $m^{-2}s^{-1}$.

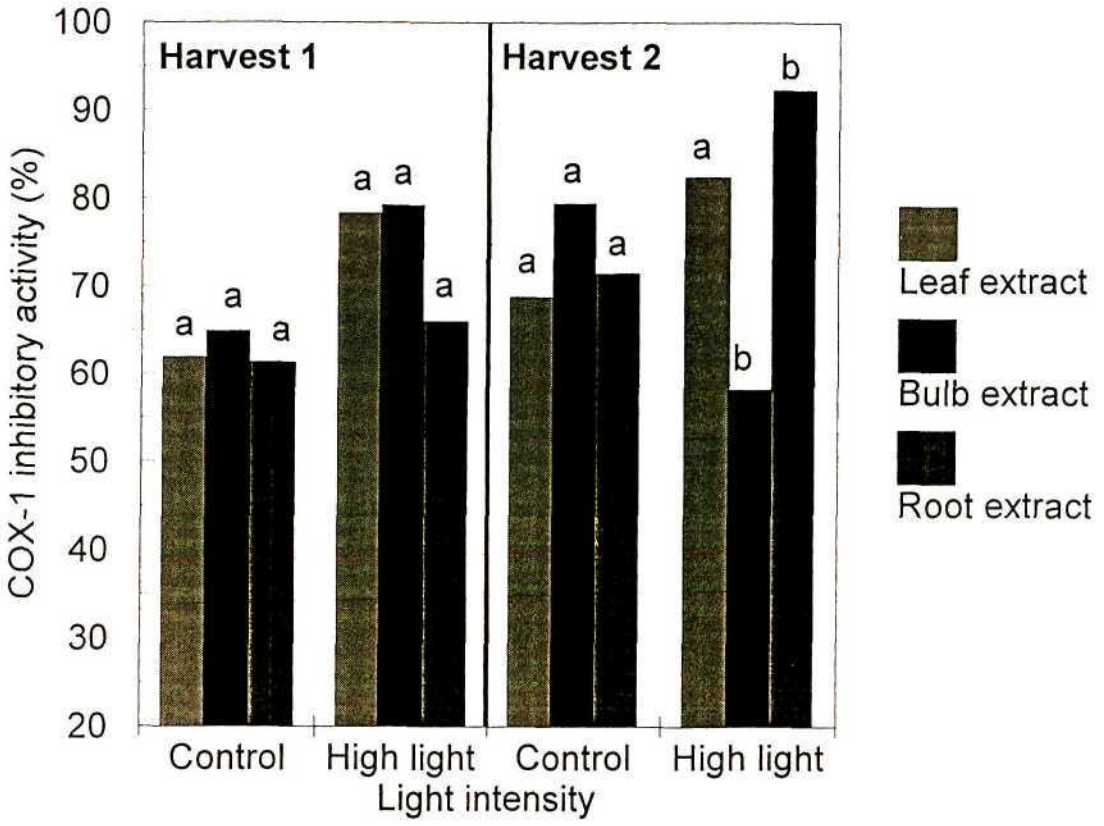


FIGURE 9.6: The % inhibition of COX-1 by ethanolic extracts of leaves, bulbs and roots of *E. autumnalis autumnalis* plants maintained under low (Control) and high light intensity. (Bars bearing different letters are significantly different, $P \leq 0.05$).

Control = 1990 μmol $m^{-2}s^{-1}$; High light = 2335 μmol $m^{-2}s^{-1}$.

9.3.3 WINTER STORAGE TREATMENT

Growth data

Cold storage of bulbs maintained their fresh mass at a higher level than did both the dry storage and leaving the bulbs in soil during winter (Figure 9.7 A). Those bulbs kept in the soil had a significantly lower dry mass than those stored dry or cold. No significant differences were observed in the root dry mass, but the fresh mass of roots harvested from plants that remained in the soil was significantly higher than that of plants stored dry or cold (Figure 9.7 B). The ratio of fresh to dry mass was highest for the bulbs and roots of those specimens kept in soil (Figure 9.8).

A second group of plants was harvested mid-way through the summer season to determine the regenerative ability of the bulbs after storage. The bulbs kept in cold storage were slower to develop leaves, with both fresh and dry leaf mass accumulation significantly lower than those of plants stored dry or in the soil (Figure 9.9 A). Specimens from the latter two categories did not differ significantly from each other in this respect. Bulb fresh mass showed no significant differences across treatments, while the dry mass of the bulbs stored cold was significantly lower compared to the bulbs stored in the soil, but not compared to those stored dry (Figure 9.9 B). Root fresh and dry mass accumulation was slower for bulbs that had been stored cold (Figure 9.9 C). The ratio of fresh to dry mass did not differ significantly for the leaves. With respect to the bulbs stored cold, the fresh to dry mass ratio for the bulbs was significantly higher than that for the plants stored in the soil or dry, while the root ratio was significantly lower (Figure 9.10).

Anti-inflammatory activity

The COX-1 inhibitory activity determined at the end of winter was significantly lower in bulbs stored in the soil, with little difference exhibited by extracts from bulbs stored dry or cold (Figure 9.11). The activity of the root extracts did not differ significantly.

Extracts prepared from the second harvest of plants (mid-way through summer) showed high levels of COX-1 inhibitory activity in the leaf extracts from plants that had undergone cold storage (Figure 9.12). Part of this increased activity could be due to the younger physiological status of the leaves (as discussed in CHAPTER 2). No significant difference was detected in the activity of the various bulb or root extracts.

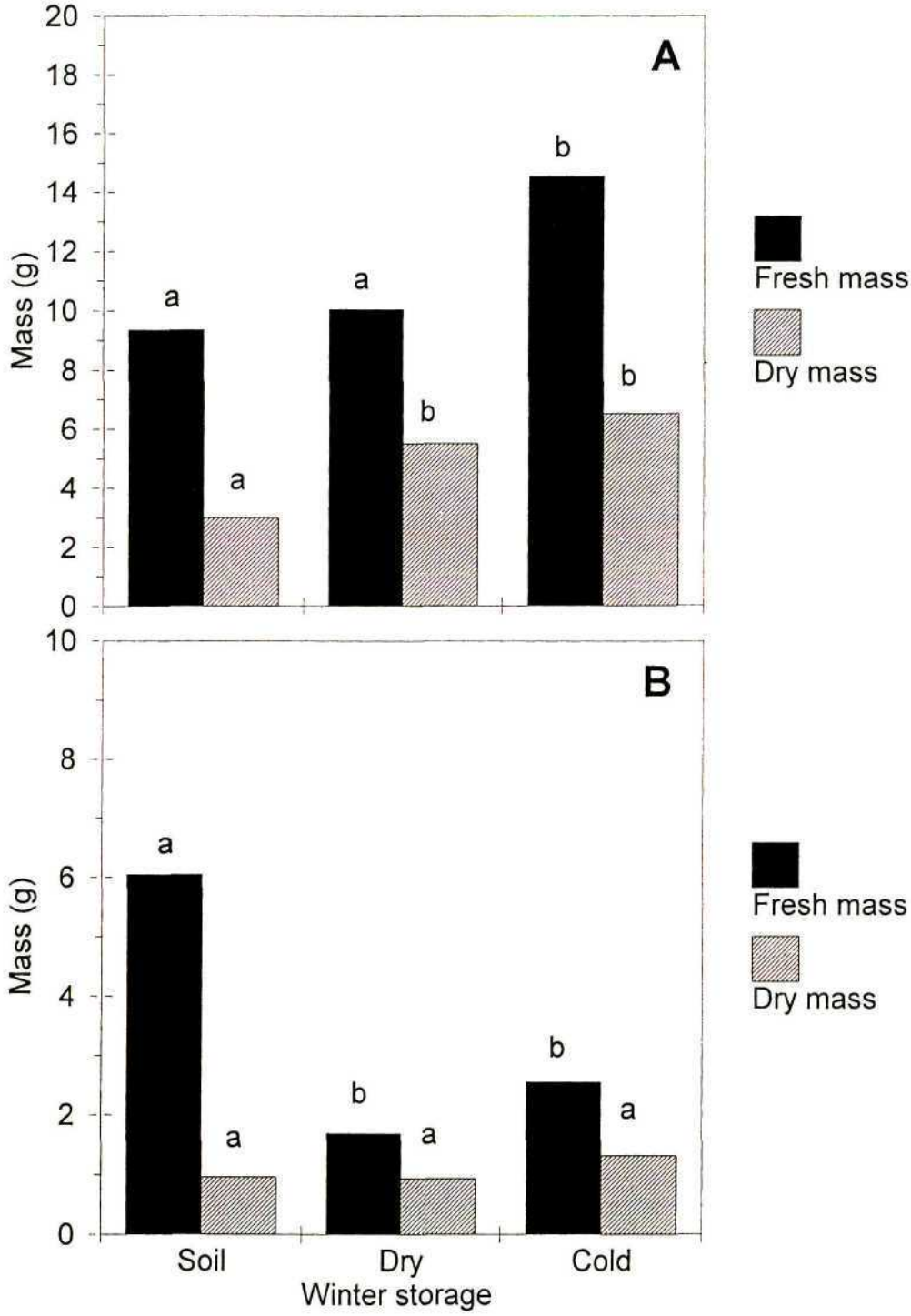


FIGURE 9.7: Variation in fresh and dry mass of *E. autumnalis autumnalis* specimens at Harvest 1. Bulbs stored dry (potting shed); in soil (greenhouse) (Control); or cold (10°C) during winter. (A) Variation in bulb growth; and (B) Variation in root growth. (Bars bearing different letters are significantly different, $P \leq 0.05$).

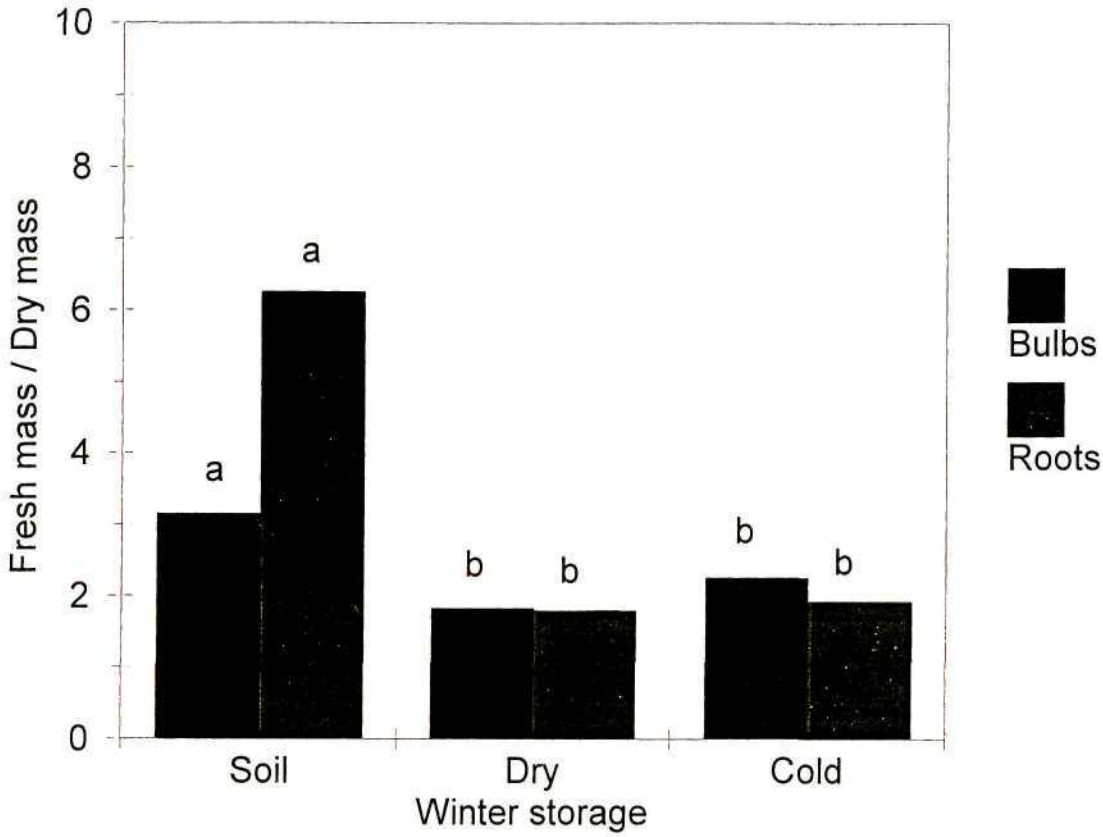


FIGURE 9.8: Variation in the ratio of fresh mass to dry mass of *E. autumnalis autumnalis* specimens at Harvest 1. Bulbs stored dry (potting shed); in soil (greenhouse) (Control); or cold (10°C) during winter. (Bars bearing different letters are significantly different, $P \leq 0.05$).

