The medicinal value of Amaryllidaceae and Asteraceae species used in male circumcision

by

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I declare that the dissertation herewith submitted for the degree of PhD (Plant Physiology) at the University of Pretoria, has not been previously submitted by me for a degree at any other university or institution of higher education.

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INTRODUCTION

1.1. BACKGROUND

1.1.1. General

The medicinal properties of plants are well known. Poppy extracts produce the pain reliever's codeine and morphine while cardiac glycosides from *Digitalis purpurea* L. (foxglove) are used as a heart medicine (Samuelsson, 1992). One of the new exciting potential drugs is galanthamine, an alkaloid from snowdrops (mainly *Narcissus confusus)* which is undergoing clinical trials for treating Alzheimer's disease (Bastida *et al.*, 1987; Tanahashi *et al.*, 1990).

Prior to modern medicine, traditional healing practices and treatment of disease by herbal remedies played a major role in the health of millions of people (WHO, 1978; Dimayuga & Garcia, 1991). In countless societies, plants feature significantly in cures for common ailments (Farnsworth, 1990; Stafford, 1991; Martin, 1995) such as coughs, colds and flu, relieving pain, treating snakebites etc. (Watt & Breyer-Brandwijk, 1962; Bolofo & Johnson, 1988).

According to Farnsworth (1990), about 64 % of the world's population use plants as drugs and hence the combined experience of such people should be taken into account if plants are to be considered as potential leads in drug development.

The plant kingdom represents an extraordinary reservoir of novel molecules. Of the estimated 250 000 plant species around the globe, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even lower. There is currently a resurgence of interest in the field (Balick, 1990; Hostettmann *et al.*, 1997). The advances in modern biology, biochemistry and biotechnology have led to the discovery of new enzymes, receptors and biotechnological pathways. The discovery of new chemical structures from natural sources, such as plants, fungi, bacteria and algae however remains the key in our search for new drugs. Furthermore, progress in separation science has facilitated the transition from crude herbal medicines to the exploitation of purified active constituents of the herbs (Glinski *et al.*, 1990).

Despite the unprecedented progress in biotechnology and other related subjects in the last two decades or so, the exact biochemistry of many plants that have been used for many years to treat diseases is still unknown (Hostettmann *et al.*, 1997). Although many plant-derived drugs are already on the market, it is still believed that many more useful compounds can still be found in the plant kingdom should the search of such entities be carried out in a logical and systematic manner. This can however be costly both in money and time (Samuelsson, 1992).

The rapid disappearance of tropical forests and other important areas of vegetation make it even more urgent to find and employ methods, which can

lead to the rapid isolation and identification of bioactive natural products (Hostettmann *et al.*, 1997). To overcome these problems, cost effective procedures must be used. Unfortunately a number of studies, which were based on the random collection procedure, turned out to be unsuccessful except for the isolation of taxol from *Taxus brevifolia*. Greater success has however been achieved when plant selection was based on their traditional use (Cox, 1990).

The mass random collection of plants might be cost effective but this approach is not very popular among researchers (Cox, 1990). It is however believed that mass random collection of plants when investigated through a number of bioassays, could result in new drug leads. However, when combined with chemotaxonomic leads, more success is usually accomplished (Elizabetsky & Wannmacher, 1993; Sauzo Brito, 1996).

Traditional healing plays an integral part in black African culture. This provides the primary health care needs of a large majority of the black South African population (Dilika & Meyer, 1997; Lindsey *et al.*, 1999). The reliance on indigenous medicinal plant use has a long history. Approximately 80 % of the black population in South Africa live in rural areas and depend upon traditional medicine to treat their common ailments (Netshiluvhi, 1999). However, bioactivity-guided fractionation of the plants to isolate active constituents has not been significantly carried out. Southern Africa has one

of the richest flora on earth with approximately 25 000 species. This constitutes about 10 % of the flowering plants known worldwide.

The value of traditional healing cannot be overestimated in South Africa as indigenous people still practice their old customs. Such customs include the traditional male circumcision. This entails a surgical operation, seclusion (both performed in the wild) and the coming-out ceremony. The surgery is followed by a period of isolation for several weeks. During this time, the wounds are treated with herbs (Wallerstein, 1980; Dilika & Meyer, 1996), as circumcision performed in the field has a high risk of infection (Green, 1994).

An observation made during previous studies, showed that plants from two families, Asteraceae and Amaryllidaceae, are used by different traditional male circumcision practicing communities to treat the wounds (Dilika & Meyer, 1996). For example, in the Eastern Cape the Xhosas apply the leaves of species of the genus *Helichrysum*: *H. pedunculatum, H. appendiculatum, H. nudifolium and H. longifolium* and the bulb scales of Amaryllidaceae species: *Boophone disticha* and *Scadoxus multiflorus,* to treat wounds.

Although these perennial herbs are widely distributed in Southern Africa, the species used by each community is usually determined by availability and locality (Hilliard, 1983; Dilika *et al.,* 1997). The Xhosas for instance, use *H. pedunculatum* mainly, whereas *H. longifolium* is applied to wounds by the Pondos in Lusikisiki (Dilika *et al.*, 1997).

Extracts from a number of *Helichrysum* species have been found to have antimicrobial properties (Tomas-Barberan *et al.,* 1990; Salie *et al.,* 1996). *H. Stoechas*, *H. decumbens, H. nitens* and *H. odarotissimum* are very good examples of species with antimicrobial activity (Tomas-Barberan *et al.,* 1988; Van Puyvelde *et al*., 1989; Rios & Villa, 1991).

H. pedunculatum has been reported in folklore to possess medicinal values, which include its ability to cure stomach ailments, anti-inflammatory properties and activity against coughs and colds (Watt & Breyer-Brandwijk, 1962; Bolofo & Johnson, 1988). Extracts from this herb have also indicated activity against a number of bacterial species, such as *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus* (Meyer & Dilika, 1996).

H. aureonitens showed antibacterial activity against *B. cereus, B. pumilus, Micrococcus kristinae* and *S. aureus* (Meyer & Afolayan, 1995). *H. caespititium,* another South African species, inhibited the growth of *Cryptococococcus neoformans, S. aureus* and *Streptococcus pyogenes* (Dekker *et al.,* 1983; Mathekga & Meyer, 1998).

In this study, *H. pedunculatum* was investigated for its antimicrobial activity and bioactive compounds. This was undertaken as crude leaf extracts showed antibacterial activity against all the tested Gram-positive bacterial species (Dilika & Meyer, 1996). In the circumcision ritual, only herbs are used as sources for antimicrobial and analgesic activity. To investigate this

traditional use, adenosine and opiate receptor binding assays were used on crude ethanolic leaf extracts to verify their pain management activity.

The Amaryllidaceae have been employed for sometime as medicine by the indigenous people of South Africa for a variety of health problems (Watt & Breyer-Brandwijk, 1962; Pettit *et al.*, 1995; Campbell *et al.*, 1998; Nair *et al.*, 1998). For example, the outer dry scales of the bulb of *B. disticha* are used as an outer covering of the wound for rapid healing by the Xhosas, whereas the Sothos use the fresh bulb scales as a wound dressing. Furthermore it has been claimed that the bulb prevents inflammation and stops the wound from becoming septic (Watt & Breyer-Brandwijk, 1962; Battern & Bokelman, 1966).

Many medicinal plants (mainly those dug up for their roots and bulbs) are endangered in South Africa. This situation is even made worse by interest shown by companies (Cunningham, 1996). A general estimation by Gossling (1998), is that 20 000 tons of plant material, worth about R270 million is harvested, processed and sold in South Africa per year (Gossling, 1998). Bulbs are used for various ailments and are generally destructively harvested (Watt & Breyer-Brandwijk, 1962; McCartan & Van Staden, 1999). About 14 % of the harvested and sold South African plant material comprises of bulbs (Mander, 1997). This poses a high threat to bulbs and many might become extinct before their medicinal potential is scientifically investigated.

B. disticha and *Scadoxus multiflorus* have been screened for their possible pain-killing effect using receptor-binding assays in this study. Also, an acetycholinesterase (AChE) inhibitory enzyme assay was used to determine if these species contain compounds with inhibitory activity against this enzyme. This assay is also of importance in the treatment of Alzheimer's disease.

1.1.2. Isolation of bioactive compounds from plants

To identify any bioactive chemical, a liquid plant extract has to be prepared first. Further processing separates the individual compounds and after chemical fingerprinting techniques have been utilized, their structures can be determined.

The main separation processes currently employed are:

(a) Thin layer chromatography (TLC)

(b) Column chromatography (normal pressure)

(c) High performance liquid chromatography (HPLC),

(d) Medium pressure liquid chromatography (MPLC),

(e) Solid phase extraction (SPE),

(f) Centrifugal partitioning chromatography (CPC).

TLC is widely used in separation. This technique is mainly utilized to determine the appropriate solvent system for other chromatographic separations e.g. HPLC and CPC. In preparative TLC, bigger quantities of test material can be separated. This has become an important technique since it permits the rapid determination of the composition of complex mixtures.

The amount of the sample to be separated in column chromatography usually determines the size of the column. Silica gel and Sephadex are the commonly used stationary phases. HPLC gives good and rapid separation and is used mainly in analytical separations. The best solvent system to employ as the mobile phase is usually determined by TLC (Harborne, 1998). HPLC is usually not suitable for separation of large quantities due to problems associated with overloading, which usually results in unsatisfactory separations. Also, some compounds might adhere to the column coating and depending on polarity, will be retained throughout the run, leading to material loss. This is not recommended for separation on preparative scale (Harborne, 1998).

The SPE technique comprises of pre-packed reusable cartridges. This is a convenient, inexpensive and time saving alternative in sample preparation to liquid/liquid extraction. This affords higher extraction efficiencies and shorter extraction times. SPE also reduces the amount of chlorinated and other organic solvents required generally in sample preparations. This affords rapid sample turnover suitable for processing large number of samples with a high reproducibility (Bennet & Larters, 2000).

MPLC is a method involving the use of longer columns with larger internal diameters than in conventional column chromatography. These columns require the higher pressures delivered by a pump for a sufficiently high flow rate. However, this method was not employed in this study.

CPC is based on liquid-liquid partitioning and presents the best alternative to circumvent the problems like the retention of plant material on the coating substance etc., associated with the other techniques mentioned above. In this method, one liquid is kept at the stationary phase by a centrifugal force while the other, which is kept in mobile phase, is pumped through at relatively high flow rates. This system offers advantages in the isolation of natural products, as it does not involve solid adsorbents such as silica. The denaturation of sensitive compounds is minimized and there is no irreversible retention. It has a high capacity and hence can be used on both analytical and preparative scales of separation (Marston *et al*, 1990; Foucault, 1995; Berthod & Amstrong, 1988; Berthod & Telaberdon, 1999).

1.1.3. Bioactivity determination

Crucial to any investigation of plants for biological activity, is the availability of suitable bioassays for monitoring the required effect. The test systems should ideally be simple, rapid, reproducible, inexpensive and sensitive enough to detect active principles even when present in small quantities in crude extracts (Farnsworth, 1990; Hostettmann *et al.,* 1997).

