EXTRACTS OF *SALVIA* SPECIES – RELATION TO POTENTIAL COGNITIVE THERAPY

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"We think we know something when it makes sense to us – sage to me does not make any of it however, I hope you like this piece of weaving"

For my parents and also for Dima, Tanay and Margherita

ABSTRACT

BACKGROUND: Dementia is a neurodegenerative disease of the brain associated with cognitive and memory impairments. Despite recognition of several types of dementia, the Alzheimer type is the most studied and understood. The cholinergic theory of Alzheimer's disease led to the development of licensed drugs based on the inhibition of the enzyme acetylcholinesterase. Extracts of *Salvia* (sage) species have been reported to have cholinergic activities relevant to the treatment of Alzheimer's disease.

AIMS: Lack of information on a chemical fingerprint of the extracts responsible for inhibition of the enzymes butyrylcholinesterase and acetylcholinesterase prompted this *in vitro* investigation of sage species for anti-cholinesterase activity. Cholinergic receptor binding activity, inhibition of β-secretase, and a pro-inflammatory cytokine suppressive activity of extracts of sage species were also studied as relevant treatment targets.

METHODS: The extracts were obtained by methods of supercritical fluid extraction using 1,1,1,2-tetrafluoroethane (Phytosol A) and steam distillation. Dose-dependant inhibition of human cholinesterases by the extracts and constituents was determined using the method of Ellman, while inhibition of β-secretase via a fluorometric method. The nicotinic acetylcholine receptors binding activity was measured as an amount of [^{3}H]-nicotine displaced from human acetylcholine receptors, whereas the muscarinic activity was assessed using the displacement of [^{3}H]-scopolamine. Determination of interleukin 8 inhibitory activity by the extracts was performed via a quantitative sandwich enzyme immunoassay using a commercially available kit.

RESULTS: Inhibition of butyrylcholinesterase by the Phytosol extracts of *S. apiana*, *S. fruticosa* and *S. officinalis* var. *purpurea* was non-competitive. In contrast, inhibition of acetylcholinesterase by *S. officinalis* var. *purpurea* oil was competitive. *S. corrugata*

extract was the most potent inhibitor of acetylcholinesterase with an IC_{50} value of 0.009±0.004 mg ml^{-1}, while *S. officinalis* var. *purpurea* oil was the most active inhibitor of butyrylcholinesterase with an IC_{50} value of 0.015±0.004 mg ml^{-1}. Time dependent increase in inhibition of butyrylcholinesterase by steam distilled oils of *S. fruticosa* and *S. officinalis* var. "*purpurea*" was also evident. IC_{50} values decreased from 0.15±0.007 and 0.14±0.007 mg ml^{-1} with 5 minutes to 0.035±0.016 and 0.06±0.018 mg ml^{-1} with 90 minutes incubation time respectively. Phytosol A extracts were more potent than steam distilled oils with respect to anti-cholinesterase activity. Minor synergy in inhibition of bovine acetylcholinesterase was apparent in 1,8-cineole/α-pinene and 1,8-cineole/caryophyllene oxide combinations, whereas a combination of camphor and 1,8-cineole was antagonistic. Oil of *S. apiana* displaced [^{3}H]-nicotine from human nicotinic acetylcholine receptors and [^{3}H]-scopolamine from muscarinic acetylcholine receptors in a dose dependent manner with IC_{50} values of 0.02 mg ml^{-1} and IC_{50} 0.1 mg ml^{-1} respectively. This oil also showed a modest suppression of interleukin 8 secretions from goblet cells. None of the tested oils and constituents had anti-β secretase activity.

CONCLUSION: These findings demonstrate that the cholinergic activity of the extracts results from a complex interaction between their constituents. Thus, inhibition of acetylcholinesterase is mainly due to the activity of the main constituents with some degree of synergy, whereas anti-butyrylcholinesterase activity is down to major synergistic interactions and identification of a chemical fingerprint responsible for the overall activity is therefore challenging. A synergistic combination of extracts or their standardised fractions with multiple activities is may be a candidate for clinical trials in Alzheimer's disease.

Contents

List of Publications

Savelev S, Okello E, Perry NSL, Wilkins RM, Perry EK (2003) Synergistic and antagonistic interactions of anti-cholinesterase terpenoids in *Salvia lavandulaefolia* essential oil. *Pharmacology Biochemistry and Behaviour*. 75(3): 661-668.

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Kennedy DO, Wake G, Savelev S, Tildesley NTJ, Perry EK, Wesnes KA, Scholey AB (2003) Modulation of Mood and Cognitive Performance Following Acute Administration of Single Doses of *Melissa Officinalis* (Lemon Balm) with Human CNS Nicotinic and Muscarinic Receptor-Binding Properties. *Neuropsycopharmacology*. 28(10):1871-1881.

Okello E, Savelev S, Perry EK (2004) *In vitro* anti-beta secretase and dual anti-cholinesterase activities of tea relevant to treatment of dementia. *Phytotherapy Research*. In press.

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
APP	Amyloid precursor protein
Aβ	Amyloid β-protein
BACE1	β-secretase
BuChE	Butyrylcholinesterase
Ch	Cholinergic
ChAT	Choline acetyltransferase
ChEI	Cholinesterase inhibitor
ChEs	Cholinesterases
CSF	Cerebro spinal fluid
CVD	Cerebrovascular disease
DLB	Dementia with Lewy bodies
DMSO	Dimethyl sulfoxide
DSM	American Psychiatry Association
FTD	Frontotemporal dementia
Glu	Glutamate acid
HAD	Human immunodeficiency virus-associated dementia
His	Histidine
IL	Interleukin
Kie	Inhibitor-enzyme constant
Kies	Inhibitor-enzyme-substrate constant
Km	Michaelis constant, the enzymatic affinity constant
M	Muscarinic
mAChR	Muscarinic acetylcholine receptor
MCI	Mild cognitive impairment
MID	Multi-infarct dementia
MMSE	Mini-Mental State Examination
nAChR	Nicotinic acetylcholine receptor
nAChR	Nicotinic acetylcholine receptor
nbM	Nucleus basalis of Meynert

nbM	Nucleus basalis of Meynert
NFTs	Neurofibrillary tangles
Phe	Phenylalanine
PS	presenilin
*Tc*AChE	*Torpedo californica* AChE
TNF-α	Tumour necrosis factor α
Tyr	Tyrosine
VaD	Vascular dementia
Vmax	Limiting value of initial enzyme velocity

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Chapter 1. General Introduction

1.1. Dementia

1.1.1. Definition

Dementia is an acquired impairment of intellectual and memory functioning caused by disease of the brain, which is not associated with disturbances in the level of consciousness (Friedland and Wilcock, 2000). The term is not used in reference to individuals with mental retardation who have not acquired an adult level of intellectual development. A practical approach to the diagnosis of dementia begins with the clinical recognition of a progressive decline in memory as well as defects in other mental abilities such as abstract thinking, judgement, personality, language, praxis, and visuospatial skills. The deficits must be of sufficient magnitude to interfere significantly with work or social activities (American Psychiatric Association, 1994 (DSM-IV)).

Dementia may have onset before the age of 65 years, known as presenile dementia, or after the age of 65 years, known as senile dementia. It is inappropriate to use the terms dementia, senile dementia, or presenile dementia as a final diagnosis in the individual patient, as they are simply symptomatic classifications similar to the terms headache or seizure disorders. It is now recognised that the disease can affect people of any adult age, although it is more common in the older age groups (Friedland and Wilcock, 2000).

The most common forms of dementia are Alzheimer's disease (AD), dementia with Lewy bodies (DLB), Vascular dementia (VaD), frontotemporal dementia (FTD), and dementia secondary to disease, such as AIDS dementia (Friedland and Wilcock, 2000;

Ritchie and Lovestone, 2002). There are many other forms of dementia which are described in detail elsewhere (Burns and Levy, 1994).

1.1.2. Clinical and pathological diagnosis

1.1.2.1 Alzheimer's disease

Alois Alzheimer (1907) reported a case of presenile dementia in a 51-year-old woman know as Auguste D. The author demonstrated the neuritic plaques (Figure 1.1) and neurofibrillary tangles (Figure 1.2.) in the brain which today are the diagnostic markers of the disease that now carries his name. Neuritic plaques are microscopic foci of extra cellular amyloid deposition and associated axonal and dendritic injury, found in large numbers in the limbic and associated cortices (Dickson, 1997). Such plaques contain extracellular deposits of amyloid β-protein (Aβ) that occur as star-shaped masses of amyloid fibrils. Much of the fibrillar Aβ found in the neuritic plaques is the species ending at amino acid 42 ($A\beta_{42}$), a hydrophobic form that is particular prone to aggregation (Selkoe, 2001). Neurofibrillary tangles (NFTs) are intracellular bundles of abnormal fibres comprising of paired filaments composed of hyperphosphorylated tau proteins and contain no Aβ deposits and neuritic plaques (Selkoe, 2001). AD is responsible for 50 to 60 per cent of cases of dementia in adults in the United States and Europe (Friedland and Wilcock, 2000).

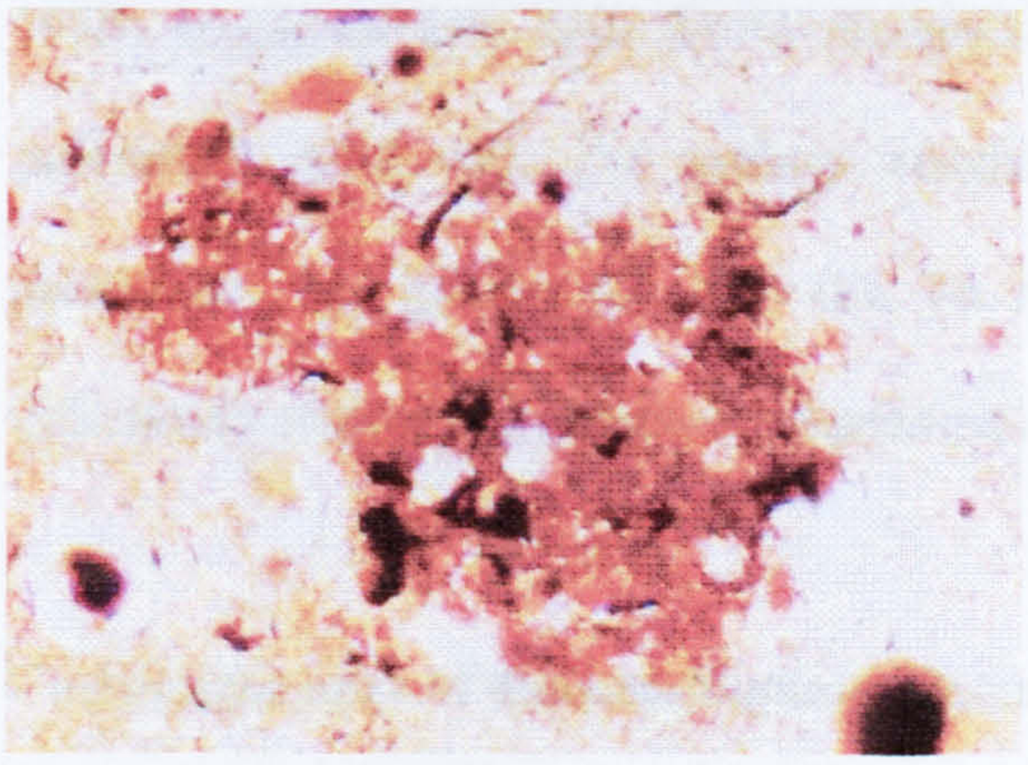


Figure 1.1. Silver-stained Alzheimer neuritic plaque.

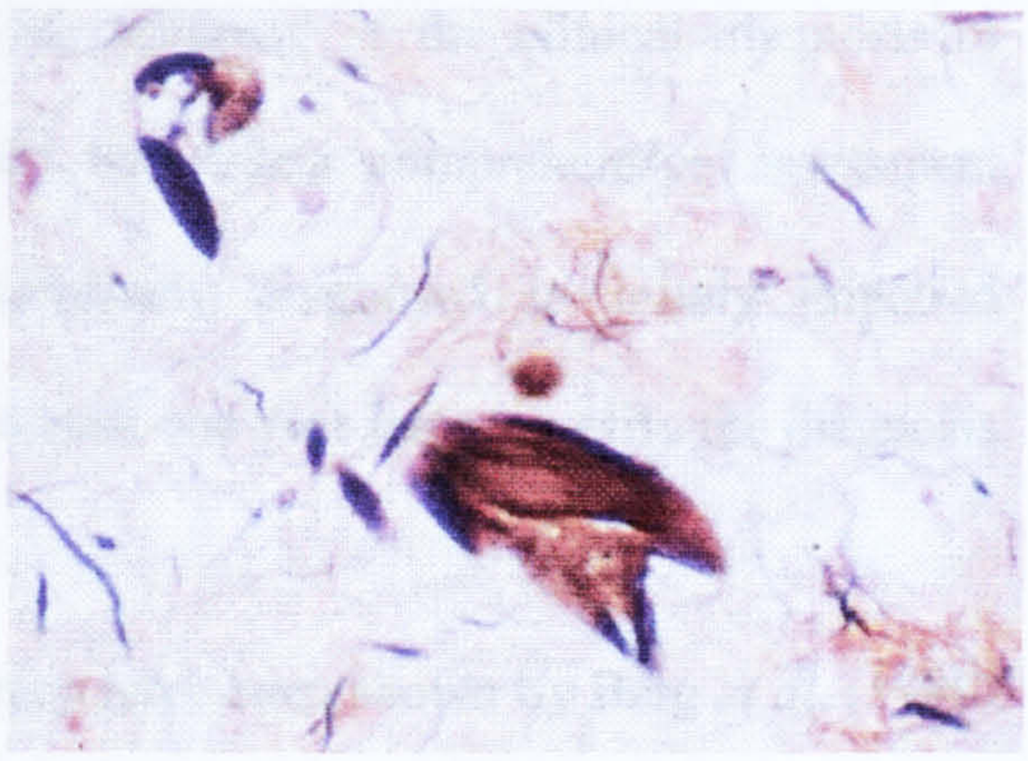


Figure 1.2. Silver-stained Alzheimer neurofibrillary tangles in cerebral cortex

The NINCDS/ADRDA standardised criteria (McKhann *et al.*, 1984) for the clinical diagnosis of AD, have improved diagnostic accuracy, and allowed comparison of results from therapeutic trials and other clinical investigations. The criteria establish probable, possible and definite AD. The NINCDS/ADRDA criteria for probable AD are equivalent to those described in DSM-IV. The reliability and validity of the NINCDS/ADRDA criteria for probable AD have been reported (Blacker *et al.*, 1995) and were adopted by the Consortium to Establish a registry for Alzheimer's Disease (CERAD) for its standardisation and reliability studies of clinical, neuropsychological and neuropathological assessments of the disease (Morris *et al.*, 1989; Mirra *et al.*, 1991). McLean *et al.* (1997) have agreed with the guidelines concerning CERAD criteria but recommended that immunohistochemistry for beta amyloid (Aβ) and tau-reactive neurofibrillary changes, in addition to hematoxylin and eosin stains, should become the basis for histological diagnosis. Marcon *et al.* (1997) have noted that for the *post-mortem* diagnosis of AD, both Aβ immunoreactivity and hyperphosphorylated tau protein immunoreactivity have to be found in the neocortex.

Salmon *et al.* (2002) used Mini-Mental State Examination (MMSE) (Folstein MF *et al.*, 1975) scores of less than 24 to ascertain the accuracy of the clinical diagnosis of individuals with AD. The authors conclude that when comprehensive assessment procedures are employed, AD can be accurately diagnosed in mildly impaired individuals with the dementia reassessment at least one year later to verify the diagnosis.

Comparable rates of clinical diagnostic accuracy have been shown by Berg *et al.* (1998). The authors studied differences between subjects with AD and cognitively intact control subjects, with respect to brain histological markers of AD. They conclude: (1) that the neurocortical neurofibrillary tangles, and to a less degree neurocortical total cored plaque densities are related to dementia severity in AD, but biological and methodological variability are important, and better histological or biochemical markers should be sought for this purpose, (2) Plaque-predominant AD may represent a developmental stage in AD, (3) An effect of Apolipoprotein E epsilon 4 as a risk factor for AD on senile plaque density is variable and complex, being confounded with age, dementia severity and methodological differences, (4) Clinical diagnostic criteria for AD, even in the very mild stage, and senile plaque-based neuropathological criteria for AD are highly accurate.

Braak and Braak, (1991) found that the pattern of senile plaque related amyloid deposition was subject to too much inter-individual variation and that analysis of amyloid in the neocortex was of only limited value in staging AD-related pathological changes. They also defined six developmental stages of neurofibrillary pathology during AD. The first four, limbic stages, correspond to clinically incipient AD whereas the last two, iscortical stages, reveal frank dementia.

A conference on diagnostic criteria for AD in 1997 (Terry *et al.*, 1999) following a workshop sponsored by the National Institute of Aging and the Ronald and Nancy Reagan Institute of the Alzheimer's Association resulted in consensus recommendations for improving the neuropathological criteria for the *post-mortem* diagnosis of AD. The conferees concluded that for the routine diagnosis of AD in the *post-mortem* brain, the semi-quantitative method of CERAD (Mirra *et al.*, 1991) in correlation with the Braaks's six stages (Braak and Braak, 1991) should be used.

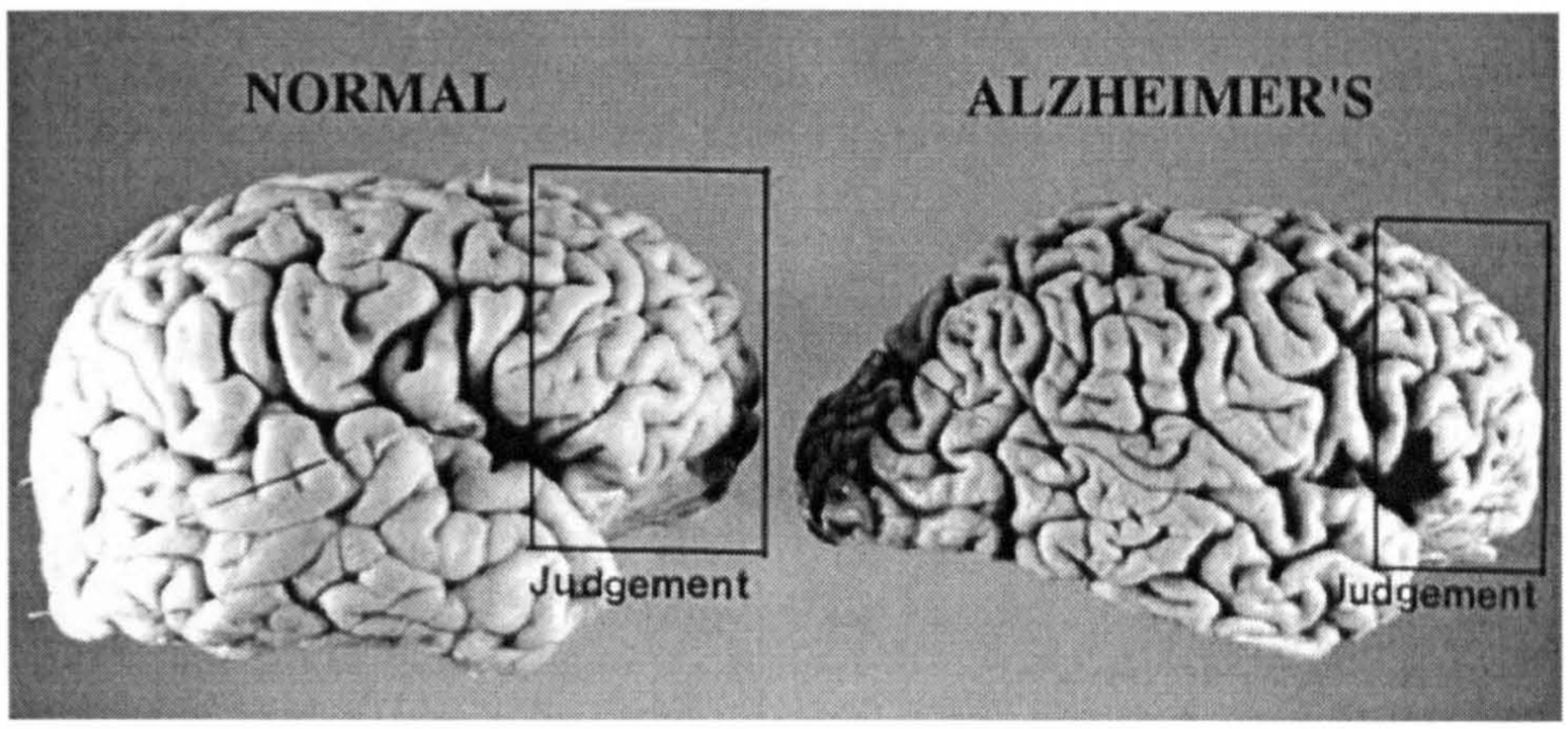


Figure 1.3. Normal and demented human brains. The frontal lobe (boxed) responsible for reasoning, judgment, personality, movement and speech is shrunk in AD sufferers

1.1.2.2. Dementia with Lewy bodies

Friedrich Lewy first described cytoplasmic inclusions in neurons in cases of parkinsonism in 1912 (Lewy, 1912) but it was not until 1961 that Okazaki *et al.* (1961) discussed their possible role in association with dementia and 1984 that Kosaka *et al.* (1984) linked the presence of cortical Lewy bodies (Figure 1.4.) to dementia. Lewy bodies (inclusions) in pigmented neurons of the brain-stem appear as dense eosinophilic cores surrounded by less densely staining peripheral halos. Neocortical Lewy bodies are more subtle and appear in the perikarya of pyramidal neurons in layers V and VI as

more subtle and appear in the perikarya of pyramidal neurons in layers V and VI as spheric, homogeneous, slightly eosinophilic inclusions lacking a concentric laminar structure (Hansen, 1999). Dementia with Lewy bodies (DLB) accounts for 15 to 25% of the cognitively impaired elderly, many of whom have received diagnoses of AD during life (McKeith *et al.*, 1996).

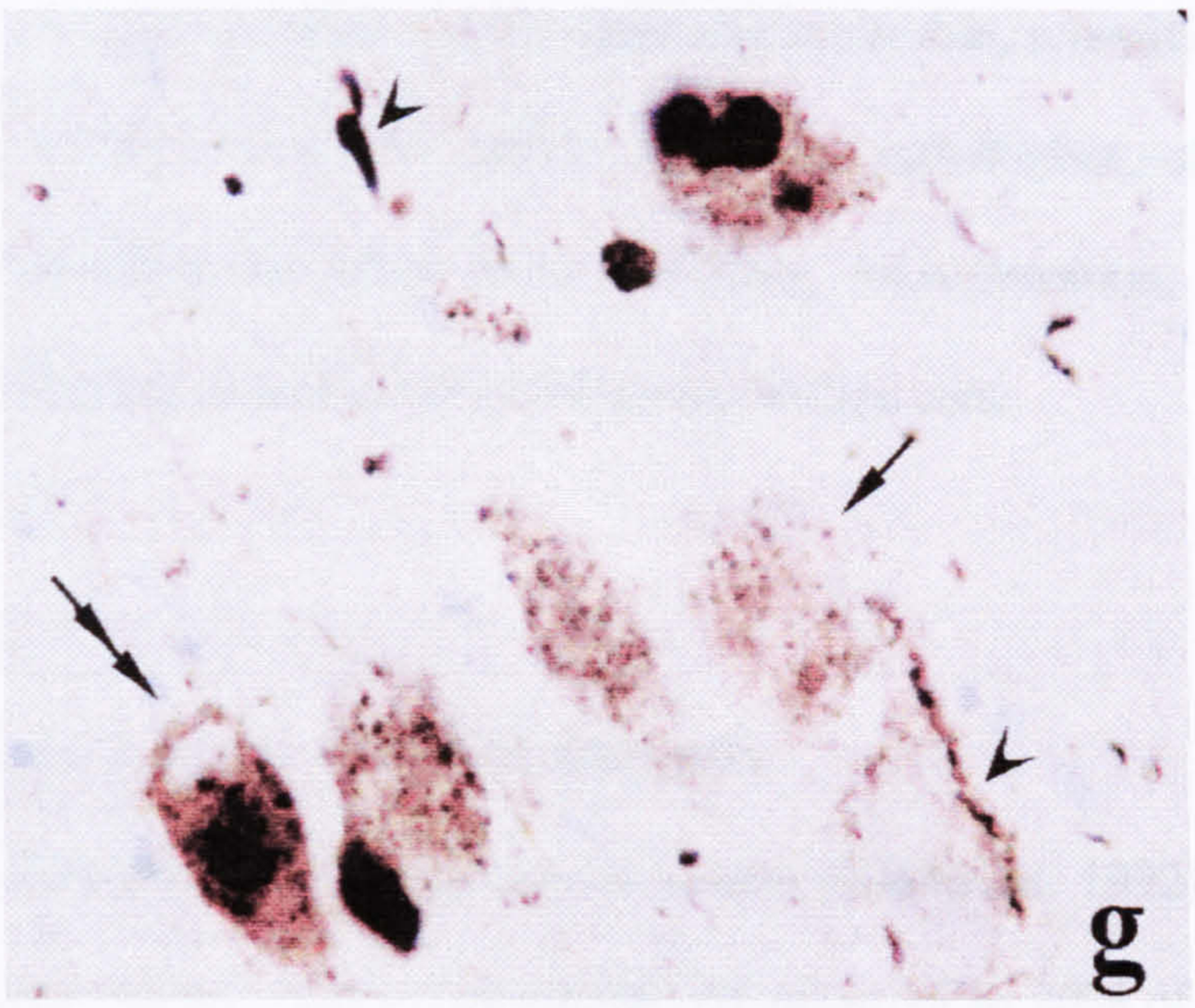


Figure 1.4. Lewy bodies

In 1996, an international consortium in Newcastle upon Tyne published consensus guidelines for the clinical and pathological diagnosis DLB (McKeith *et al.*, 1996). For the pathological diagnosis, brainstem or cortical Lewy bodies are the only features considered essential, although Lewy neurites, Alzheimer's pathology, and spongiform changes may also be seen. Later using the consensus criteria for the diagnosis of DLB McKeith *et al.* (2000) reported that 24 of 29 autopsy-confirmed cases were correctly identified antemortem with a sensitivity rate of 0.83 and a specificity of 0.91. These finding compared favourably with the corresponding sensitivity and specificity rates for NINCDS-ADRDA diagnosis of AD, 0.87 and 0.83 respectively. Although, Lopez *et al.*

low sensitivity (30.7 %) and high specificity (100%) when Lewy bodies coexist with pathological abnormalities of AD. McKeith *et al.* (2003) have also reported in a review that although the diagnostic specificity of the criteria is high (range 79-100%, mean 92%), sensitivity is lower (range 0-83%, mean 49%). The authors conclude that improved methods of case detection are therefore required. Lippa and McKeith (2003) also reviewed DLB cases with AD overlap pathology, concluding that there is a need for continuation to refine clinically applicable, scientifically founded diagnostic criteria for DLB that will satisfy clinicians, pathologists, classifiers, regulatory authorities including the USA Food and Drug Administration, and pharmaceutical companies wishing to tackle this problematic therapy area.

