

## CHAPTER 4: DISCUSSION

### 4.1 Acetylcholinesterase inhibitory and antioxidant activity

The inhibition of the enzyme, AChE and scavenging of free radicals or ROS are very important in identifying new agents or potential leads which may be useful in the treatment of AD. The dichloromethane/methanol (1:1) and ethyl acetate extracts were observed to have higher inhibition of AChE than the methanol and water extracts. This may indicate that the plants investigated in the present study contain AChE inhibitory compounds with intermediate polarity. Similar findings have been reported in literature where authors screened several extracts of different plants for AChE inhibition, and plants extracted with solvents of intermediate polarity had higher activity (Şenol et al., 2010a, b; Noridayu et al., 2011). However, in contrast to the AChE inhibitory activity, the ethyl acetate extracts showed poor radical scavenging activity, while the methanol extracts contained good antioxidant activity. A variety of bioactive compounds that could be responsible for the observed bioactivities have been reported in some of the screened medicinal plants or related genera.

Good inhibition of AChE was observed for the organic extracts of *S. tiliifolia* in the present study. It showed a 54.2% inhibition of the enzyme at the highest concentration tested, and an  $IC_{50}$  value of 1 mg/ml. Numerous species of the genus *Salvia* have been used since ancient times in folk medicine and have been subjected to extensive research intended to identify biologically active compounds. Systematic and mechanistic studies into the effects of these extracts have revealed multiple activities potentially relevant to brain function, aging and the preventive and symptomatic treatment of mild cognitive impairment and AD (Loizzo et al., 2010). The *n*-hexane extract of *S. leriifolia* has been reported to show good inhibition of AChE with an  $IC_{50}$  value of

0.59 mg/ml. This activity is attributed to the presence of monoterpenes, including sabinene,  $\delta$ -3-carene and  $\alpha$ -terpinene, different sesquiterpenes and three diterpenes (neophytadiene, phytol and vulgarol B). The essential oil and ethanol extract of *S. officinalis* as well as the essential oil of *S. lavandulaefolia* have been shown to possess anticholinesterase activity (Perry et al., 1996), as have the major components of the essential oil,  $\alpha$ -pinene, 1, 8-cineole, and camphor (Perry et al., 2000). The AChE inhibitory activity of some of the terpenes present in *Salvia* species, have been reported.  $\delta$ -3-Carene is reported to be a potent inhibitor of AChE with an  $IC_{50}$  value of 200  $\mu$ M, sabinene inhibited AChE with an  $IC_{50}$  value of 176.5  $\mu$ g/ml, while  $\alpha$ -terpinene showed inhibition with  $IC_{50}$  value of 1000  $\mu$ M (Miyazawa et al., 1997; Herholz et al., 2005; Miyazawa and Yamafuji 2005). Similar compounds may be present and responsible for the good AChE inhibition observed with the DCM/MeOH (1:1) extracts of *S. tiliifolia*.

*S. brachypetala* roots showed dose-dependent inhibition of AChE with a 62.8% inhibition of the enzyme for the organic extracts at the highest concentration tested. The organic root extracts also contained good antioxidant activity. Its neurological activity is supported by Stafford et al. (2007), who reported good monoamine oxidase (MAO) B inhibitory activity in the aqueous and ethanol extracts of the bark of this plant species. *S. brachypetala* contains stilbenes and phenolics which have been shown to have good radical scavenging activity (Glasby, 1991).

The family Caesalpiniaceae has been shown to contain several diterpenes with biological activity. The clerodane diterpenes present in fruit pulp extract of *Detarium microcarpum* Guill. & Perr. showed antifungal activity and inhibition of acetylcholinesterase (Cavin et al., 2006). The presence of clerodane or similar diterpenes in *C. mimosoides* may be responsible for the good AChE inhibitory activity seen for the organic root extracts. Several plants in the family Caesalpiniaceae, including *Caesalpinia bonducella*, *Cassia auriculata*, *C. fistula*, *Bauhinia*

*racemosa* and *B. rufescens* have also been reported to contain good antioxidant activity (Kumar et al., 2005; Kumar et al., 2008; Motlhanka, 2008; Aliyu et al., 2009; Shukla et al., 2009), which supports the present finding for the organic root extracts of *C. mimosoides*.

The genus *Buddleja* has been reported to contain various terpenoids; monoterpenes, sesquiterpenes, diterpenes and triterpenoids which have been reported to show good inhibition of AChE (Houghton et al., 2003; Loizzo et al., 2010). Some of the sesquiterpenes have also been shown to contain anti-inflammatory activity, which make it relevant in the treatment of AD (Liao et al., 1999). Various species of *Buddleja* have been found to contain luteolin, and its glycosides have been shown to contain good antioxidant and anti-inflammatory activity (López-Lázaro, 2009). It is therefore postulated that the presence of these and related compounds in *B. salviifolia* may be responsible for the antioxidant and AChEI activity shown in this study.

*Zanthoxylum davyi* roots showed good AChE inhibitory activity. Seven benzo[c]phenanthridine alkaloids have been isolated from the stem-bark of *Z. davyi* (Tarus et al., 2006), and these or similar alkaloids may be responsible for its observed inhibition of acetylcholinesterase. In addition, anticonvulsant activity has been reported for both the methanol and aqueous leaf extracts of *Z. capense* (Amabeoku and Kinyua, 2010). As convulsion is a neurologic disorder, similar compounds present in the roots of *Z. davyi* may be responsible for its activity and this supports the traditional use of the plant in the treatment of neurologic diseases. *Z. capense* leaves have also been reported to contain triterpene steroids and saponins and these compounds are known to exhibit neuroprotective activity (Chauhan et al., 1988). Organic extracts of *Z. alatum* fruits was observed to show good antioxidant activity which was reported to be due to the presence of phenolic compounds (Batoool et al., 2010). The present study showed that the roots of

*Z. davyi* contain a high level of total phenols and this may be responsible for its good antioxidant capacity.