For screening purposes, it is advised to run a number of bioassays. However it should be noted that some activities might go undetected if a small number of microorganisms, receptors and enzymes are used. Furthermore, in any biological activity, it is essential to minimize the number of false positives, if

they have not been eliminated at earlier stages in separation (Hostettmann *et al.,* 1997; Ingkaninan *et al.,* 1999a).

In this study, three bioassays have been applied:

(a) An antibacterial activity assay,

(b) A receptor-binding assay,

(c) An acetylcholinesterase inhibitory enzyme assay.

The rural communities of South Africa have gainfully used herbs exhibiting antimicrobial properties mainly for the treatment of infectious diseases. The scientific basis has been evaluated in a number of studies (Rios & Villa, 1991; Meyer & Afolayan, 1995; Meyer & Dilika, 1996; Bremner & Meyer, 1998). Such herbs can be evaluated for their antibacterial activity by agar dilution, diffusion and autobioautographic methods on TLC plates (Lund & Lyon, 1975; Hostettmann *et al,* 1997). On agar, known concentrations of crude extracts or any test material are usually added to agar before solidifying. The petri dishes are allowed to set overnight and streaked with the suspension of bacterial cultures in a growth medium proven to yield good results. The results are obtained by determining the minimum concentration that led to complete inhibition of the bacterial growth, minimum inhibitory concentration (MIC). In the agar-dilution streak assay, a number of different microorganisms can be screened simultaneously on a petri dish at a fixed concentration of extract.

Alternatively, disks soaked in crude plant extracts are placed on the top of the agar medium seeded with bacterial cultures. Wells can also be made in the agar and be filled with the test material of known concentrations. After incubation in suitable conditions for bacterial growth, the resulting clear zones around each disk or well are a measure of bacterial growth inhibition (Ghisalberti, 1993).

A commonly used method in bioassay-guided fractionation of crude extracts is direct bioautography on TLC plates. The crude extract or fractions of interest are applied as spots on TLC plates and developed in solvent systems that usually do not hinder the growth of bacteria. Thoroughly dried plates are sprayed with a bacterial suspension and incubated in humid conditions. Results are obtained after spraying the plate with *p*-iodonitrotetrazolium chloride solution, specific for detecting dehydrogenase activity. White spots against the purple/pink background show bacterial growth inhibiting compounds in the extract or fraction (Lund & Lyon, 1975; Meyer & Dilika, 1996). This technique can be used effectively in bioassay-guided fractionation as it is easy to perform and results can be obtained in a short time (Reeves & White, 1992; Ghisalberti, 1993; Rahalison *et al.*, 1994).

Harmburger & Cordell (1987), modified the method by placing a TLC plate on the agar plate already inoculated with the microorganism. The compounds diffuse from the TLC to the agar plate and after incubation, the zones of inhibition of bacterial growth are visualized by staining. However, the

differential diffusion exhibited by diverse classes of compounds became the key problem in the use of this assay.

There has been an active search for sensitive techniques to assess biological activities recently, particularly by *in vitro* assays of specific enzymes and receptors. The development of receptor-binding assays offers the possibility of rapidly increasing our knowledge on the pharmacologically active constituents of plants and to search for novel drugs (Zhu *et al.,* 1996).

Application of bioactivity-guided fractionation of extracts resulted in the isolation of a number of active compounds with selective binding to specific receptors (Zhu & Li, 1999). The sensitivity and specificity of the receptor-ligand binding assays do not necessarily predict activity *in vivo* and any positive finding would require further verification by the use of functional assays in animals or isolated organs. However, for the basic screening methodologies currently in use, these sensitive receptor-binding assays are still suitable (Phillipson, 1995).

Pain can be elicited by inflammation and requires treatment with analgesics. An opiate receptor-binding assay was introduced to evaluate potential analgesics with opiate-like properties. The aim of the adenosine A_1 receptor-binding assay is to measure the affinity of the test compounds for the receptor. Adenosine plays a physiological role in a number of systems like, platelet aggregation and analgesic properties (Vogel & Vogel, 1997).

The acetylcholinesterase inhibitory enzyme assay has been successfully employed in the screening of the Amaryllidaceae family, particularly for novel agents in the treatment of Alzheimer's disease (AD) (Ellman *et al.*, 1961). In a number of cases, this has resulted in the isolation of galanthamine as a potent natural cholinergic substance with strong acetylcholinesterase inhibiting activity (Tanahashi *et al.*, 1990).

AD is one of the most common causes of mental deterioration in elderly people, accounting for around 50 - 60% of cases of dementia among people of 65 years

of age or older. The past two decades have witnessed a considerable research effort directed towards discovering the causes of AD with the ultimate hope of developing safe and effective pharmacological treatments. Although there is no cure for AD, a large number of potential therapeutic interventions have emerged that are designed to correct loss of presynaptic cholinergic function. Few of these compounds have confirmed efficacy in delaying the deterioration of symptoms in AD (Enz *et al.*, 1993).

During the pathological process, the cholinesterase concentration increases in the perigee zones of the brain. This leads to the blocking of the synaptic conduction and finally to a temporary inactivation of that particular system. After sometime, the relative proportions of acetylcholine and cholinesterase return to normal and disturbance of function arising as a result of the temporal

disturbance of synaptic conduction clears up. This then means that the restoration of a temporally disturbed function can be accelerated if the concentration of cholinesterase in the pathologically changed areas of the brain is lowered and if it's blocking effect on acetylcholine is neutralized (Perry, 1986; Perry *et al.*, 1998; Perry *et al.*, 1999). This is easily accomplished by the administration of anticholinesterase drugs, which blocks the action of cholinesterase that prevents the breakdown of acetylcholine, allowing it to accumulate at the sites where it is liberated under natural conditions. The action of acetylcholine and restoration of the disturbed synaptic condition is then ensured.

According to Perry *et al.* (1999), the use of complementary medicine e.g. plant extracts in dementia therapy varies according to cultural conditions. Cholinergic activities have been considered relevant to the Alzheimer's disease mechanisms (Perry *et al.*, 1999). With recent major advances in understanding the neurobiology of Alzheimer's disease and the limited efficacy of rationally designed therapies, it may be timely to re-explore historic archives for new directions in drug development. Long before the current biologically based hypothesis of cholinergic derangement in AD, plants now known to contain cholinergic antagonists were recorded for their amnesia and dementia-inducing properties (Perry *et al.*, 1998; Perry *et al.*, 1999).

The expected rise in the number of AD patients has generated a great deal of attention and research interest in government and science. From such

concerns, a number of research studies were undertaken. These have resulted in medications focusing primarily on symptomatic relief of AD (Rhivaz-Vazquez *et al.*, 2000).

For many years, pharmacological evaluation of plant extracts and their isolated compounds have been limited due to lack of equipment of high resolution for identifying the active compounds in the crude extracts. The development of sensitive chromatographic and spectroscopic techniques for the isolation and structure determination of natural products has greatly enhanced phytochemical investigations. With their high resolution, the modern chemical and biological techniques have greatly improved the prospect of finding new drug entities from plants and for investigating traditional medicines. Basic phytochemical investigations should continue to be encouraged especially with the view of the rapid loss of plant species (Glinski & Caviness, 1990; Harborne, 1998).

1.2. Objectives of the study

The main aim of this study was to determine whether plants used in male circumcision practiced by the rural communities in the Eastern Cape Province of South Africa have antimicrobial, analgesic and acetylcholinesterase inhibitory activity. The plants investigated were *Helichrysum pedunculatum* (Asteraceae), *Boophone disticha* and *Scadoxus multiflorus* (Amaryllidaceae). A number of biological assays were used to verify their allegedly medicinal

properties. A receptor-binding assay was also applied on these herbs to verify the aspect of pain management as no substances exhibiting analgesic activity are used during the ritual. Other plants from the Amaryllidaceae family, which are also used in male circumcision, were investigated for their acetylcholinesterase inhibitory activity.

The specific objectives were:

1. Isolation of the antimicrobial compounds from *H. pedunculatum* using bioactivity-guided fractionation on bacteria.
2. Using centrifugal partitioning chromatography (CPC) as a pre-fractionation tool on *H. pedunculatum* extracts with receptor binding assays guiding the fractionation for the possible pain killing effect of the leaves.
3. Determining whether the Amaryllidaceae species used in male circumcision play a role in pain management using adenosine and opiate receptor-binding assays.
4. Investigating some South African Amaryllidaceae species used in male circumcision for acetylcholinesterase inhibitory activity (AChE).
5. Isolation of AChE active compounds from *S. multiflorus.*

1.3. Scope of the thesis

H. pedunculatum has been shown to contain antibacterial compounds (Meyer & Dilika, 1996). Such compounds have been found in a number of

Helichrysum species to be externally deposited on the leaves (Tomas-Lorente *et al.*, 1989; Afolayan & Meyer, 1995). Therefore, the leaves of *H. pedunculatum* were investigated for containing epicuticular antibacterial compounds and only shaken in dichloromethane to obtain extracts. Bioassay-guided fractionation using a direct bioautographic method on TLC led to the isolation of linoleic- and oleic acids, the major antibacterial compounds in the extract. These antibacterial acids both showed activity against *Bacillus cereus, Micrococcus kristinae* and *Staphylococcus aureus*. Linoleic acid was also active against *B. pumilus* and *B. subtilis* (Dilika *et al.*, 2000).

Ethanolic *H. pedunculatum* epicuticular leaf extract was also subjected to centrifugal partition chromatography (CPC) fractionation. A receptor binding-assay using adenosine A_1 and opiate was used to guide the fractionation. This led again to the isolation of linoleic acid. This compound is however considered a false positive or non-inhibitory competitor in this assay (Ingkaninan *et al.*, 1999b). After the use of solid phase extraction (SPE) to purify the active compounds, activity was observed only in the fatty acid containing fraction.

Ethanolic bulb scale extracts from *Boophone disticha* and *Scadoxus multiflorus* were also fractionated by CPC. A preliminary investigation using adenosine A_1 and opiate receptor binding assays showed activity in all the extracts but more on adenosine A_1 than opiate.

B. disticha is used in traditional male circumcision as a wound dressing. Some Amaryllidaceae species, mainly *Narcissus* species, are known for their AChE inhibitory activity (Bastida *et al.*, 1987, Selles *et al.*, 1997). *B. disticha* and *S. multiflorus* were investigated for their AChE inhibitory activity. Both bulbs showed same activity profile and *S. multiflorus* was readily available from the bulb selling company and was therefore purified further. The active compound was identified using CPC guided fractionation with the AChE inhibitory assay to monitor the activity. It was shown that galanthamine, an alkaloid undergoing clinical trials for the treatment of AD, was the isolated compound.

1.4. Structure of the thesis

This thesis consists of contributions in the form of a paper published in Fitoterapia (Chapter 2) and three manuscripts under consideration for publication (Chapters 3, 4 and 5).

