CHAPTER 3

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

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

F. Dilika[1], K. Ingkaninan[2], J.J.M. Meyer[1] & R. Verpoorte[2]

[1]Botany Department, University of Pretoria, Pretoria, 0002, South Africa

[2]Leiden/Amsterdam Center for Drug Research, Pharmacognosy Division, Box 9502, 2300 RA, Leiden, The Netherlands

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.

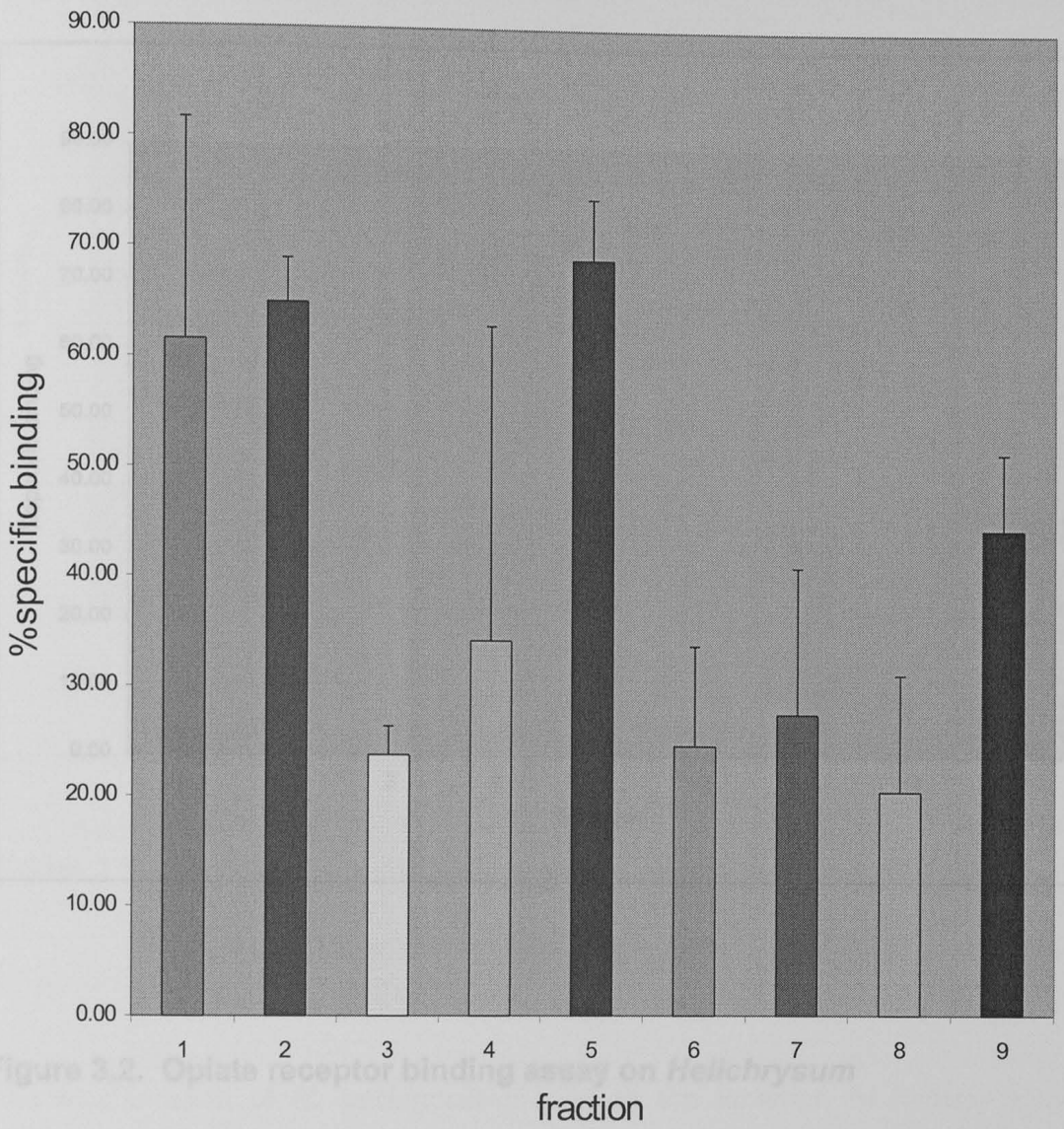

Figure 3.1. Adenosine receptor binding assay on *Helichrysum pedunculatum* leaf extract after the first CPC fractionation.