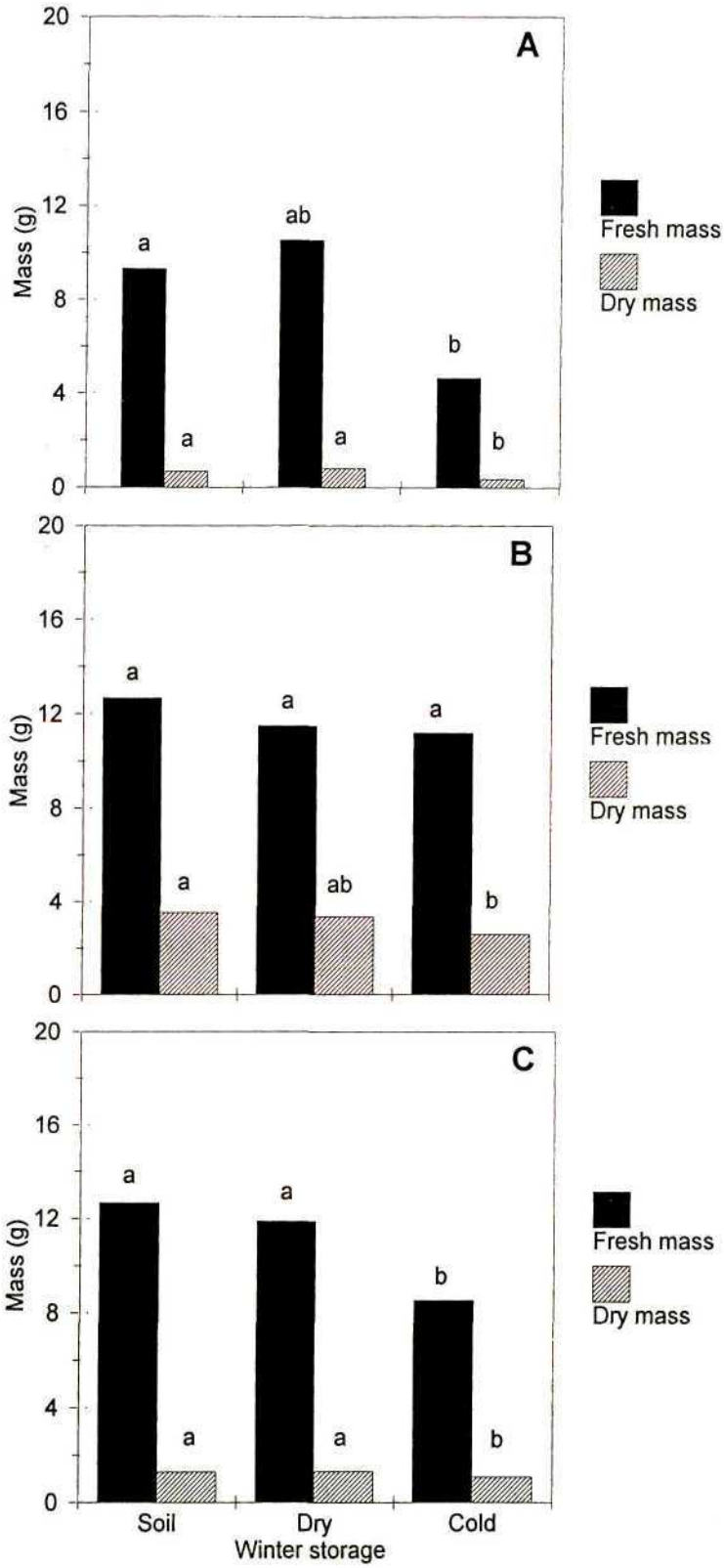


FIGURE 9.9: Variation in fresh and dry mass of *E. autumnalis autumnalis* specimens at Harvest 2. Bulbs stored dry (potting shed); in soil (greenhouse) (Control); or cold (10°C) during winter. (A) Variation in leaf growth; (B) Variation in bulb growth; and (C) Variation in root growth. (Bars bearing different letters are significantly different, $P \leq 0.05$).

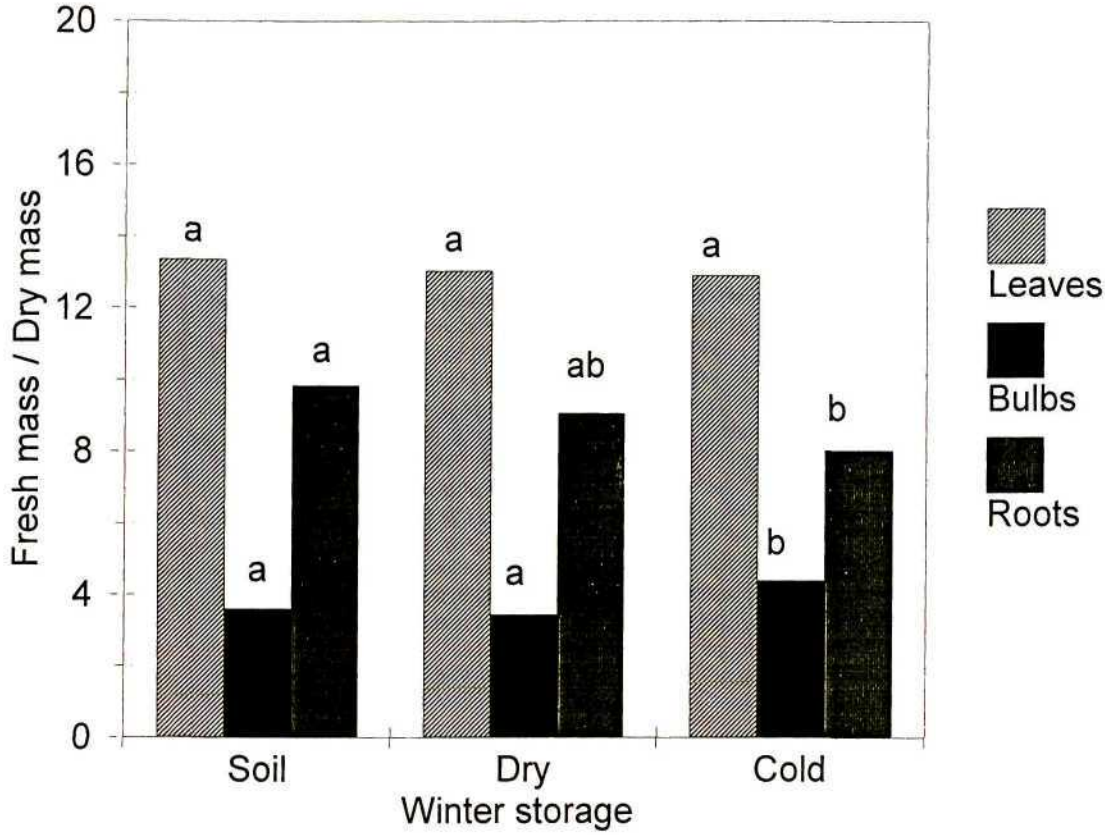


FIGURE 9.10: Variation in the ratio of fresh mass to dry mass of *E. autumnalis autumnalis* specimens at Harvest 2. Bulbs stored dry (potting shed); in soil (greenhouse) (Control); or cold (10°C) during winter. (Bars bearing different letters are significantly different, $P \leq 0.05$).

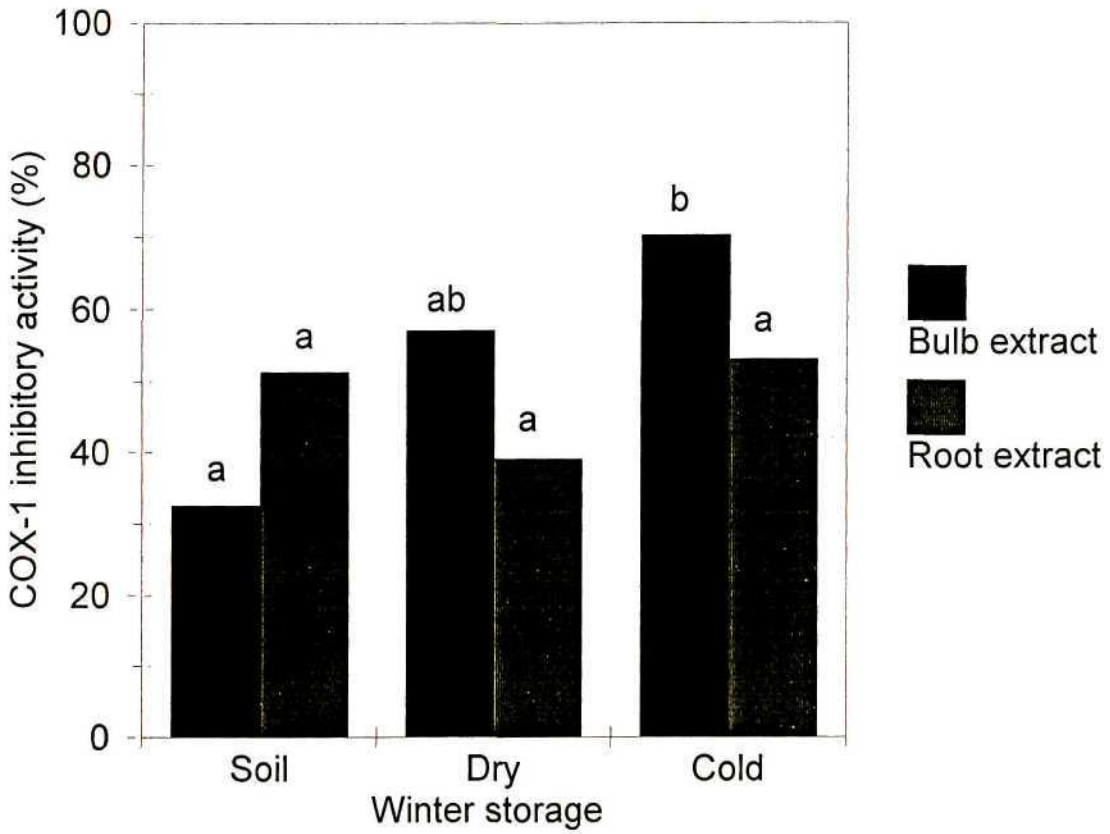


FIGURE 9.11: The % inhibition of COX-1 by ethanolic extracts of bulbs and roots of *E. autumnalis autumnalis* at Harvest 1. Bulbs stored dry (potting shed); in soil (greenhouse) (Control); or cold (10°C) during winter. (Bars bearing different letters are significantly different, $P \leq 0.05$).