The separation, purification and identification of the antibacterial compounds from the epicuticular dichloromethane leaf extract of *H. pedunculatum* are described in Chapter 2. Chapter 3 describes the fractionation of *H. pedunculatum* leaf extract using centrifugal partitioning chromatography. This chapter also deals with the application of receptor binding assays to guide the fractionation of the crude ethanolic extracts. A preliminary study on the investigation of acetylcholinesterase (AChE) inhibitory activity of two South African Amaryllidaceae species, *B. disticha* and *S. multiflorus* is dealt with in

Chapter 4. Chapter 5 describes the isolation and identification of the active compounds with the AChE inhibitory activity from *S. multiflorus.* Chapter 6 consists of the general discussion and conclusions while Chapter 7 comprises the summary of this thesis.

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CHAPTER 2

*ANTIBACTERIAL ACTIVITY OF LINOLEIC- AND OLEIC ACID ISOLATED FROM *HELICHRYSUM PEDUNCULATUM*, A PLANT USED DURING CIRCUMCISION RITES

*Antibacterial activity of linoleic- and oleic acid isolated from *Helichrysum pedunculatum*, a plant used during circumcision rites

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2.1. Abstract

Helichrysum pedunculatum (Asteraceae) is a commonly used plant to dress wounds during male circumcision rites by the Xhosas in South Africa. The antibacterial activity-guided fractionation of the dichloromethane extract of leaves of *H. pedunculatum* resulted in the isolation of linoleic- and oleic acids. These two fatty acids were then evaluated for their antibacterial activity on ten selected bacteria. Linoleic acid inhibited the growth of all the Gram-positive bacterial species tested with the minimum inhibitory concentration (MIC) varying between 0.01 and 1.0 mg/ml. Oleic acid was active against three of the five Gram-positive bacteria at a MIC of 1.0 mg/ml. Both compounds were inactive against all the Gram-negative species tested. A synergistic effect between the two fatty acids was observed against *Staphylococcus aureus* and *Micrococcus kristinae.*

*Written in the format of a paper for Fitoterapia.

Keywords: Antibacterial, Circumcision, *Helichrysum pedunculatum,*

Linoleic acid, Oleic acid

2.2. Introduction

Traditional male circumcision is a common practice among the indigenous people of South Africa. Usually, the whole ceremony takes place in a secluded area in the wild and the patients who are mainly teenagers are kept far away from families and friends throughout the period. Circumcision performed in the wild has a high risk of infection. Information obtained from various local communities has revealed a high incidence of complications arising from wound contaminations. *Staphylococcus aureus* is a bacterium which is commonly- implicated in hospitalised circumcised patients. *Helichrysum pedunculatum* Hilliard & Burtt (Asteraceae) is commonly used by the Xhosas of Transkei to bandage circumcision wounds. The folkloric use of this plant was verified by [1] when they showed that extracts of the plant have antibacterial activity. A number of other *Helichrysum* species have been reported to have antimicrobial activity [2-4]. In this study, we report on the isolation and identification of two antibacterial fatty acids from *H. pedunculatum.* Each compound was first tested individually for activity against ten bacterial species followed by an investigation of synergistic enhancement in activity against two of the bacteria.

2.3. Experimental

2.1 Plant material

Leaves of *H. pedunculatum* were collected during December 1996 from the Transkei, a region in the Eastern Cape province of South Africa. A

voucher specimen (Dilika 299) has been deposited at the H.G.W.J. Schweickerdt Herbarium (PRU) at the University of Pretoria.

2.2 Chemicals

Chromatography material and solvents were purchased from Merck-Johannesburg. Nutrient agar and broth (no 2) were obtained from Biolab. The two fatty acid standards, linoleic- and oleic acid were purchased from Aldrich.

2.3 Isolation and identification of the antibacterial compounds

Air dried *H. pedunculatum* leaves were shaken for 5 min in a orbital shaker in dichloromethane and the resulting extract was then further fractionated by column chromatography and HPLC. Column chromatography was performed on silica gel 60 using two different gradients of either hexane, chloroform, ethyl acetate and ethanol or diethyl ether, petroleum ether and methanol as eluents. Those fractions showing antibacterial activity were then rechromatographed on column chromatography using silica gel 60 (diethyl ether-light petroleum ether-methanol, 15:85:7) to separate the fraction containing oleic acid and Sephadex LH-20 (96% ethanol) for the fraction containing linoleic acid. The oleic acid fraction was further purified by HPLC utilising an analytical Phenomenex C_{18}, 250 x 4.6 mm column (methanol-water 80:20, flow rate 1.0 ml/min, 40 °C, 206nm).

GC-MS analysis (full scan mode was utilised at 280°C) of the two fatty acid fractions was conducted on a VG micromass gas chromatograph

equiped with an SE 30 capillary column (25 x 0.32 mm ID). The two isolated acids as well as their standards were also analysed by nuclear magnetic resonance spectroscopy (^{1}H- and ^{13}C-NMR).

2.4 Bioautography

Bioautography to guide fractionation was conducted on TLC plates. The chromatographic fractions were spotted and developed with chloroform-ethyl acetate (1:2) and diethyl ether-light petroleum ether-methanol (15:85:7). A 24 h old *S. aureus* culture was centrifuged at 3000 rpm for 20 minutes, the pellet resuspended in fresh sterile nutrient broth to an absorbance of 0.84 at 560 nm and sprayed on the TLC plate. After a 24 h incubation at 37 °C in humid conditions, the plates were sprayed with *p*-iodonitrotetrazolium violet (INT) and reincubated at 37 °C for 6 h [1,5].

2.5 Antibacterial testing

Standards of the two fatty acids were dissolved in acetone and mixed with autoclaved nutrient agar to final concentrations of 0.01, 0.05, 0.1 and 1.0 mg/ml. These mixtures containing 1 % acetone were then added to Petri dishes and swirled until set. The plates (including a 1% acetone control) were left overnight for the acetone to evaporate. Three replicates were used per treatment.

The ten bacterial species (Table 1) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained and recovered as described previously [1]. Bacterial species

were cultured in nutrient broth no 2 for 24 h. Before streaking onto set agar, each culture was diluted 1:100 with fresh nutrient broth. The bacteria were streaked in a radial pattern on the plates that were left overnight and then incubated at 37 °C for 24 h [6]. Complete suppression of growth by linoleic- and oleic acid was required for the acids to be declared active at a specific concentration (Table 1).

Table 1. Antibacterial activity (MIC[a] mg/ml) of linoleic- and oleic acid isolated from *H. pedunculatum*

Bacterial Species	Gram +/-	Linoleic acid	Oleic acid
Bacillus cereus	+	0.01	na[b]
B. pumilus	+	1.0	na
B. subtilis	+	0.01	1.0
Micrococcus kristinae	+	1.0	1.0
Staphylococcus aureus	+	1.0	1.0
Enterobacter cloacae	-	na	Na
Escherichia coli	-	na	Na
Klebsiella pneumoniae	-	na	Na
Pseudomonas aeruginosa	-	na	Na
Serratia marcescens	-	*na*	*na*

[a] minimum inhibitory concentration
[b] not active

2.6 Synergism of the two antibacterial compounds

The antibacterial activity of linoleic- and oleic acids was also analysed in combination to determine if there was a synergistic effect between them. This was also done by the agar dilution method. The final concentration of each compound in the mixture was 0.05, 0.1 and 0.5 mg/ml, and were tested against *M. kristinae* and *S. aureus* bacteria.

2.4. Results

The antibacterial-guided fractionation of the dichloromethane extract led to the isolation of linoleic- and oleic acid. The GC-MS spectra and the ^{1}H and ^{13}C-NMR spectra of the isolated compounds matched those of standards of these fatty acids.

Linoleic acid inhibited the growth of all the Gram-positive bacterial species tested with the MIC varying between 0.01 and 1.0 mg/ml (Table 1). Oleic acid was active against three of the five Gram-positive bacteria at a MIC of 1.0 mg/ml. None of the Gram-negative bacteria was inhibited by these fatty acids.

Both fatty acids were inhibitory to *S. aureus* and *M. kristinae* at 1.0 mg/ml when administered singularly. However, when administered in combination, the growth of both bacteria was inhibited at a concentration of 0.05 mg/ml of each fatty acid, indicating a strong synergistic effect. This is the first report of the synergistic effect of these two fatty acids on bacterial species.

2.5. Discussion

The insensitivity of the Gram-negative bacteria to fatty acids may be due to the prevention of fatty acid penetration by lipids in the cell wall [7-9]. Although there are rare strains sensitive to the inhibitory effect of the acids, most of the fatty acid sensitive strains belong to the genus *Bacillus* [7,9]. This is evident in the findings of [9; 10] on the antibacterial activity against *B. megaterium* and *B. subtilis*. This was also confirmed in this study by the activity of linoleic acid against *B. cereus, B. pumilus* and *B. subtilis,* however oleic acid acid showed no antibacterial activity against *B. cereus* and *B. pumilus.*

Linoleic acid has previously been reported for its antimicrobial properties against bacteria and fungi [11,12]. Although, [13] suggest 0.01mg/ml of the test compound for extracts to be considered active, the compounds recognised so far as constituents of *H. pedunculatum* could rationalise the use of the plant in wound treatment especially against bacterial infection. This activity might be attributed to fatty acids alone or in synergy with other compounds as they are known to have a potent antibacterial action [9].

The antibacterial activity of linoleic- and oleic acids as well as their synergistic effect to inhibit the growth of *S. aureus* might explain the use of this herb by the Xhosas of South Africa during male circumcision rituals. *S. aureus* is the most common implicated bacterial species in hospitalised circumcised patients.

2.6. Acknowledgements

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CHAPTER 3

CENTRIFUGAL PARTITIONING CHROMATOGRAPHIC ISOLATION OF BIOACTIVE COMPOUNDS FROM *HELICHRYSUM PEDUNCULATUM* (ASTERACEAE), *BOOPHONE DISTICHA AND SCADOXUS MULTIFLORUS* (AMARYLLIDACEAE), USING RECEPTOR BINDING ASSAYS

*Centrifugal Partitionling Chromatographic isolation of bioactive compounds from *Helichrysum pedunculatum* (Asteraceae), *Boophone disticha* and *Scadoxus multiflorus* (Amaryllidaceae), using receptor binding assays

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3.1. Abstract

Helichrysum pedunculatum (Asteraceae) is used in South African traditional medicine as a remedy for stomach ailments and as an anti-inflammatory agent in male circumcision. Bioassay-guided fractionation by centrifugal partition chromatography (CPC) using adenosine A_1 and opiate receptor binding assays resulted in the isolation of fatty acids from this *Helichrysum*. Solid phase extraction (SPE) on semi-pure fractions was used to investigate the possibility of other active compounds in the extract apart from the fatty acids. However, only the fatty acid fraction showed activity.

Boophone disticha and *Scadoxus multiflorus* (Amaryllidaceae) are also used in wound therapy especially by circumcising communities in South Africa.