1.1.2.3. Vascular dementia

Dementia may have vascular causes (Hachinski, 1992). In Western countries vascular dementia (VaD) is responsible for up to 15 to 20% of cases. In another 10 to 15% of cases, vascular dementia is found in coexistence with AD (Friedland and Wilcock, 2000). Many forms of VaD have been described (Fortette *et al.*, 1995): multi-infarct dementia, lacunar dementia, Binswanger's subcortical encephalopathy, cerebral amyloid angiopathy, white matter lesions associated with dementias, single infarct dementia, dementia linked to hypoperfusion and haemorrhagic dementia. These make diagnosis of VaD difficult.

Multiple infarcts (Scheinberg, 1988) and ischaemic white-matter lesions (Roman, 1987; Scheinberg, 1988) are suggested vascular causes of dementia, both of which have been associated with hypertension (Strandgaard and Paulson, 1994). Hypertension is a

powerful risk factor for all forms of VaD with the incidence rates rate ranges from 16 per 1000 person-years (Fortette *et al.*, 1995).

Vascular factors may also have a role in late-onset AD, in which white-matter lesions often occur (Brun and Englund, 1986; Blennow *et al.*, 1991). Extensive white matter lesions may affect cognitive function through a loss of axons, producing a functional disconnection of the cortex. The latter can lead to significant reductions in blood flow and metabolism in frontal, temporal and parietal cortical areas, which do not show any structural damage.

Until recently, the diagnosis of VaD was made with the assistance of the Hachinski Ischaemia Scale (Hachinski *et al.*, 1975) which had its poor rate reliability (O'Neill *et al.*, 1995). Most recently, two newer scales have been proposed. These are the State of California Alzheimer's Disease Diagnostic and Treatment Centres (ADDTC) criteria (Chui *et al.*, 1992) and the NINDS-AIREN criteria (Roman *et al.*, 1993). They require the presence of dementia and cerebrovascular disease, and a relationship between the two, for example the onset of dementia shortly after a stroke. Despite these improvements, there are reports (Holmes *et a*l., 1999; Gold *et al.*, 2002; Knopman *et al.*, 2003) highlighting problems of imperfect sensitivity and validity of the disease diagnostic criteria. As with DLB improved methods of VaD detection are also required.

1.1.2.4. Mild cognitive impairment and age-related cognitive decline

The term "mild cognitive impairment" (MCI) has been suggested to describe a condition that may or may not eventually lead to dementia (International Statistical Classification of Disease, 10th rev.). It also refers to the clinical state of individuals

whose memory is impaired but are otherwise functioning well and do not meet clinical criteria for dementia (Flicker *et al.*, 1991; Petersen *et al.*, 1999; Ritchie and Touchon, 2000). Preliminary studies suggest that the rate of progression to dementia is about 25% at two years (Tierney *et al.*, 1996) and perhaps 40% at three years (Grundman *et al.*, 1996). There are also reports indicating that individuals with MCI are not only at risk for developing AD but have neuropathologic AD (Bozoki *et al.*, 2001; Morris *et al.*, 2001; Ritchie *et al.*, 2001). These results suggest that, for many patients, MCI may represent the initial clinical presentation of AD. A current challenge is to distinguish those patients with MCI who eventually will progress in dementia severity and thus have a high probability of underlying AD from those who do not. Thus, the measurement of neuroimaging (Wolf *et al.*, 2003), the ratio of amyloid precursor protein (APP) (Padovani *et al.*, 2002) and cerebrospinal fluid (CSF) (Andersen *et al.*, 2003) may be useful markers for the identification of preclinical AD in patients with MCI.

Age-related cognitive decline is characterised by memory loss without other cognitive problems. If memory deficit is present but the other diagnostic criteria for dementia are not, a diagnosis other than dementia should be considered (American Psychiatric Association, 1994). A disorder similar to age related cognitive decline is described as "mild cognitive disorder" in the World Health Organisations ICD-10 classification (International Statistical Classification of Disease, 10th rev.). According to DSM-IV, age-related cognitive decline represents cognitive changes that are within normal limits given the person's age. Age-associated cognitive decline is characterised by a decline in only one of the five neuropsychologic domains associated with dementia: memory and learning: attention and concentration; thinking; language; and visuospatial functioning (Levy, 1994). According to the International Psychogeriatric Association (Levy, 1994)

additional criteria should be met to make a diagnosis of age-related cognitive decline. These criteria include the report of cognitive decline from a reliable source, a gradual onset of at least six months duration and the Mini-Mental State Examination test (MMSE).

1.1.3. Epidemiology and incidence of dementia

Epidemiological studies require accurate identification of dementia cases. An agreement on acceptance diagnostic criteria for identifying cases of dementia was developed in the 1980s based on the principals introduced in the third edition of the American Psychiatry Association's (DSM III) and its later revisions (DSM III-R and DSM IV)

Ueda *et al.* (1992) reported that in Japan VaD represents over 50% of dementias, whereas in another Japanese study (Asada *et al.*, 1996) of centenaries, 70% were found to have dementia, of whom AD was present in 76%. In Europe and North America, Alzheimer's disease is estimated to be more common than vascular dementia. (Ritchie and Lovestone, 2002). According to Ratnavalli *et al.* (2002) there are at least 3500 patients with frontotemporal dementia in the United Kingdom and more than 10.000 in the United States. The mean age of the onset of FTD was 52.8 years and it also appears to be more common in men.

In the United States, for healthy people between the age of 75 and 85, new cases of Alzheimer's disease are as common as myocardial infarction and more common than stroke (Katzman *et al.*, 1989). The study by Evans *et al.* (1989) of the older population of East Boston, US, concluded that over 45% of those aged 85 years or more suffered from AD. This figure was confirmed by a Swedish study (Skoog *et al.*, 1993) where

44% of a representative sample of 494 85-years-olds were considered to have AD. Interestingly, 47% received a diagnosis of vascular dementia.

In a first Shanghai study (Zhang *et al.*, 1990) it was reported that Alzheimer's disease accounted for 65% of all dementias. In the second (Zhang *et al.*, 1998), based on DSM III criteria, impact of age and education, a total of 1970 non-demented subjects who were the age of 65 years or older in 1987 were re-surveyed after five years, of whom 114 new cases of dementia were identified. This shows that with each age increase of five years for those aged 65 years and over, the likelihood of developing dementia increases nearly 74%. The incidence of dementia in total was 1.15% annually, 0.98% for males and 1.27% for females. The incidence for Alzheimer's disease, vascular dementia and dementia by other causes was 0.74, 0.33 and 0.08%, respectively. The study also showed that incidence of dementia in the Shanghai cohort is similar to that from other countries. Education had a protective effect on the development of dementia. The highest rate of 2.02% was found in illiterate individuals.

Jorm *et al.* (1987) reported national differences in the relative prevalence of AD and multi-infarct dementia (MID). MID was more common in Japanese and Russian studies rather than in Finnish and American. They also found that across the studies, AD was more common in women but MID in men.

Hofman *et al.* (1991) showed that the overall European prevalence of dementia for the five-year age groups from 60 to 94 years, were 1.0, 1.4, 4.1, 5.7, 13.0, 21.6 and 32.2%, respectively. In subjects under 75 years the prevalence was slightly higher in men than in women; in those aged 75 years or over the prevalence was higher in women. The

prevalence figures nearly doubled with every five years of increase in age.

Evidence suggests that AD may be relatively rare in the Indian sub-continent. Chandra *et al.* (1998) conducted a study to determine the prevalence of AD and other dementias in a rural elderly Hindi-speaking population in Ballabgarh in northern India. The authors performed a community survey of a cohort of 5,126 individuals aged 55 years and older, 73.3% of whom were illiterate. It was found that an overall prevalence rate of 0.84% in the population aged 55 years and older and 1.36% in aged 65 years and older. The overall prevalence rate for AD was 0.62% in the population aged 55 and over and 1.07% in the population aged 65 and over. Greater age was associated significantly with higher prevalence of both AD and all dementias. The authors concluded that the prevalence of AD and other dementias was low even with the increased age and was not associated with gender or literacy.

Results from community surveys and autopsy reports (Osuntokun *et al.*, 1992) suggest that Alzheimer's disease does not occur in Nigerians. In the survey of 932 elderly Nigerians, no subject with dementia as defined by DSM-III-R was found, although there was significant decline of cognition with the increasing age.

Differences in rates of dementia between developing and developed countries are difficult to explain, but might be attributable partly to difficulties in dementia diagnosis in areas with high rates of illiteracy, and survival bias due to high death rates at all ages (Ritchie and Lovestone, 2002). But if this is not the case, it would raise the possibility that dietary and/or life style factors may be protective. For example, a widely spread habit of chewing Areca catechu (betel nut) in Asia (Yoganathan, 2002) may have a protective effect against AD. Betel nut contains arecoline, muscarinic M1 receptor

agonist (Ghelardini *et al.*, 2001) and according to Fisher *et al.* (2003) an M1 agonist decreases brain Aβ and increases cognition in subjects with AD.

Studies of risk factors for vascular dementia, the second common cause of dementia, are fewer in number than those for Alzheimer's disease, principally because of difficulties with the definition of research criteria for this group of disorders (Ritchie and Lovestone, 2002). In Europe, estimates of vascular dementia prevalence thus vary widely from 10–50% of all cases of dementia (Rocca *et al.*, 1991). Main risk factors identified for the disease are age, male sex, hypertension, myocardial infarction, coronary heart disease, diabetes, generalised atherosclerosis, smoking, high lipid concentrations, and a history of stroke (Skoog, 1998; Erkinjuntti, 2000).

These findings may represent the changing frequencies of dementia and its causes with increasing age, but may include biases related different diagnostic criteria and also cultural or population differences. Nevertheless, because of the rapid aging of the world's population and declining incidence of heart disease and stroke (Friedland and Wilcock, 2000), it is clear that dementing illnesses, which already have a devastating impact on health-care services (Andersen *et al.*, 2003), may become an increasingly important problem in the future.

1.2. Cholinergic systems

1.2.1. Normal brain

1.2.1.1 Cholinergic neurons and axons

The mammalian brain contains several groups of cholinergic (choline acetyltransferase (ChAT)-positive) neurons located within the basal forebrain, striatum and brainstem

(Nagai *et al*., 1983; Mesulam & Geula, 1988; Mesulam *et al*., 1989; Geula & Mesulam, 1999).

The basal forebrain cholinergic system is comprised of the nucleus basalis of Meynert (nbM), the horizontal and vertical diagonal bands of Broca (HDBB and VDBB, respectively), and the medial septal nucleus (Mesulam, 1996). This neuronal system provides the primary cholinergic innervations to hippocampus, limbic neocortex and in addition to the thalamus. The nucleus basalis of Meynert provides cholinergic innervations to the amygdala and the rest of the cortical mantle. Neurons situated in the medial septal nucleus innervate predominantly the hippocampus, whereas those of the vertical and horizontal limb of diagonal band of Broca project into the anterior cingulate cortex and olfactory bulb, respectively (Mesulam, 1996).

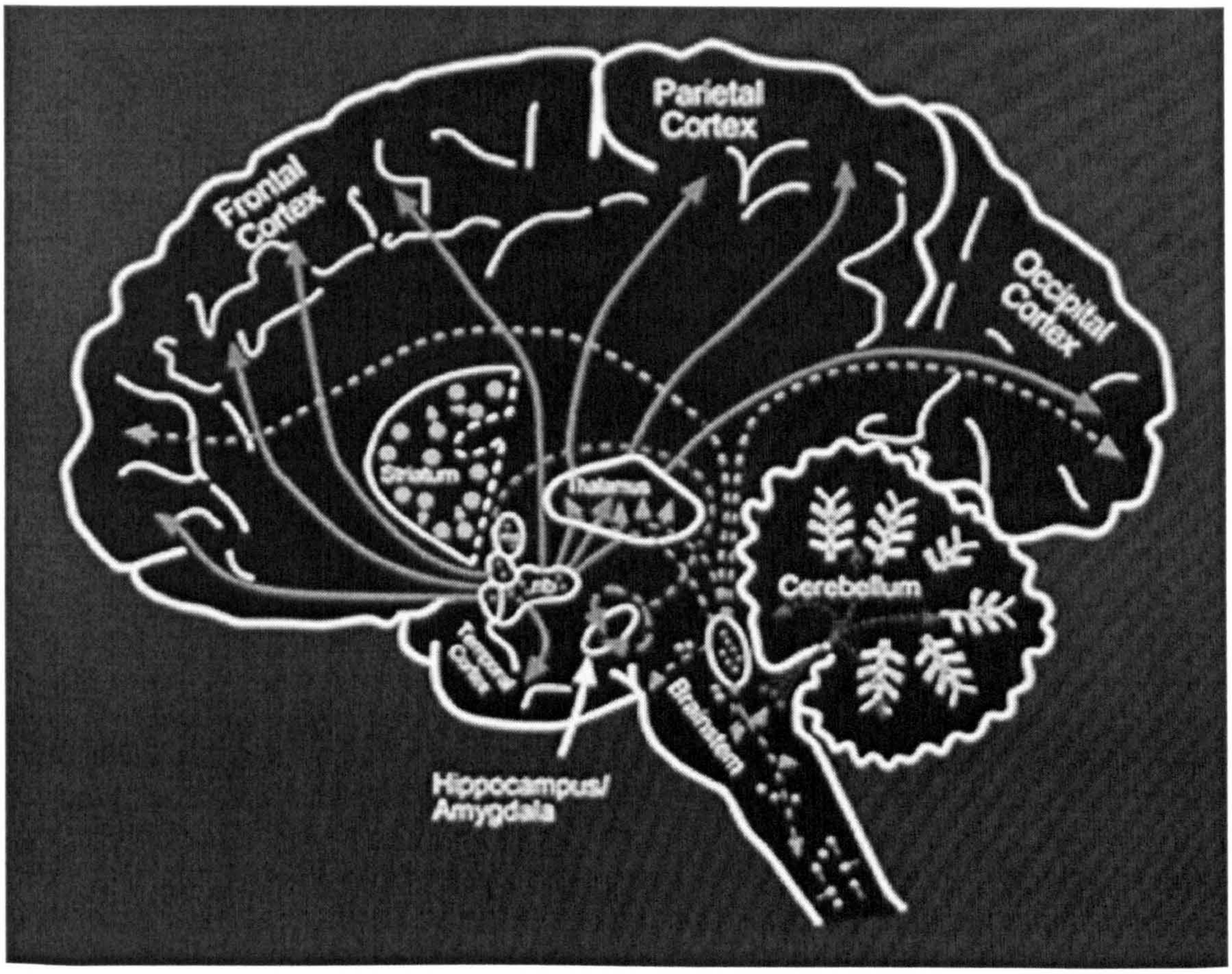


Figure 1.5. Human brain cholinergic systems

Neuroanatomical identification of cholinergic neurons occurs through the immunohistochemical demonstration of ChAT, the enzyme which synthesises the neurotransmitter acetylcholine (ACh) (Figure 1.6). A neuron is said to be cholinergic when it synthesizes ACh for the purpose of neurotransmission. Non-cholinergic or cholinoceptive neurons in the brain, which are ChAT-negative, include the glutamatergic, gabaergic, dopaminergic, histaminergic, serotonergic, and noradrenergic neurons (Mesulam, 2000), as well as many neuropeptides (personal communication with Professor E.K. Perry)

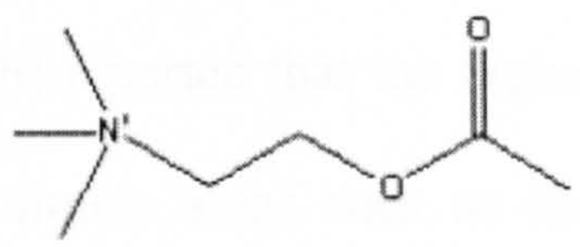


Figure 1.6. Acetylcholine ($C_7H_{16}NO_2$)

Based on anatomic location, four groups of cholinergic neurons (Ch1-Ch4) have been described in the human basal forebrain. These neurons are located around the medial septum, the vertical and horizontal limbs of the diagonal band of Broca, and the nucleus basalis of Meynert (Nagai *et al*., 1983; Hedreen *et al*., 1984; Mesulam & Geula, 1988). Among these four groups Ch4 is the most extensive (Geula *et al*., 1993). The human Ch4 can be subdivided further into another six sectors (Mesulam & Geula, 1988). The Ch1 and Ch2 neurons may provide the major cholinergic innervation of the hippocampus, the Ch3 of the olfactory bulb, and the Ch4 of the entire cortical mantle and amygdale (Geula & Mesulam, 1999).

Cholinergic cell groups are found in the upper brainstem, the pedunculopontine nucleus (Ch5), the laterodorsal tegmental nucleus (Ch6), the medial nucleus of the habenula (Ch7), the parabigeminal nucleus (Ch8) (Mizukawa *et al.*, 1986; Mesulam *et al.*, 1989). The neurons Ch5 and Ch6 provide the major cholinergic innervation of the thalamus (Hallanger *et al.*, 1987). The Ch5-Ch6 can also provide an additional, but minor source of cholinergic innervation of the interpenduncular nucleus and the Ch8 neurons project mostly to the superior colliculus but also to the thalamus (Hallanger *et al.*, 1987; Hall *et al.*, 1989). The Ch neurons of the striatum have almost entirely local connections with a lesser cholinergic input from Ch1-Ch4 (Geula & Mesulam, 1999).

Geula & Mesulam (1989, 1996) reported that the highest density of cholinergic fibers (axons) is found within core limbic areas such as the amygdala and hippocampus. Paralimbic cortical areas show the next highest density of cholinergic fibers, whereas the primary visual cortex contains the lowest. The cortical structures within the temporal lobe show a high density of cholinergic fibers, while within the occipital lobe lesser.

1.2.1.2. Cholinesterases

1.2.1.2.1. Acetylcholinesterase

All cholinergic neurons of the human basal forebrain and brainstem contain the cholinergic enzymes ChAT and acetylcholinesterase (AChE) (Mesulam & Geula, 1988; Mesulam *et al.*, 1989). AChE may also occur in non-cholinergic neurons (personal communication with Professor E. Perry). The latter is synthesized in the perikaryon and then transported to dendrites, axons and further into the cell membranes (Figure 1.7.). AChE is encoded by a gene on chromosome 3 and belongs to the Type B

carboxylesterase gene family (Gnatt *et al.*, 1991; Ballard, 2001). Analysis of the three-dimensional structure of AChE (Sussman *et al.*, 1991) and homologous lipase (Cygler *et al.*, 1993) indicates that these enzymes have a common fold termed the α/β hydrolase fold (Ollis *et al.*, 1992), in which a central β-sheet is surrounded by loops and helices. Solution of the 3D structure provided that the cholinesterase (ChEs) contain a catalytic triad, albeit with a glutamate in place of the aspartate found in the serine proteases. The active site is situated in a deep cleft, being located almost 20 Å from the surface of the catalytic subunit, at the bottom of a long and narrow cavity. This cavity was named the active-site gorge or, since over 60 % of its surface is lined by the rings of conserved aromatic residues, the aromatic gorge (Sussman *et al.*, 1991).

AChE comprises 90% of the total cholinesterases in the temporal cortex of normal brain (Perry *et al.*, 1978 a). Moreover, Shinotoh (1999) reported a positron emission tomography study of the enzyme activity in the human brain. The author found that AChE activity is predominant in the striatum followed by the cerebellum, thalamus, and the cerebral cortex.

In adult human brain AChE is mainly present as the membrane-bound globular tetramer G_4 form, and the more soluble monomer G_1 form, with minor contributions of dimeric G_2 and other asymmetric forms (Attack *et al.*, 1986). Studies (Ogane *et al.*, 1992 a; Ogane *et al.*, 1992 b) on brain fractions suggest that 60-90% of the G_4 form is intracellular and membrane located while 90% of the G_1 form is intracellular and cytoplasmic. In the cortex and hippocampus however, the G_1 form represents approximately from 30% to 40% of total AChE (Attack *et al.*, 1986). Meneguz *et al.*, (1992) reported that in rats G_4 and G_1 forms were detected in all the brain areas. Their distribution, expressed as G_4/G_1 ratio, varied in young rats from about 7.5 for the

striatum to about 2.0 for the medulla-pons and cerebellum. The age-related changes consisted in a significant and selective loss of the enzymatic activity of G4 forms in the cerebral cortex, hippocampus, striatum, and hypothalamus, which resulted in a significant decrease of the G_4/G_1 ratio. No such changes were found in the medulla-pons or the cerebellum.

The major role of the AChE is to terminate the action of ACh through catalytic hydrolysis (Volkova *et al.*, 1976; Mesulam, 2000) (Figure 1.7.). Rapid hydrolysis of acetylcholine by AChE (EC 3.1.1.7) is essential for cholinergic neurotransmission, the main feature involved in cognition. This importance is underlined by the large value of catalytic constants K_{cat}/K_m, $\approx 10^9\ M^{-1}\cdot s^{-1}$ (Nolte *et al.*, 1980), which ranks as one of the highest catalytic efficiencies known (Fersht, 1985) despite the fact that the substrate has to reach the active site at the bottom of a narrow, 20 Å deep gorge (Sussman *et al.*, 1991) by diffusion. Zhou *et al.* (1998) showed that such enzyme specificity is achieved by dynamic configuration of the five aromatic rings of tyrosine (Tyr) amino acids Tyr_{121} and $_{334}$ and phenylalanine (Phe) amino acids (Phe_{290}, $_{330}$ and $_{331}$), serving as the gate, where it can rapidly switch between the open and closed states (Figure 1.8.). One of these open states may coincide with the ACh entering state allowing the entrance of the substrate with high efficiency.

Moreover, binding between the acetyl moiety of ACh and catalytic binding site involves an interaction with three key amino acid residues. These amino acids are involved in a charge relay system within the gorge, which for AChE is centred around a serine (Ser_{200}) residue, and involves histidine (His_{447}) and glutamate acid (Glu_{334}). In this region of the gorge two large amino acids Phe_{295} and Phe_{297} may restrict a passage to the active site for larger substrates (Greig *et al.*, 2001).