The ethyl acetate extracts of *C. bulbispermum* bulbs showed a low  $IC_{50}$  value for inhibition of AChE, which may be ascribed to several alkaloids which have been isolated from the plant (Elgorashi et al., 2004). In addition, alkaloidal extracts from *Crinum jagus* and *C. glaucum* have been demonstrated to possess AChE inhibitory activity which has been ascribed to hamayne ( $IC_{50}$  - 250 $\mu$ M) and lycorine ( $IC_{50}$  - 450 $\mu$ M) (Houghton et al., 2004). Furthermore, the alkaloids; haemanthamine and lycorine, isolated from *C. ornatum*, have been shown to contain anticonvulsant activity (Oloyede et al., 2010). It is possible that the presence of these or similar alkaloids may be responsible for the activity observed. *Crinum ornatum* bulbs have been shown to contain good inhibition of DPPH radicals and hydrogen peroxide as well as being able to inhibit peroxidation of tissue lipids in the malonaldehyde test (Oloyede and Farombi, 2010). Similar to the AChE inhibitory activity, lycorine and haemanthamine have been reported to be responsible for the antioxidant activity (Oloyede et al., 2010).

Amide alkaloids with activity in the CNS have been identified from the roots of *P. guineense* (Gomes et al., 2009). *P. methysticum* has been reported to possess local anaesthetic, sedating, anticonvulsive, muscle-relaxant and sleep-stimulating effects which is due to the presence of kavopyrones (Gomes et al., 2009). *P. capense* contains the amide alkaloids; piperine and 4,5 – dihydropiperine, which have previously been shown to have CNS activity (Pedersen et al., 2009). Also, piperine has been reported to improve memory impairment and neurodegeneration in the hippocampus of animal models with AD (Chonpathompikunlert et al., 2010). The good antioxidant activity observed for *P. capense* in the present study has also been reported for other *Piper* species; *P. arboreum* and *P. tuberculatum* (Regasini et al., 2008). This activity has been

ascribed to the flavonols, quercetin and quercitrin (Williamson and Manach, 2005). In addition, *P. betle* has been reported to contain significant antioxidant activity *in vitro* and to elevate antioxidant status in animals after oral administration of the extracts (Dasgupta and De, 2004; Choudhary and Kale, 2002). Also, five phenolic amides have been isolated from *P. nigrum*, all of which contain antioxidant activity, which has been shown to be higher than alpha-tocopherol, a naturally occurring antioxidant (Nakatani et al., 1986). These findings support the AChE inhibitory and antioxidant activity observed in the roots of *P. capense*.

The present study showed the ethyl acetate extract of the roots of *X. undulatum* to have good inhibition of AChE. The ethanol extracts of the plant were found to exhibit antidepressant-like effects in three animal models (Pedersen et al., 2008). The leaves of this plant have also been reported to have selective serotonin re-uptake inhibitory activity (Nielsen et al., 2004). The neuroprotective effect of the plant has been ascribed to several glycosides (Hutchings et al., 1996), which may be responsible for the AChE inhibitory activity observed with its ethyl acetate extracts. Four pregnane glycosides; cynatroside A, cynatroside B, cynatroside C and cynascyroside D, have been isolated from *Cynanchum atratum*. These glycosides showed AChE inhibition with IC<sub>50</sub> values of 3.6 µM for cynatroside B and 152.9 µM for cynascyroside D (Lee et al., 2003; Lee et al., 2005).

Several Anacardiaceae species including *Lannea velutina*, *Sclerocarya birrea* and *Harpephyllum caffrum* have been shown to be a source of natural antioxidants. This activity has been ascribed to the high levels of proanthocyanidins and gallotannins present in the plants (Maiga et al., 2007). As *Lannea schweinfurthii*, belongs to the same family, similar compounds could be present and therefore responsible for its observed antioxidant activity.

Sericoside, the triterpenoidal saponin found in *T. sericea* has been reported to have anti-inflammatory and antioxidant activity (Mochizuki and Hasegawa, 2007). Sericoside acts by reducing neutrophil infiltration and decreasing superoxide generation due to its radical scavenging activity (Mochizuki and Hasegawa, 2007) and it may be responsible for the antioxidant activity of the plant as observed in this study.

The ethyl acetate extract of the bulb of *B. disticha* showed AChE inhibition with a low IC<sub>50</sub> value. Its observed neurological activity is supported by Pedersen et al. (2008), where the authors reported that the ethanol extract of the plant showed affinity for the serotonin transporter in the binding assay, and inhibited the serotonin, noradrenaline and dopamine transporters. This activity has been attributed to several alkaloids including buphanamine, buphanidine and distichamine, which have all been reported to have affinity for the serotonin transporter (Neergaard et al., 2009). The neurologic activity of these alkaloids has been suggested to be due to the presence of the 1,3-dioxole moiety (Sandager et al., 2005), and some of these alkaloids may be responsible for the AChE inhibitory activity of *B. disticha*.

The levels of total phenols in the roots of *S. brachypetala*, *P. capense*, *C. bulbispermum*, and bark of *S. brachypetala* were observed to be relatively high. Plants with high levels of phenols have been shown to exhibit high antioxidant activity (Hakkim et al., 2007). Most of the antioxidant potential of medicinal plants is ascribed to the redox properties of phenols, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers (Hakkim et al., 2007). Flavonoids have also been reported to be responsible for antioxidant activity, as they act on enzymes and pathways involved in anti-inflammatory processes (Araújo et al., 2008). In addition, the hydrogen-donating substituents (hydroxyl groups) attached to the aromatic ring structures of flavonoids enable them to undergo a redox reaction, which in turn, help them

scavenge free radicals (Brand-Williams et al., 1995). These phenolic compounds contribute to the antioxidant activity of the plants as was observed in this study.