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These species were also investigated for their pain killing effect using adenosine and opiate receptor binding assays. Both species were found to be active on both adenosine and opiate receptors, although not as strongly as *H. pedunculatum* extracts. However, comparing the two Amaryllidaceae species, *B. disticha* showed the most activity.

Keywords: *Adenosine A_1, Asteraceae, Amaryllidaceae, circumcision, CPC, Helichrysum pedunculatum, opiate, pain, receptor-binding assay, Scadoxus multiflorus*

3.2. Introduction

A number of common diseases affecting mankind throughout the world are in one way or another caused by central nervous system (CNS) disorders (Zhu *et al.*, 1996). Opioid drugs have been widely used in pain treatment for a long time and a limited number of such compounds like morphine is still the most commonly used in cases of severe pain treatment (Cox, 1997). Adenosine is a physiologically important nucleotide as it mediates a large variety of effects in the CNS. These include hypotention and inhibition of platelet aggregation in the cardiovascular system. Adenosine regulates such physiological functions via membrane bound receptors (Lohse *et al.*, 1984; Pirovano *et al.*, 1989). An extensive amount of research has been conducted in this area (Zhu *et al.*, 1996). Plant extracts have been investigated for their pain-killing effects and some of them have already been used for the treatment of CNS related disorders (Zhu *et al.*, 1996; Cox, 1997).

Pain can be elicited by inflammation and requires treatment with analgesics. Opiate receptor-binding assays were introduced to evaluate the potential analgesics with opiate-like properties. The aim of the adenosine A_1 receptor-binding assay is to measure the affinity of the test compounds for the receptor. Adenosine plays a physiological role in a number of systems like, platelet aggregation and analgesic properties (Vogel & Vogel, 1997). This study was undertaken as no analgesic exhibiting substances are taken during the circumcision ritual.

As part of the ongoing search for plant ingredients that are active in CNS disorders, especially on pain management, leaves of *Helichrysum pedunculatum* Hilliard & Burrt, *Boophone disticha* (L.f) Herbert and *Scadoxus multiflorus* Martyn) Raf. were investigated for activity. *H. pedunculatum* is a herb used in traditional medicine in South Africa as a cure for stomach ailments and as a dressing in male circumcision (Watt & Breyer-Brandwijk, 1962; Bolofo & Johnson, 1988). In previous studies, the leaf extracts from this herb showed inhibitory activity in a number of bacterial species (Meyer & Dilika, 1996).

B. disticha and *S. multiflorus* are also used in traditional male circumcision as a wound dressing. The alkaloids, buphanindrin and galanthamine isolated from the bulb scales of this family are reported to exhibit an analgesic effect. Since these herbs are administered traditionally in wound therapy, their pain-killing effect was investigated using receptor-binding assays on opiate and adenosine A_1.

In this study we investigated the activity of the *H. pedunculatum, B. disticha* and *S. multiflorus* leaf extracts on adenosine A_1 and opiate receptor binding assays. The efficiency of finding novel leads in the receptor binding assays used in screening, suffers from the occurrence of well-known active compounds or compounds that cause false positive reactions in the assays (Zhu *et al.,* 1996; Ingkaninan *et al.,* 1999a; Zhu & Li, 1999). Some active

compounds occur only in small quantities. The reproducible fractionation through the use of centrifugal partitioning chromatography (CPC) might help in the identification of false positive fractions and hence increase the chance of finding new drug leads. The success of such a separation depends on a suitable solvent system that can be time consuming to design (Foucault, 1995; Glinski & Caviness, 1995; Menet & Rolet-Menet, 1999).

In this study, we present our results on the investigation of leaf extracts of *H. pedunculatum* and bulb extracts of *B. disticha* and *S. multiflorus* for their possible pain relieving effect during the circumcision ritual with CPC used as a fractionation tool.

3.3. Materials and methods

Plant material and extract preparation

Leaves of *H. pedunculatum* were collected in the Eastern Cape province of South Africa and a voucher specimen (Dilika 299) deposited at the H.G.W.J. Schweickerdt Herbarium at the Botany Department, University of Pretoria, South Africa. The material was dried at room temperature to a constant mass, macerated and shaken in ethanol. The resulting ethanolic extract was filtered and the filtrate concentrated to dryness under reduced pressure.

B. disticha was collected from the grasslands of Umtata in the Eastern Cape province of South Africa. *S. multiflorus* was purchased from Wiljes & Zonnen BV, Hillegom, a bulb selling company in The Netherlands. The identity of *B. disticha* and *S. multiflorus* was confirmed through comparing the herbarium vouchers, (N. van Rooyen, 2569 and A.E van Wyk 3064, respectively from H.G.W.J. Schweickerdt Herbarium at the University of Pretoria.

The scales from the two bulbs were macerated, oven dried and the dry material was extracted using ethanol on a rotary shaker for 72 hours. The extracted material was filtered and the filtrate concentrated to dryness under reduced pressure. The resulting crude extracts were used in the receptor-binding assays.

Chemicals

All solvents used were of analytical grade and purchased from J.T. Baker, Deventer, The Netherlands or distilled in the laboratory before use. The radioligands were purchased from NEN, 's Hertogenbosch, The Netherlands. N^6-cyclopentaladenosine (CPA) was obtained from RBI, Massachusets, United States of America.

Radioligand binding assays

Adenosine A_1 and opiate receptor binding assays were used to guide the fractionation of the crude ethanolic leaf extract. The specific binding sites for both adenosine and opiate were obtained from rat cortical brain. The method employed is described by Lohse (Lohse *et al.*, 1984) and Pirovano (Pirovano *et al.*, 1989). Briefly, in the opiate receptor-binding assay, [^{3}H] naloxone (1.5 nM) was used as ligand at a K_d value of 2.1 nM. The incubation mixture was as follows, 100 µl [^{3}H], naloxone, 100 µl sample or a displacer, 100 µl 50 mM Tris/HCl buffer pH 7.4 and 100 µl rat brain homogenate containing 100 µg brain tissue. The mixture was incubated at 25 °C for 60 min. and put on ice afterwards. This was then washed with ice-cold buffer and the filters loaded with the radiolabelled bound receptor. The mixture was filtered on Glass fiber (GF/B) filters under reduced pressure. Filters were washed six times with 3 ml ice-cold 50mM Tris/HCl buffer, pH 7.4. The results of the test samples were given as counts in dpm. Nonspecific binding was determined in the presence of $1x10^{-5}$ M morphine. The filters containing samples were punched into small vials, soaked in 3.5 ml scintillation solution and the radioactivity determined using a β counter (1500 Liquid Scintillation, Hewlett Packard TriCarb). The results of the test samples were given as counts in dpm. Nonspecific binding was determined in the presence of $1x10^{-5}$ M morphine.

The adenosine A_1 receptor-binding assay was carried out on membranes of rat cortical brains prepared as described by Lohse (Lohse *et al.*, 1984). Briefly, the brain tissue (30 μg rat brain) was homogenised and the homogenate centrifuged at 1,000 x g for 10 min. The supernatant was further centrifuged at 30 000 x g for 30 min. The pellets were resuspended in water and left on ice for 30 min. The material was then centrifuged at 48 000 x g for 10 min. The membranes were resuspended in 50 mM Tris-HCl buffer pH 7.4 (Lohse *et al.*, 1984) and incubated in 2 U/ml adenosine deaminase (ADA) for 30 min before storage (Pirovano *et al.*, 1989). The protein concentration was between 6-10 mg/ml when measured with the bicinchoninic acid (BCA) method described by Smith (Smith *et al.*, 1985).

0.4 nM [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was used as a radioligand at a K_d value of 0.39 nM in the test. Nonspecific binding was determined in the presence of $1x10^{-5}$ M N^6-cyclopentayladenosine (CPA). For data analysis of the radioligand binding, a software package, Prism (Graph Pad Inc. San Diego, USA) was used.

Centrifugal Partitioning Chromatography (CPC)

The centrifugal partition chromatography experiments were performed using a modular Sanki (Kyoto, Japan), type LLN apparatus. It consists of a centrifuge (NMF model), a power supply (SPL model) and a constant flow pump (LBP-V). To monitor the separation, a Panasonic Pen recorder (VP 67222A) was

used. This was connected to the UV detector (IS 200, Linear Instruments, Reno, NV, USA). Fractions were collected using a LKB2211 Superrac fraction collector. The total internal volume was 125 ml and the pressure limited to 60 bar. Fractions were collected after every 4 min at the rate of 2 ml/min, each containing 8 ml. A maximum of six partition catridges was used and the void volume was rejected in all experiments.

The first CPC separation step was done using the solvent system, heptane/ethyl acetate/methanol/water 6/1/6/1 (v/v/v/v) (Ingkaninan *et al.*, 1999b). The dried ethanolic leaf extract (700mg) was dissolved in the mobile and stationary phases of the CPC solvent system. The resulting extract was injected into the system (maximum of 4 ml). The first 15 fractions were eluted using ascending mode and then the mode reversed to descending to collect the rest of the fractions.

The eluate was grouped into 9 fractions according to results obtained from thin layer chromatography (TLC). These fractions were subjected to adenosine A_1 and opiate receptor binding assays. From the assay results, fraction 6 was selected for further purification (second CPC separation) because it was active and had more material for further purification steps.

The resulting fraction was still very complex and contained components with a wide polarity range. Therefore, a second CPC fractionation was performed with a different solvent system. A ternary diagram approach for solvent

selection was used as described by Foucault and Menet & Rolet-Menet (Foucault, 1995; Menet & Rolet-Menet, 1999). Methanol was selected as the best possible solvent and the ethyl acetate and water proportions determined. The best results were obtained using ethyl acetate/ methanol/ water 5.5/1.5/3 (v/v/v). This was used as a solvent system at the second separation step.

TLC analysis

TLC was carried out using precoated silica gel plates (Silica gel 60 F_{254}, Merck, Darmstad, Germany). All fractions were applied as spots and developed in saturated chambers using chloroform/methanol 95/5 (v/v) as solvent system. Visual detection of compounds was done under UV light at 254 and 366 nm. The plates were sprayed with anisaldehyde reagent, heated afterwards and the colour changes of compounds noted. Fractions exhibiting similar TLC profiles were combined. The resulting subfractions were evaluated for their receptor binding activity.

Solid Phase Extraction (SPE)

This system involves prepacked silica catridges. To equilibrate these catridges, they were conditioned with consecutive elutions of three rinses of water followed by methanol and finally with water. After sample loading, the catridges were washed with 25%, 50%, 75% and 100% methanol. The resulting fractions were collected and concentrated to dryness using a

speedvac (Speed Vac Plus, 110A, New Brunswick Scientific, The Netherlands).

3.4. Results and discussions

Application of bioassay-guided fractionation of *H. pedunculatum* using CPC resulted in the isolation of fatty acid containing fractions with selective binding to adenosine A_1 and opiate receptors.

Non-specific binding was estimated in the presence of a high concentration of a receptor specific non-radioactive compound. The specifically bound ligand was determined by subtracting the non-specifically bound ligand from the total amount of radioactivity bound in the absence of any compound. This is expressed as a percentage of the total binding.