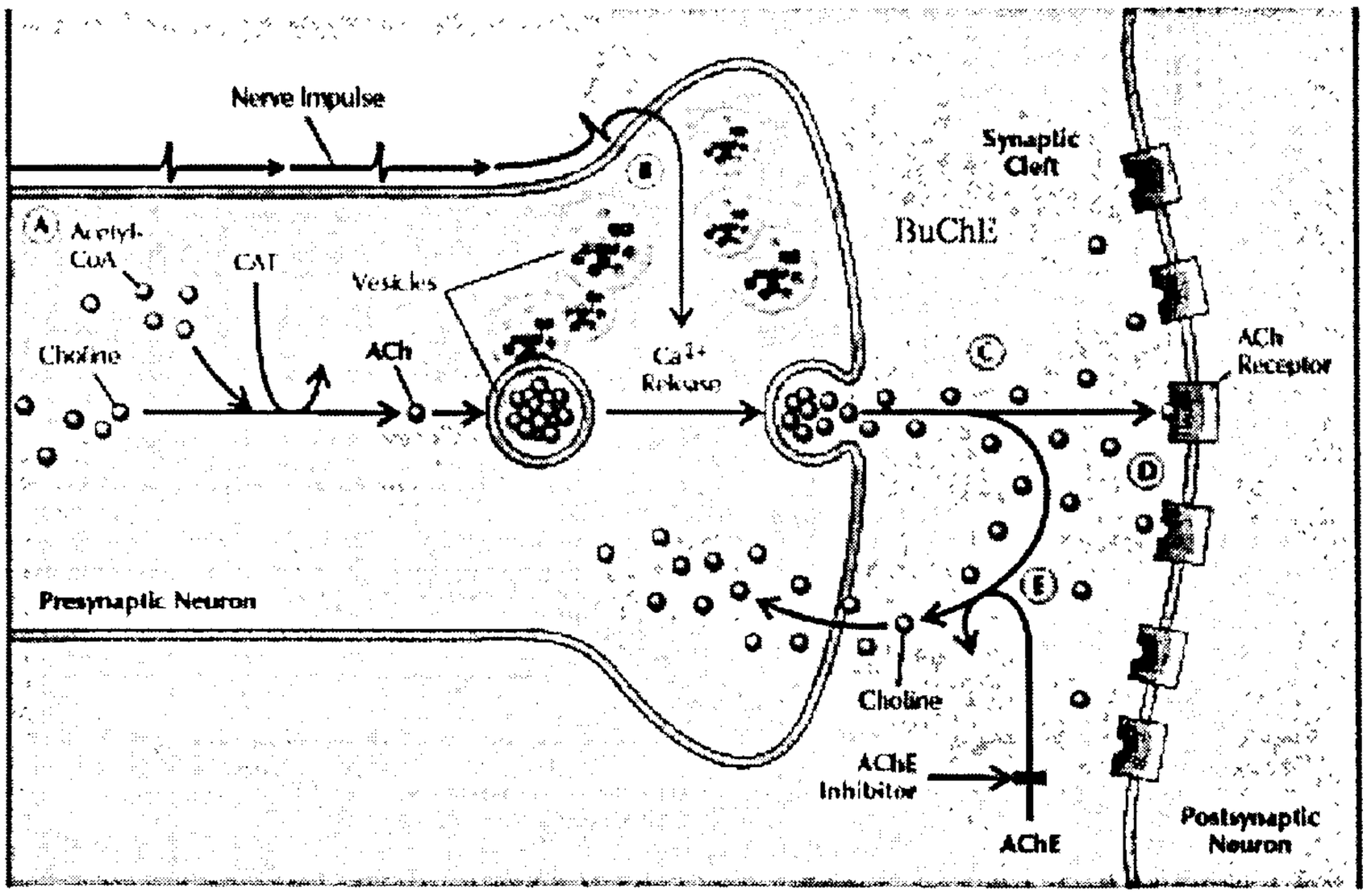


Figure 1.7. Cholinergic neurotransmission. The neurotransmitter Ach is synthesised in presynaptic cholinergic neurons by choline acetyltransferase (CAT). The process entails transfer of an acetyl group from acetyl-coenzyme A to choline (A). Until needed, the ACh molecules are stored in discrete vesicles at the ends of the presynaptic neurons. Arrival of a nerve impulse triggers the release of Ca^{2+} ions, which activate actin microfilaments that in turn pull the storage vesicles into position for ACh release. In a single event the vesicles empty their contents into the synaptic cleft (C). Most of these molecules bind to cholinergic receptors on adjacent postsynaptic neurons (D). Any that remain unbound are rapidly hydrolysed by AChE (E). The choline released in the process is reused in synthesising new ACh. Inhibition of AChE and BuChE increase the amount of ACh available for neurotransmission.

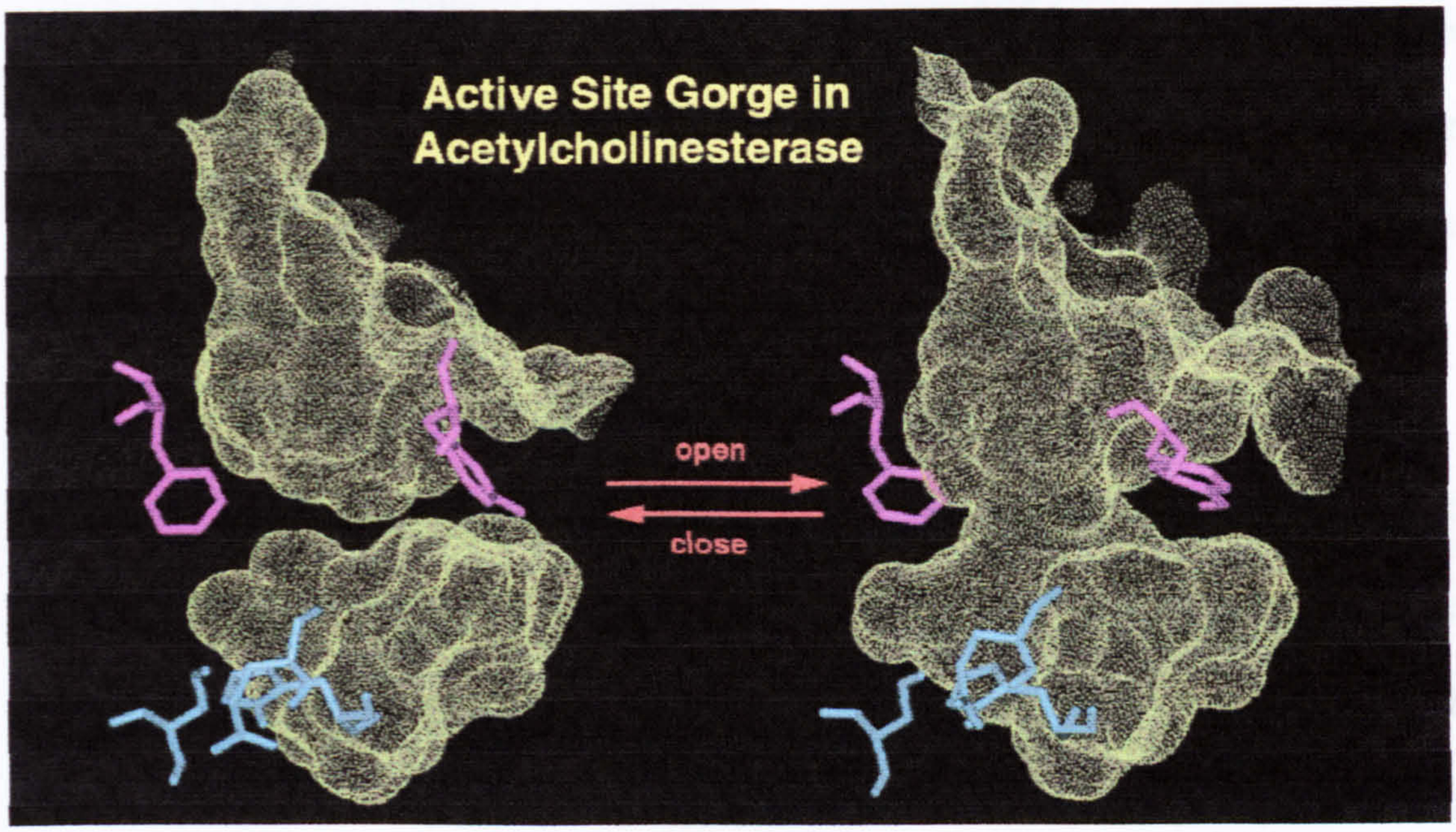


Figure 1.8. Open and close state of acetylcholinesterase with Tyr and Phe residues at the date

1.2.1.2.2. Butyrylcholinesterase

Butyrylcholinesterase (BuChE) is encoded by a gene on chromosome 3 and as AChE belongs to the Type B carboxylesterase gene family (Gnatt *et al.*, 1991). AChE and BuChE share 65% amino acid homology despite being encoded by different genes (Soreq and Zaku, 1993).

The human cerebral neurocortex also contains BuChE-rich neurons (Mesulam *et al.*, 1995; Darvesh *et al.*, 1998). The number of these neurons is approximately two orders of magnitude less than the number of AChE-rich neurons (Mesulam, 2000). In the normal brain BuChE, primary located in glial cells, accounts for 20% of ChE activity, while AChE accounts for the remaining 80% (Greig *et al.*, 2001). Most cortical BuChE-rich neurons are non-pyramidal, lie predominantly in deeper cortical layers, including

layer 6 and in the immediately adjacent juxtacortical region (Mesulam *et al.*, 1995). Limbic structures such as amygdala, hippocampus, and entorhinal cortex contain a slightly higher density of BuChE-rich neurons (Mesulam, 2000). In the hippocampal complex, BuChE-rich neurons are located mostly within a white-matter layer. There are no BuChE-rich axons in the cerebral cortex (Mesulam, 2000).

Development of normal cholinergic pathways in AChE knockout mice (Xie *et al.*, 2000;) and the hydrolysis of the ACh surrogate acetylthiocholine by widely spread BuChE, not only in the brain of a wide-type mouse (Mesulam *et al.*, 2002 b) but in areas of the human brain known to receive cholinergic input (Mesulam *et al.*, 2002 a), suggest that ACh is a functional substrate to BuChE (Figure 1.7.).

Ekholm and Konschin (1999) summarised structural differences between BuChE and AChE. In BuChE a similar channel, an aromatic gorge, as in AChE leading to the active site was found, although it was not as narrow as in AChE and it did not contain as many aromatic amino acids. Moreover, Phe_{295} and Phe_{297} in comparison with AChE are replaced with two smaller amino acids-valine and leucine-creating additional space for entering large substrates. The space of the active centre in BuChE is greater than in AChE. An intra-atomic distance analysis of the active site indicates that two hydrogen bonds easily form in AChE but only one in BuChE. The active site in BuChE is therefore less rigid than in AChE, allowing the substrate move more freely.

BuChE, like AChE, also occurs in asymmetric and globular forms where they exist as amphiphilic and hydrophilic species in different brain regions. Six major molecular forms are recognised, three globular (G_1, G_2 and G_4) and three asymmetric (A_4, A_8 and A_{12}), the latter being associated with a triple-strand collagen like tail (Mesulam, 2000).

G_4 form is the most abundant form of ChEs in the healthy human brain and is central to breakdown of ACh. In contrast, G_1 form present in smaller amounts in the healthy human brain and plays a relatively minor role in ACh degradation (Attack *et al.*, 1986; Arendt *et al.*, 1992).

The kinetics of BuChE further distinguish it from AChE. AChE is most efficient at low substrate concentrations (up to 0.5 mM, personal communication with Professor E. Perry) and becomes inhibited by a high level of ACh (above 2 mM, personal communication with Professor E. Perry). The mechanism of inhibition caused by the excess of ACh has been shown by Shafferman *et al.* (1992). It is related to a change in conformation of Tyr_{337} amino acid which lines the upper part of the catalytic gorge of AChE representing in part the peripheral site overlapping the substrate-inhibitor site. A change in conformation of Tyr_{337} or a mutation in a single amino acid may induce allosteric changes in the peripheral site which are responsible fir this inhibition. In the case of BuChE in which alanine replaces the tyrosine of AChE no substrate inhibition observed (Sussman *et al.*, 1991; Shafferman *et al.*, 1992).

In contrast, the enzymatic affinity constant (Km) for BuChE makes the enzyme less efficient at low concentrations, but very efficient at higher levels when AChE becomes substrate inhibited (Silver, 1974). It has been suggested (Giacobini, 2001) that BuChE may support the hydrolysis of excess ACh via relationship between glial BuChE, synaptic AChE and their differences in Km.

1.2.1.3. Cortical cholinergic receptors

Cholinergic signalling is mediated by nicotinic and muscarinic receptors. Nicotinic acetylcholine receptors (nAChRs) have been shown to be involved in cognitive

functions such as attention and memory and changes in nAChR expression are associated with brain development, aging and neurodegenerative diseases such as AD, DLB and Parkinson's disease (Jones *et al.*, 1999).

nAChRs are members of the ligand-gated ion channel family that includes γ-amino-butyric acid A ($GABA_A$), glycine and 5-HT_3 receptors. They are formed by the pentameric association of α and β subunits, of which at least 12 nAChR subunit genes (α2- α10 and β2-β4) have been identified to date in the rat and chick nervous system, while human α2- α7, α9, α10 and β2-β4 nAChR subunits have been cloned (Sargent, 1993; McGehee and Role, 1995; Lukas *et al.*, 1999; Lustig *et al.*, 2001;). Different combination of subunits generate subtypes of nAChRs with diverse functional and pharmacological properties (Chavez-Noriega *et al.*, 1997) which in vivo may have selective roles in specific brain pathways (Lena *et al.*, 1999). The main nAChRs subtypes in mammalian brain have been shown to be those containing α4β2 subunits which bind nicotine with high affinity or α-bungarotoxin-sensitive α7 subunits (Goldman *et al.*, 1986, 1987; Gotti *et al.*, 1997; Pacheco *et al.*, 2001). nAChRs act presynaptically in the hippocampus to influence the release of various neurotransmitters including glutamate (Gray *et al.*,1996), GABA (Radcliffe *et al.*, 1999) and noradrenaline (Clarke and Reube, 1996), whilst α7nAChRs and α4β2 may also have a postsynaptical role in fast synaptic transmission of the hippocampus (Frazier *et al.*, 1998).

The highest density of α4β2 and α7 receptor subunits is found in the subiculum, followed by the thalamus (Rubboli *et al.*, 1994; Jones *et al.*, 1999). Graham *et al.* (2003) reported differential distribution of nAChR subunits on various cell types within the

human hippocampus and entorhinal cortex and emphasises their potential role in neuronal modulation, astrocyte activity and vascular function.

Five subtypes of muscarinic cholinergic receptors (m1-m5), localised on both pre- and post-synaptic membrane, have been recognised, each the product of a different gene (Brann *et al.*, 1993). The receptors couple to multiple G proteins to modulate signal transduction pathways. While the m1, m2 and m4 proteins are abundant in the brain, the m3 and m5 are expressed only in small quantities (Levey, 1996). The m1-m3 receptors refer to the pharmacologically identified M1-M3 (transcribed from the m1-m3 genes) subunits (Geula and Mesulam, 1999). In the human brain high densities of muscarinic M1 and M4 cholinergic receptors occur within the striatum and, hypothalamus, intermediate to high densities in the amygdala, hippocampus, and cerebral cortex, and the lowest density in the cerebellum (Lin *et a*l., 1986; Cortes *et al.*, 1987) The M1 receptor is mainly located postsynaptically (on cholinoceptive neurons and other cells) in the cortex, hippocampus and brainstem. The M1 receptors are much more numerous than the M2 subtype and display peak densities in almost all limbic and paralimbic regions (Kasa *et al.*, 1997).

In the cerebral cortex, nicotinic and muscarinic receptors are localised predominantly on pyramidal neurons in layers III and V and a smaller population on polymorphic neurons (Geula and Mesulam, 1999). Approximately 30% of pyramidal neurons display reactivity for both muscarinic and nicotinic receptors (Schroder *et al.*, 1989; Graham *et al.*, 2003).

1.2.2. Demented brain

1.2.2.1. Alzheimer's disease

Davies and Maloney (1976), Bowen *et al.* (1976) and Perry *et al.* (1977) reported a significant loss in levels of biochemically determined ChAT activity in *post-mortem* tissue from cerebral cortex of AD. Since that time the loss of cortical ChAT activity, especially within the nucleus basalis of Meynert (nbM) and other regions of the basal forebrain complex (Whitehouse *et al.*, 1982), has become established as one of the most consistent findings in AD (Geula and Mesulam, 1999). Thus, it has been suggested (Bartus *et al.*, 1982) that the degeneration of basal forebrain neurons and the accompanying loss of cholinergic projections to the neocortex and hippocampus provide an important contribution to emerging cognitive deficits. The greatest cortical deficits in acetylcholine occur in those brain areas concerned with memory and cognition, the hippocampus and temporal, frontal and parietal cortices (Geula and Mesulam, 1996; Perry *et al.*, 1998). There is a strong correlation between cortical cholinergic deficits (especially in temporal and parietal lobe) and dementia scores (Perry *et al.*, 1978 b).

In the human cerebral cortex, ChAT is found only in pre-synaptic cholinergic axons, whereas AChE is found in cholinoceptive (ChAT-negative) neurons as well as in cholinergic axons. AChE, therefore, is a marker for both the presynaptic and the post-synaptic component of cholinergic pathways. Some studies (Davies, 1979; Zubenko, *et al.*, 1989) reported up to a 90% loss in the activity of cortical AChE in AD. This loss was restricted to the membrane-bound detergent soluble G_4 form of the enzyme, mainly in cortical and subcortical brain regions (Attack *et al.*, 1983; Fishman *et al.*, 1986; Seik *et al.*, 1990). The G_4 form of AChE is preferentially expressed at cholinergic synapses and the G_4/G_1 ratio is a marker for these sites (Seik *et al.*, 1990). It was noted (Fishman

et al., 1986) that the G_4/G_1 ratio is correlated with the activity level of the presynaptic cholinergic marker ChAT in normal human brain. The G_4 form of AChE represents more than 80% of total AChE activity transported in axons. In severe AD patients, the G_4 form is decreased in the frontal and parietal cortices by 71% and 45 % respectively and in the caudate putamen by 47% as compared with control levels (Giacobini, 2000).

In contrast, in the brains of patients with AD, the G_1 form of BuChE shows a 20% increase while the G_4 form remains the same as in non-diseased brains (Giacobini, 2000). As AD progresses, levels of the G_1 forms of AChE and BuChE in neuritic plaques and neurofibrillary tangles increase according to plaque density (Greig *et al.*, 2001). Although, the level of collagen-tailed forms is very low in normal brain, A_{12} and A_8 forms showed an increase of three- to four-fold in the cortex of AD patients reaching about 15 % of the total AChE activity (Younkin *et al.*, 1989). Thus, the cortical cholinergic depletion and the concomitant loss of axonal AChE are among the earliest and most severe markers of the neuropathology in AD. However, Mesulam, (2000) reported that the biochemical determination of AChE is a weak indicator of cortical cholinergic innervations because the enzyme is not only found in cholinergic axons but also in cholinoceptive neurons. A more accurate assessment of cortical cholinergic denervation may require the microscopic analysis of AChE-rich or ChAT-rich cortical axons (Geula and Mesulam, 1989). On a basis of this method Geula and Mesulam (1989) showed that cortical cholinergic depletion starts even before the onset of clinical dementia and that it becomes especially severe in advanced AD.

Kasa *et al.* (1997) reviewed evidence that the most affected areas of the cholinergic innervation of the diseased brain include the cortex, the entorhinal area, the hippocampus, the ventral striatum and the basal part of the forebrain, whereas other

areas are less affected. Geula and Mesulam (1996) demonstrated that the areas with the greatest loss (greater than 75%) of cholinergic innervation were all in the temporal lobe and least pronounced in primary sensory-motor areas. For instance, within the hippocampal formation, AChE-rich axons showed reduction in all sectors, whereas fiber density in the amygdala was high but relatively preserved in the central nucleus and in the entorhal cortex (Emre *et al.*, 1993), showing that the cortical cholinergic denervation in AD is selective and not part of general cholinergic deficiency. The loss of axonal AChE is also most severe in cortical structures within the temporal lobe (Mesulam, 2000) and in subcortical areas (Geula and Mesulam, 1999).

Pilleri (1966) reported a loss of nbM-Ch4 neurons in AD. Later, Whitehouse *et al.* (1982) found a 75% decrease in the number of nbM-Ch4 neurons in AD patients. Later reports have produced support for a consistent loss not only of nbM-Ch4 neurons, ranging from 30% to 95% but also, ChAT activity in the latter (30-90%) of AD patients (Geula and Mesulam, 1999). Moreover, mean mRNA expression per ChAT-positive neuron was reduced by 50% in the Ch4 in those with AD (Strada *et al.*, 1992).

Mesulam (2000) reviewed evidence that histopathological markers of AD, such the amyloid plaques, neurofibrillary tangles and vessels with amyloid angiopathy, express intense AChE and BuChE activity. The AChE in these lesions displays enzymatic properties that differ from that of normal neurons and that are similar to those of glial AChE. Furthermore, the AD-related histopathological lesions with BuChE activity tend to be more abundant in cortical layers which display higher densities of BuChE-positive glia. The AD-related ChEs therefore seem to have a glial origin with a possible role in development of the disease.

Geula and Mesulam (1999) also reviewed evidence that neuronal loss occurs in practically all the basal forebrain cholinergic cell groups and in all nbM-Ch4 subsectors. The greater reduction of nbM-Ch4 neurons is consistent with the greater loss of ChAT activity within at least the temporal cortex. In addition, Doucette and Ball (1987) showed that nbM-Ch4 neuronal loss in AD has no left-right asymmetry.

Arendt *et al.* (1984; 1985) reported correlation between the number of neuritic plaques in five neocortical areas and loss of neurons in subsectors of the nbM, which give rise to the cholinergic innervation of the affected cortical areas. It was suggested that the degeneration of cortical cholinergic afferents from the neurons of the nbM is an important feature in the pathogenesis of neuritic plaques. A significant relationship has been reported between cortical plaques and nbM-Ch4 neuronal loss (Mann *et al.*, 1984; Rasool *et al.*, 1986). Many of the reaming nbM-Ch4 neurons have been shown to contain neurofibrillary tangles as well as moderate density of amyloid plaques (Arendt *et al.*, 1988). However, the relationship between plaques and tangles within the nbM and the loss of Ch4 neurons needs further investigation (Geula and Mesulam, 1999). In contrast the brainstem Ch5 and Ch6 cholinergic neurons, major innervation for the thalamus and minor for the cerebral cortex, show almost no neuronal loss in AD but presence of neurofibrillary tangles (Geula and Mesulam, 1999).

Greig *et al.* (2001) also reviewed a role of cholinesterases in AD. In advanced AD AChE activity may be reduced to 55-67% of normal levels in certain brain areas, while BuChE activity increases (Perry *et al.*, 1978 a). Levels of AChE and BuChE G_1 forms have been positively correlated with plaque density and pathogenicity (Arendt *et al.*, 1992). Both enzymes may also contribute to beta-amyloid peptide (Aβ) aggregation in plaques formation, as they accumulate within the amyloid plaques and are found in

neurofibrillary tangles in AD (Perry *et al.*, 1978 a; Inesterosa *et al.*, 1996). Increasing levels of BuChE are also associated with the development of cortical and neocortical neuritic plaques (Greig *et al.*, 2001).

Geula *et al.* (1998) showed in an *in vivo* study that fibrillar Aβ is toxic to neurons and can induce phosphorylation of tau characteristic of neurofibrillary tangles. Injections of the fibrillar form of Aβ have been shown to cause the degeneration of basal forebrain cholinergic neurons (Harkany *et al.*, 1995) and a decrease in ACh release (Abe *et al.*, 1994). The soluble form of Aβ, which is produced by cells under normal conditions (Geula and Mesulam, 1999), decreased ACh synthesis in a cell line derived from cholinergic neurons of the basal forebrain (Pederson *et al.*, 1996), together with ACh release (Kar *et al.*, 1996) and transduction through the muscarinic receptor (Auld *et al.*, 1998; Geula and Mesulam, 1999).

Perry *et al.* (2001) reviewed evidence that reduction of nAChRs, α4 subunit expression in the cortex and α7 subunit in the thalamus and in some cases in hippocampus, occurs in areas with the pathological changes associated with AD. The α3 and α4 subunits has been shown to be reduced in the hippocampus (Guan *et al.*, 2000) and either reduced (Guan *et al.*, 2000) or unaltered in the cortex (Martin-Ruiz *et al.*, 1999) in AD. Therefore, the receptors are selectively involved in the symptoms and pathological changes associated with the disease. Perry *et al.* (2001) also noted that dementia rating has been positively correlated with loss of high affinity nicotinic agonist binding in the cerebral cortex. The decline in nAChRs in Alzheimer's disease may be a consequence of synaptic or dendritic loss (or loss of both), rather than of neuronal loss, from the neocortex.