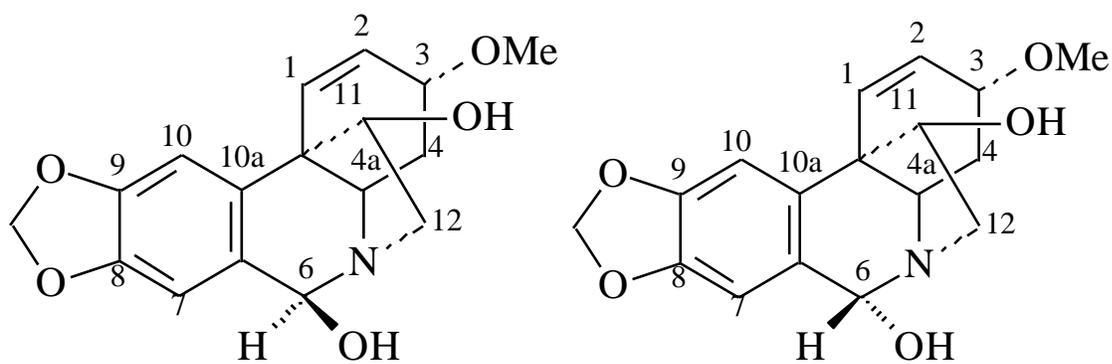
## **4.2 Isolation and structural elucidation of compounds from *Boophane disticha***

Nuclear Magnetic Resonance (NMR) spectroscopy and Quadrupole Time-of-Flight (QTOF) are the techniques most often used in the structural elucidation of compounds. NMR exploits the magnetic properties of nuclei present in the atoms which results in a characteristic spectrum and the information obtained is useful in determining the structure of compounds isolated. Various 1D and 2D experiments were used (such as proton -  $^1\text{H}$ , carbon -  $^{13}\text{C}$ , Heteronuclear Multiple Bond Correlation - HMBC, Heteronuclear Single Quantum Coherence - HSQC, Correlation Spectroscopy - COSY and Distortionless Enhancement by Polarisation Transfer - DEPT) to determine the proton to carbon relation and the chemical environment they are in for structure elucidation (Silverstein et al., 2005). High resolution Time-of-Flight Mass Spectroscopy (HRTOFMS) can be used to confirm the molecular mass of the compound and together with QTOF, it provides information of the accurate mass of the compounds under investigation and the resulting fragmentation pattern obtained is used in conjunction with NMR data for structure elucidation. QTOF is a technique used to determine the mass of the compound under investigation using the principle that smaller (lighter) ions will travel faster through a flight tube than larger (heavier) ions. The velocity of an ion depends on its mass-to-charge ratio. The velocity that an ion obtains in the TOF analyser is therefore used to determine the mass of the ion and is a more accurate estimate (Silverstein et al., 2005; Pavia et al., 2009).

#### 4.2.1 Compound 1 (6 – hydroxycrinamine)

The signals obtained from the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were complex (Appendix E; Figures 1 and 2), suggesting that compound 1 was a mixture of two epimers; epimer A and epimer B (Figure 3.6). The data obtained from the integration of the  $^1\text{H}$  spectra confirmed the compound to be a 3:1 mixture of two epimers, with epimer A, 6 $\alpha$ -hydroxycrinamine as the major epimer. This was different from the same mixture of epimers isolated from *Brunsvigia orientalis*, which was isolated as a 2:1 mixture of two epimers A/B (Viladomat et al., 1996). These epimers are difficult to separate and a 3:1 mixture of the epimers A and B have also been isolated from *Crinum zeylanicum* (Tsuda et al., 1984). However, we observed from our extensive literature search that 6 $\alpha$ -hydroxycrinamine and its epimer have not been isolated from any species of *Boophane* and it appears that it is the first time the compound is isolated from *B. disticha*. Epimer A was the major epimer and the epimeric difference is found at the benzylic position (C-6). Epimer A shows H-6 in the  $\alpha$ - position and hydroxyl group in the  $\beta$ - position while epimer B shows H-6 in the  $\beta$ - position and hydroxyl group in the  $\alpha$ - position. In both epimers, the small coupling constant between H-2 and H-3, the large one between H-4 $\alpha$  and H-3, and the NOE contour correlation between H-3 and H-4a, shows that there is a *cis*-relationship between the C-3 pseudoequatorial substituent and the 5, 10b-ethanobridge. The 5, 10b-ethanobridge which occurs in the  $\alpha$ - position shows that 6-hydroxycrinamine is a crinine-type alkaloid. A C-11 hydroxyl substituent is observed at the *exo*-position and this is due to the de-shielding effect on H-11, the NOE effect between H-10 and H-11 and the long range W-coupling between H-11 and H-4a. Epimer A was observed to have a benzylic proton H-6 $\alpha$  as a singlet at  $\delta$  5.01 and spatial proximity between H-6 and H-12 *endo* was observed. In epimer B, the proton H-6 $\beta$  was observed at a lower field, as a singlet at  $\delta$  5.60, and a NOE contour correlation between H-6 $\beta$  and

H-4a was seen, and this confirms the hydroxyl group assigned at C-6. In both epimers, C-9 was assigned at lower fields than C-8 because of its three-bond correlation with the methane proton H-7. The quaternary carbons C-6a and C-10a were ascribed by means of their correlations with the methine protons H-10 and H-7, respectively. Also, the singlets at  $\delta$  51.1 and 50.7 were assigned to C-10b of both epimers, considering their three-bond connectivity with H-2, H-4 and H-10.



