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Review

Medicinal plants with cholinesterase inhibitory activity: A Review

E. A. Adewusi[1], N. Moodley[2] and V. Steenkamp[1]*

[1]Department of Pharmacology, School of Medicine, Faculty of Health Sciences, University of Pretoria, South Africa.
[2]Biosciences, Council for Scientific and Industrial Research, South Africa.

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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

*Corresponding author. E-mail: Vanessa.Steenkamp@up.ac.za. Tel: +27 12 319 2547. Fax: +27 12 319 2411.

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

E.A. Adewusi [a], N. Moodley [b], V. Steenkamp [a,*]

[a] *Department of Pharmacology, School of Medicine, Faculty of Health Sciences, PO Box 2034, University of Pretoria, Pretoria 0001, South Africa*
[b] *Biosciences, Council for Scientific and Industrial Research, Pretoria, South Africa*

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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

* Corresponding author. Tel.: +27 12 3192457; fax: +27 12 3192411.
E-mail address: vanessa.steenkamp@up.ac.za (V. Steenkamp).

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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

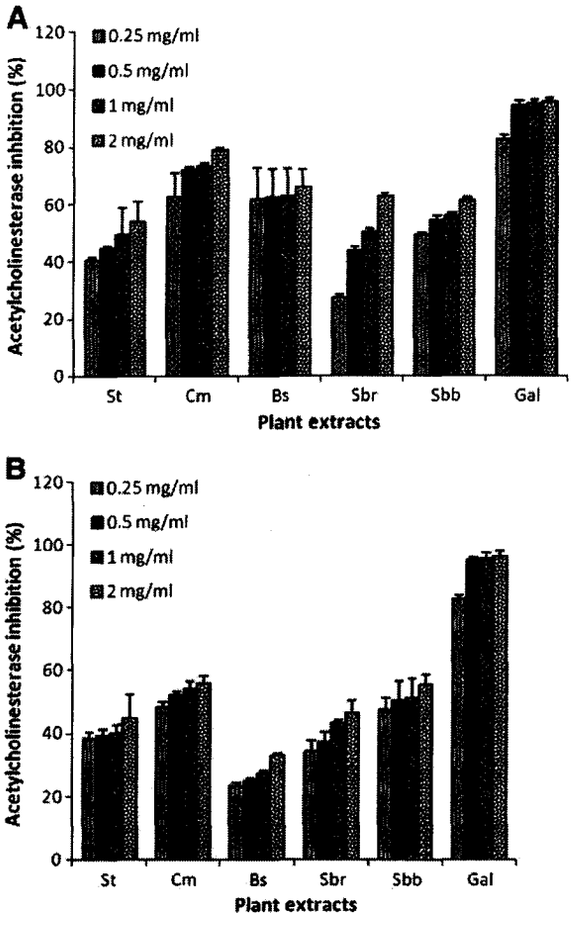

Fig. 1. AChE inhibitory activity (%) of (A) DCM:MeOH (1:1) extracts and (B) water extracts, of the plants investigated. St, *Salvia tiliifolia* whole plant; Cm, *Chamaecrista mimosoides* root; Bs, *Buddleja salviifolia* whole plant; Sbr, *Schotia brachypetala* root; Sbb, *Schotia brachypetala* bark;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

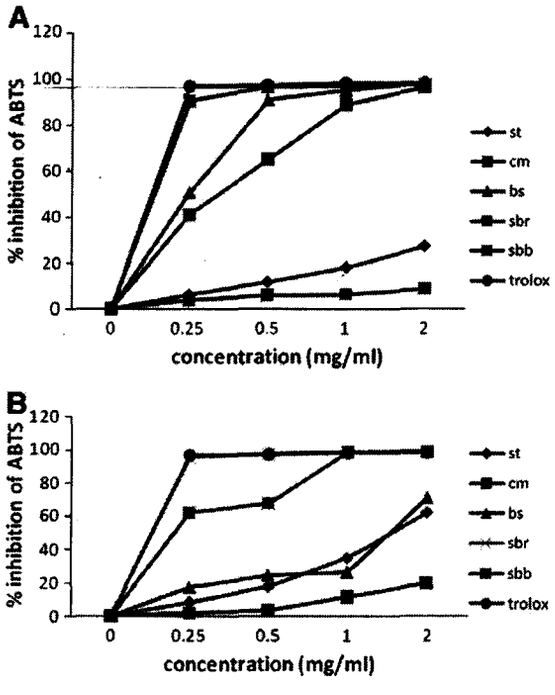

Fig. 2. ABTS radical scavenging activity of (A) DCM:MeOH (1:1) extracts and (B) water extracts, of the plants investigated. St, *Salvia tiliifolia* whole plant; Cm, *Chamaecrista mimosoides* root; Bs, *Buddleja salviifolia* whole plant; Sbr, *Schotia brachypetala* root; Sbb, *Schotia brachypetala* bark;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

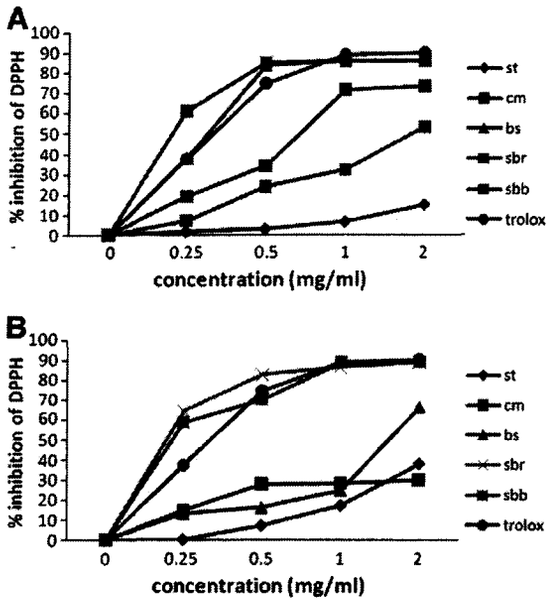

Fig. 3. DPPH radical scavenging activity (%) of (A) DCM:MeOH (ABTS) (1:1) extracts and (B) water extracts, of the plants investigated. St, *Salvia tiliifolia* whole plant; Cm, *Chamaecrista mimosoides* root; Bs, *Buddleja salviifolia* whole plant; Sbr, *Schotia brachypetala* root; Sbb, *Schotia brachypetala*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

EA Adewusi, V Steenkamp*

Department of Pharmacology, School of Medicine, Faculty of Health Sciences, University of Pretoria, South Africa

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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

*Corresponding author: Prof. Vanessa Steenkamp, Department of Pharmacology, Faculty of Health Sciences, University of Pretoria, Private Bag X323, Arcadia, 0007, South Africa.
Tel: +27123192547
Fax: +27123192411
E-mail: Vanessa.steenkamp@up.ac.za

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

♦Tel:012-3541330 ♦Fax:012-3541367 / 0866515924 ♦E-Mail: manda@med.up.ac.za
♦Web: //www.healthethics-up.co.za ♦H W Snyman Bld (South) Level 2-34 ♦P.O.BOX 667, Pretoria, S.A., 0001

MS: dd 2011/09/05: C:\Documents and Settings\user\My Documents\A Letter\z-Commercial Cell Lines\Cell Lines~8-2011 Adewusi.doc

APPENDIX E

Data for Compound 1 (6-hydroxycrinamine)