The screening showed that the leaf extract of *Helichrysum pedunculatum* possessed binding affinity to opiate and adenosine A_1 receptors. Both assays showed the same activity profile. Fractions with higher than 70% inhibition were considered active. The following fractions showed activity, 3, 4, 6, 7 and 8 (adenosine A_1) and 4, 6, 7 and 8 (opiate). This is clearly illustrated in Figures 3.1 and 3.2 respectively.

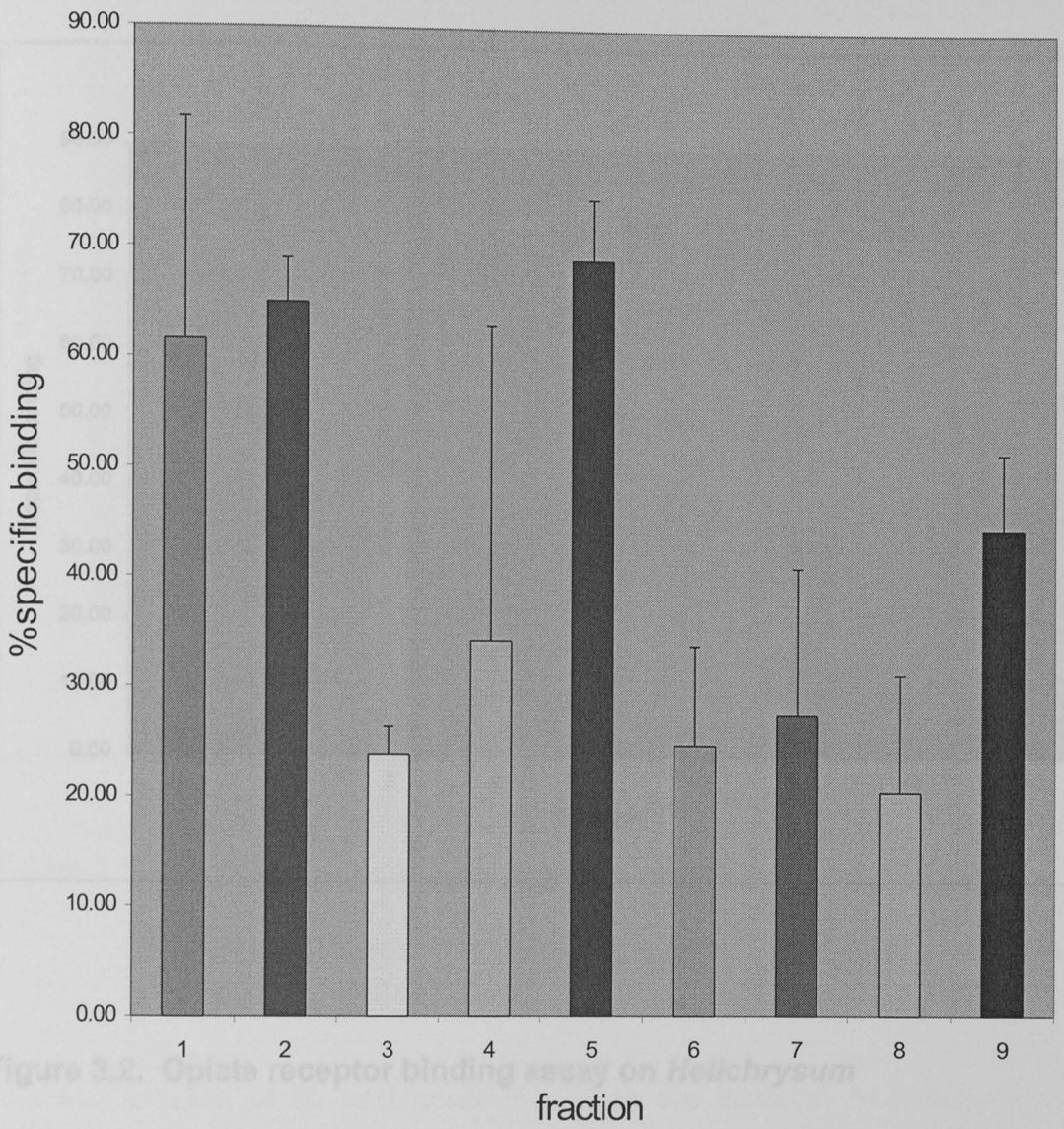

Figure 3.1. Adenosine receptor binding assay on *Helichrysum pedunculatum* leaf extract after the first CPC fractionation.

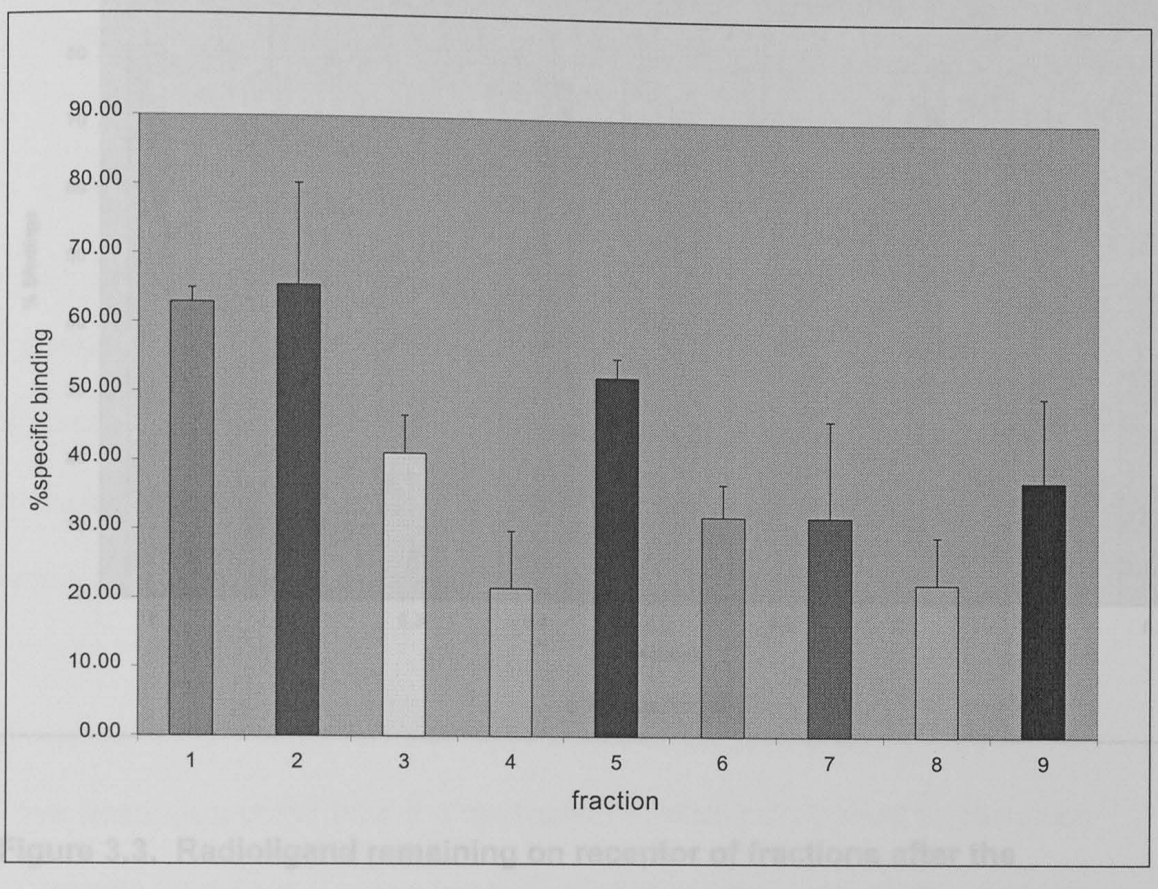

Figure 3.2. Opiate receptor binding assay on *Helichrysum pedunculatum* leaf extract after the first CPC fractionation

However, fractions 3 and 4 were not considered for further investigations as they were shown to contain fatty acids. After the second separation of fraction 6, the adenosine A_1 activity was only present in fractions 6.2, 6.3 and 6.9 (Figure 3.3). TLC analysis of these fractions however indicated that fatty acids still remained from the first fractionation.

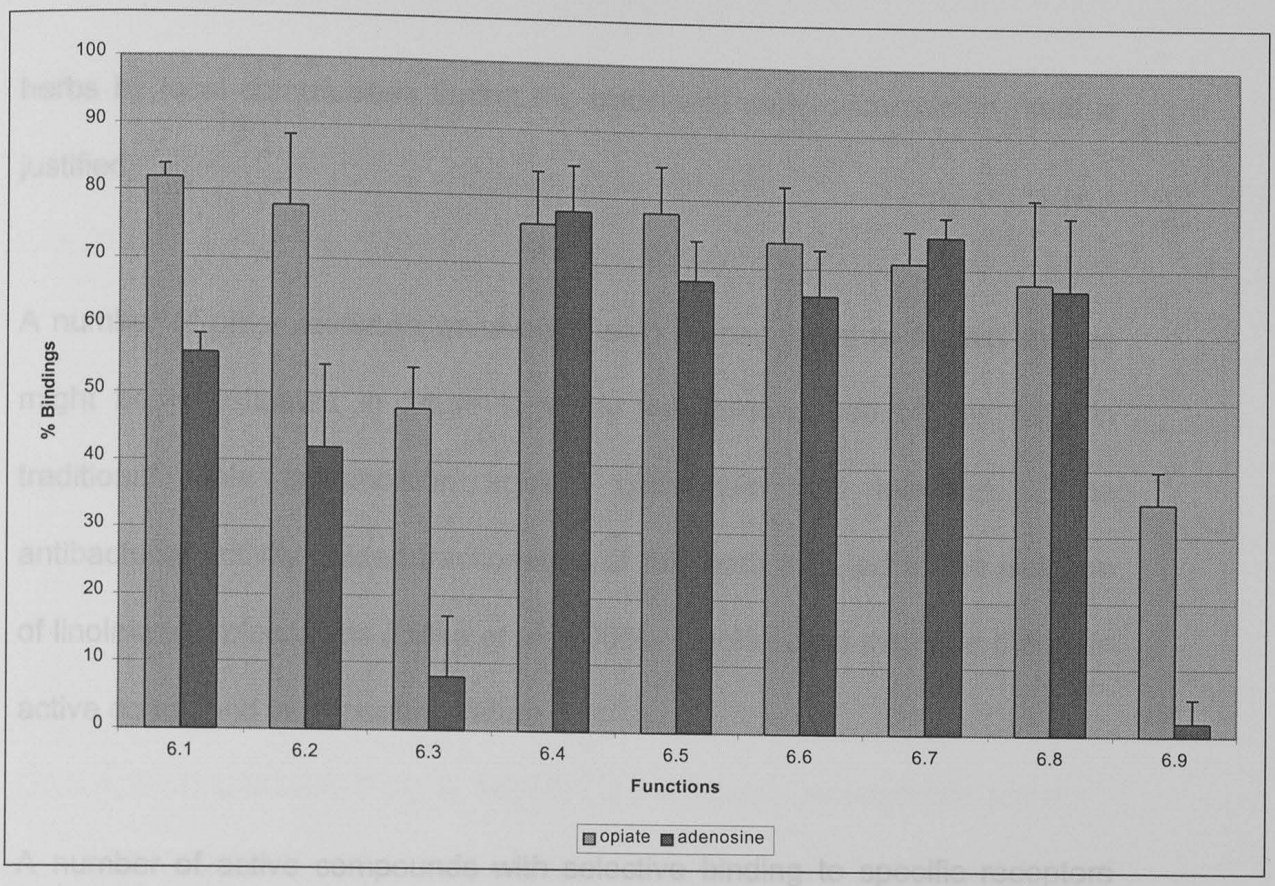

Figure 3.3. Radioligand remaining on receptor of fractions after the second CPC fractionation of fraction 6.