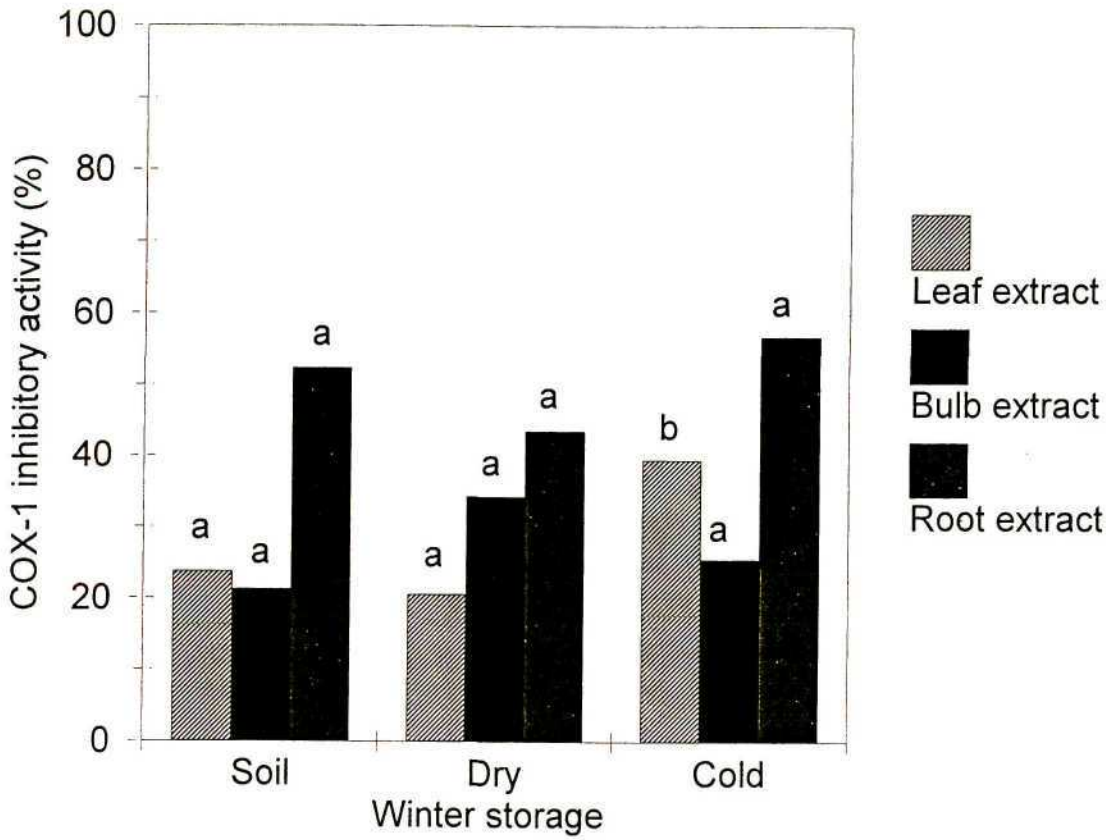


FIGURE 9.12: The % inhibition of COX-1 by ethanolic extracts of leaves, bulbs and roots of *E. autumnalis autumnalis* at Harvest 2. Bulbs stored dry (potting shed); in soil (greenhouse) (Control); or cold (10°C) during winter. (Bars bearing different letters are significantly different, $P \leq 0.05$).

9.4 DISCUSSION

9.4.1 KELPAK APPLICATION

Commercial Kelpak ® products are formed from seaweed concentrate prepared from *Ecklonia maxima* (Osbeck) Papenfuss. The application of seaweed concentrates to plants has been found to significantly increase both vegetative growth and root initiation and growth (FEATONBY-SMITH AND VAN STADEN, 1984; CROUCH AND VAN STADEN, 1992). Kelpak has been used with great success as a soil drench at the acclimitization step in the production of tissue cultured plants such as *Scilla* and *Kniphofia* (LINDSEY *et al.*, 1998). This study reflected these trends but did not show significantly higher growth rates for the fertilized plants (Figures 9.1 and 9.2). This might have been reflected if the experiment had been continued over a longer period.

9.4.2 EFFECT OF LIGHT INTENSITY

The high light intensity (and associated higher temperatures) to which the plants were exposed over a growing season tended to decrease fresh and dry mass accumulation in *E. autumnalis autumnalis*, although this was only significant for the bulb data (Figure 9.4). The decrease in the fresh to dry mass ratio observed for the leaves of the control plants at the second harvest could be due to the onset of dormancy, with the leaves beginning to die back and lose water. The higher temperature and light intensity in the high light intensity greenhouse might have delayed this process, with the result that there was no significant difference in terms of the ratios of leaf, bulb or root masses between the two harvests of plants grown in this greenhouse (Figure 9.5). It is probable that plants grown under these conditions were stressed and this was reflected in a higher accumulation of mass in the bulbs. This experiment could be extended to compare growth data obtained from plants grown in open field conditions, with associated higher light intensity, and greater fluctutions in temperature and water availability.

Although the formation of taxon-specific compounds is controlled by genetic principles, variations in the chemical composition of plant tissues do occur, primarily due to the impact of ecological factors (MÁTHÉ, 1988). Variability in natural product content and composition is a function of growth and development. This factor is

important in determining the optimal time to harvest medicinal plants (MÁTHÉ, 1988).

Fertilization with a Kelpak preparation tended to decrease the level of activity shown by the extracts, especially those prepared from the bulbs. This decrease in activity was significant for the leaf and bulb extracts (Figure 9.3), but could not be correlated with increased growth.

The light intensity under which the plants were grown had no significant effect on the accumulation of the active principle(s) in the leaves or bulbs, although high levels of activity were detected in root extracts from plants harvested at the end of the growing season (Figure 9.6). This was associated with a decrease in growth. The anti-inflammatory activity of the leaf and root extracts was high relative to plants grown under field conditions, with the anti-inflammatory activity of the bulb extracts notably lower (CHAPTER 2). This could be indicative of stress in plants grown in the high temperature, high light intensity greenhouse.

9.4.3 WINTER STORAGE

Storing the bulbs dry, at 10°C (in a coldroom) during winter significantly increased the levels of anti-inflammatory activity shown by the bulb extracts (but not the root extracts) at the end of the winter season (Figure 9.11). After a period of growth during summer, this activity dropped to a level comparable to extracts from plants stored in the soil and those stored dry. At this stage, however, higher levels of activity were observed in the leaf extracts from these plants (Figure 9.12).

9.5 CONCLUSION

From the series of experiments conducted, it would appear that the factor significantly affecting the accumulation of the active principle(s) in *E. autumnalis autumnalis* is cold storage of the bulbs during winter. Growing the plants under conditions of stress (high temperature, high light intensity), or with addition of fertilizer, resulted in lower levels of these compounds being accumulated. The accumulation of the anti-inflammatory compounds can thus not be correlated with alterations in primary metabolism as exhibited by changes in growth rate.

CHAPTER 10

MOLECULAR STUDIES FOR DNA FINGERPRINTING

10.1 INTRODUCTION

DNA molecular markers, such as random amplified polymorphic DNA (RAPD) markers have been applied to the detection of genetic polymorphism in plants (NEWBURY AND FORD-LLOYD, 1993; GOTO, 1998). Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequences of plants. Furthermore, RAPD techniques have been found to be efficient and reliable in comparison with analyses obtained with restriction fragment length polymorphism (RFLP) and isozymes (GOTO *et al.*, 1998).

The advantages of RAPD markers lie in that they do not involve radioactive labelling, are less time consuming and require much less starting material than other DNA-based fingerprinting techniques (GOTO, 1998). Further advantages lie in the quick DNA extraction procedures that can be used, and in that the technique yields true genetic markers (GOTO *et al.*, 1998). The aim of this technique is to identify primers that will give clearly identifiable polymorphic banding patterns that are sharply defined and reproducible to facilitate clonal differentiation (GOTO, 1998).

Genetic analysis using Random Amplified Polymorphic DNA markers

Single, short primer-based DNA amplification techniques have been successfully applied to plant genomes. This technique involves the use of a short, arbitrarily chosen oligonucleotide primer, which anneals to the plant DNA and directs DNA amplification of multiple genome regions. The amplification products are separated, resulting in a linear array. This profile is representative for the target DNA and is specified by the DNA sequence of the primer. Molecular polymorphisms are generated by variations in primer sites on the target DNA, length variations between primer sites, and possibly changes in the secondary structure of target DNA between

or flanking the primer recognition sites (GRESSHOFF, 1995). These polymorphisms are detected as DNA segments which amplify from only one specimen (species). The polymorphisms function as genetic markers, and can be used to construct genetic maps (WILLIAMS *et al.*, 1990).

RAPD Techniques

PCR reactions involve the *in vitro* synthesis of millions of copies of a specific DNA segment, based on the annealing and extension of two oligonucleotide primers that flank the target region in duplex DNA. After denaturation of the genomic DNA, each primer hybridizes to one of the two separated strands such that extension from each 3' hydroxyl end is directed toward the other. The annealed primers are then extended on the template strand with a DNA polymerase (ERLICH *et al.*, 1991). Amplification of genomic DNA can be directed by only one oligonucleotide primer of arbitrary sequence to produce a characteristic profile of short DNA products of varying complexity (INNIS AND GELFAND, 1990). A single PCR cycle comprises the three steps: denaturation, primer binding and DNA synthesis, carried out at discrete temperatures. A reaction cycle between the denaturation and the primer binding temperatures allows sufficient time for the polymerase activity to amplify short PCR products (ERLICH *et al.*, 1991).

Raising the temperature to 94°C separates the strands of genomic DNA. The sequence in the genome to be copied is defined by the chosen primer (short sequences of DNA) used to anneal to specific regions of a particular gene, and is arbitrary but not random in nature. This occurs when the temperature is lowered to ±45°C. Primers that have not tightly annealed to the DNA strands are removed by raising the temperature to 72°C (the optimal temperature for *Taq*), preventing other unwanted regions of the genome being copied (NEWBURY AND FORD-LLOYD, 1993). The enzyme only copies from double-stranded DNA, which occurs in the form of the primer-genomic DNA duplex. The exponential increase in the specific unit length pieces of DNA results in them becoming the most abundant DNA sequence in the reaction tube (NEWBURY AND FORD-LLOYD, 1993).

This amplified sequence is then resolved on agarose gels using electrophoresis, and visualized as a DNA band with ethidium bromide (NEWBURY AND FORD-LLOYD, 1993; GRESSHOFF, 1995). Consistent results are obtained with different DNA preparations from the same source, provided the DNA concentration is optimized (CAETANO-ANOLLÉS *et al.*, 1991).

The RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region. Detection of these polymorphisms is by the presence or absence of an amplification product from a single locus (TINGEY AND DEL TUFO, 1993). The banding patterns obtained in this manner are repeatable for a plant genotype using a particular primer, on the condition that all the reaction characteristics remain the same. Variation in any one of several parameters in the reaction can result in different banding patterns (NEWBURY AND FORD-LLOYD, 1993).

The advantages of this technique lie in its speed, technical simplicity and frequency of identification of polymorphisms (ARNHEIM AND ERLICH, 1992; NEWBURY AND FORD-LLOYD, 1993). Most importantly, this polymorphism can be mapped as a standard genetic marker. It is impossible to determine directly whether an individual is homozygous or heterozygous for a particular RAPD locus since most RAPD markers are dominant, and individuals containing two copies of an allele cannot be distinguished quantitatively from those containing only one (NEWBURY AND FORD-LLOYD, 1993;TINGEY AND DEL TUFO, 1993).

Application to *Eucomis* species

Eucomis species show very similar gross morphological features, making identification, especially in the absence of flower characteristics, a complex process. In addition, the genus has proved especially susceptible to hybridization. This has proved a distinct advantage in the breeding of the species for ornamental purposes, but the formation of hybrids complicates identification. This was the motivation behind this particular study. The aim was to develop a molecular fingerprint to aid in the rapid and conclusive identification of a species. In addition, it should be possible to determine genotype relationships using arbitrary primers (DE LANGE *et al.*, 1993), a particularly useful tool in terms of hybrid production.