Moreover, there are several different interactions between Aβ peptides and nAChRs, especially with the α7 subtype (Auld *et al.*, 2002). These interactions may be involved in the disruption of normal cholinergic neurotransmission and the possible enhancement of sensitivity to Aβ neurotoxicity in AD. Wang *et al.* (2000) reported an interaction between Aβ and α7 nicotinic receptors and their precipitation in human brain tissue, particularly in the AD brain. Especially, $A\beta_{1-42}$ and α7 nAChRs were co-localised on cortical neurons and neurotic plaques. *In vitro*, the formation of $A\beta_{1-42}$/α7 nicotinic receptor complexes was inhibited by the $A\beta_{12-28}$ fragment, suggesting that this sequence competes for the binding site and is therefore likely to contain the active site (Wang *et al.*, 2000). Thus, as with the effects of Aβ on ACh release (Kar *et al.*, 1996) and synthesis (Pedersen *et al.*, 1996), this sequence of the peptide may be important for its effects on the cholinergic system.

Pettit *et al.* (2001) provided evidence of a novel physiological role for $A\beta_{1-42}$ as an inhibitor of postsynaptic nAChRs in rat hippocampal inter-neurons. In this study $A\beta_{1-42}$ blocked nAChR-mediated current and reduced open channel probability. The authors suggested that chronic inhibition of cholinergic signaling by $A\beta_{1-42}$ could contribute to the cognitive deficits and loss of cholinergic function associated with AD.

Kasa *et al.* (1997) also reviewed evidence that the M1 receptor, locating post-synaptically on cholinergic neurons, binding parameters were generally unaltered in brain tissue samples from AD patients, whereas the M2 pre-synaptic receptor density in the brain tissues of the diseased patients is significantly decreased in numerous areas of the brain, including cortical areas and the hippocampal formation. The authors also noted that the densities of muscarinic receptors, and proportions of the M1 and M2 subtypes, were not significantly different in sections of hippocampal tissue samples

between AD and age-matched, non-demented controls. However, most studies report robust reductions in G-protein signaling and/or phosphoinositide generation after muscarinic receptor stimulation (e.g. with carbachol) in AD brain tissue (Jope, 1996). Rodrigues-Puertas *et al.* (1997) found that M1 and M3 receptors are reduced in severe AD in entorhinal cortex and hippocampus, M1 and total mAChRs reduced in visual and frontal cortex, with unchanged M3 and M4 receptors.

Nitsch *et al.* (1992) suggested that cholinergic neurotransmission may also influence the processing of the amyloid precursor protein (APP) through activation of muscarinic acetylcholine receptors. They showed *in vitro* that such stimulation with carbachol increased the basal release of APP derivatives through the α-secretase pathway. Geula and Mesulam (1999) raised the possibility that AD may be associated with a cycle wherein the cholinergic depletion promotes the production of Aβ, which in turn, further reduces the effectiveness of cholinergic neurotransmission.

1.2.2.2. Dementia with Lewy bodies

Oh, call it by some better name. -Thomas Moore

In DLB cortical cholinergic neurotransmission is compromised to a greater extent than in AD and this is thought to contribute to cognitive decline and the neuropsychiatric symptoms experienced by these patients (Perry *et al.*, 1978 b; Perry *et al.*, 1990). Neuropathological studies have found that up to 60% of the patients with AD can have Lewy bodies in the neocortex and brainstem (Lopez, 2003). Nevertheless, the losses of ChAT in DLB occur in temporal and parietal neocortex and some thalamic niclei (Perry *et al.*, 1998; Ballard *et al.*, 2000). ChAT is also moderatly reduced in striatum (Piggott and Marshal, 1996). Perry *et al.* (1993) also found that hallucinations in DLB are

associated with marked cortical cholinergic deficit. Liberini *et al.* (1996) reviewed evidence that the neuronal damage in the nbM of patients with DLB is greater than that of AD cases. Moreover, an extensive cholinergic deficit in frontal, parietal and temporal cortices, with reduction in ChAT activity is greater than those in AD. Minoshima *et al.*, (2001) reported significant metabolic reductions in the occipital cortex, particularly in the primary visual cortex, which distinguished DLB versus AD with 90% sensitivity and 80% specificity.

M1 receptors are elevated in temporal and parietal cortex (Ballard *et al.*, 2000). Piggott *et al.*, (2003) reviewed reports suggesting that in the cerebral cortex post-synaptic target neurons are undamaged in DLB, with severe degeneration of presynaptic cholinergic projections provoking upregulation of M1 receptors in cholinoceptive neurons in temporal and parietal cortex. Piggott *et al.* (2003) also reported significant reduction of M1 receptors in striatum. Using epibatidine binding assay, Martin-Ruiz *et al.*, (2000) found a selective loss of cortical nicotinic receptor α4 subunit but no change in α7 subunits in DLB.

DLB can also be suspected clinically on the basis of the early occurrence of a cognitive decline resembling a chronic confusional state with fluctuating cognitive sings and visual and/or auditive hallucinations in a patient with mild Parkinsonism (Dubois, 2003). The main difference between DLB and AD is the increased persistence of visual hallucinations in DLB (Ballard, 2003). The rapidly progressive dementia is accompanied by aphasis, dyspraxia, spatial disorientation and severe visuospatial disorders suggestive of temporoparietal dysfunction. The neuropsychological profile differs from that of patients with AD: cognitive deficits are more acute, attentional

fluctuations more intense, and psychotic features more precocious. Moreover, patients with DLB present less severe memory deficits than those with AD (Dubois, 2003).

1.2.2.3. Vascular dementia

Mesulam *et al.* (2003) reported that subcortical ischemic lesions can cause cortical cholinergic denervation in the absence of primary nucleus basalis or neocortical pathology. The greatest cholinergic loss occurred in the posterior parietal, dorsal frontal, and occipital cortices. Even in the areas there were some residual AChE-rich fibers. The hippocampus, entorhinal cortex, and much of the temporal neocortex displayed a relatively normal density of cholinergic axons. This pattern is the exact opposite of the AD pattern where the temporal lobe displays the greatest cholinergic denervation. The authors concluded that the physiologic impact of cholinergic denervation is therefore likely to differ in VaD and AD.

Grantham and Geerts (2002) reviewed evidence that *in post-mortem* studies patients with VaD have decreased brain ChAT activity in the cortex, hippocampus and striatum. Levels of ChAT are typically reduced when nAChR numbers are decreased leading to a general degeneration of cholinergic neurotransmission. Moreover, ACh concentrations in cerebral spinal fluid (CSF) correlated with the severity of the dementia, as measured using the Mini-Mental State Evaluation and the Hasegawa revised version of the Dementia Scale.

In contrast to DLB, Martin-Ruiz *et al.* (2000) found no reduction in epibatidine binding or immunoreactivity of cortical nicotinic receptor α4 and α7 subunits in VaD. Nordberg

et al. (1992) reported that M1 and M2 receptors were decreased in multi-infarct dementia, while marked loss of nicotinic receptors was observed in cortical tissue.

1.2.2.4. Mild cognitive impairment

Volumetric magnetic resonance imaging has shown (Du *et al.*, 2000) that the entorhinal cortex and hippocampal volumes are reduced in mild cognitive impairment, and that there is loss of entorhinal cortex neurones (Kordower *et al.*, 2001). In PET studies, reduction of glucose metabolism in the hippocampal formation has been seen without any significant neocortical change (De Santi *et al.*, 2001). Imaging results therefore indicate that at a structural and metabolic level the first pathological changes develop in the entorhinal and hippocampal areas. Mufson *et al.* (1999) found *in-vitro* that cholinergic nbM neurons are relatively preserved and the remaining neurons are not hyperinnervated by galaninergic fibers in subjects with MCI and mild AD. Padovani *et al.* (2002) demonstrated that alteration in APP metabolism is an early event in sporadic AD and that most patients with MIC have undergone such changes. The APP changes may predate the onset of clinical dementia and be detected when cognitive impairment is barely measurable. In addition, Rinne *et a*l. (2003) reported *in-vivo* that hippocampal acetylcholinesterase activity is only slightly reduced in MCI and early AD. There is also a report (O'Brien *et al.*, 2003) that BuChE did not affect attentional performance in non-demented individuals with mild cognitive impairment.

1.3. Current and prospective treatment of dementia

1.3.1. Cholinergic theraputical agents

The cholinergic theory of AD, originally derived from *post-mortem* studies of the brain (Davies and Maloney, 1976; Bowen *et al.*, 1977; Perry *et al.*, 1977; Whitehouse *et al.*,

1982; Perry 1986), led to the development of licensed drugs based on the inhibition of AChE (Thomsen and Kewitz., 1990; Enz *et al.*, 1991; Sugimoto *et al.*, 1995). Treatment with such drugs results in a significant improvement in cognitive function (Farlow *et al.*, 1992; Knapp *et al.*, 1994; Wilkinson, 1997; Rogers *et al.*, 1998; Rosler *et al.*, 1999; Raskind *et al.*, 2000) and may also retard progression of the disease (Farlow, 2002; Ott and Lapane, 2002) by prolonging the action of ACh at the post-synaptic receptors.

Numerous cholinesterase inhibitors have undergone development since Perry *et al.* (1978 b) reported that the severity of a cholinergic deficit in AD correlates with the degree of cognitive decline. Because of the severe cortical cholinergic denervation in DLB (1.2.2.2.), VaD or mixed AD and VaD (1.2.2.3.) concomitant with cognitive decline, cholinergic therapy may be relevant to these disorders (Giacobini, 2000; Fisher and Bowler, 2003). Moreover, individuals with MCI, who are at risk of AD, may also represent a suitable target for the therapy (Giacobini, 2000). Patients who develop AD start with normal cognitive function and slowly progress to a stage of MCI. In addition, Fisher and Bowler (2003) noted that for symptomatic treatment of dementia, a precise diagnosis is not essential and that individual responsiveness should determine whether treatment continues or not.

To date, AChE inhibitors approved by the U.S. Food and Drug Administration (FDA) for the symptomatic treatment of patients with AD include tacrine, donepezil, rivastigmine and galanthamine. There are no FDA-approved treatments for DLB and VaD (Giacobini, 2000).

1.3.1.1. Licensed drugs

1.3.1.1.1. Tacrine (Cognes®)

Tacrine (Figure 1.9.) (Shaw and Bentley, 1953) was the first marketed acetylcholinesterase inhibitor approved by FDA in 1993 (Thal, 1999). Harel *et al.* (1993) demonstrated that the three-ring structure of tacrine is stacked against the indole tryptophan (Trp84) at the aromatic gorge and that the N-methylacridinium forms a charge-transfer complex with a tryptophan in the active site of AChE. They also reported that in tacrine/AChE complex, the only residue undergoing significant conformational change is phenylalanine (Phe_{330}). Tacrine thus binds between the rings of Phe330 and Trp84. The authors highlighted the important role of aromatic groups of the enzyme as binding sites for quaternary ligands.

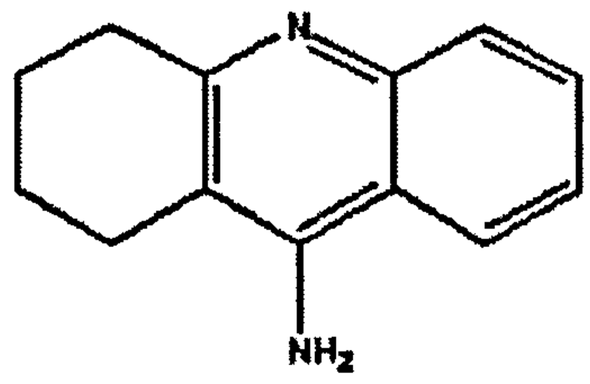


Figure 1.9. Tacrine ($C_{13}H_{14}N_2$). Synonyms: 9-Amino-1,2,3,4-tetrahydroacridine; 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate; Cognex; THA

Tacrine preferentially inhibits AChE in the hippocampus and cortex (Enz *et al.*, 1993). Moreover, Zhao and Tang (2002) reported that tacrine also preferentially inhibits the G_1 human form of AChE of hippocampal, striatum and cortical origins, exhibiting significant differences in Ki values between G_4 and G_1 forms. The G_1 form is predominant in the brain of patients with AD. Tacrine is a non-competitive type of inhibitor not only with the enzyme but also the G_1 and G_4 forms. In addition, Pacheco *et*

al. (1995) showed that tacrine readily inhibits both AChE and BuChE in a mixed, non-competitive way. Giacobini (2000) also summarised evidence confirming that tacrine is a non-selective inhibitor of cholinesterases.

Increased binding of [^{11}C]-nicotine has been observed in the temporal cortex of AD patients treated with tacrine, 80 mg/day for 3 months (Nordberg, 2000). Nordberg (2000) also suggested that the restoration of cortical nicotinic receptors, following tacrine treatment, may be due to a stimulatory effect of the inhibitor on these receptors via an allosteric site which is separately located from ACh binding site. Nevertheless, Prince *et al.* (2002) reported on an *in vitro* study of tacrine on nAChRs expressed in human adult cells. They found that the mean channel open time decreased with increasing tacrine concentration in a voltage-dependent manner, suggesting that tacrine acts as an open channel blocker.

Herrera *et al.* (2001) reported that tacrine acts as an antagonist on rat intestinal smooth muscle and it also antagonist to M1 receptor (Snape *et al.*, 1999). Giacobini (2000) also noted that 3 months treatment with tacrine in those with AD may cause down-regulation of the muscarinic receptors in the temporal cortex, which may be normalised after 10 months of the treatment. Snape *et al.* (1999) found using *in vivo* microdialysis in cerebral cortex of rats that orally administrated tacrine produce a marked (at least 30-fold) increase in extracellular acetylcholine which remained elevated for more than 2 hours.

In addition, Perry *et al.* (1994) reported that two of the Lewy body cases with extremely low cholinergic activity were responders in therapeutic trials of tacrine, whereas Lebert *et al.* (1998) also showed that tacrine increased cognitive performances (verbal initiation

and digit span) in 22 patients with DLB out of 31. These data suggest that cholinergic therapy may be particularly relevant to patients with LDB. Although, tacrine was marketed in 1993, it never achieved widespread use in AD patients because of hepatotoxicity. In the pivotal trials, almost half the subjects discontinued therapy because of severe side-effects (Thal, 1999).

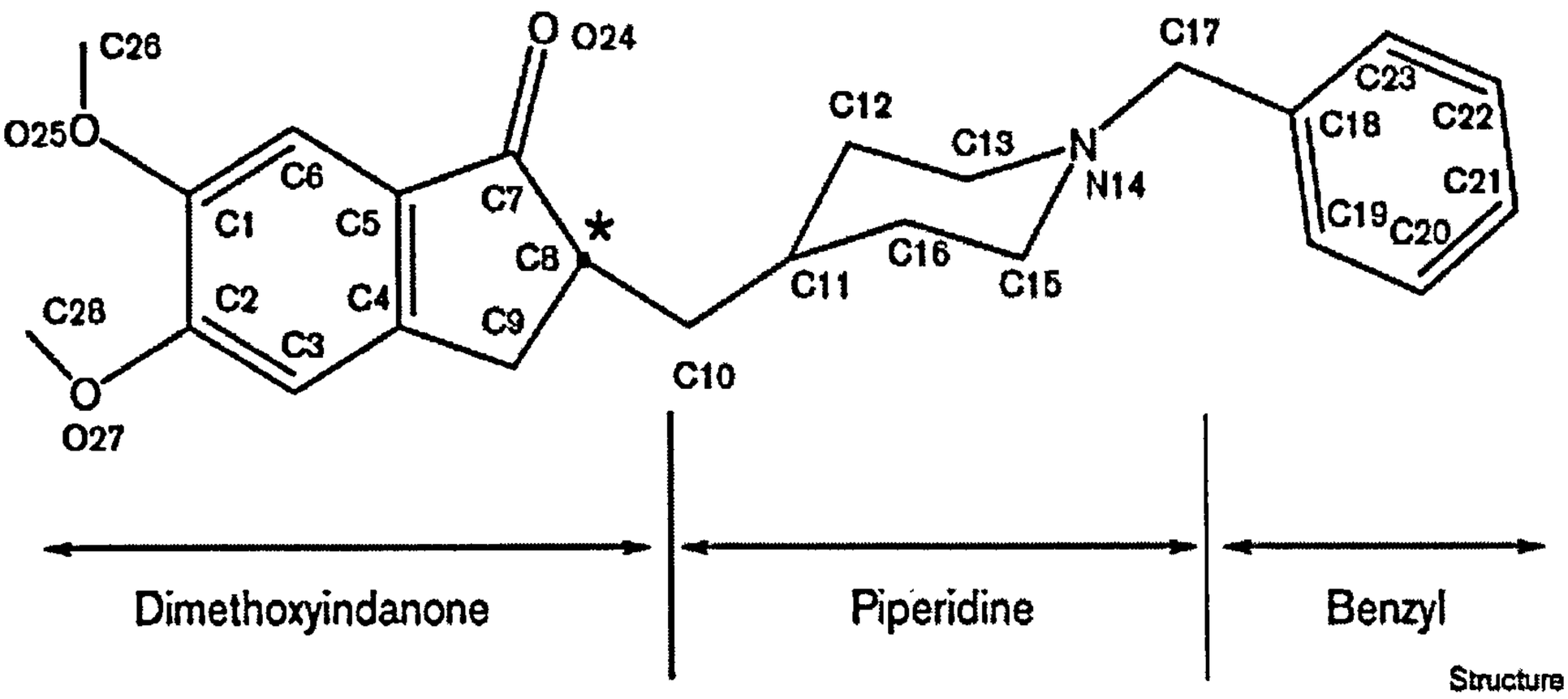
1.3.1.1.2. Donepezil (Aricept®)

In 1996 donepezil (Figure 1.10.) received FDA approval for marketing (Nightingale, 1997). Donepezil (E2020) is a member of a large family of *N*-benzylpiperidine-based AChE inhibitors that were developed, synthesized and evaluated by Eisai in Japan (Kawakami *et al.*, 1996).

Kryger *et al.* (1999) reported that the inhibitor has a unique orientation along the active-site gorge, extending from the anionic sub-site of the active site, at the bottom near Trp_{84}, to the peripheral anionic site, at the top near Trp_{279}. E 2020 does not interact with the catalytic triad (glutamate-histidine-serine or Glu-His-Ser) at the bottom of the gorge but binds to the free and the acylated forms of AChE. In the middle of the gorge, Phe_{330} may serve as an additional quaternary binding site, midway down the gorge, between the peripheral site and the anionic sub-site of the active site. At the top of the gorge the indanone ring stacks against the indole ring of Trp_{279}, in the peripheral binding site, by a classical π–π interaction.

E2020 is a drug with both high affinity and a high degree of selectivity for AChE, as opposed to butyrylcholinesterase (Snape *et al.*, 1999), with the BuChE/AChE ratio of inhibition is around 1000 (Giacobini, 2000). The high affinity and selectivity results

from a fact that Phe330 and Trp279 residues are conserved in AChE, but absent in BuChE.



Structure

Figure 1.10. Donepezil($C_{24}H_{29}NO_3$). Synonyms: E2020, Aricept, (R,S)-1-benzyl-4-[(5,6-dimethoxy-1-indanon) 2-yl] methyl piperidine. *C8 is a chiral carbon

Zhao and Tang (2002) reported that E2020 is more selective for G_1 human form of AChE in striatum and hippocampus than G_4 form. In contrast, in cortex both forms were inhibited to similar degree, namely Ki values for G_1 and G_4 forms were $3.5\pm1.2\times10^{-9}$ M and $4.0\pm1.5\times10^{-9}$ M respectively. In addition, E2020, as tacrine, shows non-competitive type of inhibition not only with the enzyme but its both forms, indicating that it is down to the inhibitor to determine the nature of inhibition, not the molecular form of the enzyme or its tissue source.

Donepezil readily penetrates the blood brain barrier (BBB) in rats (Kosasa *et al.*, 2000) and has non-competitive, reversible type of inhibition of AChE (Snape *et al.*, 1999). Donepezil, like tacrine, is antagonist to M1 receptor (Snape *et al.*, 1999) with binding activity at nicotinic sites in both the hippocampus and prefrontal cortex of aged rats (Barnes *et al.*, 2000). Moreover, Takada *et al.*, (2003) showed that the drug protects

cortical neurons against glutamate neurotoxicity via α4β2- and α7-nAChRs and also protects apoptotic neuronal death.

The cholinergic deficits seen in VaD are less pronounced and of a different pattern than those of AD (1.2.2.3.). There is, as yet, no convincing evidence that acetylcholinesterase inhibitors have any significant extent in "pure" VaD. A trial of donepezil (Pratt and Perdono, 2002) did show a benefit in VaD, but this study used the NINDS-AIREN criteria and that the study population probably included many patients with mixed dementia

Lopez-Pousa and Lombardia (1999) also reported that donepezil is a specific, reversible AChE inhibitor of close to 100% absorption and a half-life of 70 hours, achieving stable concentrations at approximately 3 weeks. AD patients treated with single daily doses of 5 or 10 mg improve in the ADAS-Cog scale. The medication is initiated with a dose of 5 mg per day. The drug is administrated once daily and appears to be well tolerated, with gastrointestinal side-effects occurring in fewer than 20% of subjects placed on the 10 mg dose (Thal, 1999). Giacobini (2000) also summarised side-effects caused by donepezil treatment. The overall percentage of side-effects was 6-13%, compare with 40-58% for tacrine. Hepatotoxicity is virtually non-existent with the drug. Longer half-life (70 hours) and once-a-day dosing schedule have enabled donepezil to quickly surpass tacrine in clinical use. This drug has received widespread acceptance in the U.S for AD patients (Thal, 1999).

1.3.1.1.3. Rivastigmine (Exelon ®)

Rivastigmine (Figure 1.11.) is a member of the carbamate class of AChEIs and was approved by the FDA in 2000 (Bar-On *et al.*, 2002). Bar-On *et al.* (2002) reported kinetic and structural studies on interaction of ChEs with rivastigmine. They found that the carbamyl moiety of the inhibitor is covalently linked to the active site serine (Ser_{200}), with the leaving group, (-)-*S*-3-[1-(dimethylamino)ethyl]phenol (NAP), being retained in the "anionic" site without causing any conformational changes.

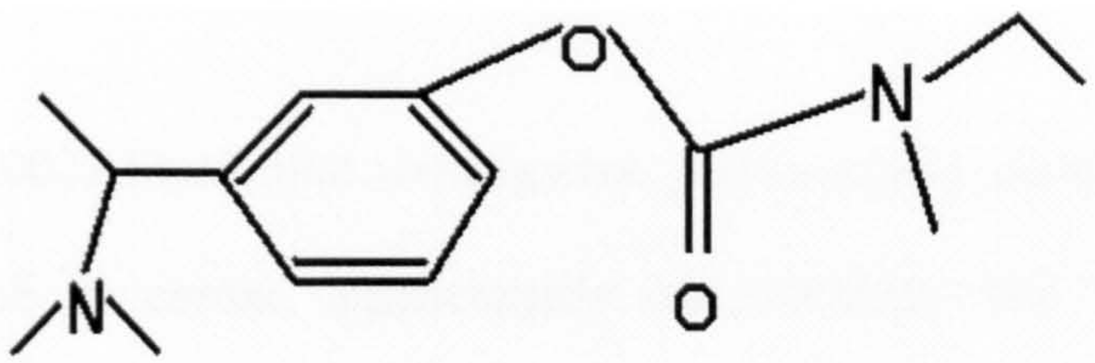


Figure 1.11. Rivastigmine ($C_{14}H_{22}N_2O_2$). Synonyms: ENA-713, Exelon, (-)-S-N-ethyl-3-[(1-dimethyl-amino) ethyl]-*N*-methylphenylcarbamate hydrogen tartrate

Reaction with rivastigmine resulted in disruption of the catalytic triad not only within *Torpedo californica* AChE (*Tc*AChE) but also recombinant human AChE. Bar-On *et al.* (2002) suggested that this is due to NAP causing a disturbance of His_{440} away from its catalytic triad. Alternatively, the disturbance of His_{440} may occur by NAP orienting the transition state such that the *N*-ethylmethyl group crowds the histidine. NAP is a competitive inhibitor of AChE but when administrated to rats, no inhibition of brain AChE is detected, probably due to poor BBB permeability and rapid clearance.

Moreover, Bar-On *et al.* (2002) found that rivastigmine inhibits recombinant human AChE much faster than *Tc*AChE in a slow reversible manner. It was also a more potent inhibitor of human BuChE than AChE. The last result contradicts to other reports (Enz

et al., 1991; Giacobini, 2000), where rivastigmine had a slight predominance for AChE. Bar-On and colleagues (2002) noted that possible differences in molecular structure (e.g., degrees of oligomerization and glycosylation) between CSF AChE and recombinant enzymes may be responsible for these differences. Nevertheless, from all these reports (Enz *et al.*, 1991; Giacobini, 2000; Bar-On *et al.*, 2002) it is apparent that rivastigmine is a non-selective inhibitor of ChEs and has a dual mode of action. Bar-On and colleagues (2002) also cautioned that the origin of ChEs may affect the kinetic results.

