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IN VITRO EFFECT OF SELECTED MEDICINAL PLANTS ON β-AMYLOID INDUCED TOXICITY IN NEUROBLASTOMA CELLS

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Declaration

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Publications and Presentations

Publications

Adewusi EA, Moodley N, Steenkamp V (2010). Medicinal plants with cholinesterase inhibitory activity: A Review. African Journal of Biotechnology 9: 8257-8276. (Appendix A).

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Abstract

Neurodegenerative diseases occur as a result of the breakdown and deterioration of the neurons of the central nervous system (CNS). They are commonly found in elderly people and are a major cause of morbidity and mortality, thereby imposing severe strains on the social welfare systems. Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder. Cholinergic deficit, senile plaque/amyloid-β peptide deposition and oxidative stress have been identified as three main pathogenic pathways which contribute to the progression of AD. The current therapeutic options cause several side-effects and have problems associated with bioavailability. Therefore, the need arises to search for new compounds from natural products with potential to treat AD.

Seventeen plants were selected for this study based on their documented ethno-medicinal use in improving memory, to treat insomnia, calm agitated people, and other neurological disorders. The plants were screened for inhibition of acetylcholinesterase (AChE) using the TLC and microtiter plate method. A dose-dependent inhibition of the enzyme was observed and 4.5% of all the plants showed low (<30% inhibition) AChE inhibition. The ethyl acetate extracts of the roots of *Crinum bulbispermum*, *Xysmalobium undulatum*, *Lannea schweinfurthii*, *Scadoxus puniceus* and bulbs of *Boophane disticha* had the best AChE inhibition. Although the IC_{50} of these plant extracts were higher than that of the positive control, galanthamine (0.00053 mg/ml), they showed good AChE inhibitory activity considering they are still mixtures containing various compounds.

The antioxidant activity of the plant extracts was determined by their ability to scavenge ABTS (2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (1,1-diphenyl-2-picryl-

hydrazyl) radicals. The dichloromethane/methanol (1:1) extracts of *Chamaecrista mimosoides* (root), *Buddleja salviifolia* (whole plant), *Schotia brachypetala* (root and bark), water extracts of *Chamaecrista mimosoides* (root), *Buddleja salviifolia* (whole plant), *Schotia brachypetala* (root and bark) and methanol extracts of the roots of *Crinum bulbispermum*, *Piper capense*, *Terminalia sericea*, *Lannea schweinfurthii* and *Ziziphus mucronata* all showed good antioxidant activity (>50%), in both assays.

B. disticha contained very promising AChE inhibition and was subjected to isolation of active compounds using thin layer chromatography, column chromatography and preparative thin layer chromatography. Two compounds, 6-hydroxycrinamine (a crinine-type alkaloid) and cycloeucalenol (a cycloartane triterpene), were isolated for the first time from the bulbs of this plant. 6-Hydroxycrinamine, and two fractions, EAM 17-21 21,22 and EAE 11 (which could not be purified further due to low yield), were found to inhibit AChE with IC_{50} values of 0.445 ± 0.030 mM, 0.067 ± 0.005 mg/ml and 0.122 ± 0.013 mg/ml, respectively.

Cytotoxicity of the isolated compounds and two active fractions was determined on human neuroblastoma (SH-SY5Y) cells using the MTT and neutral red uptake assays. 6-hydroxycrinamine and fraction EAM 17-21 21,22 were found to be toxic with IC_{50} values of 54.5 µM and 21.5 µg/ml as determined by the MTT assay. The isolated compounds and fractions did not show any protective effect against cell death induced by $A\beta_{25-35}$ possibly due to the poor antioxidant activity of *B. disticha* bulbs.

Cytotoxicity was also determined for the methanol extracts of the roots of *C. bulbispermum*, *T. sericea*, *L. schweinfurthii* and *Z. mucronata*, as they contained promising antioxidant activity. *C. bulbispermum* was the most toxic, reducing cell viability by <40% at the highest concentration

tested. *Z. mucronata* and *L. schweinfurthii* were the least toxic with IC_{50} values exceeding 100 µg/ml, the highest concentration tested. Three concentrations of the plant extracts that were not toxic, or presented low toxicity, were selected to evaluate their possible protective effect against cell death induced by $A\beta_{25-35}$. Pretreatment with *Z. mucronata* and *T. sericea* roots showed a dose dependent inhibition of cell death caused by $A\beta_{25-35}$. Pre-treatment with *L. schweinfurthii* roots resulted in an optimum dose for inhibition of $A\beta_{25-35}$ induced cell death at 25 µg/ml, while still maintaining 80% viability. The roots of *C. bulbispermum* at non-toxic dose still maintained >50% viability.

This study confirms the neuroprotective potential of some of the plants which had AChE inhibitory and antioxidant activity. In addition, four of the plants were shown to prevent cell death caused by $A\beta_{25-35}$. These plants can serve as potential leads in developing drugs relevant to treatment of AD. 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 structure of the alkaloid with the aim of reducing its observed toxicity.

Keywords: Acetylcholinesterase, Alzheimer's disease, amyloid-β, antioxidant, cytotoxicity, plant extracts, SH-SY5Y cells.

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List of Abbreviations

^{13}C	carbon 13
1D	one dimensional
^{1}H	proton
2D	two dimensional
5-HT	5-hydroxytryptamine
Aβ	Amyloid-β
ABTS	2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEIs	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
APP	Amyloid-β precursor protein
ATCC	American cell type collection culture
ATCI	Acetylthiocholine iodide
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxyl toluene
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
CNS	Central Nervous System
COSY	Correlation Spectroscopy
COX-2	Cyclooxygenase-II
CSIR	Council for Scientific and Industrial Research
DCM	Dichloromethane

DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
DTNB	5,5´-bisdithionitrobenzoic acid
ESI^-	Electrospray negative mode
ESI^+	Electrospray positive mode
EtOAc	Ethyl acetate
FCS	Fetal calf serum
GABA	γ-aminobutyric acid
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRTOFMS	High resolution time-of-flight mass spectroscopy
HSQC	Heteronuclear Single Quantum Coherence
IC_{50}	50% Inhibitory concentration
J	spin-spin coupling in Hertz
LC-MS	Liquid chromatography-mass spectrometry
m/z	mass to charge ratio
MAO	Monoamine oxidase
MeDi	Mediterranean diet
MeOH	Methanol
MMP	1-methyl-4-phenylpyridinium ion
MS	Mass spectrometry
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
NIST	National Institute of Standards and Technology

NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NOE	Nuclear Overhauser Effect
NR	Neutral red
NSAIDs	Non-steroidal anti-inflammatory drugs
OSI	Oxidative stability instrument
PBS	Phosphate Buffered Saline
PC-12	Rat pheochromocytoma cells
Ppm	parts per million
QSAR	Quantitative structure activity relationship
QTOF	Quadrupole Time-of-Flight
R_f	Retention value
ROS	Reactive oxygen species
SANBI	South African National Biodiversity Institute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SH-SY5Y	Human neuroblastoma cells
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
WHO	World Health Organisation
XO	Xanthine oxidase

CHAPTER 1: LITERATURE REVIEW

1.1 Neurodegeneration

Neurodegenerative disease is a term applied to a variety of conditions which result from a chronic breakdown and deterioration of neurons, particularly those of the central nervous system (CNS). These neurons may accumulate aggregated proteins which cause dysfunction (Houghton and Howes, 2005). Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis and spongiform encephalopathy are some of the common forms of neurodegenerative diseases (Chiba et al., 2007). These diseases are commonly found in elderly people and in advanced industrialized societies where life expectancy is long. They are a major cause of morbidity, mortality and impose severe strains on the social welfare systems, and as a result are gaining increased recognition by the World Health Organisation (WHO) (Houghton and Howes, 2005).

Neurodegenerative diseases are characterized by a gradual onset of progressive symptoms including loss of memory and tremor, difficulty in learning or retaining information, inability to handle complex tasks, impaired spatial orientation and abilities, language deficits and behavioral changes. These symptoms have been recognized as a feature of increasing age for a long time and are acknowledged in many traditional medical systems. However, it is only recently that they have been recognized and received attention from mainstream medicine as distinctive diseases (Houghton and Howes, 2005).

1.2 Alzheimer's disease

In 1906, during the 37th Conference of German psychiatrists in Tübigen, the Bavarian neuropsychiatrist, Alois Alzheimer described for the first time the symptoms of "a particular disease of the cerebral cortex", characterized by a gradual and irreversible degeneration of intellectual functions such as memory, orientation, judgement, language and the capacity to acquire new knowledge (Hostettmann et al., 2006). This disease became known as Alzheimer's disease (AD).

AD is the most common age-related neurodegenerative disorder and is also the most common cause of progressive mental deterioration in persons aged 65 or older (Shah et al., 2008). The clinical symptoms result from the deterioration of selective cognitive domains, particularly those related to memory. Memory decline initially manifests as a loss of episodic memory, which is considered as a subcategory of declarative memory. The dysfunction in episodic memory impedes recollection of recent events including autobiographical activities (LaFerla et al., 2007). Patients with AD also suffer from marked reduction of cholinergic neuronal function in those areas of the brain responsible for higher mental functions which partially accounts for the impairments in activities of daily life (Brodaty et al., 1993; Coyle and Kershaw, 2001). The estimated global dementia prevalence in people aged over 60 is approximately 3.9% with regional prevalence being 1.6% in Africa, 3.9% in Eastern Europe, 4.0% in China, 4.6% in Latin America, 5.4% in Western Europe and 6.4% in North America (Qiu et al., 2009). Rates of increase are not uniform; numbers in developed countries are estimated to double between 2001 and 2040, and increase by more than 300% in India, China, south Asia and western Pacific (Ferri et al., 2005).

Hence, the global trend in the phenomenon of population aging has dramatic consequences for public health, healthcare financing and delivery systems in the world and especially in developing countries (Qiu et al., 2007).

1.3 Pathology of Alzheimer's disease

The pathology of AD is complex and three main pathogenic pathways are believed to contribute to the progression of the disease; cholinergic deficit, senile plaque/amyloid-β peptide deposition and oxidative stress (Small and Mayeux, 2005). These three pathogenic pathways are discussed below:

i. Cholinergic hypothesis

In the late 1970s, White and his colleagues discovered that the brains of patients with neurodegenerative diseases including AD were deficient in acetylcholine (ACh) (White et al., 1977), and this became a consistent report (Hollander et al., 1986). As a result, the cholinergic hypothesis was developed, which essentially states that a loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age (Bartus, 2000; Terry and Buccafusco, 2003; Heinrich and Teoh, 2004) (Figure 1.1).

ACh is critical for an adequately functioning memory. ACh is stored in the nerve terminals in structures called vesicles. The contents of these vesicles are released from the nerve endings when the nerve terminal is depolarized, and the ACh released enters into the synapse and binds to the receptor. The ACh released has a very short half-life due to the presence of large amounts of acetylcholinesterase (AChE), an enzyme which hydrolyses the ester bond in the molecule, thus leading to loss of stimulatory activity. Current therapeutic strategies for the symptomatic

treatment of AD and other related disorders such as vascular dementia, dementia with Lewy bodies, senile dementia and Parkinson's disease are aimed at enhancing the associated cholinergic deficit by inhibiting AChE (Rösler et al., 1999; Brenner, 2000; Rahman and Choudhary, 2001), resulting in a boost in endogenous level of ACh in the brain and an improvement of cognitive function (Elufioye et al., 2010).

ii. Amyloid Cascade Hypothesis

Postmortem examinations of the brains of people with AD show characteristic structures termed amyloid plaques and neurofibrillary tangles. Amyloid-β precursor protein (APP) is a type-1 transmembrane protein of unknown function. It is cleaved by two proteases to form amyloid-β (Aβ). Aβ is also secreted by mammalian cells and occurs normally in plasma and cerebrospinal fluid. The Amyloid Cascade Hypothesis, suggests that improper metabolism of APP is the initiating event in AD pathogenesis, leading to the aggregation of Aβ (Shah et al., 2008). It has also been proposed that Aβ peptide deposits, or even the partially aggregated soluble form are responsible for triggering a neurotoxic cascade of events which ultimately results in neurodegeneration (Castro et al., 2002; Dastmalchi et al., 2007). The Aβ peptide is a prime target for developing therapies for neurodegenerative diseases including AD (Jayaprakasam et al., 2010) and so, modulating the chain of events starting from the production of Aβ peptide fragments from APP to its deposition in the form of extracellular plaques are believed to be possible approaches towards the treatment of AD (Dastmalchi et al., 2007).

Recent evidence indicates that AChE may be involved in the pathogenesis of plaques. AChE appears to enhance the aggregation of the Aβ peptide, a major event in the process of plaque formation (Inestrosa et al., 1996). It also increases the toxicity of Aβ (Reyes et al., 2004).

Administration of AChE inhibitors has been shown to interfere with Aβ production, most likely by reducing the levels of the APP from which Aβ is cleaved (Lahiri et al., 1994; Greig et al., 2001; Shaw et al., 2001). This evidence gives rise to the fact that in addition to amelioration of cholinergic deficit, AChE inhibition may also retard the disease process as it may reduce the toxicity of Aβ and subsequently reduce plaque formation (Eskander et al., 2005).

iii. Oxidative stress and antioxidant activity

Aging in most species studied to date is accompanied by the progressive accumulation of oxidative damage in many tissues (Head, 2009). Oxidative stress is the imbalance between cellular production of free radical species and the ability of the cells to eliminate them employing endogenous antioxidant defense mechanisms (Sanvicens et al., 2006). Oxidative stress causes cellular damage and subsequent cell death especially in organs such as the brain. The brain in particular is highly vulnerable to oxidative damage as it consumes about 20% of the body's total oxygen, has a high content of polyunsaturated fatty acids and lower levels of endogenous antioxidant activity relative to other tissues (Halliwell and Gutteridge, 1985; Floyd and Hensley, 2002; Shulman et al., 2004). Oxidative stress is involved in the propagation of cellular injury that leads to neuropathology in several neurodegenerative diseases. It is intimately linked with an integrated series of cellular phenomena, which all seem to contribute to neuronal demise (Andersen, 2004). Several lines of evidence have also suggested that Aβ-induced oxidative stress plays an important role in the pathogenesis or progression of AD (Butterfield et al., 2001). Aβ induces oxidative stress (Hensley et al., 1994), and oxidative stress promotes the production of

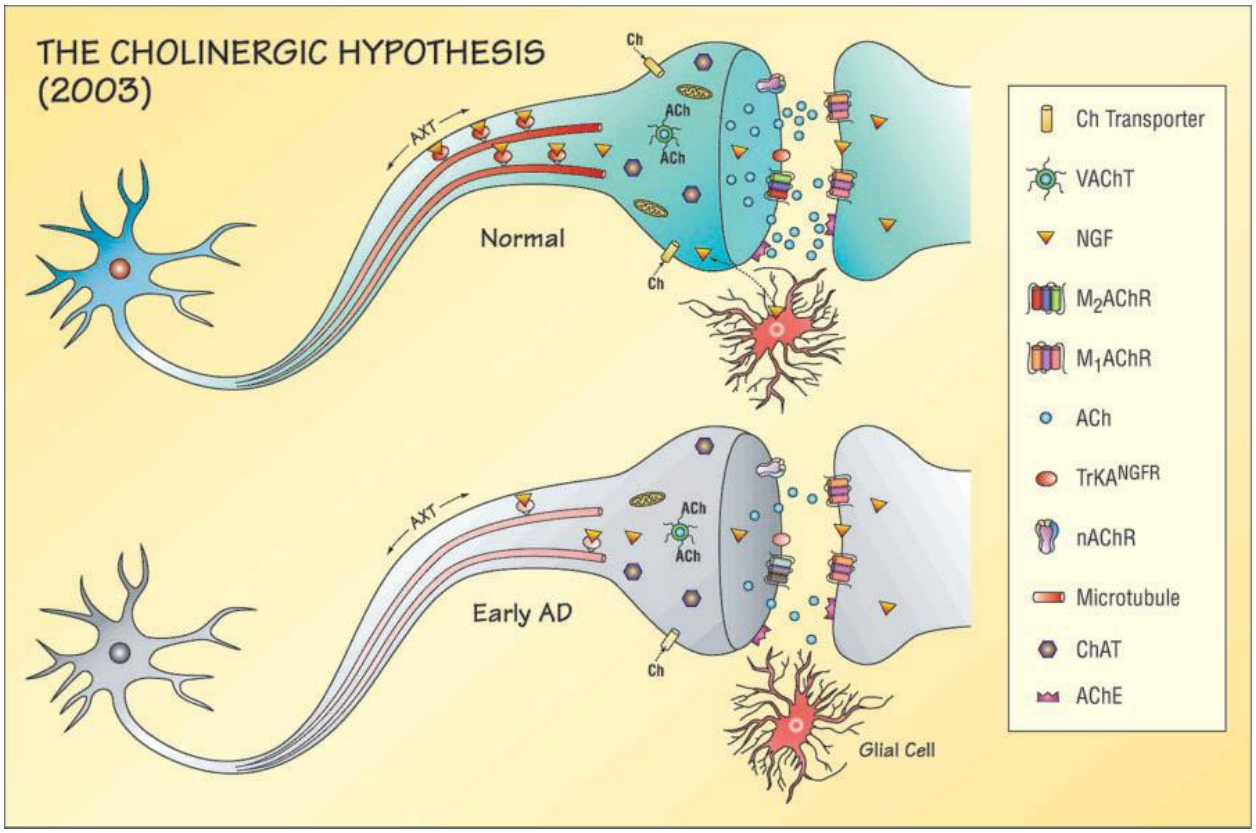


Figure 1.1 Schematic representation of the known and proposed changes in cholinergic neurons that occur in the aged and early AD brain compared with healthy young neurons. Alterations in high-affinity choline uptake, impaired acetylcholine release, deficits in the expression of nicotinic and muscarinic receptors, dysfunctional neurotrophin support (i.e., NGF receptors), and deficits in axonal transport are represented in the early AD neuron either by a decrease in the number of symbols presented or by reduced color intensity (From Terry and Buccafusco, 2003).

Aβ (Tamagno et al., 2008). The relevance of oxidative stress to the progression of many neurodegenerative disorders has generated an interest in the potential use of radical scavengers and their natural biological counterparts for protecting cells and tissues from oxidative damage (Behl, 2000; Chen et al., 2003; Prokai et al., 2003; Deng et al., 2004). The radical scavengers help to scavenge free radicals before they can bring about their deleterious effects (Dastmalchi et al., 2007). *In vitro* studies have demonstrated the neuroprotective activities of antioxidants (Sanvicens et al., 2006), and data from clinical trials appear very promising and have shown potential benefit from treatment with radical scavengers in pathologies such as AD (Grundman, 2000; Doraiswamy, 2002; Shults, 2003; Nagayama et al., 2004). The deleterious effect of oxidative stress and reactive oxygen species in neurodegeneration is provided in Figure 1.2.

It is thus evident that the cholinergic hypothesis, amyloid cascade hypothesis and oxidative stress are three very important pathologic pathways which contribute to the progression of several neurodegenerative diseases. These three pathways occur simultaneously and form a chain reaction leading to neurodegeneration (Figure 1.3). These pathways are the target for drug development.

1.4 Current therapeutic approaches for AD

AD sufferers have a longer life expectancy if they are not institutionalized and if properly cared for by spouses or family members in a known environment. It is therefore ideal to delay institutionalization for as long as the spouse and/or family members can cope and within the borders of self-dignity of the patient and overall affordability (Greeff, 2009). Drug development for AD is challenging, however, several therapeutic approaches have been adopted. Drugs that are currently in use and the role of dietary factors in the treatment of AD are discussed in detail.

1.4.1 Acetylcholinesterase inhibitors

Acetylcholinesterase inhibitors (AChEIs), are the best developed therapy currently used in the treatment of mild to moderate AD (Shah et al., 2008). AChEIs slow the progression of the disease by decreasing levels of Aβ protein. Tacrine was the first widely used AChEI (Summers, 2006). A 30-week randomized control clinical trial showed a significant dose related improvement in cognitive function with tacrine (Whitehouse, 1993; Knapp et al., 1994). However, subsequent studies were less impressive and a short half-life, hepatotoxicity and cholinergic side-effects, have limited the use of the drug. Second generation AChEIs, including donepezil, galanthamine and rivastigmine have since been developed. These drugs have fewer side effects, longer half-lives and greater efficacy (Shah et al., 2008).

AChEIs are usually started at low doses to minimize side effects such as facial flushing, dyspepsia, nausea, vomiting and diarrhea. The dose is then titrated up to the maximum tolerated dose (Mulugeta et al., 2003).

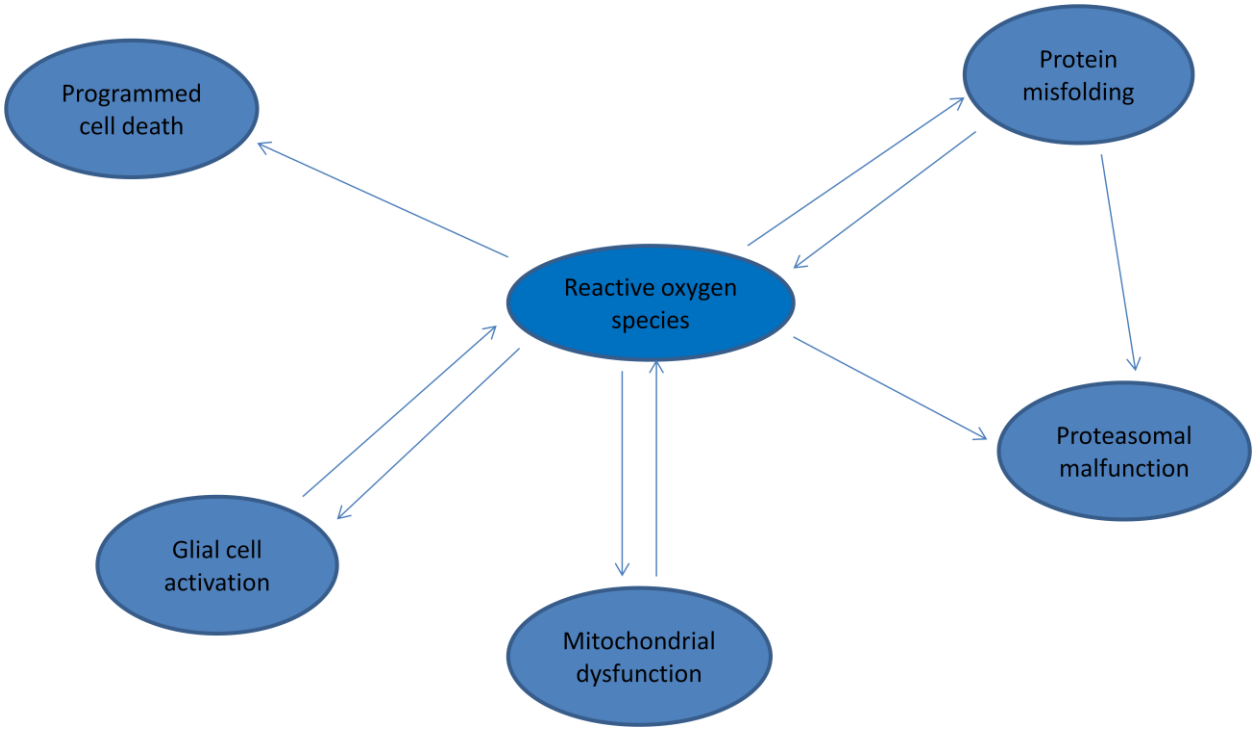


Figure 1.2 ROS production as a player in the cycle of events leading to neurodegeneration (From Anderson, 2004).

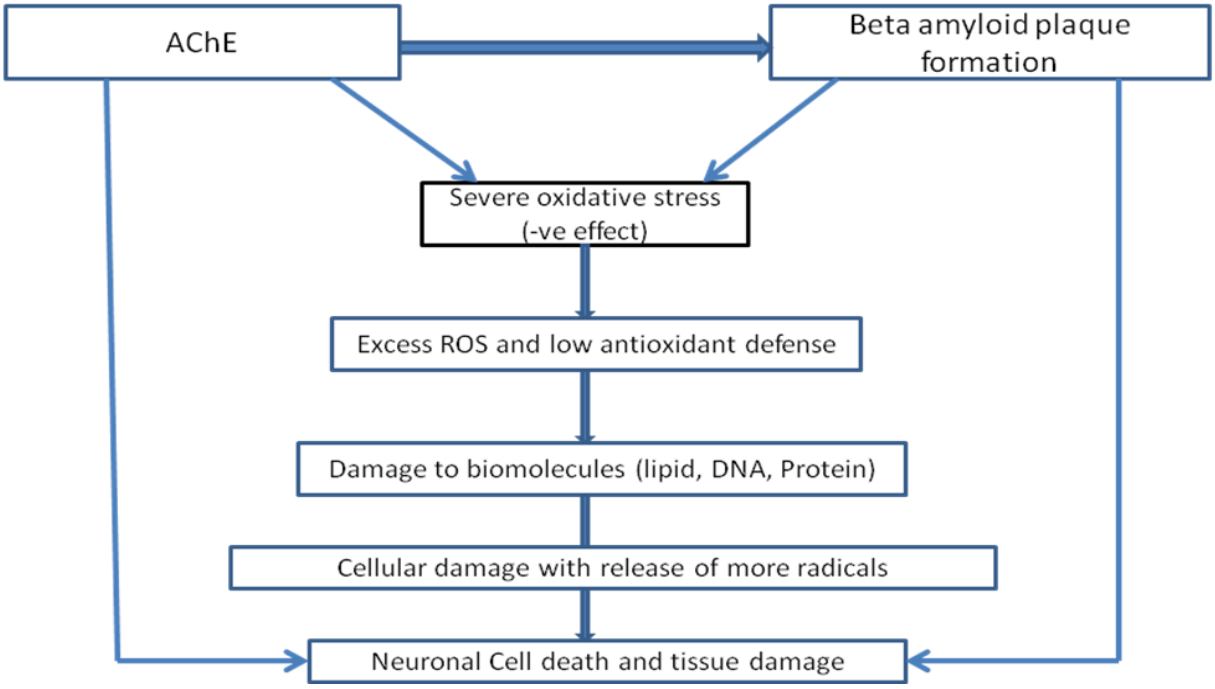


Figure 1.3 Acetylcholinesterase, beta amyloid plaque formation and oxidative stress as part of a chain reaction leading to neurodegeneration.

1.4.2 Antioxidants

Multiple lines of evidence indicate that oxidative stress is an important pathogenic process associated with aging and AD. In addition, markers of oxidative stress have been shown to precede pathological lesions in AD, including senile plaques and neurofibrillary tangles (Sayre et al., 1997; Nunomura et al., 1999; Nunomura et al., 2001; Castellani et al., 2006). Antioxidants act by slowing the progression of the disease or preventing cognitive decline (Shah et al., 2008). Vitamin E in combination with vitamin C is reported to be associated with a decrease in the prevalence and incidence of AD (Zandi et al., 2004; Shah et al., 2008). Also, in controlled clinical trials conducted with α-tocopherol, AD patients with moderate impairment taking high doses of the antioxidant were observed to display some beneficial effect with respect to the rate of deterioration of cognitive functions (Sano et al., 1997; Ramassamy, 2006).

1.4.3 Statins

Cerebral Aβ levels have been shown to be decreased *in vivo* with simvastatin (Fassbender et al., 2001). The first study to show neuropathologic change in statin use was published by Li et al. (2007). Several epidemiologic studies have indicated that the use of statins significantly reduces the risk of AD (Jick et al., 2000; Wolozin et al., 2000; Rockwood et al., 2002; Yaffe et al., 2002). These studies suggest that statins may slow progression of AD, but may not be able to reverse neuronal degeneration once it has occurred (Shah et al., 2008)

1.4.4 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs), act by down-regulating pro-inflammatory signals, microglia and astrocytes and may reduce the risk of AD by lowering Aβ production (Breitner et al., 1994). The Baltimore Longitudinal Study of Aging showed reduced risk for AD

with NSAID use proportional to the duration of use (Stewart et al., 1997). In addition, case control studies of individuals taking NSAIDs for arthritis, a small clinical trial of indomethacin, and a number of other similar studies also indicated protection from development of AD or progression of the disease (Rogers et al., 1993; Andersen et al., 1995). However, a randomized controlled primary prevention trial of NSAIDs in AD, the AD Anti-inflammatory Prevention Trial (ADAPT), was terminated in 2004 due to concerns regarding cardiovascular risks (Shah et al., 2008). As a result of the associated risks, cyclooxygenase-II (COX-2) inhibitors and NSAIDs are currently not recommended for the treatment or prevention of AD.

1.4.5 Diet

Diet may play an important role in the causation and prevention of AD (Solfrizzi et al., 2003; Luchsinger and Mayeux, 2004). The Mediterranean diet (MeDi), has received increased attention in recent years because of converging ecological and interventional evidence relating it to lower risk for cardiovascular disease, several forms of cancer, and overall mortality (Lagiou et al., 1999; de Lorgeril et al., 1999; Singh et al., 2002). The diet is characterized by high intake of vegetables, legumes, fruits, and cereals; high intake of unsaturated fatty acids (mostly in the form of olive oil), low intake of saturated fatty acids; a moderately high intake of fish; a low-to-moderate intake of dairy products (mostly cheese or yogurt); a low intake of meat and poultry; and a regular but moderate amount of ethanol, primarily in the form of wine and generally during meals (Trichopoulou et al., 2003). Therefore, the MeDi appears to include many of the components reported as potentially beneficial for cognitive performance.

1.5 Traditional Medicine

The use of plants as medicine predates written human history and almost all cultures in the world have a body of expertise concerned with the therapeutic properties of local flora (Houghton, 1995). Herbal medicines are an important part of the culture and traditions of people, as many in urban and rural communities, are reliant on them for their health care needs. This is because in addition to their cultural significance, herbal medicines are generally more accessible and affordable (Mander, 1998; Fennell et al., 2004). As a result, there is an increasing trend worldwide, to integrate traditional medicine with primary health care.

Interaction between different cultures has resulted in the expansion of the pharmacopoeia of each group due to the adoption of the plants used by the other. Thus, northern European herbal medicines use many plants that originate from North America (Houghton, 1995). Also, renewed interest in these pharmacopoeias has meant that researchers are concerned not only with determining the scientific rationale for the plant's usage, but also with the discovery of novel compounds of pharmaceutical value. This has enabled scientists to target plants that may be medicinally useful (Cos and Balick, 1994). The last two decades have witnessed remarkable change in attitude toward plants as a source of pharmaceuticals within the scientific and industrial communities, which have been primarily concerned with the search for molecules with new structures and biological activity. By the 19th century, an estimated 122 drugs from 94 plant species had been discovered through ethno-botanical leads (Fabricant and Farnsworth, 2001).

1.6 Plants used traditionally to treat age-related/neurological disorders

Plants have been used since antiquity in traditional medicinal systems for the treatment of memory dysfunction and several other age-related diseases. An ethno-pharmacological approach

and bio-assay guided isolation have provided leads in identifying compounds which are potential AChE inhibitors, inhibitors of Aβ induced cell death, and antioxidants from plant sources, including those for memory disorders which are currently either in clinical use or templates for further drug discovery (Dastmalchi et al., 2007; Mukherjee et al., 2007).

Celastrus paniculatus seeds and oil have been used in Ayurvedic medicine for stimulating intellect and sharpening memory (Nadkarni, 1976; Warrier et al., 1995). Oral administration of the seed oil to rats resulted in a decrease in levels of noradrenaline, dopamine and 5-hydroxytryptamine (5-HT) in the brain, and this correlated with an improvement in the learning and memory process (Nalini et al., 1995). In addition, an antioxidant effect in the CNS observed with the aqueous seed extract, may explain the reputed benefits on memory since it enhanced cognition *in vivo* (Kumar and Gupta, 2002a). Studies on the inflorescences of the plant have shown the methanol extract to have anti-inflammatory effect which may also have some relevance in the management of neurodegenerative disorders (Ahmad et al., 1994).

The leaves of *Centella asiatica* have been used in Ayurvedic medicine for revitalizing and strengthening nervous function and memory. An ayurvedic formulation composed of four herbs, including *C. asiatica*, is used as a restorative and for the prevention of dementia (Manyam, 1999). An alcoholic extract of the plant has been shown to possess tranquilising and potentially cholinomimetic activities *in vivo*, which may be due to the presence of the triterpenoid brahminoside (Sakina and Dandiya, 1990). Also, aqueous extracts of the whole plant enhanced cognitive function in rats, which was associated with the *in vivo* antioxidant activity of the extract (Kumar and Gupta, 2002b). In addition, the essential oil from the plant is reported to contain monoterpenes including β-pinene and γ-terpinene (Brinkhaus et al., 2000), which have demonstrated AChE inhibitory activity (Perry et al., 2000).

The roots of the Indian medicinal plant *Clitoria ternatea*, have been reported to promote intellect (Warrier et al., 1995; Misra, 1998). A study investigating both the aerial parts and the roots of *C. ternatea,* showed its alcoholic root extracts to be more effective in attenuating memory deficits in rats compared to its aerial parts (Taranalli and Cheeramkuzhy, 2000). An aqueous extract of the root was observed to increase ACh levels in rat hippocampus following oral administration, and it was hypothesized that this effect may be due to an increase in ACh synthetic enzymes (Rai et al., 2002; Howes and Houghton, 2003).

Aqueous and ethanol extracts of several plants including *Malvia parviflora*, *Albizia adianthifolia, A. suluensis* and *Crinum moorei*, used in southern Africa to treat memory loss, have been screened for AChE inhibitory activity. The extracts of *C. moorei* showed good activity (Risa et al., 2004; Stafford et al., 2008). Several other species of *Crinum* including *C. campanulatum*, *C. graminicola*, *C. macowanii* and *C. variabile* have also been investigated for inhibition of AChE. Leaves of these plants were found not to be very active while bulbs and roots, were observed to contain several compounds with inhibitory activity (Jäger et al., 2004; Bay-Smidt et al., 2011).

Among the natural phytochemicals identified from plants, flavonoids represent one of the most important and most interesting classes of biologically active compounds. Evidence suggests that flavonoids are effective in the protection of various cell types from oxidative injury (Zou et al., 2010). It has been reported that the antioxidant activities of flavonoids such as quercetin, luteolin and catechins are stronger than the antioxidant nutrients; vitamin C, vitamin E and β-carotene. Flavonoids from *Scutellaria baicalensis,* including baicalein and baicalin, have been reported to reduce the cytotoxicity of Aβ by a reduction of oxidative stress (Heo et al., 2004).

Galanthus species have been used traditionally in Bulgaria and Turkey for neurological conditions (Mukherjee et al., 2007). Galanthamine is an Amaryllidaceae alkaloid first isolated in the 1950s from *Galanthus nivalis* (Shu, 1998). It also occurs in other genera of the Amaryllidaceae family, *Narcissus* spp. and *Lycoris* spp. Galanthamine increases the availability of ACh in the cholinergic synapse by competitively inhibiting the enzyme responsible for its breakdown, AChE. The binding of galanthamine to AChE slows down the catabolism of ACh and, as a consequence, ACh levels in the synaptic cleft are increased (Thomsen et al., 1991a, b; Bores et al., 1996; Heinrich and Teoh, 2004). In addition to amelioration of cholinergic deficit, galanthamine also helps prevent neurodegeneration due to its inherent antioxidant activity, by reducing the toxicity of Aβ and subsequently reducing plaque formation (Eskander et al., 2005). This effect was determined in human neuroblastoma cell cultures which were exposed to Aβ peptide, and where galanthamine (300 nM) reduced cell death significantly (Arias et al., 2004; Geerts, 2005).

Physostigma venenosum has been used traditionally in Africa as a ritual poison, claimed to determine the guilt or innocence of a person accused of a crime. Treatment with the indole alkaloid physostigmine, an AChE inhibitor isolated from *P. venenosum* has been shown to improve cognitive function in several *in vivo* studies (Mukherjee et al., 2007). The chemical structure of physostigmine has provided a template for the development of rivastigmine (Foye et al., 1995), a carbamate derivative that reversibly inhibits the metabolism of AChE in the CNS (Williams et al., 2003). It binds to both the esteratic and ionic sites of AChE, preventing the enzyme from metabolizing ACh (Polinsky, 1998).

Huperzia serrata (Lycopodiaceae) has been used in Chinese traditional medicine for memory impairment (Hostettmann et al., 2006). Huperazine A, a lycopodium alkaloid related to the

quinolizidines and isolated from *H. serrata* is a potent inhibitor of AChE with a long duration of action (Ashani et al., 1992). In a multi-center, double blind trial, huperazine A significantly improved memory and behavior in AD patients, and was reported to be a more selective inhibitor for AChE and less toxic than the synthetic AChE inhibitors, donepezil and tacrine (Raves et al., 1997; Mukherjee et al., 2007). Huperzine A is also a strong antioxidant that has been demonstrated to inhibit Aβ-induced neurotoxicity (Xiao et al., 2002). Studies using cell cultures have shown that huperazine A decreases neuronal death and protects neurons against Aβ-induced apoptosis (Xiao et al., 2002; Hostettmann et al., 2006).

Ginkgo biloba has been used for the improvement of memory loss associated with abnormalities in blood circulation (Samuelsson, 2004). Administration of plant extracts to both AD and non-AD patients in various randomized, double-blind, placebo controlled, multicentre trials resulted in improvement of cognitive function (Hofferberth, 1994; Kanowski et al., 1997; Le Bars et al., 1997; Rigney et al., 1999). Since early pharmacological data revealed that the flavonoids from *G. biloba* modulated contractile motion of vascular smooth muscles, attempts were made to prepare a standardized extract rich in flavonoids, the outcome of which was EGb 761 (Kumar, 2006). EGb 761 showed cognitive enhancing activity in a number of clinical studies (Dastmalchi et al., 2007). The extract also showed neuroprotective effect against Aβ and nitric oxide (NO) induced toxicity in neuronal cell cultures (Bastianetto et al., 2000a, 2000b). Furthermore, apoptosis was reduced both *in vitro* and *in vivo* (Schindowski et al., 2001; Yao et al., 2001) and antioxidant activities were reported (Barth et al., 1991; Marcocci et al., 1994; Topic et al., 2002).

Hypericum perforatum commonly known as St. John's Wort has been used for treatment of neurological disorders (Ross, 2001). The dried crude herb standardized to hypericins was shown to improve memory and learning dysfunction (Widy-Tyszkiewicz et al., 2002; Trofimiuk et al.,

2005). Lu et al. (2001), reported that a standard extract of the plant possessed neuroprotective activity. Hydro-alcoholic extracts of the aerial parts of the plant demonstrated nootropic activity *in vivo*, which may be due to adrenergic (α and β receptor) and serotonergic (5HT1A) antagonistic activity (Khalifa, 2001; Kumar et al., 2000, 2002). In addition, the hydro-alcoholic extract of the plant have also been reported to reduce the rate of degradation of ACh (Re et al., 2003).