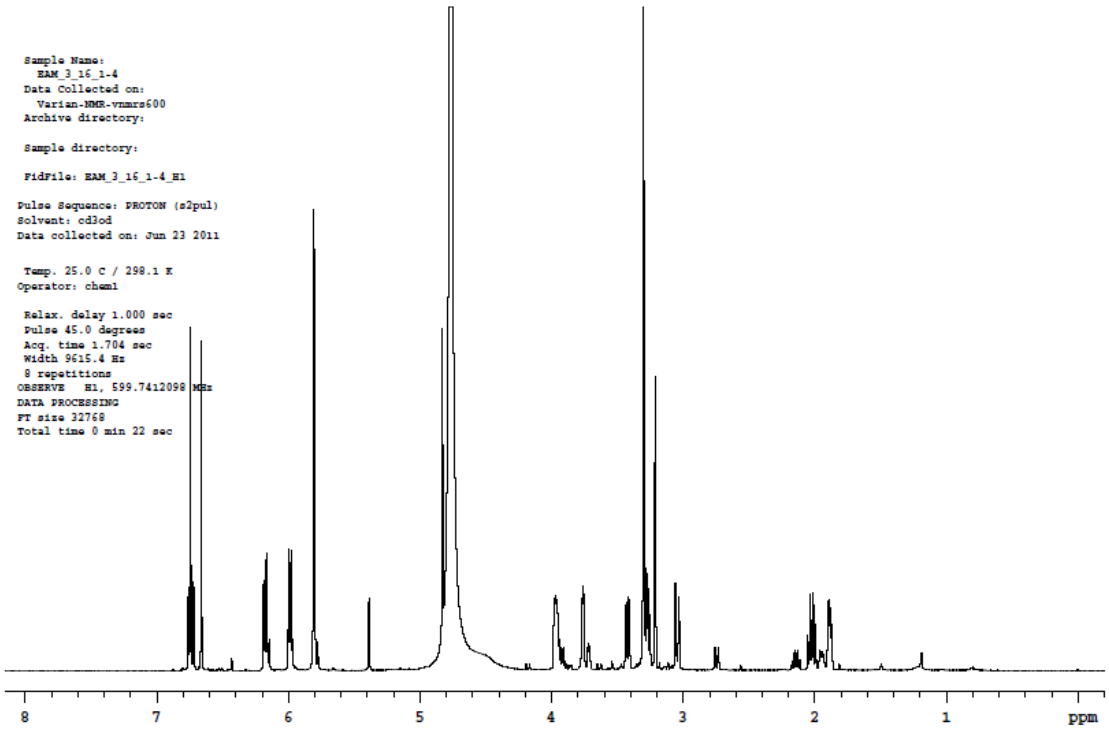

Figure 1 ^{1}H NMR spectrum for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

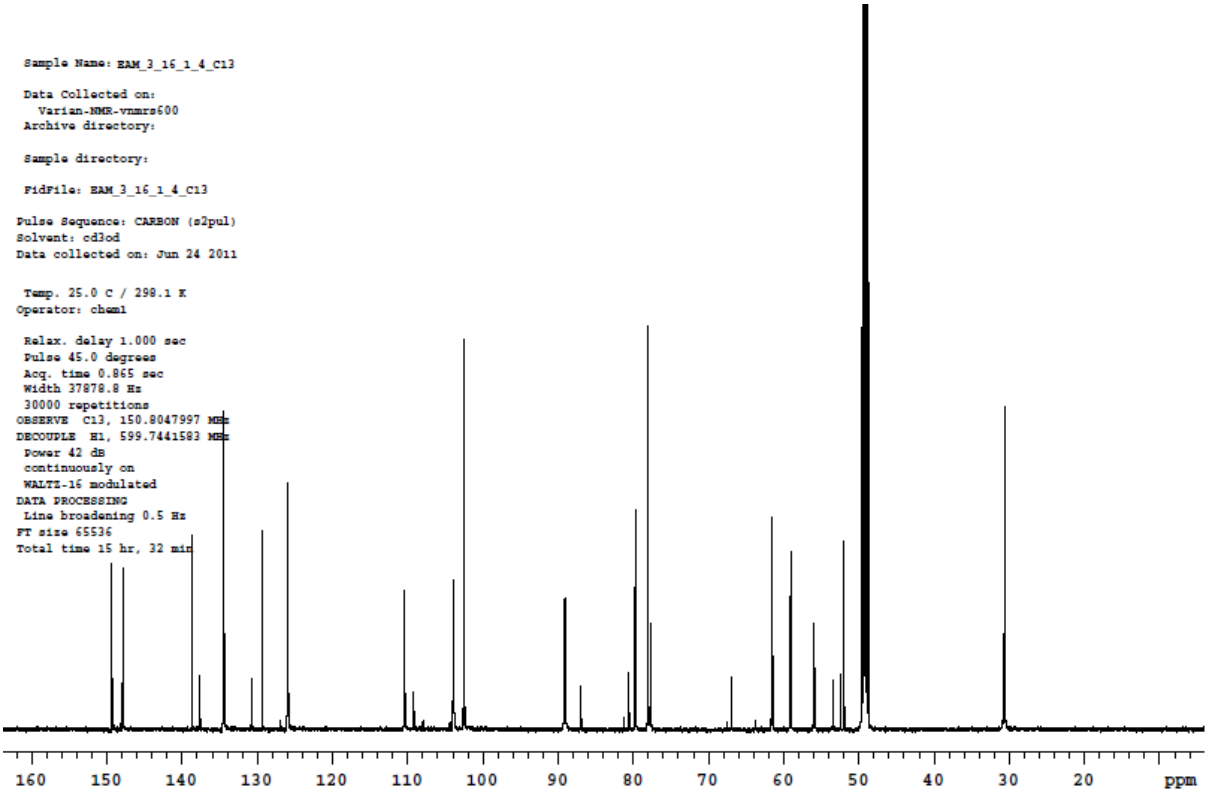

Figure 2 ^{13}C NMR spectrum for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

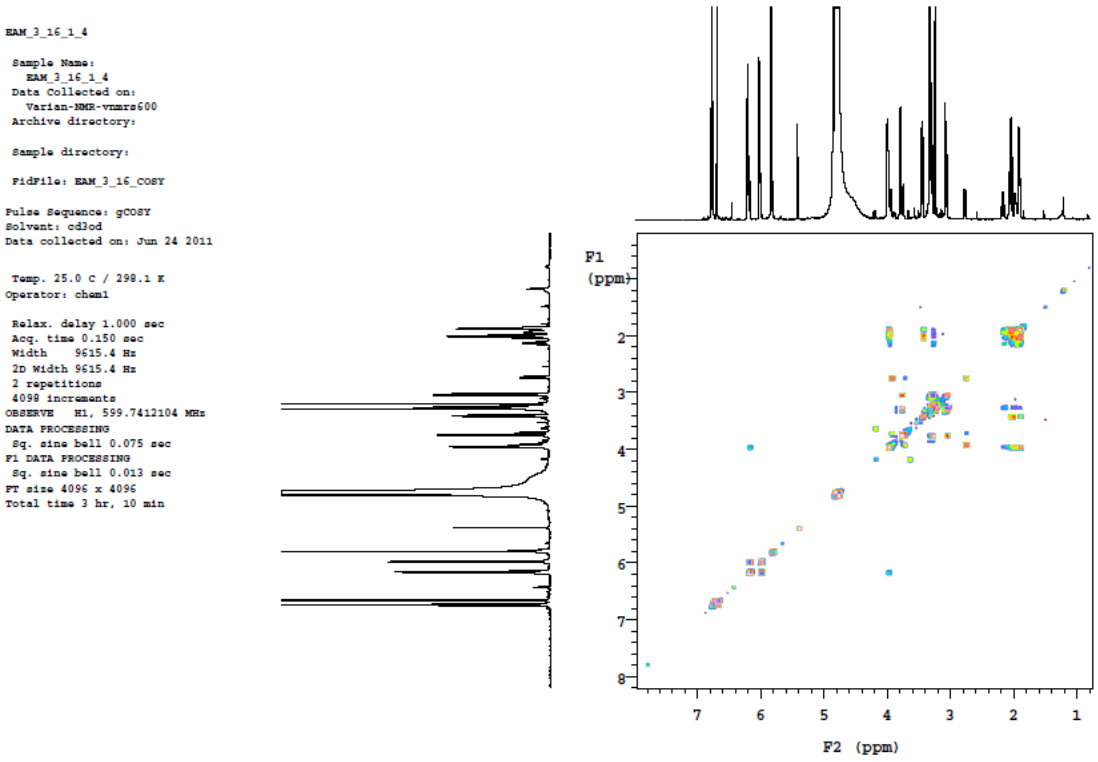

Figure 3 COSY (2D NMR spectrum) for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