Zhao and Tang (2002) found that rivastigmine preferentially inhibits the G_1 form of human brain AChE in cortex, hippocampus and striatum. The G_4 form was also inhibited in these brain areas but to a lesser degree. The level of G_1 form in AD does not decline in the cerebral cortex and it found to be present in plaques and tangles (Greig, *et al.*, 2001).

Lopez-Pousa and Lombardia (1999) also found that rivastigmine is a competitive, slowly reversible inhibitor of AChE with a half-life of 2 hours and the total of approximately 10 hours. They also found that AD patients treated with two daily doses of 6 or 12 mg improve in the ADAS-Cog scale. Cutler *et al.* (1998) demonstrated *in-vivo* that administration of rivastigmine is associated with significant inhibition of AChE and BuChE in the CSF and plasma of patients with AD. Moreover, Giacobini *et al.*, (2002) reported *in-vivo* strong and consistent association between the inhibition of BuChE in CSF by the drug and improved cognitive performance in subjects with mild to moderate stages of AD. Inhibition of BuChE in CSF in healthy young volunteers was less apparent.

Rosler *et al.* (1999) reported a clinical trial in 725 patients with mild to moderately severe probable AD, which was conducted at 45 centres in Europe and in North America for 26 weeks. Data from the trial showed that rivastigmine is a well tolerated and effective drug in the treatment of the disease. At doses of 6-12 mg pre day it improves cognition, participation in activities of daily living, and global evaluation ratings in patients with AD. In another trial, Farlow *et al.* (2000) reported the efficacy of rivastigmine in patients with mild to moderately severe AD in an open-label extension of a 26-week, double-blind, placebo-controlled study. By 52 weeks, patients originally treated with 6-12 mg per day rivastigmine had significantly better cognitive function than patients originally treated with placebo.

In the largest placebo controlled trial (McKeith *et al.*, 2000) of rivastigmine (12 mg per day) in 120 DLB patients over 20 weeks, followed by a 3-week withdrawal period, patients taking the drug were significantly less apathetic and anxious, and had fewer delusions and hallucinations compared to placebo controls. It was concluded that rivastigmine produces statistically and clinically significant behavioural effects in patients with DLB, and seems safe and well tolerated if titrated individually. It was suggested that cholinesterase inhibitors could be a more rational choice of treatment for Lewy-body dementia patients than neuroleptics, both on an efficacy and safety basis.

1.3.1.1.4. Galanthamine (Reminyl ®)

Galanthamine (Figure 1.12.) is a naturally occurring alkaloid in plant species of *Galanthus and Narcissus* (Azoeva *et al.*, 1968; Hanks 2002). It is the most recently (February, 2001) FDA approved drug for the treatment of AD (Alzheimer Research Forum). Greenblatt *et al.* (1999) reported that the inhibitor binds at the base of the

active site gorge of *Tc*AChE, interacting with both the choline-binding site (Trp_{84}) and the acyl-binding pocket (Phe_{288}, Phe_{290}). The tertiary amine group of galanthamine does not interact closely with Trp_{84}; rather, the double bond of its cyclohexene ring stacks against the indole ring. The tertiary amine appears to make a non-conventional hydrogen bond, via its N-methyl group, to Asp_{72}, near the top of the gorge. The hydroxyl group of the inhibitor makes a strong hydrogen bond (2.7 Å) with Glu_{199}. The binding of galanthamine to *Tc*AChE is tight due the rigid chemical structure of the inhibitor.

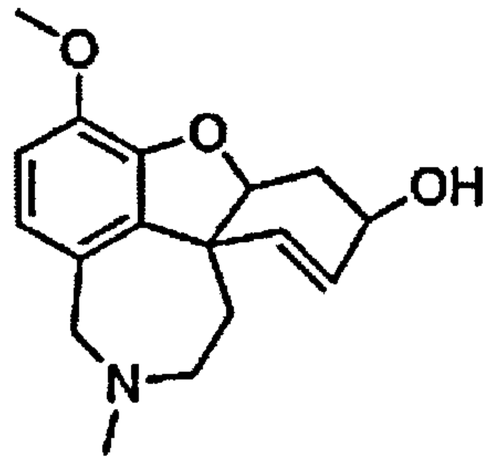


Figure 1.12. (-)-Galanthamine ($C_{17}H_{21}NO_3$). Synonyms: Reminyl, (4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro[3a,3,2-ef][2]-benzazepin-6-ol

Galanthamine, like donepezil, is not selective for any cholinesterase iso-forms (Inglis, 2002). The drug displays 53-fold selectivity for human AChE over BuChE, with IC_{50} values of 0.35 μM for erythrocyte AChE and 18.6 mM for plasma BuChE (Thomsen and Kewitz, 1990). Interestingly, it displays 10-fold lower potency towards human brain AChE than towards the erythrocyte enzyme (Thomsen *et al.*, 1991). Giacobini (2000) also showed that galanthamine is more selective for human erythrocyte AChE than for human plasma BuChE, with BuChE/AChE ratio of 5.3.

Moreover, galanthamine is a non-competitive, allosteric ligand for nAChRs (Pereira *et al.*, 1993; Storch *et al.*, 1995; Schrattenholz *et al.*, 1996). Samochocki *et al.* (2003) also reported that galanthamine is a potent allosteric ligand of human α3β4, α4β2, and α6β4

nicotinic receptors in a range of concentrations from 0.1μM to 1 μM. This range of concentration correlates with the cerebrospinal fluid concentration of the drug at the recommended daily dosage of 16 to 24 mg. At concentrations more than 10 μM, galantamine inhibits nAChRs. Samochocki *et al.*, (2003) also found that galantamine at concentrations up to 100 μM was inactivate on human M1, M2, M3, M4, and M5 mAChRs. In addition, Wevers *et al.* (1999) provided evidence suggesting that stimulation of nicotinic receptors may help to reduce amyloid plaques in the demented brain.

Scott and Goa (2000) and Corey-Bloom (2003) reviewed evidence that galantamine is an effective well tolerated symptomatic treatment for AD which improves cognition, function and activities of daily living in patients with mild to moderate AD. The cognition benefits are sustained for at least 12 months at baseline levels and apparent versus no treatment for at least 24 months. In addition, it delays the development of behavioural disturbances and psychiatric symptoms, and reduces caregiver burden (as measured by caregiver time). Side-effects associated with galantamine are mainly cholinergic, usually mild to moderate in intensity and transient. Galantamine is being considered as one of the first-line pharmacological treatments in patients with mild to moderate AD (Corey-Bloom, 2003).

Furthermore, Erkinjuntti *et al.* (2002) reported a randomised, double-blind, placebo controlled 6-month trial where 24 mg per day galanthamine was assigned to individuals with probable VaD and AD combined with cerebrovascular disease (CVD). They found that the drug significantly improves cognition, activities of daily living, behaviour, and global function in these individuals. Regarding Erkinjuntti *et al.* (2002) study, Fisher and Bowler (2003) noted that mixed dementia-*i.e.*, the coexistence of CVD with AD- is

now well recognised form of dementia. Both the NINDS-AIREN criteria for possible VaD and NINCDS-ADRDA criteria for possible AD were prepared before the mixed dementia was widely recognised. The authors suggested that the trial probably does not verify the true demented state of the individuals and in reality the theraputic effect of galanthamine may be attributed to mixed dementia They also emphasised the importance of early identification of cases with vascular characteristics in cognitive decline, as these individuals are candidates for preventative therapy rather than just symptomatic treatment. They conclude that for symptomatic treatment, a precise diagnosis is not essential and that is individual responsiveness should determine whether treatment continues or not.

1.3.1.2. Unlicensed plant derived drugs

The prototypical inhibitor of AChE physostigmine (Figure 1.13.) (Holmstedt, 2000), together with huperzine A (Figure 1.14.) are naturally occurring chemicals in plant species of *Physostigma venenosum* (Jobst, 1863), *Huperzia serrata* (Liu *et al.*, 1986) respectively.

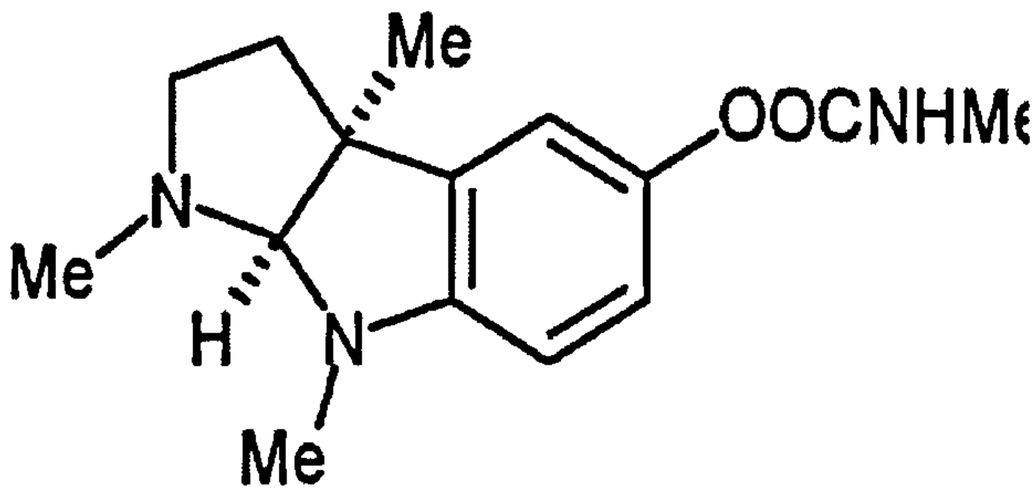


Figure 1.13. Physostigmine

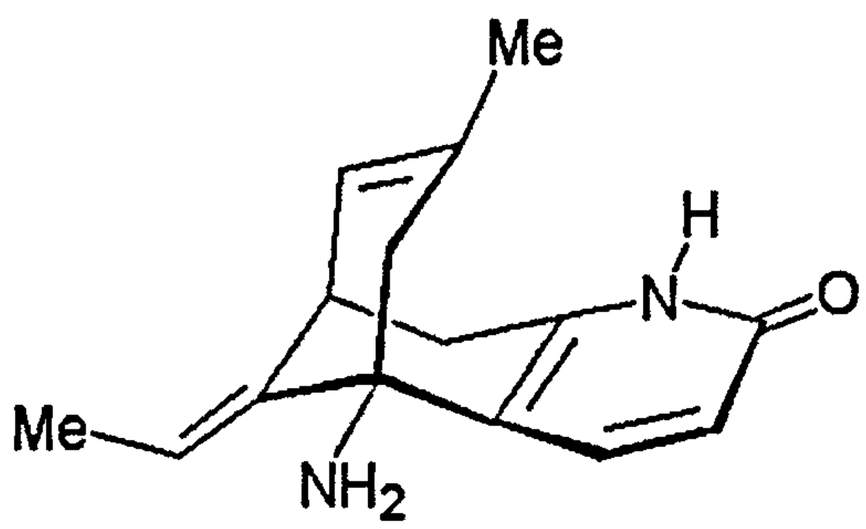


Figure 1.14. Huperzine-A

Severe adverse effects observed in the clinical trials of physostigmine (Brufani and Filocamo, 2000) led for the development of its synthetic or semi-synthetic analogues for the possible treatment of AD, *i.e.*, MF201 and MF286 (Perola *et al.*, 1997), phenserine (Al-Jafari *et al.*, 1998) and tolserine (Kamal *et al.*, 2000). Physostigmine is a non-competitive inhibitor of AChE (Perola *et al.*, 1997) and it is more selective for human erythrocyte AChE than for human plasma BuChE, with a BuChE/AChE ratio of 6.5 (Giacobini, 2000). It shows no AChE form selectivity in any brain region (Zhao and Tang, 2002) and has less affinity for muscarinic as well as for nicotinic receptors than tacrine (Svensson and Giacobini, 2000).

Huperzine A is a non-competitive (Zhao and Tang, 2002), slow reversible inhibitor of AChE (Liu *et al.*, 1986). It inhibits mammalian AChE more than BuChE with a BuChE/AChE ratio of 908 (Giacobini, 2000). It also inhibits the G_4 form of human AChE more than the G_1 form in cortex with Ki values of $7.0\pm3.5\times10^{-9}$ M and $3.5\pm1.5\times10^{-7}$ M respectively, whereas in hippocampus and striatum this selectivity is less apparent (Zhao and Tang, 2002).

Ashani *et al.* (1994) reported that the inhibition of AChE by huperzine A occurs through association with residues located inside the active site gorge, rather than at the rim of the gorge. Tyrosine at position 337 (Y337) is essential for inhibition of recombinant human AChE by huperzine A (Ki = 26 nM). Lack of the aromatic side chain in the position homologous to Y337 explains the poor inhibitory potency of huperzine A towards human BuChE (Ki > 20,000 nM).

The synthesis and pharmacological evaluation of a series of compounds that combine the structural skeletons of (-)-huperzine A and tacrine have been reported (Camps *et al.*,

2000). Certain hybrids display more powerful anti-AChE activity than either tacrine or (-)-huperzine A. These hybrids were named huprines (Camps *et al.*, 2000), and huprine X the most powerful of the series, inhibited human AChE with an inhibition constant, K_i, of 26 pM, being 40-fold more potent than donepezil, 180-fold more potent than (-)-huperzine A, and 1200-fold more potent than tacrine (Camps *et al.*, 2000).

Sun *et al.* (1999) found that orally administrated 100 μg of huperzine A for one month enhances the memory and learning performance of adolescent students. Moreover, Zhang *et al.* (2002) reported results of a clinical trial of huperzine A on 202 patients with probable mild to moderate AD. They found that the drug is safe and effective. It significantly improves the cognition, behaviour, activity of daily life, and mood of AD patients. Mild and transient adverse events such as edema of bilateral ankles and insomnia were observed in 3% of huperzine A treated patients.

1.3.1.3. Cholinergic anti-inflammatory pathway

There is evidence (Arkiyama *et al.*, 2000; Selkoe, 2001) of a profound inflammatory processes in limbic and associated cortices in AD. The inflammatory cytopathology is thought to represent a secondary response to the early accumulation of Aβ in the brain. This innate immune response leads to the accumulation of pro-inflammatory mediators such as tumour necrosis factor α (TNF-α), interleukin 1 (IL-1), IL-6, free radicals and microglia activation. Microglia are involved in the Aβ-mediated pathogenic cascade, because they represent monocyte/macrophages-derived cells in the nervous system (Weiner and Selkoe, 2002). Clinical studies suggest that anti-inflammatory drugs may delay or slow the progression of AD (Arkiyama *et al.*, 2000).

Exposure of human macrophages to nicotine or ACh inhibits the release of pro-inflammatory cytokines TNF, IL-1 and IL-18 but not anti-inflammatory cytokine IL-10 (Tracy, 2003). Tissue macrophages produce most of the TNF that appears during an ongoing inflammatory response (Tracey, 2003). Moreover, the mechanism that regulates inflammation has been advanced by identification that the nAChR α7 subunit of macrophages is essential for cholinergic suppression of TNF (Wong *et al.*, 2003). Thus, anti-ChE agents with agonistic properties for α7 subunit may not only inhibit the release of TNF but increase a level of ACh which also suppresses the cytokine.

1.3.1.4. *Salvia*. From ethnobotany to clinical trials

1.3.1.4.1. Basis for the use of plants as medicine

The origin of plant medicine lies in the evolution and ecological interactions in which animals, plants and their ancestors have participated. Plants are sessile organisms and flight from predators is not an option (Wake, 2001). Plants that synthesise and store molecules, which are distasteful and/or toxic to animals, will have an evolutionary advantage over those, which do not. These molecules may disrupt the normal activity of animal nervous and other metabolic systems (Farnsworth, 1990; Schmeller *et al.*, 1997).

There are also reports (Gnagey *et al.*, 1987; Marcel *et al.*, 1998) that ChEs in invertebrates of *Drosophila melanogaster* and *Caenorhabditis elegans* may exist in an intermediate form, which is able to hydrolyse both acetyl and-butyryl-cholines. This form may share some characteristics with both human ChEs, *i.e.*, AChE and BuChE. Plants which use bioactive molecules intervening in herbivore biochemical pathways as enzyme inhibitors or neuroreceptor activators or blockers, may use less energy and

resources for defence than those which relay on structural defences such as lignification, production of spines and adhesive lattices (Balick, 1996; Wake, 2001).

1.3.1.4.2. Ethnobotanical outlook

Plant families throughout history supplied the fundamental *materia medica* for human cultures (Farnsworth, 1990). A plant, rejected as food because of objectionable taste or undesirable physiological effects after ingestion may have been looked for other potential uses such as in religion or medicine (Schultes and Raffauf, 1990). Such plants are likely to have entered early pharmacopoeias first and the great medicines of ancient times, such as the Solanaceous drugs, stimulants, sedatives and pain-killers such as coca and opium, and the hallucinogens found in the Cactaceae, Malpighiaceae and Convolvulaceae are all of this type (Balick, 1996). More than a hundred of these prototype drugs or unmodified compounds from them are still used in allopathic medicine (Farnsworth, 1990).

Many tribal cultures have no tradition of writing or only a small number of people in the group may be literate. Medicinal knowledge of plants, which often overlaps with ritual information, may therefore only be passed verbally to the next generation. Westernisation of the tribal cultures may lead to a decline in young people being apprenticed to the tribal healers and if the adept does not pass that knowledge further, the corpus of it is lost. Deforestation may also lead to the extinction of useful plant species forcing indigenous groups to seek alternative materials for use as medicines. Thus, plant species containing compounds of therapeutic potential may be lost before anything is known about them (Wake, 2001).

Salvia (sage) is one of the most numerous genera within the family Lamiaceae which grows in many parts of the world. *Salvia* comes from the Latin *salvare* meaning to save. Dweck (2000) summarised sayings which emphasise the value of the plant throughout the last twelve centuries, namely "why should a man die, if sage grows in his garden?", "he that would live for aye, must eat sage in May", "for a ripe old age, in May you eat sage", "sage helps the nerves and by its powerful might, Palsy is cured and fever put to flight", "sage is singularly good for the head and brain, it quickeneth the senses and memory, strengtheneth the sinews, restoreth health to those that have the palsy, and taketh away shakey trembling of the members". The plant has the reputation as one which wards off evil spirits and increase the fertility of women. It helps against snake bites and was used by country women in church not to fall asleep.

Dweck (2000) also reviewed traditional use of *Salvia* species. Thus, *S. officinalis* L. or "common sage" is said to be anti-inflammatory in small doses. Its suggested uses included those as a general treatment to enhance "head and brain" functioning, improve the memory, quicken the senses, and delay age associated cognitive decline (Perry *et al*, 1999). A tea made of either the dried or fresh leaves and drunk in small doses may reduce perspiration. It is known as a carminative, spasmolytic, antiseptic and is used for treatment of oral inflammation. The use of the plant has been recommended for varicose veins and ulcers, as well as warts on the legs. Infusion of sage has been used as a hair tonic and also in cases of alopecia, hair loss.

The Native American tribes of south–western USA and northern Mexico believe that as little as a tablespoon of the seed of *S. columbariae* Benth. known as "Golden Chia" would sustain a tribesman for a day on a laborious march. A hot water extract of *S. coccinea.* Juss. ex Murr. is used in South America for treating blood clots and

varicosities. The root of *S. miltiorrhiza* or "Danshen" which is native to northern China has been used as a tonic for at least to the first millennium B.C. to stimulate the blood circulation and regulate the menses, relieve abdominal pain, depression and insomnia. *S. plebeia* (Brown) has been used in the Indo-Chinese region to relieve toothache and also treat cholera and dysentery. The seeds of *S. plebeia* have long been used to in native medicine of Asia to treat gonorrhoea and menorrhagia. In China *S. plebeia* is known as a detoxifier, diuretic, blood cooler, haemostatic, and for the reduction of swelling. *S sclarea* L. or "Clary sage" has been used by the native Jamaicans to treat inflammation of the eyes. A decoction of the leaves boiled in coconut oil is beneficial for the stings of scorpion. The dried roots of Clary sage, crushed and powdered, are used to clear the head and ease a headache (Dweck, 2000).

S. africana-lutea L. known as "golden African sage", has been used in South Africa for cold and is said to be diaphoretic. The Nama, north Americans, use a decoction of the plant for coughs, cold and female ailment. Traditional medicine practitioners in the Western Cape Province use infusion of the leaves to treat headache, fever and digestive disorders (Watt and Breyer-Brandwijk, 1962). *S. apiana* (Jepson) or "white sage" has long been used as important ceremonial incense by many Native Americans for ritual purification and cleansing. The smoke is said to smell wonderful and has a soothing effect (Epling, 1947). A hot water extract of *S. transsylvanica* Schur ex Griseb. has been used in central parts of Europe to treat inflammation of the throat (Prokopenko and Litvinenko, 1982). An infusion of dry or fresh leaves of *S fruticosa* Mill. or "Greek sage" is used as spasmolytic, hypoglycaemic, diuretic, choleretic and also for treatment of common cold (Bellomaria *et al.*, 1992). In Ethiopia and Eritrea the fruits of *S. schimperi* Benth. are used locally for treatment of liver disorders (Tadesse and

Demissew, 1992) and *S. divinorum* is used by the Mazatec people of Oaxaca in Mexico to induce visions (Valdes, 1994).

1.3.1.4.3. CNS activities relevant to dementia

Botanical descriptions with plant images and biological activities of extracts from *Salvia* species are included in the *Salvia* database (Appendix 1). The database can also be accessed via MPRC web-site, *i.e.*, http://www.ncl.ac.uk/medplant.

As mentioned above, the classical inhibitor of AChE, physostigmine (1.3.1.2), together with huperzine-A. (1.3.1.2.) and galanthamine (1.3.1.1.4) are naturally occurring molecules in plants. The search for other plant derived inhibitors of ChEs has accelerated in view of the benefits of these drugs not only in the treatment of AD but other forms of dementia, such as dementia with Lewy bodies (Perry *et al.*, 1994), vascular dementia (Erkinjuntti *et al.*, 2002) and Down's syndrome (Kishnani *et al.*, 1999).

Howes and Houghton (2003) reviewed CNS activities of *S. miltiorrhiza* Bung. (Dan Shen) which are relevant to memory and cognition. The root of the plant may inhibit neuronal cell death by inhibition of presynaptic glutamate release. Glutamate may induce neuronal degeneration by overstimulation of NMDA receptors. Memantine, an NMDA receptor antagonist, treatment leads to functional improvement and reduces care dependence in severely demented patients. Dan Shen also inhibits nitric oxide formation, which may be involved the effects of excitatory amino acids, including their effects on brain development, learning and memory. Zhang *et al.* (1994) showed that the plant may protect neuronal cell damage in the hippocampus after brain injury. Ji *et al.* (2000)

reported that Dan Shen increases coronary blood flow and scavenges free radicals in ischemic diseases, leading to improvement of heart functions. Ji *et al.* (2000) also reported that Dan Shen is effective against stroke, which is a common feature in VaD or mixed dementia (1.1.2.3.), while Hong *et al.* (1997) showed its anti-AChE activity. Thus, the plant may have dual mode of action in the treatment of VaD, inhibition of AChE and protection against stroke.

Du *et al.* (2000) demonstrated that salvianolic acid B (Figure 1.15.), a constituent of Dan Shen root, ameliorates learning and memory impairments induced by cerebral transient ischemia in mice. Moreover, salvianolic acid B may not only prevent the β-amyloid peptide toxicity *in vitro* (Zheng and Juntian, 2000) by decreasing nitric oxide release in a dose dependent manner but also inhibit its aggregation and fibril formation (Tang and Zhang, 2001).

Tanshinone I (Figure 1.16.) and Tanshinone II (Figure 1.17) are the major lipid soluble constituents of the dried root of *Salvia miltiorrhiza* used in traditional Chinese medicine for the treatment of cerebrovascular diseases including stroke (Lam *et al.*, 2003). Lam *et al.* (2003) showed that Tanshinone II (16 mg kg^{-1}) readily penetrates the blood brain barrier in mice and reduces brain infarct volume after middle cerebral artery occlusion. The authors concluded that tanshinones may have potential for further development as neuroprotective drugs. Moreover, Liu and Wu (1999) demonstrated that 20 mg kg^{-1} of Tanshinone II improved learning memory in mice. Howes and Houghton (2003) also reviewed anti-inflammatory and anti-oxidant properties of Tanshinones which are relevant to the treatment of dementia.

Perry *et al.* (1996) reported that essential oil of *S officinalis* inhibits human brain AChE in a dose dependent manner. An IC_{50} of the oil was 0.07 mg ml^{-1}. Moreover, Akhondzadeh *et al.* (2003 a) reported a double blind, randomised and placebo-controlled clinical trail of 45% EtOH extract of *Salvia officinalis* in individuals with mild to moderate stages of AD. The study showed that 4 months administration of 60 drops of the extract a day significantly enhance cognition and may reduce agitation in those with the disease.