Salvia lavandulaefolia (Spanish Sage), has been used for the enhancement of memory (Perry et al., 1998). Volatile oil from the plant showed strong AChE inhibitory activity (Perry et al., 1996), which is likely due to the presence of the cyclic monoterpenes; 1,8-cineole and α-pinene, and other constituents which act synergistically (Perry et al., 2001). Administration of the volatile oil also decreases AChE activity *in vivo* (Perry et al., 2001). An ethanol extract of the plant showed *in vitro* anti-inflammatory activity, while the essential oil resulted in mood elevation and improvement of memory in clinical studies (Perry et al., 2001; Tildesley et al., 2005; Dastmalchi et al., 2007).

Several other plants have been shown in literature to contain cholinesterase inhibitory activity and a list of these plants together with their scientific names, plant part, solvent extract, percentage inhibition and concentration at which the enzyme is inhibited, have been reported in a review article (Adewusi et al., 2010; Appendix A).

Problem Statement

Selective cholinesterase inhibitors, free of dose-limiting side effects, are not currently available, and current compounds may not allow sufficient modulation of acetylcholine levels to elicit the full therapeutic response (Felder et al., 2000). In addition, some of the synthetic medicines used

have been reported to cause gastrointestinal disturbances and problems associated with bioavailability (Melzer, 1998; Schulz, 2003). Therefore, the search for new AChE inhibitors, particularly from natural products, with higher efficacy continues.

1.7 Study Aim

To determine the *in vitro* acetylcholinesterase, antioxidant activity, cytotoxicity and amyloid-β inhibition of selected medicinal plants.

1.8 Objectives

1. To screen selected medicinal plants for AChE inhibitory activity using a TLC and microtiter plate assay based on Ellman's method.
2. To evaluate the antioxidant activity of selected medicinal plants using the DPPH and ABTS radical scavenging assays.
3. To determine the level of phenols and flavonoids in various extracts from the different selected medicinal plants with good antioxidant activity.
4. To isolate active compounds from the plant with promising AChE inhibitory activity using bio-assay guided fractionation.
5. To determine the effect of the isolated compounds and most promising plant extracts on SH-SY5Y cells.
6. To determine the effect of the isolated compounds and most promising plant extracts with good antioxidant activity, on inhibition of β-amyloid induced cell injury in SH-SY5Y cells using the neutral red uptake and MTT assays.

CHAPTER 2: MATERIALS AND METHODS

2.1 Reagents and Chemicals

Acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE) type VI-S from electric eel, 5,5´-dithiobis [2-nitrobenzoic acid] (DTNB), galanthamine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), trolox, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), neutral red dye, dimethyl sulfoxide (DMSO), amyloid beta ($A\beta_{25-35}$), catechin, butylated hydroxytoluene (BHT), ascorbic acid, tannic acid, tris base, fetal bovine serum, sodium chloride, magnesium chloride hexahydrate and quercetin were all purchased from Sigma. Vanillin was procured from BDH chemicals Ltd., whereas Folin-Ciocalteus's phenol reagent and sodium carbonate were obtained from Merck Chemical supplies (Damstadt, Germany). Human neuroblastoma (SH-SY5Y) cells were purchased from American cell type collection culture (ATCC CRL-2266, Rockville, MD, USA). Ham's F-12 medium, fetal calf serum (FCS) and other cell culture reagents were obtained from Gibco Invitrogen Corporation. Methanol and all other organic solvents (analytical grade) were purchased from Merck. All chemicals for UPLC-MS work were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany) while ultra-pure solvents were purchased from Honeywell (Burdick & Jackson, Muskegon, USA). Ultra-pure water was generated from a Millipore Elix 5 RO system and Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France).

2.2 Plant material

2.2.1 Plant collection

Plants were collected from different places all over South Africa. These include the Council for Scientific and Industrial Research (CSIR), Pretoria {plants with voucher numbers starting with 'P'}; the South African National Biodiversity Institute (SANBI), Pretoria {*}; Venda, Limpopo {plants with voucher numbers starting with 'LT'}; Makhado, Limpopo {plants with voucher numbers starting with 'NH'}; and University of Witwatersrand Botanical Gardens, Johannesburg {plant with voucher number starting with 'HANKEY'}. The identities of the plants were confirmed by a botanist. Voucher specimens of plants with voucher numbers starting with 'P' and * were deposited at SANBI, 'LT' at the Herbarium in Onderstepoort, 'NH' at Soutpanbergensis Herbarium and 'HANKEY' at the University of Witwatersrand Herbarium.

Selection was based on their documented ethno-medicinal use in improving memory, to treat insomnia, calm agitated people, and other neurological disorders (Table 2.1). In all, 44 extracts from 17 different plants were screened for activity.

Table 2.1 Plants investigated in the present study with documented ethno-medicinal use in treatment of neurological disorders.

Species	Family	Plant part	Voucher number	Traditional use
Adenia gummifera (Harv.) Harms.	Passifloraceae	Root	NH1912	Infusions made from the root are administered for depression and to treat madness and epilepsy (Bryant, 1966; Gelfand et al., 1985)
Boophane disticha (L.f.) Herb.	Amaryllidaceae	Root and bulb	*	Weak decoctions of bulb scales are given to sedate violent, psychotic patients; bulb infusions are used to treat mental illness (van Wyk and Gericke, 2000; Sobiecki, 2002)
Buddleja salviifolia (L.) Lam.	Buddlejaceae	Whole plant	P01281	*Buddleja* species are used together with *Heteromorpha trifoliate* and *Cussonia paniculata* by Sotho in South Africa to treat early nervous and mental illnesses (Watt and Breyer-Brandwijk, 1962)
Chamaecrista mimosoides L. Greene	Caesalpiniaceae	Root	P08814	Cold water root infusions of *C. mimosoides* are reported to be taken to remember forgotten dreams by Zulu (Hulme, 1954)
Crinum bulbispermum (Burm.f.) Milne-Redh. & Schweick.	Amaryllidaceae	Root and bulb	*	*Crinum* species are used as anticonvulsants (Oloyede and Farombi, 2010)
Ficus capensis Thunb.	Moraceae	Fruits	LT14	*Ficus* species are used to treat epilepsy, and to calm agitated people, and have been studied for behavioural effects in psychotic patients (Hutchings et al., 1996; Gamaliel et al., 2000)
Lannea schweinfurthii (Engl.) Engl.	Anacardiaceae	Root	LT19	Decoctions from the root are used to improve memory and as a sedative (Mabogo, 1990; van Wyk and Gericke, 2000)
Piper capense L.f.	Piperaceae	Root	LT16	Tuber or root reported to cause sleepiness (van Wyk and Gericke, 2000)

Salvia tiliifolia Vahl.	Lamiaceae	Whole plant	P03649	*Salvia* species have been reported to be used for memory-enhancing purposes in European folk medicine (Perry et al., 2003)
Scadoxus puniceus (L.) Friis & I. Nordal.	Amaryllidaceae	Root and bulb	*	Known to cause CNS excitation or depression (Veale et al., 1992)
Schotia brachypetala Sond.	Fabaceae	Root and bark	P06300 (root); P08514 (bark)	The bark and roots of *S. brachypetala* are reported to be used for nervous conditions (van Wyk and Gericke, 2000)
Tabernaemontana elegans Stapf.	Apocynaceae	Root	NH1920	*Tabernaemontana* species have been used as traditional rejuvenation remedies, to improve memory and as a central nervous system stimulant (Taesotikul et al., 1998; Ingkaninan et al., 2003)
Terminalia sericea Burch. ex DC.	Combretaceae	Root	NH1878	Roots are used for general weakness and epilepsy (Hutchings et al., 1996; Gelfand et al., 1985)
Tulbaghia violacea Harv.	Alliaceae	Root and bulb	*	Rhizome infusion is administered for fits (Hutchings et al., 1996)
Xysmalobium undulatum (L.)W.T.Aiton.	Apocynaceae	Root	HANKEY 1653	Roots are administered to treat hysteria (Hutchings et al., 1996)
Zanthoxylum davyi (I. Verd.) P.G. Watermann	Rutaceae	Root	LT4	Used as infusions and decoctions to treat epilepsy and febrile conditions (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996)
Ziziphus mucronata Willd.	Rhamnaceae	Root	NH1909	*Ziziphus* species are used to nourish the heart and calm the spirit. It is often used to aid sleep or calm the mind (Gomes et al., 2009)

*obtained from SANBI, Pretoria

2.2.2 Extract preparation

Plant material was cut into small pieces and air-dried at room temperature. Dried material was ground to a fine powder using an Ika Analytical Mill (Staufen, Germany), and stored at ambient temperature in the dark till use. Six grams of the powdered plant material was extracted with 60 ml of either methanol or ethyl acetate for 24 h while shaking. The extracts were filtered, concentrated using a rotary vacuum evaporator and then further dried *in vacuo* at ambient temperature for 24 h. All extracts were stored at -20 °C prior to analysis. The residues were re-dissolved in either MeOH or ethyl acetate to the desired test concentrations.

Plant specimens obtained from the CSIR were already prepared as an extract in dichloromethane/methanol (1:1) or water. These specimens were diluted to the desired test concentrations using DCM/MeOH (1:1) or water, respectively.

2.3 Determination of acetylcholinesterase inhibition

2.3.1 Micro-plate assay

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method as modified by Eldeen et al. (2005). Three buffers were prepared for the assay; Buffer A (50 mM Tris-HCl, pH 8), Buffer B (50 mM, pH 8, containing 0.1 % bovine serum albumin) and Buffer C (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$). Into a 96-well plate was placed: 25 µl of 15 mM ATCI in water, 125 µl of 3 mM DTNB in Buffer C, 50 µl (72.5 µl for isolated compounds/fractions) of Buffer B and 25 µl (2.5 µl for isolated compounds/fractions) of plant extract. Absorbance was measured spectrophotometrically (Labsystems Multiscan EX type 355 plate reader) at 405 nm every 45 s, three times

consecutively. Thereafter, AChE (0.2 U/ml) was added to the wells and the absorbance measured five times consecutively every 45 s. Galanthamine served as the positive control. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the absorbance before adding the enzyme from the absorbance after adding the enzyme. The percentage inhibition was calculated using the equation:

$$\text{Inhibition (\%)} = 1 - (A_{sample}/A_{control}) \times 100$$

Where A_{sample} is the absorbance of the sample extract and $A_{control}$ is the absorbance of the blank [extraction solvent in Buffer A (50 mM Tris-HCl, pH 8)]. Extract concentration providing 50% inhibition (IC_{50}) was obtained from the graph of the percentage inhibition against extract concentration.

2.3.2 TLC assay

Plant extracts (10 µl), fractions or isolated compounds (3 µl) were applied to TLC plates (silica; 10 cm × 10 cm). After developing, the plates were sprayed with 5 mM ATCI and 5 mM DTNB in 50 mM Tris-HCl buffer, pH 8 until the silica was saturated with the solvent. The plates were then sprayed with 3 U/ml AChE which was dissolved in 50 mM Tris-HCl buffer (pH 8). Galanthamine was used as a positive control, as it is a known AChE inhibitory compound (Heinrich and Teoh, 2004). A yellow background with white spots was considered indicative of AChE inhibiting fractions/compounds.

False-positive reactions were eliminated by the method of Rhee et al. (2003). A TLC plate identical to the one described above was prepared. The developed TLC plate was sprayed with 5 mM DTNB in 50 mM Tris-HCl (pH 8). Plates were then sprayed with 5 mM ATCI and 3 U/ml

AChE in Buffer A. A yellow background with white spots was indicative of false positive reactions.

2.4 Antioxidant activity

2.4.1 DPPH radical scavenging activity

The effect of the extracts on DPPH radical scavenging was estimated using the method of Liyana-Pathirana and Shahidi (2005), with minor modifications. A solution of 0.135 mM DPPH in methanol was prepared and 185 µl of this solution was mixed with 15 µl of varying concentrations of the extract in a 96-well plate. The reaction mixture was vortexed and left in the dark for 30 min (room temperature). The absorbance of the mixture was determined at 570 nm using a microplate reader (Labsystems Multiscan EX type 355 plate reader). Trolox was used as the reference antioxidant compound. The ability to scavenge the DPPH radical was calculated using the equation:

DPPH radical scavenging activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

where $A_{control}$ is the absorbance of DPPH radical + methanol and A_{sample} is the absorbance of DPPH radical + sample extract/standard. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage versus extract concentration.

2.4.2 ABTS radical scavenging activity

The method of Re et al. (1999) was followed for determination of radical scavenging ability. The stock solution which was allowed to stand in the dark for 16 h at room temperature contained equal volumes of 7 mM ABTS salt and 2.4 mM potassium persulfate. The resultant $ABTS^{*+}$

solution was diluted with methanol until an absorbance of 0.706 ± 0.001 at 734 nm was obtained. Varying concentrations of the extract were allowed to react with 2 ml of the $ABTS^{*+}$ solution and the absorbance readings were recorded at 734 nm using a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer. The $ABTS^{*+}$ scavenging capacity of the extract was compared with that of trolox and the percentage inhibition calculated as:

ABTS radical scavenging activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

where $A_{control}$ is the absorbance of ABTS radical + methanol and A_{sample} is the absorbance of ABTS radical + sample extract/standard. All tests were carried out on three separate occasions. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage versus extract concentration.

2.5 Phytochemical screening

The plants which showed good antioxidant activity (>50%) in both assays, were further evaluated for determination of level of phenolic compounds.

2.5.1 Determination of total phenolics

Total phenolic content in the extracts was determined using the modified Folin-Ciocalteu method of Wolfe et al. (2003). The extract (1 mg/ml) was mixed with 5 ml Folin-Ciocalteu reagent (diluted with water 1:10 v/v) and 4 ml (75 g/l) sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40^{o}C for colour development. Absorbance was measured at 765 nm using the Perkin-Elmer UV/Vis Lambda 2 spectrophotometer. Total phenolic content was expressed as mg/g tannic acid equivalent using the equation based on the calibration curve: y = 0.1216x, where x is the absorbance and y is the tannic acid equivalent (mg/g).

2.5.2 Determination of total flavonoids

Total flavonoid content was determined using the method of Ordonez et al. (2006). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample (1 mg/ml). After 1 h incubation period at room temperature, the absorbance was measured at 420 nm using the Perkin-Elmer UV/Vis Lambda 2 spectrophotometer. A yellow colour is indicative of the presence of flavonoids. Total flavonoid content was calculated as quercetin equivalent (mg/g), using the equation based on the calibration curve: y = 0.025x, where x is the absorbance and y is the quercetin equivalent (mg/g).

2.6 Compound isolation

Boophane disticha bulb contained very promising AChE inhibitory activity, was available in sufficient amounts and was therefore selected for isolation of active compounds.

2.6.1 Column chromatography

Different sized columns, ranging from 1.5-8 cm in diameter, were used depending on the amount of sample available and the purification stage. Separation of crude extracts was generally carried out on a column using silica gel 60 (0.063-0.2 mm).

2.6.2 Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out on pre-coated glass plates (Merck, SIL G-25 UV_{254}, 20 cm x 20 cm). 10 µl (1 mg/ml) of the extract was loaded onto the plates and eluted with chloroform: methanol (9:1 or 8:2) for the methanol extracts and hexane: ethyl acetate (9:1 or 8:2) for the ethyl acetate extracts. The plates were first viewed under ultraviolet (UV) light (short-

wave 254 nm and long-wave 366 nm) and then sprayed with a vanillin-H_2SO_4 (1 g vanillin in 100 ml H_2SO_4) solution, and heated at 100ºC for few minutes, as a general qualitative test to detect the compounds present. The TLC plates were also sprayed with Dragendorff's reagent to detect the presence of alkaloids.

2.6.3 Preparative thin-layer chromatography

Compounds which were visible under UV light were further purified using preparative TLC. The glass plates were lined with the sample, 1.5 cm from the bottom of the plate. The samples were loaded onto the plates using a Pasteur pipette, from one end of the plate to the other. Plates were developed using the solvent systems described above (2.6.2). Compounds of interest were visualized under UV light. The bands were scraped off, dissolved in methanol or ethyl acetate and then filtered to remove the silica gel from the filtrate.

2.6.4 Isolation of compounds from the methanol extract

The methanol extract (1.4 g) was subjected to silica gel column chromatography (66.7 g; particle size 0.063 - 0.2 mm). The separation and purification was carried out using a stepwise gradient mixture of chloroform, $CHCl_3$: MeOH starting from 100:0 until 70:30 as eluent to give 50 fractions. Fractions were collected every 5 min at a rate of 1 ml/min. The fractions were pooled together based on the similarity in their R_f values on a thin-layer chromatography plate to give four sub-fractions. Each sub-fraction was tested for their inhibition of AChE on the TLC plate as described in section 2.3.2 above. Sub-fractions 2 (3-16) and 3 (17-21), were the only active sub-fractions.

Sub-fraction 2 was further chromatographed on a silica gel column using a stepwise gradient mixture of $CHCl_3$: MeOH starting from 95:5 until 75:25 as eluent to give another set of 30 fractions and tested for activity. Of this sub-fraction, only fractions 1, 4 and 8 were active.

Sub-fraction 3 was also further chromatographed on a silica gel column using a stepwise gradient mixture of $CHCl_3$: MeOH starting from 95:5 until 75:25 as eluent, to give 23 fractions which were also tested for activity. Fractions 5, 14, 15 and 16 were active and had similar R_f values as fractions 1, 4 and 8 from sub-fraction 2, and so these seven fractions were combined and further purified. Preparative TLC yielded **compound 1** or **EAM 3,16 1-4** (20 mg). Compound 1 was analysed using the UPLC-QTOF (mass spectroscopy determination) and Nuclear Magnetic Resonance spectroscopy (1D and 2D experiments) for its structure determination.

Fractions 21 and 22 from sub-fraction 3 were combined as they had similar R_f values and showed activity for inhibition of AChE on the TLC plate. However, the yield (0.5 mg) was very low and this fraction could not be purified further. It was named **EAM 17-21 21,22** and reserved for the quantitative assay.

A schematic diagram of the fractionation and isolation of the methanol extract is provided in Figure 2.1.

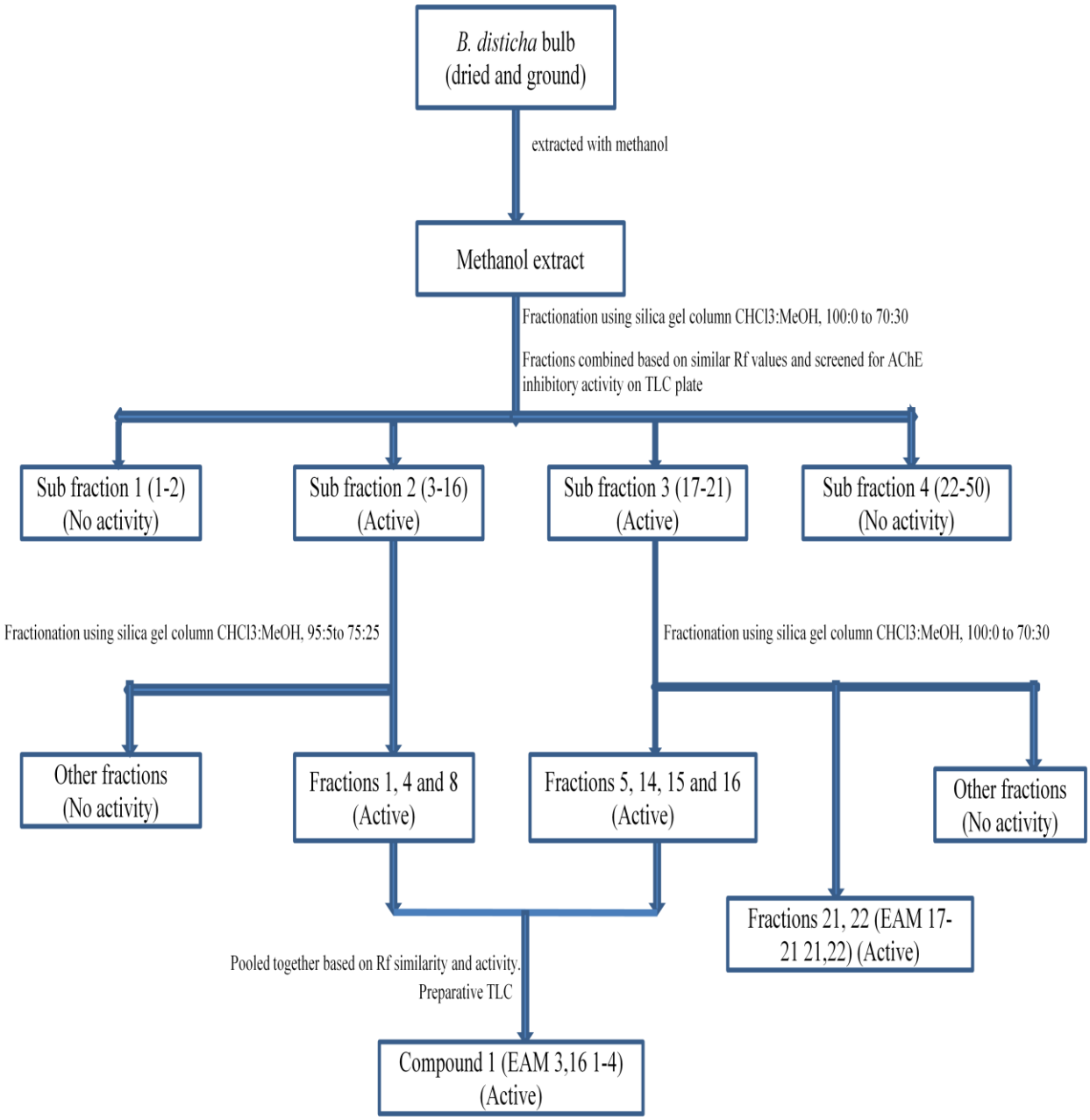


Figure 2.1 Schematic diagram of the fractionation and isolation of compounds from the methanol extracts of *Boophane disticha*.

2.6.5 Isolation of compounds from the ethyl acetate extract

The ethyl acetate extract (1.4 g) was subjected to silica gel column chromatography (65 g; particle size 0.063 - 0.2 mm). The separation and purification was done using a stepwise gradient mixture of hexane: ethyl acetate starting from 100:0 until 0:100 as eluent to give 70 fractions. Fractions were collected every 5 min at a rate of 1 ml/min. The fractions were pooled together based on the similarity in their R_f values on a thin-layer chromatography plate to give four sub-fractions. All four sub-fractions were tested for activity and only sub-fraction 2 (20-41) was active.

Sub-fraction 2 was subjected to further silica gel column chromatographic purification and subsequently eluted using a stepwise gradient mixture of hexane: ethyl acetate, starting from 90:10 until 0:100, to give another set of 120 fractions. These fractions were pooled together based on the similarity in their R_f values on a thin-layer chromatography plate and again tested for activity. Fraction 104 (EAE 104) showed a major spot on TLC but it was not active in the AChE inhibition assay, and the NMR data showed it to be impure so structural elucidation could not be carried out. Furthermore, the yield obtained was very low (0.2 mg), making further purification impossible.

Fractions 82-100 were active and were combined and loaded onto a column for further purification and isolation of the compounds. Compounds were eluted using a stepwise gradient mixture of hexane: ethyl acetate, starting from 80:20 until 0:100. Fraction 1 obtained from this separation was found to be a pure and active compound (**compound 2** or **EAE 1** or **F1**). However, this compound was very unstable, degraded, and lost its activity before NMR analysis was done. Fraction 9 (**compound 3** or **EAE 9**) was found to be pure, but inactive. This fraction

was further analysed using the UPLC-QTOF (mass spectroscopy determination) and Nuclear Magnetic Resonance spectroscopy (1D and 2D experiments) for its structure elucidation. Fraction 11 (**EAE 11**) was active for inhibition of AChE, but could not be further purified because of its very low yield (0.4 mg). However, it was reserved for the quantitative assay.

A schematic diagram of the fractionation and isolation of the ethyl acetate extract is provided in Figure 2.2.

The active compound and fractions were evaluated quantitatively, for their AChE inhibitory activity using the method described in section 2.3.1.

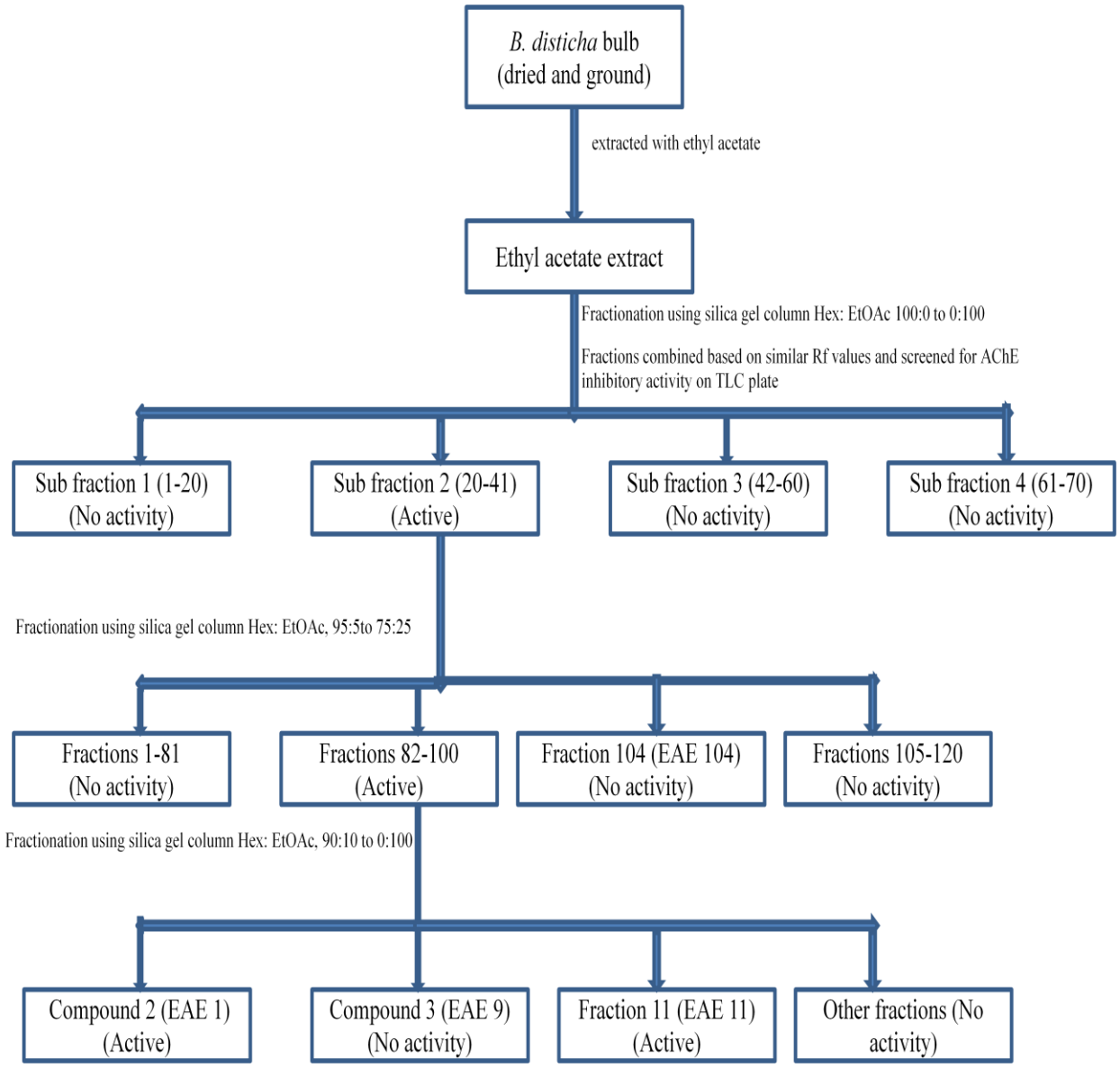


Figure 2.2 Schematic diagram of the fractionation and isolation of compounds from the ethyl acetate extracts of *Boophane disticha*.

2.7 Structural elucidation of compounds

2.7.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy was performed using a 600 MHz Varian NMR. All spectra were recorded at room temperature in either deuterated chloroform or deuterated methanol. The chemical shifts were reported in ppm relative to tetramethylsilane (TMS). Structural characterizations were carried out using a combination of 1D (^{1}H, ^{13}C) and various 2D (Distortionless Enhancement by Polarisation Transfer - DEPT, Correlation Spectroscopy - COSY, Heteronuclear Single Quantum Coherence - HSQC and Heteronuclear Multiple Bond Correlation - HMBC) experiments.

2.7.2 Mass Spectrometry

The various samples were analysed using a WATERS 2695 HPLC separation module. Two Atlantis T3 columns (10 x 250 mm, 5 µ particle size) connected in series were used for the separation. UV-VIS detection was done on a WATERS PDA scanning from 200 – 600 nm. Mass spectrometry detection was performed using a WATERS SQD scanning from 100 – 1200 *m/z* with polarity (+/-) switching.

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 3 mDalton. The mass spectrometer was operated in positive and negative mode with a capillary voltage of 2.0 kV, the sampling cone at 30 V and the extraction cone at 4 V. The scan time was 0.1 s covering the 100 to 1000 Dalton mass range. The source temperature was 120ºC and the desolvation temperature was set at 450ºC. Nitrogen gas was used as the

nebulisation gas at a flow rate of 800 L/h. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.1 (SCN 704).

2.8 Cytotoxicity studies

Compounds and fractions isolated from the bulbs of *B. disticha*, together with four very promising plants {*Ziziphus mucronata* (roots), *Lannea schweinfurthii* (roots), *Terminalia sericea* (roots) and *Crinum bulbispermum* (roots)}, selected based on their good antioxidant activity, were further investigated for their cytotoxicity and determination of their ability to prevent cell death induced by $A\beta_{25-35}$. *Piper capense* had good antioxidant activity but was not selected for further studies as the extracts were very toxic to the SH-SY5Y cells (results not shown). Methanol extracts of these plants were prepared as described in section 2.2.2. Residues were re-dissolved in DMSO to the desired test concentrations.

2.8.1 Cells and cell culture

SH-SY5Y (ATCC CRL-2266) cell lines were used for the cytotoxicity studies. Ethical clearance was obtained to carry out studies on the commercially purchased cell line (Appendix D). Cells were cultured in Ham's F-12 supplemented with 2% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37^{o}C in a humidified incubator at 95% air and 5% CO_2. For use in the assay, the cells were trypsin-treated for 10 min, decanted from culture flasks and centrifuged (200*g*, 10 min). The pellet was re-suspended in 1 ml FCS-supplemented Ham's F-12 medium and enumerated by staining with trypan blue. The cells were diluted to a concentration of 1×10^5 cells/well in Ham's F-12 medium, and 100 µl of the cell suspension plated into each of the wells of a 96-well microtiter plate. 80 µl of Ham's F-12

medium was added and plates were then incubated for 1 h at 37°C in a humidified incubator at 95% air and 5% CO_2 to allow for cellular reattachment.

2.8.2 Cell viability

2.8.2.1 MTT assay

The MTT assay as described by Mossmann (1983) was used to measure cell viability. The principle of the assay is based on generation formazan (a blue product), in the mitochondria of active cells which is measured by photometric techniques (Hansen et al., 1989). The cells were plated into 96-well culture plates, as described in section 2.8.1 above, and treated with various concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml) of the plant extracts for 72 h. Thereafter, 20 µl of MTT solution (5 mg/ml) was added to the wells and further incubated for 3 h. 50 µl of solution containing 30% (w/v) *N*,*N*- dimethylformamide and 20% SDS in water was then added to breach the cells and dissolve the formazan crystals. The plates were incubated overnight at 37°C, after which absorbance was measured at 570-630 nm using a microtiter plate reader (Labsystems Multiscan EX type 355 plate reader). Wells without cells were used as blanks and were subtracted as background from each sample. Cell viability was expressed as a percentage of the control values. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage versus extract concentration.

2.8.2.2 Neutral red assay

The neutral red uptake assay as described by Borenfreund and Puerner (1984), was also used to assess cell viability. This method is based on the determination of the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells. After treatment and incubation of the cells (as

described above for the MTT assay), 150 µl of neutral red dye (100 µg/ml) dissolved in serum free medium (pH 6.4), was added to the culture medium for 3 h at 37°C. Cells were washed with Phosphate Buffered Saline (PBS), and 150 µl of elution medium (EtOH/AcCOOH, 50%/1%) was added followed by gentle shaking for 60 min, so that complete dissolution could be achieved. Absorbance was recorded at 540-630 nm using a microtiter plate reader (Labsystems Multiscan EX type 355 plate reader). Cell viability was expressed as a percentage of the control values. A graph of percentage cell viability against extract concentration was plotted, and extract concentration providing 50% inhibition (IC_{50}) of cell death was calculated from the graph.

2.8.3 Treatment with $A\beta_{25-35}$

$A\beta_{25-35}$ was reconstituted in sterile water at a concentration of 1 mM. Aliquots were incubated at 37°C for 72 h to form aggregated amyloid. During the experiment, $A\beta_{25-35}$ was directly added to cultured medium to achieve a final concentration of 20 µM. Three concentrations of each of the plant extracts, compound or fractions that were not toxic or presented low toxicity (as determined from the tests above), were selected to assess their protective effects. The cells were plated as described above, pre-treated with the plant extracts for 2 h before $A\beta_{25-35}$ treatment and then further incubated for 72 h. The viability of the cells was determined by the MTT and neutral red assays. Cell viability was expressed as a percentage of the control values.

2.9 Statistical analysis

Tests were carried out where possible at least in triplicate and on three different occasions. The results are reported as mean ± standard deviation (S.D.). Standard curves were generated and calculation of the 50% inhibitory concentration (IC_{50}) values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc.). Cytotoxicity results are expressed as the

percentage cell survival compared to the untreated control using a dose response curve. The curve was created and IC_{50} calculated using GraphPad Prism. Data obtained from mass spectroscopy were analysed using MassLynx 4.1 (SCN 704) software and the fragmentation patterns of the compounds isolated were identified with the Agilent ChemStation software which has a National Institute of Standards and Technology (NIST) library of mass fragmentations.

CHAPTER 3: RESULTS

3.1 Acetylcholinesterase inhibitory activity

The results of the plant extracts showing moderate to good inhibition of AChE in various solvents, are provided in Figures 3.1 and 3.2. At the highest concentration tested, 50% showed good (>50% inhibition), 45.5% showed moderate (30-50% inhibition) and 4.5% low (<30% inhibition) AChE inhibition. Percentage activity as described by Vinutha et al. (2007) was used. Generally, inhibition was dose-dependent and the higher activity of the organic extracts may suggest that organic solvents are able to extract more active compounds with possible AChE inhibitory activity than water. No false positive reactions were observed with the assay on the TLC plates (results not shown).

The IC_{50} values of the plant extracts indicating AChE inhibitory activity are presented in Table 3.1. Ethyl acetate extracts of the roots of *C. bulbispermum*, *X. undulatum*, *L. schweinfurthii*, *S. puniceus* and bulbs of *B. disticha* all had very low IC_{50} values. Although the IC_{50} of these plant extracts were higher than that of galanthamine (0.00053 mg/ml), they possess good AChE inhibitory activity considering they are still mixtures containing various compounds.

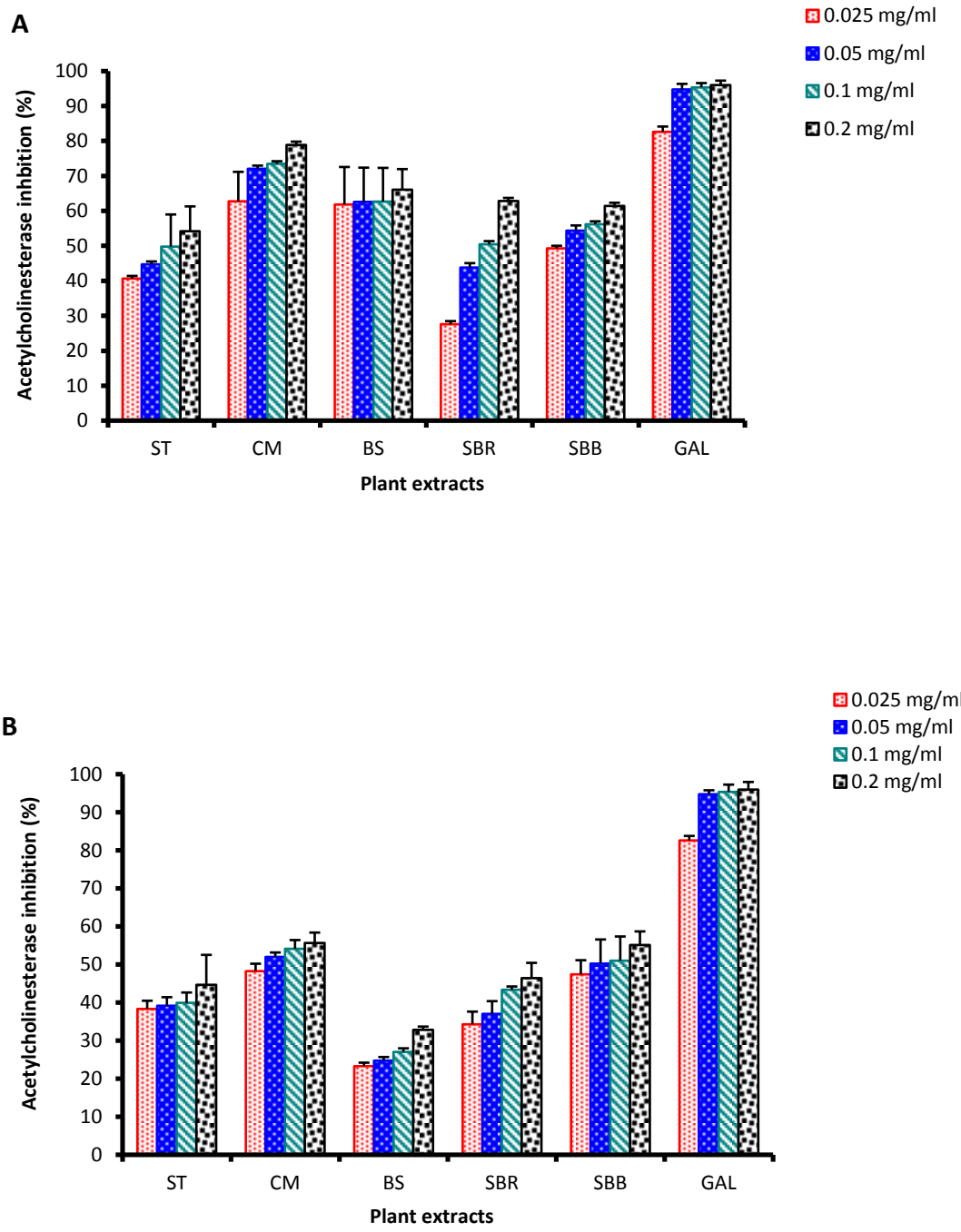


Figure 3.1 AChE inhibitory activity (%) of (A) DCM: MeOH (1:1) extracts and (B) water extracts of plants with moderate to good activity. ST, *Salvia tiliifolia* (whole plant); CM, *Chamaecrista mimosoides* (root); BS, *Buddleja salviifolia* (whole plant); SBR, *Schotia brachypetala* (root); SBB, *Schotia brachypetala* (bark); GAL, Galanthamine (positive control).