The fractionation of *H. pedunculatum* led to the isolation of linoleic acid. Although this compound is reported in literature to be a false positive in this assay, the presence of this compound in *H. pedunculatum* used during the circumcision ritual might have a pain-killing effect.

The investigation of the two Amaryllidaceae species, *B. disticha* and *S. multiflorus* showed that the ethanolic extracts possessed binding affinity to opiate and adenosine A_1 receptors. More activity was observed on adenosine A_1 than on opiates in the receptor binding assays. Because the two receptors involved in this study are involved in pain management, the use of these

herbs by local communities during the traditional male circumcision ritual is justified.

A number of other receptors involved in pain management or *in vivo* studies might be investigated in future to verify the folkloric use of this herb in traditional male circumcision against both pain and infection. The antibacterial activity guided fractionation of this herb also led to the isolation of linoleic and oleic acids (Dilika *et al.*, 2000). Linoleic acid might be the main active compound in *H. pedunculatum.*

A number of active compounds with selective binding to specific receptors have been discovered from the application of bioactivity-guided fractionation of extracts from various plants in previous studies (Phillipson, 1995). The use of functional assays in animals or isolated organs would be required for further verification as the sensitivity and specificity of the receptor-ligand binding assays do not necessarily predict activity *in vivo*.

3.5. Acknowledgements

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CHAPTER 4

PRELIMINARY INVESTIGATION OF *BOOPHONE DISTICHA* AND *SCADOXUS MULTIFLORUS* FOR ACETYLCHOLINESTERASE INHIBITORY ACTIVITY

*Preliminary investigation of *Boophone disticha* and *Scadoxus multiflorus* for acetylcholinesterase inhibitory activity

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4.1. Abstract

Boophone disticha and *Scadoxus multiflorus* are herbs used as wound dressing in traditional male circumcision in South Africa. Only the outer scales of the bulbs, fresh or dry depending on the community, are used to dress wounds. The bulbs of these species were investigated for acetylcholinesterase (AChE) inhibitory activity. The AChE inhibitory enzyme assay has been gainfully employed in the screening of the Amaryllidaceae family, particularly for novel agents in the treatment of Alzheimer's disease (AD).

Centrifugal partitioning chromatography (CPC) was used as a fractionation tool and the AChE inhibitory enzyme assay was used to guide the fractionation of *B. disticha* and *S. multiflorus* extracts. Four of the CPC collected fractions of *B. disticha* had AChE inhibitory activity higher than 70% at 0.1 mg/ml.

**Written in the format of a paper for the South African Journal of Botany*

Keywords: *Acetylcholinesterase, alkaloid, Boophone disticha, Centrifugal partitioning chromatography, Alzheimer's disease.*

4.2. Introduction

Age related diseases are gradually becoming a problem mainly in the western world as the population continues to grow older. One good example is Alzheimer's disease (AD). The search for causes and possible treatment of AD is becoming more urgent as no effective therapy exists currently. Although the pathogenesis of AD remains unknown, acetylcholinesterase (AChE) inhibition seems to show some symptomatic improvements in a number of clinical trials (Enz *et al.*, 1993; Selles *et al.*, 1997a; Nordberg & Svensson, 1998).

The enzyme AChE controls the breakdown of acetylcholine and is therefore of great interest. AChE reverses the cholinergic deficit in the brain and hence its importance in the treatment of AD (Enz *et al.,* 1993; Kihara *et al.*, 1995). Some form of AChE may possibly be related to the enzyme providing a major proportion of the substrate choline in the release of the newly synthesised acetylcholine (Perry, 1986).

Besides their ornamental value (McCartan & Van Staden, 1999), Amaryllidaceae species are of great interest because of their rich alkaloid content (Tanahashi *et al.,* 1990; Selles *et al.*, 1999). The alkaloids have shown a number of biological activities and some are already used widely in drugs e.g. morphine. They also play an important role in both plant defenses

against herbivory and serve as a storage form of nitrogen (Selles *et al.*, 1997b; Campbell *et al.,* 1998; Selles *et al.*, 1999). A number of South African Amaryllidaceae species are reported to have been used in traditional healing practices for various ailments (Watt & Breyer-Brandwijk, 1962; Campbell *et al.*, 1998). Amaryllidaceae alkaloids exhibit various pharmacological effects such as antiviral, antitumour, anticholinergic and anti-inflammatory activity (Bastida *et al.*, 1987, Han *et al.*, 1992; Cakici *et al.*, 1997). An alkaloid isolated from this family, galanthamine, is among the number of cholinesterase inhibitors currently undergoing clinical trials (Nordberg & Svensson, 1998).

We present in this study our results of an AChE inhibitory activity investigation on *B. disticha* (Lf) Herbert and *S. multiflorus* (Martyn) Raf. A photometric method was used to measure acetylcholinesterase inhibitory activity by following a colour change, which is produced by thiocholine as it reacts with the dithiobisnitrobenzoate ion.

4.3. Materials and methods

Plant material

B. disticha was collected from the grasslands of Umtata in the Eastern Cape province of South Africa and *S. multiflorus* was purchased from Wiljes & Zonnen BV, Hillegom, a bulb selling company in The Netherlands.

Chemicals

Acetylthiocholine iodide (ACTI), AChE, bovine serum albumin (BSA), 5,5'dithiobis-[2]- nitrobenzoic acid (DTNB) and acetylcholine esterase type VI-S (from electric eel-lyophillised powder) were obtained from Sigma (St Louis, MO, USA). All organic solvents were of analytical grade and purchased from J.T. Baker (Deventer, The Netherlands). The buffer used throughout the experiments was 50 mM Tris-HCl pH 8.0. The AChE esterase (480 U/mg) contained 530 protein units prepared to 1130 U/ml stock solution using buffer. This was kept at –80 °C after the dilution to 1/5000 using 0.1% BSA. 3 mM DTNB was dissolved in buffer containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_20$ and 15 mM ACTI dissolved in "millipore" water.

General extraction

The scales of the two bulbs were macerated, oven dried and the dry material was extracted using ethanol on a rotary shaker for 72 hours. The extracted material was filtered and the filtrate concentrated to dryness under reduced pressure. The resulting crude extracts were used in the AChE inhibitory activity assays.

CPC fractionation

The extracts were fractionated with CPC using heptane/ethyl acetate /methanol/water 6/1/6/1 (v/v/v/v). The first 15 fractions were eluted using the ascending mode and the rest using descending mode. Fractions were pooled based on similarity on TLC plates. All resulting fractions were tested for their AChE activity.

From the results, the most active fraction from each of the two species was subjected to further CPC fractionation. In the second fractionation step (CPC2), the solvent system was changed to ethyl acetate/methanol/water 43/22/35 (v/v/v). After TLC analysis, similar fractions were tested for their AChE inhibitory activity.

TLC analysis

All fractions collected were applied individually as spots on a TLC plate (Silica gel 60 $F_{254,}$ No. 5554, Merck, Darmstad, Germany) and developed with chloroform/methanol, 9/1 (v/v). After development in a saturated chamber, the dry plates were observed and marked under UV 254 and 366 nm. The plates were then sprayed with Dragendorff's reagent to visualise alkaloids as orange spots (Stahl, 1967). Similar fractions were pooled together and the resulting subfractions tested for their activity.

AChE activity determination

A microtitre plate assay was used on a 96-well microplate reader to determine AChE activity. The method described by Ellman *et al.*, (1961), was modified to measure the AChE activity. Briefly, the assay mixture constituted of 125 µl of 3 mM DTNB and 25 µl of 15 mM ATCI. The lyophilised enzyme was dissolved in buffer to obtain a 1130 U/ml stock solution. This stock solution was kept at –80 °C. 0.1% BSA in buffer was used for further enzyme dilutions. To dissolve DTNB, a buffer containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$ was used. ATCI was dissolved in "millipore" water.

1 mg of dry extract was dissolved in 100 µl of methanol. Samples not dissolving in methanol were brought into solution with DMSO. The final concentration of the organic solvent was always below 10 %. This was made to 1 ml with 50 mM Tris-HCl (pH 8.0) buffer and tested at the final concentration of 0.1 mg/ml.

The reagents were put into the microtitre plate wells for reading at the following volumes:

15 mM ATCI	25 µl
3 mM DTNB	125 µl
50 mM Tris/HCl pH 8	50 µl
Extract material (1 mg/ml)	25 µl

The extract was stirred to obtain a completely homogenous suspension. The reaction rate was measured at 405 nm for every 20 seconds. This was repeated 5 times to obtain background count.

Finally 25 μl of the enzyme (1/5000 AChE) was added, stirred and the activity determined at 405 nm every 20 seconds. This measurement was repeated 10 times allowing 3 seconds mixing prior to each measurement. The mixing time also helps to stabilise the photometer to new light conditions.

The reaction can presented as follows,

Acetylthiocholine $+H_2O \rightarrow$ acetate+ thiocholine (1)

Thiocholine+ dithiobisnitrobenzoate $\longleftrightarrow$ thiocholine-thionitrobenzoate disulphide +thionitrobenzoate (2)

The reaction rates were recorded with Biorad microplate reader model 3550 UV. The experiments were performed in duplicate.

4.4. Results and discussion

Background staining was reduced, by spraying the plates with a $NaNO_2$ solution.

We observed from the TLC plates that some fractions were likely to contain alkaloids because after staining with Dragendorff's reagent, some of them turned deep orange. The sensitivity of the method by Ellman, which is based

on the measurement of the rate of production of thiocholine when acetylcholine is hydrolyzed, makes it possible to analyse low concentrations of enzyme (Ellman *et al.*, 1961). With this method the kinetic study of AChE activity is also possible to be obtained.

Activity was determined by using the velocities of the reactions before and after adding the enzyme by a Microplate Manager software version 4.0. from Biorad laboratories. From the resulting CPC fractionation, the activity was maintained. The percentage of inhibition was calculated by comparing the velocities of the extracts to the velocity of the control (containing only the buffer). All the ethanolic bulb extracts tested showed inhibitory activity to acetylcholinesterase in the microtitre plate assay. This was shown to increase with the increasing number of fractionation steps. Results from *B. disticha* are shown in Figure 4.1. The same activity profile was observed in *S.multiflorus*.