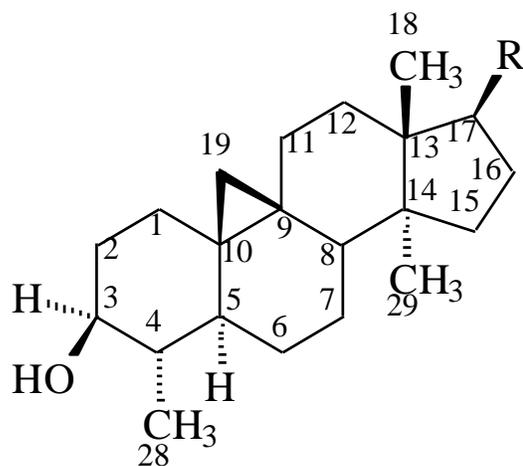
Epimer A (6 $\alpha$ -hydroxycrinamine) (**3.1**)    Epimer B (6 $\beta$ -hydroxycrinamine) (**3.2**)

**Figure 3.6** Structure of 6-hydroxycrinamine showing its two epimers.

#### 4.2.2 Compound 3 (cycloeucaenol)

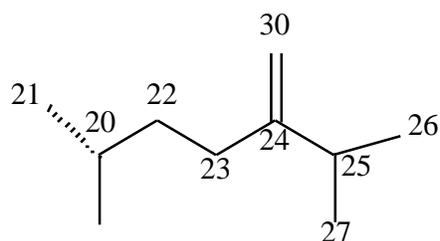
The  $^1\text{H-NMR}$  spectrum of cycloeucaenol (Appendix E; Figure 9), revealed the presence of two exomethylene or olefinic protons which appeared as broad singlets at  $\delta_{\text{H}}$  4.70 and  $\delta_{\text{H}}$  4.64, one methine proton which was seen as a multiplet next to the hydroxyl group at  $\delta_{\text{H}}$  3.19 and six methyl protons. The methyl protons include: Me-26 and 27 appeared as doublets ( $\delta_{\text{H}}$  1.02 and 1.00,  $J = 6.6$  Hz), Me-29 and Me-21 as broad singlets ( $\delta_{\text{H}}$  0.97 and 0.94), while Me-18 and Me-28 appeared as singlets ( $\delta_{\text{H}}$  0.95 and 0.86). Also, two methylene protons of a cyclopropyl group which appeared as broad singlets ( $\delta_{\text{H}}$  0.12 and 0.37) were observed in the  $^1\text{H-NMR}$  spectrum. The  $^{13}\text{C-NMR}$  spectrum (Appendix E; Figure 10) showed 30 carbon signals including a double bond between C-24 and C-30. C-24 is an olefinic quaternary carbon at  $\delta_{\text{C}}$  157.2 while C-30 is an exomethylene carbon at  $\delta_{\text{C}}$  106.2. It also shows a methylene carbon (C-19) of cyclopropyl ( $\delta_{\text{C}}$  27.5), a methylene carbon (C-3) next to the hydroxyl group ( $\delta_{\text{C}}$  76.8), and six methyl signals ( $\delta_{\text{C}}$  22.2, 22.1, 19.4, 18.0, 18.6 and 14.6). The NMR data was compared to that of the published data on cycloeucaenol (Liu et al., 2011).

The  $^1\text{H-NMR}$  spectrum of the stereo-isomer is very similar to that of cycloeucaenol. The only difference observed is in the side chain as the position of the double bond differs from that of cycloeucaenol. The methyl protons of the stereo-isomer; Me-27, Me-28 and Me-21 appeared as broad singlets ( $\delta_{\text{H}}$  4.64, 0.95 and 0.86), Me-26 appeared as a multiplet ( $\delta_{\text{H}}$  1.64), while Me-29 was observed to appear as a singlet ( $\delta_{\text{H}}$  0.88). A hexet was observed at  $\delta_{\text{H}}$  2.22 ( $J = 7.0$  Hz), while an olefinic proton which appeared as a doublet was observed at  $\delta_{\text{H}}$  1.00 ( $J = 6.6$  Hz). These  $^1\text{H-NMR}$  data compares with data reported by Akihisa et al. (1997). The  $^{13}\text{C-NMR}$  spectrum of the stereo-isomer also compares well with cycloeucaenol from C-1 to C-21.

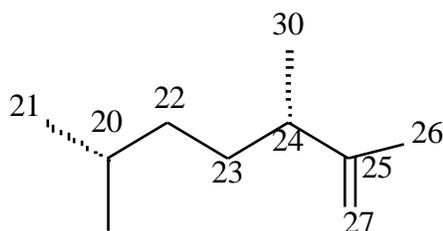


3.3

Side chain (R)



3.3a



3.3b

Figure 3.8 Structure of cycloecalenol with its stereo-isomer.

The only difference observed is in the side chain from C-22, because of the change in position of the double bond in the stereo-isomer, which appears between C-25 and C-27. C-25 is an olefinic quaternary carbon at  $\delta_C$  150.5 while C-27 is an exomethylene carbon at  $\delta_C$  109.6. The other differences are found at C-22, C-24, C-26 and C-30 which appear at  $\delta_C$  34.0, 41.8, 18.9 and 22.1. The data obtained for the  $^{13}\text{C}$ -NMR spectrum of the stereo-isomer on the side-chain, also compares with data reported by Akihisa et al. (1997).

Cycloeucaleanol and its stereoisomer are cycloartane triterpenes, and it appears that they are isolated for the first time from *B. disticha*. Literature searches conducted on this plant, did not reveal any published information for triterpenes isolated from *B. disticha*. Integration of the  $^1\text{H}$ -NMR spectra showed that cycloeucaleanol was slightly more abundant than the stereo-isomer, as they were both present in a ratio of 1.04:1 from integration of signals in the  $^1\text{H}$ -NMR spectra.

