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.

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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*.

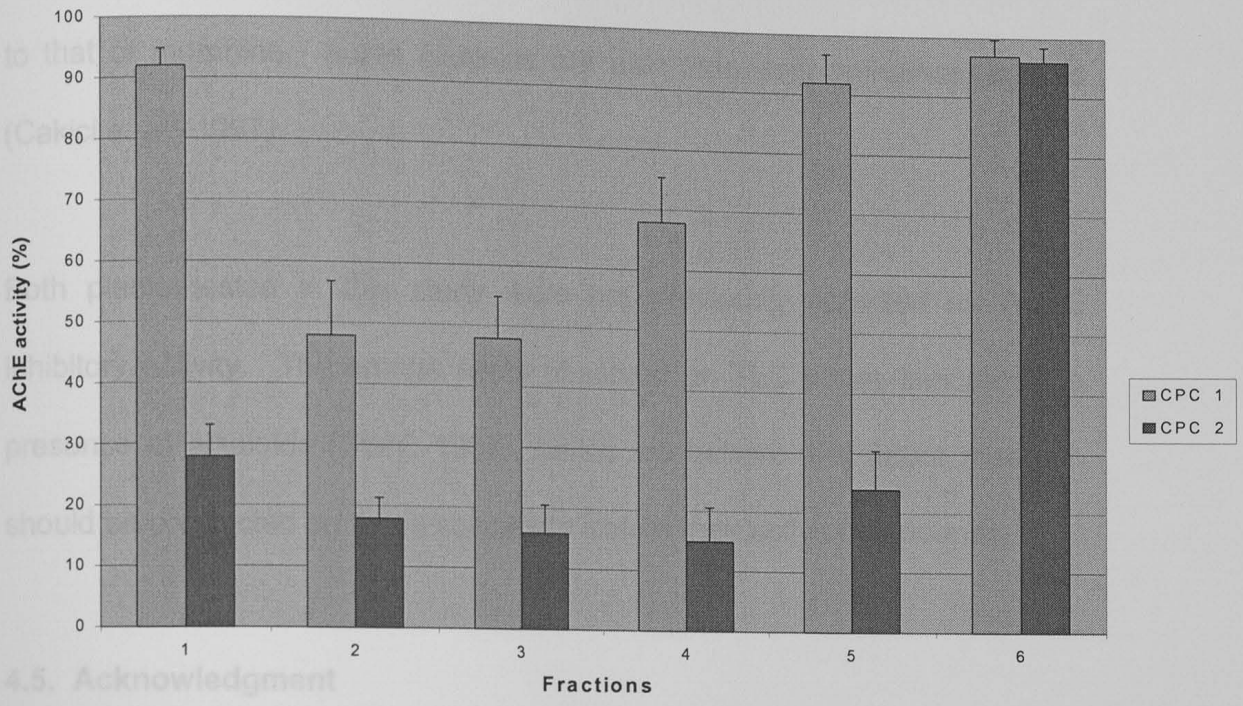

Fig 4.1. AChE activities of *Boophone disticha* subfractions of CPC1 and CPC 2 (where CPC2 is fractionation of fractions 2,3 &4)

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

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4.6. References

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