10.2 MATERIALS AND METHODS

10.2.1 DNA EXTRACTION PROCEDURE

Leaf material was ground in liquid nitrogen and transferred to centrifuge tubes with 20 mℓ of urea extraction buffer (Appendix III). This was left on ice for 30 min and then incubated at 65°C for 30 min. The tubes were centrifuged at 12 000 g at 10°C for 20 min to remove cell debris. The supernatant was transferred to clean tubes. Potassium acetate (1.5 M) was added (6 mℓ per 20 mℓ) to co-precipitate proteins (with salt from the buffer). The sample was left on ice for 30 min before centrifuging at 12 000 g at 10°C for 20 min. The supernatant was transferred to clean tubes and the DNA precipitated overnight at 10°C with 0.7 volumes isopropanol.

The DNA was then transferred to microfuge tubes. Spooled DNA was washed in 70% ethanol and resuspended in 500 $\mu\ell$ deionised, distilled water (ddH_2O). Sodium chloride (100 $\mu\ell$ of 1 M NaCℓ) was added followed by 50 $\mu\ell$ CTAB (1%). This was incubated for 1 h at 65°C. Chloroform-isoamyl alcohol (24:1 v/v) extractions were conducted until the interphase was clean i.e. any remaining protein was removed, and the aqueous phase was retained.

This suspension was transferred to a clean microfuge tube and the DNA was precipitated with 0.7 volumes (±400-500 $\mu\ell$) isopropanol on ice for +2hrs. The DNA was washed with 70 % ethanol and centrifuged for 10 min before being resuspended in 500$\mu\ell$ ddH_2O. The DNA samples were stored at -20°C until needed.

10.2.2 DNA QUANTIFICATION

The DNA concentration in each sample was determined using an ultraviolet-visible recording spectrophotometer (Pharmacia GeneQuant RNA/DNA calculator). A blank of 1 mℓ ddH_2O was used. The sample (1 $\mu\ell$) was added to 1 mℓ water and the RNA/DNA and percentage purity were recorded. The DNA stock was then diluted to a concentration of 25 ng $\mu\ell^{-1}$.

10.2.3 PCR REACTION

Various primers were screened to find those giving a minimum of 5 clear bands of suitable intensity. A cocktail (Table 10.1) was developed for 10 reactions. The cocktail (23.8 $\mu\ell$) was added to each microfuge tube. The *Taq* polymerase and 10x Buffer (10 mM Tris-HCℓ; 1.5 mM $MgC\ell_2$; 50 M KCℓ; [pH 8.3]), were obtained from Boehringer-Mannheim. Mineral oil (2 drops) was added and the mixture was centrifuged briefly (Sigma 113 Benchtop Microcentrifuge). The DNA (1 $\mu\ell$) and the different primers (0.2 $\mu\ell$) were added to the respective microfuge tubes and the tubes were again centrifuged briefly. The preparation steps were conducted on ice.

TABLE 10.1: The constituents of the cocktail prepared for primer screening.

Cocktail	Volume ($\mu\ell$)
10x Buffer	27.5
dNTP's (0.4 mM)	22.0
dH_2O	210.1
Taq	2.2
TOTAL	**261.8**

PCR amplification was conducted in a Hybaid Thermal reactor using the following cycling profile: 1 cycle of 1 min at 94°C, 20 s at 36°C and 2 min at 72°C with a 2,4°C s^{-1} ramp; 75 cycles of 10 s at 94°C, 20 s at 36°C and 2 min at 72°C with a 2,4°C s^{-1} ramp; and 1 cycle of 5 min at 72°C and 1 min at 35°C.

10.2.4 AGAROSE ELECTROPHORESIS

Agarose gels (1.5% w/v) were prepared by dissolving molecular biology grade agarose in 0.5x TAE (Appendix III) using a microwave (0.7g in 40 mℓ). The gel was allowed to cool slightly (below 50°C) before adding Ethidium bromide (1μg $m\ell^{-1}$). The sample (25 $\mu\ell$) was added to 5 $\mu\ell$ gel-loading buffer (Appendix III), using a repliplate. This mixture was loaded into the set gel. The molecular weight marker (2.5 $\mu\ell$) used was Boehringer-Mannheim MWM III (Lamda phage (λ) DNA cut with *Eco*RI and *Hind*III).

Electrophoresis was conducted at 40 V in 0.5x TAE buffer. The DNA products separated in the gels were visualized under UV light (Spectraline TC 312A Transilluminator, 312 nm). The banding patterns were recorded using black and white photography.

10.3 RESULTS

10.3.1 DNA QUANTIFICATION

No single DNA extraction protocol is appropriate to all situations, and each new PCR application requires optimization (INNIS AND GELFAND, 1990). The concentrations of genomic DNA extracts were determined using a spectrophotometer, followed by dilution of the extracts to 25 ng $\mu\ell^{-1}$.

TABLE 10.2: Results of the DNA quantification.

SPECIES	Volume ($\mu\ell$)	RNA/DNA (μg mℓ^{-1})	Purity (%)	Concentration (μg mℓ^{-1})
E. autumnalis autumnalis	1	0.2	66	0.2
E. autumnalis amaryllidifolia	5	0.2	70	0.04
E. bicolor	1	1.0	86	1.0
E. comosa-comosa	2	0.2	81	0.1
E. comosa-punctata striata	4	0.2	66	0.05
E. comosa-punctata	5	0.1	98	0.02
E. pole-evansii	5	0.1	62	0.02
E. zambesiaca	2	0.9	83	0.45
Hybrid	5	0.3	78	0.06

10.3.2 RAPD AMPLIFICATION OF DNA

A total of 100 10-base primers (OPERON Technologies Inc.) were screened for their ability to detect polymorphisms in the genomic DNA, each primer used singly. The primer sequences were randomly generated, with the only requirements being a GC content of 60-70%, and that they had no self-complementary ends. The primers screened were OPA-01 → OPA-20; OPB-01 → OPB-20; OPC-01 → OPC-20; OPD-01 → OPD-20 and OPE-01 → OPE-20, where OP refers to OPERON Technologies, and the letters A-E refer to the primer series. The number specifies the primer.

Of these primers, eight were selected on the basis that more than 5 distinct bands were resolved. These primers were used in the PCR reactions for 10 of the species under study. The sequences and molecular weights of the primers used are given in TABLE 10.3.

TABLE 10.3 Random primers used for DNA amplification.

Primer	Sequence	M_r
OPB-08	$^{5'}$GTCCACACGC$^{3'}$	3004
OPB-10	$^{5'}$CTGCTGGGAC$^{3'}$	3035
OPC-08	$^{5'}$TGGACCGGTG$^{3'}$	3075
OPC-11	$^{5'}$AAAGCTGCGG$^{3'}$	3068
OPC-15	$^{5'}$GACGGATCAG$^{3'}$	3068
OPD-03	$^{5'}$GTCACCGTCA$^{3'}$	2995
OPD-11	$^{5'}$AGCGCCATTG$^{3'}$	3019
OPE-3	$^{5'}$CCAGATGCAC$^{3'}$	2998
OPE-6	$^{5'}$AAGACCCCTC$^{3'}$	2948

These gels were photographed (PLATES 10.1 and 10.2) and the profiles compared across species. Excessive smearing (visible for the first three samples) could be due to inappropriate DNA concentration or purity. Alternatively the primer concentration for those reactions may be incorrect. The latter factor can also cause poorly visualized bands. Problems such as the absence of a detectable product or low yield can be

caused by incorrect concentrations of the *Taq* enzyme (INNIS AND GELFAND, 1990).

Bands resulting from the RAPD amplification were scored based on their approximate size. This was calculated using regression analysis of the (log) size in base pairs of the bands produced by the molecular weight marker (MWM III) versus distance for each gel. These results are presented in TABLE 10.4. The bands detected for each primer were assigned codes (a-m) according to their size. Bands of identical size were assumed to be homologous across species (SMITH *et al.*, 1996).

TABLE 10.4: Approximate sizes (base pairs) of bands amplified in the PCR reactions.

BAND	PRIMER							
	OPB-08	OPB-10	OPC-08	OPC-15	OPD-03	OPD-11	OPE-03	OPE-06
a	2792	2589	4181	2142	3829	2531	2461	6055
b	2636	2208	3899	1856	3169	1492	2348	1849
c	2220	1243	1753	1667	2793	1251	2240	1374
d	1979	771	1374	1444	1310	1049	1856	1230
e	1765	678	1077	1027	1230	641	1503	1022
f	1574	636	1005	814	1051	556	1305	914
g	1444	493	971	785	-	519	1134	818
h	1116	-	816	332	-	378	875	759
i	995	-	761	-	-	-	660	732
j	862	-	537	-	-	-	616	436
k	647	-	436	-	-	-	-	376
l	594	-	-	-	-	-	-	-
m	514	-	-	-	-	-	-	-

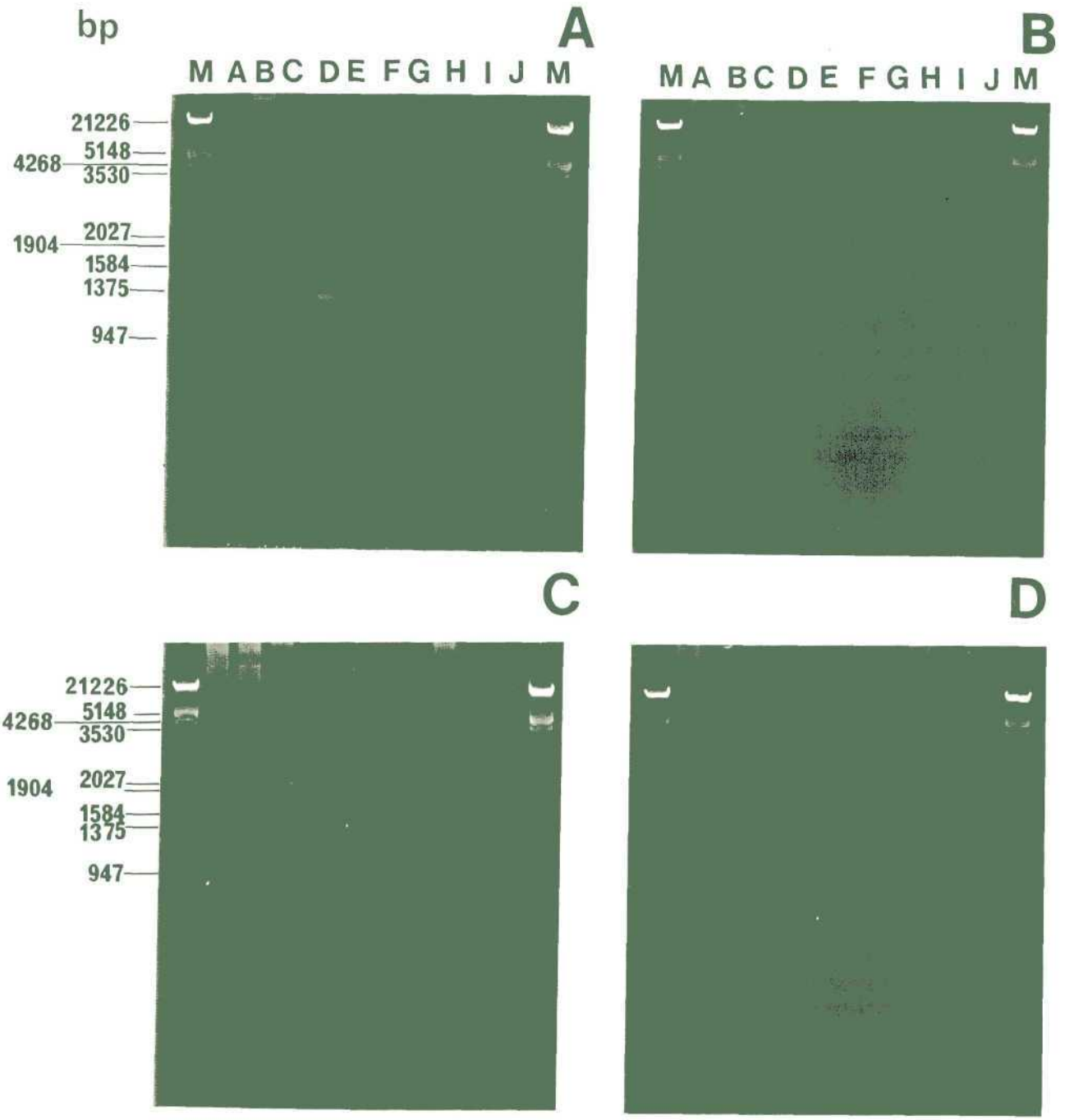


PLATE 10.1: Amplification of RAPD bands obtained from different species of *Eucomis*, separated using agarose gel electrophoresis, and stained with ethidium bromide. Random primers used: (A) OPB-08; (B) OPB-10; (C) OPC-08 and (D) OPC-15.