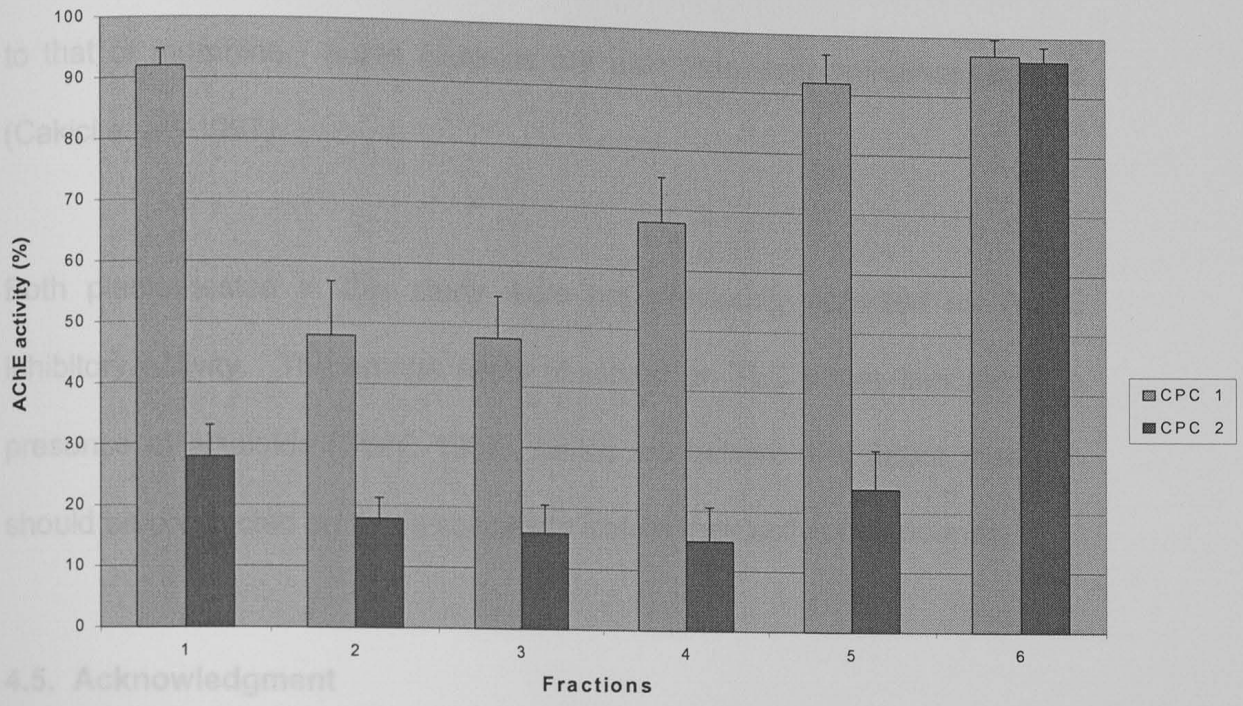

Fig 4.1. AChE activities of *Boophone disticha* subfractions of CPC1 and CPC 2 (where CPC2 is fractionation of fractions 2,3 &4)

Some of the alkaloids have been reported for their AChE inhibitory activity (Bastida *et al.*, 1987; Kihara *et al.*, 1995; Campbell *et al.*, 1998; Nordberg, & Svensson, 1998). Crude bulb extracts from *Narcissus* "Sir Winston Churchill" and "Carlton" gave high AChE inhibitory activity at 0.1 mg/ml (Ingkaninan, 1999).

Amaryllidaceae alkaloids are mainly found in their bulbs although some are also present in the aerial parts. Galanthamine (GAL) isolated mainly from *Narcissus* species has been used medicinally in Russia for various diseases (Harvey, 1995). GAL is also reported to exhibit analgesic activity comparable

to that of morphine. Some alkaloids are also promising anticancer agents (Cakici *et al.*, 1997).

Both plants tested in this study were not previously reported for AChE inhibitory activity. The orange spots observed on TLC plates indicated the presence of alkaloids (Stahl, 1967), hence we believe that future research should be conducted on these species to isolate their active compounds.

4.5. Acknowledgment

The authors acknowledge the National Research Foundation of South Africa for financial support. Special thanks to Wim Snoeijier for providing the plant material. E. Cloete and S. G. Cawe of the Botany Department, University of Transkei are also acknowledged for providing the bulbs of *Boophone disticha*.

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CHAPTER 5

ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF GALANTHAMINE ISOLATED FROM *SCADOXUS MULTIFLORUS*

*Acetylcholinesterase inhibitory activity of galanthamine isolated from *Scadoxus multiflorus*

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5.1. Abstract

Scadoxus multiflorus is used in traditional healing practises of South Africa, especially in wound treatment. A number of Amaryllidaceae species are rich in alkaloids. This group of compounds is known for a number of biological activities including acetylcholinesterase inhibitory activity. This activity is of interest for developing drugs for the treatment of Alzheimer's disease. The acetylcholinesterase inhibitory activity-guided fractionation of ethanolic extracts from the dried outer bulb scales of *S. multiflorus* led to the isolation of galanthamine. Centrifigal partition chromatography was mainly used as the fractionation tool. Galanthamine isolated from *S. multiflorus* showed acetylcholinesterase inhibitory activity at 0.1 mg/ml.

*Written in the format of the South African Journal of Botany

Keywords: *Scadoxus multiflorus*, galanthamine, acetylcholinesterase inhibitory activity.

5.2. Introduction

A number of South African Amaryllidaceae species have been used in traditional healing practices (Watt & Breyer-Brandwijk, 1962; Van Wyk *et al.*, 1997). The family is generally known to be rich in alkaloids. These have shown a wide range of activities (Campbell *et al.*, 1998). However, such alkaloids are deemed to be highly toxic (Watt & Breyer-Brandwijk, 1962; Bruneton, 1995). Bruneton (1995) stated that plants containing such alkaloids have limited medicinal value and their indiscriminate use is potentially lethal. *Scadoxus multiflorus* (Martyn) Raf. (Amaryllidaceae) is medicinally used in wound therapy, against colds and asthma as well (Watt & Breyer-Brandwijk, 1962, Van Wyk *et al.*, 1997).

Bulbs of *S. multiflorus* are regarded as highly poisonous and alkaloids have been isolated from the bulbs of the plant (Van Wyk *et al*, 1997). Galanthamine and other alkaloids like haemanthamine, haemultine and lycorine were isolated from the bulbs of *S. multiflorus* by Von Jaspersen-Schib in 1970 (Von Jaspersen-Schib, 1970; Hutchings *et al.*, 1996; Van Wyk *et al.*, 1997). Galanthamine, an alkaloid typically found in this family has recently been approved for the treatment of Alzheimer's disease (Bastida *et al.*, 1987, Selles *et al.*, 1997).

In this paper we report the acetylcholinesterase inhibitory effect of extracts of the bulbs of *S. multiflorus*. Bioassay-guided fractionation resulted in the isolation of galanthamine as the major active compound in the bulb.

5.3. Material and methods

Plant material

Bulb material of *S. multiflorus* were obtained from Wiljes & Zonnen BV, Hillegom, a bulb selling company in The Netherlands.

Chemicals

Acetylthiocholine iodide (ACTI), bovine serum albumin (BSA), 5,5'dithiobis-[2]-nitrobenzoic acid (DTNB) and acetylcholine esterase type VI-S from electric eel-lyophillised powder were obtained from Sigma (St Louis, MO, USA). All organic solvents were of analytical grade and purchased from J.T. Baker (Deventer, The Netherlands). The buffer used throughout the experiments was 50 mM Tris-HCl, pH 8.0. The acetylcholinesterase (AChE) source was electric eel lyophilised VI-S powder of 480 U/mg containing 530 protein units prepared to 1130 U/ml stock solution using buffer and were obtained from Sigma (St Louis, MO, USA). This was kept at –80 °C after the dilution to 1/5000 using 0.1% BSA. [DTNB (3 mM) was dissolved in buffer

containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_20$ and 15 mM ACTI dissolved in "millipore" water].

Extract preparation

The air dried macerated bulb scales were shaken in methanol for 48 hours, concentrated to dryness and dissolved in heptane/ethyl acetate/methanol/water 6/1/6/1(v/v/v/v). The resulting extract was then subjected to centrifugal partitioning chromatography.

Centrifugal Partitioning Chromatography (CPC)

The CPC experiments were performed using a modular Sanki (Kyoto, Japan), type LLN apparatus. This apparatus consists of a centrifuge (NMF model), a power supply (SPL model) and a constant flow pump (LBP-V). To monitor the chromatograms, a Panasonic Pen recorder (VP 67222A) was used. This was connected to the UV detector (IS 200, Linear Instruments, Reno, NV, USA). Fractions were collected using a LKB2211 Superrac fraction collector. The total internal volume was 125 ml and the pressure limited to 60 bar. Fractions were collected after every 4 min and the flow rate was 2 ml/min. A maximum of six partition catridges was used and the void volume was rejected in all experiments.

The first CPC separation step was done using the solvent system, heptane/ethyl acetate/methanol/water 6/1/6/1 (v/v/v/v) (Ingkaninan *et al.*, 1999). The extract was injected into the CPC system (maximum of 4 ml) and the first 15 fractions were eluted using ascending mode and then the mode was reversed to descending to collect the rest of the fractions.

The resulting fractions were still very complex and contained components with a wide polarity range. Therefore, a second CPC fractionation was performed with a different solvent system. A ternary diagram approach for solvent selection was used as described by Foucault and Menet & Rolet-Menet (Foucault, 1994; Menet & Rolet-Menet, 1999). Methanol was selected as the best solvent and the ethyl acetate and water proportions determined. The best results were obtained using ethyl acetate/methanol/water 5.5/1.5/3 (v/v/v). This was used as a solvent system at the second separation step.

TLC analysis

The different fractions obtained were checked for similarity using TLC on Silica gel F_{254} (Merck, Darmstad) with chloroform/methanol 9/1 (v/v) as an eluting solvent. Dried plates were observed and marked under UV light at 254 and 366 nm. These plates were then sprayed with Dragendorff's reagent for the visual detection of alkaloids (Stahl, 1967). Similar fractions from the TLC analysis were pooled together. The resultant fractions were tested for their acetylcholinesterase inhibitory activity.

Acetylcholinesterase inhibitory activity (AChE)

A microtitre plate assay was used to detect the AChE inhibitory activity. This was modified from the method by Ellman (Ellman *et al.*, 1961; Ingkaninan *et al.*, 1999). For the assay, the content of the well consisted of 125 µl of 3 mM DTNB, 25 µl of 15 mM ATCI, 50 µl of buffer and finally 25 µl of extract dissolved to 1mg/ml in buffer containing less than 10 % MeOH.