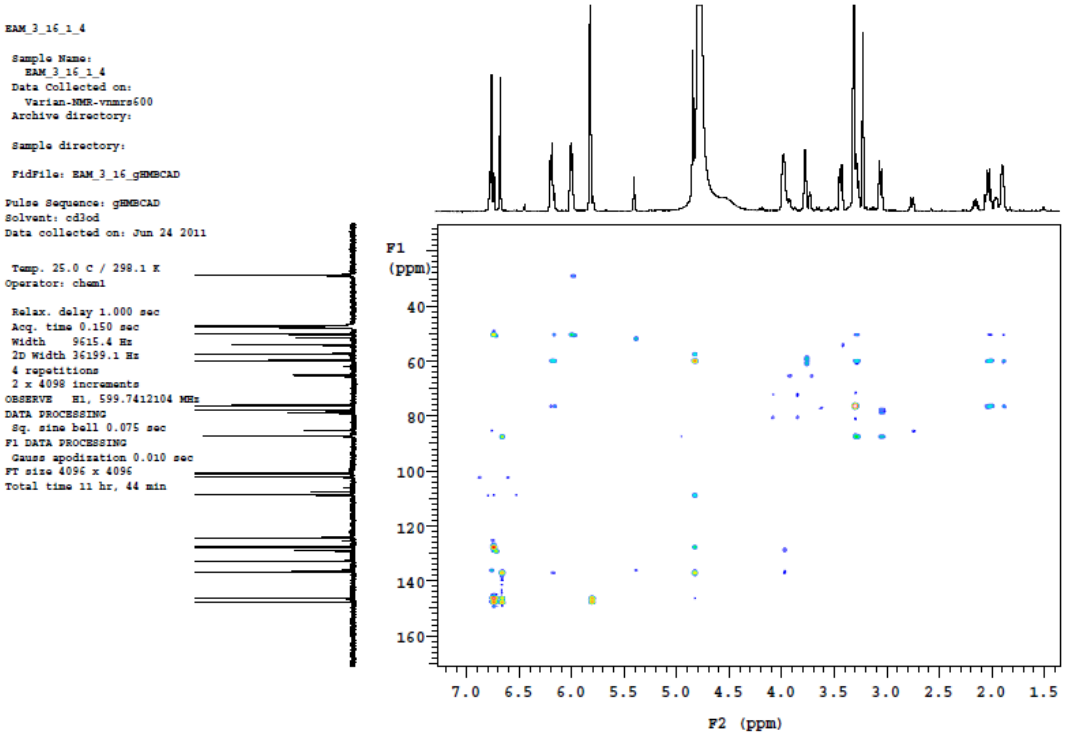

Figure 4 HMBC (2D NMR spectrum) for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

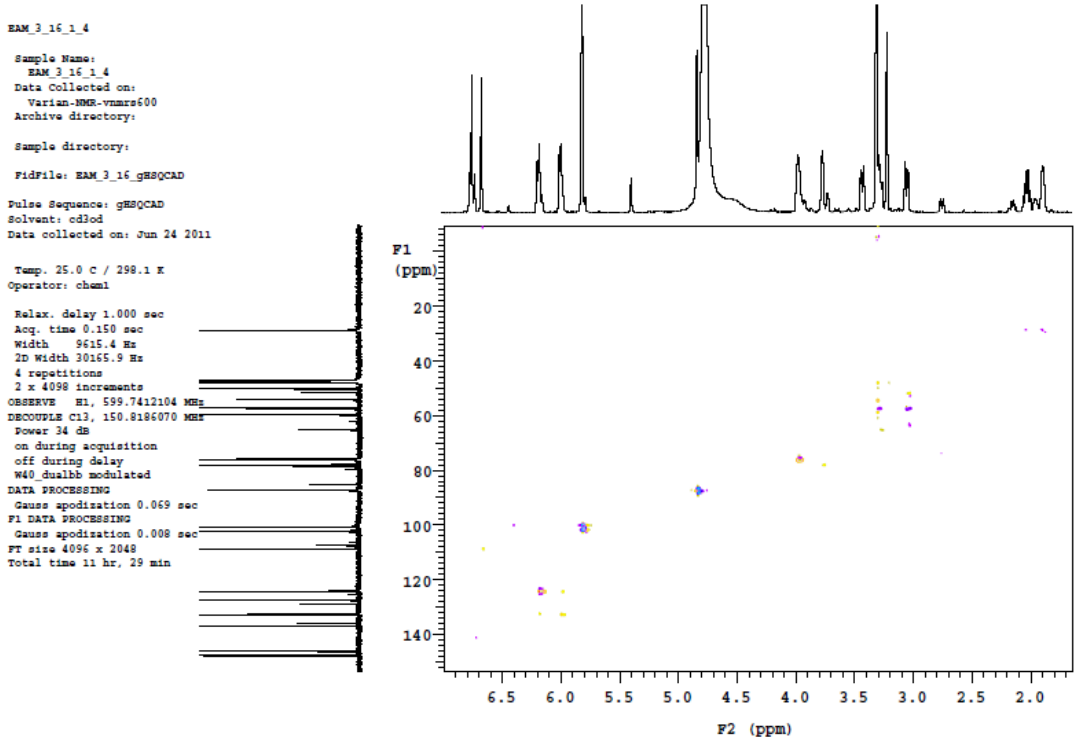

Figure 5 HSQC (2D NMR spectrum) for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

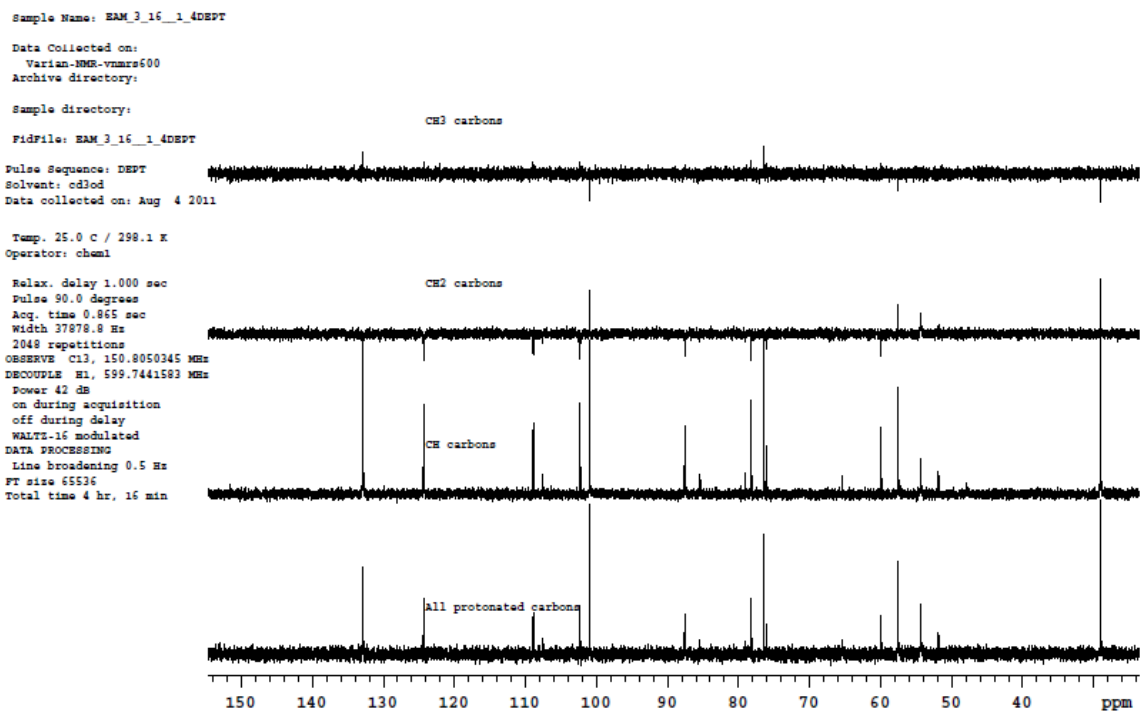

Figure 6 DEPT (2D NMR spectrum) for 6-hydroxycrinamine (600 MHz Varian NMR in d-methanol).