Lanes A = *E. autumnalis autumnalis*; B = *E. autumnalis amaryllidifolia*; C = *E. autumnalis clavata*; D = *E. bicolor*; E = *E. comosa-comosa*; F = *E. comosa-punctata striata*; G = *E. comosa-punctata*; H = *E. pole-evansii*; I = *E. zambesiaca*; J = Hybrid. M = Molecular weight marker III.

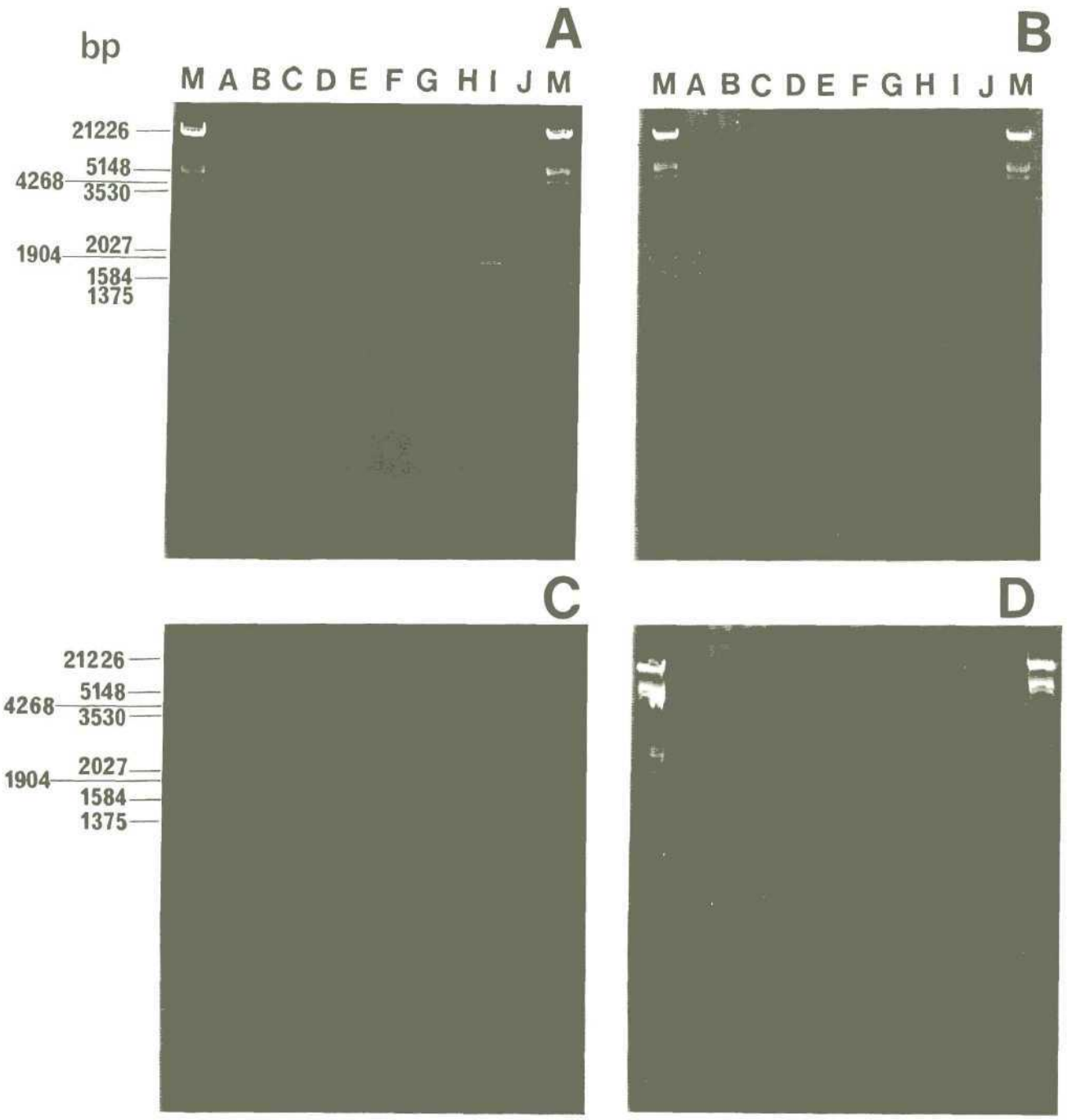


PLATE 10.2: Amplification of RAPD bands obtained from different species of *Eucomis*, separated using agarose gel electrophoresis, and stained with ethidium bromide. Random primers used: (A) OPD-03; (B) OPD-11; (C) OPE-03 and (D) OPE-06.

Lanes A = *E. autumnalis autumnalis*; B = *E. autumnalis amaryllidifolia*; C = *E. autumnalis clavata*; D = *E. bicolor*; E = *E. comosa-comosa*; F = *E. comosa-punctata striata*; G = *E. comosa-punctata*; H = *E. pole-evansii*; I = *E. zambesiaca*; J = Hybrid. M = Molecular weight marker III.

10.3.3 DNA FINGERPRINTS

DNA was isolated from the ten species under study with varying success (in terms of purity). Agarose gel electrophoresis with ethidium bromide staining of the amplification products detects the major fragments (CAETANO-ANOLLÉS *et al.*, 1991). Fingerprints produced from the electrophoresis of the amplified DNA revealed several bands common to more than one species (OPB-08b; OPC-08k; OPD-11e and OPE-06f). In addition, bands that were unique to a species were identified, which could be used to characterize the species. The different base composition of the individual primers was responsible for a unique fingerprint for the amplified DNA of each species (DE LANGE *et al.*, 1993).

The results of the DNA fingerprinting are presented in TABLE 10.5. Empty columns in this table indicate lanes in which the amplification products were not distinguishable.

TABLE 10.5: Detection of amplification products produced by RAPD primers.
Presence (+) or absence (-) of RAPD bands amplified from *Eucomis* species.

PRIMER	SPECIES									
	A	B	C	D	E	F	G	H	I	J
OPB-08a	-		-	-	-	-	+	-	-	-
OPB-08b	+		+	+	+	+	+	+	+	+
OPB-08c	-		-	-	-	-	+	-	-	-
OPB-08d	-		-	-	-	-	-	-	+	-
OPB-08e	-		-	-	-	+	-	+	-	-
OPB-08f	+		-	-	-	+	-	+	-	-
OPB-08g	-		-	-	-	-	-	-	-	+
OPB-08h	+		-	+	-	-	-	-	-	-
OPB-08i	-		-	-	+	+	-	-	+	-
OPB-08j	-		+	-	-	+	+	-	+	-
OPB-08k	-		-	-	-	+	-	-	-	-
OPB-08l	-		-	-	+	-	-	-	-	-
OPB-08m	-		-	-	-	-	+	-	-	-

PRIMER	SPECIES									
	A	B	C	D	E	F	G	H	I	J
OPB-10a			-	-	-	+	-	-	-	-
OPB-10b			-	-	+	+	-	-	-	+
OPB-10c			-	+	-	+	-	-	-	-
OPB-10d			-	+	+	-	-	+	+	+
OPB-10e			-	+	-	-	+	-	+	-
OPB-10 f			-	-	-	-	+	-	-	-
OPB-10g			+	-	+	-	-	+	-	+
OPC-08a			-	+	-	-	-	-	-	-
OPC-08b			-	+	-	-	-	-	-	-
OPC-08c			-	-	+	-	+	-	-	-
OPC-08d			-	-	+	-	-	-	-	-
OPC-08e			-	-	-	-	-	+	-	-
OPC-08f			+	-	-	-	-	+	+	-
OPC-08g			-	-	-	-	-	-	+	-
OPC-08h			-	-	-	-	-	+	-	-
OPC-08i			+	+	-	-	-	-	-	+
OPC-08j			-	-	-	-	+	-	-	-
OPC-08k			+	+	+	+	+	-	+	+
OPC-15a				+	-	-	-	-	-	-
OPC-15b				+	-	-	-	-	-	-
OPC-15c				+	+	+	-	-	+	-
OPC-15d				+	+	-	+	-	+	-
OPC-15e				+	+	+	+	-	-	-
OPC-15f				-	-	-	-	-	-	+
OPC-15g				-	-	-	+	+	+	-
OPC-15h				-	-	+	-	-	-	-
OPD-03a				-	+	-	-	-	-	-
OPD-03b				+	-	-	-	-	-	-
OPD-03c				+	+	+	-	-	-	+
OPD-03d				-	-	-	-	-	+	-
OPD-03e				-	+	-	-	-	-	-
OPD-03f				+	+	+	+	-	+	+
OPD-03g				-	-	+	-	-	-	-

PRIMER	SPECIES									
	A	B	C	D	E	F	G	H	I	J
OPD-11a			-	-	-	+	-	-	-	-
OPD-11b			-	+	+	+	-	-	-	-
OPD-11c			-	+	-	-	-	-	-	+
OPD-11d			-	+	-	+	-	-	-	-
OPD-11e			-	+	+	-	+	+	+	+
OPD-11f			-	+	-	-	+	-	-	-
OPD-11g			-	-	-	-	+	-	-	-
OPD-11h			+	-	+	-	-	+	-	+
OPE-03a				+	-		-	-	-	-
OPE-03b				-	-		+	-	-	-
OPE-03c				-	-		+	-	-	-
OPE-03d				-	-		+	-	-	-
OPE-03e				-	-		-	+	+	-
OPE-03f				-	-		+	-	-	-
OPE-03g				+	-		-	-	-	-
OPE-03h				-	-		-	-	+	+
OPE-03i				-	-		-	-	+	-
OPE-03j				+	+		+	+	-	+
OPE-06a	+		-	-	-	+	-	-	-	-
OPE-06b	-		-	+	+	-	-	+	+	+
OPE-06c	-		-	+	+	-	+	-	-	-
OPE-06d	-		-	+	+	+	-	+	-	+
OPE-06e	-		+	-	+	-	-	+	+	-
OPE-06f	-		+	+	+	+	+	+	+	+
OPE-06g	-		-	+	-	-	-	-	-	-
OPE-06h	-		-	-	-	-	+	+	+	+
OPE-06i	-		-	-	-	-	+	-	-	-
OPE-06j	-		-	-	-	+	+	-	-	-
OPE-06k	-		-	-	-	+	+	-	-	-

10.4 DISCUSSION

The absorbance of a sample for DNA quantification is measured using several different wavelengths to assess the purity and concentration of the DNA. Absorbance measurements at 260 nm are quantitative for relatively pure nucleic acid preparations in microgram quantities. Absorbancy readings cannot discriminate between DNA and RNA, however the ratio of absorbance at 260 and 280 nm can be used as an indication of nucleic acid purity. Proteins, for example have a peak absorbance at 280 nm that will reduce the A_{260}/A_{280} ratio. Absorbance at 320 nm indicates particulates in the solution or dirty cuvette, while contaminants containing peptide bonds or aromatic moieties e.g. protein and phenol, absorb at 230 nm (AUSUBEL *et al.*, 1989).