#### **4.2.3 Acetylcholinesterase inhibitory activity of isolated compound and fractions**

6-Hydroxycrinamine had an  $\text{IC}_{50}$  value of  $445 \pm 30 \mu\text{M}$  for inhibition of AChE (Table 3.8). The results obtained in the present study are similar to that obtained by Elgorashi et al. (2004), who isolated a similar compound (a 2:1 mixture of two epimers) with an  $\text{IC}_{50}$  value of  $490 \pm 7 \mu\text{M}$ . Concentrations of AChE inhibitory activity of  $\leq 500 \mu\text{M}$  are considered active (Wink, 2000), and according to this criterion, the alkaloid can be said to inhibit AChE activity. However, the high  $\text{IC}_{50}$  value indicates that 6-hydroxycrinamine has a weak activity. As mentioned in section 4.2.1, the compound is a crinine-type alkaloid. Several other crinine-type alkaloids have been isolated from other plants in the Amaryllidaceae family; crinine, epibuphanisine, crinamidine and epivittatine have been isolated from *Crinum moorei* (Elgorashi et al., 2001a); hamayne from *C. macowanii* (Elgorashi et al., 2001b); 3-O-acetylhamayne and crinamine from *C. bulbispermum*.

Each of these alkaloids were screened for inhibition of AChE and they showed weak inhibition with  $IC_{50}$  values of  $461 \pm 14 \mu\text{M}$ ,  $547 \pm 5 \mu\text{M}$ ,  $300 \pm 27 \mu\text{M}$ ,  $553 \pm 3 \mu\text{M}$ ,  $594 \pm 8 \mu\text{M}$ ,  $239 \pm 9 \mu\text{M}$  and  $697 \pm 12 \mu\text{M}$  for crinine, epibuphanisine, crinamidine, hamayne, 3-O-acetylhamayne, epivittatine and crinamine, respectively (Elgorashi et al., 2004). However, further studies on the structure activity relationship could be carried out on 6-hydroxycrinamine and other crinine-type alkaloids to modify their structures, with the aim of developing more potent AChE inhibitors.

EAM 17-21 21,22 a fraction obtained from the methanol extract had a significant AChE inhibitory activity with a low  $IC_{50}$  value. It showed good inhibition of the enzyme with a percentage inhibition of 67.96% and 54.31% at 250  $\mu\text{g/ml}$  and 125  $\mu\text{g/ml}$ , respectively. Its NMR spectra (Appendix E; Figure 16), indicates it is a mixture of alkaloids and it showed a positive result when sprayed with Dragendorff's reagent. Buphanidrine and buphanamine, isolated from *B. disticha* have been tested for their affinity to the serotonin transporter in the rat brain (Sandager et al., 2005). Both alkaloids showed slight affinity which indicates their potential in the development of neuroprotective agents. It is possible that these or similar alkaloids are present and responsible for the AChE inhibitory activity observed in fraction EAM 17-21 21,22.

Fraction EAE 11 obtained from the ethyl acetate extract also showed inhibition of AChE as observed by its low  $IC_{50}$  value. A dose-dependent inhibition of the enzyme was observed with a 54.8% inhibition at 250  $\mu\text{g/ml}$ . The NMR spectra and chromatogram of EAE 11 (Appendix E; Figures 17-19), shows that it contains three major compounds which include cycloartane triterpenes but not similar to cycloeucalenol and its stereoisomer. Several terpenoids including ursolic acid from *Origanum majorana* and argentatin A from *Parthenium argentatum* have been reported to exhibit AChE inhibitory activity with  $IC_{50}$  values of 7.5 nM and 42.8  $\mu\text{M}$ , respectively. Tashinones, a group of diterpenes, have also been shown to contain AChE

inhibitory activity, with dihydrotanshinone and cryptotanshinone reported to have IC<sub>50</sub> values of 1.0 µM and 7.0 µM for inhibition of AChE (Ren et al., 2004). These or similar compounds may be responsible for the activity shown by fraction EAE 11.

Cycloeucalenol did not show any activity for inhibition of AChE. However, it has been reported to show anti-inflammatory, cardiotonic and spasmolytic effects (Kongkathip et al., 2002), which also indicates that it could be studied further as a potential lead in developing drugs useful in treating inflammation and with cardioprotective properties.

### **4.3 Cytotoxicity studies**

The continuous use and the growing demand for herbal therapies have invigorated the quest for validating the efficacy and safety or toxic implications of medicinal plants. This is very important, as it helps in developing safe and cheap alternative medicines.

One of the fundamental *in vitro* toxicological assays performed is the direct assessment of the effects of a plant extract or compound on the viability of a human cell line. Data obtained in these assays are very useful in selecting the most promising candidate for further drug development and data for future pre-clinical studies (Cos et al., 2006). Numerous assays have been employed for the determination of the toxic cellular effects of xenobiotics on cells, assessing functions of cellular physiology such as membrane integrity, mitochondrial function or protein synthesis as a surrogate for cell viability (Weyermann et al., 2005). While this approach to determine cell viability has been shown to be accurate and reproducible, each assay has been associated with certain limitations. In order to overcome these limitations and improve the reliability of the *in vitro* data, a number of cell viability assays should be run in parallel,

providing a more comprehensive picture of the potential cellular toxicity through different mechanisms.