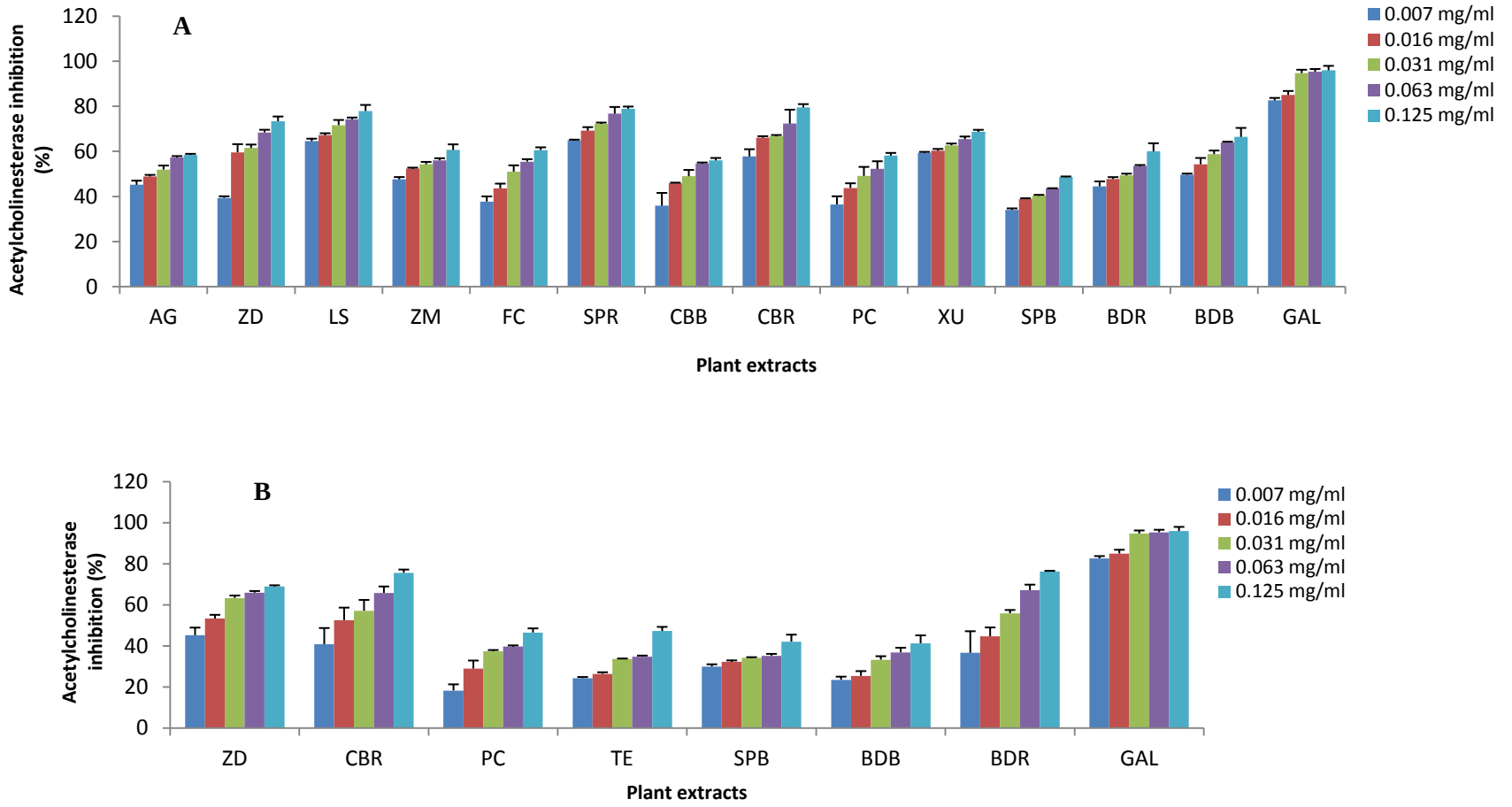


Figure 3.2 AChE inhibitory activity (%) of (A) ethyl acetate extracts and (B) methanol extracts, of plants with moderate to good activity. AG, *Adenia gummifera* (root); ZD, *Zanthoxylum davyi* (root); LS, *Lannea schweinfurthii* (root); ZM, *Ziziphus mucronata* (root); FC, *Ficus capensis* (fruit); SPR, *Scadoxus puniceus* (root); SPB, *S. puniceus* (bulb); CBB, *Crinum bulbispermum* (bulb); CBR, *C. bulbispermum* (root); PC, *Piper capense* (root); XU, *Xysmalobium undulatum* (root); BDR, *Boophane disticha* (root); BDB, *B. disticha* (bulb); *Tabanaemontana elegans* (root); GAL, Galanthamine (positive control).

Table 3.1 Acetylcholinesterase inhibitory activity of the plant extracts as represented by their IC_{50} values.

Species	Plant part analyzed	Extraction solvent	AChE inhibition IC_{50} (mg/ml)
Salvia tiliifolia	Whole plant	DCM:MeOH (1:1)	1.0000 ± 0.010
		Water	12.0000 ± 1.200
Chamaecrista mimosoides	Root	DCM:MeOH (1:1)	0.0300 ± 0.080
		Water	0.3500 ± 0.020
Buddleja salviifolia	Whole plant	DCM:MeOH (1:1)	0.0500 ± 0.020
		Water	*
Schotia brachypetala	Root	DCM:MeOH (1:1)	0.8900 ± 0.010
		Water	3.4000 ± 0.500
	Bark	DCM:MeOH (1:1)	0.2700 ± 0.070
		Water	0.4900 ± 0.040
Adenia gummifera	Root	Methanol	*
		Ethyl acetate	0.0189 ± 0.005
Piper capense	Root	Methanol	*
		Ethyl acetate	0.0407 ± 0.012
Zanthoxylum davyi	Root	Methanol	0.0100 ± 0.004
		Ethyl acetate	0.0116 ± 0.002
Xysmalobium undulatum	Root	Methanol	*
		Ethyl acetate	0.0050 ± 0.000
Lannea schweinfurthii	Root	Methanol	*
		Ethyl acetate	0.0030 ± 0.000
Terminalia sericea	Root	Methanol	*
		Ethyl acetate	*
Ziziphus mucronata	Root	Methanol	*
		Ethyl acetate	0.0112 ± 0.003
Tabernaemontana elegans	Root	Methanol	*
		Ethyl acetate	*
Ficus capensis	Fruits	Methanol	*
		Ethyl acetate	0.0319 ± 0.005
Scadoxus puniceus	Bulb	Methanol	*
		Ethyl acetate	*
	Root	Methanol	*
		Ethyl acetate	0.0030 ± 0.000
Crinum bulbispermum	Bulb	Methanol	*
		Ethyl acetate	0.0393 ± 0.014
	Root	Methanol	0.0148 ± 0.039
		Ethyl acetate	0.0021 ± 0.007
Boophane disticha	Bulb	Methanol	*
		Ethyl acetate	0.0073 ± 0.002

	Root	Methanol	0.0199 ± 0.009
		Ethyl acetate	0.0230 ± 0.007
Tulbaghia violacea	Bulb	Methanol	*
		Ethyl acetate	*
	Root	Methanol	*
		Ethyl acetate	*
Control			
Galanthamine			$5.3 \times 10^{-4} \pm 1.0 \times 10^{-5}$

* represents extracts with maximum inhibition below 50% at the highest concentration tested

3.2 Antioxidant activity and polyphenolic content

The dose-dependent ABTS and DPPH radical scavenging activity of the plant extracts with good antioxidant activity is depicted in Figures 3.3 and 3.4, respectively. Antioxidant activity is expressed as a percentage of the ratio of the decrease in absorbance of the test solution to that of ABTS or DPPH solution without the plant extracts. The plants with good activity showed a propensity to quench the free radicals, as indicated by the dose-dependent increase in percentage inhibition. The ethyl acetate extracts of all the plants with the exception of *T. sericea* showed either no activity or very low radical scavenging activity in both the DPPH and ABTS assays. This may indicate that solvents of high polarity are able to extract more antioxidant compounds than intermediate polar solvents, for the plants investigated in the study.

The IC_{50} values (concentration of the extract that is able to scavenge half of the DPPH or ABTS radical) are presented in Table 3.2. The DCM: MeOH (1:1) extracts of the root of *S. brachypetala* and the methanol extract of the root of *L. schweinfurthii* and *T. sericea* showed the highest radical scavenging activity.

The plant extracts which showed good antioxidant activity (>50%), in both assays, were further screened for determination of the level of total phenols and flavonoids (Table 3.3). All these extracts contained phenols, with the highest amounts in the DCM: MeOH (1:1) and water extracts of *S. brachypetala* root and bark, and methanol extract of *P. capense* root. The plant extracts also contained some flavonoids with the highest found in methanol extracts of *T. sericea* roots.

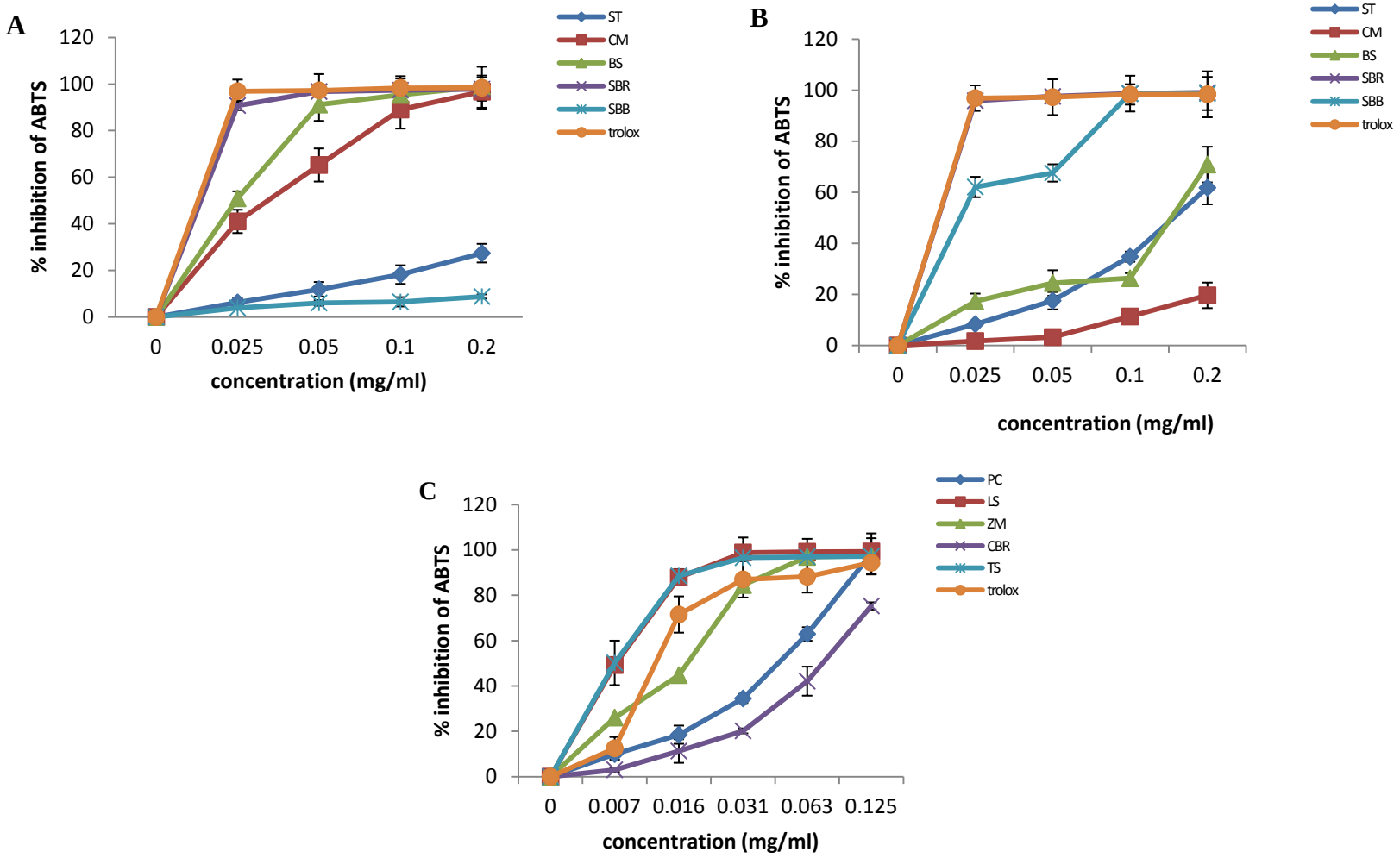


Figure 3.3 ABTS radical scavenging activity of (A) DCM: MeOH (1:1) extracts, (B) water extracts, and (C) methanol extracts, of plants with good activity. ST, *Salvia tiliifolia* (whole plant); CM, *Chamaecrista mimosoides* (root); BS, *Buddleja salviifolia* (whole plant); SBR, *Schotia brachypetala* (root); SBB, *S. brachypetala* (bark); PC, *Piper capense* (root); LS, *Lannea schweinfurthii* roots; ZMM, *Ziziphus mucronata* roots; CBR, *Crinum bulbispermum* (roots); TS, *Terminalia sericea* roots; trolox (positive control).

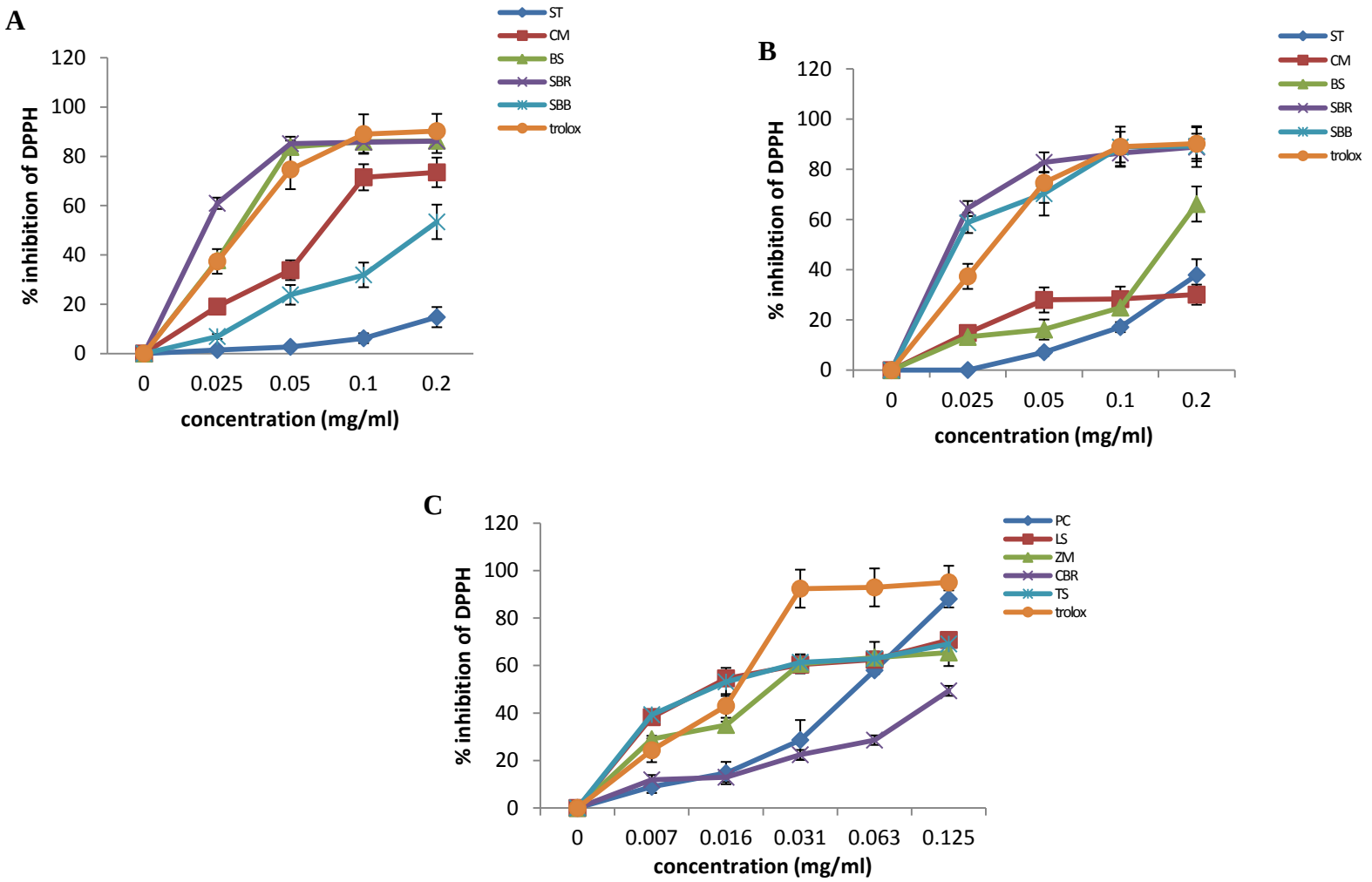


Figure 3.4 DPPH radical scavenging activity of (A) DCM: MeOH (1:1) extracts, (B) water extracts and (C) methanol extracts, of plants with good activity. ST, *Salvia tiliifolia* (whole plant); CM, *Chamaecrista mimosoides* (root); BS, *Buddleja salviifolia* (whole plant); SBR, *Schotia brachypetala* (root); SBB, *Schotia brachypetala* (bark); PC, *Piper capense* (root); LS, *Lannea schweinfurthii* roots; ZM, *Ziziphus mucronata* roots; CBR, *Crinum bulbispermum* (roots); TS, *Terminalia sericea* roots; trolox (positive control).

Table 3.2 Antioxidant activity of the plant extracts as represented by their IC_{50} values.

Species	Plant part analyzed	Extraction solvent	ABTS radical inhibition IC_{50} (mg/ml)	DPPH radical inhibition IC_{50} (mg/ml)
Salvia tiliifolia	Whole plant	DCM:MeOH (1:1)	*	*
		Water	1.5100 ± 0.230	*
Chamaecrista mimosoides	Root	DCM:MeOH (1:1)	0.3000 ± 0.050	0.7200 ± 0.030
		Water	*	*
Buddleja salviifolia	Whole plant	DCM:MeOH (1:1)	0.1400 ± 0.080	0.2300 ± 0.010
		Water	1.0000 ± 0.050	1.6000 ± 0.510
Schotia brachypetala	Root	DCM:MeOH (1:1)	$3.2600 \times 10^{-7} \pm 0.100 \times 10^{-9}$	0.0500 ± 0.020
		Water	$3.7000 \times 10^{-7} \pm 0.210 \times 10^{-9}$	0.0500 ± 0.020
	Bark	DCM:MeOH (1:1)	*	1.9000 ± 0.500
		Water	0.1500 ± 0.030	0.1300 ± 0.030
Adenia gummifera	Root	Methanol	*	*
		Ethyl acetate	*	*
Piper capense	Root	Methanol	0.0402 ± 0.003	0.0443 ± 0.010
		Ethyl acetate	*	*
Zanthoxylum davyi	Root	Methanol	0.0752 ± 0.021	*
		Ethyl acetate	*	*
Xysmalobium undulatum	Root	Methanol	*	*
		Ethyl acetate	*	*
Lannea schweinfurthii	Root	Methanol	0.0076 ± 0.001	0.0151 ± 0.004
		Ethyl acetate	*	*
Terminalia sericea	Root	Methanol	0.0071 ± 0.001	0.0147 ± 0.006
		Ethyl acetate	0.0746 ± 0.017	*
Ziziphus mucronata	Root	Methanol	0.0187 ± 0.020	0.0291 ± 0.051
		Ethyl acetate	*	*
Tabernaemontana elegans	Root	Methanol	*	*
		Ethyl acetate	*	*
Ficus capensis	Fruits	Methanol	*	*
		Ethyl acetate	*	*
Scadoxus puniceus	Bulb	Methanol	*	*
		Ethyl acetate	*	*
	Root	Methanol	*	*
		Ethyl acetate	*	*

Crinum bulbispermum	Bulb	Methanol	*	*
		Ethyl acetate	*	*
	Root	Methanol	0.0685 ± 0.041	*
		Ethyl acetate	*	*
Boophane disticha	Bulb	Methanol	*	*
		Ethyl acetate	*	*
	Root	Methanol	0.0913 ± 0.017	*
		Ethyl acetate	*	*
Tulbaghia violacea	Bulb	Methanol	*	*
		Ethyl acetate	*	*
	Root	Methanol	*	*
		Ethyl acetate	*	*
Control				
Trolox			0.1310 ± 0.0050	$0.96 \times 10^{-4} \pm 0.40 \times 10^{-6}$

* represents extracts with maximum inhibition below 50% at the highest concentration tested

Table 3.3 Total phenol and flavonoid contents of plant extracts with antioxidant activity (>50%) in both DPPH and ABTS assays.

Species	Plant part analyzed	Extraction solvent	Total Phenols[a]	Total Flavonoids[b]
Chamaecrista mimosoides	Root	DCM:MeOH (1:1)	141.53 ± 0.21	16.86 ± 0.35
		Water	64.16 ± 0.13	5.32 ± 0.38
Buddleja salviifolia	Whole plant	DCM:MeOH (1:1)	169.66 ± 0.33	23.95 ± 0.11
		Water	77.92 ± 0.91	12.11 ± 0.26
Schotia brachypetala	Root	DCM:MeOH (1:1)	303.91 ± 0.92	4.24 ± 0.23
		Water	291.80 ± 0.12	13.44 ± 0.08
	Bark	DCM:MeOH (1:1)	305.52 ± 0.21	10.97 ± 0.17
		Water	337.66 ± 0.12	17.71 ± 0.54
Crinum bulbispermum	Root	Methanol	202.38 ± 5.03	9.18 ± 0.50
Piper capense	Root	Methanol	237.60 ± 11.69	18.14 ± 1.97
Terminalia sericea	Root	Methanol	36.73 ± 2.07	73.05 ± 4.70
Lannea schweinfurthii	Root	Methanol	101.27 ± 1.60	13.58 ± 3.52
Ziziphus mucronata	Root	Methanol	73.86 ± 2.52	17.76 ± 2.29

[a]Expressed as mg tannic acid/g of extract

[b]Expressed as mg quercetin/g of extract

3.3 Isolation of compounds from *Boophane disticha*

Two compounds were isolated from the bulbs of *B. disticha*; 6-hydroxycrinamine from the methanol extract and cycloeucalenol from the ethyl acetate extract. In addition, two fractions which were active but could not be purified further because of their very low yield, were reserved and together with the isolated compounds, were screened for AChE inhibition. Studies on the cytotoxicity of the compounds and fractions were also carried out (section 3.4.1).

3.3.1 Structural elucidation of compound 1 (6 – hydroxycrinamine)

Compound 1 (6-hydroxycrinamine, $C_{17}H_{19}NO_5$), was isolated from the methanol extract of the bulbs of *B. disticha*. It was isolated as yellow crystals. The MS chromatogram (Figure 3.5; Appendix E: Figure 8), shows the (M + 1) ion at *m/z* 318 which corresponds to the reported molecular weight of 6-hydroxycrinamine (Viladomat et al., 1996). The fragment (M + 1 – 32) at *m/z* 286 corresponds to the loss of methanol, which confirms the presence of a methoxy group in the molecule. The molecular fragment ion peaks were determined as shown in Figure 3.5, (M + 1, $C_{17}H_{20}NO_5$) at *m/z* 318 with 300 (M + 1 – H_2O), 286 (– CH_4O), 268 (– CH_6O_2), 250 (– CH_8O_3), 240 (– $C_2H_6O_3$), 227 (– $C_4H_{11}O_2$) and 199 (– $C_5H_{11}O_3$).

The compound is very polar and was dissolved in deuterated methanol for NMR analysis (^{1}H, ^{13}C and 2D experiments). The ^{1}H and ^{13}C NMR (Appendix E: Figure 1 and 2), provided further evidence for the structure of the isolated compound. The NMR data was compared to that of the published data on 6-hydroxycrinamine (Viladomat et al., 1996), and the comparison is given in Table 3.4 (^{1}H NMR) and Table 3.5 (^{13}C NMR). The signals obtained from both NMR spectra were complex suggesting that compound 1 was a mixture of two epimers, epimer A (6α-hydroxycrinamine) (**3.1**) and epimer B (6β-hydroxycrinamine) (**3.2**) (Figure 3.6). From the data

obtained and comparison with literature data, the two epimers could be identified in the NMR spectra (Figure 3.6).

3.3.2 Structural elucidation of compound 3, 24-methylenecycloartan-3β-ol (cycloeucalenol)

Compound 3 (cycloeucalenol, $C_{30}H_{50}O$), was isolated from the ethyl acetate extracts of the bulbs of *B. disticha* as white crystals. The MS chromatogram (Figure 3.7; Appendix E: Figure 14), shows the (M + 1) ion at *m/z* 427, which corresponds to the reported molecular weight of cycloeucalenol (Deng et al., 2009). The molecular fragment ion peaks were determined (M + 1, $C_{30}H_{50}O$) at *m/z* 427 with 409 (M + 1 – H_2O), 343 (– C_6H_{12}), 327 (– $C_6H_{12}O$), 285 (– $C_9H_{18}O$), 219 (– $C_{14}H_{24}O$), 177 (– $C_{17}H_{30}O$) and 163 (– $C_{18}H_{32}O$).

The compound is non-polar and was dissolved in deuterated chloroform for NMR analysis (^1H, ^{13}C and 2D experiments). The NMR spectra of the compound after comparison with reported literature data confirmed it to be cycloeucalenol, as the major compound, together with its stereo-isomer. The two compounds co-chromatographed together and were thus not possible to separate with the available solvent systems. Similarly, Knapp and Nicholas (1970), isolated both compounds as a mixture and the authors were unable to separate the compounds. Cycloeucalenol and its stereo-isomer have the same skeletal structure (**3.3**). The only difference in their structures is in the side chain (**3.3a** and **3.3b**) (Figure 3.8). Cycloeucalenol has a double bond between position C-24 and C-30 while its stereo-isomer has a double bond between C-25 and C-27. The NMR data was compared to that of the published data on cycloeucalenol (Liu et al., 2011), and the comparison is given in Table 3.6 (^1H NMR) and Table 3.7 (^{13}C NMR). In addition, the data obtained for the ^{13}C-NMR spectrum of the stereo-isomer on the side-chain, also compares with data reported by Akihisa et al. (1997).

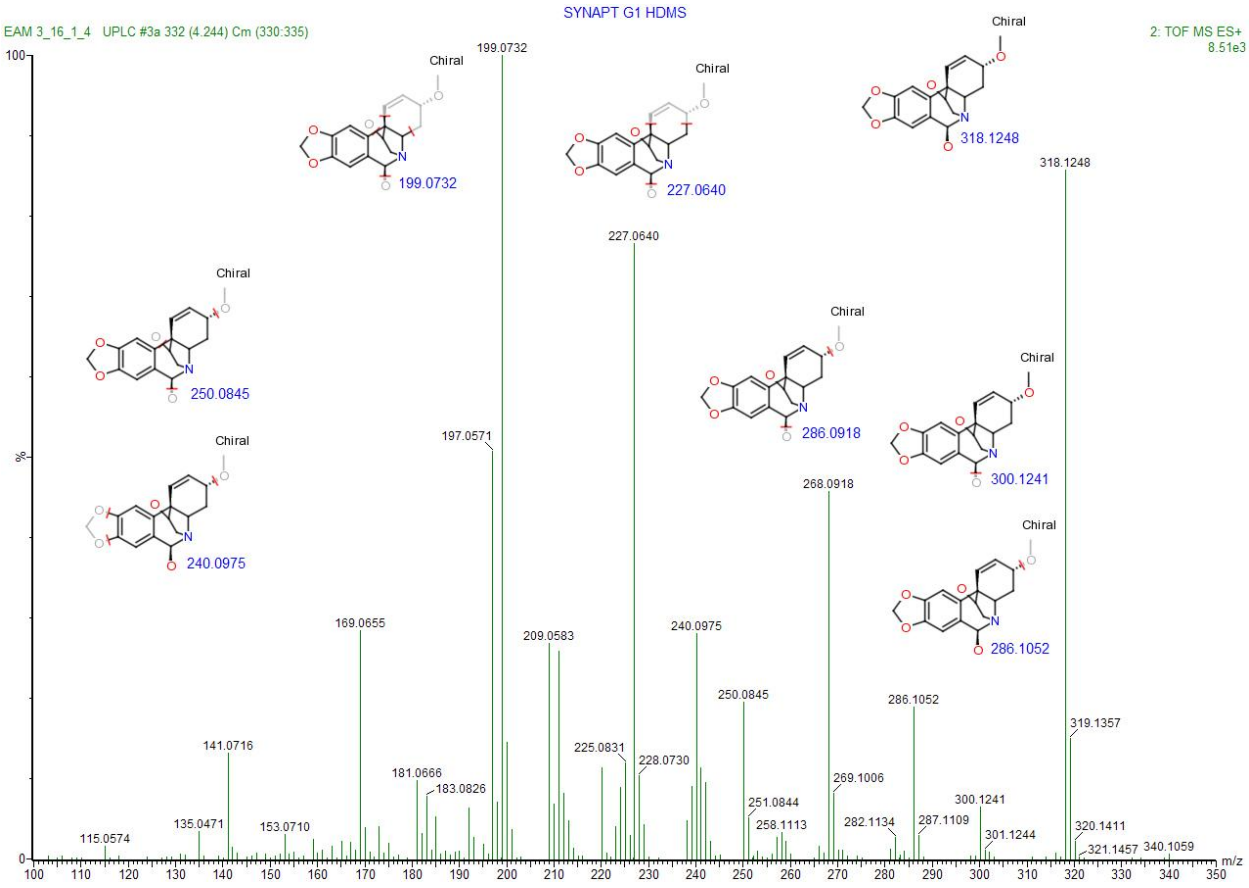
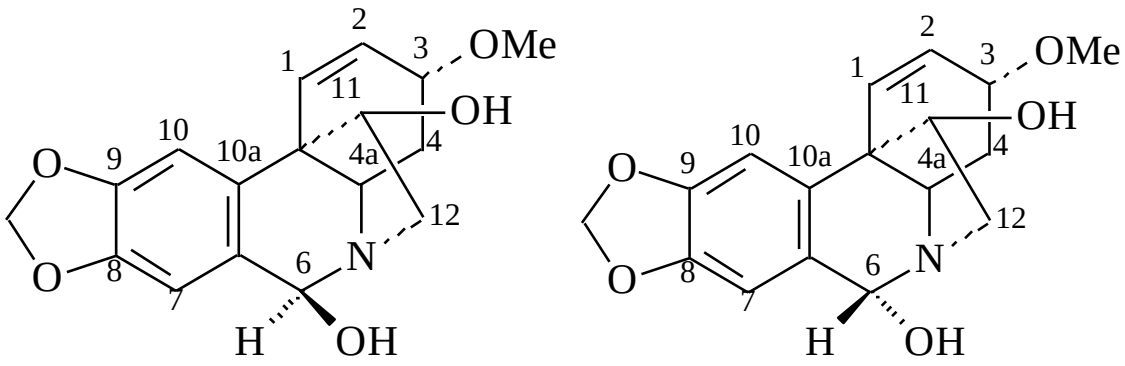


Figure 3.5 HRTOFMS (ESI^+) spectra for compound 1 (6-hydroxycrinamine).



Epimer A (6α-hydroxycrinamine) (**3.1**) Epimer B (6β-hydroxycrinamine) (**3.2**)

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

Table 3.4 ^{1}H NMR data for 6-hydroxycrinamine in methanol-d_4 (CD_3OD) compared to literature.

H	**6α-hydroxycrinamine (Epimer A), δ_H (*J* in Hz)**	**Reference compound A*, δ_H (*J* in Hz)**	**6β-hydroxycrinamine (Epimer B), δ_H (*J* in Hz)**	**Reference compound B*, δ_H (*J* in Hz)**
1	6.22 *m*	6.23 *d* (10.5)	6.22 *m*	6.21 *d* (10.5)
2	6.22 *m*	6.19 *dd* (10.5, 2.0)	6.22 *m*	6.17 *dd* (10.5, 1.5)
3	4.02 *m*	4.02 *ddd* (9.0, 6.5, 2.0)	4.02 *m*	3.96 *ddd* (10.5, 6.0, 1.5)
4α	2.14 *m*	2.11 *ddd* (13.0, 12.5, 9.1)	2.26 *m*	2.26 *ddd* (13.5, 12.5, 10.0)
4β	2.07 *m*	2.08 *ddd* (12.5, 6.5, 5.0)	2.16 *m*	2.16 *ddd* (12.5, 6.0, 5.0)
4a	3.71 *brdd* (12.9, 5.0)	3.73 *ddd* (13.0, 5.0, 1.0)	3.42 *m*	3.41 *ddd* (13.0, 5.0, 1.0)
6	5.01 *s*	5.01 *s*	5.60 *s*	5.59 *s*
7	6.80 *s*	6.80 *s*	6.96 *s*	6.96 *s*
10	6.75 *s*	6.74 *s*	6.73 *s*	6.72 *s*
11	3.89 *m*	3.90 *ddd* (6.5, 3.0, 1.0)	3.89 *m*	3.87 *ddd* (7.0, 2.5, 1.0)
12 endo	3.36 *brd*	3.35 *dd* (14.0, 6.5)	4.19 *dd* (14.1, 6.5)	4.19 *dd* (14.0, 7.0)
12 exo	3.33 *dd* (14.1, 2.9)	3.30 *dd* (14.0, 3.0)	3.02 *brd*	3.01 *dd* (14.0, 2.5)
OCH_2O	5.90 *brd*	5.89 *d*-5.91 *d* (1.5)	5.90 *brd*	5.88 *d*-5.90 *d* (1.5)
3-OMe	3.39 *s*	3.37 *s*	3.39 *s*	3.38 *s*

*Literature data from Viladomat et al., 1996

Table 3.5 ^{13}C NMR data for 6-hydroxycrinamine in methanol-d_4 (CD_3OD) compared to literature.

Carbon atom	**6α-hydroxycrinamine (Epimer A), δ_C/ppm**	**Reference compound A*, δ_C/ppm**	**6β-hydroxycrinamine (Epimer B), δ_C/ppm**	**Reference compound B*, δ_C/ppm**
1	136.9	136.4	136.8	136.2
2	123.3	123.0	123.1	123.2
3	76.2	75.9	75.8	75.6
4	29.8	29.4	29.6	29.4
6	88.4	88.0	85.9	85.5
6a	127.5	127.3	127.5	128.8
7	109.7	109.5	108.5	108.3
8	147.0	146.5	146.8	146.7
9	148.1	147.8	147.9	147.5
10	103.1	102.8	103.0	102.7
10a	136.2	135.8	134.9	134.6
10b	51.1	50.4	50.7	50.8
11	78.4	78.1	79.3	79.0
12	58.1	57.7	52.1	51.8
-OCH_2O	102.9	101.1	101.3	101.1
3-OMe	56.2	55.9	56.2	55.9

*Literature data from Viladomat et al., 1996

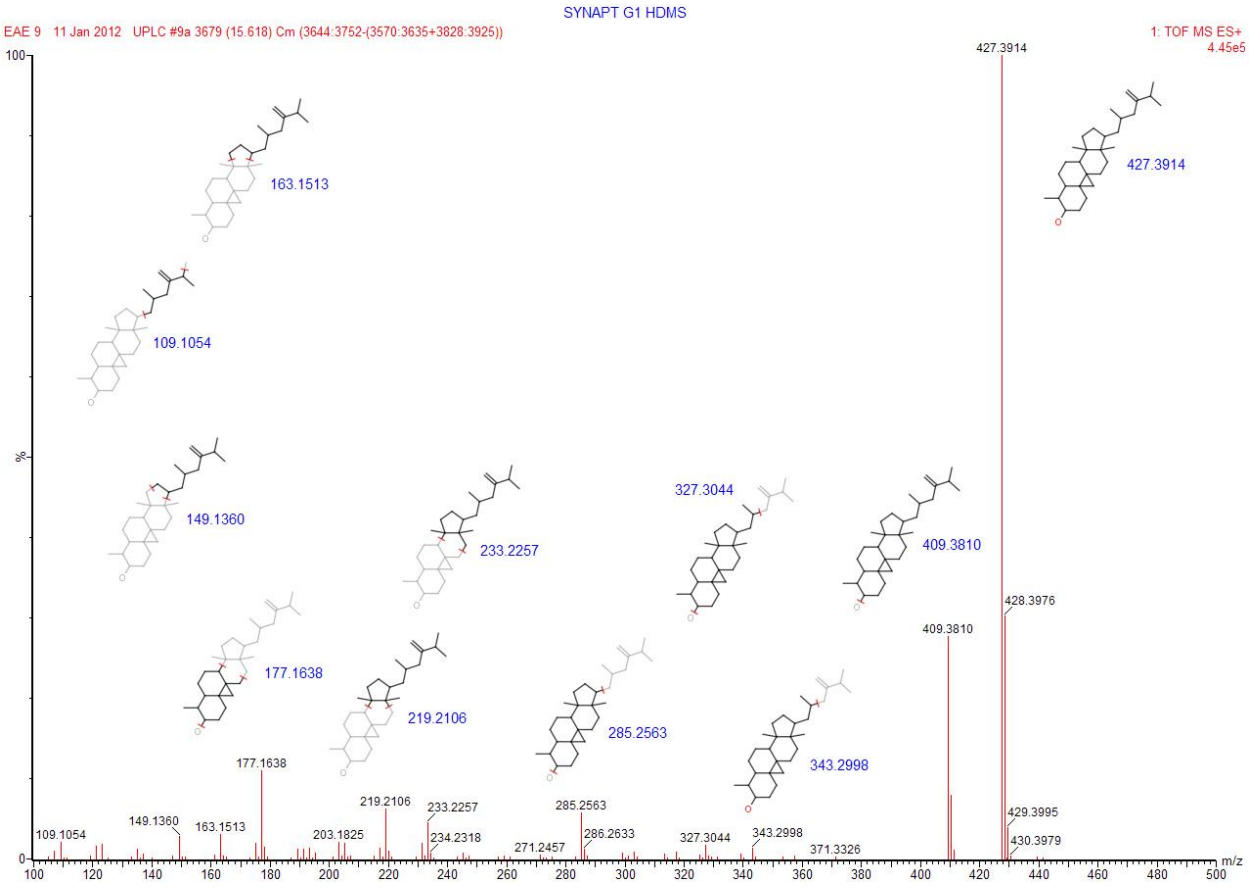
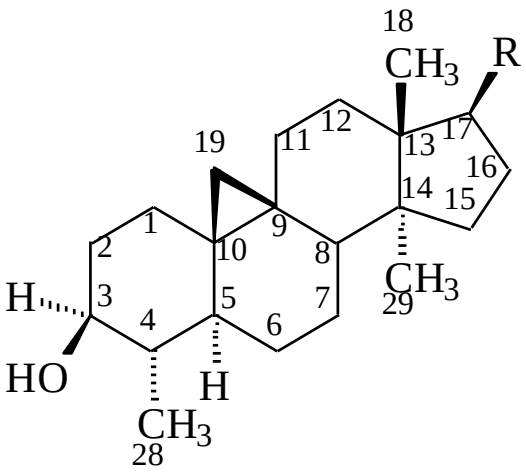
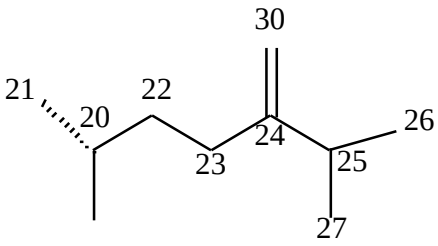


Figure 3.7 HRTOFMS (ESI^+) spectra for compound 3 (cycloeucalenol).