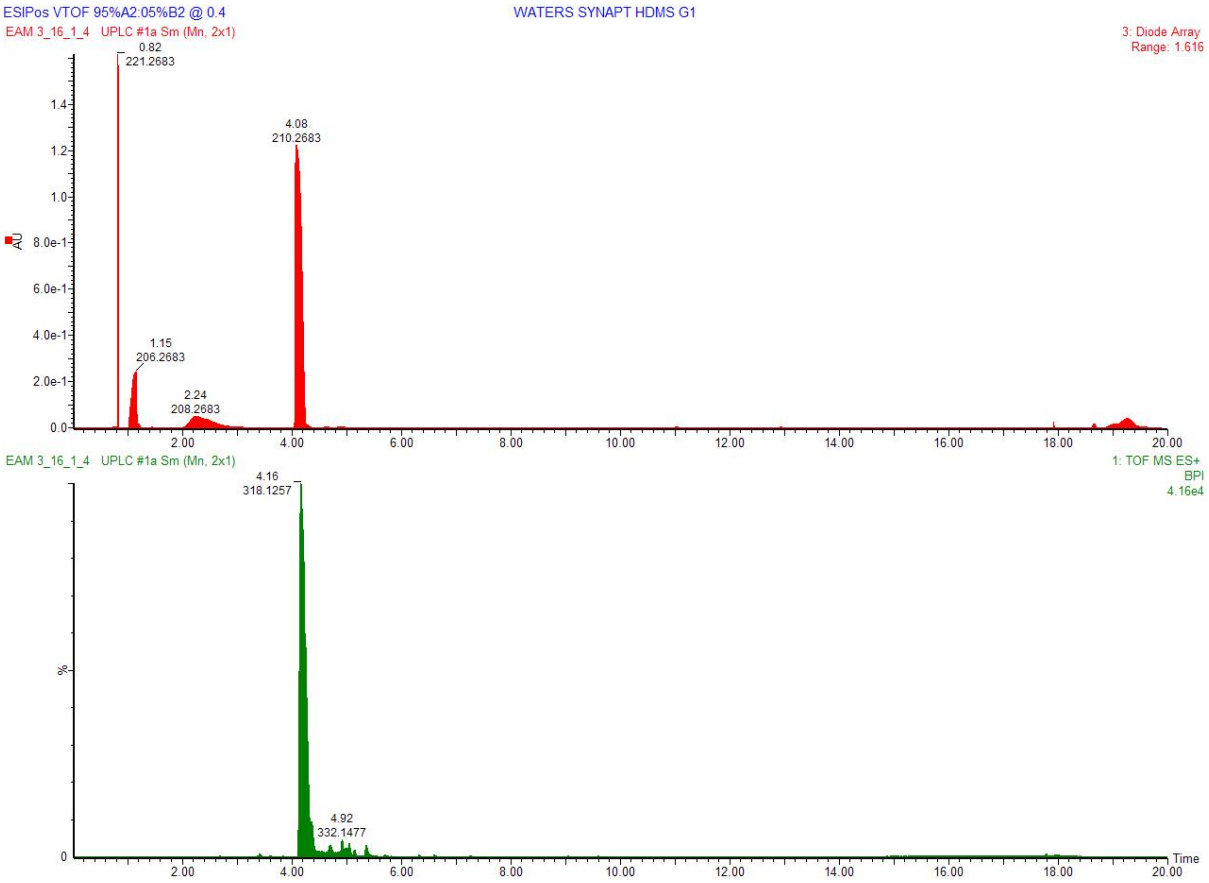

Figure 7 UPLC chromatogram for 6-hydroxycrinamine.

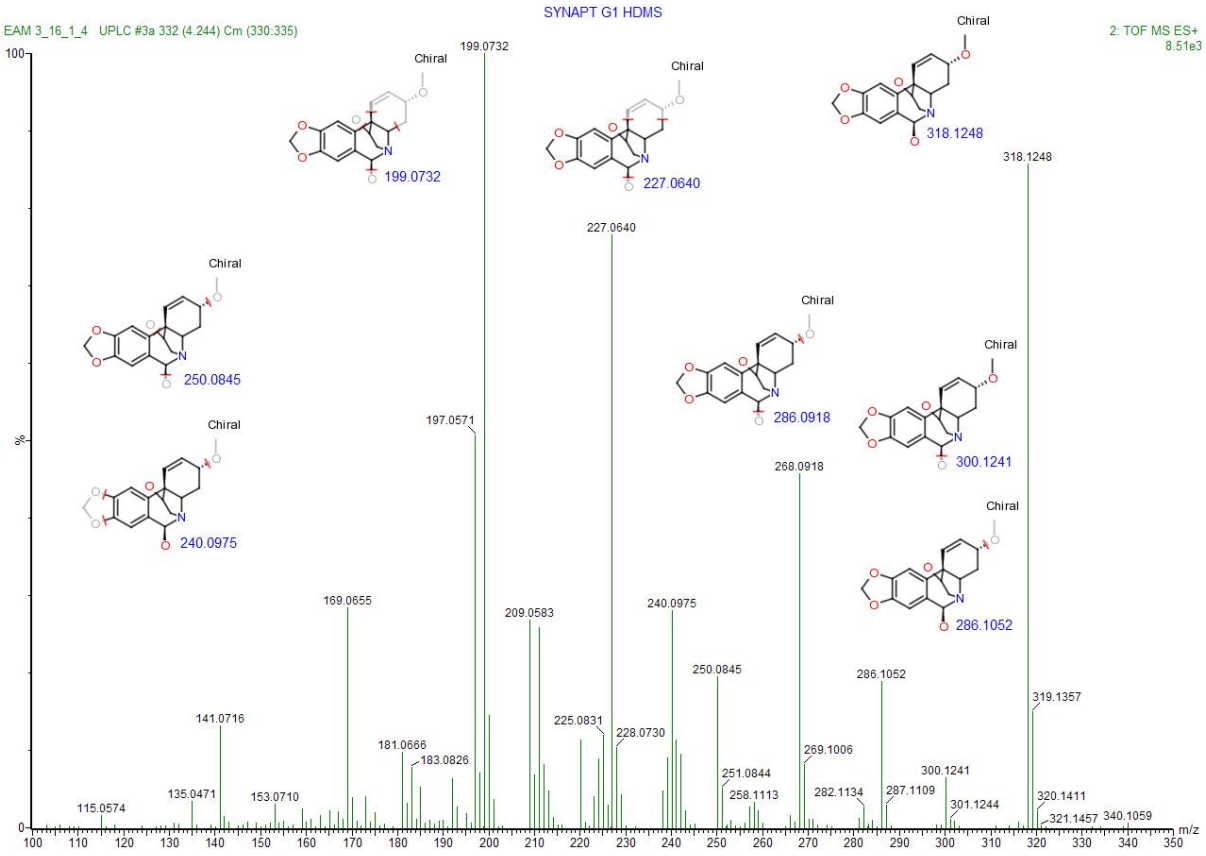

Figure 8 HRTOFMS (ESI^+) spectra for 6-hydroxycrinamine.

Data for Compound 3 (cycloeucalenol)