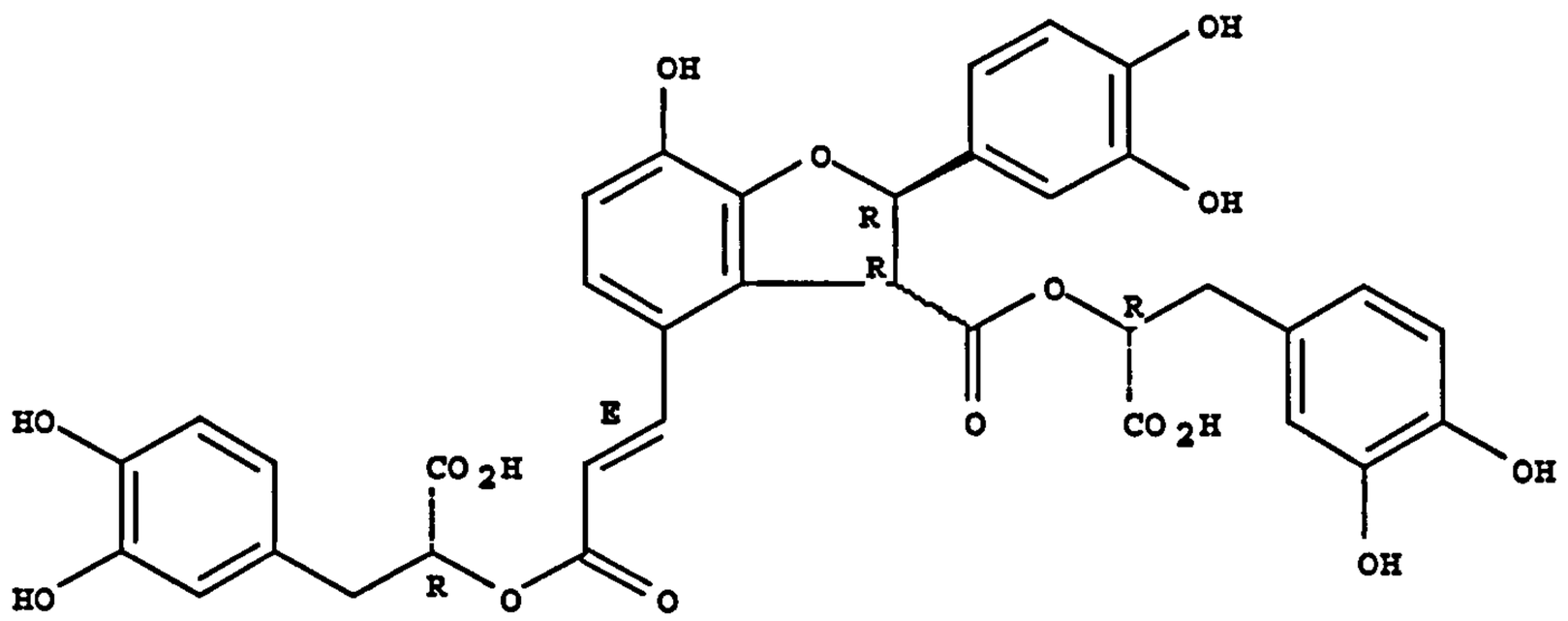


Figure 1.15. Salvianolic acid B (C36 H30 O16)

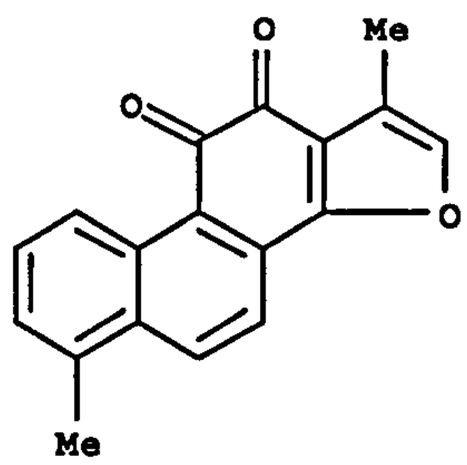


Figure 1.16. Tanshinone I (C18 H12 O3).

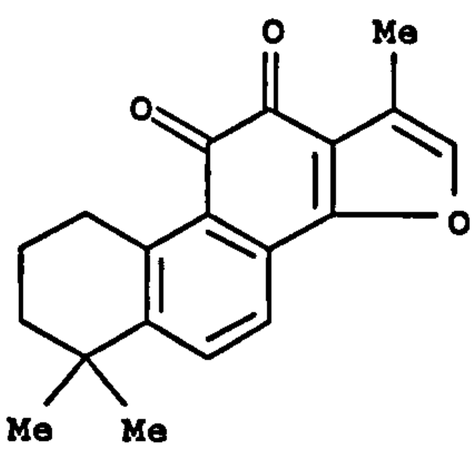


Figure 1.17. Tanshinone II (C19H18O3).

Essential oil of *S. lavandulaefolia* Vahl. (Spanish sage) showed (Perry *et al.*, 2000) to exhibit human erythrocyte anti-AChE activity with an IC_{50} value of 0.03 µl ml^{-1} (approx., 0.04 mg ml^{-1}). Perry also reported *et al.*, (2002) that *in vivo* essential oil of Spanish sage inhibits the rat brain AChE. There was a decrease in AChE activity in the

striatum and hippocampus, though not in the cortex, at doses of 20 µl and 50 µl suggesting that orally administrated constituents of the oil or their metabolites reach the brain and inhibit AChE in select areas. The inhibition of the enzyme seen in the striatum may be of relevance to AD as this part of the brain is thought to participate in emotional and motivational behaviour and AD patients experience behavioural dysfunction (Seldon *et al.*, 1994). One of the symptoms of AD is short-term memory loss and the hippocampus that is severely affected in the disease plays a major role in short term memory (1.2.2.1.). Furthermore, the improvements in mood and performance were apparent in healthy young volunteers after oral administration of 50 µl dose of Spanish sage oil (Tildesley *et al.*, 2003). Essential oil of Spanish sage lacks thujone-a toxic component (Hold *et al.*, 2000) of *S. officinalis*, hence Spanish sage may be a more appropriate species to investigate for dementia treatment. Some of the major individual constituents of essential oil *Salvia* species such as 1,8-cineole (Figure 1.18), α-pinene (Figure 1.19) were also reported (Perry *et al.*, 2000) *in-vitro* to inhibit human erythrocyte AChE with IC_{50} values of 0.67 mM and 0.63 mM respectively.

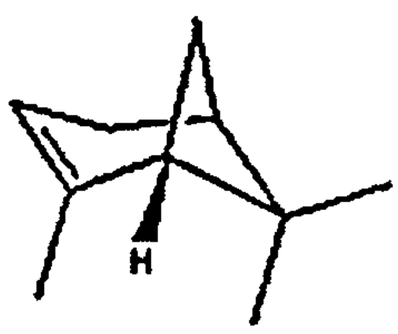


Figure 1.18. α-pinene: $C_{10}H_{16}$

(1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene)

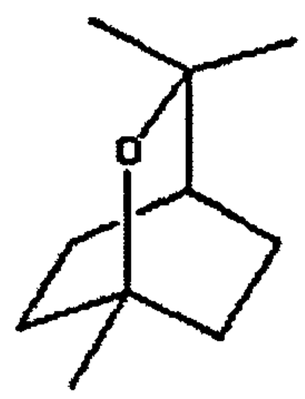


Figure 1.19. 1,8-cineole: $C_{10}H_{18}O$

(1,3,3-trimethyl-2-oxabicyclo[2.2.2.] octane)

Wake *et al.* (2000) reported nicotinic and muscarinic activities of 80% EtOH extracts of *Salvia coccinea* Juss. ex Murr., *S. verticillata* L. and *S. elegans* var "Scarlet Pineapple".

The genus also has other properties relevant to dementia therapy, namely antiinflammatory (Moretti *et al.*, 1997; Hosseinzadeh and Yavary, 1999; Baricevic *et al.*, 2001), antioxidant (Lionis *et al.*, 1998; Mantle *et al.*, 2000; Zupko *et al.*, 2001), oestrogenic (Bartram, 1995) and glutamergic activity (Kuang and Xiang, 1994). Volatile constituents of essential oils of *Salvia* species are likely to readily cross the blood brain barrier (Perry *et al.*, 2002) due to their small molecular size and lipophilicity. Their volatile nature may also enable their administration as inhaled vapour avoiding the alimentary system with its attendant denaturing of active molecular species.

In addition, another plant of the family Lamiaceae, *Melissa officinalis* (lemon balm) showed *in vivo* to improve cognitive performance and mood in young people at single doses of 600, 1000 and 1600 mg encapsulated dried leaf. Ethanol extract of the dried leaf plant *in vitro* showed no inhibition of human AChE but was able *in-vitro* to displace [^{3}H]-(*N*)-nicotine and [^{3}H]-(*N*)-scopolamine from human nicotinic and muscarinic receptors, with IC_{50} values of 0.18 mg ml^{-1} and 3.47 mg ml^{-1} respectively (Kennedy *et al.*, 2003). In a four month double blind, randomised, placebo controlled trial 60 drops a day of 45% EtOH extract of lemon balm significantly improved cognitive function, using ADAS-cog and CDR diagnostic criteria, and reduced agitation in patients with mild to moderate AD (Akhodzadeh *et al.*, 2003 b).

Thus, the genus *Salvia* (family Lamiaceae) is a warranted candidate in endorsing herbal medicine as treatment of age related disorders.

1.3.1.4.4. Guidelines for clinical trials of botanicals

Current USA guidelines for clinical trials of botanicals were highlighted by Berman (2003 a) at a three day conference on medicinal plants and their uses at Imperial College, London. According to the speaker, botanical agents are characterised by the FDA according to their use, not their composition. If the intended use is to "promote health" and preserve the "structure and function" of the body, the agent is viewed as a dietary supplement. If the intended use is to treat or prevent a disease, the agent is considered to be a drug. Botanical agents used as dietary supplements are regulated by the FDA under the Dietary Supplement health and Education Act of 1994 (DSHEA). In contrast, botanical agents used as drugs are regulated by the FDA's centre for Drug Evaluation and Research (CDER).

Botanical drugs have two unique characteristics compared with to conventional agents. Firstly, they are mixtures of uncharacterised constituents whose therapeutic effect occurs through likely synergistic interaction of unknown constituents with the known ones. Such interaction may provide more efficacy than by the known constituent along. Nevertheless, being a mixture is a disadvantage in that it is challenging to assay a product for quality if the active ingredients are unknown. Secondary, they have had substantial prior human use to formal clinical trials. Prior human use is an advantage as it gives some assurance that the product may be safe and effective.

For botanical drugs *in-vitro* information, although encouraged, is not absolutely required. As far as clinical data are concerned neither phase 1, 2 nor 3 trials should be omitted in a product evaluation program. Phase 3 trials should not be undertaken until dose optimisation with respect to safety and efficacy has been accomplished in prior 1 and 2 trials.

1.4. Neuroactive phytochemical project

Despite the strong ethnobotanical reputation of sage as a plant with memory enhancing properties not enough has been studied towards establishing the clinical potential of the genus. There are no reports on anti-BuChE, anti-secretase and pro-inflammatory cytokines suppressive activities of sage, although nicotinic and muscarinic binding properties of ethanol extracts were reported (Wake, 2001).

This Ph.D project was designed as a follow up of previous positive findings on anti-acetylcholinesterase activity of Spanish sage oil (Perry *et al.*, 2000). *In vitro* activities of non-polar extracts of *Salvia* species relevant to current and prospective treatment of neurodegenerative disorders have been evaluated. Studies on chemical interactions of low molecular weight compounds have also been included, as these add to understanding of a pharmacological mechanism of interaction and may contribute towards standardisation of bioactive extracts for clinical trials.

The main question to be addressed is: could a multiple drug therapy for treatment of dementia be based on a standardised herbal preparation comprising a number of synergistically interactive molecules with multiple activities relevant to treatment of dementia?

1.5. Aims of the project

i) To screen a range of non-polar extracts of *Salvia* species and their individual chemical compounds for anti-AChE, anti-BuChE and anti-β secretase activities.

ii) To develop and standardise a novel supercritical fluid extraction process based on the proprietary solvent Phytosol A for the purpose of isolation of bioactive extracts which could be applied clinically for treatment of dementia.

iii) To investigate and characterise chemical interactions of single compounds present in sage species towards inhibition of AChE, hence to conclude if the activity of a single compound accounts for the activity of a whole extract.

Chapter 2. Materials and Methods

2.1. Steam distillation. Anti-cholinesterase activity of oils of *Salvia* species

2.1.1. Chemicals

Acetylcholinesterase (EC 3.1.1.7;) from human erythrocytes (Ott *et al.*, 1975), acetylthiocholine iodide (ATChI), butyrylcholinesterase (EC 3.1.1.8) from human serum, butyrylthiocholine (BTChI), 5:5-dithiobis-2-nitrobenzoic acid (DTNB), diethyl ether (Analar, boiling point 34-35 ^{0}C), physostigmine, quinidine, anhydrous magnesium sulphate and ethanol (Analar) were purchased from Sigma, U.K.

2.1.2. Plant material

Aerial parts of *Salvia officinalis* L. "*purpurea*" were harvested at flowering (6/02) and vegetative (9/02) stages from Jesmond Dene garden, Newcastle upon Tyne, U.K. Fresh leaves of the plant were steam distilled on the day of harvest.

Aerial parts of *Salvia officinalis* L. (broad leaf sage) were collected on 19th August, 2002 (8/02) from Dilston Physic Garden, Corbridge, Northumberland. Fresh leaves of the plant were steam distilled on the day of harvest.

Air-dried, aerial parts of *Salvia lavandulaefolia* Vahl. (Spanish sage) were supplied and verified by Advanced Phytonics Ltd., U.K. The plants were harvested in the wild in October, 2001 in Granada, Spain. Leaves of the plant were used in steam distillation.

Air-dried, aerial parts of *Salvia fruticosa* Mill. "Greek sage" were supplied and verified by Advanced Phytonics Ltd., U.K. The plant, growing wild, was harvested in the

Troodos area of Cyprus in August, 2002 (8/02). The material contained no seeds or floral remains. Leaves of the plant were used in steam distillation.

Aerial parts of *Salvia fruticosa* Mill. were also harvested in the Troodos region of Cyprus in late July, 2001 (7/01). The plant was verified by Dr Djakouri in Cyprus and sent to U.K as dried material. Leaves of the plant were used in steam distillation.

Verified voucher specimens of each plant (001; 002; 003; 004; 005; 006 as listed above) were deposited in the herbarium of Medicinal Plant Research Centre, University of Newcastle, U.K.

2.1.3. Extraction process

The essential oils were extracted for 2.5 hours by a method of steam distillation in an all-glass apparatus (Houghton and Raman, 1998). Nine parts of diethyl ether was used to separate oil from one part of water-oil phase in a three batch extraction process. Anhydrous magnesium sulphate was added to the diethyl ether-oil mixture to a saturation level in order to absorb any remaining water. The oil-ether phase was filtered and the ether evaporated at room temperature using a rotary evaporator to obtain the oils, which were stored in sealed amber glass vials under oxygen free nitrogen at 5 ^{0}C for less than one month before analyses.

2.1.4. Assays

2.1.4.1. Acetylcholinesterase activity

2.1.4.1.1. Human erythrocyte AChE

Assessment of human AChE inhibition was carried out using the method of Ellman *et al.* (1961) as modified by Nostrandt *et al.* (1993). A typical run consisted of 5 µl of AChE solution, at a final assay concentration of 0.03 U/ml; 200 µl of 0.1 M phosphate buffer pH 8; 5 µl of DTNB, at a final concentration of 0.3 mM prepared in 0.1 M phosphate buffer pH 7 with 0.12 M of sodium bicarbonate; and 5 µl of a sample solution (oils) in 53% ethanol (EtOH). The reactants were mixed in a 96-well, flat bottom polystyrene microtiter plate. The mixture was pre-incubated at 30 ^{0}C over a range of times. The reaction was initiated by adding 5 µl of ATChI at a final concentration of 0.5 mM. Each sample was assayed in triplicate.

A measurement of the hydrolysis was started after 6 minutes maintenance time. This time was necessary to overcome the problem with the inhibition of the human enzyme by EtOH (see below: EtOH interference). As a control 5 µl of the inhibitor solution was replaced with 5 µl of 53% ethanol. The control was also assayed in triplicate. To monitor any non-enzymatic hydrolysis in the reaction mixture two blanks for each run were prepared in triplicate. One blank consisted of 205 µl buffer (replacing 5 µl enzyme), 5 µl DTNB, 5 µl 53% EtOH and 5 µl substrate. A second blank also had 5 µl enzyme, 205 µl buffer (replacing 5 µl substrate), 5 µl DTNB and 5 µl 53% EtOH. A kinetic run absorbance at 412 nm was measured on a Titertek Multiskan MCC/340 microplate reader for a period of six minutes at 30 ^{0}C with a Genesis-Lite Windows microplate software (Labsystem International).

The thiocholine, formed during hydrolysis of ATCh, rapidly reacts with DTNB and releases a yellow 5-thio-2-nitrobenzoic acid anion (Figure 2.1.). The production of this coloured anion was recorded at 412 nm over 6 min run. Any inhibition of AChE by an inhibitor would reduce the release of such anion, as a result of binding to the enzyme, and that would result in reduction of hydrolysis of the enzyme. The spectrophotometer reads the changes in releasing of the acid anion and expresses it as a change of absorbance per minute (abs/min) in a slope value. A percentage of the inhibition was calculated as:

$$\text{Inhibition} = \frac{\text{abs/min with no inhibitor} - \text{abs/min with inhibitor}}{\text{abs/min with no inhibitor}} \times 100\%$$

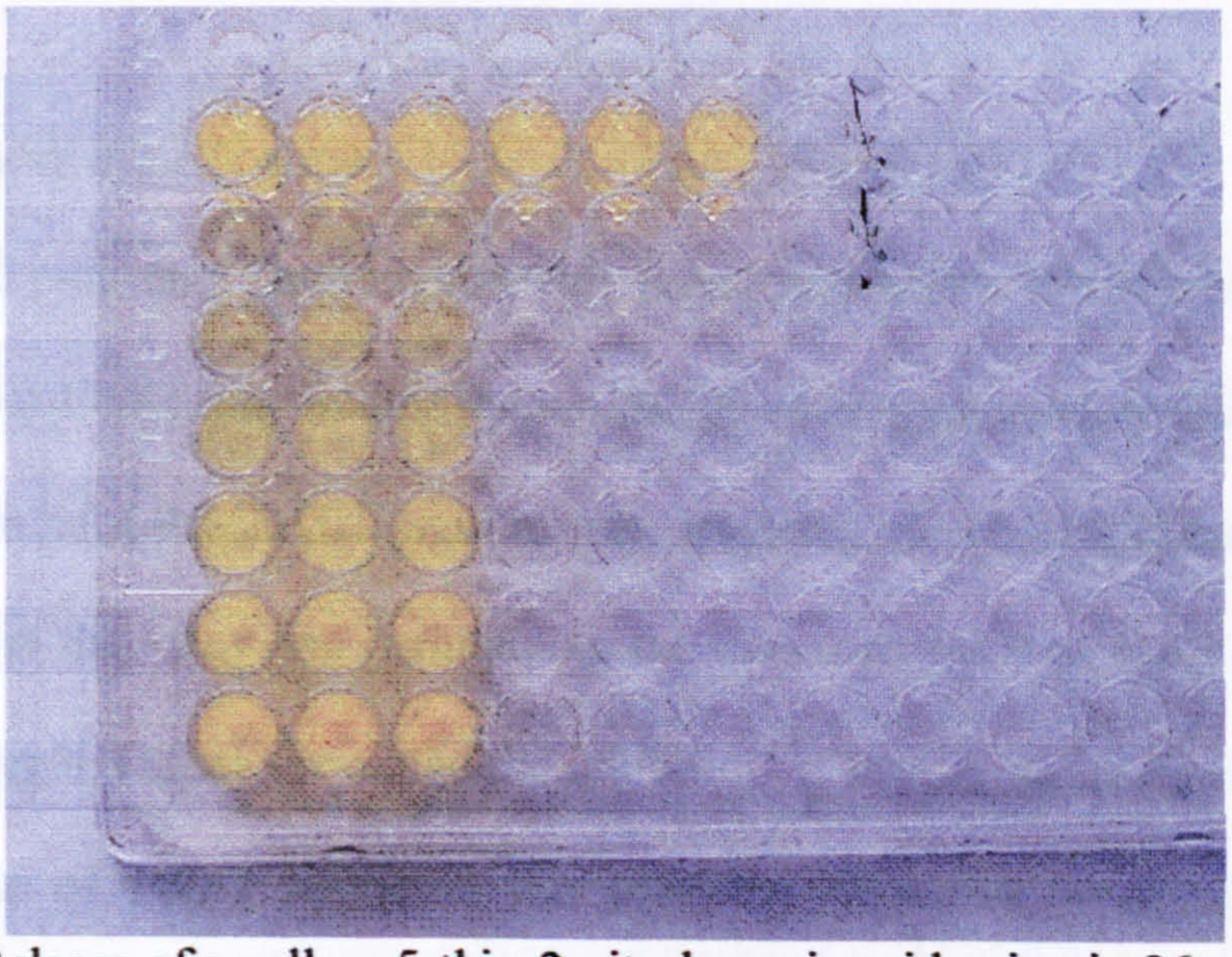


Figure 2.1. Release of a yellow 5-thio-2-nitrobenzoic acid anion in 96-well plate

2.1.4.1.2. Bovine erythrocyte AChE

A measurement of hydrolysis of bovine AChE was carried out as described in 2.1.4.1.1. with a final concentration of the enzyme at 0.008 U/ml. There was no six minutes maintenance time as the enzyme was not inhibited in the assay by 1.9% EtOH (see

below: EtOH interference) and 84% EtOH was therefore used in preparation of inhibitor stock solutions.

2.1.4.1.3. Butyrylcholinesterase activity

Assessment of BuChE (human serum) inhibition was performed as described in 2.1.4.1.1 with the exception of: a final enzyme concentration of 0.01 U/ml; BTChI was used at a final concentration of 0.5 mM; the enzyme was not inhibited by 1.9% EtOH in the assay, thus 84% EtOH was used in preparation of inhibitor stock solutions; there was no 6 minutes maintenance time (see below: EtOH interference).

2.1.5. Ethanol interference

Reports (Haboubi and Thurnham, 1986; Baker and Chen, 1989; Dudek *et al.*, 1995) indicating that EtOH may inhibit human AChE were confirmed in this study. An assay concentration of 1.9% EtOH significantly inhibited (53±2.6%) human AChE. Using 1.2% EtOH in the assay and maintaining the mixed reagents in the plate for 6 minutes before running the assay prevented the inhibition of human AChE by EtOH. Such a low concentration of EtOH restricted the solubility limits of plant extracts and their individual constituents. 1.9% EtOH did not however inhibit bovine erythrocyte AChE or human BuChE.

Because of a low solubility range of monoterpenoids and sesquiterpenoids in 53% EtOH solution the initial screening was performed using bovine AChE and chemicals showing activity were re-analysed using human AChE.

2.1.6. Dose-response curves and equations

Each inhibitor was tested in triplicate over a range of concentrations. A triplicate of each concentration was equivalent to n=1. Using a Microsoft Excel software dose-response curves were fitted to the data points. There were six triplicate sets for the oils (n=6), resulting in the same number of dose-response equations, which were used for statistical analysis.

A concentration of the compounds which gave 50% inhibition of the enzymes was expressed as an IC_{50} value. The concentration of compounds which did not achieve 50% inhibition was expressed as the percent inhibition, i.e. maximum inhibition of the enzymes within the solubility limit of a chemical.

2.1.7. Gas Chromatography-Mass Spectrometry (GC/MS)

GC/MS analysis of aromatic compounds was performed on Hewlett-Packard 6890 GC with a split/splitless injector (280 ^{0}C) linked to a Hewlett-Packard 5973 mass selective detector (electron voltage 70eV, filament current 220 uA, source temperature 230 ^{0}C, quad temperature 150 ^{0}C, multiplier voltage 2400V, interface temperature 300 ^{0}C). Data acquisition was controlled by a HP Kayak xa pc chemstation computer, initially in full scan mode 35-550 amu/sec for greater sensitivity. The total peak areas were calculated on the basis of 1 % of the major peak.

A sample (1µl) in dichloromethane (Analar) was injected by an HP6890 auto sampler and the split opened after one minute. Separation was performed on Zebron (ZB) ZB-5 (30m x 0.25mm ID x 0.25µmdf) capillary column coated with 5% phenyl polysiloxane. The GC was temperature programmed from 40-300 ^{0}C at 4 ^{0}C per min and held at a final temperature for 20 minutes with Helium as the carrier gas at flow rate of 1ml/min (initial pressure of 50 kPa, split at 30 mls/min).