The SH-SY5Y cell line has been widely used as a model of neurons since the early 1980s, as the cells possess many biochemical and functional properties of neurons. It is a comparatively homogenous neuroblast-like cell line, and it exhibits neuronal marker enzyme activity, specific uptake of norepinephrine and expresses one or more neurofilament proteins. In addition, the SH-SY5Y cells possess the capability of proliferating in culture for long periods without contamination, which is a prerequisite for the development of an *in vitro* cell model. Consequently, the SH-SY5Y cell line has been widely used in experimental neurological studies, including analysis of neuronal differentiation, metabolism and function related to neurodegenerative and neuroadaptive processes, neurotoxicity and neuroprotection (Xie et al., 2010).

Several lines of evidence suggest that A $\beta$ - induced oxidative stress plays an important role in the pathogenesis or progression of AD (Butterfield et al., 2001; Wang et al., 2009). A $\beta$  induces oxidative stress which in turn promotes the production of A $\beta$  and this results in a vicious cycle leading to generation of free radicals and oxidative stress (Tamagno et al., 2008). Several antioxidants such as huperzine A (Xiao et al., 2002), alpha-tocopherol, flavonoids, hydroquinones and estrogens (Bastianetto and Quirion, 2002; Behl and Moosman, 2002; Mook-Jung et al., 2002), have been demonstrated to inhibit A $\beta$ - induced neurotoxicity, which implies that plants with good antioxidant activity may possibly have neuroprotective properties.

### 4.3.1 Cytotoxicity assessment and effect of isolated compounds and active fractions on A $\beta$ -induced neurotoxicity

6-Hydroxycrinamine, a crinine-type alkaloid and fraction EAM 17-21 21,22, which is also a mixture of alkaloids were found to be toxic with low IC<sub>50</sub> values (Table 3.9). This is not surprising as the toxic effect of *B. disticha* is documented in literature and this is mainly due to its alkaloidal content (Botha et al., 2005). The cytotoxic effect of several crinine-type and similar alkaloids, have been reported. Lycorine, crinamine and augustine have been reported to demonstrate significant cytotoxic activity in 12 different cell lines (Likhitwitayawuid et al., 1993). Crinafolidine and crinafoline were observed to produce significant reduction in the viability and *in vivo* growth of S-180 ascites tumor cells (Tram et al., 2002). Amaryllidaceae alkaloids and flavan isolated from *Crinum augustum* and *C. bulbispermum* have been shown to exhibit cytotoxic activity on human leukemic Molt4 cells (Zvetkova et al., 2001). Criasiaticidine A and lycorine have been shown to be toxic to Meth-A (mouse sarcoma) and Lewis lung carcinoma (mouse carcinoma) tumor cell lines (Min et al., 2001). In addition, crinamine, hemanthamine and papyramine have been shown to be toxic to non-tumoral fibroblastic LMTK cells (Bastida et al., 2011).

However, despite the toxicity of these crinine-type alkaloids, quantitative structure-activity relationship (QSAR) studies could be carried out to modify the structures in order to make them less toxic and improve their activity. QSAR development provides a powerful tool to correlate the biological activities of compounds to their structural or physicochemical parameters and extends the correlated parameters for the prediction of new active ligands (Viswanadhan et al., 1989).

The triterpenoids, cycloeucalenol (a cycloartane triterpene) and fraction EAE 11 (a mixture of triterpenoids) were least toxic and had higher IC<sub>50</sub> values than 6-hydroxycrinamine and the alkaloidal fraction (EAM 17-21 21,22). Other triterpenoids – anagallisin C, heterogenoside E and F have been shown to have low toxicity against HeLa, KB-3-1 and HepG<sub>2</sub> cells (Huang et al., 2011). In addition, five triterpenoids - 20*S*, 24*S*-epoxy-23*S*, 25-dihydroxy-dammarane-3-one; 20*S*, 25-epoxy-24*R*-hydroxydammarane-3-one; 20*S*, 24*S*-epoxydammarane-3β, 25-diol; betulinic acid and morolic acid acetate had no toxic effects on human epidermoid cancer (KB) cells and human epithelial carcinoma (A2780) cells (Nan et al., 2004). Furthermore, two cycloartane triterpenoids; 25-*O*-acetylcimigenol-3-*O*-β-D-glucopyranosyl(1"→2')-β-D-xylopyranoside and 25-*O*-acetylcimigenol-3-*O*-β-D-galactopyranoside showed low toxicity when tested against mouse hepatocytes with IC<sub>50</sub> values >100 μM (Tian et al., 2006). All these support the findings in the present study.

The low toxicity of cycloeucalenol and other similar triterpenoids make them useful agents which can be studied further with the aim of developing effective AChE inhibitors, anti-inflammatory and cardioprotective agents (Kongkathip et al., 2002).

The isolated compounds and fractions did not prevent cell death caused by Aβ<sub>25-35</sub>. Antioxidants have been reported to play a major role in ensuring protection against Aβ-induced neurodegeneration (Xiao et al., 2002). Therefore, the poor activity observed is likely due to the very low radical scavenging activity of *B. disticha* bulbs in both the DPPH and ABTS radical scavenging assays (Table 3.2).