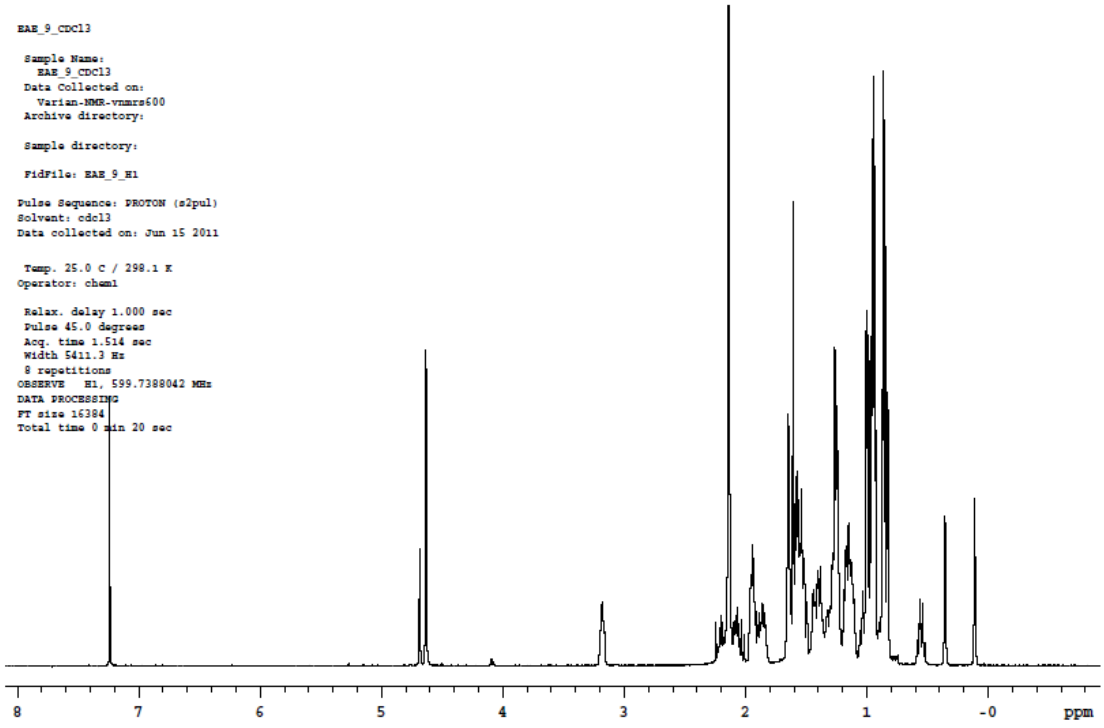

Figure 9 ^{1}H NMR spectrum for cycloeucalenol (600 MHz Varian NMR in d-chloroform).

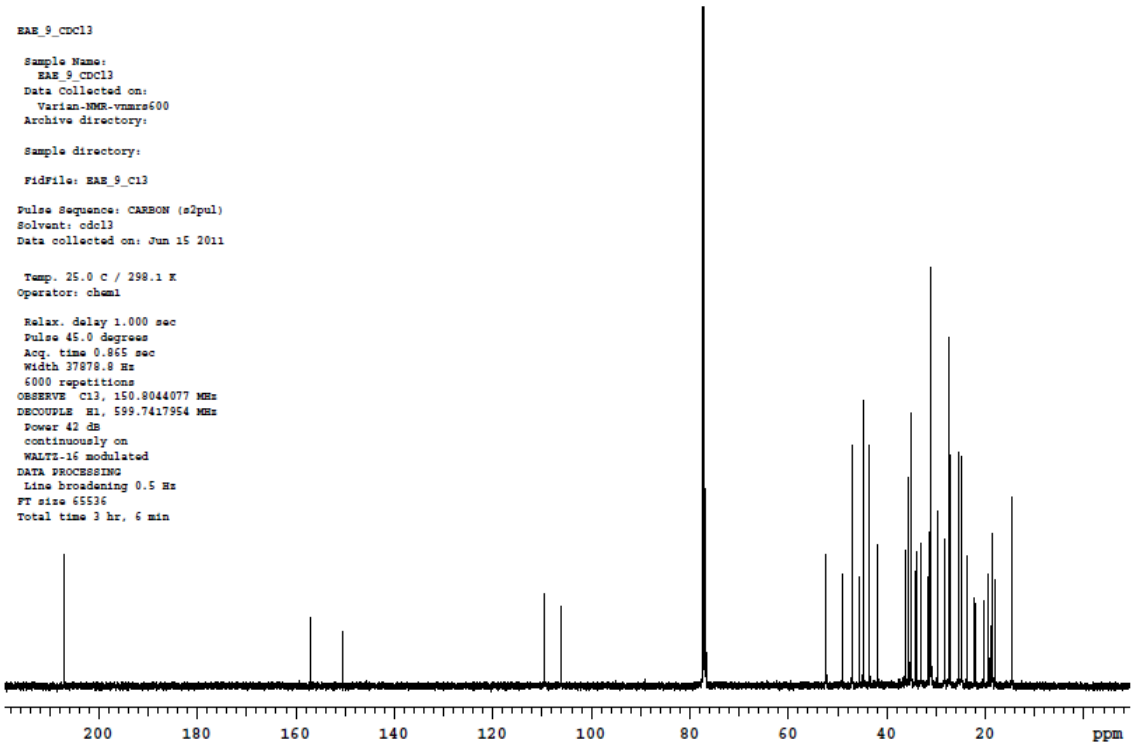

Figure 10 ^{13}C NMR spectrum for cycloeucalenol (600 MHz Varian NMR in d-chloroform).

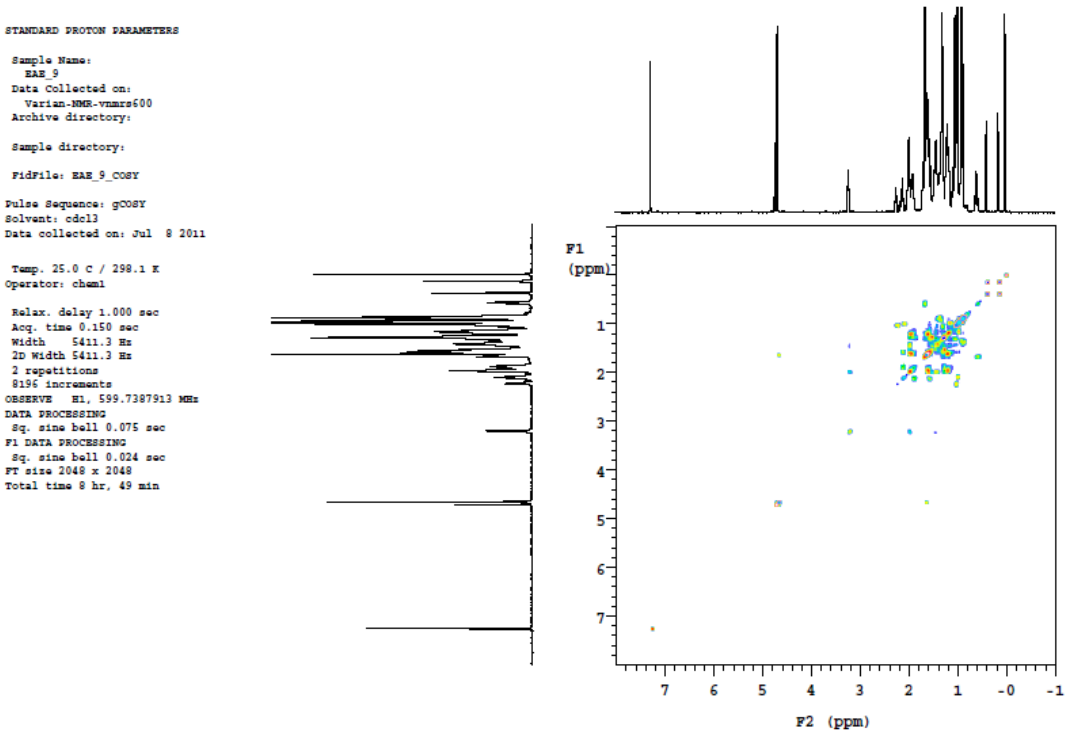

Figure 11 COSY (2D NMR spectrum) for cycloeucalenol (600 MHz Varian NMR in d-chloroform).