2.2. Supercritical fluid extraction. Anti-cholinesterase activity of extracts of *Salvia* species

2.2.1. Chemicals/materials

Phytosol A (1,1,1,2-tetrafluoroethane) and a hand-set extraction kit (Figure 2.2.) were provided by Advanced Phytonics Ltd., U.K. Acetylcholinesterase (EC 3.1.1.7;) from human erythrocytes (Ott *et al.*, 1975), acetylthiocholine iodide , butyrylcholinesterase (EC 3.1.1.8) from human serum, butyrylthiocholine , 5:5-dithiobis-2-nitrobenzoic acid (DTNB), physostigmine, quinidine, hexane (analar) and ethyl acetate (analar) were purchased from Sigma, U.K.

2.2.2. Plant material

Aerial parts of *S. africana-lutea* Linn. (syn. *S. aurea*), *S. apiana* Jepson "white sage", *S. argentea* Linn. (silver sage), S. *atrocyanea*, *S. confertiflora*, *S. corrugata* Vahl., *S. discolor* Kunth., *S. divinorum* Epling (Pipilzintzinti), *S. glutinosa* Linn. (hardy sage), *S. haematodes* Linn., *S. involucrata* Cav., *S. Jamensis* var. *la luna*, *S. keerlii* Benth., *S. longistyla* Benth., *S. microphylla* var. *neurepia* Epling, *S. napifolia* Jaquin., *S. sclarea* Linn. (clary sage), *S. stenophylla* Burch. ex Benth., *S. verbenaca* Linn., *S. verticilata* Linn., were harvested in June, 2002 (*S. yunnanensis* June, 2001) from Chelsea Physic Garden, London, U.K. Freeze-dried material, which had no seeds or floral remains, were used to prepare the extracts. The freeze-drying was performed on the day of harvesting.

Aerial parts of *S. officinalis* Linn. "*purpurea*" were harvested at flowering stage (18/07/02) from Jesmond Dene garden, Newcastle upon Tyne, U.K. The material contained no seeds or floral remains. Freeze-dried leaves were used in the extraction process.

Aerial parts of *S. officinalis* Linn. (broad leaf sage) were collected in July, 2001 from Dilston Physic Garden, Corbridge, Northumberland. The material contained no seeds or floral remains. Freeze-dried leaves were used in the extraction process.

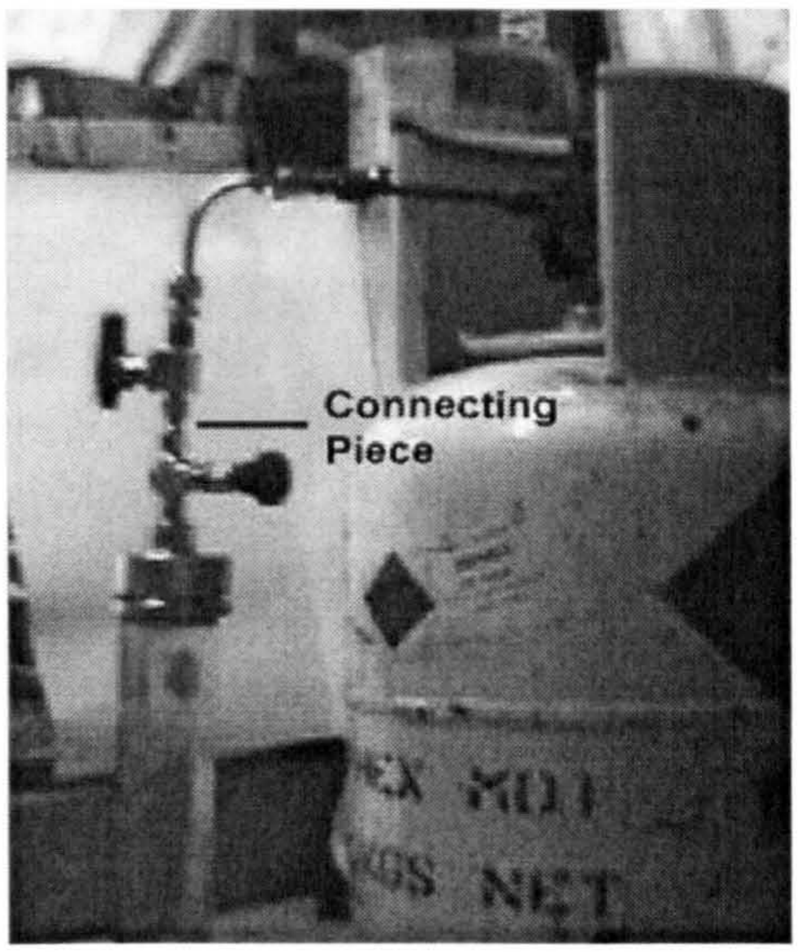
Aerial parts of *S. lavandulaefolia* Vahl. (Spanish sage) were supplied and verified by Advanced Phytonics Ltd., U.K. The plants were harvested in the wild in October, 2001 in Granada, Spain. Freeze-dried leaves were used in the extraction process.

Air-dried, aerial parts of *S. fruticosa* Mill. "Greek sage" were supplied and verified by Advanced Phytonics Ltd., U.K. The plant, growing wild, was harvested in the Troodos area of Cyprus in August, 2002. The material contained no seeds or floral remains. Freeze-dried material were used in the extraction process

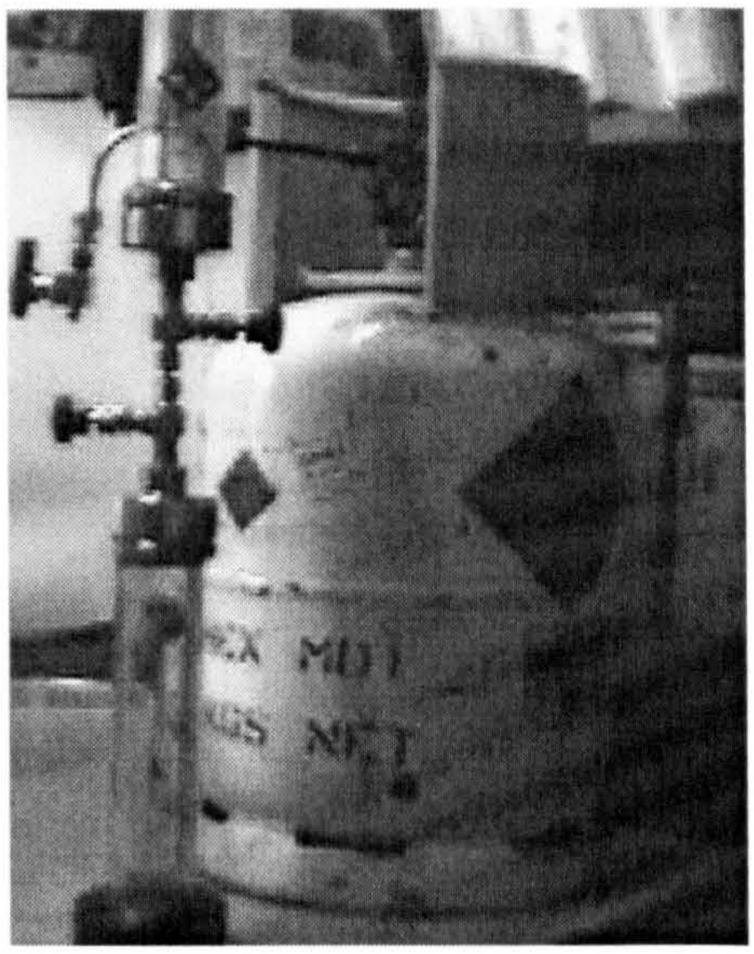
2.2.3. Extraction process using Phytosol A

A batch extraction process was performed using a hand-set extraction kit provided by Advanced Phytonics Ltd., U.K (Figure 2.2). One part of ground plant material was mixed with eight parts of the solvent (Kusova and Vetrov, 1987) at room temperature and left for 1hour and 15 minutes (consistent shaking) in a pressurised plastic/rubber-free extraction vessel (Figure 2.2. a). After the time elapsed, the extraction vessel was inverted and connected to a vertically positioned evaporation vessel (Figure 2.2 b). The solvent-extract phase was filtered using a plastic/rubber free filter (Figure 2.2. d) into an evaporation vessel (Figure 2.2. b) by opening valves of each of the vessels (Figure 2.2. c). It may be necessary to release some of the positive pressure from the evaporator during the filtration to allow full transportation of the solvent-extract phase from the extractor to the evaporator. After the filtration process, the valves were closed and the

vessels detached. The solvent was boiled off by carefully opening the valve of the vertically positioned evaporator, leaving a plant extract.

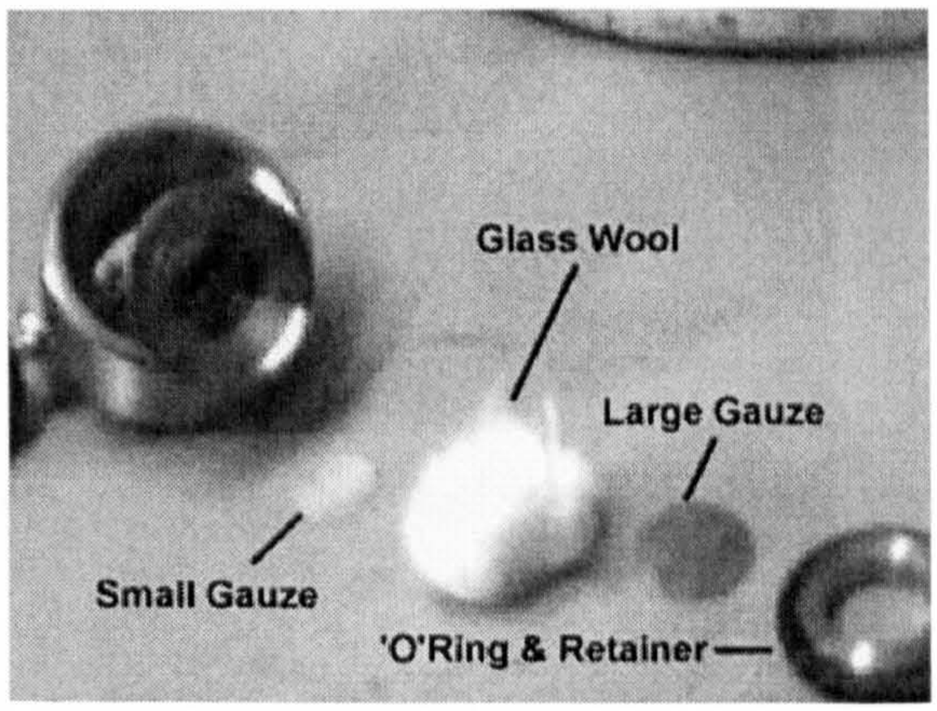


a. Transferring Phytosol A into a glass extraction vessel from a pressurised cylinder.



b. Filtering the solvent-extract phase from the pressurised extraction vessel (top) into a glass evaporation vessel (bottom).

c. A lid to the extraction and evaporation vessels. It consists of a valve which controls a pressurised flow of the solvent and a filter which prevents plant material passing through from the extractor to the evaporator.



d. Parts of the filter. Cotton wool was used instead of the glass wool. The "O" ring was made of polytetrafluoroethylene (PTFE) to avoid a cross contamination with plasticizers.

Figure 2.2. A hand-set extraction kit.

The plant extract was collected using a glass Pasture pipette into an eppendorf tube and centrifuged on an Eppendorf 5417R centrifuge machine for 30 minutes at 5 ^{0}C with a speed of 1200 rpm. After the centrifugation process, the extract was visually examined

for its possible separation, namely a solid residue and oil (or resin). The oil part was carefully pipetted off and stored in sealed amber glass vials under oxygen free nitrogen at 5 ^{0}C. 1 ml of hexane was added to the residue (only three residues were analysed; *S. argentea*, *S. napifolia*, *S. yunnanensis*) to dissolve non-polar traces of the oil. The mixture was sonicated in Decon F5 minor sonication bath for 15 min and then centrifuged for 10 minutes at 5 ^{0}C with a speed of 1200 rpm. After that a supernatant was pipetted off and new 1 ml hexane was added. The process was repeated three times. At the end of the process, remained hexane was dried off with oxygen free nitrogen and the residue stored at 5 ^{0}C.

2.2.4. Assays

Inhibition of human AChE and human BuChE by Phytosol A extracted botanicals were carried out as described in 2.1.4.1.1. and 2.1.4.1.3. respectively.

2.2.5. Gas Chromatography-Mass Spectrometry (GC/MS)

Analysis of chemical composition of extracts of *Salvia* species was performed as described in 2.1.7.

2.2.6. Extraction with ethanol

3 grams of air dried plant (ground) were mixed with 30 ml 70% EtOH for 5 days in a dark with constant gentle shaking. After this the liquid part was filtered off and centrifuged (14000 rpm, 10 min × 2) using Eppendorf an centrifuge machine (5417 R). 1 ml of the supernatant was transferred into an eppendorf (3 tubes to calculate the mean) and freeze-dried at –55 ^{0}C, 0.5 mBar to a constant weight (±0.05% variation).

2.3. Anti-cholinesterase activity of terpenoids present in extracts of *Salvia* species

2.3.1. Chemicals/Reagents

As in 2.1. with addition of;

(1S)-(+)-3-carene, manool, sclareol, (-)-guaiol, (R)-(+)-limonene were purchased from Aldrich Chem. Co., U.K. (-)-globulol, (-)-alpha-copaene, (-)-bornyl acetate, (-)-β-pinene, (-)-trans-caryophyllene, (-)-alpha-thujone were obtained from Fluka, U.K. and Neryl acetate, alpha-terpineol from Acros Organics, U.K. Sabinene was purchased from Apin Chem. Ltd., U.K. (+)-α-pinene, 1,8-cineole, (1R)-(+) camphor, (±)-linalool, (-)-borneol, caryophyllene oxide, α-caryophyllene (α-humulene) from purchased from Sigma, U.K.

2.3.2. Assays

2.3.2.1. Acetylcholinesterase activity

2.3.2.1.1. Bovine erythrocyte AChE

As described in 2.1.4.1.2

Lack of inhibition of the enzyme activity by 1.9% EtOH allowed initial screening of single chemicals, present in species of *Salvia*, for their anti-AChE activity at high concentrations at five minutes incubation time. Those compounds showing marked potency were further analysed using the human enzyme.

2.3.2.1.2. Human erythrocyte AChE

As described in section 2.1.4.1.1.

2.3.2.1.3. Butyrylcholinesterase activity

As described in section 2.1.4.1.3.

2.3.3. Dose-response curves and equations

As described in section in 2.1.6.

There were four triplicate sets of concentrations for individual compounds (n=4) resulting in the same number of dose-response equations, which were used for statistical analysis.

2.4. Synergistic and Antagonistic Interactions of Anticholinesterase Terpenoids in *Salvia lavandulaefolia* Essential Oil

2.4.1. Chemicals/Reagents

Acetylcholinesterase (AChE; EC 3.1.1.7) from bovine erythrocytes, acetylthiocholine iodide (ATChI), 5:5-dithiobis-2-nitrobenzoic acid (DTNB), (+)-α-pinene, (-)-β-pinene, 1,8-cineole, (1R)-(+) camphor, (±)-linalool, (-)-borneol, (-)-bornyl acetate, caryophyllene oxide (car., oxide), ethanol (Analar), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium bicarbonate ($NaHCO_3$) were purchased from Sigma, UK.

Salvia lavandulaefolia (fresh leaves) steam distilled oil was purchased from Baldwins & Co., London, UK.

2.4.2. Bovine erythrocyte AChE

A measurement of hydrolysis of bovine AChE was carried out as described in section 2.1.4.1.2.

2.4.3. Dose-response curves

Each inhibitor was tested over a range of concentrations in triplicate to obtain mean inhibition data. Using a Microsoft Excel programme a dose-response curve was fitted to

the data points. There were four replicate sets of concentrations for each inhibitor (n=4), consequently producing four dose-response equations, which were used for calculations and statistical analysis of individual compounds and their combinations (Appendix 4). A concentration of an inhibitor corresponding to 50% inhibition of AChE (IC_{50} value) was calculated using the dose-response curve equations. A concentration of each inhibitor was substituted into the dose-response curve equation of each inhibitor as X (concentration). Hence Y would represent inhibition, *i.e.*, an IC_{50} value or the activity of an inhibitor at its particular concentration (Appendix 4).

2.4.4. Calculation of expected inhibition of combined chemicals

The method used is described by Berenbaum (1985) and based on an assumption that the agents in a combination do not interact, producing a zero-interactive response. This is expressed in the following equation:

$$\sum_{i=1}^{n} \frac{d_i}{D_i} = 1 \qquad (1)$$

where n is a number of agents in a combination with i=1,2,3,…,n; d_i is the actual dose (concentration) of the individual agents in a combination and D_i is the dose (concentration) of the agents that individually would produce the same effect as the individual compounds in the combination.

If equation 1 gives a value of more than 1, >1 – synergy effect, if less than 1, <1 – antagonism occurs. For a combination of two agents equation (1.1) can be written as:

$$da/Da + db/Db = 1 \qquad (1.1)$$

Where da and db are known and represent values of final concentrations of two agents in a combination. On a graph of the dose-response curves of agents A and B, a horizontal line intersects these two curves at points corresponding to Da and Db (X-axis co-ordinates). Da, Db and the combination (da,db) are isoeffective (Equation 1.1). Therefore, a solution is to find the horizontal, isoeffective straight line, which would determine doses of A (Da) and B (Db) to satisfy equation 1.1. This line must have one value on the Y-axis and two on the X-axis. A value on the vertical axis (Y) represents an inhibition and values on the horizontal axis (X) represent concentrations. These concentrations are expressed as Da and Db. It is therefore a matter of finding which isoeffective Y value, inserted into the dose-response curve equations of each agent, produces values of its corresponding concentrations (X) for Da and Db, which satisfy equation 1.1. The horizontal isoeffective line locating these values indicates the response of the combination if there is no interaction, and represents the value of the expected inhibition of a non-interactive combination. If a calculated value of the combination is significantly less than obtained experimentally, synergy can be inferred, if more - antagonism. The same approach can be applied to any number of agents in a combination (Equation 1). The method is illustrated in Appendix 5.

2.4.5. Calculation of the interaction index of a combination

The value of observed inhibition of a combination, comprising a number of compounds, was inserted into the dose-response curve equations of each individual compound as Y (vertical axis for an inhibition) to calculate the corresponding value of X (horizontal axis for concentrations), Da and Db in equation (2). If the equation for zero-interaction response resulted in a value significantly less than 1, synergism was inferred, if more then antagonism. The calculation of the interaction index is shown in Appendix 5.

2.4.6. Statistics

The data was presented as mean ± standard deviation of the mean. Group comparisons was analysed using a two sample Student's *t*-test with a probability value of < 0.05 as the level of statistical significance.

2.5. Extracts of *Salvia* species in relation to β-secretase, interleukin 8 and cholinergic receptor activities

2.5.1. β-secretase assay

2.5.1.1. Materials

Secretase activity kit and BACE-1 was purchased from R&D systems Europe, LTD. UK. The EDANS/DABCYL substrate corresponds to the amino acid sequence associated with β-secretase cleavage of amyloid precursor protein (aa 668-675) and includes substitution of Asn and Leu for amino acids Lys_{670} and Met_{671} known as the Swedish mutation.

Recombinant human BACE-1 (β-secretase):

A cDNA sequence encoding the ectodomain (amino-acid residues 1-460) of the recombinant human β-secretase was expressed as secreted protein with a COOH-terminal His tag in a murine myeloma cell line, NS0. Purity > 90%.

Extracts of *S. fruticosa*, *S. officinalis* var. *purpurea* and *S. apiana* were obtained using Phytosol A (2.2.3.). 1,8-cineole and 3-carene were purchased from Sigma Co., U.K. and Fluka Co., U.K. respectively.

2.5.1.2. Method

Principle of the test:

Cleavage of the substrate, conjugated to the reporter molecules EDANS and DABCYL, by BACE-1 separates the EDANS and DABCYL allowing the release of a fluorescent signal. The level of secretase enzymatic activity is proportional to the fluorometric reaction. A measurement of fluorescence was performed on a Tecan SPECTRAFluoro Plus fluorometer using Magellan software.

BACE-1 preparation:

Reconstitute 100 µg of product in 1000 µl distilled water to make a stock solution 1 [100µg/ml]. Take 187.5 µl from stock 1 and add 1312.5 µl distilled water to prepare a stock solution 2 [18.75 µg BACE/1.5ml or 0.0125 µg/µl]. This is a working stock solution. Using 40 µl of the stock for the assay or 0.5 µg BACE per well would make 4.8 µg/ml BACE final concentration.

Assay procedure:

40 µl BACE-1 were mixed with 50 µl reaction buffer (supplied) and 10 µl test solution (proposed inhibitor). The mixture was incubated over 30, 60, 90 and 120 minutes in dark at 37 ^{0}C. 5 µl substrate were added after a period of incubation time. A 96 flat bottom micro titter plate was read on a fluorescence microplate reader in endpoint mode at an excitation wavelength of 360 nm and emission at 535 nm.

Negative control: as above with the exception that 10 µl test solution was replaced with the 10 µl 84% EtOH (8% EtOH in the assay). Run in triplicate.

Blank 1 as above with the exception that 10 µl inhibitor was replaced with the 10 µl water. Run in triplicate.

Blank 2 as above with the exception that 40 µl BACE were replaced with 40 µl distilled water and 10 µl 84% EtOH (8% EtOH in the assay) were used instead of inhibitor. Run in triplicate.

Blank 3 as above with the exception that 5 µl substrate were replaced with 5 µl distilled water and 10 µl 84% EtOH (8% EtOH in the assay) were added instead of inhibitor. Run in triplicate.

2.5.2. Neuroinflammation as a target in the treatment of AD

2.5.2.1. Materials

Pelikine Compact Human IL-8 ELISA kit and IL-8 was purchased from Research Diagnostics Inc. HT29-MXT cell line was supplied Dr. Smirnova (Mucin research group, School of Cell and Molecular Bioscience, University of Newcastle). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffer saline (PBS), sulphuric acid (H_2SO_4) and 96-well polysterene plates was purchased from Sigma Co, UK. Universal microplate spectrophotometer mQuant (Bio-Tek Instrument Ink).

2.5.2.2. Method

2.5.2.2.1. Media and sample preparation

Oils of *S. apiana* and *S. fruticosa* have been tested on the HT29-MXT cell line. The HT29-MXT cell line, a well characterised human mucin-secreting cell subpopulation of polarised goblet cells (Lesuffleur *et al.*, 1991; Lesuffleur *et al.*, 1993) secretes a constitutive level of the secondary pro-inflammatory cytokine IL-8 (≈0.3 ng ml^{-1}) (Smirnova *et al.*, 2002 a).

Cells were seeded ($5x10^5$ cells/well) in 6-well plates and grown in 4ml of Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a 10%CO_2/90% air atmosphere for 7 days to the pre-confluent stage. Before exposure to oils, cells were pre-incubated for 24 hours in serum-free medium to stabilise the stage of cell growth and washed twice with PBS at room temperature.

9 mg oils were diluted in 100 µl 40% EtOH to make a stock solution. After 1 hour (h), 4h and 24h exposure to oils (a final concentration of 0.9 mg ml^{-1}) the cell culture media was subjected to IL-8 immunoassay. Level of IL-8 secretion in oil treated cells was measured against control (EtOH treated) cells.

2.5.2.2.2 IL-8 immunoassay

Measurements of IL-8 in cell culture media were performed by a quantitative sandwich enzyme immunoassay using a commercially available "Pelikine Compact Human IL-8 ELISA kit" (Research Diagnostics Inc). In brief, the monoclonal anti-human IL-8 antibody was coated onto the 96-well polystrene plates and 100µl of cell culture media (or standard) was added to the wells and incubated for one hour at room temperature. After one hour plates were washed four times with phosphate buffer saline (PBS), containing 0.005% Tween 20. The secondary biotinylated (colour reagent) monoclonal anti-human IL-8 antibody was added and incubated for one hour at room temperature. After washing plates (as described above) horseradish peroxidase (HRP) conjugated streptavidin, (serving as a substrate) was added. After 30 minutes of incubation at room temperature plates were washed four times with PBS. A colour reaction was developed with 3,3',5,5'–tetramethylbenzidine (TMB) peroxidase (enzyme hydrolysing biotinyl-streptavidin complex) solution and stopped with addition of 0.5 M H_2SO_4. Absorbance,

using universal microplate spectrophotometer mQuant, was read at 450 nm. A standard curve was constructed using an IL-8 standard from the "Human IL-8 ELISA kit". The IL-8 standard was calibrated against WHO interim International Standard, 1 pg of IL-8 standard is equivalent to 0.56 pg of the WHO standard. The sensitivity of the IL-8 assay was 50 pg ml^{-1}.