An advantage of the PCR technique (as opposed to RFLPs) is that the template DNA need not be ultra-pure in terms of protein contamination, and need not be intact provided some molecules exist that contain sequences complementary to the primers (ARNHEIM AND ERLICH, 1992). In addition, only small amounts of tissue are required for this technique, making it viable from a conservation standpoint.

RAPD markers are well suited for DNA fingerprinting (WILLIAMS *et al.*, 1990). Short primers of arbitrary nucleotide sequence can be used to reproducibly amplify segments of genomic DNA. Polymorphisms detected among the amplification products can be used as genetic markers (WELSH AND McCLELLAND, 1990; WILLIAMS *et al.*, 1990). A single primer used in the arbitrarily primed-PCR (AP-PCR) reaction produces discrete and reproducible set of products characteristic of the genome (WELSH AND McCLELLAND, 1990). The information content of a single RAPD marker is, however, low, making the use of multiple markers necessary (WILLIAMS *et al.*, 1990). The AP-PCR conditions encompass a rapid, reproducible and effective method for the preliminary identification of a species (WELSH AND McCLELLAND, 1990).

Not all amplification products arise from perfect pairing between the primer and DNA template. Mispairing may occur, giving rise to amplification products that are still

reproducible under carefully standardized conditions, and these may be used as genetic markers. These markers are, however, more susceptible to slight changes in temperature and are thus not as reliable. It is important that the genomic DNA is complete, and relatively free of single-strand breaks, since this will prevent amplification of the DNA.

Genetic clones produce identical product profiles, with possible variation in band intensity (CAETANO-ANOLLÉS *et al.*, 1991). This conserved pattern is useful in identification at the species level, with detection of polymorphisms between cultivars (CAETANO-ANOLLÉS *et al.*, 1991). Establishing fingerprint reference libraries with reliable markers is necessary to avoid incorrect identification of a clone (GOTO, 1998). *Eucomis* clones, produced in tissue culture, can thus be used (in future studies) as an additional test of the protocol reliablity, and primer suitability.

The specificity of the *Taq* DNA polymerase-mediated amplifications can be affected by the concentration of enzyme and primers, as well as the annealing time, extension time and number of cycles used in the reaction (SAIKI *et al.*, 1988; ERLICH *et al.*, 1991). A distinct disadvantage to this technique is that it is not possible to distinguish between target DNA and contaminants. This necessitates great care in maintaining standardized conditions and in the prevention of contamination of the sample and reaction by foreign DNA.

The gels presented in PLATES 10.1 and 10.2 show excessive smearing for lanes A, B and C. These represent the three subspecies of *E. autumnalis.* Generally, smearing can be converted to discrete bands by adjusting the concentration of polymerase, primer or DNA. The specificity of the interaction between the primer and the template DNA is temperature and salt dependent, requiring the empirical optimization of reaction conditions (WILLIAMS *et al.*, 1993). Smearing is often indicative of too high a concentration of primer (or too much DNA) (WILLIAMS *et al.*, 1993). This problem was, however, largely confined to these three samples, suggesting a specific problem in the extraction of the DNA. This problem can be caused by a high polysaccharide content in the DNA sample, resulting in poor annealing of the primers to the DNA in the PCR reaction. Further modification of the extraction protocol for these samples is thus necessary.

Reaction constituents that may require further optimization include the dNTP concentration and magnesium ion concentration. The optimal concentration of dNTPs is influenced by the $MgC\ell_2$ concentration, reaction stringency, primer concentration, length of amplification product and the number of cycles. The nucleotide concentration must be sufficient to saturate the enzyme, but not too low, or unbalanced, to promote misincorporation (ARNHEIM AND ERLICH, 1992). The magnesium ions form a soluble complex with the dNTPs, essential for dNTP incorporation. In addition, the magnesium ions stimulate polymerase activity, and increase the temperature at which correctly base-paired DNA and primer/template interactions dissociate. Too low a concentration thus results in low yields, and too high in the accumulation of non-specific products (ARNHEIM AND ERLICH, 1992). Both the magnesium ion concentration and the annealing temperature can also affect the relative intensity of the bands. High magnesium ion concentrations can cause some DNA segments to be amplified more efficiently, and some less efficiently (WILLIAMS *et al.*, 1993).

The use of PCR-enhancers can also improve the quality of reactions. These include dimethyl sulphoxide (DMSO), spermidine, formamide, glycerol and polyethylene glycol (ALKAMI BIOSYSTEMS, 1999). Studies have shown that DMSO improved the amplification of some products, decreased the amplification of others, and for some loci had no effect. Other results have, however, indicated that DMSO may inhibit *Taq* polymerase. Spermidine is supposed to reduce non-specific reactions between the polymerase and template DNA (ALKAMI BIOSYSTEMS, 1999). These are factors that can be investigated further in future.

Several of the primers produced amplification products (Table 10.5) that appear characteristic of the genus (OPB-08b; OPC-08k; OPD-11e and OPE-06f). In general, the amplification products produced for each species differed, with a distinctive pattern produced for each primer. Bands shared between species could indicate that they are more closely related phylogenetically. Further experimental work is necessary to distinguish the relationships between species, and in particular, to establish the relationship between the hybrid specimen and particular individual species. Morphogenetically, the hybrid resembles *E. autumnalis* and *E. comosa-comosa*. Although some primers produced common bands for these species and the

hybrid, no conclusions could be drawn from the data reported here.

Wild populations of medicinal plants are characterized by high degrees of genetic variability as a consequence of the unrestricted genetic recombination, gene flow, and mutation that occur in natural habitats (MÀTHÉ, 1988). The conservation of these genetic resources (both species and ecotypes) is vital to maintain stocks for future breeding, and to manipulate the plant morphology and chemistry (MÀTHÉ, 1988). These techniques (RAPD fingerprinting) can be used to monitor this variation, and to identify specific genotypes of interest.

10.5 CONCLUSION

The AP-PCR technique coupled to agarose gel electrophoresis, results in the production of DNA fragments of different sizes, with each individual potentially having a unique set of different-sized DNA fragments. These can be used to distinguish between individuals (species) (DE LANGE *et al.*, 1993). To ensure the reproducibility and reliability of this technique, the reactions need to be repeated and unreliable primers discarded. In addition, several individuals from a species need to be sampled. Once this is achieved, these results can be modelled using computer-generated programmes to determine phylogenetic relationships. The results presented in this chapter show that the various species do produce different banding patterns when visualized on agarose gels, and the species can be distinguished in this manner. The protocols reported can provide the basis for further work on this genus.

These results formed a preliminary study into the development of DNA fingerprints for the different species, and hybrids, of *Eucomis*. Differences were detectable between the amplification products visualized on agarose gels for the different species. The results for the different species need to be further replicated using a number of different individuals. This can be extended using clones obtained from tissue culture. Although no reliable conclusions can be drawn directly from this study, a suitable protocol for future work has been developed and tested.

CHAPTER 11

GENERAL CONCLUSIONS

11.1 INTRODUCTION

The search for new medicinal, as well as other types of bioactive compounds, has recently focussed on the ethnobotanical uses of plant drugs, with the recognition and study of the synergistic effects of herbal remedies forming a new area of research (NIGG, 1992). The study of the immuno-regulatory effects produced by plant products, and the modification of prostaglandin synthesis, are examples of the change in focus of scientific research, and it is proposed that such effects will explain many of the uses of crude plant extracts by traditional healers (LOZOYA, 1994). New scientific evidence supports a future increase in the use of herbal remedies, resulting in the development of a new global pharmacology of natural products (LOZOYA, 1994).

Despite the successful introduction of many plant-derived drugs into therapy, their development has been restricted by several factors (KINGHORN, 1992). The low concentrations of biologically active natural compounds, and their sensitivity (the compounds may be thermally or hydrolytically unstable, or have unfavourable solubility properties) have served to limit investigative studies.

The work presented in this thesis encompassed four major lines of study focussed around a traditionally-used medicinal plant indigenous to southern Africa. The first investigation centred on the screening of the available species of *Eucomis* for potential anti-inflammatory activity. This initial study was extended to include comparisons of anti-inflammatory activity in extracts prepared from the different plant parts. The second aspect entailed the investigation and identification of the active principle in *Eucomis autumnalis autumnalis*. This study was supplemented by further investigation of the effect of specific factors on the levels of the anti-inflammatory compound(s) in this species. A third aspect of this project centred on the development of a micropropagation protocol applicable to the different species of *Eucomis*. This

was again supplemented by a study of the levels of anti-inflammatory compound(s) produced *in vitro*. The fourth aspect of this investigation entailed the production of TLC fingerprints. This was conducted in an attempt to identify the presence of the active principle in extracts prepared from the plants. A final investigation included in the thesis was the development of DNA fingerprints for the different species. This was performed in an attempt to provide a clearer identification of the different species, and to form the basis for future investigations into the phylogenetic relationships between species and hybrids.

11.2 SCREENING AND IDENTIFICATION OF THE ANTI-INFLAMMATORY PRINCIPLE

The results presented in CHAPTERS 2 and 3 indicate the highly complex nature of the plant extracts prepared from *Eucomis* species. It is evident that there are several compounds in these extracts exhibiting anti-inflammatory activity. These compounds may be present in different proportions in the different plant parts, and probably all play a role in the efficacy of the traditional remedies prepared from this plant. The complexity of these extracts is further enhanced by the presence of compounds such as saponins (CHAPTER 2), which can affect the pharmacological activity of the other constituents in the preparation.

Bioassay-guided fractionation of the ethyl acetate bulb extract produced several highly active fractions. The compounds in these fractions are still in the process of being fully identified.

Homoisoflavanones, isolated from *Eucomis* species in the past, and reported to have anti-inflammatory, anti-allergic, anti-histamine and angioprotective activity, were isolated from the bulbs of *E. autumnalis autumnalis* in this study. The two compounds that were identified, however, exhibited significantly different pharmacological activity. Eucomin was shown to possess only COX-1 inhibitory activity (not COX-2), while 5,7-dihydroxy-6-methoxy-3-(4-methoxy benzyl)-chroman-4-one showed insignificant activity in both assays (CHAPTER 4). This illustrates the importance of directly linking pharmaceutical activity to specific compounds in an extract.

Two highly active compounds, with both COX-1 and COX-2 inhibitory activity were isolated and characterized. A potent, selective COX-2 inhibitor was identified as a spirostane triterpenoid (eucosterol). Bioassay-guided fractionation directed the isolation of a potent COX-1 inhibitor (a phenol-hydrocarbon chain) that is undergoing further analysis at the Cape Technicon. This investigative method has great potential for the isolation of highly active, and possibly novel, natural products from traditional medicinal plants. Although the potential for the discovery of substances with valuable pharmacological activity is considerable, whether such compounds ever play a significant role in the development of new drugs (as templates for synthesis / semi-synthesis, and as biochemical tools) depends on a wide variety of factors, including financial restraints (VLIETINCK, 1987).

While such an investigation cannot fully evaluate the pharmacological activity of a plant used in medicinal preparations, the value of this study lies firstly in the application of scientific methods in substantiating the efficacy of the preparation, and secondly in identifying specific compounds, that, in a purified state, could form the basis of new drug development. One of the most successful approaches for the investigation of compounds with medicinal value from higher plants, includes the pharmacological screening of plant extracts, followed by a bioassay-guided fractionation of the active extracts leading to the isolation of pure constituents (VLIETINCK, 1987).

11.3 ENVIRONMENTAL FACTORS AND ANTI-INFLAMMATORY ACTIVITY

Levels of anti-inflammatory activity exhibited by *E. autumnalis autumnalis* were affected primarily by the environmental conditions during the period of winter dormancy. Bulbs stored at low temperatures (8-10°C) showed a limited period in which the anti-inflammatory activity of the bulb extracts was higher than that of the control. This effect was evident for a longer period in the leaves.