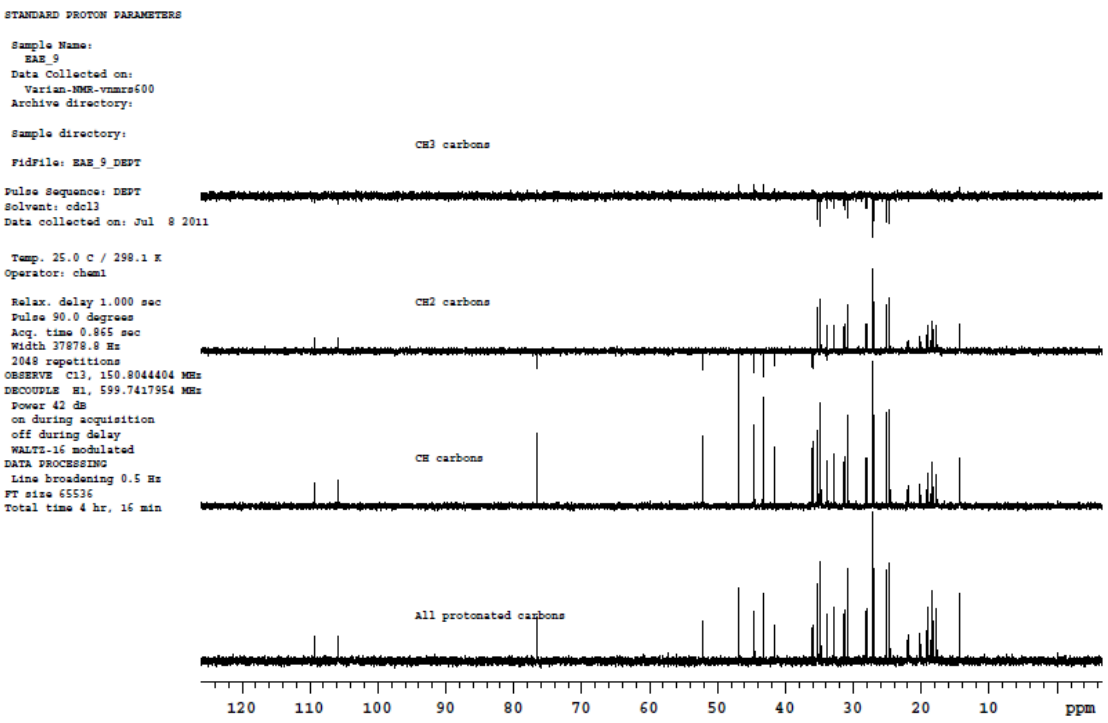

Figure 12 DEPT (2D NMR spectrum) for cycloeucalenol (600 MHz Varian NMR in d-chloroform).

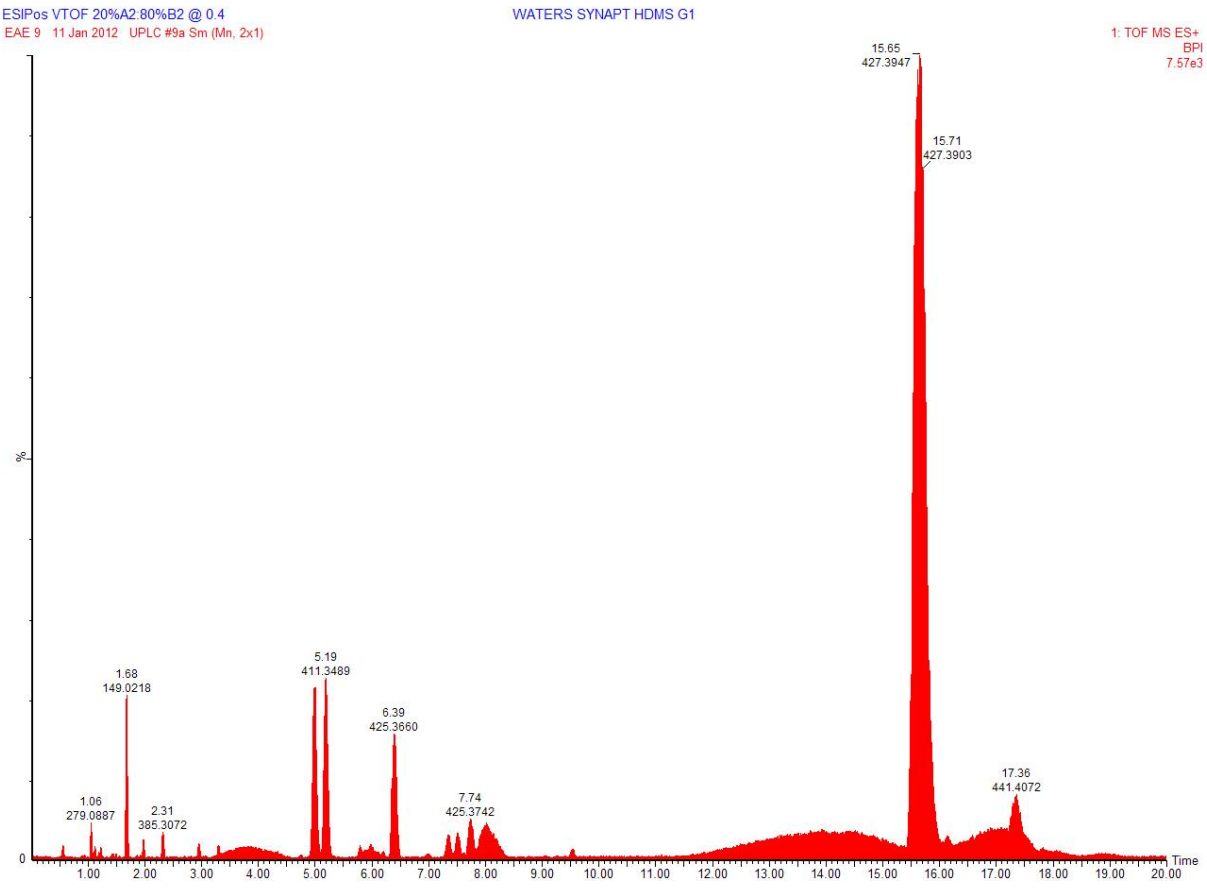

Figure 13 UPLC chromatogram for cycloeucalenol.

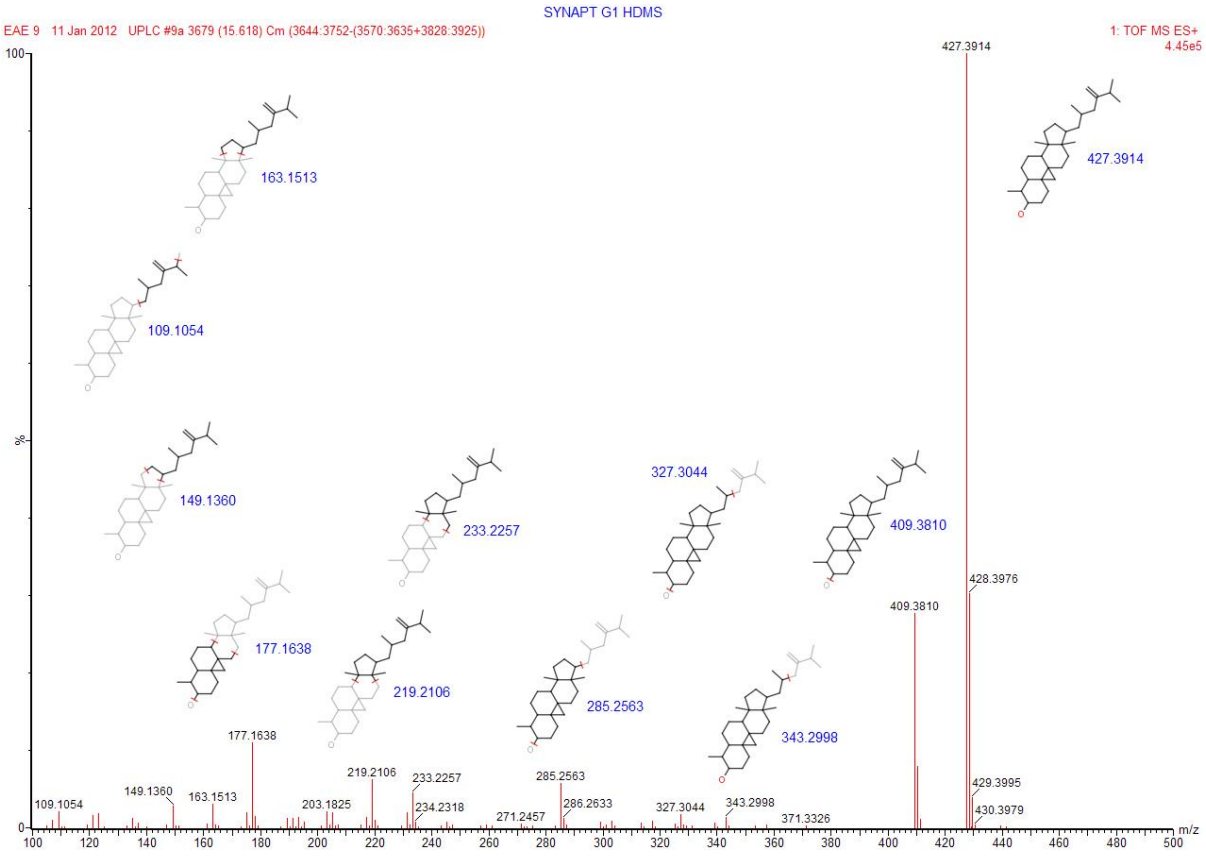

Figure 14 HRTOFMS (ESI^+) spectra for cycloeucalenol.