### 4.3.2 Cytotoxicity assessment and effect of several medicinal plants on A $\beta$ -induced neurotoxicity

The low toxicity of *Z. mucronata* as observed in the present study is supported by McGaw et al. (2007). The authors tested the hexane, methanol and water extracts of the bark and leaf of the plant and obtained IC<sub>50</sub> values >100  $\mu$ g/ml. Extracts of the roots of *Z. mucronata* have been reported to maintain the viability of cervical carcinoma (HeLa), colon adenocarcinoma (HT29) and skin carcinoma (A431) cell lines by 75% at 100  $\mu$ g/ml (Kamuhabwa et al., 2000). In addition to its low toxicity, *Z. mucronata* showed good neuroprotective effect at the highest concentration tested. *Z. spinosa* has been reported to demonstrate anxiolytic activity in the elevated plus-maze assay and this neuroprotective effect is attributed to spinosin, its active constituent (Wang et al., 2008; Koetter et al., 2009). *Z. jujuba* has been shown to improve memory (Mitchell, 1995). It has also been reported to be used to treat mental problems including neurasthenia and schizophrenia and this activity is likely due to its active ingredients; stepharine and asimilobine (Chen, 1998; Qian, 1996; Wing, 2001). In addition, *Z. jujuba* is reported to contain quercetin, kaempferol and phloretin derivatives which have been shown to help ameliorate ethanol-induced memory deficit and improve cognitive function in rats (Pawlowska et al., 2009; Taati et al., 2011). Several plants from the Rhamnaceae family: *Z. hajarensis*, *Z. mucronata*, *Z. lotus* and *Rhamnus alaternus* have been documented to be a source of natural antioxidants. This activity has been ascribed to high levels of flavonoids, gallic catechin, quercetin glycosides and flavonol glycosides (Marwah et al., 2007; Benammar et al., 2010). Two cyclopeptide alkaloids have been isolated from the roots of *Z. mucronata* (Barboni et al., 1994), which are also important sources of antioxidants (Zanolari et al., 2003; Hara et al., 2006; Shirwaikar et al., 2006). A possible underlying mechanism of the

neuroprotection observed with *Z. mucronata* may be associated with the presence of these compounds and polyphenols.

*Lannea stuhlmanii* has been reported to maintain viability of HeLa, HT29 and A431 cell lines by 75% at 100 µg/ml (Kamuhabwa et al., 2000). The hexane, methanol and water extracts of the bark and leaves of *Rhus lancea*, a member of the Anacardiaceae family, were shown to be non-toxic in the brine shrimp mortality assay (McGaw et al., 2007). Methanol extracts of the leaves were observed to have an IC<sub>50</sub> of 1 mg/ml (McGaw et al., 2007). Our study shows *L. schweinfurthii* to have low cytotoxic and good protective effect against Aβ induced toxicity (Table 3.10 and Figure 3.12). Similar neuroprotective activity has been reported for other plants in the Anacardiaceae family including *Magnifera indica* and *Rhus verniciflua*. Polyphenolic compounds present in *M. indica* including gallic acid, epicatechin gallate and epigallocatechin gallate have been shown to have neuroprotector effects against Aβ induced toxicity in primary cultures of hippocampal cells (Bastianetto et al., 2006). Their neuroprotective effect is enhanced by their ability to cross the blood-brain barrier as they are water soluble, and have high bioavailability (Passwater and Kandaswami, 1994). Stimaosterols isolated from *R. verniciflua* are reported to demonstrate neuroprotective effects against kainic acid-induced excitotoxicity (Byun et al., 2010). In addition, 1-docosanoyl cafferate isolated from *R. verniciflua* is an ester derivative of caffeic acid that has been shown to protect against Aβ-induced neurotoxicity (Sul et al., 2009; Lee et al., 2011). Several other plants in the Anacardiaceae family including *L. velutina*, *L. barteri*, *L. acida* and *L. microcarpa* have also been shown to be good radical scavengers due to high levels of polyphenolic compounds like tannin polyflavonoids, hydroquinones, alkylphenols and dihydroalkylhexenones (Koné et al., 2011; Ouattara et al.,

2011). Similar compounds could be present in *L. schweinfurthii* and may be responsible for its good neuroprotective activity.

Extracts from the stem-bark of *T. spinosa* have been tested for their cytotoxic effect in the brine shrimp assay (Mbwambo et al., 2011). Dichloromethane/methanol (1:1) and 80% ethanol extracts from the plant showed IC<sub>50</sub> values of 99.5 µg/ml and 75.8 µg/ml, respectively, and this finding supports the low toxicity of *T. sericea* observed in the present study. Methanol and water extracts of *T. chebula* have been reported to show effective neuroprotective activity against H<sub>2</sub>O<sub>2</sub> – induced cell death in PC12 cells which is ascribed to its OH and H<sub>2</sub>O<sub>2</sub> radical scavenging activity and its total phenol and tannin content (Chang and Lin, 2012). In addition, oral administration of *T. chebula* extracts have been found to protect neurons from ischemic damage induced by transient cerebral ischemia and this has been attributed to its polyphenolic compounds which include tannic acid, chebulagic acid, chebulinic acid and corilagin (Park et al., 2011). Several pentacyclic triterpenoids have been isolated from *Terminalia* species of which sericic acid and a sericoside are the main compounds in the root extract (Eldeen et al., 2005). Sericoside also found in *T. sericea*, has been reported to have good antioxidant activity (Mochizuki and Hasegawa, 2007), and its presence and other similar compounds may account for the observed neuroprotective effect of *T. sericea*.

*C. bulbispermum* root extract reduced cell viability to 16% at 50 µg/ml. Extracts of the whole plant of *C. papillosum* have been reported to decrease viability in HeLa and HT29 cell lines by 25%. In A431 cells a 50% reduction in viability was found at 100 µg/ml (Kamuhabwa et al., 2000). These results are similar to those obtained in the present study. Methanol extracts of the roots of *C. bulbispermum* showed modest neuroprotective activity at a non-toxic dose. The aqueous leaf extracts of the plant have been reported to possess moderate antioxidant activity,

attributed to the presence of flavonoids and tannins (Ratnasooriya et al., 2005). In addition, lycorine and haemanthamine have been isolated from *C. ornatum* bulbs, both of which have good antioxidant activity (Oloyede et al., 2010). The observed neuroprotective effect of *C. bulbispermum* root extract may be due to the presence of these compounds and polyphenols.

## CHAPTER 5: CONCLUSION

The treatment of diseases using plant remedies is part of human culture, and it continues to play a major role in the health care systems worldwide. Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies. The World Health Organization (WHO) estimates that 80% of the world's population presently use herbal remedies for some aspect of primary health care (WHO, 2009).