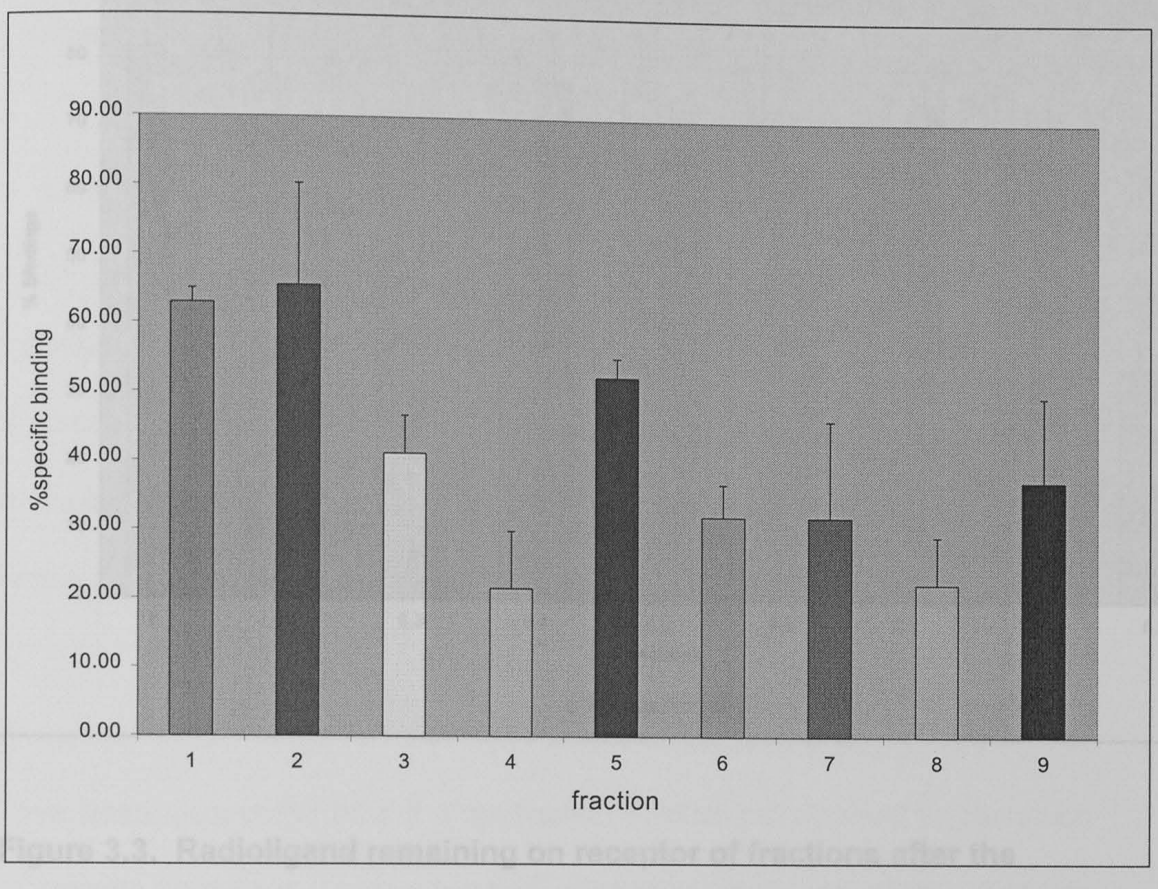

Figure 3.2. Opiate receptor binding assay on *Helichrysum pedunculatum* leaf extract after the first CPC fractionation

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.