The effects of fertilization, and a growth environment characterized by a higher temperature and light intensity, were less obvious. The causes underlying the lower levels of anti-inflammatory activity associated with these plants could not be

ascertained from this study. These answers could be linked to the metabolic pathways from which the anti-inflammatory compounds are derived. The interaction between these specific pathways and primary metabolism would have to be determined to produce these solutions.

11.4 MICROPROPAGATION

Protocols have been successfully established for all eleven species of *Eucomis* under study. Previous publications described *in vitro* propagation methods using twin-scales. The protocols presented here utilize leaf explants, providing a renewable, non-destructive source of explants for the periodic re-establishment of cultures in long-term propagation schemes. This is important since the long-term production of *in vitro* plantlets in general has been found to be economically unviable in the absence of a fixed market demand (REES, 1992).

Rapid rates of propagation using these techniques enables the rapid and economic bulk production of plantlets for use in commercial or conservation efforts. To further increase the commercial efficiency of this venture, it is possible firstly to standardize the initiation media, and secondly to eliminate the root initiation medium. While these modifications do not maximize adventitious shoot production (or root production), an acceptable number of shoots are produced by the cultures for propagation. The elimination of the rooting medium is accompanied by a longer duration of the plantlets on the shooting medium.

Eucomis species are hardy geophytes, bearing attractive inflorescences. The plants show great potential horticulturally, and would conceivably form a profitable small scale crop for both the horticultural market and the medicinal plant trade.

11.5 *IN VITRO* PRODUCTION OF COX INHIBITORS

A basic MS medium, supplemented with NAA and BA, forms an optimal medium for the initiation and growth of *in vitro* plantlets of *Eucomis* species. Plantlets produced *in vitro* exhibit high levels of anti-inflammatory activity. Extracts, prepared using ethanol,

possess high levels of COX-1 and COX-2 inhibitory activity, with a COX-2/COX-1 ratio slightly less than that of the bulb extract prepared from the adult plants.

Altering the levels of nitrogen and sucrose added to the basic tissue culture medium essentially affected the primary metabolic systems, evident in a change in growth rate. Low levels of sucrose, however, impacted negatively on the levels of anti-inflammatory activity exhibited by the plant extracts.

The development of callus cultures for the accumulation of secondary metabolites *in vitro* represents a potentially valuable system for the reliable and economic procurement of medicinal compounds. Callus cultures initiated from *E. autumnalis autumnalis* produced relatively high levels of anti-inflammatory compounds, exhibiting both COX-1 and COX-2 inhibitory activity. This field of study shows potential for further research centred on the optimization of callus growth, and additional investigation of the active compounds produced by these cells.

11.6 CONSERVATION

An important aspect of the conservation of medicinal plants is the identification of marketable plant products which can be harvested sustainably (COTTON, 1996). The development of projects involving the sustainable use of medicinal plants is especially important for plants such as *Eucomis* species, which are perceived by traditional medical practitioners and herb traders as becoming rare in their natural environment (COTTON, 1996). The leaves of *Eucomis* species show potential as a substitute plant part for harvest, thus forming a renewable resource, with extracts showing high COX-1 and COX-2 inhibitory activity. The success of projects aimed at sustainable development is dependent on both economic viability and ecological sustainability.

The supply of such plants on a reliable basis is of great importance, and is dependent on some form of bulk propagation. Plantlets produced using *in vitro* techniques can be used both to re-introduce the species into natural habitats and to relieve the stress on existing populations by providing an alternate resource.

11.7 DNA FINGERPRINTING

The conclusive identification of both species and hybrids based on DNA fingerprinting is an established procedure. This process requires the optimization of several steps including that of the extraction of the DNA and of the PCR reaction conditions. Once this has been achieved, species can be reliably identified based on the presence of molecular markers. The protocols presented in CHAPTER 10 form the basis of this work. Differences in amplification products were detected using eight oligonucleotide primers with random sequences. Further work needs to be done to establish phylogenetic relationships between the species and hybrids.

11.8 CONCLUSION

The potential for the discovery of new, biologically active natural products from plants and other natural sources is substantial, and this type of multi-disciplinary research has the capacity to discover chemical compounds that may cure some of the most threatening forms of disease known to mankind (KINGHORN, 1992), as well as providing alternate medical preparations, carrying minimal side-effects, for common ailments. The historical treatment of pain and fever with traditional medicinal plants provided the initiative for this investigation, focussing on *Eucomis* species, a genus indigenous to southern Africa, and commonly used for this purpose.

The results presented in this thesis represent an extensive investigation into the medicinal properties and biology of this valuable medicinal plant. In addition, this study provides scientific verification for the use of *Eucomis* species in medicinal preparations. The discovery of both COX-1 and COX-2 inhibitors in the plant extracts demonstrates both the efficiency of these remedies, and the potential for further drug development based on the relative absence of undesirable side-effects associated with COX-2 inhibitors. *Eucomis* plants represent a valuable resource that has been exploited by African traditional healers for centuries, and that shows great potential for future profit to the horticultural, floricultural and pharmaceutical industries.

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Standard curves for the commercial NSAID indomethacin in the COX assays

The commercial NSAID used as a positive control was indomethacin (1-[p-Chlorbenzoyl]-5-methoxy-2-methylindole-3-acetic acid) with a molecular weight of 357.8 ($C_{19}H_{16}ClNO_4$). This standard was included in each COX-1 assay test at a concentration of 5 μM. Each COX-2 assay test included indomethacin at a concentration of 200 μM and nimesulide at a concentration of 200 μM. A dilution curve (Figure D and E) was prepared for the standard in each assay. The IC_{50} values were calculated using regression analysis of the Log concentrations.

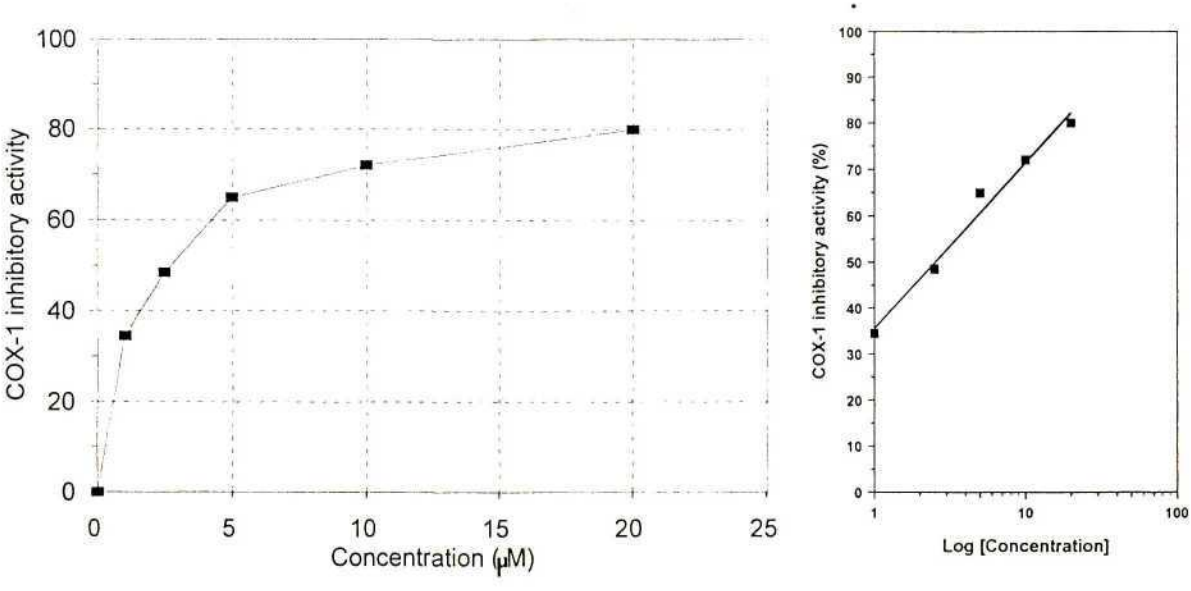

FIGURE D: Standard curve for the inhibition of COX-1 by the commercial NSAID indomethacin. Dilution curve (Left) and regression analysis (Right).

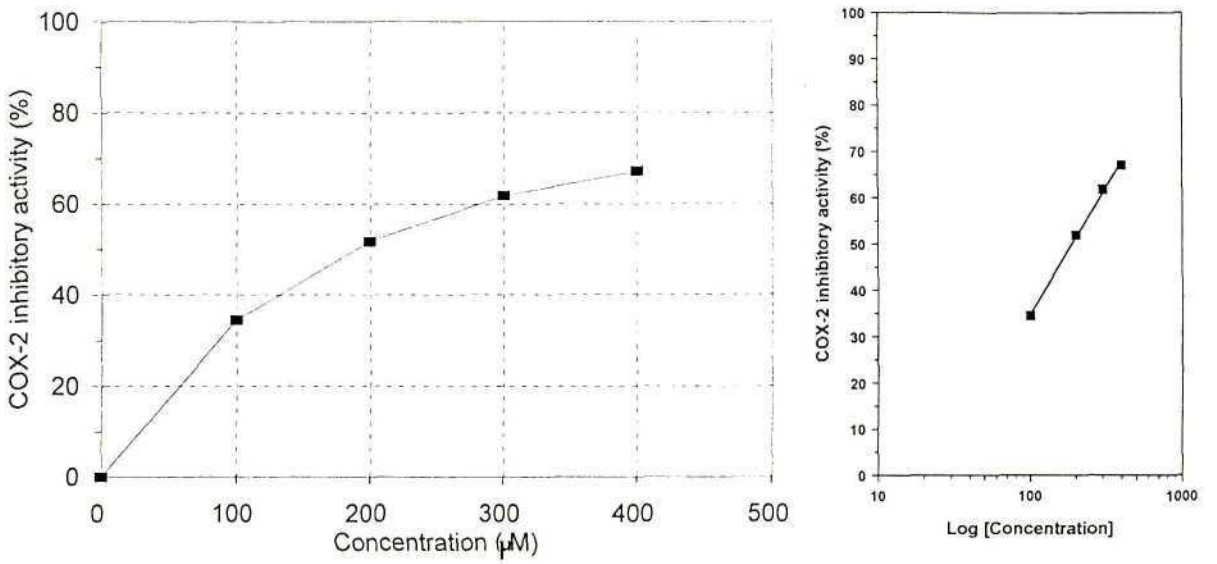

FIGURE E: Standard curve for the inhibition of COX-2 by the commercial NSAID indomethacin. Dilution curve (Left) and regression analysis (Right).

The IC_{50} for indomethacin in the COX-1 assay was calculated by regression analysis of the Log concentrations to be 3.1μM and in the COX-2 assay to be 188 μM, giving a COX-2/COX-1 ratio of 60.

APPENDIX I

COX-1

The COX-1 enzyme was prepared fresh from sheep seminal vesicles. Preparation took place in the Botany Department, University of Natal Pietermaritzburg.

Enzyme standardization: COX-1

Isolation of sheep seminal microsomes

Eight seminal vesicles (± 4 cm in diameter) were obtained from 1-2 year old rams from the Cato Ridge Abattoir outside Pietermaritzburg (Abakor). The vesicles were kept on ice until they could be frozen with liquid nitrogen and stored at -70°C (overnight). The frozen vesicles were cut on a glass petri dish placed on ice. The cut vesicles (64.6 g) were then transferred to a beaker containing 150 mℓ 0.1 M potassium phosphate buffer (pH 7.4) supplemented with 1 mM EDTA (ethylene diamine tetra-acetic acid disodium salt). This mixture was macerated at medium speed for 10 min using an UltraThurax, and then sonicated in an ultrasound bath for 5 min.

The homogenate was centrifuged at 4 000 g for 15 min (Avanti J-251 Centrifuge) to remove cell debris. The supernatant was decanted and centrifuged at 17 000 g for 10 min to remove mitochondria. The microsomes were isolated by ultracentrifugation of 60 mℓ of the supernatant at 100 000 g for 1 h (Beckman L7-5S Centrifuge). The resulting microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4).