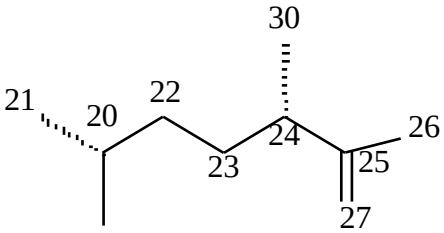


3.3

Side chain (R)



3.3a



3.3b

Figure 3.8 Structure of cycloeucalenol (3.3a) with its stereo-isomer (3.3b).

Table 3.6 ^{1}H NMR data for cycloeucalenol in chloroform-d_1 ($CDCl_3$) compared to literature.

H	**Reference compound*, δ_H (*J* in Hz)**	**(24,30)-cycloeucalenol (3.3a), δ_H (*J* in Hz)**	**Stereoisomer, (25,27)-cycloeucalenol (3.3b), δ_H (*J* in Hz)**	**Reference compound stereoisomer#, δ_H (*J* in Hz)**
3	3.22 *m*	3.19 *m*		
18	0.97 *s*	0.95 *s*		
19a	0.39 *d* (3.8)	0.37 *brs*		
19b	0.14 *d* (3.8)	0.12 *brs*		
21	0.91 *brs*	0.94 *brs*	0.86 *brs*	0.86 *d* (6.6)
24			2.22 *hextet* (7.0)	2.09 *hextet* (7.0)
26	1.03 *d* (6.8)	1.02 *d* (6.6)	1.64 *m*	1.64 *t* (1.4)
27	1.03 *d* (6.8)	1.00 *d* (6.6)	4.64 *brs*	4.67 *t* (1.4)
28	0.89 *s*	0.86 *s*	0.95 *brs*	0.94 *d* (7.1)
29	0.98 *d* (5.9)	0.97 *brs*	0.88 *s*	0.89 *s*
30a	4.72 *brs*	4.70 *brs*	1.00 *d* (6.6)	1.00 *d* (6.9)
30b	4.67 *brs*	4.64 *brs*		

* Literature data for cycloeucalenol from Liu et al., 2011.

Literature data for stereoisomer from Akihisa et al., 1997.

Table 3.7 ^{13}C NMR data for cycloeucalenol in chloroform-d_1 ($CDCl_3$) compared to literature.

Carbon atom	**Reference compound* δ_C/ppm**	**(24,30)-cycloeucalenol (3.3a), δ_C/ppm**	**Stereoisomer, (25,27)-cycloeucalenol (3.3b), δ_C/ppm**
1	30.8	31.0	31.0
2	34.8	35.0	35.0
3	76.6	76.8	76.8
4	44.6	44.8	44.8
5	43.3	43.6	43.6
6	24.7	24.9	24.9
7	28.1	28.3	28.3
8	46.9	47.1	47.1
9	23.5	23.8	23.8
10	29.5	29.8	29.8
11	25.2	25.4	25.4
12	35.3	35.6	35.6
13	45.3	45.5	45.6
14	48.9	49.1	49.1
15	32.9	33.1	33.1
16	27.0	27.2	27.2
17	52.2	52.4	52.4
18	17.8	18.0	18.0
19	27.2	27.5	27.5
20	36.1	36.3	36.4
21	18.3	18.6	18.6
22	35.0	35.2	34.0
23	31.3	31.5	31.7
24	156.9	157.2	41.8
25	33.8	34.2	150.5
26	22.0	22.2	18.9
27	21.9	22.1	109.6
28	14.4	14.6	14.6
29	19.1	19.4	19.4
30	105.9	106.2	22.1

* Literature data for cycloeucalenol from Liu et al., 2011.

3.3.3 Acetylcholinesterase inhibitory activity of isolated compound and fractions

In the bioassay guided purification of the compounds, AChE inhibitory activity of each fraction was assessed using the TLC method as described in section 2.3.2. The concentration of the isolated compounds and fractions that showed a 50% inhibition of enzyme activity (IC_{50}) was determined by a microtiter plate assay based on Ellman's method (Eldeen et al., 2005), as described in section 2.3.1.

From the TLC bioautographic assay, compound 1 or 6-hydroxycrinamine (EAM 3,16 1-4), compound 2 (EAE 1) and fractions EAM 17-21 21,22 and EAE 11 all showed activity (results not shown). No false positive reactions were seen (results not shown). However, compound 2 was unstable and degraded on storage before NMR analysis could be carried out. Compound 3 or cycloeucalenol (EAE 9), was not active for inhibition of AChE.

The compound and fractions which showed activity were further screened using the microtiter plate assay to determine their IC_{50} values for inhibition of AChE. The IC_{50} values obtained for inhibition of AChE by compound 1 and fractions EAM 17-21 21,22 and EAE 11 are provided in Table 3.8. The IC_{50} value of 6-hydroxycrinamine was expressed as a molar concentration since its structure and molecular weight could be determined while that of the fractions were expressed as mg/ml.

Table 3.8 *In vitro* AChE inhibitory activity of the isolated compound and fractions.

Compound/Fractions	**AChE inhbitory activity (IC_{50} values)**
6-hydroxycrinamine (compound 1)	0.445 ± 0.030 mM
EAM 17-21 21,22	0.067 ± 0.005 mg/ml
EAE 11	0.122 ± 0.013 mg/ml

3.4 Cytotoxicity studies

3.4.1 Cytotoxicity assessment and effect of isolated compounds and active fractions on Aβ-induced neurotoxicity

The effect of the isolated compounds and active fractions, on viability of SH-SY5Y cells are presented in Figure 3.9. Both the compounds and active fractions had a dose-dependent effect on viability. Results obtained from both cytotoxicity assays (MTT and neutral red uptake), were comparable. 6-hydroxycrinamine was the more toxic of the two compounds isolated with IC_{50} values of 61.7 µM and 54.5 µM for the neutral red and MTT assays, respectively (Table 3.9). EAM 17-21 21,22 was also the more toxic of the two active fractions with IC_{50} values of 36.9 µg/ml and 21.5 µg/ml for the neutral red and MTT assays, respectively.

The compounds and fractions were further tested at non-toxic doses to evaluate their possible protective effect against $A\beta_{25-35}$ induced cell death. None of the four samples showed any protective effect (results not shown).

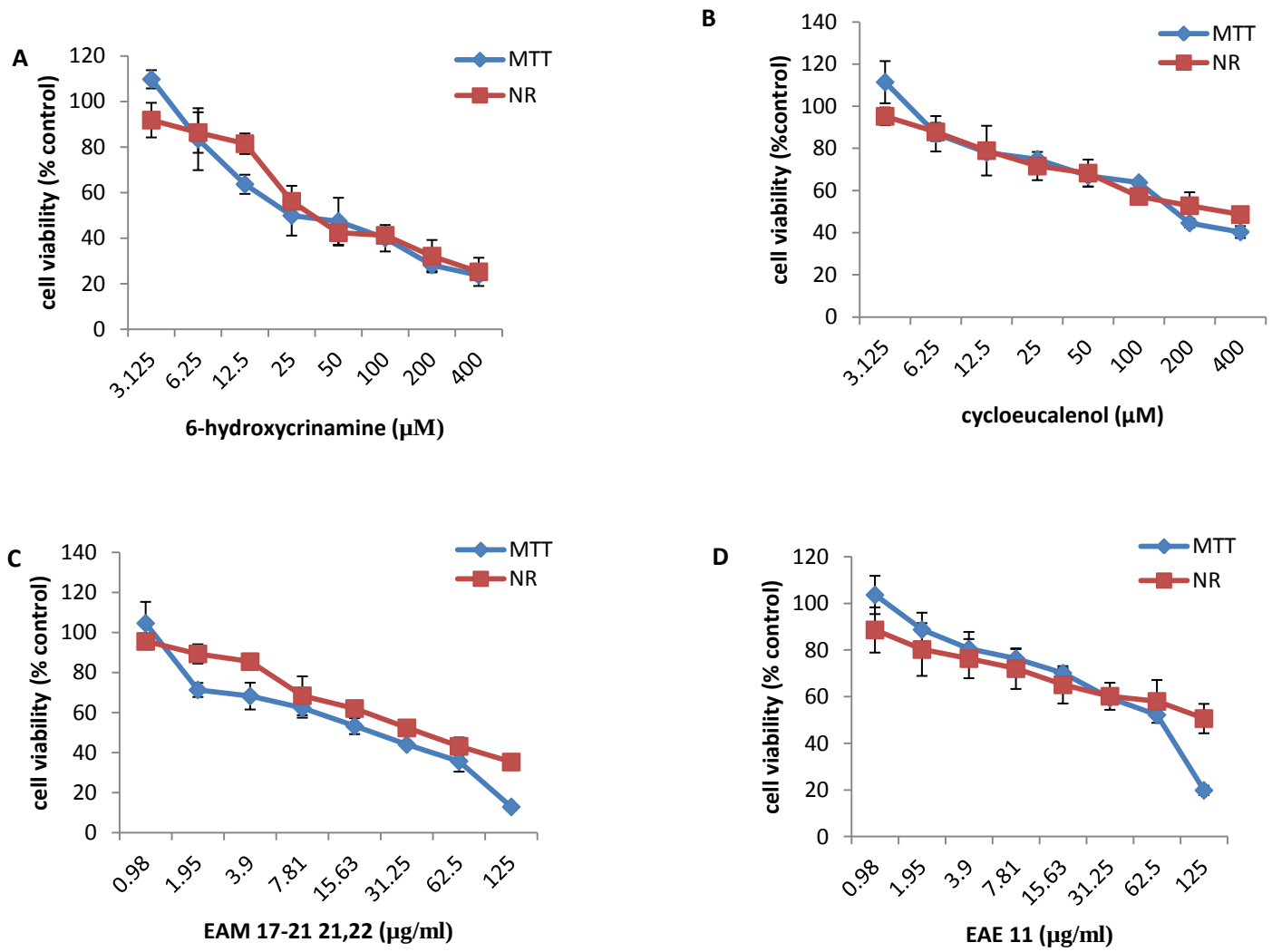


Figure 3.9 Effect of (A) 6-hydroxycrinamine, (B) cycloeucalenol, (C) EAM 17-21 21,22 and (D) EAE 11 on the viability of SH-SY5Y cell lines as measured by the MTT and neutral red uptake assays after 72 h of incubation.

Table 3.9 IC_{50} values of isolated compounds and fractions on SH-SY5Y cell lines.

Compound/Fraction	**Neutral Red assay (IC_{50} values)**	**MTT assay (IC_{50} values)**
6-hydroxycrinamine	61.7 ± 4.5 µM	54.5 ± 2.6 µM
cycloeucalenol	223.0 ± 6.4 µM	173.0 ± 5.1 µM
EAM 17-21 21,22	36.9 ± 3.5 µg/ml	21.5 ± 1.6 µg/ml
EAE 11	99.2 ± 5.8 µg/ml	83.7 ± 4.1 µg/ml

3.4.2 Cytotoxicity assessment and effect of several medicinal plants on Aβ-induced neurotoxicity

All the four plant extracts tested affected cell viability. The results, presented as percentage of cell growth compared to the untreated control for SH-SY5Y cells are presented in Figure 3.10. The cytotoxic effect of the four plant extracts, as represented by their IC_{50} values are shown in Table 3.10. The results obtained from both the neutral red and MTT assays were comparable. *C. bulbispermum* root extract was the most toxic, reducing cell viability by <40% at the highest concentration tested. Root extracts of *Z. mucronata* and *L. schweinfurthii* were the least toxic with IC_{50} values exceeding 100 µg/ml, the highest concentration tested.

Three concentrations that were not toxic, or presented low toxicity (from the results obtained in the cytotoxicity assays), were selected to evaluate their possible protective effect against cell death induced by $A\beta_{25-35}$. Treatment with $A\beta_{25-35}$ decreased cell viability to 16% of viable cells at the highest concentration (20 µM) tested (Fig. 3.11). Pretreatment with *Z. mucronata* and *T. sericea* roots showed a dose dependent inhibition of cell death caused by $A\beta_{25-35}$ (Fig. 3.12). Pre-treatment with *L. schweinfurthii* roots resulted in an optimum dose for inhibition of $A\beta_{25-35}$ induced cell death at 25 µg/ml, while still maintaining 80% viability (Fig. 3.12). The roots of *C. bulbispermum* at non-toxic dose still maintained >50% viability.

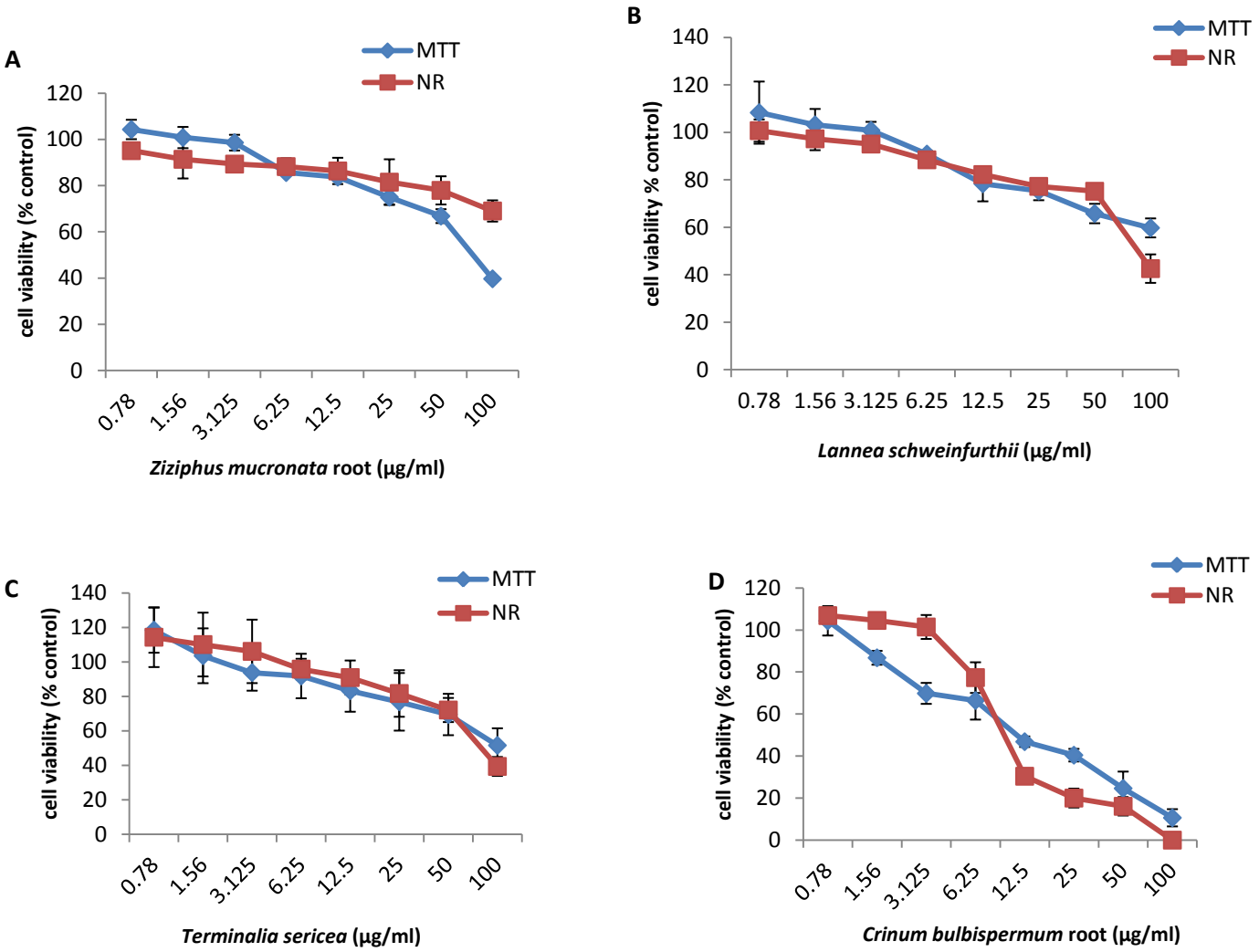


Figure 3.10 Effect of the methanol extract of the investigated plant extracts on viability of SH-SY5Y cell lines as measured by the MTT and neutral red uptake assays after 72 h of incubation (A) *Z. mucronata* roots; (B) *L. schweinfurthii* roots; (C) *T. sericea* roots; (D) *C. bulbispermum* roots.

Table 3.10 IC_{50} values of the methanol extracts of the investigated plants on SH-SY5Y cell lines.

Plant and part	**Neutral red assay IC_{50} (µg/ml)**	**MTT assay IC_{50} (µg/ml)**
Z. mucronata roots	>100	>100
L. schweinfurthii roots	>100	>100
T. sericea roots	100.72 ± 3.77	95.14 ± 2.45
C. bulbispermum roots	10.71 ± 0.26	12.53 ± 0.89

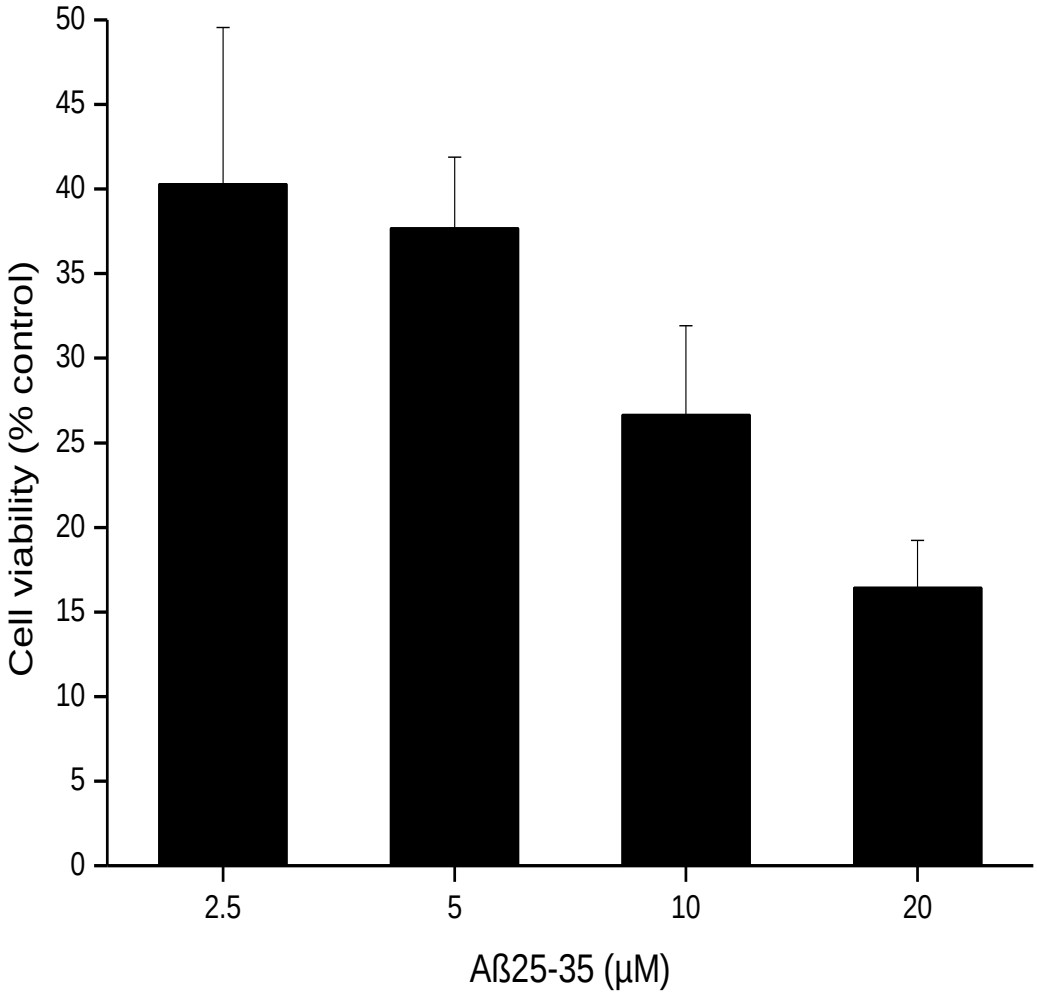


Figure 3.11 Effect of $A\beta_{25-35}$ on SH-SY5Y cell viability.

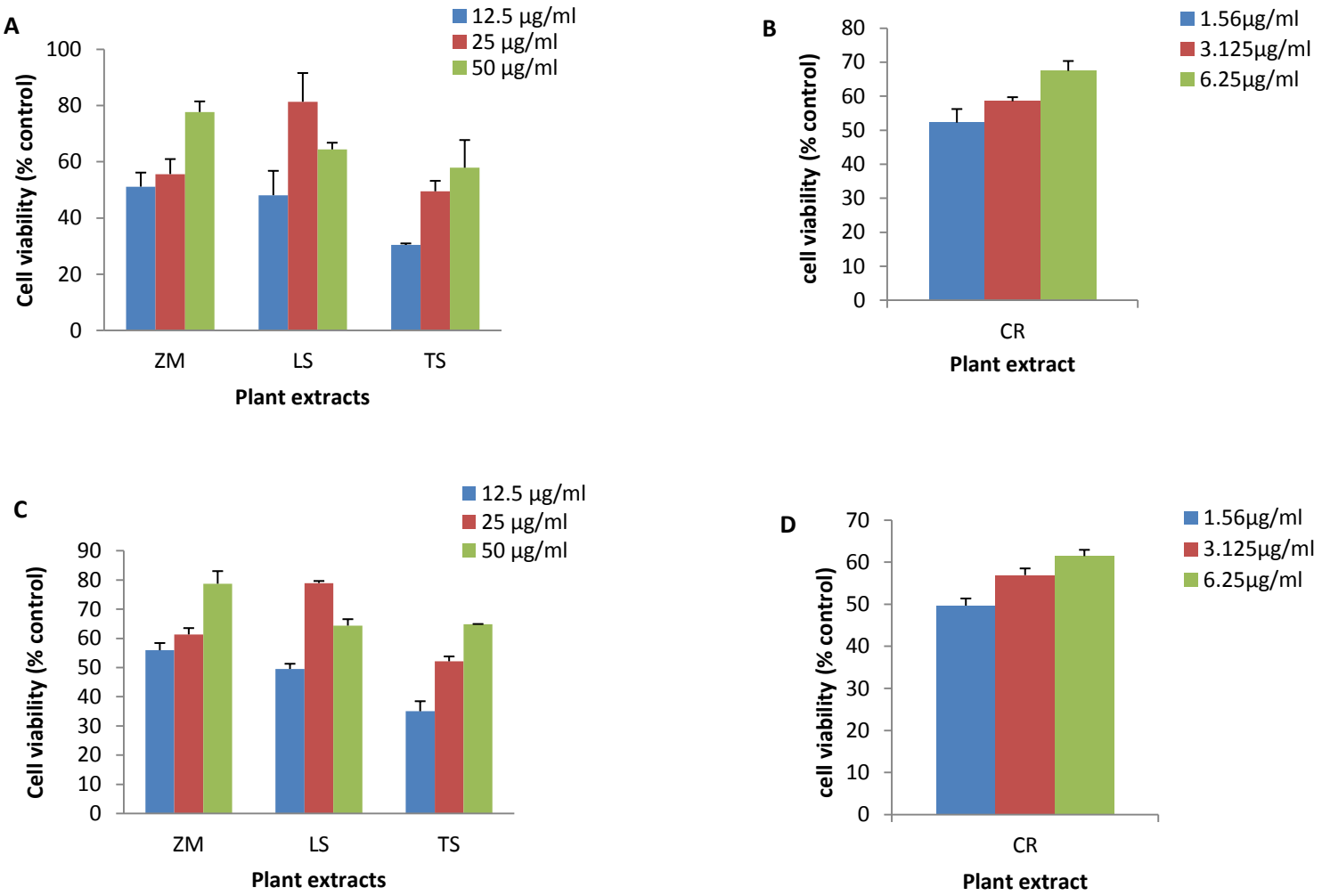


Figure 3.12 Effect of different plant extracts on Aβ25-35 induced toxicity. (A) and (B) Neutral red assay; (C) and (D) MTT assay. ZM, *Z. mucronata* root; LS, *L. schweinfurthii* root; TS, *T. sericea* root; CR, *C. bulbispermum* root.

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, δ-3-carene and α-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, α-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. δ-3-Carene is reported to be a potent inhibitor of AChE with an IC_{50} value of 200 µM, sabinene inhibited AChE with an IC_{50} value of 176.5 µg/ml, while α-terpinene showed inhibition with IC_{50} value of 1000 µM (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 (Batool 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} - 250µM) and lycorine (IC_{50} - 450µM) (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_{50} 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_{50} 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, buphanidrine 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.

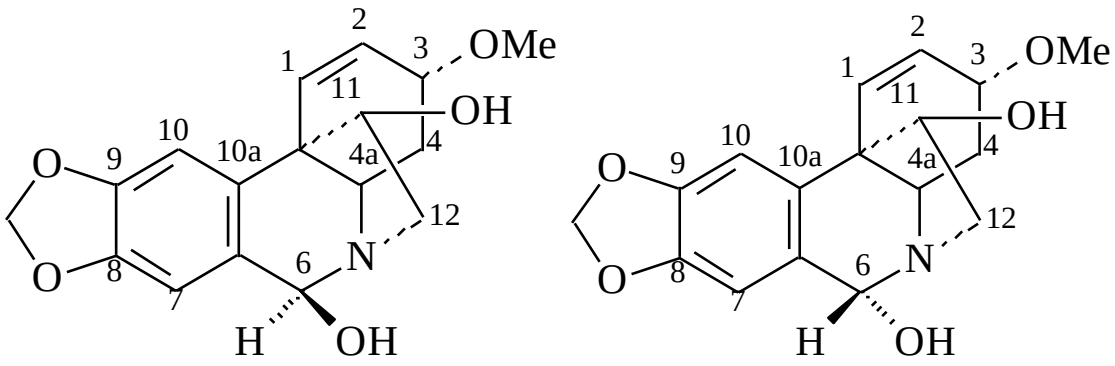
4.2 Isolation and structural elucidation of compounds from *Boophane disticha*

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}H, carbon - ^{13}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}H and ^{13}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}H spectra confirmed the compound to be a 3:1 mixture of two epimers, with epimer A, 6α-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 6α-hydoxycrinamine 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 α- position and hydroxyl group in the β- position while epimer B shows H-6 in the β- position and hydroxyl group in the α- position. In both epimers, the small coupling constant between H-2 and H-3, the large one between H-4α 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 α- 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-6α as a singlet at δ 5.01 and spatial proximity between H-6 and H-12 *endo* was observed. In epimer B, the proton H-6β was observed at a lower field, as a singlet at δ 5.60, and a NOE contour correlation between H-6β 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 δ 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.



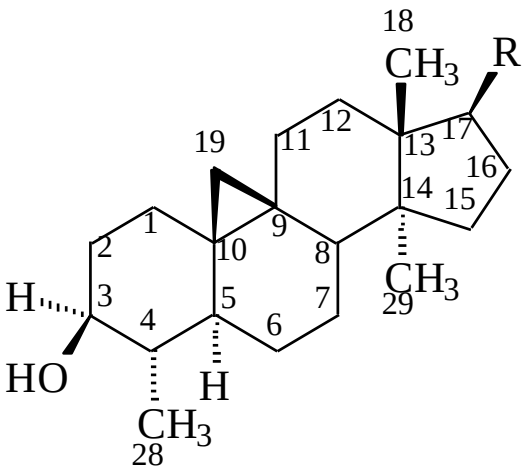
Epimer A (6α-hydroxycrinamine) (**3.1**) Epimer B (6β-hydroxycrinamine) (**3.2**)

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

4.2.2 Compound 3 (cycloeucalenol)

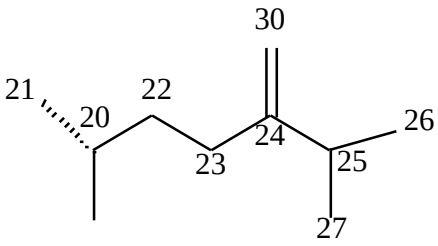
The ^{1}H-NMR spectrum of cycloeucalenol (Appendix E; Figure 9), revealed the presence of two exomethylene or olefinic protons which appeared as broad singlets at δ_H 4.70 and δ_H 4.64, one methine proton which was seen as a multiplet next to the hydroxyl group at δ_H 3.19 and six methyl protons. The methyl protons include: Me-26 and 27 appeared as doublets (δ_H 1.02 and 1.00, J = 6.6 Hz), Me-29 and Me-21 as broad singlets (δ_H 0.97 and 0.94), while Me-18 and Me-28 appeared as singlets (δ_H 0.95 and 0.86). Also, two methylene protons of a cyclopropyl group which appeared as broad singlets (δ_H 0.12 and 0.37) were observed in the ^{1}H-NMR spectrum. The ^{13}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 δ_C 157.2 while C-30 is an exomethylene carbon at δ_C 106.2. It also shows a methylene carbon (C-19) of cyclopropyl (δ_C 27.5), a methylene carbon (C-3) next to the hydroxyl group (δ_C 76.8), and six methyl signals (δ_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 cycloeucalenol (Liu et al., 2011).

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

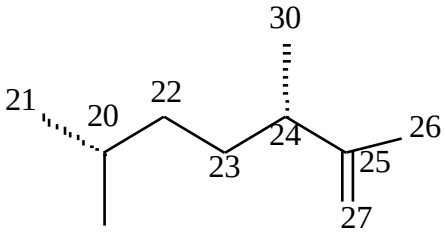


3.3

Side chain (R)



3.3a



3.3b

Figure 3.8 Structure of cycloeucalenol 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 δ_C 150.5 while C-27 is an exomethylene carbon at δ_C 109.6. The other differences are found at C-22, C-24, C-26 and C-30 which appear at δ_C 34.0, 41.8, 18.9 and 22.1. The data obtained for the ^{13}C-NMR spectrum of the stereo-isomer on the side-chain, also compares with data reported by Akihisa et al. (1997).

Cycloeucalenol 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}H-NMR spectra showed that cycloeucalenol 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}H-NMR spectra.

4.2.3 Acetylcholinesterase inhibitory activity of isolated compound and fractions

6-Hydroxycrinamine had an IC_{50} value of 445 ± 30 µM 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 IC_{50} value of 490 ± 7 µM. Concentrations of AChE inhibitory activity of ≤ 500 µM are considered active (Wink, 2000), and according to this criterion, the alkaloid can be said to inhibit AChE activity. However, the high 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 ± 14 µM, 547 ± 5 µM, 300 ± 27 µM, 553 ± 3 µM, 594 ± 8 µM, 239 ± 9 µM and 697 ± 12 µM 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 µg/ml and 125 µg/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 µg/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 µM, respectively. Tashinones, a group of diterpenes, have also been shown to contain AChE

inhibitory activity, with dihydrotanshinone and cryptotanshinone reported to have IC_{50} 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 Aβ- induced oxidative stress plays an important role in the pathogenesis or progression of AD (Butterfield et al., 2001; Wang et al., 2009). Aβ induces oxidative stress which in turn promotes the production of Aβ 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 Aβ- 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 Aβ-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_{50} 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_{50} 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 $HepG_2$ 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*-epoxdammarane-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_{50} 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 $A\beta_{25-35}$. 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 Aβ-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_{50} values >100 µg/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 µg/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, gallocatechin, 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_{50} 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). Stimasterols 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_{50} 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_2O_2 – induced cell death in PC12 cells which is ascribed to its OH and H_2O_2 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 ± 30 µM. 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 acitivities 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 Aβ 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, cycloeucalenol 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_{50} values exceeding 100 µg/ml, the highest concentration tested, while *C. bulbispermum* root extracts reduced cell viability to 16% at 50 µg/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 $A\beta_{25-35}$ 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 $A\beta_{25-35}$. 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.

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Review

Medicinal plants with cholinesterase inhibitory activity: A Review

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Alzheimer's disease (AD), a common neurodegenerative disease, is characterized by low levels in the brain of the neurotransmitter, acetylcholine (ACh). Clinical treatment of this disease is palliative and relies mostly on enhancing cholinergic function by stimulation of cholinergic receptors or prolonging the availability of ACh released into the neuronal synaptic cleft by use of agents which restore or improve the levels of acetylcholine. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), enzymes which breakdown acetylcholine, are considered as a promising strategy for the treatment of AD. A potential source of AChE and BChE inhibitors is provided by the abundance of plants in nature, and natural products continue to provide useful drugs and templates for the development of other compounds. The present work constitutes a review of the literature on 123 species of medicinal plants that have been tested for AChE inhibitory activity and 42 plant species which have been tested for BChE inhibitory activity. The plant species listed are potential cholinesterase inhibitors and may aid researchers in their study of natural products which may be useful in the treatment of AD.

Key words: Alzheimer's disease, acetylcholine, acetylcholinesterase, butyrylcholinesterase and medicinal plants.

INTRODUCTION

Neurodegenerative disease is a term applied to a variety of conditions arising from a chronic breakdown and deterioration of the neurons, particularly those of the central nervous system (Houghton and Howes, 2005). Alzheimer's disease (AD) was first described in 1906 by a Bavarian neuropsychiatrist Alois Alzheimer (Hostettmann et al., 2006). It is a complex, multifactoral, progressive, neurodegenerative disease primarily affecting the elderly population and is estimated to account for 50 - 60% of dementia cases in persons over 65 years of age (Frank and Gupta, 2005). The pathological features identified in the central nervous system (CNS) in AD are amyloid plaques, neurofibrillary tangles, inflammatory processes and disturbance of neurotransmitters (Selkoe, 2001; Bossy-Wetzel et al., 2004). There is also a progressive loss of neurons in the basal forebrain, which is the major source of cholinergic innervations of the neocortex and hippocampus. These changes involve progressive and irreversible impairment of cognitive function, resulting mainly in a loss of memory, with neurological and neuropsychiatric disorders (Hostettmann et al., 2006).

The pathophysiology of AD is complex and involves several different biochemical pathways. The first neurotransmitter defect discovered in AD involved acetylcholine (ACh), which plays an important role in memory and learning. In the CNS, ACh stimulation of the nicotinic receptors appears to be associated with cognitive

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Abbreviations: AD, Alzheimer's disease; **CNS,** central nervous system; **ACh,** acetylcholine; **AChE,** acetylcholinesterase; **BChE,** butyrylcholinesterase; **DTNB,** 5,5´-bisdithionitrobenzoic acid; **ATCI,** acetylthiocholine iodide; **BTCCI,** butyrylthiocholine chloride; **TLC,** thin layer chromatography; **DPPH,** diphenyl picryl hydrazine; **XO,** xanthine oxidase; **EtOAc,** ethylacetate; **BHA,** butylated hydroxyanisole; **OSI,** oxidative stability instrument; **BHT,** butylated hydroxytoluene.

function. Normally, ACh is stored in the nerve terminals, in structures called vesicles and is released from the nerve endings when the nerve terminal is depolarized, thereby entering the synapse and binding to the receptor (Houghton et al., 2006). However, in patients with AD, the ACh which is released has a very short half-life due to the presence of large amounts of the enzymes; acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which are both present in the brain and are detected among neurofibrillary tangles and neuritic plaques (Beard et al., 1995; Orhan et al., 2004). These enzymes hydrolyse the ester bond in the ACh molecule, leading to loss of stimulatory activity.

The increase in life expectancy during the 20th century has concomitantly increased the number of people suffering from the disease. There are considerable financial, social and emotional burdens associated with the caring for patients with this disease (Akhondzadeh and Noroozian, 2002). In fact, in advanced industrialized and post-industrialized societies, where life expectancy is long, this disease is a major cause of morbidity and it imposes severe strains on the social welfare systems. It is estimated that in the USA, at least 5 million people are affected by AD (Houghton and Howes, 2005).

Approaches to enhance cholinergic function in AD have included stimulation of cholinergic receptors or prolonging the availability of ACh released into the neuronal synaptic cleft by use of agents which restore the level of acetylcholine through inhibition of both AChE and BChE. BChE, primarily associated with glial cells and specific neuronal pathways cleaves ACh in a similar manner to AChE to terminate its physiological action. Such studies, together with a statistically slower decline in the cognitive performance of dementia patients possessing specific BChE polymorphisms that naturally lower BChE activity, have targeted BChE as a new approach to intercede in the progression of AD (Loizzo et al., 2009). Recently, Hodges (2006) demonstrated that the inhibition of AChE holds a key role not only to enhance cholinergic transmission in the brain but also to reduce the aggregation of β-amyloid and the formation of the neurotoxic fibrils in AD. Therefore, AChE and BChE inhibitors have become remarkable alternatives in treatment of AD (Orhan et al., 2004). Existing anticholinesterase drugs (example, tacrine, donepezil, physostigmine, galantamine and heptylphysostigmine) for the treatment of dementia are reported to have several dangerous adverse effects such as hepatotoxicity, short duration of biological action, low bioavailability, adverse cholinergic side effects in the periphery and a narrow therapeutic window (Hung et al., 2008; Sancheti et al., 2009).