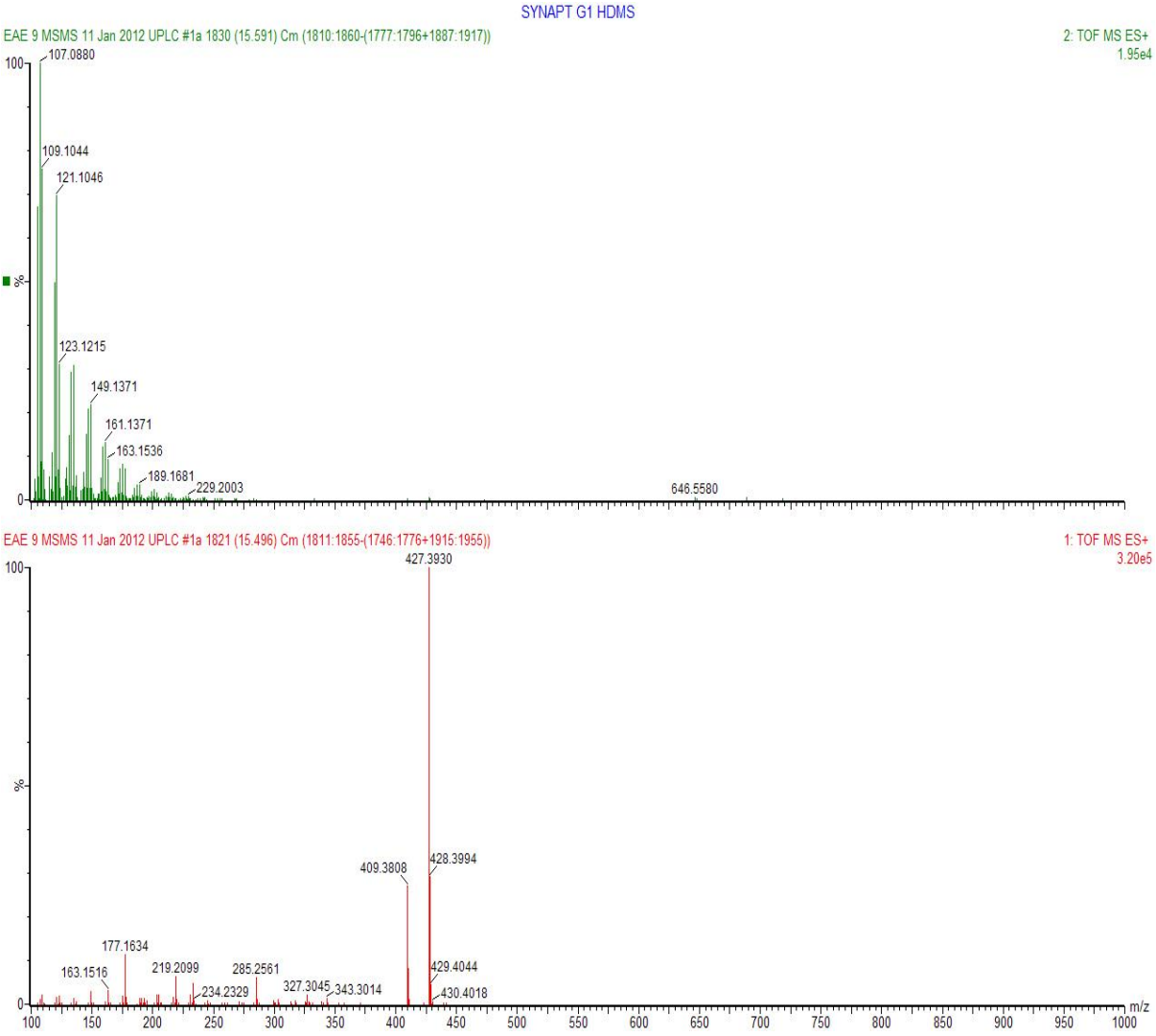

Figure 15 HRTOFMS (ESI^+) spectra for cycloeucalenol.

Data for Fractions

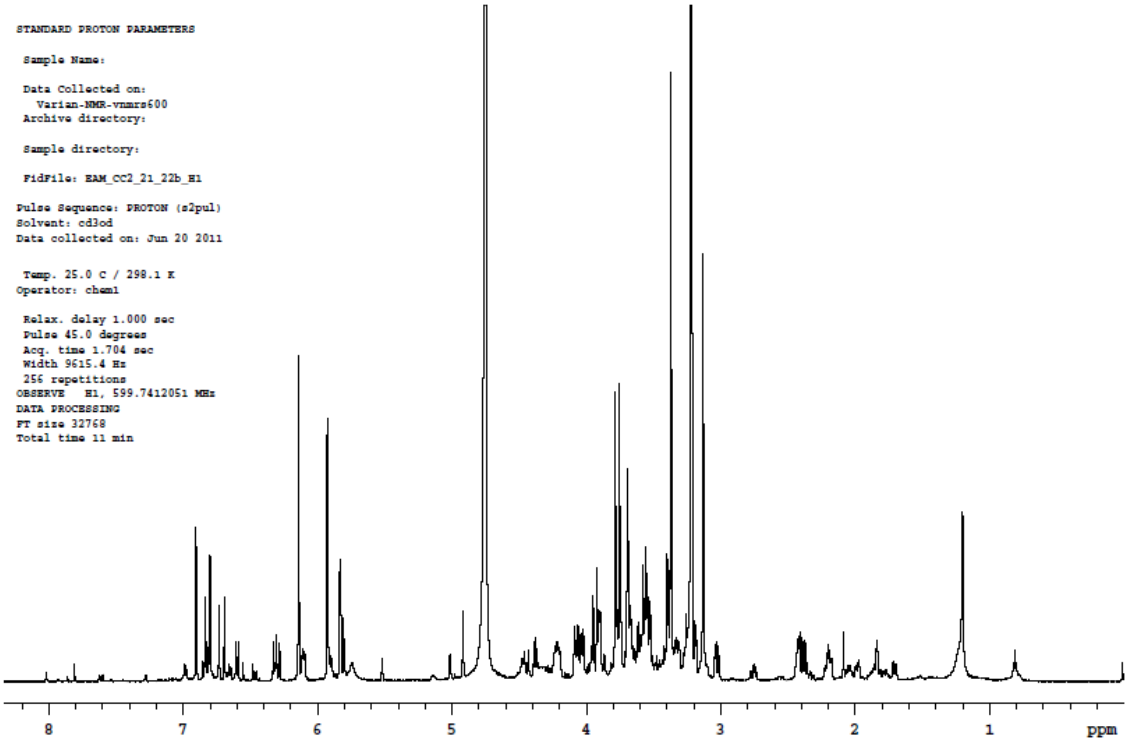

Figure 16 ^{1}H NMR spectrum for fraction EAM 17-21 21,22 (600 MHz Varian NMR in d-methanol).