One of the many ailments which have long been treated with herbal remedies are neurodegenerative diseases. Seventeen plants were selected for this study based on documented ethno-medicinal use in improving memory, to treat insomnia, calm agitated people, and other neurological disorders.

The plants were screened for inhibition of AChE. The TLC method was used as a qualitative assay while the microplate method was used to determine percentage inhibition and  $IC_{50}$  values. Ethyl acetate extracts of the roots of *Crinum bulbispermum*, *Xysmalobium undulatum*, *Lannea schweinfurthii*, *Scadoxus puniceus* and bulbs of *Boophane disticha* showed the best inhibition of AChE. Though the  $IC_{50}$  of these plants was higher than that of galanthamine (0.00053 mg/ml), they possess good AChE inhibitory activity considering they are still mixtures containing various compounds.

The ABTS and DPPH radical scavenging assays were employed to assess the antioxidant capacity of the plant extracts. The roots of *Schotia brachypetala*, *L. schweinfurthii*, *Terminalia sericea*, *Ziziphus mucronata* and *C. bulbispermum* all had very good radical scavenging activity in both assays with low  $IC_{50}$  values. These plants contain high levels of phenols and flavonoids which may be responsible for the observed antioxidant activity. Results obtained are supported

by literature and these plants can serve as leads in developing potential antioxidant compounds which are relevant in the treatment of AD.

The bulbs of *B. disticha* showed good inhibition of AChE and were further selected for isolation and purification of compounds. Two compounds - 6-hydroxycrinamine and cycloeucalenol were isolated from the methanol and ethyl acetate extracts, respectively. The crinine alkaloid, 6-hydroxycrinamine was isolated as a 3:1 mixture of two epimers, and it showed weak inhibition of AChE with an  $IC_{50}$  value of  $445 \pm 30 \mu\text{M}$ . Cycloeucalenol, a cycloartane triterpene, did not show any inhibition of AChE. However, it has been reported to have cardioprotective effects in addition to its anti-inflammatory, spasmolytic and cardioprotective activity. Its reported anti-inflammatory activity makes it relevant in the development of potential leads for AD, as inflammation is one of the pathological features of the disease. It appears that this is the first report of the presence of these compounds in *B. disticha*. Fraction EAM 17-21 21,22 obtained from the methanol extract could not be purified further because of its very low yield. It showed good inhibition of AChE and its NMR spectra indicated that it is a mixture of alkaloids. Further studies need to be carried out to identify these alkaloids which contain AChE inhibitory activity. Fraction EAE 11 obtained from the ethyl acetate extract also showed good inhibition of AChE but its very low yield meant it could not be purified further. Its NMR spectra and chromatogram indicates that it is also a mixture of several triterpenes. Since several triterpenoids have been reported to contain AChE inhibitory activity, this fraction needs to be studied further to identify these triterpenes with inhibitory activity.

The studies on the bulbs of *B. disticha* shows that it contains alkaloids and triterpenoids with a variety of biological activities including AChE inhibition, anti-inflammatory and cardio-

protective activity, which can serve as attractive targets for future studies to uncover new alternatives to the existing therapies for neurodegenerative diseases including AD.

A predominant feature of the brain of patients with AD is the presence of A $\beta$  plaques which have been linked to increased oxidative stress and presence of ROS in the brain of sufferers. The compounds isolated and active fractions of *B. disticha*, and four very promising plants (*Z. mucronata*, *T. sericea*, *L. schweinfurthii* and *C. bulbispermum*), with good antioxidant activity were studied further to assess their cytotoxicity and evaluate their ability to protect against cell death induced by A $\beta_{25-35}$ .

6-Hydroxycrinamine and the alkaloidal fraction EAM 17-21 21,22 were found to exhibit significant cytotoxic activity, which is similar for many other alkaloids which have been isolated. In contrast however, cycloeucaleanol and the triterpenoid fraction EAE 11 showed low toxicity. QSAR studies may be required to modify the structures of the alkaloids to make them less toxic while maintaining or enhancing their potency. 6-hydroxycrinamine and fractions EAM 17-21 21,22 and EAE 11 did not prevent A $\beta_{25-35}$  induced neurotoxicity possibly due to the poor antioxidant activity of *B. disticha*. This may indicate that their mechanism of neuroprotection is possibly only through inhibition of AChE.

All four plant extracts tested had effects on cell viability. Extracts of the roots of *Z. mucronata* and *L. schweinfurthii* were the least toxic with IC<sub>50</sub> values exceeding 100  $\mu$ g/ml, the highest concentration tested, while *C. bulbispermum* root extracts reduced cell viability to 16% at 50  $\mu$ g/ml. Pre-treatment with all five extracts at the non-toxic dose showed a dose-dependent inhibition of cell death caused by A $\beta_{25-35}$ . The neuroprotective effect of the plants can be

attributed in part to their antioxidant activity. These plants can serve as potential leads in developing drugs relevant to preventing A $\beta$ <sub>25-35</sub> mediated neuronal degeneration.

The results obtained in the study have provided scientific support for the ethno-medicinal use of some of the plants which showed promising activity, in the treatment of neurological disorders. This study confirmed the acetylcholinesterase inhibitory and antioxidant activity of some of the medicinal plants used to treat neurodegenerative disorders. In addition, four of the plants were shown to prevent cell death caused by A $\beta$ <sub>25-35</sub>. Furthermore, two new compounds present in the bulbs of *B. disticha* were identified. Additional investigations need to be carried out by applying QSAR studies to modify the structures of the alkaloid and triterpene isolated from the plant with the aim of reducing the observed toxicity and developing more potent AChE inhibitors.