The history of drug discovery has shown that plants contain active compounds that have become new sources to investigate for the pharmaceutical industry. Plant constituents may not only act synergistically with other constituents from the same plant but may also enhance the activity of compounds or counteract toxic effects of compounds from other plant species (Howes and Houghton, 2003). In traditional practices, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases and different neuropharmacological disorders (Mukherjee et al., 2007a). Yokukansan, a Chinese herbal remedy which is used to treat various neurological states has been reported as being effective with no adverse effects (De Caires and Steenkamp, 2010). Also, galanthamine, an alkaloid from snowdrop, has been approved by the Food and Drug Administration in the United States for use in the treatment of Alzheimer's disease (Ingkaninan et al., 2003; Heinrich and Teoh, 2004). Since AD has become a public health burden, and the commonly available synthetic drugs have undesirable side-effects, new treatment strategies based on medicinal plants have been the subject of current focus. This article summarizes the plants so far reported to have AChE and BChE inhibitory activity.

EXPERIMENTAL APPROACHES

The colorimetric method of Ellman et al. (1961) which is based on determining the amount of thiocholine released when acetylthiocholine or butyrylthiocholine is hydrolysed by AChE or BChE is widely used. The thiocholine released is quantified by its reaction with 5,5´-bisdithionitrobenzoic acid (DTNB), which produces a yellow 5-thio-2-nitrobenzoate anion. Several 96-well microplate assays have been derived from Ellman's method with some modifications which have enabled determinations to be performed with a much higher throughput (Houghton et al., 2006).

The Ellman reaction for detecting AChE and BChE inhibitory activity has also been adapted for thin layer chromatography (TLC) plates. Samples are spotted on the plate before standard development, after which a solution of DTNB and acetylthiocholine iodide (ATCI) or butyrylthiocholine chloride (BTCCl) is sprayed until the plate is saturated. Thereafter the enzyme solution is sprayed on the plate and it is incubated for 5 min. A yellow coloration with white spots is indicative of inhibitory activity. This provides an extremely rapid method to screen large numbers of samples to discover new inhibitors of AChE and BChE (Hostettmann et al., 2006). However, this method is known to give a number of false-positive effects. To rule out such results, plates are first sprayed with DTNB, followed by a mixture of the enzyme and ATCI where the occurrence of white spots is indicative of false positive results (Adsersen et al., 2006; Houghton et al., 2006). A similar method for TLC detection has been introduced which uses acetylnaphthol as the substrate and measures the amount of naphthol, the reaction product formed, by its chromogenic reaction with Fast Blue B salt (Giovanni et al., 2008).

Other methods and assays used for detection and

quantification of AChE and BChE inhibition include high performance liquid chromatography (HPLC) with on-line coupled UV-MS-biochemical detection (Ingkaninan et al., 2000), fluorometry, radiometric assay, mass spectrometry, and assays based on immobilized enzyme (Hostettmann et al., 2006). Fluorometric methods are reported to be more sensitive than colorimetric methods for the assay of AChE and BChE. Fluorogenic substrates such as 2-naphthyl acetate, indoxyl acetate, resorufin butyrate and fluorofore coumarinylphenylmaleimide have been utilized (Rhee et al., 2003). A limitation of the flow assay could be that the presence in the plant extract of fluorescent compounds with similar excitation and emission wavelengths as the product of the enzyme reaction results in a positive peak which might obscure inhibitory activity.

CHOLINESTERASE INHIBITORY ACTIVITY OF PLANT EXTRACTS

Acetylcholinesterase inhibitory activity

Extracts of several medicinal plants have been reported to show AChE inhibitory activity. A summary of screening studies of these plants is provided in Table 1 in alphabetical order of their family, together with their scientific names, plant part, solvent extract, percentage inhibition and concentration at which the enzyme is inhibited.

Aqueous and methanol extracts of 11 plants, used in Danish folk medicine for improvement of memory and cognition, and three *Corydalis* species have been tested for their AChE inhibitory activity (Adsersen et al., 2006). The authors reported significant dose-dependent inhibitory activity for extracts of the *Corydalis* species whereas only moderate inhibition of the enzyme was observed for extracts of *Ruta graveolens* L., *Lavandula augustifolia* Miller, *Rosmarinus officinalis* L., *Petroselinum crispum* (Mil.) Nym. ex A. W. Hill., and *Mentha spicata* L. The latter five species contain essential oils with terpenes, a group of compounds reported to have AChE inhibitory activity (Perry et al., 2000; Adsersen et al., 2006).

Ferreira et al. (2006) reported the AChE inhibitory activity of the essential oil, ethanol extract and decoction of ten plant species from Portugal. Among the plant extracts screened, *Melissa officinalis*, *Paronychia argentea*, *Sanguisorba minor*, *Hypericum undulatum* and *Malva silvestris* are used in herbal medicine, *Laurus nobilis* and *Mentha suaveolens* as condiments, and *Lavandula augustifolia* and *Lavandula pedunculata* as aromatics. *M. officinalis* and *M. suaveolens* showed AChE inhibitory capacity higher than 50% in the essential oil fraction. The ethanol extract of *L. nobilis*, *H. undulatum* and *S. minor* exhibited AChE inhibition of 64% (1 mg/ml), 68% (0.5 mg/ml) and 78% (1 mg/ml), respectively. In addition decoctions of *L. pedunculata*, *M. suaveolens* and *H. undulatum* at 5 mg/ml, exhibited percentage inhibitions of 68, 69 and 82%, respectively.

Mukherjee et al. (2007b) reported the AChE inhibitory activity of the hydroalcohol extracts of six herbs used in Indian system of medicine. The hydroalcohol extract from *Centella asiatica, Nardostachys jatamansi, Myristica fragrans, Evalvulus alsinoides* inhibited 50% of AChE activity (IC_{50}) at concentrations of 100 - 150 µg/ml. The AChE inhibitory activity of petroleum ether, chloroform, ethyl acetate and methanol extracts obtained from 14 *Salvia* species growing in Turkey has been reported (Orhan et al., 2007). Most of the extracts did not show any activity against AChE at 0.2 mg/ml. The most active extracts at 1 mg/ml for AChE inhibition were the petroleum ether extract of *S. albimaculata* (89.4%) and chloroform extract of *S. cyanescens* (80.2%). In a recent study by Khadri et al. (2010), the aqueous extract, proanthocyanidin rich extract and organic extracts of *Cymbopogon schoenanthus* shoots from South Tunisia all showed good AChE inhibitory activity.

Butyrylcholinesterase inhibitory activity

BChE has been shown to be implicated in the progression of AD as it also reduces the availability of ACh which is an important neurotransmitter in AD. A summary of medicinal plants which have been screened and reported to have BChE inhibitory activity are listed in Table 1.

The chloroform: methanol (1:1) extracts of 21 plant species were screened for their anticholinesterase activity on BChE enzyme by the *in vitro* method of Ellman (Orhan et al., 2004). The extracts did not show any noticeable inhibitory activity against the enzyme at 10 µg/ml, however, extracts of *Rhododendron ponticum* subsp. *ponticum*, *Corydalis solida* subsp. *solida* and *Buxus semperivirens* showed inhibition at 1 mg/ml. Loizzo et al. (2009) evaluated the essential oils of *Origanum ehrenbergii* and *Origanum syriacum* both collected from Lebanon for their BChE inhibitory activity when using a modification of Ellman's method, with *O. ehrenbergii* showing the highest activity. The data obtained from this and other studies on both oils showed that they could be used as a valuable new flavor with functional properties for food or nutriceutical products with particular relevance to supplements for the elderly.

Petroleum ether, chloroform, ethyl acetate and methanol extracts of 14 *Salvia* species have also been screened for possible BChE inhibitory activity. At 1 mg/ml, the ethyl acetate extracts of *Salvia frigida* and *Salvia migrostegia*, chloroform extracts of *Salvia candidissima* ssp. *occidentalis* and *Salvia ceratophylla*, as well as petroleum ether extract of *Salvia cyanescens* inhibit BChE by more than 90% (Orhan et al., 2007).

DISCUSSION

Inhibition of AChE, the key enzyme in the breakdown of

Table 1. Medicinal Plants with cholinesterase inhibitory activity

Family and botanical name	Parts used	Type of extract (solvent)	AChE Method; Activity (% inhibition) (concentration of plant extract tested)	BChE Method; Activity (% inhibition) (concentration of plant extract tested)	References
Acanthaceae					
Acanthus ebracteatus Vahl.	Aerial part	Methanol	TLC and 96 well plate; 36.19 ± 8.00 (0.1mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007a)
Andrographis paniculata Nees.	Aerial part	Hydroalcohol	96 well plate; 50% (222.41µg/ml)*	ND	Mukherjee et al. (2007b)
Anacardiaceae					
Magnifera indica L.	Bark	Methanol	TLC and 96 well plate; 8.15 ± 0.77 (100 µg/ml)	ND	Vinutha et al. (2007)
	Bark	Water	TLC and 96 well plate; 6.29 ± 0.37 (100µg/ml)	ND	Vinutha et al. (2007)
Semecarpus anacardium Linn. f.	Bark	Methanol	TLC and 96 well plate; 69.94 ± 0.75 (100 µg/ml)	ND	Vinutha et al. (2007)
	Bark	Water	TLC and 96 well plate; 1.09 ± 0.37 (100 µg/ml)	ND	Vinutha et al. (2007)
Apiaceae					
Carum carvi L.	Radix	Methanol	TLC and 96 well plate; 11.00 ± 0.00 (0.1mg/ml)	ND	Adsersen et al. (2006)
Petroselinum crispum (Mil.) Nym. ex A. W. Hill.	Radix	Methanol	TLC and 96 well plate; 21.00 ± 0.00 (0.1mg/ml)	ND	Adsersen et al. (2006)
Pimpinella anisum L.	Fructus	Methanol	TLC and 96 well plate; 3.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
Apocynaceae					
Tabernaemontana divaricata L.	Roots	Ethanol	96 well plate; 50% (2.56 mg/l)*	96 well plate; 50% (76.95 mg/l)*	Chattipakorn et al. (2007)
Araceae					
Acorus calamus L.	Rhizomes	Methanol	96 well plate; 50% (791.35µg/ml)*	ND	Ahmed et al. (2009)
Asteraceae					
Carthamus tinctorius L.	Flower	Methanol	TLC and 96 well plate; 30.33 ± 9.22 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Brassicaceae					
Capsella bursa-pastoris (L.) Medik.	Whole plant	Methanol	96 well plate; 10.00 ± 2.00 (5 mg/ml)	96 well plate; 13.00 ± 1.00 (5 mg/ml)	Sancheti et al. (2009)

Table 1. Cont'd

Buxaceae					
Buxus sempervirens L.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 61.76 ± 0.76 (1 mg/ml)	ND	Orhan et al. (2004); Mukherjee et al. (2007 a)
Caesalpinaceae					
Robinia pseudoacacia L.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 26.32 ± 0.82 (1 mg/ml)	96 well plate; 31.47 ± 0.99 (1mg/ml)	Orhan et al. (2004)
Caryophyllaceae					
Paronychia argentea Lam.	Aerial parts	Water	UV spectophotometry; 26.10 ± 1.20 (5 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Essential oil	UV spectophotometry; 49.50 ± 1.00 (1 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Ethanol	UV spectophotometry; 48.70 ± 6.10 (0.5 mg/ml)	ND	Ferreira et al. (2006)
Celastraceae					
Euonymus sachalinensis (F. Schmidt.) Maxim.	Leaf	Methanol	96 well plate; 10.00 ± 3.00 (5 mg/ml)	96 well plate; 43.00 ± 1.00 (5 mg/ml)	Sancheti et al. (2009)
Combretaceae					
Combretum kraussii Hochst.	Leaf	Ethyl acetate	TLC and 96 well plate; 96.00 ± 4.60 (1 mg/ml)	ND	Eldeen et al. (2005)
	Leaf	Ethanol	TLC and 96 well plate; 88.00 ± 3.10 (1 mg/ml)	ND	Eldeen et al. (2005)
	Bark	Ethyl acetate	TLC and 96 well plate; 82.00 ± 6.10 (1 mg/ml)	ND	Eldeen et al. (2005)
	Bark	Ethanol	TLC and 96 well plate; 83.00 ± 4.50(1 mg/ml)	ND	Eldeen et al. (2005)
	Root	Ethyl acetate	TLC and 96 well plate; 81.00 ± 4.10 (1 mg/ml)	ND	Eldeen et al. (2005)
	Root	Ethanol	TLC and 96 well plate; 82.00 ± 5.20 (1 mg/ml)	ND	Eldeen et al. (2005)
Terminalia bellirica (Gaertn.) Roxb.	Fruit	Methanol	TLC and 96 well plate; 39.68 ± 8.15 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007a)
Terminalia chebula Retz.	Fruit	Methanol	96 well plate; 89.00 ± 1.00 (5 mg/ml)	96 well plate; 95.00 ± 1.00 (5 mg/ml)	Sancheti et al. (2009)
Convolvulaceae					
Convolvulus pluricaulis Choisy.	Whole plant	Methanol	TLC and 96 well plate; 2.22 ± 1.17 (100 µg/ml)	ND	Vinutha et al. (2007)
Evalvulus alsinoides L.	Whole plant	Hydro alcohol	96 well plate; 50% (141.76 µg/ml)*	ND	Mukherjee et al. (2007 b)

Table 1. Cont'd

Crassulaceae					
Rhodiola rosea L.	Root	Methanol	96 well plate; 42.00 ± 3.20 (10 g/l)	ND	Hillhouse et al. (2004); Mukherjee et al. (2007 a)
Cupressaceae					
Chamaecyparis pisifera (Siebold and Zuccarini) Endlicher	Whole plant	Methanol	96 well plate; 59.00 ± 2.00 (5 mg/ml)	96 well plate; 62.00 ± 2.00 (5 mg/ml)	Sancheti et al. (2009)
Cyperaceae					
Cyperus rotundus L.	Whole plant	Methanol	TLC and 96 well plate; 44.19 ± 2.27 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Dioscoreaceae					
Dioscorea bulbifera L.	Whole plant	Methanol	96 well plate; 79.00 ± 2.00 (5 mg/ml)	96 well plate; 82.00 ± 2.00 (5 mg/ml)	Sancheti et al. (2009)
Ericaceae					
Rhododendron luteum Sweet.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 76.32 ± 0.58 (1 mg/ml)	96 well plate; 69.14 ± 1.89 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Rhododendron ponticum L. subsp. *Ponticum*	Whole plant	Chloroform: methanol (1:1)	96 well plate; 93.03 ± 1.12 (1 mg/ml)	96 well plate; 95.23 ± 1.28 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Rhododendron schlippenbachii Maxim.	Whole plant	Methanol	96 well plate; 67.00 ± 1.00 (5 mg/ml)	96 well plate; 63.00 ± 2.00 (5 mg/ml)	Sancheti et al. (2009)
Euphorbiaceae					
Euphorbia antiquorum L.	Stem	Methanol	TLC and 96 well plate; 42.31 ± 9.10 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Fabaceae					
Albizia adianthifolia (Schumach.) W.F. Wight.	Bark	Ethyl acetate	TLC and 96 well plate; 61.00 ± 5.10 (1 mg/ml)	ND	Eldeen et al. (2005)
	Bark	Ethanol	TLC and 96 well plate; 53.00 ± 2.20 (1 mg/ml)	ND	Eldeen et al. (2005)
	Root	Ethyl acetate	TLC and 96 well plate; 45.00 ± 2.10 (1 mg/ml)	ND	Eldeen et al. (2005)
	Root	Ethanol	TLC and 96 well plate; 51.00 ± 3.40 (1 mg/ml)	ND	Eldeen et al. (2005)
Vicia faba L.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 45.23 ± 1.03 (1 mg/ml)	96 well plate; 55.85 ± 0.48 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)

Table 1. Cont'd

Fumariaceae					
Fumaria asepala Boiss.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 91.99 ± 0.70 (1 mg/ml)	96 well plate; 93.12 ± 0.28 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria capreolata L.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 96.89 ± 0.17 (1 mg/ml)	96 well plate; 89.24 ± 0.83 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria cilicica Hausskn.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 88.03 ± 0.65 (1 mg/ml)	96 well plate; 80.03 ± 0.28 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria densiflora DC.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 93.42 ± 0.92 (1 mg/ml)	96 well plate; 85.66 ± 1.24 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria flabellata L.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 92.14 ± 1.01 (1 mg/ml)	96 well plate; 87.91 ± 0.61 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria judaica Boiss.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 96.47 ± 0.63 (1 mg/ml)	96 well plate; 98.43 ± 0.39 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria kralikii Jordan	Whole plant	Chloroform: methanol (1:1)	96 well plate; 84.98 ± 1.07 (1 mg/ml)	96 well plate; 75.43 ± 0.98 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria macrocarpa Boiss. ex Hausskn.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 93.43 ± 0.64 (1 mg/ml)	96 well plate; 88.74 ± 0.34 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria parviflora Lam.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 87.02 ± 0.31 (1 mg/ml)	96 well plate; 87.09 ± 1.45 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria petteri Reichb subsp. *thuretii* (Boiss.)	Whole plant	Chloroform: methanol (1:1)	96 well plate; 89.45 ± 0.86 (1 mg/ml)	96 well plate; 87.32 ± 0.76 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Fumaria vaillantii Lois.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 94.23 ± 0.47 (1 mg/ml)	96 well plate; 99.32 ± 0.25 (1 mg/ml)	Orhan et al. (2004); Mukherjee et al. (2007 a)
Ginkgoaceae					
Ginkgo biloba L.	Whole plant	Ethanol	96 well plate; 50% (268.33 µg)*	ND	Perry et al. (1998); Das et al. (2002); Mukherjee et al. (2007 a)
Guttiferae					
Mammea harmandii Kosterm.	Flower	Methanol	TLC and 96 well plate; 33.63 ± 8.00 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Hypericaceae					
Hypericum undulatum Shoubs. ex Willd.	Flower	Water	UV spectophotometry; 81.70 ± 3.40 (5 mg/ml)	ND	Ferreira et al. (2006)
	Flower	Essential oil	UV spectophotometry; 30.30 ± 19.70 (1 mg/ml)	ND	Ferreira et al. (2006)
	Flower	Ethanol	UV spectophotometry; 68.40 ± 4.70 (0.5 mg/ml)	ND	Ferreira et al. (2006)

Table 1. Cont'd

Lamiaceae					
Lavandula augustifolia Miller	Whole plant	Methanol	TLC and 96 well plate; 34.00 ± 0.00 (0.1 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Essential oil	UV spectophotometry; 39.50 ± 8.60 (1 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Ethanol	UV spectophotometry; 64.30 ± 9.00 (1 mg/ml)	ND	Ferreira et al. (2006)
Lavandula pedunculata (Miller) Cav.	Aerial parts	Water	UV spectophotometry; 67.80 ± 10.70 (5 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Essential oil	UV spectophotometry; 56.50 ± 4.90 (0.5 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Ethanol	UV spectophotometry; 42.00 ± 16.80 (1 mg/ml)	ND	Ferreira et al. (2006)
Mentha spicata L.	Whole plant	Methanol	TLC and 96 well plate; 15.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
Mentha suaveolens Ehrh.	Aerial parts	Water	UV spectophotometry; 68.90 ± 2.50 (5 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Essential oil	UV spectophotometry; 52.40 ± 2.50 (1 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Ethanol	UV spectophotometry; 27.10 ± 2.70 (1 mg/ml)	ND	Ferreira et al. (2006)
Origanum vulgare L.	Whole plant	Methanol	TLC and 96 well plate; 3.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
Origanum ehrenbergii Boiss	Aerial parts	Essential oil	UV spectophotometry; 50% (0.3 µg/ml)*	UV spectophotometry; 50% (0.3 µg/ml)*	Loizzo et al. (2009)
Origanum syriacum L.	Aerial parts	Essential oil	UV spectophotometry; 50% (1.7 µg/ml)*	UV spectophotometry; 50% (1.6 µg/ml)*	Loizzo et al. (2009)
Rosmarinus officinalis L.	Whole plant	Methanol	TLC and 96 well plate; 17.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
Salvia albimaculata Hedge and Hub	Whole plant	Petroleum ether	96 well plate; 89.40 ± 2.07 (1 mg/ml)	96 well plate; 73.90 ± 0.76 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Chloroform	NI	96 well plate; 87.90 ± 0.22 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 51.70 ± 3.22 (1 mg/ml)	96 well plate; 69.80 ± 1.99 (1 mg/ml)	Orhan et al. (2007)

Table 1. Cont'd

	Whole plant	Methanol	96 well plate; 38.90 ± 3.22 (1 mg/ml)	96 well plate; 27.40 ± 1.32 (1 mg/ml)	Orhan et al. (2007)
Salvia aucheri Bentham var. *canescens* Boiss and Heldr	Whole plant	Chloroform	96 well plate; 64.50 ± 1.03 (1 mg/ml)	96 well plate; 77.60 ± 3.76 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 53.40 ± 1.59 (1 mg/ml)	96 well plate; 69.60 ± 2.15 (1mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 39.90 ± 1.17 (1 mg/ml)	96 well plate; 12.60 ± 1.05 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 27.30 ± 0.98 (1 mg/ml)	96 well plate; 59.90 ± 378.00 (1 mg/ml)	Orhan et al. (2007)
Salvia candidissima Vahl. *ssp. occidentalis*	Whole plant	Chloroform	96 well plate; 48.60 ± 5.13 (1 mg/ml)	96 well plate; 91.10 ± 1.98 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 46.10 ± 1.28 (1 mg/ml)	96 well plate; 77.80 ± 0.93 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 39.40 ± 4.31 (1 mg/ml)	96 well plate; 55.60 ± 0.28 (1 mg/ml)	Orhan et al. (2007)
Salvia ceratophylla L.	Whole plant	Chloroform	96 well plate; 30.80 ± 5.25 (1 mg/ml)	96 well plate; 91.10 ± 1.98 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 19.30 ± 1.57 (1 mg/ml)	96 well plate; 29.20 ± 0.77 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 27.80 ± 2.82 (1 mg/ml)	96 well plate; 34.90 ± 6.50 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	NI	96 well plate; 38.80 ± 4.94 (1 mg/ml)	Orhan et al. (2007)
Salvia cryptantha Montbret and Bentham	Whole plant	Chloroform	96 well plate; 24.90 ± 1.65 (1 mg/ml)	NI	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 73.30 ± 2.55 (1 mg/ml)	96 well plate; 53.60 ± 0.67 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 47.20 ± 5.18 (1 mg/ml)	96 well plate; 36.30 ± 2.79 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 71.80 ± 2.62 (1 mg/ml)	96 well plate; 92.00 ± 0.41 (1mg/ml)	Orhan et al. (2007)

Table 1. Cont'd

Salvia cyanescens Boiss and Bal.	Whole plant	Chloroform	96 well plate; 80.20 ± 4.35 (1 mg/ml)	96 well plate; 91.80 ± 0.54 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 51.20 ± 3.78 (1 mg/ml)	96 well plate; 56.90 ± 1.03 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 9.00 ± 0.88 (1 mg/ml)	96 well plate; 13.10 ± 0.70 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 37.70 ± 5.35 (1 mg/ml)	96 well plate; 67.40 ± 3.59 (1 mg/ml)	Orhan et al. (2007)
Salvia forskahlei L.	Whole plant	Chloroform	96 well plate; 41.30 ± 2.91 (1 mg/ml)	96 well plate; 60.20 ± 4.42 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 47.00 ± 2.31 (1 mg/ml)	96 well plate; 62.90 ± 0.67 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 35.80 ± 2.46 (1 mg/ml)	96 well plate; 46.70 ± 3.69 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 25.20 ± 4.46 (1 mg/ml)	96 well plate; 69.30 ± 1.65 (1mg/ml)	Orhan et al. (2007)
Salvia frigida Boiss	Whole plant	Chloroform	96 well plate; 53.70 ± 2.25 (1 mg/ml)	96 well plate; 77.80 ± 0.21 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 59.50 ± 0.45 (1 mg/ml)	96 well plate; 92.20 ± 0.29 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 32.60 ± 0.01 (1 mg/ml)	96 well plate; 59.90 ± 2.30 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 6.20 ± 0.24 (1 mg/ml)	96 well plate; 54.90 ± 1.95 (1 mg/ml)	Orhan et al. (2007)
Salvia halophila Hedge	Whole plant	Chloroform	NI	96 well plate; 53.90 ± 2.16 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 36.10 ± 1.21 (1 mg/ml)	96 well plate; 37.20 ± 3.88 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 18.90 ± 1.21 (1 mg/ml)	96 well plate; 50.90 ± 4.20 (1 mg/ml)	Orhan et al. (2007)
Salvia lavandulaefolia Vahl.	Whole plant	Steam distilled oil	96 well plate; 63.00 ± 3.70 (0.1 µg/ml)	ND	Perry et al. (1996, 2000, 2001); Mukherjee et al. (2007 a)
Salvia migrostegia Boiss and Bal.	Whole plant	Chloroform	96 well plate; 36.40 ± 5.45 (1 mg/ml)	96 well plate; 62.50 ± 1.31 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 37.10 ± 3.15 (1 mg/ml)	96 well plate; 89.60 ± 0.67 (1 mg/ml)	Orhan et al. (2007)

Table 1. Cont'd

	Whole plant	Methanol	96 well plate; 23.60 ± 0.61 (1 mg/ml)	96 well plate; 32.60 ± 3.40 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	NI	96 well plate; 22.10 ± 2.70 (1 mg/ml)	Orhan et al. (2007)
Salvia multicaulis Vahl.	Whole plant	Ethyl acetate	NI	96 well plate; 64.30 ± 1.02 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 47.70 ± 3.58 (1 mg/ml)	96 well plate; 36.20 ± 0.93 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 21.40 ± 3.91 (1 mg/ml)	96 well plate; 68.80 ± 3.80 (1 mg/ml)	Orhan et al. (2007)
Salvia officinalis L.	Whole plant	Ethanol	96 well plate; 68.20 ± 15.60 (2.5 mg/ml)	ND	Perry et al. (1996, 2000, 2001); Mukherjee et al. (2007 a)
	Whole plant	Steam distilled oil	96 well plate; 52.40 ± 0.80 (0.1 µg/ml)	ND	Perry et al. (1996, 2000, 2001); Mukherjee et al. (2007 a)
Salvia sclarea L.	Whole plant	Chloroform	96 well plate; 55.30 ± 0.98 (1 mg/ml)	96 well plate; 59.90 ± 0.50 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 33.50 ± 4.94 (1 mg/ml)	96 well plate; 75.70 ± 1.83 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 25.30 ± 1.86 (1 mg/ml)	96 well plate; 15.10 ± 1.76 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 25.80 ± 4.51 (1 mg/ml)	96 well plate; 52.60 ± 2.92 (1 mg/ml)	Orhan et al. (2007)
Salvia syriaca L.	Whole plant	Chloroform	96 well plate; 66.90 ± 2.49 (1 mg/ml)	96 well plate; 87.30 ± 1.99 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	96 well plate; 49.80 ± 2.41 (1 mg/ml)	96 well plate; 70.90 ± 2.69 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Methanol	96 well plate; 12.10 ± 1.22 (1 mg/ml)	96 well plate; 12.30 ± 1.10 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 33.40 ± 2.98 (1 mg/ml)	96 well plate; 63.50 ± 2.12 (1 mg/ml)	Orhan et al. (2007)
Salvia triloba L.	Aerial parts	Ethanol	96 well plate; 54.30 ± 3.20 (2 mg/ml)	ND	Orhan et al. (2007)
Salvia verticillata L. ssp. *amasiaca*	Whole plant	Chloroform	NI	96 well plate; 55.70 ± 0.55 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Ethyl acetate	NI	96 well plate; 53.30 ± 5.50 (1 mg/ml)	Orhan et al. (2007)

Table 1. Cont'd

	Whole plant	Methanol	96 well plate; 39.10 ± 3.10 (1 mg/ml)	96 well plate; 72.00 ± 2.99 (1 mg/ml)	Orhan et al. (2007)
	Whole plant	Petroleum ether	96 well plate; 45.60 ± 4.17 (1 mg/ml)	96 well plate; 85.00 ± 53.10 (1 mg/ml)	Orhan et al. (2007)
Teucrium polium L.	Aerial parts	Ethanol	96 well plate; 65.80 ± 3.70 (2 mg/ml)	ND	Orhan et al. (2009)
Lauraceae					
Laurus nobilis L.	Leaf	Decoction	UV spectophotometry; 56.10 ± 5.50 (5 mg/ml)	ND	Ferreira et al. (2006)
	Leaf	Essential oil	UV spectophotometry; 51.30 ± 1.70 (0.5 mg/ml)	ND	Ferreira et al. (2006)
	Leaf	Ethanol	UV spectophotometry; 64.30 ± 9.00 (1 mg/ml)	ND	Ferreira et al. (2006)
Leguminosae					
Albizia procera (Roxb.) Benth.	Bark	Methanol	TLC and 96 well plate; 40.71 ± 0.46 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Butea superba Roxb.	Root barks	Methanol	TLC and 96 well plate; 55.87 ± 5.83 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Cassia fistula L.	Root	Methanol	TLC and 96 well plate; 54.13 ± 3.90 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Mimosa pudica L.	Whole plant	Water	TLC and 96 well plate; 1.68 ± 0.22 (100 µg/ml)	ND	Vinutha et al. (2007)
Trigonella foenum graecum L.	Seeds	Hydro alcohol	TLC and 96 well plate; 50% (140.26 µg)*	ND	SatheeshKumar et al. (2009)
	Seeds	Ethyl acetate	TLC and 96 well plate; 50% (53 µg)*	ND	SatheeshKumar et al. (2009)
	Seeds	Chloroform	TLC and 96 well plate; 50% (146.94 µg)*	ND	SatheeshKumar et al. (2009)
Lycopodiaceae					
Lycopodium clavatum L.	Whole plant	Chloroform: methanol (1:1)	96 well plate; 49.85 ± 1.33 (1 mg/ml)	ND	Orhan et al. (2004); Mukherjee et al. (2007 a)
Magnoliaceae					
Michelia champaca L.	Leaf	Methanol	TLC and 96 well plate; 34.88 ± 4.56 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Malvaceae					
Abutilon indicum L.	Whole plant	Methanol	TLC and 96 well plate; 30.66 ± 1.06 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)

Table 1. Cont'd

Malva silvestris L.	Aerial parts	Aqueous	UV spectophotometry; 25.00 ± 5.70 (5 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Essential oil	UV spectophotometry; 28.10 ± 2.90 (0.1 mg/ml)	ND	Ferreira et al. (2006)
Meliaceae					
Azadirachta indica A. juss.	Bark	Aqueous	TLC and 96 well plate; 5.89 ± 0.33 (100 µg/ml)	ND	Vinutha et al. (2007)
Trichilia dregeana Sond.	Bark	Ethyl acetate	TLC and 96 well plate; 55.00 ± 4.40 (1 mg/ml)	ND	Eldeen et al. (2005)
Menispermaceae					
Stephania suberosa Forman.	Roots	Methanol	TLC and 96 well plate; 91.93 ± 10.80 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Tiliacora triandra (Colebr.) Diel	Root	Methanol	TLC and 96 well plate; 42.29 ± 2.89 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Tinospora cordifolia Miers	Stem	Methanol	TLC and 96 well plate; 69.43 ± 0.37 (100 µg/ml)	ND	Vinutha et al. (2007)
	Stem	Aqueous	TLC and 96 well plate; 12.92 ± 0.26 (100 µg/ml)	ND	Vinutha et al. (2007)
Mimosaceae					
Acacia nilotica (L.) Willd. ex Del. spp. *kraussiana* (Benth.) Brenan	Leaf	Ethyl acetate	TLC and 96 well plate; 53.00 ± 3.70 (1 mg/ml)	ND	Eldeen et al. (2005)
	Leaf	Ethanol	TLC and 96 well plate; 56.00 ± 6.30 (1 mg/ml)	ND	Eldeen et al. (2005)
	Bark	Ethyl acetate	TLC and 96 well plate; 41.00 ± 2.10 (1 mg/ml)	ND	Eldeen et al. (2005)
Acacia sieberiana Dc. var. *woodii* (Burtt Davy) Keay & Brenan	Root	Ethyl acetate	TLC and 96 well plate; 60.00 ± 4.30 (1 mg/ml)	ND	Eldeen et al. (2005)
	Root	Ethanol	TLC and 96 well plate; 62.00 ± 4.1 (1 mg/ml)	ND	Eldeen et al. (2005)
Moraceae					
Ficus religiosa L.	Bark	Methanol	TLC and 96 well plate; 54.47 ± 1.28 (100 µg/ml)	ND	Vimutha et al. (2007)
Streblus asper Lour.	Seed	Methanol	TLC and 96 well plate; 30.51 ± 4.21 (0.1 µg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)

Table 1. Cont'd

Moringaceae					
Moringa oleifera Lam.	Bark	Methanol	TLC and 96 well plate; 4.99 ± 2.74 (100 µg/ml)	ND	Vinutha et al. (2007)
Musaceae					
Musa sapientum L.	Fruit	Methanol	TLC and 96 well plate; 29.14 ± 4.73 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Myristicaceae					
Myristica fragrans Houtt.	Seed	Hydroalcohol	96 well plate; 50% (133.28 µg/ml)*	ND	Mukherjee et al. (2007 b)
Myrsinaceae					
Embelia ribes Burm. f.	Fruit	Methanol	TLC and 96 well plate; 15.70 ± 1.19 (100 µg/ml)	ND	Vinutha et al. (2007)
	Root	Methanol	TLC and 96 well plate; 50.82 ± 0.71 (100 µg/ml)	ND	Vinutha et al. (2007)
Nelumbonaceae					
Nelumbo nucifera Gaertn.	Stamen	Methanol	TLC and 96 well plate; 23.77 ± 2.83 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Nyctaginaceae					
Boerhavia diffusa L.	Whole plant	Methanol	TLC and 96 well plate; 23.78 ± 1.17 (100 µg/ml)	ND	Vinutha et al. (2007)
Olacaceae					
Ptychopetalum olacoides Benth.	Root	Ethanol	Dose dependent activity at doses of 50 and 100 mg/kg, i.p.	ND	Siqueira et al. (2003); Mukherjee et al. (2007 a)
Papaveraceae					
Corydalis cava (L.) Schw. et K.	Whole plant	Water	TLC and 96 well plate; 62.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Whole plant	Methanol	TLC and 96 well plate; 85.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Tuber	Water	TLC and 96 well plate; 92.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Tuber	Methanol	TLC and 96 well plate; 92.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
Corydalis intermedia (L.) Mérat	Whole plant	Water	TLC and 96 well plate; 57.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Whole plant	Methanol	TLC and 96 well plate; 84.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)

Table 1. Cont'd

	Tuber	Water	TLC and 96 well plate; 78.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Tuber	Methanol	TLC and 96 well plate; 97.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
Corydalis solida (L.) Swartz ssp. *laxa*	Whole plant	Water	TLC and 96 well plate; 78.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Whole plant	Methanol	TLC and 96 well plate; 89.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Tuber	Water	TLC and 96 well plate; 85.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Tuber	Methanol	TLC and 96 well plate; 96.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
Corydalis solida (L.) Swartz ssp. slivenensis	Whole plant	Water	TLC and 96 well plate; 48.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Whole plant	Methanol	TLC and 96 well plate; 82.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Tuber	Water	TLC and 96 well plate; 87.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
	Tuber	Methanol	TLC and 96 well plate; 97.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al. (2006)
Piperaceae					
Piper interruptum Opiz	Stems	Methanol	TLC and 96 well plate; 65.16 ± 8.13 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Piper nigrum L.	Seeds	Methanol	TLC and 96 well plate; 58.02 ± 3.83 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Plumbaginaceae					
Plumbago indica L.	Root	Methanol	TLC and 96 well plate; 30.14 ± 3.28 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Poaceae					
Cymbopogon schoenanthus (L.) Spreng	Whole plant	Hexane	96 well plate; 50% (0.55 mg/ml)*	ND	Khadri et al. (2010)
	Whole plant	Dichloromethane	96 well plate; 50% (0.41 mg/ml)*	ND	Khadri et al. (2010)
	Whole plant	Ethyl acetate	96 well plate; 50% (0.35 mg/ml)*	ND	Khadri et al. (2010)
	Whole plant	Methanol	96 well plate; 50% (0.29 mg/ml)*	ND	Khadri et al. (2010)

Table 1. Cont'd

	Whole plant	Proanthocyanidin	96 well plate; 50% (0.75 mg/ml)*	ND	Khadri et al. (2010)
	Whole plant	Aqueous	96 well plate; 50% (0.42 mg/ml)*	ND	Khadri et al. (2010)
Pyrolaceae					
Pyrola japonica Klenze ex Alefeld	Whole plant	Methanol	96 well plate; 37.00 ± 2.00 (5 mg/ml)	96 well plate; 36.00 ± 3.00 (5 mg/ml)	Sancheti et al. (2009)
Rosaceae					
Sanguisorba minor Scop.	Aerial parts	Water	UV spectophotometry; 7.10 ± 1.60 (1 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Essential oil	UV spectophotometry; 46.10 ± 9.70 (1 mg/ml)	ND	Ferreira et al. (2006)
	Aerial parts	Ethanol	UV spectophotometry; 77.50 ± 2.20 (1 mg/ml)	ND	Ferreira et al. (2006)
Rubiaceae					
Paederia linearis Hook. f.	Whole plant	Methanol	TLC and 96 well plate; 29.31 ± 6.39 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Rubia cordifolia L.	Stem	Methanol	TLC and 96 well plate; 22.12 ± 2.22 (100 µg/ml)	ND	Vinutha et al. (2007)
	Stem	Aqueous	TLC and 96 well plate; 5.86 ± 0.37 (100 µg/ml)	ND	Vinutha et al. (2007)
Rutaceae					
Aegle marmelos (L.) Correa ex Roxb.	Fruit pulp	Methanol	TLC and 96 well plate; 44.65 ± 3.04 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Ruta graveolens L.	Whole plant	Water	TLC and 96 well plate; 22.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al.(2006)
	Whole plant	Methanol	TLC and 96 well plate; 39.00 ± 0.00 (0.1 mg/ml)	ND	Adsersen et al.(2006)
Sabiaceae					
Meliosma oldhamii Miq. ex. Maxim.	Whole plant	Methanol	96 well plate; 12.00 ± 2.00 (5 mg/ml)	96 well plate; 19.00 ± 2.00 (5 mg/ml)	Sancheti et al. (2009)
Saliaceae					
Salix mucronata Thunb.	Bark	Ethyl acetate	TLC and 96 well plate; 82.00 ± 3.90 (1 mg/ml)	ND	Eldeen et al. (2005)

Table 1. Cont'd

Sapotaceae					
Mimusops elengi L.	Flower	Methanol	TLC and 96 well plate; 32.81 ± 5.36 (0.1 mg/ml)	ND	Ingkaninan et al. (2003); Mukherjee et al. (2007 a)
Scrophulariaceae					
Bacopa monniera L.	Whole plant	Ethanol	96 well plate; 42.90 ± 1.20 (0.1 mg/ml)	ND	Das et al. (2002); Mukherjee et al. (2007 a)
Solanaceae					
Withania somnifera Dunal.	Root	Methanol	TLC and 96 well plate; 75.95 ± 0.16 (100 µg/ml)	ND	Vinutha et al. (2007)
	Root	Aqueous	TLC and 96 well plate; 24.60 ± 0.38 (100 µg/ml)	ND	Vinutha et al. (2007)
Symplocaceae					
Symplocos chinensis (Lour.) Druce	Whole plant	Methanol	96 well plate; 74.00 ± 2.00 (5 mg/ml)	96 well plate; 75.00 ± 2.00 (5 mg/ml)	Sancheti et al. (2009)
Tamariacaceae					
Myriacaria elegans Royle	Aerial parts	Methanol	96 well plate; 74.80 ± 0.00 (0.2 µg/ml)	ND	Ahmad et al. (2003); Mukherjee et al. (2007 a)
Umbelliferae					
Centella asiatica (L.) Urban	Whole plant	Hydroalcohol	96 well plate; 50% (106.55 µg/ml)*	ND	Mukherjee et al. (2007 b)
Valerianaceae					
Nardostachys jatamansi DC	Rhizomes	Methanol	96 well plate; 50% (562.21 µg/ml)*	ND	Ahmed et al. (2009)
Verbanaceae					
Lantana camara L.	Aerial parts	Aqueous	TLC and 96 well plate; 3.63 ± 1.20 (100 µg/ml)	ND	Vinutha et al. (2007)
Zingiberaceae					
Alpinia galanga Willd.	Rhizomes	Methanol	TLC and 96 well plate; 16.98 ± 0.37 (100µg/ml)	ND	Vinuta et al. (2007)
Zygophyllaceae					
Tribullus terrestris L.	Whole plants	Chloroform: methanol (1:1)	96 well plate; 37.89 ± 0.77 (1 mg/ml)	96 well plate; 78.32 ± 1.27 (1 mg/ml)	Orhan et al. (2004)
Zygophyllum fabago L.	Whole plants	Chloroform: methanol (1:1)	96 well plate; 13.25 ± 0.45 (1 mg/ml)	96 well plate; 78.37 ± 0.95 (1 mg/ml)	Orhan et al. (2004)

NI, no inhibition; ND, not done; * represents IC_{50}

acetylcholine, is considered one of the treatment strategies against several neurological disorders including AD. Several medicinal plants tested have been shown to have an inhibitory effect on AChE. Notable among such plants are several species belonging to the genus *Corydalis* (Adsersen et al., 2006). Kim et al. (1999) found that a methanolic extract of the tuber of *Corydalis ternata* showed significant inhibition of AChE. They isolated protopine, determined the IC_{50} value to be 50 µM and showed that mice treated with protopine exhibited diminished scopolamine-induced dementia measured in a passive avoidance task. Protopine has also been isolated from the tubers of *Corydalis cava* (Preininger et al., 1976), and the aerial parts of *Corydalis solida* ssp. *tauricola* (Şener and Temizer, 1990). Berberine has been isolated from *C. ternata* and at 2.5 µM, this compound was found to have a reversible and specific AChE inhibitor effect (90%) (Hwang et al., 1996). It has been concluded that protoberberine- and protopine-type alkaloids, common compounds in *Corydalis spp.*, are potent inhibitors of AChE (Adsersen et al. 2006). Plant species belonging to Fumariaceae, Papaveraceae and Ericaceae families have also been shown to have very strong activity against AChE and BChE. Since most of the acetylcholinesterase inhibitors are known to contain nitrogen, the strong activity of plants belonging to these families may be due to their rich alkaloid content (Orhan et al., 2004). Plant extracts having activities where percentage inhibition of the enzyme is 60% or more are considered to possess strong inhibitory activity (Khan et al., 2006), while moderate activity refers to percentage inhibition between 15 to 50% (Adsersen et al., 2006) and extracts having percentage inhibition of less than 15% do not show any significant inhibition of the enzyme.