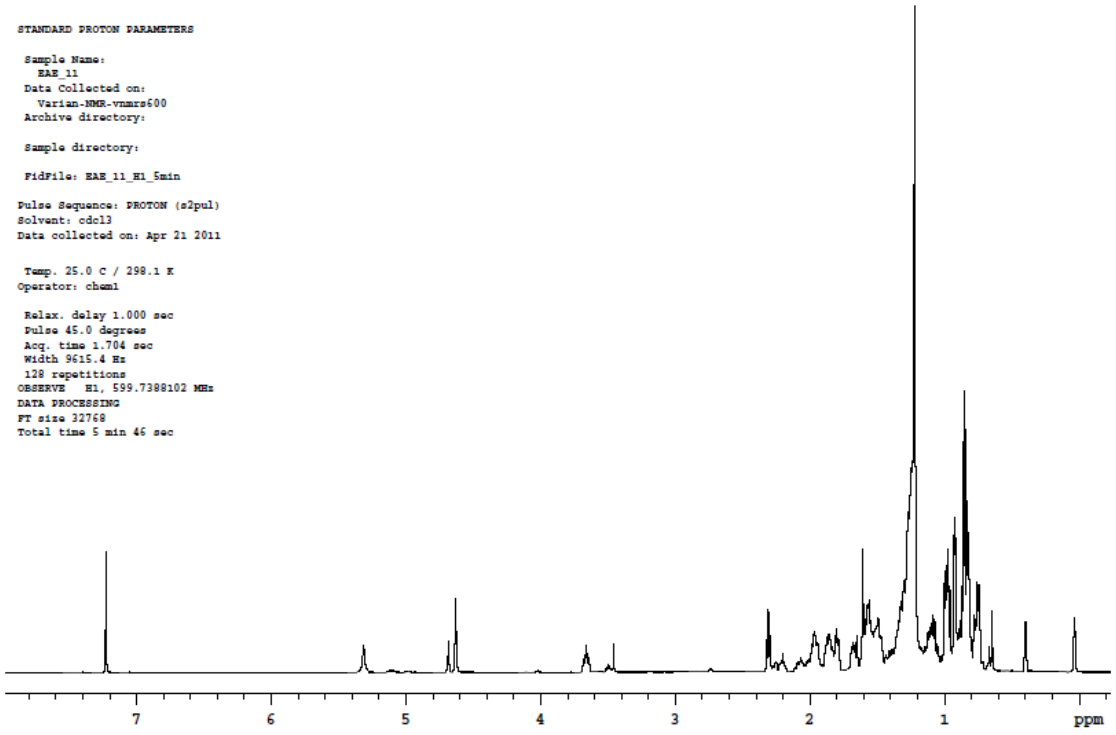

Figure 17 ^{1}H NMR spectrum for fraction EAE 11 (600 MHz Varian NMR in d-chloroform).

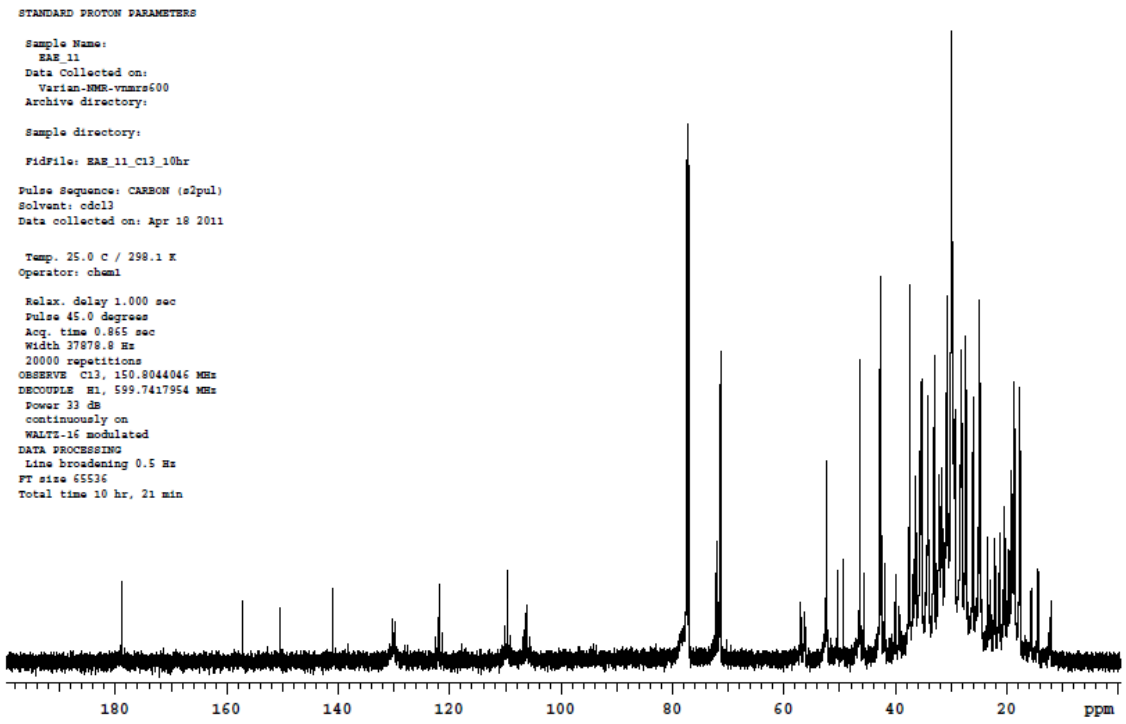

Figure 18 ^{13}C NMR spectrum for fraction EAE 11 (600 MHz Varian NMR in d-chloroform).

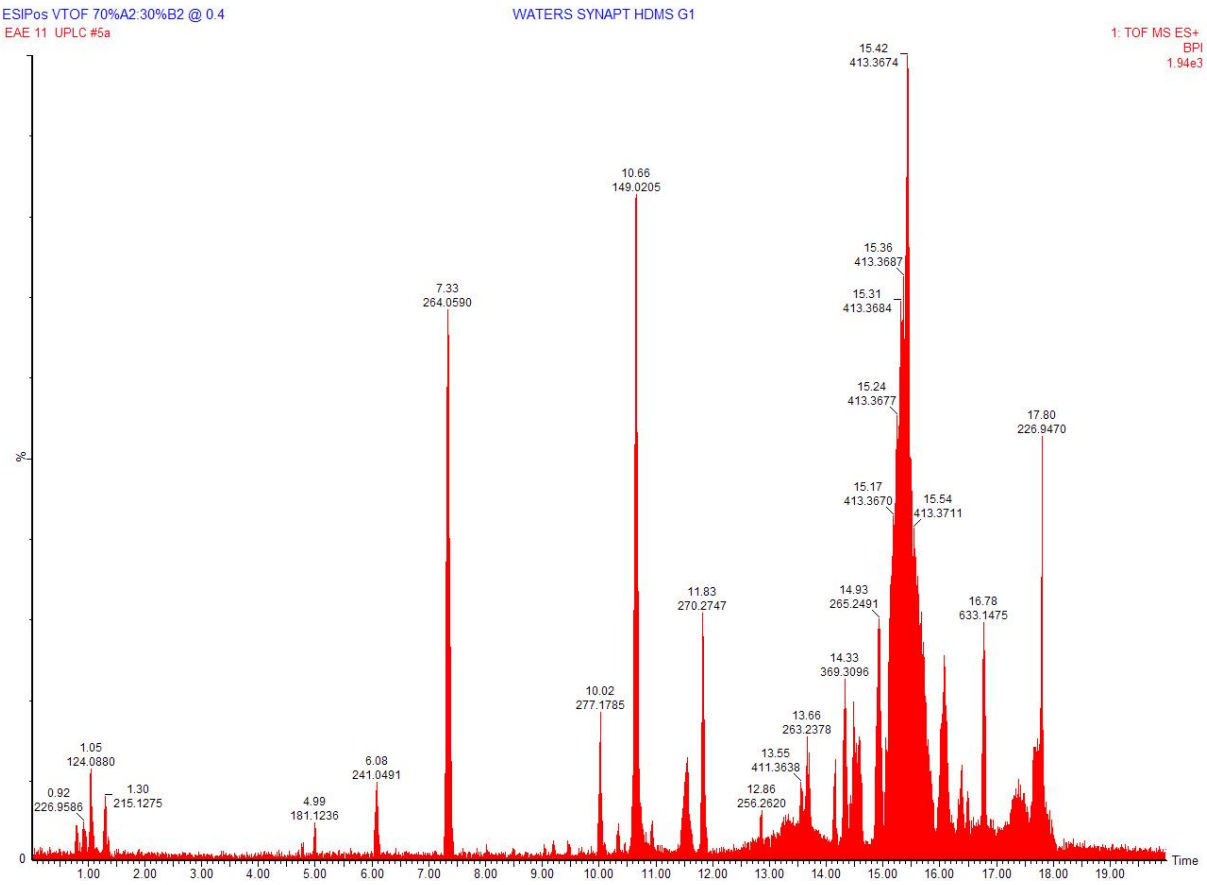

Figure 19 UPLC chromatogram for fraction EAE 11.

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