CHAPTER 1

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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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