A large amount of evidence has demonstrated that oxidative stress is intimately involved in age-related neurodegenerative diseases and there have been a number of studies which have examined the positive benefits of antioxidants to reduce or to block neuronal death occurring in the pathophysiology of these disorders (Ramassamy, 2006; Loizzo et al., 2009).

The anticholinesterase activities of 14 *Salvia* species were evaluated by Orhan et al. (2007). These plants were further screened for their antioxidant activity using the diphenyl picryl hydrazine (DPPH) and the xanthine oxidase (XO) inhibition assay. It was observed that the ethylacetate (EtOAc) extracts had high antioxidant activity against XO, ranging between 66.1% and 162.4%.

The EtOAc and methanol (MeOH) extracts exhibited good DPPH radical-scavenging activity, similar to that of butylated hydroxyanisole (BHA), the reference drug used. *Salvia* species have been shown to contain phenolic compounds and its antioxidant activity has been ascribed to the presence of carnosic and rosmarinic acids (Cuvelier et al., 1996; Orhan et al., 2007). Salvianolic acid, a rosmarinic acid dimer isolated from *Salvia officinalis*, had a very strong free radical-scavenging activity for DPPH and superoxide anion radicals (Lu and Foo, 2001; Orhan et al., 2007). In addition, β-sitosterol isolated from *Salvia plebeia* was also found to be a strong antioxidant by the oxidative stability instrument (OSI) (Weng and Wang, 2000; Orhan et al., 2007). Several studies on the AChE inhibitory activity of some *Salvia* species have also been reported. The essential oil of *Salvia lavandulaefolia*, together with its major components, α-pinene, 1,8-cineone, and camphor have been shown to have uncompetitive and reversible acetylcholinesterase inhibitory activity due to its monoterpenoids (Perry et al., 2000; Orhan et al., 2007). In another study, four diterpenes, dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA, were isolated from the acetone extract of the dried root of *Salvia miltiorrhiza* and it was concluded that these compounds contributed to the anticholinesterase activity of the plant (Ren et al., 2004; Orhan and Aslan, 2009). These data indicate that terpenoids and monoterpenes in *Salvia* species may be responsible for their anticholinesterase activity. Several other bioactive isolated compounds with cholinesterase inhibitory activity have been reported by Houghton et al. (2006) and Hostettmann et al. (2006).

Khadri et al. (2010) evaluated the water, methanol and proanthocyanidin extracts of *C. schoenanthus* for its total phenolic content, total flavonoids and ability to scavenge the DPPH radical. The results obtained showed a high phenolic content in the three extracts with slightly higher values in the proanthocyanidinrich extracts. In addition, all the extracts were rich in flavonoids and they had very good antioxidant activity comparable to butylated hydroxytoluene (BHT), a known standard. The antioxidant activity of the plant together with its moderate inhibitory activity of acetylcholinesterase supports its medicinal use by local populations for treatment of neurodegenerative diseases. Antioxidant activity (DPPH and β-carotene-linoleic acid assays), and acetylcholinesterase inhibitory activity were determined for 10 Portuguese plants (*H. undulatum*, *M. officinalis*, *L. nobilis*, *L. pedunculata, S. minor, M. suaveolens, L. augustifolia, M. silvestris, P. argentea* and *S. officinalis)* (Ferreira et al., 2006). The authors concluded that these plants may help in treating patients suffering from AD, as they showed inhibition of AChE and have very good antioxidant activity.

Loizzo et al. (2009) further carried out studies on the antioxidant, and anti-inflammatory activities of the essential oils of *O. ehrenbergii* and *O. syriacum* to further validate their use in the treatment of AD. Both plants exhibited significant antioxidant activity and the chemical composition of *O. syriacum* essential oil indicated that it contained antioxidant compounds such as carvacrol, carvacrol methyl ether and thymol methyl ether (Mastelić et al., 2008; Loizzo et al., 2009). Inhibition of nitric oxide (NO) production may result in anti-inflammatory activity and this was studied *in vitro* by analyzing the effect of the essential oils on chemical mediators released from macrophages. The oil of *O. ehrenbergii* showed good

anti-inflammatory activity which is probably due to the presence of thymol, one of the major components of the oils which has a phenolic structure, and has been credited with a series of pharmacological properties, including antimicrobial, antioxidant and anti-inflammatory effects (Braga et al., 2006; Loizzo et al., 2009). These results showed that both oils provide interesting properties from a functional perspective in the prevention of neurodegenerative disorders.

Chattipakorn et al. (2007) carried out additional *in vivo* studies on *Tabernaemontana divaricata* using male Wistar rats, after confirming its cholinesterase inhibitory activity *in vitro*. The major finding of this study was that the plant can inhibit neuronal AChE activity in an animal model and that it has cortical AChE inhibitory effects. According to the authors, there are several possible active compounds with AChE inhibitory activity in *T. divaricata* which include at least forty-four alkaloids and non-alkaloid constituents such as triterpenoids, steroids, flavonoids, phenyl propanoids and phenolic acids. The inhibitory effects of AChE activity in the animal model could be due to the effects of mixed alkaloids in *T. divaricata*.

The 96-well microplate and thin layer chromatography assays based on Ellman's method were the two most commonly used methods for detecting AChE and BChE inhibitory activity in the studies conducted. This is probably because these two methods ensure the possibility of running several replicates for each determination, to improve statistical treatment of results, and are both economical, as only small amounts of reagents and test substances are used.

Methanol was observed to be the most commonly used solvent in extracting the plants. This may indicate that most of the compounds which show anticholinesterase activity are polar in nature. The plant part most commonly investigated was the aerial parts or whole plant (in case of herbs), indicating that roots or bark do not contain sufficient anticholinesterase inhibitory activity.

CONCLUSION

Present efforts aimed at the treatment of Alzheimer's disease, senile dementia, ataxia, myasthenia gravis and Parkinson's disease are centered around the reduction of cholinergic deficit by the use of AChE and BChE inhibitors. Several drugs are on the market, including the plant alkaloid galanthamine. However, a search for more efficient agents with fewer side effects has resulted in the screening of several medicinal plants for possible activity as shown in this review. It is easy to perceive the potential in these plants as attractive targets for future studies, to identify the active constituents and possibly to uncover new alternatives to the existing therapies for neurodegenerative diseases. Furthermore, *in vivo* activity of the active compounds needs to be determined in animal models and human subjects, so as to determine their efficacy in a metabolic environment. Such future studies will be necessary to expand the existing, limited therapeutic arsenal for the majority of neurodegenerative diseases, especially for those therapies with side effects that limit their effectiveness.

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Antioxidant and acetylcholinesterase inhibitory activity of selected southern African medicinal plants

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Abstract

Alzheimer's disease (AD) is the most common type of dementia in the aging population. Enhancement of acetylcholine levels in the brain is one means of treating the disease. However, the drugs presently used in the management of the disease have various drawbacks. New treatments are required and in this study, extracts of *Salvia tiliifolia* Vahl. (whole plant), *Chamaecrista mimosoides* L. Greene (roots), *Buddleja salviifolia* (L.) Lam. (whole plant) and *Schotia brachypetala* Sond. (root and bark) were evaluated to determine their polyphenolic content, antioxidant and acetylcholinesterase inhibitory (AChEI) activity. The DPPH and ABTS assays were used to determine antioxidant activity and Ellman colorimetric method to quantify AChEI activity. Although all four plants showed activity in both assays, the organic extracts of *C. mimosoides* root was found to contain the highest AChEI activity ($IC_{50}=0.03\pm0.08$ mg/ml) and *B. salviifolia* whole plant had the highest antioxidant activity (ABTS; $IC_{50}=0.14\pm0.08$ mg/ml and DPPH; $IC_{50}=0.23\pm0.01$ mg/ml). The results suggest that the tested plant species may provide a substantial source of secondary metabolites, which act as natural antioxidants and acetylcholinesterase inhibitors, and may be beneficial in the treatment of AD.

Keywords: Acetylcholinesterase; Alzheimer's disease; Antioxidant; Medicinal plants; Neurodegeneration

1. Introduction

Dementia is characterized by the gradual onset and continuing decline of higher cognitive functioning (Dhingra et al., 2005). Alzheimer's disease (AD), the most common form of dementia (Nie et al., 2009), is a progressive age-related disorder that is characterized by the degeneration of neurological function. The latter is due to the reduction in levels of the neurotransmitter acetylcholine, in the brains of the elderly as the disease progresses, resulting in loss of cognitive ability (Felder et al., 2000). Acetylcholinesterase inhibitors (AChEIs) have been shown to function by increasing acetylcholine within the synaptic region, thereby restoring deficient cholinergic neurotransmission (Giacobini, 1998; Krall et al., 1999).

Selective cholinesterase inhibitors, free of dose-limiting side effects, are not currently available, and current compounds may not allow sufficient modulation of acetylcholine levels to elicit the full therapeutic response (Felder et al., 2000). In addition, some of the synthetic medicines used e.g. tacrine, donepezil and rivastigmine have been reported to cause gastrointestinal disturbances and problems associated with bioavailability (Melzer, 1998; Schulz, 2003). Therefore, the search for new AChEIs, particularly from natural products, with higher efficacy continues.

Oxidative stress, caused by reactive oxygen species (ROS), is known to result in the oxidation of biomolecules, thereby leading to cellular damage and it plays a key pathogenic role in the aging process (Zhu et al., 2004). In recent years, there has been growing interest in finding natural antioxidants in plants because they inhibit oxidative damage and may consequently

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prevent aging and neurodegenerative diseases (Fusco et al., 2007).

In an effort to discover new sources which can potentially be used in the treatment of AD, four plants — *Salvia tiliifolia* Vahl. (Lamiaceae), *Chamaecrista mimosoides* L. Greene (Caesalpiniaceae), *Buddleja salviifolia* (L.) Lam. (Buddlejaceae) and *Schotia brachypetala* Sond. (Fabaceae), traditionally used in the treatment of neurodegenerative diseases (Orhan et al., 2007; Stafford et al., 2008), were evaluated for their AChEI and antioxidant capacity.

2. Material and methods

2.1. Chemicals

Acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE) type VI-S, from electric eel, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), galanthamine, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and trolox were purchased from Sigma. Methanol and all other organic solvents (analytical grade) were purchased from Merck.

2.2. Plant collection and extract preparation

The plant species; *S. tiliifolia* (whole plant; P03649), *C. mimosoides* (root, P08814), *B. salviifolia* (whole plant, P01281), *S. brachypetala* (bark, P08514) and *S. brachypetala* (root, P06300) were collected in Gauteng Province, South Africa. Identities of the specimens were confirmed by the South African National Biodiversity Institute (SANBI), Tshwane and voucher specimens are deposited at this institution. The plant samples were cut into small pieces and dried in an oven at 30–60 °C for 48 h. Dried material was ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction. Six grams of the powdered plant material was extracted with 60 ml of either dichloromethane/methanol (1:1) or distilled water for 24 h. Organic extracts were concentrated using a rotary vacuum evaporator and then further dried *in vacuo* at ambient temperature for 24 h. The aqueous extracts were concentrated by freeze-drying. All extracts were stored at −20 °C prior to analysis. The residues were redissolved in DCM:MeOH or distilled water, respectively to the desired test concentrations.

2.3. Micro-plate assay for inhibition of acetylcholinesterase

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method as modified by Eldeen et al. (2005). Into a 96-well plate was placed: 25 µl of 15 mM ATCI in water, 125 µl of 3 mM DTNB in Buffer C (50 mM Tris–HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$), 50 µl of Buffer B (50 mM, pH 8, containing 0.1% bovine serum albumin) and 25 µl of plant extract (0.25, 0.5, 1 or 2 mg/ml). Absorbance was measured spectrophotometrically (Labsystems Multiscan EX type 355 plate reader) at 405 nm every 45 s, three times consecutively. Thereafter, AChE (0.2 U/ml) was added to the wells and the absorbance measured five times consecutively every 45 s. Galanthamine served as the positive control. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the absorbance before adding the enzyme from the absorbance after adding the enzyme. The percentage inhibition was calculated using the equation:

$$\text{Inhibition } (\%) = 1-\left(A_{\text{sample}} / A_{\text{control}}\right) \times 100$$

where A_{sample} is the absorbance of the sample extracts and A_{control} is the absorbance of the blank [methanol in Buffer A (50 mM Tris–HCl, pH 8)]. Extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting the percentage inhibition against extract concentration.

2.4. Determination of total phenolics

Total phenolic content in the extracts were determined by the modified Folin–Ciocalteu method of Wolfe et al. (2003). The extract (1 mg/ml) was mixed with 5 ml Folin–Ciocalteu reagent (diluted with water 1:10 v/v) and 4 ml (75 g/l) sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40 °C for color development. Absorbance was measured at 765 nm using the Hewlett Packard UV–VIS spectrophotometer. Total phenolic content is expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: $y=0.1216x$, where x is the absorbance and y is the tannic acid equivalent (mg/g).

2.5. Determination of total flavonoids

Total flavonoid content was determined using the method of Ordonez et al. (2006). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample (1 mg/ml). After one hour at room temperature, the absorbance was measured at 420 nm. A yellow color is indicative of the presence of flavonoids. Total flavonoid content was calculated as quercetin equivalent (mg/g), using the following equation based on the calibration curve: $y=0.025x$, where x is the absorbance and y is the quercetin equivalent (mg/g).

2.6. Determination of total proanthocyanidins

The procedure reported by Sun et al. (1998) was used to determine the total proanthocyanidin content. A volume of 0.5 ml of 1 mg/ml extract solution was mixed with 3 ml of a 4% vanillin–methanol solution and 1.5 ml hydrochloric acid. The mixture was allowed to stand for 15 min after which the absorbance was measured at 500 nm. Total proanthocyanidin content is expressed as catechin equivalents (mg/g) using the following equation based on the calibration curve: $y=0.5825x$, where x is the absorbance and y is the catechin equivalent (mg/g).

2.7. Antioxidant activity

2.7.1. DPPH radical scavenging activity

The effect of the extracts on DPPH radical was estimated using the method of Liyana-Pathiranan and Shahidi (2005), with minor modifications. A solution of 0.135 mM DPPH in methanol was prepared and 185 µl of this solution was mixed with 15 µl of varying concentrations of the extract (0.25, 0.5, 1 and 2 mg/ml), in a 96-well plate. The reaction mixture was vortexed and left in the dark for 30 min (room temperature). The absorbance of the mixture was determined at 570 nm using a micro plate reader. Trolox was used as the reference antioxidant compound. The ability to scavenge the DPPH radical was calculated using the equation:

$$\text{DPPH radical scavenging activity}(\%) = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

where $A_{control}$ is the absorbance of DPPH radical + methanol and A_{sample} is the absorbance of DPPH radical + sample extract/ standard. The extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting inhibition percentage versus extract concentration.

2.7.2. ABTS radical scavenging activity

The method of Re et al. (1999) was adopted for the ABTS assay. The stock solution which was allowed to stand in the dark for 16 h at room temperature contained equal volumes of 7 mM ABTS salt and 2.4 mM potassium persulfate. The resultant $ABTS^{*+}$ solution was diluted with methanol until an absorbance of 0.706±0.001 at 734 nm was obtained. Varying concentrations (0.25, 0.5, 1 and 2 mg/ml) of the extract were allowed to react with 2 ml of the $ABTS^{*+}$ solution and the absorbance readings were recorded at 734 nm. The $ABTS^{*+}$ scavenging capacity of the extract was compared with that of trolox and the percentage inhibition calculated as:

$$\text{ABTS radical scavenging activity}(\%) = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

where $A_{control}$ is the absorbance of ABTS radical + methanol and A_{sample} is the absorbance of ABTS radical + sample extract/ standard. All tests were carried out on three separate occasions. The extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting inhibition percentage versus extract concentration.

2.8. Statistical analysis

All determinations were done in triplicate, and the results reported as mean±standard deviation (S.D.). Calculation of IC_{50} values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc).

3. Results and discussion

Four plants — *S. tiliifolia* Vahl. (Lamiaceae), *C. mimosoides* L. Greene (Caesalpiniaceae), *B. salviifolia* (L.) Lam. (Buddlejaceae) and *S. brachypetala* Sond. (Fabaceae), traditionally used in the treatment of neurodegenerative diseases (Orhan et al., 2007; Stafford et al., 2008) were the focus of the current study. Cold water root infusions of *C. mimosoides* are reported to be taken to remember forgotten dreams by the Zulu (Hulme, 1954). *Buddleja* species are used together with *Heteromorpha trifoliate* and *Cussonia paniculata* by Sotho in South Africa to treat early nervous and mental illnesses (Watt and Breyer-Brandwijik, 1962). The bark and roots of *S. brachypetala* are reported to be used for nervous conditions (Van Wyk and Gericke, 2000), whereas *Salvia* species have been reported to be used for memory-enhancing purposes in European folk medicine (Perry et al., 2003). The inclusion of *S. tiliifolia* and *B. salviifolia* was a taxonomically informed selection as both *Salvia* and *Buddleja* species have been reported to be useful in treatment of neurodegenerative diseases (Perry et al., 2003; Watt and Breyer-Brandwijik, 1962).

The results of the AChE inhibitory activities of the tested plant extracts as well as the positive control, galanthamine, are provided in Fig. 1. All the plant extracts contained some level of inhibitory activity against AChE. Water was used as one of the solvents as the plants investigated are traditionally prepared as either infusions or decoctions (Hulme, 1954; Hutchings et al., 1996; Watt and Breyer-Brandwijik, 1962). However, the DCM: MeOH (1:1) extracts had better activity than the water extracts with *C. mimosoides* root showing the highest percentage inhibition of AChE. The higher activity of the DCM:MeOH (1:1) extracts may suggest that organic solvents are able to extract more active compounds with possible AChE inhibitory activity than water. The IC_{50} values of the plant extracts indicating AChE inhibitory activity are presented in Table 1. A Low IC_{50} value is indicative of good inhibition of the enzyme. The organic extracts of *C. mimosoides* had the lowest IC_{50} value, indicating that it contained the best inhibition of the enzyme.

Since a large amount of evidence demonstrates that oxidative stress is intimately involved in age-related neurodegenerative diseases, there have been a great number of studies which have examined the positive benefits of antioxidants to reduce or to block neuronal death occurring in the pathophysiology of these disorders (Ramassamy, 2006). In addition, the antioxidant potential of a compound can be attributed to its radical scavenging ability, and in order to evaluate the ability of the plant extracts to serve as antioxidants, two activities were measured; ability to scavenge DPPH and ABTS radicals. Figs. 2 and 3 depict the dose-dependent ABTS and DPPH radical scavenging activity of the plant extracts expressed as a percentage of the ratio of the decrease in absorbance of the test solution to that of DPPH or ABTS solution without the plant extracts, respectively. All the plant extracts showed a propensity to quench the free radicals, as indicated by the dose-dependent increase in percentage inhibition. This corresponded to a rapid decrease in absorbance in the presence of a plant

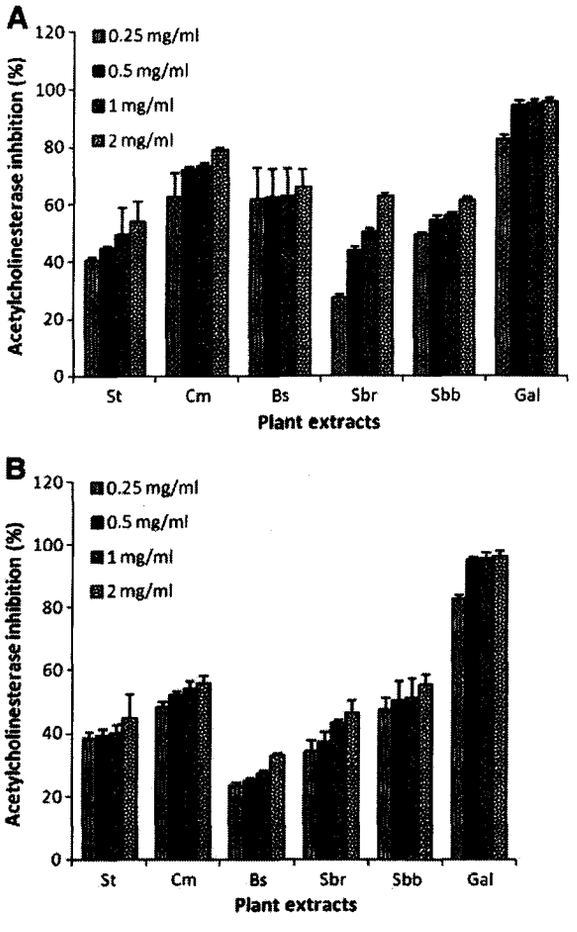

Fig. 1. AChE inhibitory activity (%) of (A) DCM:MeOH (1:1) extracts and (B) water extracts, of the plants investigated. St, *Salvia tiliifolia* whole plant; Cm, *Chamaecrista mimosoides* root; Bs, *Buddleja salviifolia* whole plant; Sbr, *Schotia brachypetala* root; Sbb, *Schotia brachypetala* bark; Gal, galanthamine (positive control).

extract, indicating high antioxidant potency of the extracts in terms of electron or hydrogen atom-donating capacity. The IC_{50} values (concentration of the extract that is able to scavenge half of the DPPH or ABTS radical) are presented in Table 3. The organic extracts of the root of *S. brachypetala* had the lowest IC_{50} values in both antioxidant assays, indicative of its good antioxidant potential.

All five extracts contained phenols with the highest amount in the water extract of the bark of *S. brachypetala* (Table 2). The lowest phenolic content was found in the water extract of the roots of *C. mimosoides*. Antioxidant activity of plants has been partly ascribed to phenolic compounds (Robards et al., 1999). Most of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers (Hakkim et al., 2007). The plant extracts also contained some flavonoids with the highest found in the organic extracts of *S. tiliifolia* (Table 2). Flavonoids have also been reported to be responsible for antioxidant activity, as they act on enzymes and pathways involved in anti-inflammatory processes

Table 1
Acetylcholinesterase inhibitory activity, represented by IC_{50} of plant extracts as determined by the microplate assay.

Extract	AChE inhibition	
	IC_{50} (mg/ml)	
	DCM:MeOH (1:1)	Water
S. tiliifolia	1±0.01	12±1.20
C. mimosoides	0.03±0.08	0.35±0.02
B. salviifolia	0.05±0.02	ND
S. brachypetala root	0.89±0.01	3.40±0.50
S. brachypetala bark	0.27±0.07	0.49±0.04

ND, not determined, represents extracts with maximum inhibition below 50% at the highest tested concentration of 2 mg/ml.
The IC_{50} value for the positive control, galanthamine, was 5.3×10^{-4} mg/ml.

(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). The tannins found in proanthocyanidins are also good antioxidant components, as they can reduce metallic ions such as Fe^{3+} to the Fe^{2+} form and can inhibit the 5-lipoxygenase enzyme in arachidonic acid metabolism, which is important in inflammation physiology (Okuda, 2005). The highest level of proanthocyanidins was contained in water extracts of the bark of *S. brachypetala* (Table 2).

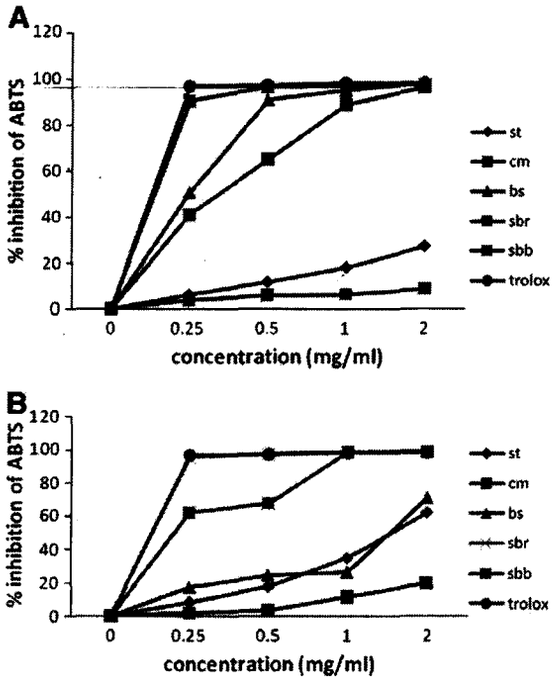

Fig. 2. ABTS radical scavenging activity of (A) DCM:MeOH (1:1) extracts and (B) water extracts, of the plants investigated. St, *Salvia tiliifolia* whole plant; Cm, *Chamaecrista mimosoides* root; Bs, *Buddleja salviifolia* whole plant; Sbr, *Schotia brachypetala* root; Sbb, *Schotia brachypetala* bark; trolox (positive control).

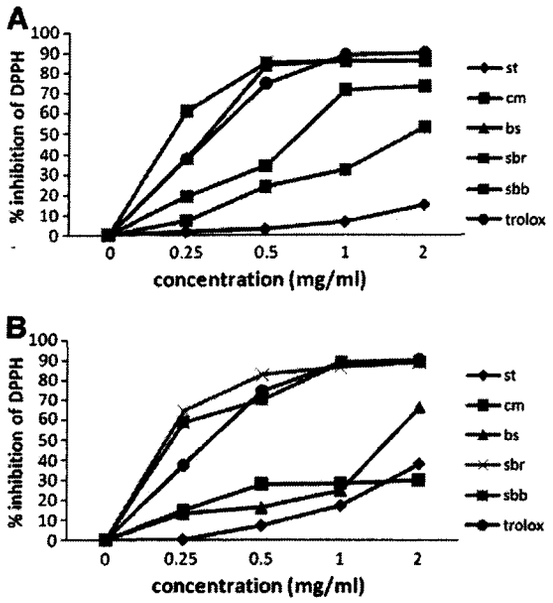

Fig. 3. DPPH radical scavenging activity (%) of (A) DCM:MeOH (ABTS) (1:1) extracts and (B) water extracts, of the plants investigated. St, *Salvia tiliifolia* whole plant; Cm, *Chamaecrista mimosoides* root; Bs, *Buddleja salviifolia* whole plant; Sbr, *Schotia brachypetala* root; Sbb, *Schotia brachypetala* bark; trolox (positive control).

A variety of bioactive compounds that could be responsible for the observed bioactivities has been reported in some of the screened medicinal plants or related genera. 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, α-pinene, 1, 8-cineole, and camphor (Perry et al., 2000). *S. brachypetala* showed dose-dependent inhibition of AChE and high antioxidant activity for the organic extracts of the root. This finding 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 both 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 have also been reported to contain good antioxidant activity (Motlhanka, 2008), 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 (Houghton et al., 2003). Some of the sesquiterpenes have been shown to contain anti-inflammatory activity (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.

4. Conclusion

Since AD is pathologically complex, the use of multifunctional drugs is a more rational approach to treatment. Overall, the DCM:MeOH extracts of *C. mimosoides*, *B. salviifolia* and *S. brachypetala* roots showed good antioxidant and cholinesterase inhibitory activity. These plant extracts and their active components could emerge as natural antioxidants, alternative anticholinesterase drugs or serve as starting points for synthesizing more effective AChE inhibitors.

Table 2
Total phenols, flavonoids and proanthocyanidin contents of the plant extracts investigated.

Plant	Extract	Total phenols [a]	Total flavonoids [b]	Total proanthocyanidins [c]
S. tiliifolia	DCM:MeOH (1:1)	129.75±0.02	35.98±0.08	64.08±0.02
	Water	72.02±0.01	10.65±0.01	17.86±0.10
C. mimosoides	DCM:MeOH (1:1)	141.53±0.21	16.86±0.35	98.83±0.01
	Water	64.16±0.13	5.32±0.38	16.19±0.05
B. salviifolia	DCM:MeOH (1:1)	169.66±0.33	23.95±0.11	92.42±0.63
	Water	77.92±0.91	12.11±0.26	51.80±0.34
S. brachypetala root	DCM:MeOH (1:1)	303.91±0.92	4.24±0.23	19.65±0.82
	Water	291.80±0.12	13.44±0.08	12.17±0.07
S. brachypetala bark	DCM:MeOH (1:1)	305.52±0.21	10.97±0.17	24.54±0.47
	Water	337.66±0.12	17.71±0.54	163.04±0.86

Data represent mean±SD.
[a] Expressed as mg tannic acid/g of dry plant material.
[b] Expressed as mg quercetin/g of dry plant material.
[c] Expressed as mg catechin/g of dry plant material.

Table 3
Antioxidant activity, represented by IC_{50} of the plant extracts, measured by the DPPH and ABTS radical scavenging tests.

Extract	DPPH test		ABTS test	
	IC_{50} (mg/ml)		IC_{50} (mg/ml)	
	DCM:MeOH (1:1)	Water	DCM:MeOH (1:1)	Water
S. tiliifolia	ND	ND	ND	1.51±0.23
C. mimosoides	0.72±0.03	ND	0.3±0.05	ND
B. salviifolia	0.23±0.01	1.60±0.51	0.14±0.08	1±0.05
S. brachypetala root	0.05±0.02	0.05±0.02	$3.26\times10^{-7}\pm0.1\times10^{-9}$	$3.7\times10^{-7}\pm0.21\times10^{-9}$
S. brachypetala bark	1.90±0.50	0.13±0.03	ND	0.15±0.03

ND, not determined represents extracts with a maximum inhibition below 50% at the highest tested concentration of 2 mg/ml.

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In vitro screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from southern Africa

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Phenols

ABSTRACT

Objective: To determine the acetylcholinesterase inhibitory (AChEI) and antioxidant activity of the ethyl acetate and methanol extracts of 12 traditional medicinal plants used in the treatment of neurological disorders. **Methods:** AChEI activity was determined spectrophotometrically using the Ellman's colorimetric method. Antioxidant activity was carried out by determining the ability of the extracts to scavenge 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals. The levels of total phenols, flavonoids and flavonols were determined quantitatively using spectrophotometric methods. **Results:** AChEI was observed to be dose-dependent. *Lannea schweinfurthii* (*L. schweinfurthii*) (Engl.) Engl. and *Scadoxus puniceus* (*S. puniceus*) (L.) Friis & I. Nordal. root extracts showed the lowest IC_{50} value of 0.000 3 mg/mL for the ethyl acetate extracts while *Zanthoxylum davyi* (*Z. davyi*) (I. Verd.) P.G. Watermann had the lowest IC_{50} value of 0.01 mg/mL for the methanol extracts in the AChEI assay. The roots of *Piper capense* (*P. capense*) L.f., *L. schweinfurthii*, *Ziziphus mucronata* (*Z. mucronata*) Willd., *Z. davyi* and *Crinum bulbispermum* (*C. bulbispermum*) (Burm.f.) Milne-Redh. & Schweick. showed noteworthy radical scavenging activity and good AChEI activity. **Conclusions:** Five plants show good antioxidant and AChEI activity. These findings support the traditional use of the plants for treating neurological disorders especially where a cholinesterase mechanism and reactive oxygen species (ROS) are involved.

1. Introduction

Neurological disorders primarily affect the elderly population. Alzheimer's disease (AD), the most common neurodegenerative disorder is characterized clinically by progressive memory deficits and impaired cognitive function[1,2]. AD is estimated to account for between 50 and 60% of dementia cases in persons over 65 years of age and according to the United Nations, the number of people suffering from age-related neurodegeneration, particularly from AD, will exponentially increase from 25.5 million in 2000 to an estimated 114 million in 2050[3]. It is a major public health concern in developed countries due to the increasing number of sufferers, placing strains on caregivers as well as on financial resources[2].

A deficiency in levels of the neurotransmitter acetylcholine (ACh) has been observed in the brains of AD patients, and inhibition of acetylcholinesterase (AChE), the key enzyme which hydrolyses ACh, is a major treatment option for AD[4]. Traditionally used plants have been shown to be good options in the search for AChE inhibitors. Galantamine, originally isolated from plants of the Amaryllidaceae family, has become an important treatment of AD[5]. The AChE inhibitory activity of this drug is the principal mode of action to provide symptomatic relief. Galantamine increases the availability of ACh in the cholinergic synapse by competitively inhibiting the enzyme responsible for its breakdown, AChE. The binding of galantamine to AChE slows down the catabolism of ACh and, as a consequence, ACh levels in the synaptic cleft are increased[6-9]. It is licensed in Europe for AD treatment and was well tolerated and significantly improved cognitive function when administered to AD patients in multi-center randomized-controlled trials[10]. To date, several plants

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have been identified as containing acetylcholinesterase inhibitory (AChEI) activity[11].

Reactive oxygen species (ROS) generated from activated neutrophils and macrophages have been reported to play an important role in the pathogenesis of various diseases, including neurodegenerative disorders, cancer and atherosclerosis[12,13]. Oxidative processes are among the pathological features associated with the central nervous system in AD. Oxidative stress causes cellular damage and subsequent cell death especially in organs such as the brain. The brain in particular is highly vulnerable to oxidative damage as it consumes about 20% of the body's total oxygen, has a high content of polyunsaturated fatty acids and lower levels of endogenous antioxidant activity relative to other tissues[14-16]. The brain of patients suffering from AD is said to be under oxidative stress as a result of perturbed ionic calcium balances within their neurons and mitochondria[17,18]. Herbal products are reported to possess the ability to act as antioxidants, thereby reducing oxidative damage[19]. Among the natural phytochemicals identified from plants, flavonoids together with flavonols, and phenols represent important and interesting classes of biologically active compounds. Evidence suggests that these compounds are effective in the protection of various cell types from oxidative injury[20].

The aim of the present study was to determine the AChEI and antioxidant activity of the ethyl acetate and methanol extracts of 12 plants, traditionally used in the treatment of neurological disorders.

2. Material and methods

2.1. Chemicals

Acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE) type VI-S, from electric eel, 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB), galanthamine, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and trolox were purchased from Sigma. Methanol and all other organic solvents (analytical grade) were purchased from Merck.