The main active fraction was further purified by preparative TLC (Silica gel $60F_{254}$) (Merck, Darmstad) with chloroform/methanol (9/1), (v/v). The distinct separated chromatographs were scraped and extracted in methanol. The resulting eluate was concentrated to dryness and tested for AChE activity. The most active fractions were analysed with nuclear magnetic resonance (nmr) for structure elucidation.

5.4. Results and discussion

The crude ethanolic extract from *S. multiflorus* showed an inhibitory activity on AChE. The AChE activity was observed to be increasing with the increasing number of fractionation steps. The alkaloid-containing fraction showed more activity and was hence subjected to further purification by preparative TLC. After the final purification step, only the alkaloid-containing subfraction showed activity.

The isolated alkaloid was analysed by nmr spectroscopy (^{1}H). The structure of the active compound was identified as galanthamine (Figure 5.1).

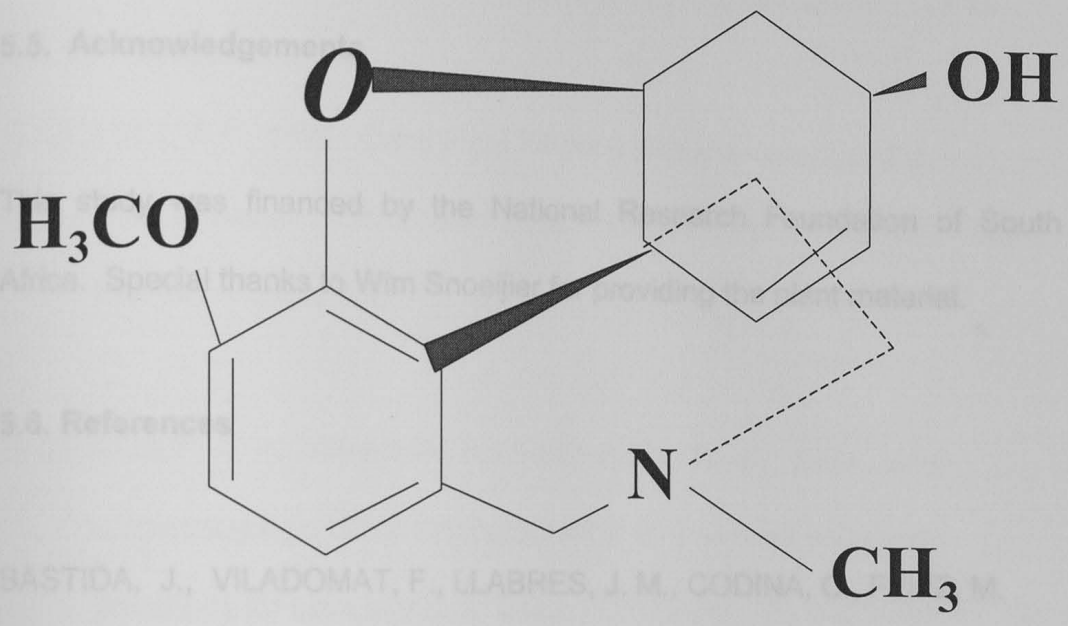

Figure 5.1. Galanthamine

The strong AChE activity found in this extract could thus be attributed to galanthamine isolated using bioassay-guided fractionation. This finding is in agreement with earlier reports on the occurrence of this compound in this plant. The high analgesic activity comparable to morphine of galanthamine could be the key factor in the verification of the reported traditional uses of this plant.

Ethanolic bulb extracts from *Narcissus tarzertta* showed inhibition of abdominal constrictions on mice (Caciki *et al.*, 1997) and AChE inhibitory activity (Hazenkamp *et al.*, 1999). Fractionation of *Narcissus confuss* also led

to the isolation of galanthamine, N-formylnorgalanthamine, haemanthamine and tazettine.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1. Introduction

Traditional healing practices and treatment of diseases using herbal remedies featured significantly in the health of millions of people for a long time (Martin, 1995). Among the sources of new drug development, plant based medicine is the most widely used one (Zhu, 1999). About 64 % of the world's population use plants as drugs, hence, the combined effect of such people make the consideration of plants as potential leads in drug development a viable choice. Furthermore, greater success has been achieved when plant selection was based on their traditional use (Farnsworth, 1990) (Farnsworth, 1990). Approximately, 80 % of South Africa's black population living in rural areas depend upon traditional medicine to treat their common ailments (Jager & Van Staden, 1995). According to Salie (1996), the indigenous people of the Western Cape use traditional medicines mainly from plants belonging to the Asteraceae family (Salie *et al.*, 1996). A number of *Helichrysum* species have been reported to have medicinal value (Watt & Breyer-Brandwijk, 1962; Tomas-Barberan *et al.* 1990; Meyer & Afolayan, 1995; Dilika, *et al.*, 1997; Mathekga & Meyer, 1998).

Some South African Amaryllidaceae species are also used in traditional healing practices (Watt & Breyer-Brandwijk, 1962). The plant family is known for its rich alkaloid content (Tanahashi *et al.*, 1990). This group of compounds has shown activity in a number of biological activities including

antitumour, antiviral, antimalarial and acetylcholinesterase inhibitory activity (Tanahashi *et al.*, 1990; Cakici, *et al.*, 1997; Campbell, *et al.*, 1998).

Tropical forests and other important areas of vegetation are rapidly disappearing. This and other factors make establishing the discovery methods for rapid isolation and identification of bioactive natural products an urgent need (Samuelsson, 1992; Hostettmann *et al.*, 1997). The destructive harvesting of bulbs used in traditional healing practices in South Africa (Mander, 1997), also poses a high threat as some might become extinct before the scientific basis of their use is investigated and perhaps verified.

6.2. Antibacterial activity of linoleic- and oleic acids isolated from *Helichrysum pedunculatum* used during circumcision rites

The crude extracts of *H. pedunculatum* were reported for their antibacterial activity in a previous study (Meyer & Dilika, 1996). The bioassay-guided fractionation using direct bioautography on TLC led to the isolation of linoleic- and oleic acids. The two acids were active against *Bacillus subtilis, Micrococcus kristinae* and *Staphylococcus aureus* with the MIC varying between 0.01 and 1.0 mg/ml. Linoleic acid additionally inhibited the growth of *B. cereus* and *B. pumilus.* None of the Gram-negative bacteria was inhibited by these fatty acids. Furthermore, the two acids showed a synergistic effect against *S. aureus*, a bacterial species commonly implicated in hospitalised circumcised patients.

6.3. Centrifugal partitioning chromatographic isolation of bioactive compounds from *Helichrysum pedunculatum* (Asteraceae) and *Boophone disticha* and *Scadoxus multiflorus* (Amaryllidacae) using receptor binding assays

H. pedunculatum leaf extract was found to be active in both adenosine and opiate receptor binding assays. Centrifugal partitioning chromatography was used to fractionate the ethanolic leaf extract of *H. pedunculatum*. Bioasay-guided fractionation on adenosine A_1 and opiate receptor binding assays resulted in the isolation of linoleic acid. Fractions containing linoleic acid were eliminated from the first stages of separation as this compound is regarded as a false positive (non-competitive inhibitor) in this assay (Ingkaninan *et al.*, 1999). This makes the identification of active compounds from such extracts difficult (Zhu *et al.*, 1996; Zhu *& Li*, 1999). This might be due to the interactive mechanisms between plant constituents found in the crude extract. The presence of this compound might however validate the use of the herb in pain relief and as an antibiotic.

6.4. Preliminary investigation of *Boophone disticha* and *Scadoxus multiflorus* for acetylcholinesterase inhibitory activity

Age related diseases, for example Alzheimer's disease (AD), are gradually becoming a problem especially in the western world as the population continues to grow older. Acetylcholinesterase inhibition seems to show

symptomatic improvement in some clinical trials in the treatment of Alzheimer's disease (Enz *et al.*, 1993; Nordberg & Svensson, 1998). Ethanolic extracts from bulb material of *Boophane disticha* and *Scadoxus multiflorus* showed an inhibitory effect on the AChE enzyme assay at concentrations of 0.1 mg/ml.

6.5. Acetylcholinesterase inhibitory activity of galanthamine isolated from *Scadoxus multiflorus*

The genus *Narcissus* is known for the possession of compounds with AChE inhibitory activity (Nordberg & Svensson, 1998). This is mainly due to the presence of galanthamine (GAL), an alkaloid already undergoing clinical trials for the treatment of Alzheimer's disease. GAL has been isolated from a number of species in this genus.

Ethanolic extracts of *S. multiflorus* were further purified as it showed more activity compared to the other tested bulb extracts. Bioassay-guided fractionation using the AChE inhibitory activity method of Ellman. (1961), led to the isolation of galanthamine (Ellman *et al.*, 1961). This compound might also account for the pain killing effect of the bulb material as used during the traditional circumcision ritual.

In conclusion, the use of plants as dressings in the traditional male circumcision is not a coincidence. The indications of antibacterial activity and

pain management of the herbs analysed in this study have showed this. However, precautions should be taken into account during the ritual as is the case in any surgery. There is also a need for regulating the cultural practice to ensure continuity and sustainable utilization of the natural resources.

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CHAPTER 7

SUMMARY

THE MEDICINAL VALUE OF AMARYLIDACEAE AND ASTERACEAE SPECIES USED IN MALE CIRCUMCISION

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The indigenous people of South Africa still practise their cultural traditions widely. Traditional male circumcision is a common ritual and mainly performed in the wild. *Helichrysum pedunculatum (*Asteraceae) is the most commonly used herb in wound dressing during these rituals. Scales of *Boophone disticha* and *Scadoxus multiflorus* (Amaryllidaceae) are also applied as a dressing in both fresh and dry forms, to wounds, depending on community.

Dry leaf extracts of *H. pedunculatum* showed antibacterial activity against the Gram-positive bacterial species. *Staphylococcus aureus,* the most common cause of bacterial infection in male circumcised patients, was also inhibited. Antibacterial activity-guided fractionation of the dichloromethane leaf extract led to the isolation of linoleic and oleic acids. These two acids were active on Gram-positive bacterial species.

The advancement of high throughput screening has overcome the barrier of large numbers of fractions to be tested in assays. Crude *H. pedunculatum* ethanolic extract was subjected to CPC fractionation and the resulting fractions were tested on adenosine A_1 and opiate receptor binding assays. All crude extracts showed activity. The receptor binding-guided fractionation of led to the isolation of linoleic acid. This compound is however regarded as false positive in this assay.

The Amaryllidaceae species used during circumcision rituals were investigated using the acetylcholinesterase inhibitory enzyme assay. It was found that *B. disticha* and *S. multiflorus* showed the same activity profile at a concentration of 0.1 mg/ml. The bioassay-guided fractionation led to the isolation of galanthamine from *S. multiflorus,* a compound already undergoing clinical trials for the treatment of Alzheimer's disease.

Finally, with the right resources and effort, plant based drugs can be the way forward in our search for new leads in drug discovery. The herbs used in

traditional male circumcision in South Africa might also yield good results in pain management investigations.

CHAPTER 8

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