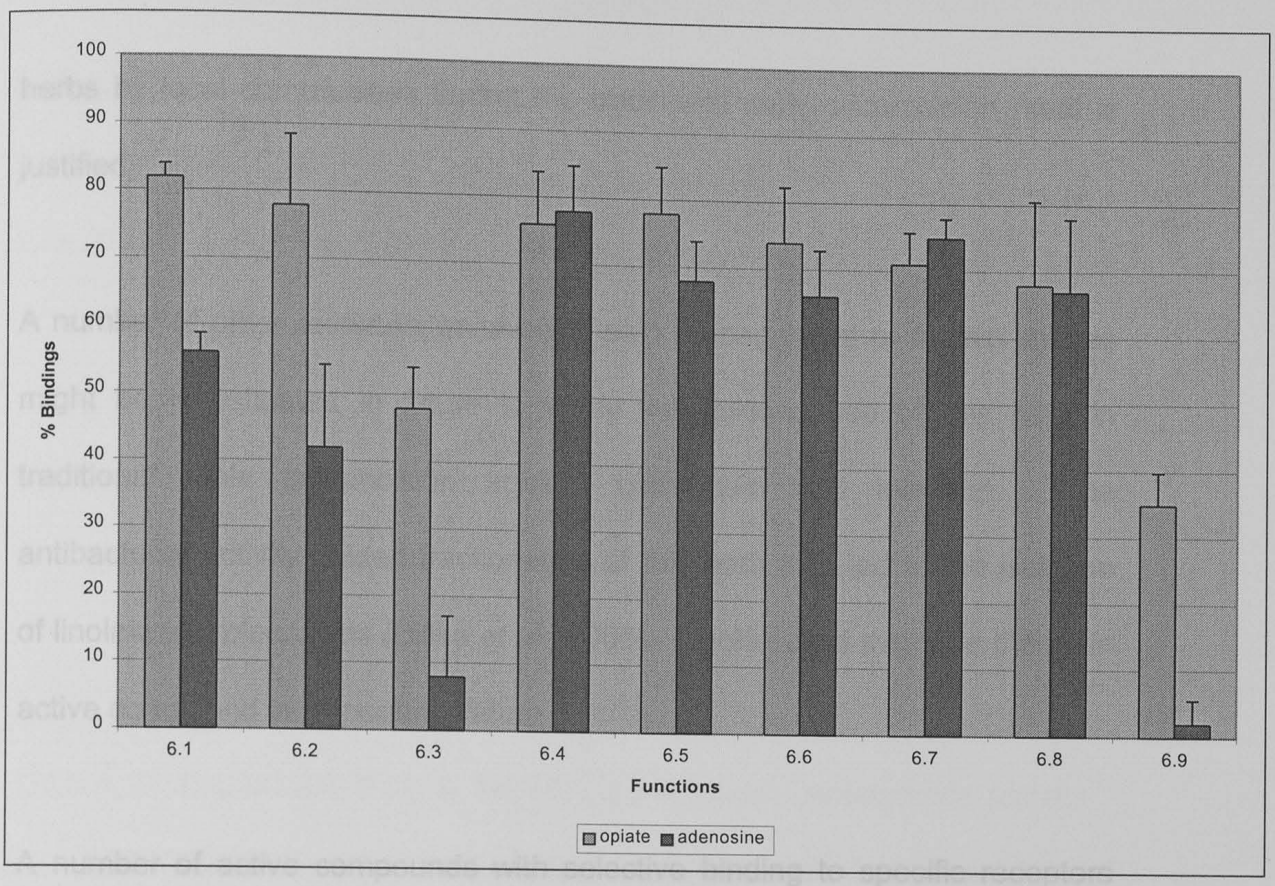

Figure 3.3. Radioligand remaining on receptor of fractions after the second CPC fractionation of fraction 6.

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*.

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