2.2. Plant collection and extract preparation

Specimens investigated in this study were identified and voucher specimens deposited at the South African National Biodiversity Institute (SANBI), Tshwane. The plant samples were cut into small pieces and air-dried at room temperature. Dried material was ground to a fine powder and stored at ambient temperature till use. Six grams of the powdered plant material was extracted with 60 mL of either methanol or ethyl acetate for 24 h while shaking. The extracts were filtered, concentrated using a rotary vacuum evaporator and then further dried in vacuo at ambient temperature for 24 h. All extracts were stored at −20 ℃ prior to analysis. The residues were redissolved in either MeOH or ethyl acetate to the desired test concentrations.

2.3. Micro-plate assay for inhibition of acetylcholinesterase

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method[21] as modified by Eldeen *et al*[22]. Into a 96-well plate was placed: 25 μL of 15 mmol/L ATCI in water, 125 μL of 3 mmol/L DTNB in Buffer A (50 mmol/L Tris-HCl, pH 8, containing 0.1 mol/L NaCl and 0.02 mol/L $MgCl_2 \cdot 6H_2O$), 50 μL of Buffer B (50 mmol/L, pH 8, containing 0.1 % bovine serum albumin) and 25 μL of plant extract (0.007 mg/mL, 0.016 mg/mL, 0.031 mg/mL, 0.063 mg/mL or 0.125 mg/mL). Absorbance was determined spectrophotometrically (Labsystems Multiscan EX type 355 plate reader) at 405 nm at 45 s intervals, three times consecutively. Thereafter, AChE (0.2 U/mL) was added to the wells and the absorbance measured five times consecutively every 45 s. Galantamine served as the positive control. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the absorbance before adding the enzyme from the absorbance after adding the enzyme. The percentage inhibition was calculated using the equation:

$$\text{Inhibition } (\%) = 1 - (A_{sample}/A_{control}) \times 100$$

Where A_{sample} is the absorbance of the sample extracts and $A_{control}$ is the absorbance of the blank [methanol/ethyl acetate in 50 mmol/L Tris-HCl, (pH 8)]. Extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting the percentage inhibition against extract concentration.

2.4. Determination of total phenolics

Total phenolic content was determined using the modified Folin-Ciocalteu method of Wolfe *et al*[23]. The extract (1 mg/mL) was mixed with 5 mL Folin-Ciocalteu reagent (diluted with water 1:10 v/v) and 4 mL (75 g/L) sodium carbonate. The mixture was vortexed for 15 s and allowed to stand for 30 min at 40 ℃ for color development. Absorbance was measured at 765 nm using a Hewlett Packard UV-VIS spectrophotometer. Total phenolic content is expressed as mg/g gallic acid equivalent and was determined using the equation based on the calibration curve: Y = 6.993X + 0.037, where X is the absorbance and Y is the gallic acid equivalent (mg/g).

2.5. Determination of total flavonoids

Total flavonoid content was determined using the method of Ordonez *et al*[24]. A volume of 0.5 mL of 2% $AlCl_3$ ethanol solution was added to 0.5 mL of sample solution (1 mg/mL). After one hour at room temperature, the absorbance was measured at 420 nm using a Hewlett Packard UV-VIS spectrophotometer. A yellow color is indicative of the presence of flavonoids. Total flavonoid content was

calculated as quercetin equivalent (mg/g), using the equation based on the calibration curve: Y = 0.025X, where X is the absorbance and Y is the quercetin equivalent (mg/g).

2.6. Determination of total flavonols

Total flavonol content was assessed using the method of Kumaran and Karunakaran[25]. To 2 mL of sample (1 mg/mL), 2 mL of 2% $AlCl_3$ ethanol and 3 mL (50 g/L) sodium acetate solution were added. The samples were incubated for 2.5 h at 20 ℃ after which absorbance was determined at 440 nm. Total flavonoid content was calculated using the equation based on the calibration curve: Y = 0.0255X, where X was the absorbance and Y is the quercetin equivalent (mg/g).

2.7. Antioxidant activity

2.7.1. DPPH radical scavenging activity

The effect of the extracts on DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi[26], with minor modifications. A solution of 0.135 mmol/L DPPH in methanol was prepared and 185 μL of this solution was mixed with 15 μL of varying concentrations of the extract (0.007 mg/mL, 0.016 mg/mL, 0.031 mg/mL, 0.063 mg/mL or 0.125 mg/mL), in a 96-well plate. The reaction mixture was vortexed and left in the dark for 30 min (room temperature). The absorbance of the mixture was determined at 570 nm using a microplate reader. Trolox was used as the reference antioxidant compound. The ability to scavenge the DPPH radical was calculated using the equation:

DPPH radical scavenging activity (%) =

$[(A_{control} - A_{sample})/A_{control}] \times 100$

Where $A_{control}$ is the absorbance of DPPH radical + methanol and A_{sample} is the absorbance of DPPH radical + sample extract/standard. The extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting inhibition percentage versus extract concentration.

2.7.2. ABTS radical scavenging activity

The method of Re *et al*[27] was adopted for the ABTS assay. The stock solution which was allowed to stand in the dark for 16 h at room temperature contained equal volumes of 7 mmol/L ABTS salt and 2.4 mmol/L potassium persulfate. The resultant $ABTS^{*+}$ solution was diluted with methanol until an absorbance of 0.706 ± 0.001 at 734 nm was obtained. Varying concentrations (0.007 mg/mL, 0.016 mg/mL, 0.031 mg/mL, 0.063 mg/mL or 0.125 mg/mL) of the extract were allowed to react with 2 mL of the $ABTS^{*+}$ solution and the absorbance readings were recorded at 734 nm. The $ABTS^{*+}$ scavenging capacity of the extract was compared with that of trolox and the percentage inhibition calculated as:

ABTS radical scavenging activity (%) =

$[(A_{control} - A_{sample})/A_{control}] \times 100$

where $A_{control}$ is the absorbance of ABTS radical + methanol and A_{sample} is the absorbance of ABTS radical + sample extract/standard. The extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting inhibition percentage versus extract concentration.

2.8. Statistical analysis

All determinations were carried out on three occasions in triplicate. The results are reported as mean ± standard deviation (S.D.). Calculation of IC_{50} values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc.).

3. Results

Twelve plant species: roots of *Adenia gummifera* (*A. gummifera*) (Harv.) Harms (Passifloraceae), *Piper capense* (*P. capense*) L.f. (Piperaceae); *Zanthoxylum davyi* (*Z. davyi*) (I. Verd.) P.G. Watermann (Rutaceae), *Xysmalobium undulatum* (*X. undulatum*) (L.)W.T.Aiton. (Apocynaceae), *Lannea schweinfurthii* (*L. schweinfurthii*) (Engl.) Engl. (Anacardiaceae), *Terminalia sericea* (*T. sericea*) Burch. ex DC. (Combretaceae), *Ziziphus mucronata* (*Z. mucronata*) Willd. (Rhamnaceae), *Tabernaemontana elegans* (*T. elegans*) Stapf. (Apocynaceae), *Crinum bulbispermum* (*C. bulbispermum*) (Burm.f.) Milne-Redh. & Schweick. (Amaryllidaceae), *Scadoxus puniceus* (*S. puniceus*) (L.) Friis & I. Nordal. (Amaryllidaceae), *Tulbaghia violacea* (*T. violacea*) Harv. (Alliaceae) and fruits of *Ficus capensis* (*F. capensis*) Thunb. (Moraceae) were investigated for AChEI as these plants have been reported to treat various neurological conditions[28-39]. Ten of the plant species showed some level of inhibitory activity against AChE as indicated by their IC_{50} values (Table 1). At the highest concentration (0.125 mg/ml), 40% showed good (>50% inhibition), 50% moderate (30-50% inhibition) and 10% low (<30% inhibition) AChE inhibition[40]. *L. schweinfurthii* and *S. puniceus* root extracts showed the lowest IC_{50} values for the ethyl acetate extracts while *Z. davyi* had the lowest IC_{50} value for the methanol extracts (Table 1). Generally, inhibition of AChE was dose dependent and the ethyl acetate extracts were more active than the methanol extracts.

The ethyl acetate extracts of all the plants with the exception of *T. sericea* showed either no activity or very low radical scavenging activity in both the DPPH and ABTS assays as indicated by their IC_{50} values (Table 1). As the methanol extract showed higher activity, it would appear as if very polar solvents are able to extract compounds containing antioxidant activity. Methanol extracts of the roots of five plants and ethyl acetate of one plant showed radical scavenging activity < 50%.

The extracts which showed good DPPH and ABTS radical scavenging ability (> 60%) were further evaluated for their phenolic composition (Table 2). The levels of these phenolic

Table 1
AChEI, ABTS and DPPH radical scavenging activity of methanol and ethyl acetate extracts.

Species	Extraction solvent (plant part)	AChE inhibition IC_{50} (mg/mL)	ABTS radical inhibition IC_{50} (mg/mL)	DPPH radical inhibition IC_{50} (mg/mL)
A. gummifera	Ethyl acetate (root)	0.018 9±0.005	*	*
P. capense	Methanol (root)	*	0.040 2±0.003	0.044 3±0.010
	Ethyl acetate (root)	0.040 7±0.012	*	*
Z. davyi	Methanol (root)	0.010 0±0.004	0.075 2±0.021	*
	Ethyl acetate (root)	0.011 6±0.002	*	*
X. undulatum	Ethyl acetate (root)	0.000 5±0.000	*	*
L. schweinfurthii	Methanol (root)	*	0.003 6±0.001	0.015 1±0.004
	Ethyl acetate (root)	0.000 3±0.000	*	*
T. sericea	Methanol (root)	*	0.003 1±0.001	0.014 7±0.006
	Ethyl acetate (root)	*	0.074 6±0.017	*
Z. mucronata	Methanol (root)	*	0.018 7±0.020	0.029 1±0.051
	Ethyl acetate (root)	0.011 2±0.003	*	*
F. capensis	Ethyl acetate (fruit)	0.031 9±0.005	*	*
S. puniceus	Ethyl acetate (bulb)	0.000 3±0.000	*	*
C. bulbispermum	Ethyl acetate (root)	0.039 3±0.014	*	*
	Methanol (bulb)	0.014 8±0.039	0.068 5±0.041	*
	Ethyl acetate (bulb)	0.002 1±0.007	*	*
Galanthamine	N/A	5.3×10^{-5}	N/A	N/A
Trolox	N/A	N/A	0.013 1	9.6×10^{-6}

*Represents extracts with maximum inhibition below 50% at the highest tested concentration of 0.125 mg/mL.

Table 2
Total phenol, flavonoid and flavonol contents of the methanolic plant extracts with antioxidant activity (> 60%).

Plant and part	Total phenol[a]	Total flavonoid[b]	Total flavonol[b]
Z. davyi roots	97.26±0.40	8.66±0.40	22.84±0.10
L. schweinfurthii roots	101.27±0.10	13.58±0.30	17.29±0.60
T. sericea roots	36.73±0.21	73.05±0.40	28.78±0.50
Z. mucronata roots	73.86±0.25	17.76±0.20	15.53±0.30
C. bulbispermum roots	202.38±0.50	9.18±0.50	20.79±0.10
P. capense roots	237.60±0.12	18.14±0.20	12.90±0.10

[a]Expressed as mg tannic acid/g of extract. [b]Expressed as mg quercetin/g of extract.

compounds are an indication of the potential antioxidant activity of the plant extracts. The methanol extracts of *T. sericea* roots contained the highest flavonoid and flavonol content.

4. Discussion

Z. davyi roots showed good AChEI with IC_{50} values of 0.01 mg/mL and 0.012 mg/mL for the methanol and ethyl acetate extracts respectively. Seven benzo[c]phenanthridine alkaloids have been isolated from the stem-bark of *Z. davyi*[41], and these or similar alkaloids may be responsible for its observed inhibition of acetylcholinesterase. Also, anticonvulsant activity has been reported for both the methanol and aqueous leaf extracts of *Z. capense*[42]. 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 shown to contain triterpene steroids and saponins and these compounds are known to exhibit neuroprotective activity[43]. The ethyl acetate extracts of *C. bulbispermum* bulbs showed an IC_{50} value of 0.039 mg/ml for AChEI, which may be ascribed to several alkaloids which have been isolated from the plant[44]. In addition alkaloidal extracts from *Crinum jagus* and *C. glaucum* have been demonstrated to possess AChEI activity which

has been ascribed to hamayne (IC_{50}-250 μ mol/L) and lycorine (IC_{50}-450 μ mol/L)[45]. Furthermore, the alkaloids; haemanthamine and lycorine, isolated from *C. ornatum*, have been shown to contain anticonvulsant activity[46]. It is possible that the presence of these or similar alkaloids may be responsible for the activity observed. The ethyl acetate extract of *Piper capense* was observed to show inhibition of AChEI with an IC_{50} value of 0.041 mg/mL. Amide alkaloids with activity in the CNS have been identified from the roots of *P. guineense*[28]. *P. methysticum* has been reported to possess local anaesthetic, sedating, anticonvulsive, muscle-relaxant and sleep-stimulating effects which are due to the presence of kavopyrones[28]. *P. capense* contains the amide alkaloids; piperine and 4,5-dihydropiperine, which have previously been shown to have CNS activity[47]. Also, piperine has been reported to improve memory impairment and neurodegeneration in the hippocampus of animal models with AD[48]. The ethanol extracts of *X. undulatum* were found to exhibit good antidepressant-like effects in three animal models[49]. The leaves of this plant have also been reported to have good selective serotonin re-uptake inhibitory activity[50]. The neuroprotective effect of the plant has been ascribed to several glycosides[29], which may be responsible for its observed activity as its ethyl acetate extracts showed inhibition of the enzyme with IC_{50} value of 0.000 5 mg/mL. Glycosides are among the class of compounds which show neuroprotective activity. Four pregnane glycosides; cynatroside A, cynatroside B, cynatroside C and cynascyroside D, have been isolated from *C. atratum*[51-53]. These glycosides showed AChE inhibition with IC_{50} values varying between 3.6 μ mol/L for cynatroside B and 152.9 μ mol/L for cynascyroside D[51-53].

Polar solvents have been reported to extract compounds including alkaloids which show cholinesterase inhibitory activity[22]. This explains the use of methanol and ethyl acetate as solvents for extraction in this study. As the ethyl acetate extracts showed better activity for most of the plants, it may appear as if the solvent is able to extract more of the compounds which inhibit AChE.

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[54]. As *L. schweinfurthii*, belongs to the same family, similar compounds could be present and therefore responsible for its good antioxidant activity, as its methanol extracts showed an IC_{50} value of 0.003 6 mg/mL for inhibition of ABTS radicals. *P. capense* showed good antioxidant activity (IC_{50} value of 0.040 2 mg/mL and 0.044 3 mg/mL for inhibition of ABTS and DPPH radicals) which has also been reported for other *Piper* species; *P. arboreum* and *P. tuberculatum*[55-57]. This activity has been ascribed to the flavonols; quercetin and quercitrin[58]. The leaves and roots of *T. sericea* are reported to be used traditionally in treating several infections and diseases. Sericoside, the triterpenoidal saponin found in *T. sericea* has been reported to have anti-inflammatory and antioxidant activity[59]. Sericoside acts by reducing neutrophil infiltration and decreasing superoxide generation due to its radical scavenging activity[60] and it may be responsible for the antioxidant activity of the plant as observed in the study. *C. 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[30]. Similar to the AChEI activity, lycorine and haemanthamine have been reported to be responsible for the antioxidant activity[46].

The total phenolic content of the methanol extracts of *P. capense* and *C. bulbispermum* roots were relatively high for both solvents tested. Phenolic compounds contribute to the antioxidant activity of plant extracts and they are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors and singlet oxygen quenchers[60].

Flavonoids have been reported to be partly responsible for antioxidant activity, as they act on enzymes and pathways involved in anti-inflammatory processes[61]. Furthermore, the hydrogen-donating substituents (hydroxyl groups) attached to the aromatic ring structures of flavonoids enable them to undergo a redox reaction, which in turn, helps them scavenge free radicals[62].

Flavonols are phytochemical compounds found in high concentrations in a variety of plant-based foods and beverages[58]. Consumption of flavonols has been associated with a variety of beneficial effects including an increase in erythrocyte superoxide dismutase activity, decrease in lymphocyte DNA damage, decrease in urinary 8-hydroxy-2′-deoxyguanosine, and an increase in plasma antioxidant capacity[58].

The roots of *P. capense*, *Z. capense*, *L. schweinfurthii*, *Z. mucronata* and *C. bulbispermum* showed good antioxidant and cholinesterase inhibitory activity. These findings support the traditional use of the plants for treating neurological disorders especially those where a cholinesterase mechanism and reactive oxygen species are involved. These novel leads require further investigation.

Conflict of interest statement

We declare that we have no conflict of interest.

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Appendix D

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

DATE: 31/08/2011

TO:
Prof V Steenkamp
Dept of Pharmacology

Best Prof V Steenkamp

RE.: Commercial Lines: The use of Commercial lines ~ Mr E A Adewusi

During the meeting held on 31/08/2011, the use of Commercial Lines were discussed.

The Faculty of Health Science Ethics Committee approved the use of the cell lines as an in vitro study.

With regards

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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APPENDIX E

Data for Compound 1 (6-hydroxycrinamine)

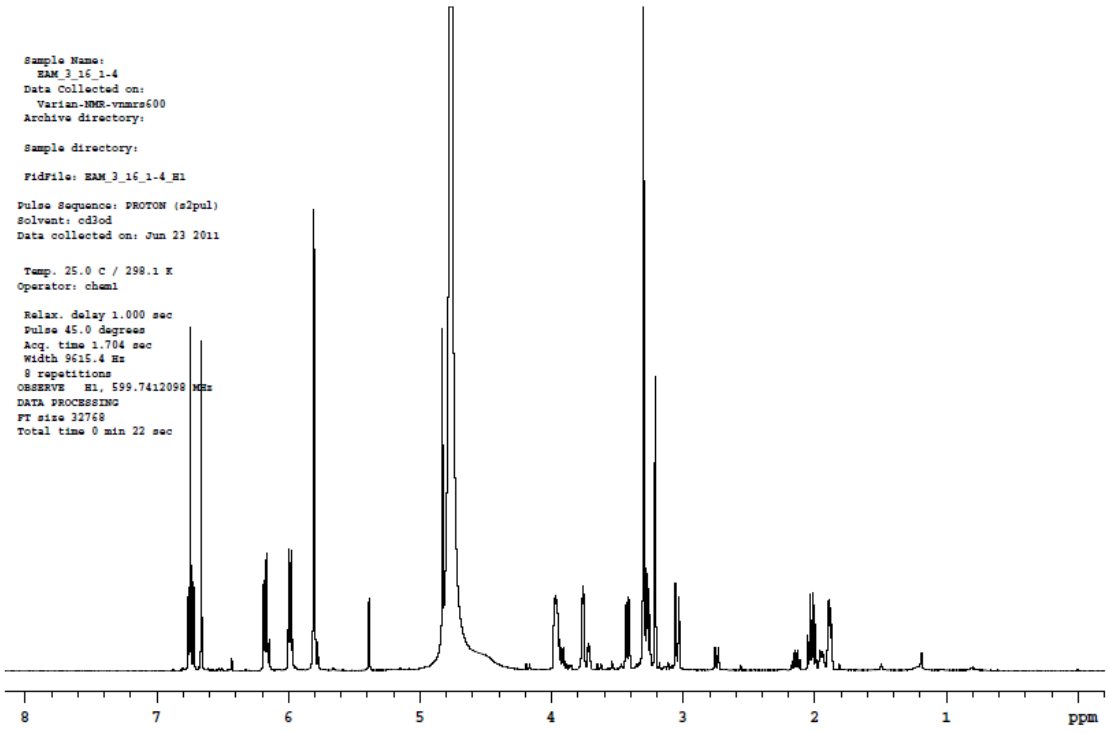

Figure 1 ^{1}H NMR spectrum for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

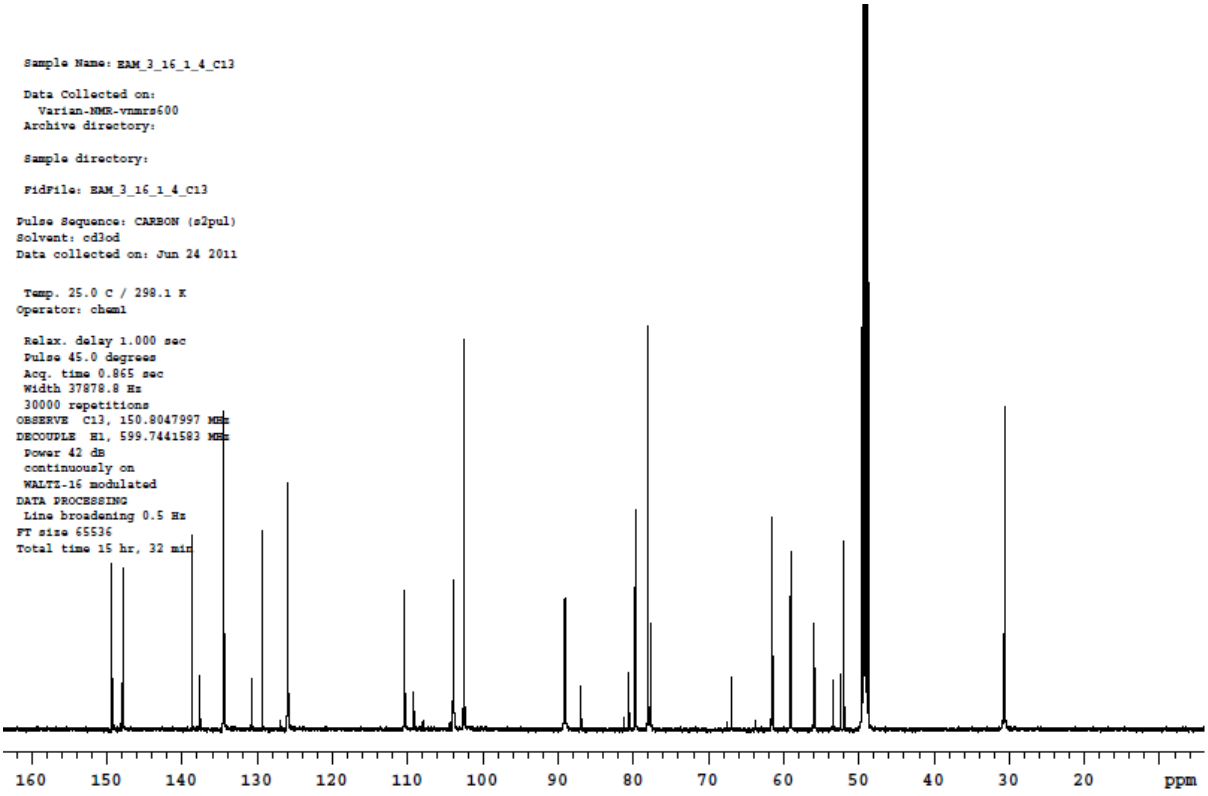

Figure 2 ^{13}C NMR spectrum for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

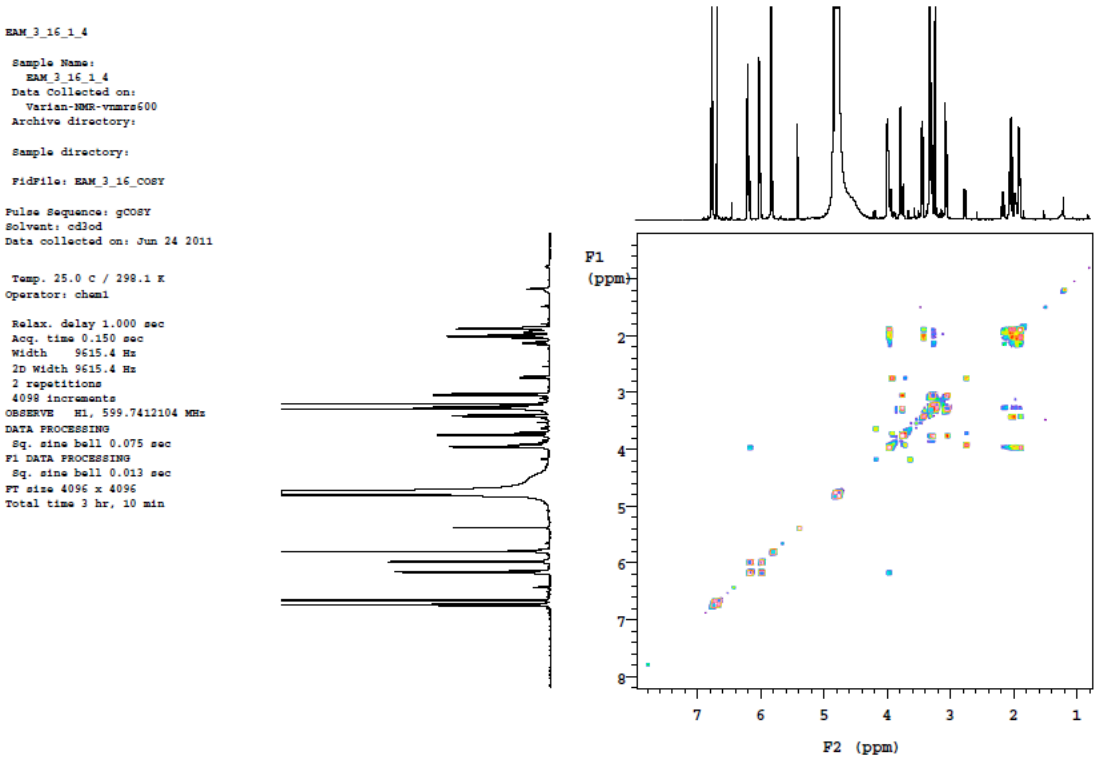

Figure 3 COSY (2D NMR spectrum) for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

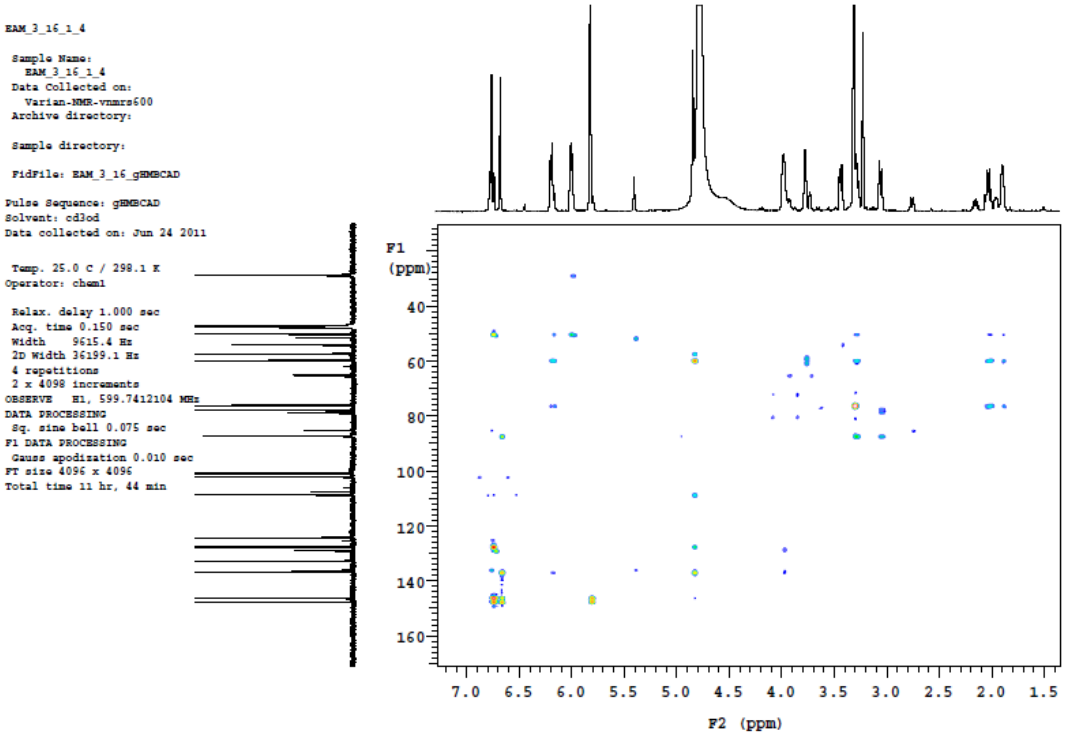

Figure 4 HMBC (2D NMR spectrum) for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

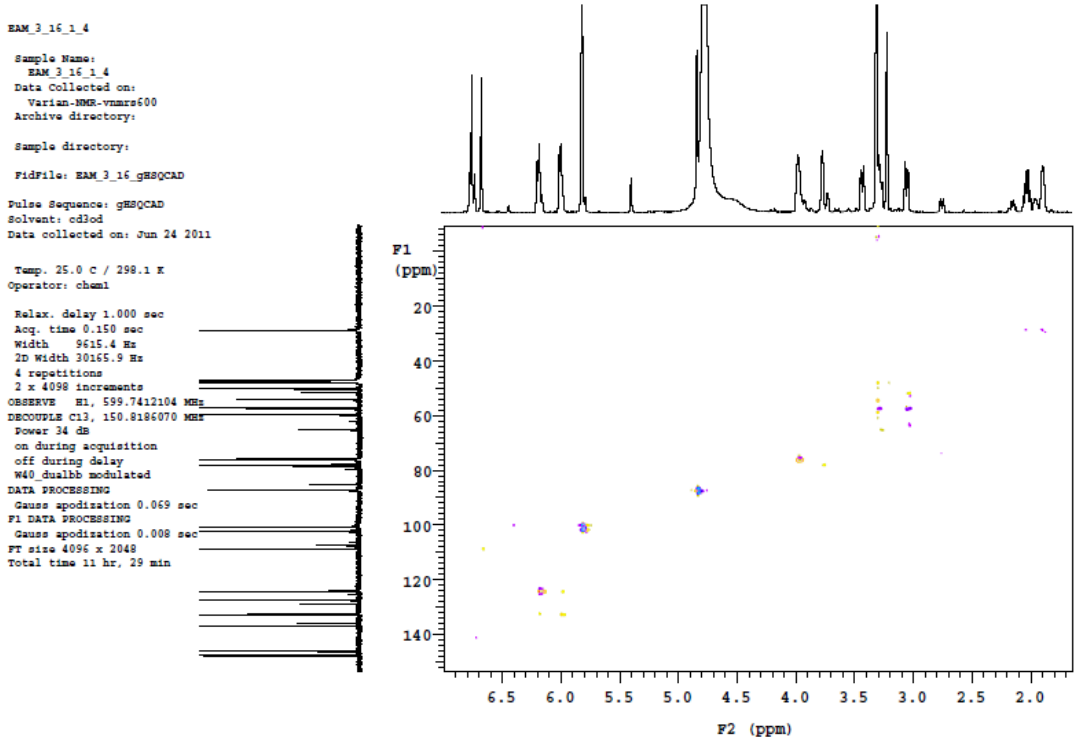

Figure 5 HSQC (2D NMR spectrum) for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

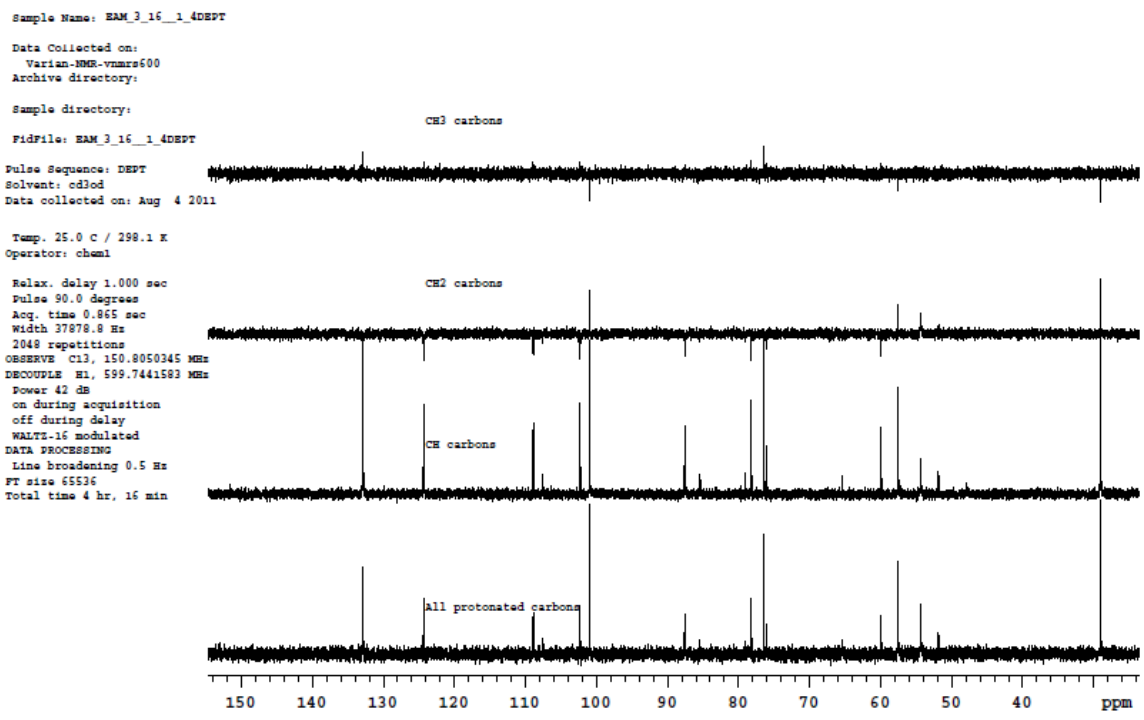

Figure 6 DEPT (2D NMR spectrum) for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

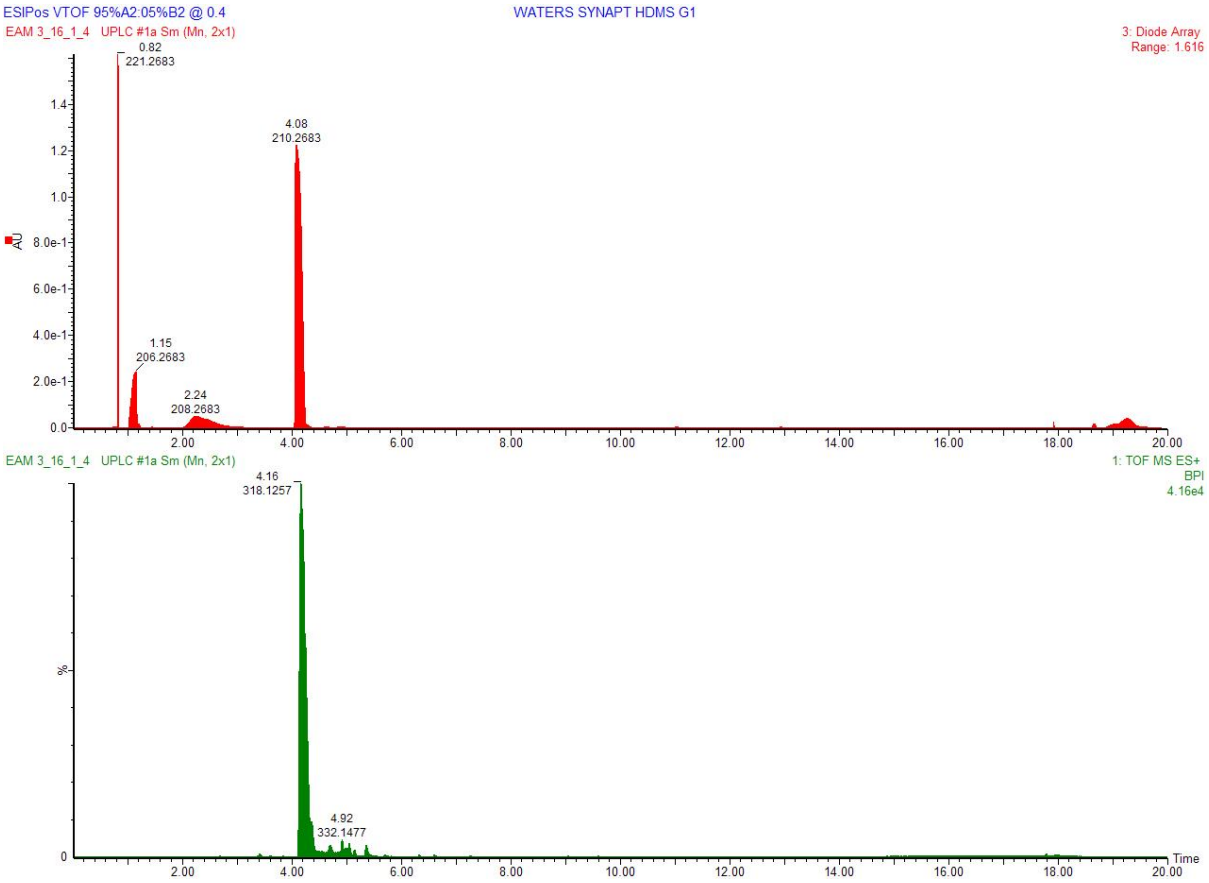

Figure 7 UPLC chromatogram for 6-hydroxycrinamine.