Determination of enzyme concentration

The enzyme concentration was determined using the Bio-Rad Protein Assay Kit. The standard procedure was altered in that 20 $\mu\ell$ of each standard or sample solution, and 1 mℓ of the diluted dye reagent, were pipetted directly into the cuvettes. The standard curve (Figure A) prepared from the assay revealed the protein concentration of the enzyme preparation to be 0.83 mg mℓ^{-1}. Aliquots of 10 $\mu\ell$ were pipetted into Eppendorf tubes and stored at -70°C.

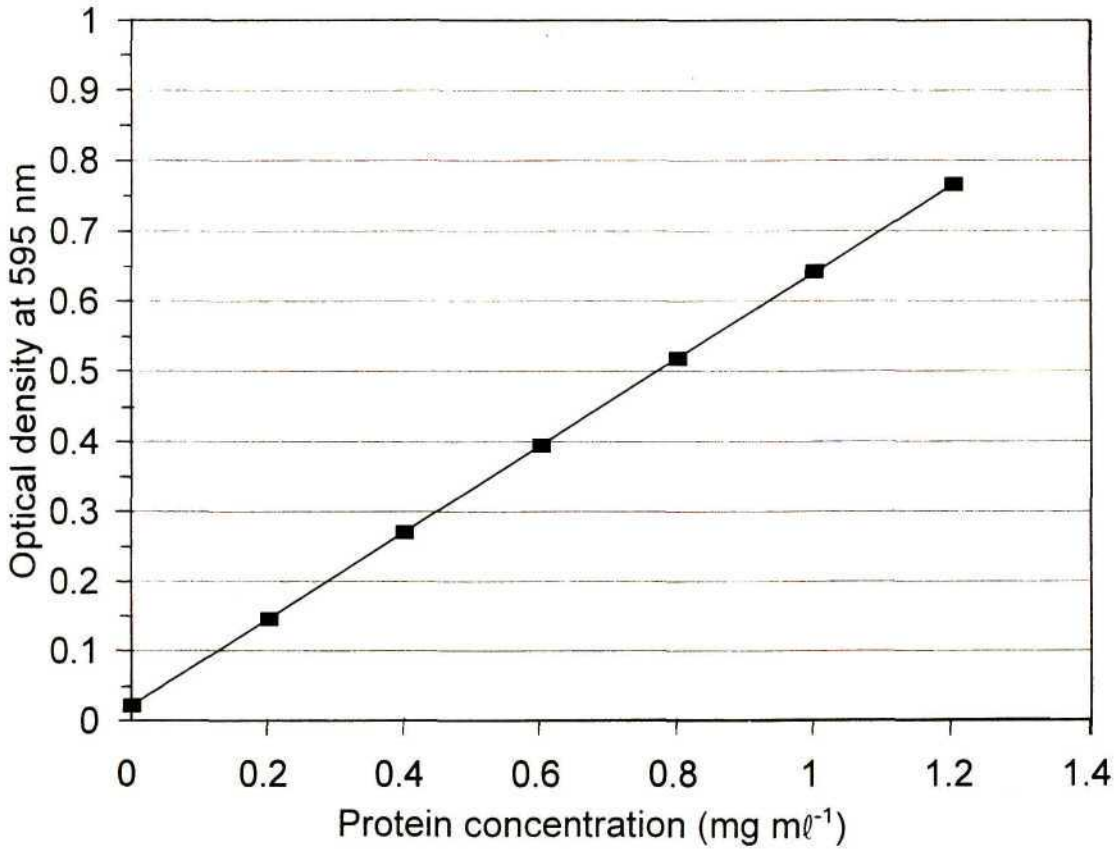

FIGURE A: Standard curve for the determination of the protein concentration of the microsomal preparation.

Standardization of enzyme concentration for assay

Concentrations varying from 0 to 1% of the isolated microsomal fraction were tested in the bioassay. Solvent blanks (2.5 $\mu\ell$ ethanol + 17.5 $\mu\ell$ water) were used as the test solutions (Figure B) with an incubation period of 10 min. The optimal incubation time for the arachidonic acid combined with the enzyme was also tested (Figure C).

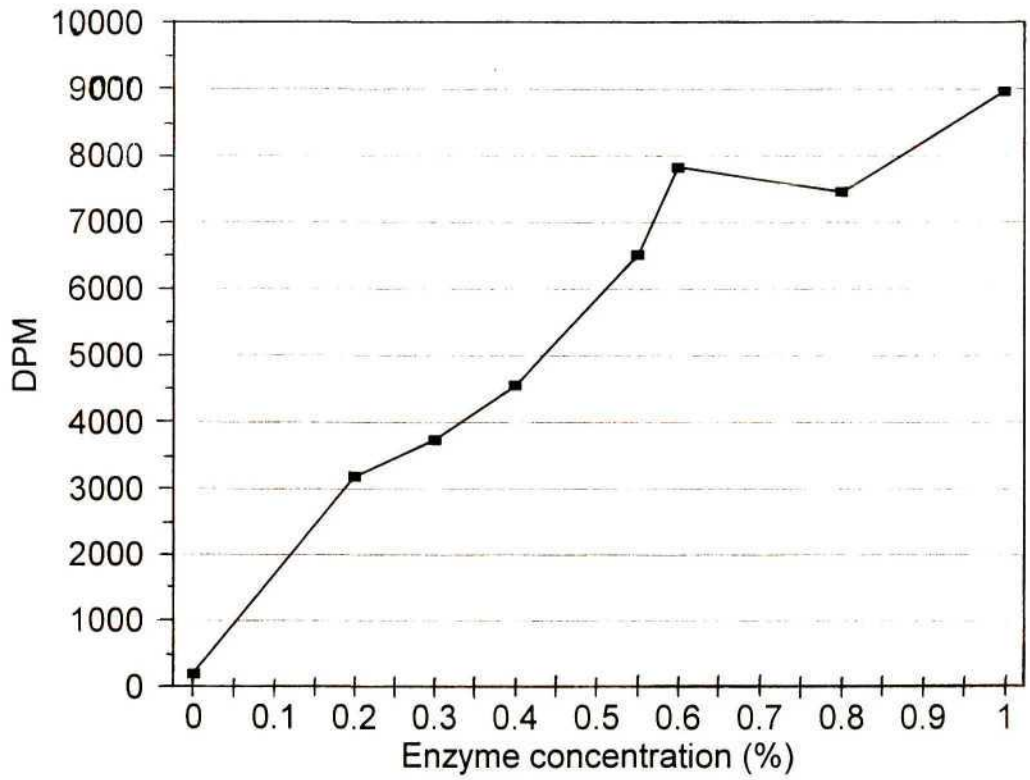

FIGURE B: Standard curve for the determination of enzyme concentration for the COX-1 assay.

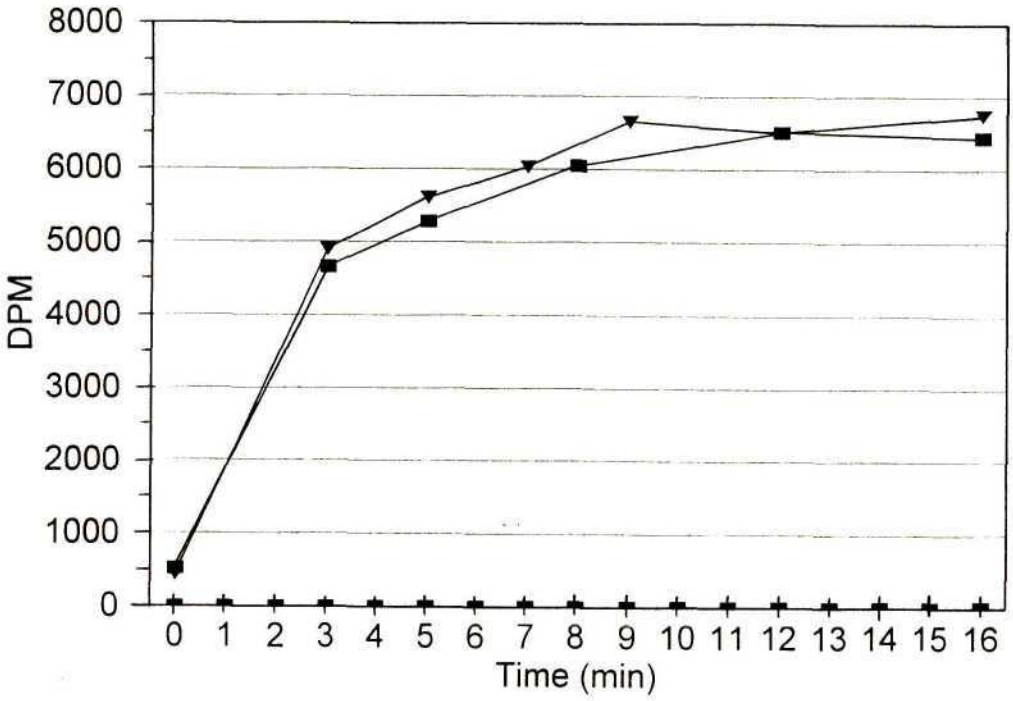

FIGURE C: Standard curve for the determination of the optimal incubation time for the COX-1 assay. [Enzyme dilutions: ▼ 0.55%; ■ 0.45%].

In order to fulfill the requirement for a repeatable assay, in which many samples can be tested together (NOREEN *et al.*, 1998), a reaction time of 10 minutes was chosen. At this time, the activity of the enzyme was approximately steady, with the graph becoming level. From these curves it was decided to use a 0.55 % dilution and an incubation time of 10 min. Using these parameters, the DPM count is sufficiently high (6 000 - 8 000 dpm) and the values fall within the region of increment on the curves.

COX-2

The COX-2 enzyme was purchased from Cayman chemicals. Aliquots of 10 $\mu\ell$ were prepared and stored at -70°C. Enzyme batches prepared and stored in this way remain stable for one year.

APPENDIX II

TABLE A : Nutrient composition of Murashige and Skoog (1962) Basic culture medium.

STOCK	SALT	MASS (g) / 1ℓ STOCK	VOLUME STOCK (mℓ/ℓ)
i	NH_4NO_3	165	10
ii	KNO_3	95	20
iii	$CaCl_2.2H_2O$	44	10
iv	$MgSO_4.7H_2O$	37	10
v	NaFe EDTA	4	10
vi	KH_2PO_4	17	10
vii	H_3BO_3 $MnSO_4.4H_2O$ $ZnSO_4.7H_2O$ KI	0.62 2.23 0.86 0.083	10
viii	$Na_2MoO_4.2H_20$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$	0.025 0.0025 0.0025	10
ix	Thiamin HCl Nicotinic acid (Niacin) Pyridoxin HCl Glycine	0.01 0.05 0.05 0.2	10

APPENDIX III

TABLE A: Constituents of the basic extraction buffer used for the extraction of DNA from *Eucomis* species (Total volume 150 mℓ).

CHEMICAL	STOCK CONCENTRATION	VOLUME / MASS
Tris	100 mM	15 mℓ
EDTA	50 mM	15 mℓ
NaCℓ	500 mM	15 mℓ
STS	1 %	15 mℓ
Urea	8 M	72 g

TABLE B: Constituents of the loading buffer used for non-denaturing Agarose gel electrophoresis of DNA from *Eucomis* species (Total volume 10 mℓ).

CHEMICAL	VOLUME / MASS
NaOH	0.02 g
EDTA [0.5 M]	20 μℓ
Glycerol	0.5 mℓ
Bromophenol Blue	0.04 g

TABLE C: Constituents of TAE stock (50x) used for non-denaturing Agarose gel electrophoresis of DNA from *Eucomis* species (Total volume 500 mℓ; pH 8.5).

CHEMICAL	VOLUME / MASS
Tris-acetate	121 g
EDTA [0.5 M]	20 $\mu\ell$
Glacial acetic acid	28.55 mℓ
$Na_2.EDTA.2H_2O$ [0.5 M]	50mℓ