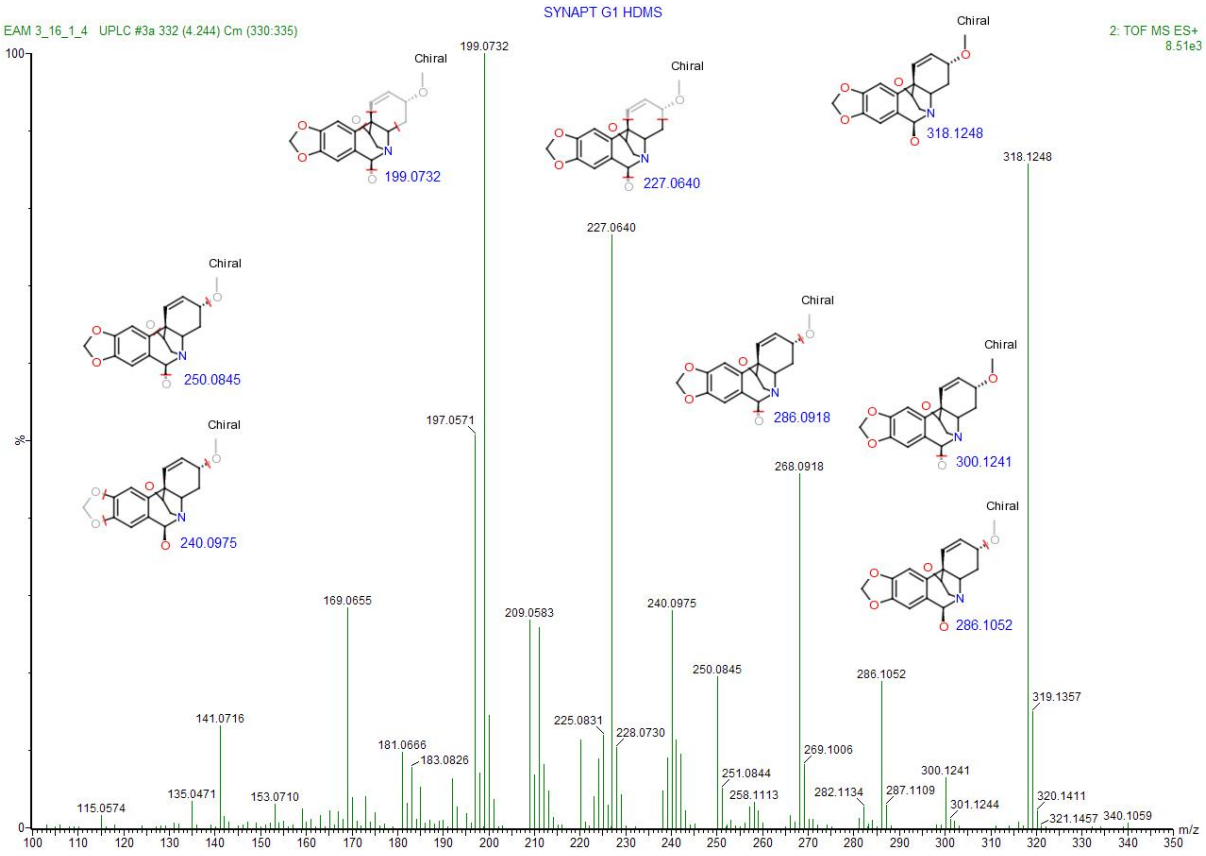

Figure 8 HRTOFMS (ESI^+) spectra for 6-hydroxycrinamine.

Data for Compound 3 (cycloeucalenol)

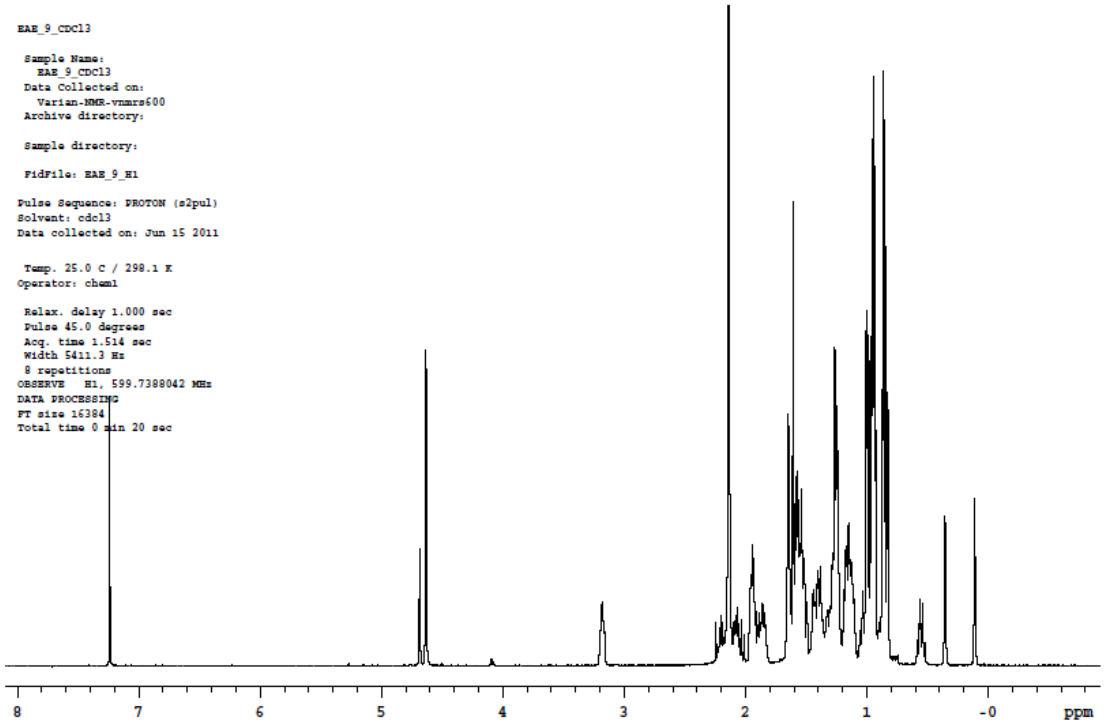

Figure 9 ^{1}H NMR spectrum for cycloeucalenol (600 MHz Varian NMR in d-chloroform).

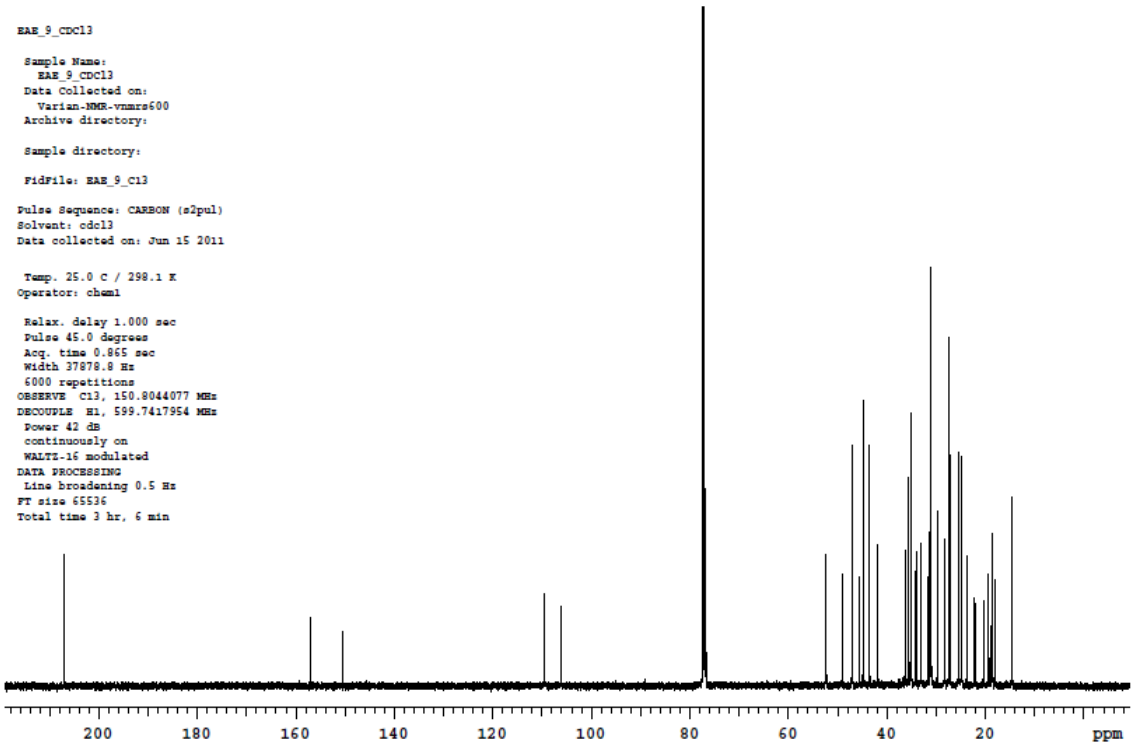

Figure 10 ^{13}C NMR spectrum for cycloeucalenol (600 MHz Varian NMR in d-chloroform).

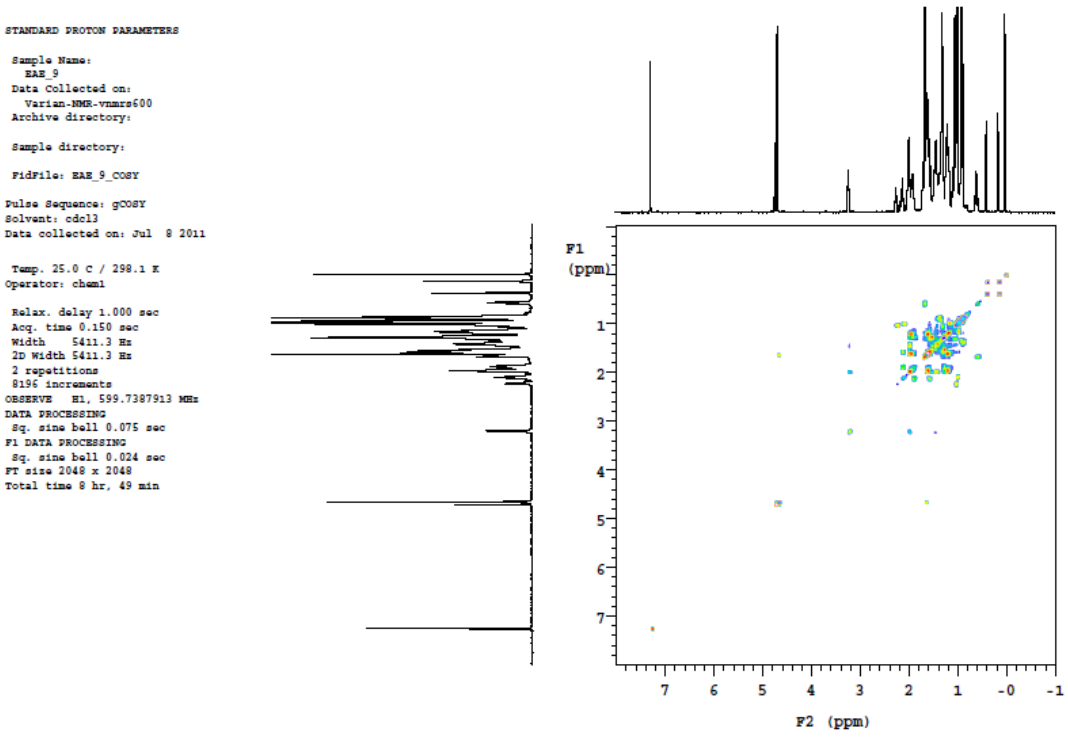

Figure 11 COSY (2D NMR spectrum) for cycloeucalenol (600 MHz Varian NMR in d-chloroform).

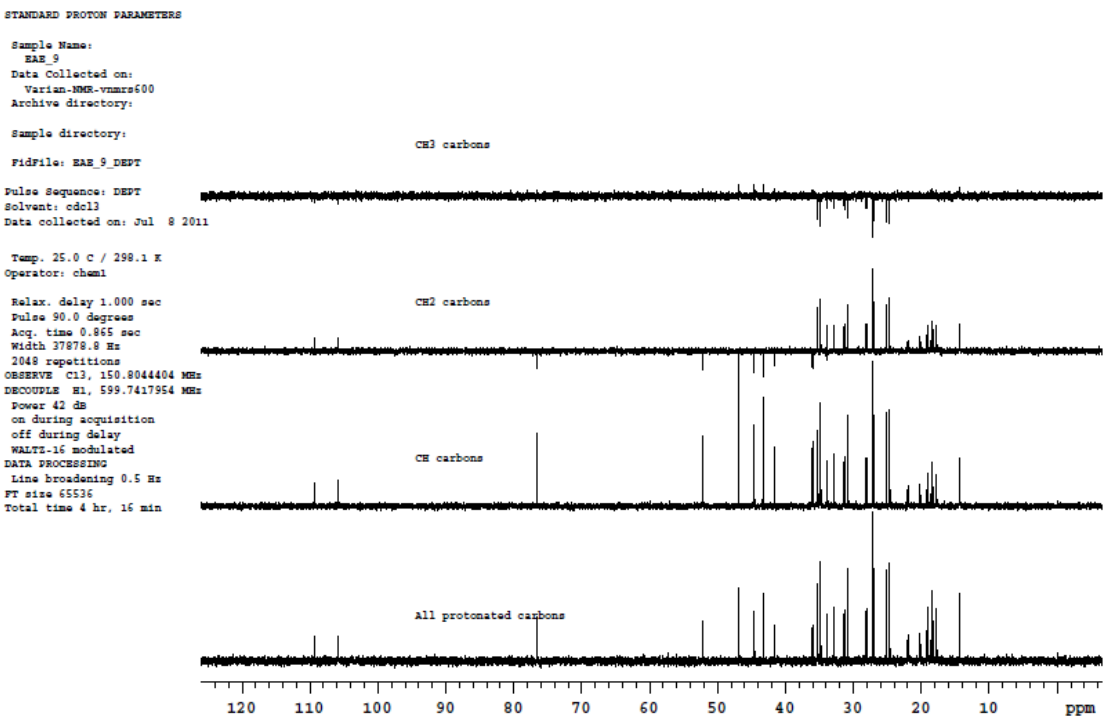

Figure 12 DEPT (2D NMR spectrum) for cycloeucalenol (600 MHz Varian NMR in d-chloroform).

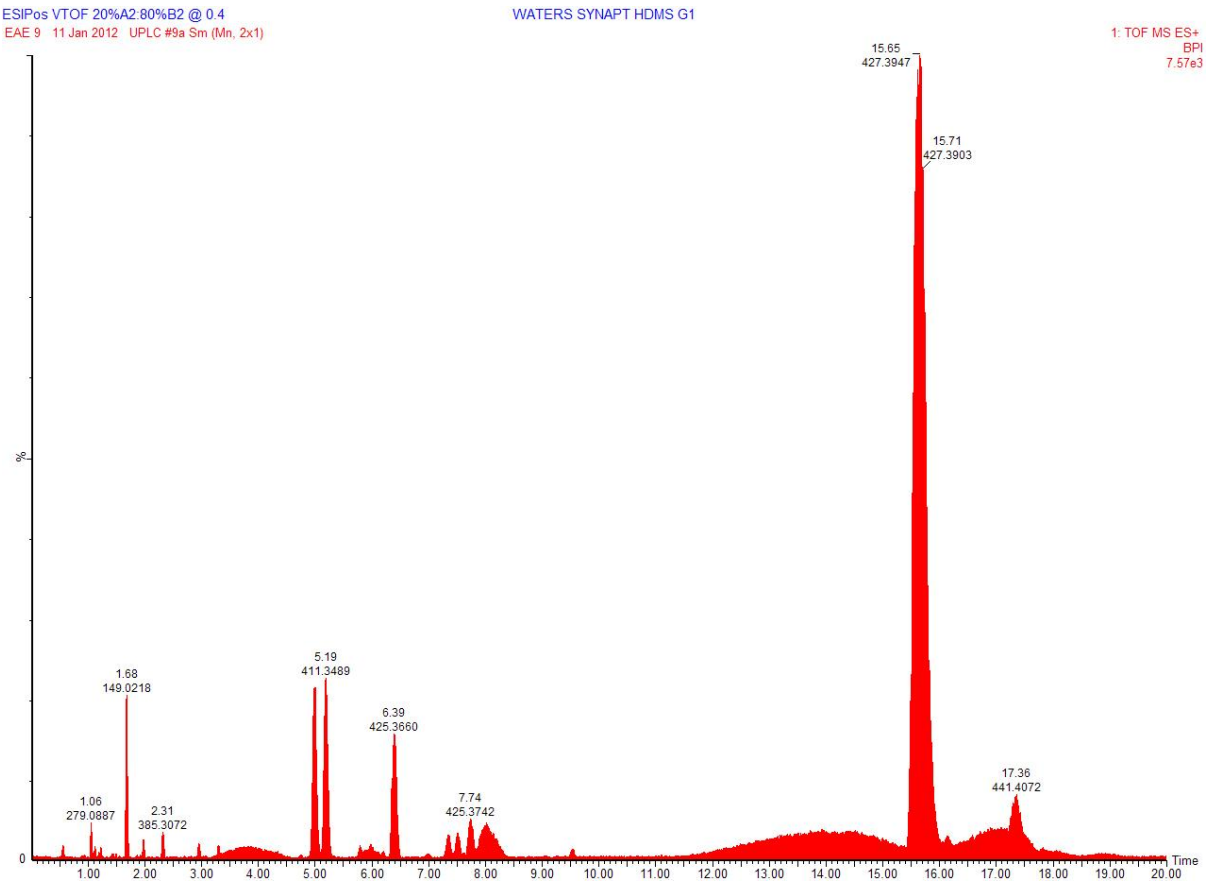

Figure 13 UPLC chromatogram for cycloeucalenol.

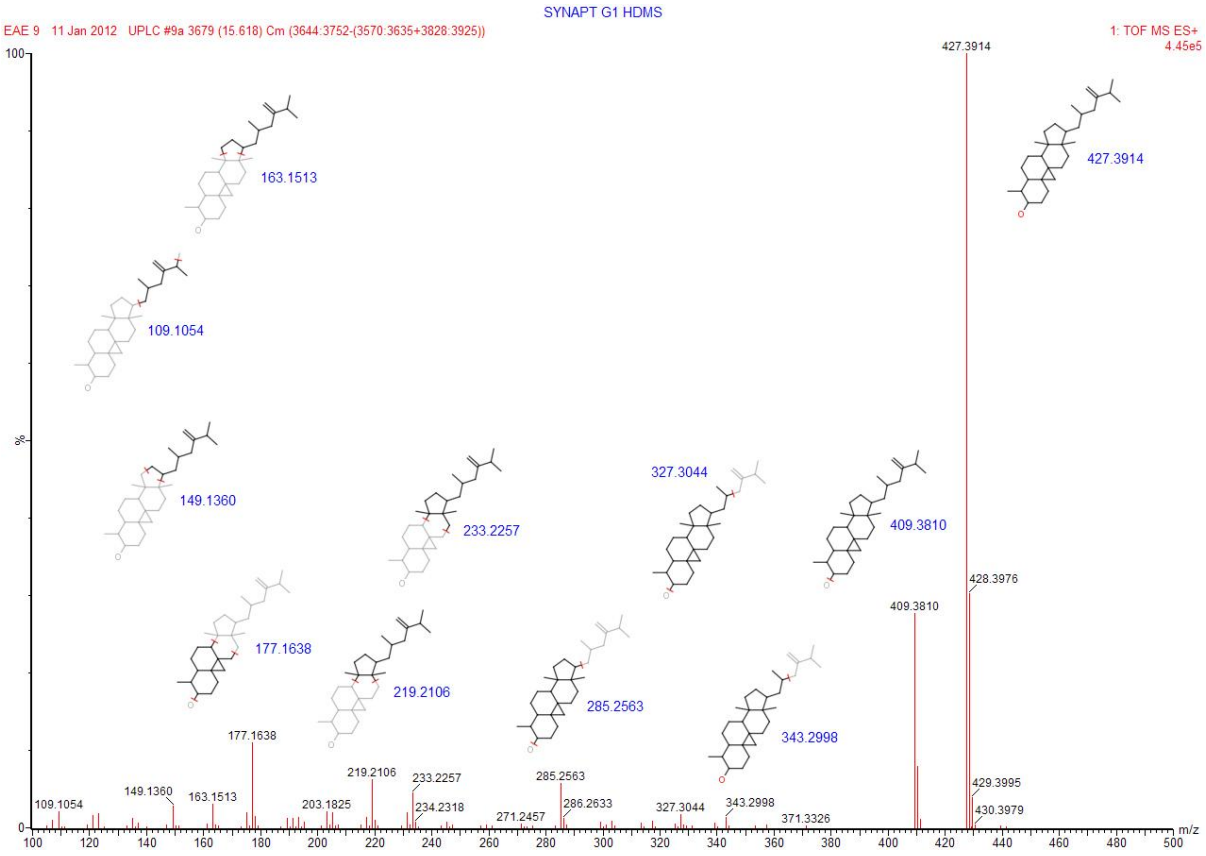

Figure 14 HRTOFMS (ESI^+) spectra for cycloeucalenol.

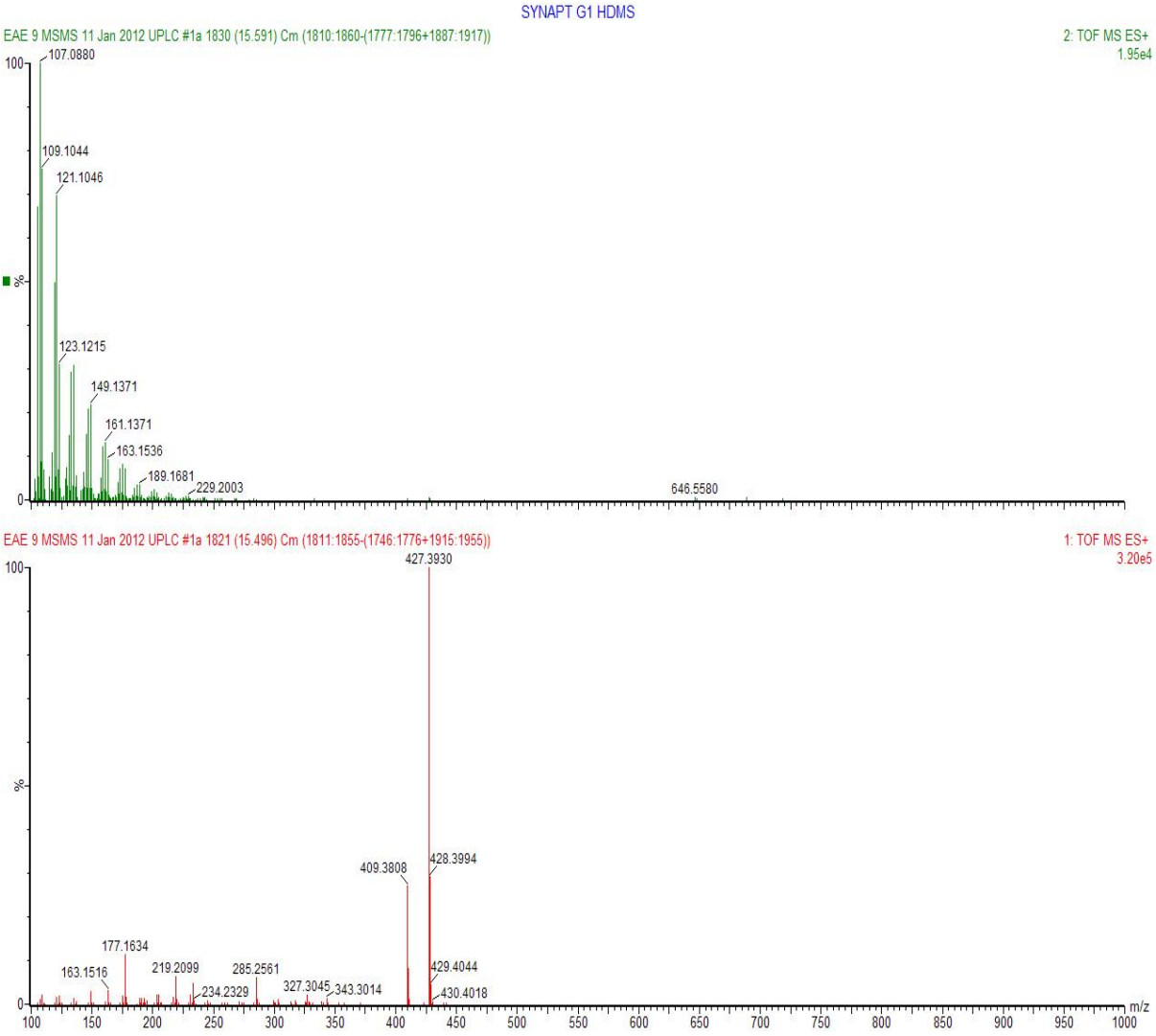

Figure 15 HRTOFMS (ESI^+) spectra for cycloeucalenol.

Data for Fractions

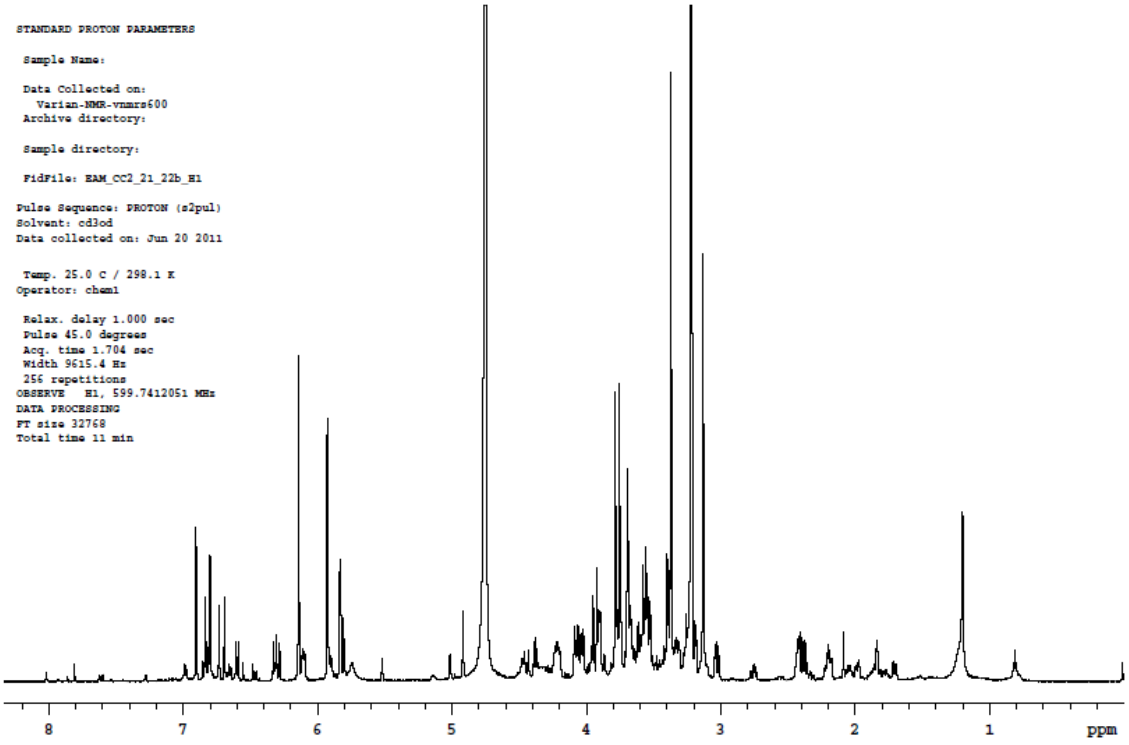

Figure 16 ^{1}H NMR spectrum for fraction EAM 17-21 21,22 (600 MHz Varian NMR in d-methanol).

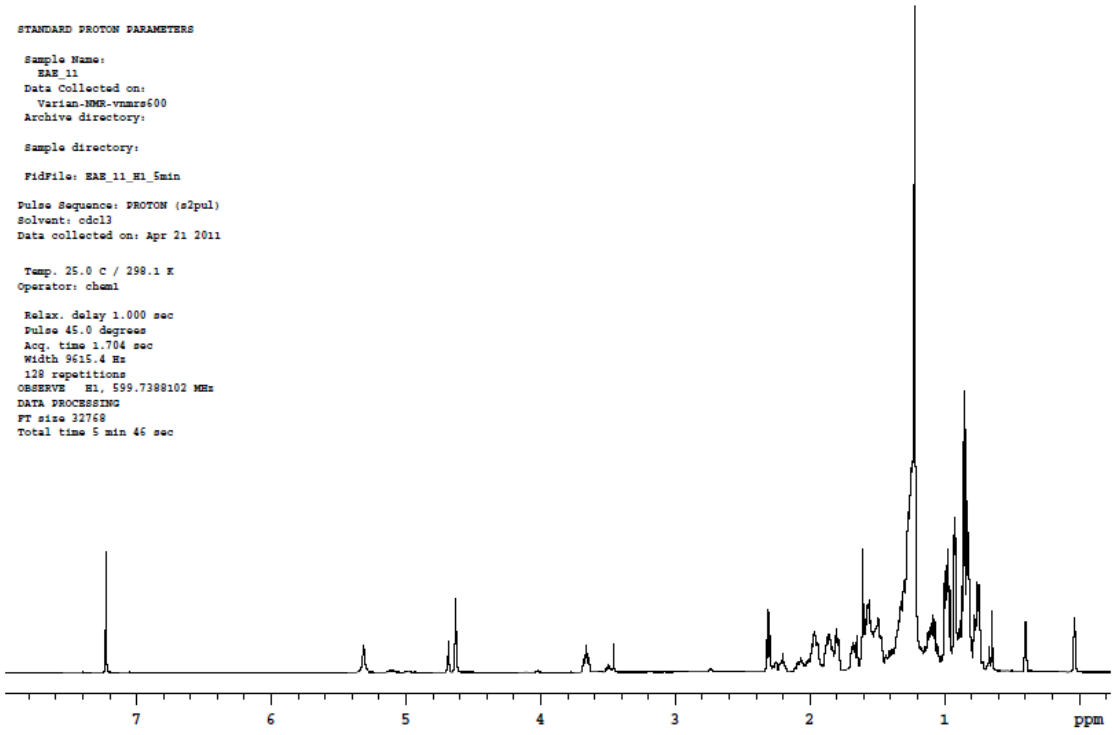

Figure 17 ^{1}H NMR spectrum for fraction EAE 11 (600 MHz Varian NMR in d-chloroform).

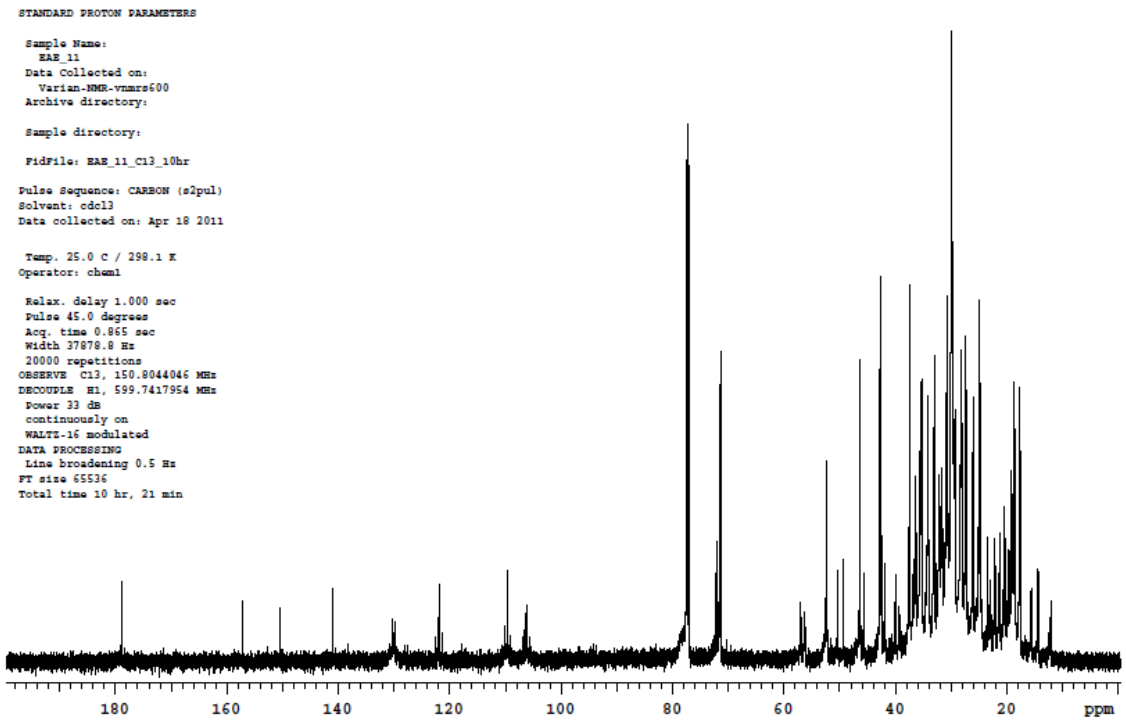

Figure 18 ^{13}C NMR spectrum for fraction EAE 11 (600 MHz Varian NMR in d-chloroform).

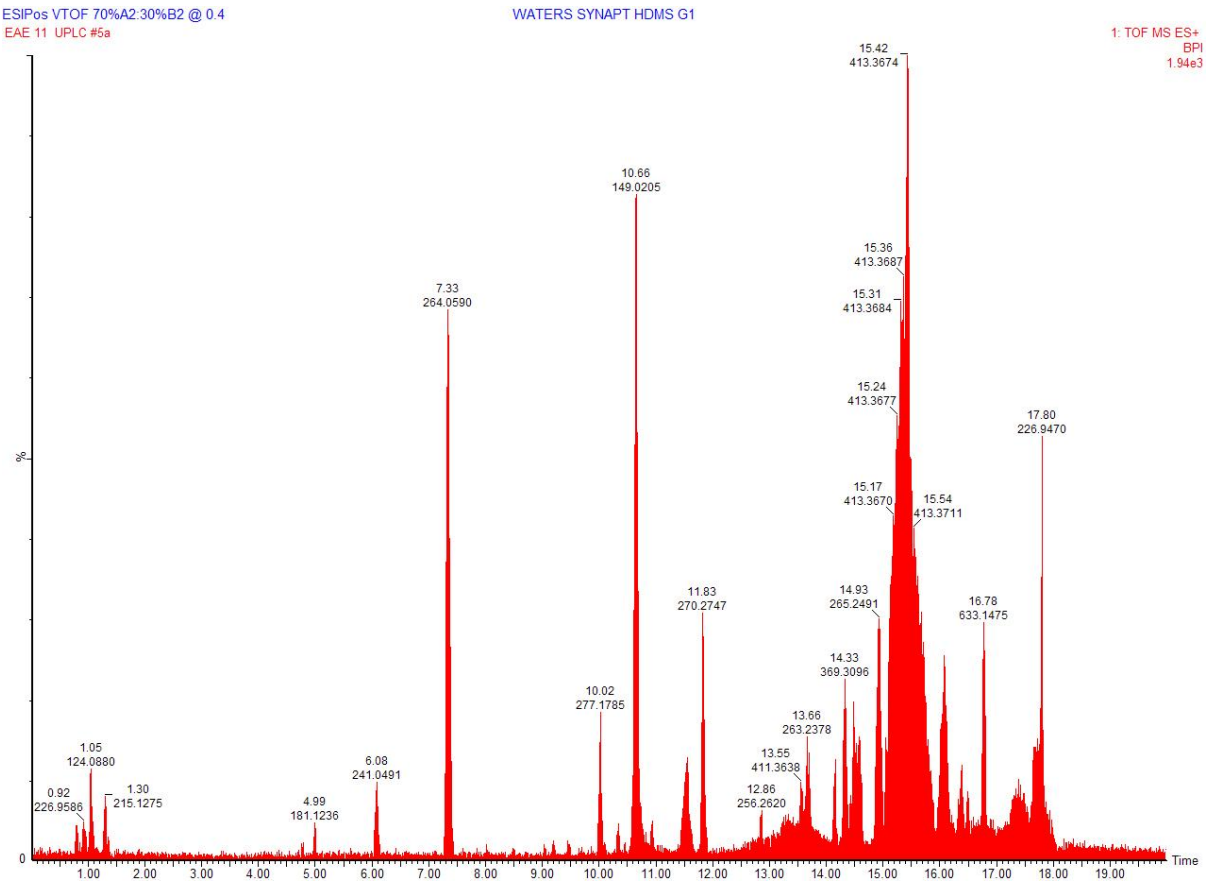

Figure 19 UPLC chromatogram for fraction EAE 11.

Summary

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder. Cholinergic deficit, senile plaque/amyloid-β peptide deposition and oxidative stress have been identified as three main pathogenic pathways which contribute to the progression of AD. The current therapeutic options cause several side-effects and therefore the need to search for new compounds from natural products with potential to treat AD.

Seventeen plants were selected for this study based on their documented ethno-medicinal use in treating age-related disorders. The plants were screened for inhibition of acetylcholinesterase (AChE). The ethyl acetate extracts of the roots of *Crinum bulbispermum*, *Xysmalobium undulatum*, *Lannea schweinfurthii*, *Scadoxus puniceus* and bulbs of *Boophane disticha* had the best AChE inhibition. Although the IC_{50} of these plant extracts were higher than that of the positive control, galanthamine (0.00053 mg/ml), they showed good AChE inhibitory activity considering they are still mixtures containing various compounds.

The antioxidant activity of the plant extracts was determined by their ability to scavenge ABTS and DPPH radicals. The dichloromethane/methanol (1:1) extracts of *Chamaecrista mimosoides*, *Buddleja salviifolia*, *Schotia brachypetala*, water extracts of *Chamaecrista mimosoides*, *Buddleja salviifolia*, *Schotia brachypetala* and methanol extracts of the roots of *Crinum bulbispermum*, *Piper capense*, *Terminalia sericea*, *Lannea schweinfurthii* and *Ziziphus mucronata* all showed good antioxidant activity (>50%), in both assays.

B. disticha contained very promising AChE inhibition and was subjected to isolation of active compounds. 6-hydroxycrinamine and cycloeucalenol, were isolated for the first time from the

bulbs of this plant. 6-Hydroxycrinamine, and two fractions, EAM 17-21 21,22 and EAE 11, were found to inhibit AChE with IC_{50} values of 0.445 ± 0.030 mM, 0.067 ± 0.005 mg/ml and 0.122 ± 0.013 mg/ml, respectively.

Cytotoxicity of the isolated compounds and two active fractions was determined on SH-SY5Y cells using the MTT and neutral red uptake assays. 6-hydroxycrinamine and fraction EAM 17-21 21,22 were found to be toxic with IC_{50} values of 54.5 µM and 21.5 µg/ml as determined by the MTT assay. Cytotoxicity was also determined for the methanol extracts of the roots of *C. bulbispermum*, *T. sericea*, *L. schweinfurthii* and *Z. mucronata*, as they contained promising antioxidant activity. *Z. mucronata* and *L. schweinfurthii* were the least toxic with IC_{50} values exceeding 100 µg/ml, the highest concentration tested. Pretreatment with *Z. mucronata* and *T. sericea* roots showed a dose dependent inhibition of cell death caused by $A\beta_{25-35}$. Pre-treatment with *L. schweinfurthii* roots resulted in an optimum dose for inhibition of $A\beta_{25-35}$ induced cell death at 25 µg/ml, while still maintaining 80% viability.

This study confirms the neuroprotective potential of some of the plants which had AChE inhibitory and antioxidant activity. In addition, four of the plants were shown to prevent cell death caused by $A\beta_{25-35}$. These plants can serve as potential leads in developing drugs relevant to treatment of AD. Furthermore, two new compounds present in the bulbs of *B. disticha* were identified. Additional investigations need to be carried out by applying quantitative structure activity relationship studies to modify the structure of the alkaloid with the aim of reducing its observed toxicity.

Keywords: Acetylcholinesterase, Alzheimer's disease, amyloid-β, antioxidant, cytotoxicity, galanthamine, MTT, Neutral red, plant extracts, SH-SY5Y cells