2.5.3. Oil of *S. apiana* in relation to nicotinic and muscarinic receptors

2.5.3.1. Preparation of brain neuron membranes for receptor assay

(Court *et al.*, 1994)

Membrane homogenates were prepared by paring grey matter from human occipital cortical tissue (supplied by the Brain Bank at the Medical Research Council's Neurochemical Pathology Unit, Newcastle General Hospital). The tissue was homogenised in Na/K phosphate buffer, 50 mM, pH 7.4 (1 g tissue per 10 ml buffer), on ice, using an Altratuttax® in a biological safety cabinet. The homogenate was centrifuged at 20000 rpm for 20 minutes at 4 ^{0}C, the supernatants discarded and the membrane pellets re-suspended and centrifuged twice more in similar volumes of ice-cold buffer to remove cytoplasmic elements from the membranes. The membranes were then re-suspended in a similar volume of buffer and aliquots of 1 ml of the suspension were pipetted into 1.5 ml Eppendorf tubes which were centrifuged at 5000 rpm for 10 minutes to pellet the membranes. All supernatants were removed and discarded after treatment with sodium hypochlorite, and the membrane pellets stored at -70 ^{0}C until use.

2.5.3.2. Nicotinic receptor displacement assay

2.5.3.2.1. Materials

Buffer was prepared as;

Tris-hydroxymethyl methylamine (Sigma, U.K), 50 mM (60.55g)

Calcium chloride hexahydrrate (Sigma, U.K), 8 mM (17.58 g)

Adjust pH to 7.4 with concentrated HCL (Sigma, UK)

Distilled water: bring the total volume to 1 litter.

Radioligand; L-(-)-N-methyl-[^{3}H]-nicotine (DuPont, specific activity 81.5 Ci/mM), Carbachol (carbamylcholine chloride), Ultima Gold MV (Canberra-Packard) scintillation fluid, and 6x 30cm strips of Whatman GF/C filter sheets were purchased from Sigma Co., U.K.

Oil of *S. apiana* was obtained as described in section 2.2.3.

2.5.3.2.2. Method

Pelleted human occipital cerebral cortical cell membranes bearing nicotinic acetylcholine receptors as prepared in 2.4.3.1. were used. Each membrane pellet, contained in a 1.5 ml Eppendorf tube, was suspended in 1.2 ml of ice cold assay buffer and 50 µl of membrane suspension was added to each assay tube. Carbachol, a displacer for assessment of a non-specific binding, was prepared as a 1 mM solution. The samples were run in triplicate and displacement values were calculated from the mean of these triplicates.

A run for the assay;

i. 350 µl buffer + 50µl of the membrane suspension, vortex mix for 10 seconds

ii. 50 µl carbachol (or oil), vortex mix for 10 seconds

iii. 30 minutes incubation at 25 ^{0}C in a water bath

iv. 50 µl L-(-)-N-methyl-[^{3}H]-nicotine, vortex mix for 10 seconds

v. 60 minutes incubation at 25 ^{0}C

The membranes were then filtered from the reaction mixture onto 6x 30cm strips of Whatman GF/C filter sheet which was priory soaked in 0.2% polyethylene imine solution overnight, using a Brandel Cell Harvester and washed 3 times with cold buffer. The filter sheet was then removed from the harvester and the discs, which held the harvested membranes, were separated from the filter sheet with forceps. After that the discs were transferred to 'Pony' brand plastic scintillation vials and 2 ml of Ultima Gold MV (Canberra-Packard) scintillation fluid was added. The vials were allowed to stand for 2 hours art room temperature (22 ^{0}C) and the assays were then counted for decomposition per minute (dpm) in a Canberra Packard 1900 CA Tri-carb LSC Counter, 5 minutes per vial. Dose response curves were plotted for dilution series using Microsoft Excel 2000 computer software.

Controls for the assay;

Control 1

i. 400 µl buffer + 50µl the membrane suspension, vortex 10 seconds

ii. 50 µl L-(-)-N-methyl-[^{3}H]-nicotine, vortex mix for 10 seconds

Control 1 values on the scintillator print-out represent the dpm count of non-specific bindings of L-(-)-N-methyl-[^{3}H]-nicotine to the membranes.

Control 2

i. 350µl buffer + 50µl the membrane suspension, vortex 10 seconds

ii. 50µl the non-specific radioligand and 50µl the specific displacer, vortex 10 seconds

Control 2 dpm values represent the portion of the total bound ligand which is occupying sites other than the nicotinic receptor. A dpm value for all nicotinic receptor sites

available for radioligand binding (control 3) was calculated as dpm values of control 1 minus the dpm values of control 2.

A percentage of displacement produced by oil was calculated as

$$\frac{\text{dpm control 3} - \text{dpm sample}}{\text{dpm control 3}} \times 100\%$$

2.5.3.3. Muscarinic receptor displacement assay

2.5.3.3.1. Materials

As in section 2.5.3.2.1. except that the assay buffer was HEPES, containg 10 mM $MgCl_2$ and 100 mM NaCl, pH 7.4. The radioligand was 1 nM L-methyl-[3H]-scopolamine (Dupont specific activity 84.5 Ci/mM, Sigma Co., U.K), atropine and 6x 30cm strips of Whatman GF/B filter sheets (Sigma Co., U.K) were used.

2.5.3.3.2. Method

Muscarinic displacement was measured using a similar protocol to that described in section 2.5.3.2.2. (Court *et al.*, 1994). Non-specific binding was defined in the presence of 1 μM atropine.

Controls for the assay;

Control 1

iii. 400 μl buffer + 50μl the membrane suspension, vortex 10 seconds

iv. 50 μl L-methyl-[3H]-scopolamine, vortex mix for 10 seconds

Control 1 values on the scintillator print-out represent the dpm count of non-specific bindings of L-methyl-[3H]-scopolamine to the membranes.

Control 2

iii. 350µl buffer + 50µl the membrane suspension, vortex 10 seconds

iv. 50µl the non-specific radioligand and 50µl the specific displacer (atropine), vortex 10 seconds

A percentage of displacement produced by oil was calculated as in section 2.5.3.2.2.

Chapter 3. Steam distillation. Anti-cholinesterase activity of oils of *Salvia* species

3.1. Introduction

S. fruticosa, *S. officinalis* and *S. lavandulaefolia* are some of the most common species of the genus widely growing throughout the Mediterranean and Middle East (Giannouli and Kintzios, 2000; Appendix 1). The plants have aromatic leaves and have been used for production of essential oils. The oils of sage are chemically complex mixtures, often containing more than 100 individual components that can be grouped into volatile and non-volatile fractions. A volatile fraction constitutes 90-95% of the whole oil and contains monoterpenoids and sesquiterpenoids hydrocarbons and their oxygenated derivatives along with aliphatic aldehydes, alcohols and esters. A non-volatile residue contains fatty acids and carotenoids (Waterman, 1993; Giannouli and Kintzios, 2000). In addition to flavouring foods, the oils act as antioxidants and preservatives against food spoilage, while a broad range of application in aromatherapy and health care has been observed during the last fifteen years (Hay and Waterman, 1993).

Gas chromatography, combined with mass spectrometry, is used to analyse the chemical composition of oils. The major constituents of oil of *S. lavandulaefolia* are α-pinene, 1,8-cineole, camphor (Marcos *et a*l., 1988) whereas oil of *S. officinalis* is abundant in thujone, camphor, 1,8-cineole, borneol, and α/β pinenes (Holla and Vaverkova, 1993; Bellomaria *et al.*, 1992) and oil of *S. fruticosa* is rich in 1,8-cineole, camphor, borneol, β-caryophyllene and α/β pinenes (Bayrak and Akgul, 1987). The chemical profile of oils within a particular species may vary. Thus, according to Perry *et al.* (1999) a content of thujones and camphor in oil of *S. officinalis* at the flowering stage is the minimum but

the amount of β-pinene and 1,8-cineole is the maximum. Stems have low oil contents in comparison with leaves and flowering parts of the plant. Moreover, Langer *et al.* (1996) reported that oils of aged leaves of *S. officinalis* and *S. fruticosa* contain less α-thujone but more camphor. Bellomaria *et al.* (1992) reported that oil of *S. fruticosa* may contain 42% camphor and 20% 1,8-cineole if collected in the Troodos area of Cyprus but 55% 1,8-cineole and 6% camphor if harvested in the Anglisidhes and Stavros areas of the island. Oils of *S. fruticosa* from western Crete contain low amounts of camphor and high of 1,8-cineole, while the oils from the central part of Crete are equally abundant in camphor and 1,8-cineole (Karousou *et al.*, 2000). In addition, Venskutonis (1997) reported that drying fresh leaves of *S. officinalis* at 30 ^{0}C does not affect the total contents of volatile compounds, as compared with freeze-drying, whereas drying at 60 ^{0}C gives losses of 31%, mainly of non-oxygenated monoterpenoids. Fadel and El-Massry (2000) also reported that the yield of monoterpene hydrocarbons increases after drying, while monoterpene esters decreases from 24.8% in fresh leaves to 1.8% in dry leaves.

Oils of the plants exhibit a wide range of bioactivities (Appendix 1) including those of CNS relevant to treatment of dementia (1.3.1.4.4.). A role of acetyl-and butyl-cholinesterases in the normal and demented brain has been described in Chapter 1 (1.2.1.2.; 1.3.). The Chapter (1.3) also explains the significance of inhibition of cholinesterases in the treatment of the disease. As there are no reports on anti-BuChE activity of any species of *Salvia*, the purpose of this study was to determine *in vitro* whether essential oils of the genus contribute to inhibition of BuChE. Parallel AChE inhibitory activity was also investigated.

3.2. Results

Anti-BuChE activity of the oils over a range of pre-incubation times (5 to 90 minutes) is shown in Table 3.1. Quinidine, a selective inhibitor of BuChE used as a positive control, inhibited BuChE (5 min incubation) with an IC_{50} value of 6.8×10^{-4} mg ml^{-1} (0.9μM).

Table 3.1. Anti-butyrylcholinesterase activity of oils of *Salvias* in relation to incubation time

Oil	IC_{50}, mg/ml[a] or Incubation, %[b] mean±SD (n=6)[c]			
	5 min	30 min	60 min	90 min
S. fruticosa (7/01)	0.15±0.007†	0.06±0.01†	0.04±0.01†	0.035±0.016†
S. fruticosa (8/02)	10±4 (0.3 mg/m)‡	14±5 (0.3 mg/ml)‡	17±4 (0.3 mg/ml)‡	21±8 (0.3 mg/ml)‡
S. officinalis "*purpurea*" (6/02)	35±3 (0.3 mg/ml)‡	0.22±0.015†	0.24±0.02†	0.2±0.017†
S. officinalis "*purpurea*" (9/02)	0.14±0.007†	0.09±0.01†	0.074±0.015†	0.06±0.018†
S officinalis L.	0.33±0.01†	0.24±0.014†	0.21±0.017†	0.23±0.02†
S. lavandulaefolia	26±5 (0.2 mg/ml)‡	23±4 (0.2 mg/ml)‡	26±5 (0.2 mg/ml)‡	18±6 (0.2 mg/ml)‡

[a]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [b]Inhibitory activity of oils which did not reach 50% enzyme inhibition. The percent activity corresponds to the solubility limit of oil. [c]Each oil was tested in triplicate. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of six sets of triplicate using a dose response equation of each triplicate. †-corresponded to [a]. ‡-corresponded to [b].

There was an increase in the potency of oils during this period. The IC_{50} value of *S. fruticosa* oil (7/01) decreased from 0.15±0.007 mg/ml with 5 minutes incubation to 0.035±0.016 mg/ml with 90 minutes incubation, whereas *S. fruticosa* oil (8/02) had no marked activity. The potency of oil of *S. officinalis* var. "*purpurea*" (9/02) increased from 0.14±0.007 to 0.06±0.018 mg/ml at 5 and 90 minutes respectively. Oil of *S.*

officinalis var. "*purpurea*" (6/02) reached 50% inhibition after 30 minutes of incubation. Oils of *S. officinalis* and *S. lavandulaefolia* showed no increase in inhibition during any period of pre-incubation tested. Extracts of *S. fruticosa* (7/01) and *S. officinalis* var. "*purpurea*" (9/02) had the most obvious time dependent increase in inhibition of the enzyme as compared with other extracts. Anti-AChE activity of the oils over a range of incubation time is shown in Table 3.2. Physostigmine, a selective inhibitor of AChE used as a positive control, inhibited the enzyme (5 min incubation) with an IC_{50} value of 1.4×10^{-5} mg ml^{-1} (0.04 μM).

Table 3.2. Human anti-acetylcholinesterase activity of oils of *Salvias* in relation to incubation time

Oil	IC_{50}, mg/ml[a] mean±SD (n=6)[b]			
	5 min	30 min	60 min	90 min
S. fruticosa (7/01)	0.05±0.005	0.06±0.004	0.05±0.008	0.06±0.013
S. fruticosa (8/02)	0.04±0.003	0.055±0.006	0.06±0.01	0.06±0.015
S. officinalis "*purpurea*" (6/02)	0.1±0.006	0.14±0.01	0.18±0.01	0.24±0.02
S. officinalis "*purpurea*" (9/02)	0.04±0.008	0.03±0.01	0.02±0.01	0.03±0.015
S officinalis L.	0.07±0.01	0.05±0.014	0.065±0.01	0.08±0.02
S. lavandulaefolia	0.053±0.007	0.06±0.01	0.08±0.013	0.12±0.023

[a]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [b]Each oil was tested in triplicate. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of six sets of triplicate using a dose response equation of each triplicate.

Oils of *S fruticosa* (7/01) and *S. officinalis* var. "*purpurea*" (9/02) had apparent dual cholinergic activity, namely they were active on both enzymes within the incubation time, while the duality of oil *S. officinalis* was less apparent (Table 3.1 and 3.2).

In contrast to inhibition of BuChE, there was no increase in inhibitory activity within these periods although the oils of *S. officinalis* var. "*purpurea*" (6/02) and *S. lavandulaefolia* showed apparent decrease from 0.1±0.006 and 0.053±0.007 mg/ml at 5 minutes to 0.24±0.02 and 0.12±0.023 mg/ml at 90 minutes incubation respectively. Oil of *S. officinalis* var. "*purpurea*" (9/02) had the lowest IC_{50} values throughout the course of incubation.

Table 3.3. Chemical composition of oil of *Salvia* species

Compound[a]	RT[b], min	*S. fruticosa* % in oil[c]		*S. officinalis purpurea,* % in oil[c]		*S.officinalis* L. % in oil[c]	*S .lavandu laefolia* % in oil[c]
		7/01	8/02	6/02	9/02		
α-pinene	12.61	--	2.4	--	--	--	--
Camphene	13.2	--	5.0	--	--	1.2	--
β-pinene	14.42	--	1.6	0.5	1.2	3.5	--
β-mycrene	15.16	0.6	1.5	--	--	0.8	--
D-limonene	16.66	1.2	3.6	--	--	0.8	2.7
1,8-cineole	16.78	17.6	21.5	2.7	2.8	5.4	17.4
(+)-4-carene	18.81	--	2.2	--	--	--	--
trans-sabinenehydrate	19.6	--	--	0.4	--	--	--
Thujone, isomers	19.8	--	--	8.4	12.7	6.2	--
Unidentified	21.17	--	--	--	--	--	5.9
Camphor	21.29	49.2	49.3	4.8	12.3	11.0	42.5
Bicyclo (3,1,1) heptan-3-one, 2,6,6,-trimehtyl -2α; 5α	21.92	--		2.0	3.1	1.0	--
Borneol	22.1	4.6	1.7	3.7	3.2	8.7	6.1
3-cyclohexen-1-ol, 4 methyl-1-(1-methylethyl)-, (R)	22.55	--	--	--	--	--	2.1
α-terpineol	23.05	--	--	--	--	--	2.1
Unidentified	25.36	--	--	--	--	--	1.2
Bornyl acetate	26.45	2.8	1.5	2.4	2.3	3.1	1.0
Unidentified	26.68	--	--	--	--	--	4.3
(+)-2-carene	28.59	--	--	--	--	--	8.8

		Steam distilled oils					
α-cubenene	28.62	--	--	0.7	--	--	--
Ylangene	29.36	--	--	0.4	--	--	--
Copaene	29.51	--	--	1.0	--	--	--
1-H-cycloprop(e)-azulene, 1a,2, 3,4,4a,5,6,7b-octadydro-1,4,7-tetra methyl-,[1aR-(1aRα...)]	30.63	--	--	1.0	--	1.1	--
Caryophyllene	30.95	11.9	6.6	3.0	2.6	2.4	1.0
Germacrene D	31.24	--	--	1.3	1.0	1.3	--
1H-cycloprop(e)-azulene,deca-hydro 1,1,7-trimethyl-4-methylene -, (1a,a)	31.56	2.8	1.1	2.0	1.4	5.3	--
α-caryophyllene	32.02	0.8	--	32.5	24.2	23.2	--
Naphthalene: 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-(1α., 4a.α., 8a.α)	32.8	--	--	2.8	2.3	2.1	--
1H-cycloprop(e) azulene, 1a,2,3, 5, 6,7,7a,7b-octahydro-1;1,4,7-tetra methyl-, (1aR-(1a.α,7α,7aβ,7bα)	33.3	1.2	--	2.3	1.8	3.5	--
Naphthalene: 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-(1α., 4aβ., 8a.α)	33.87	--	--	1.4	1.0	1.0	--
Naphthalene: 1,2,3,5,6,8a,-hexa hydro -4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	34.14	--	--	3.9	3.0	2.7	--
Caryophyllene oxide	34.95	--	0.6	--	--	--	
4aH-cycloprop(e)azulene-4a-ol	35.51	--	--	2.1	1.6	1.4	--
(-)-Spathulenol	35.79	0.8	--	1.0	--	1.0	--
Ledol (isomers)	36.2	--	--	9.4	6.8	1.5	--
Guaiol	36.4	1.2	0.7	--	--	--	--
Unidentified	48.18	--	--	3.4	2.1	0.8	--

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Relative percentage as calculated from a total area of peaks on a basis of 1 % of a major peak

-- not detected

Table 3.3 shows the chemical composition of essential oils of *Salvia* species obtained by steam distillation. The major constituents of the oils of *S. fruticosa* from different origins were 1,8-cineole, camphor and caryophyllene. Oils of *S. officinalis* var. "*purpurea*" had α-caryophyllene (α-humulene), camphor and thujone as the main constituents. Oil of *S. officinalis* was also rich in α-caryophyllene, camphor, borneol and thujone, whilst camphor, 1,8-cineole and 2-carene were abundant in oil of *S. lavandulaefolia.*

3.3. Discussion

3.1. Anti-cholinesterase activity of oils

The main finding of the present study was that oils obtained from species of *Salvia* inhibit BuChE in a time dependant manner. Anti-BuChE activity of the oils differed among the species investigated. Oils of *Salvia fruticosa* (7/01) and *Salvia officinalis* var. *"purpurea"* (9/02) showed an apparent increase in the inhibition of BuChE during the course of time. Their potencies increased four and two folds respectively. Volatility and thermo-instability of the oils restricted the course of incubation time at 30 ^{0}C, although Ogura *et al.* (2000) reported that rivastigmine, a non-volatile chemical, reached its maximum inhibition of BuChE after six hours.

Perry *et al.* (2000 b) reported *in vitro* uncompetitive, reversible inhibition of erythrocyte AChE by essential oil of *S. lavandulaefolia* and Perry *et al.* (2002) also showed *in-vivo* that the oil inhibits brain AChE in rats. This study confirmed that the oils of *Salvia* species readily inhibit human AChE. The activity of the oils did not significantly change in the course of the incubation times with the exception of oils of *S.*

lavandulaefolia and *S. officinalis* var. "*purpurea*" (6/02) for which inhibition declined within the time. In contrast, physostigmine increases inhibition of AChE within this period of incubation time (Perola *et al.*, 1997; Ogura *et al.*, 2000). Molecules of the oils may not only dynamically modulate accessibility of the substrate to the active site (McCammon and Northrup, 1981) but also cause steric and/or allosteric effects on the latter (Tai *et al.*, 2002).

The presence of dual anti-cholinesterase activity in plants may be explained as a defensive mechanism against herbivores (Schmeller *et al.*, 1997). Li *et al.* (2000) demonstrated that BuChE is not a minor but an abundant cholinesterase in mammalian tissues. Gnagey *et al.* (1987) and Marcel *et al.* (1998) also reports that the ChE in invertebrates of *Drosophila melanogaster* and *Caenorhabditis elegans* may exist in an intermediate form, which would resemble some characteristics with both human cholinesterases.

Extracts with preferential cholinergic activity could potentially be used therapeutically according to severity of AD. Thus, oils of *S. fruticosa* (7/01) and *S. officinalis* var. "*purpurea*" (9/02), which have dual anti-ChE activity, may be appropriate to patients with those forms of the disease, where the level of AChE has not yet significantly declined (Davies *et al.*, 1999) but a possibility that BuChE could hydrolyse ACh exists (Mesulam *at al.*, 2002 b), i.e., a moderate stage. The oils of *Salvia lavandulaefolia* and *Salvia officinalis* "*purpurea*" (6/02) may have potential for therapeutic use by dementia patients with mild stage of the disease, where Km of ACh is low enough to keep BuChE inactivated.

A double blind, randomised and placebo-controlled clinical trail of 45% EtOH extract of *S. officinalis* showed improvement in cognition and agitation in patients with mild to moderate AD (Akhondzadeh *et al.*, 2003). Moreover, Perry *et al.* (2002) reported *in–vivo* that essential oil of *S. lavandulaefolia* inhibits brain AChE activity in the striatum and hippocampus in rats at doses of 20 µl and 50 µl. Thus, if the effect of the trial is attributed to inhibition of cholinesterases then dual cholinergic activity of the oils may become clinically relevant.

3.3.2. Species variation

Anti-BuChE activity of oils of *S. fruticosa* (7/01) and *S. fruticosa* (8/02) differed significantly, although anti-AChE activity did not. The GC/MS analysis of chemical composition of these oils shows lack of certain monoterpenoids, namely α-pinene, camphene, β-pinene and (+)-4-carene in the oil of the plant collected in July, 2001. Oil of *S. fruticosa*, as confirmed in this study, may contain 42% camphor and 20% 1,8-cineole if the foliage is collected in the Troodos area of Cyprus (Bellomaria *et al.*, 1992). The amounts of these major constituents were similar in both oils. The structural differences of the enzymes (1.2.1.2.; 1.3) allow the suggestion that anti-BuChE activity of *S. fruticosa* (7/01) oil is attributed to synergistic interactions between its minor constituents rather than camphor or 1,8-cineole only, while anti-AChE activity may be down to one of these molecules alone. This suggestion can also be supported by the fact that oil of *S. lavandulaefolia* did not inhibit BuChE, despite being abundant in camphor and 1,8-cineole, but AChE.

Oils of *S. officinalis* and *S. officinalis* var. "*purpurea*" (6/02) and (9/02) were rich in α-caryophyllene (syn., α-humulene) but had evenly less camphor and 1,8-cineole than oils

of *S. fruticosa* and *S. lavandulaefolia*. The GC/MS analysis of these oils shows no striking differences in their chemical profiles. Dual anti-ChE activity of oil of *S. officinalis* var. "*purpurea*" (9/02), but not the others, may also be explained via synergistic interactions of its minor constituents. Anti-AChE activity of all three oils, despite a low amount of one of the main compounds of *Salvia* species camphor and 1,8-cineole (Karousou *et al.*, 2000; Appendix 1, *Salvia*'s database), may be explained via abundant presence of α-caryophyllene. In addition, all oils of *S. officinalis* contained from 6.2% to 12.7% toxic thujone isomers. β-thujone is regarded as less toxic than α-thujone (Hold *et al.*, 2000). Hold *et al.* (2000) reported that α-thujone acts as a non-competitive agonist to the γ-aminobutyric acid type A ($GABA_A$) receptor in mice. Nevertheless, the International Organisation for Standardisation allows 18-43% α-thujone in oil of *S. officinalis* (ISO, 1997).

Chapter 4. Supercritical fluid extraction. Anti-cholinesterase activity of extracts of *Salvia* species

4.1. Introduction

Traditional methods of extraction of essential oils such as steam distillation and organic solvent extraction have several limitations. Organic solvents are flammable, toxic and environmentally unfriendly, while steam distillation requires conditions where some components may be lost through thermal degradation, hydrolysis or volatilization (Luque de Castro *et al.*, 1999). Misharina *et al.* (2003) showed that storage of steam distilled oil of *Majorana hortensis* M. in the light causes chemical transformation of terpenoids. This transformation may be initiated by high temperature during the extraction process and further supported via photo-oxidation. The implementation of new extraction techniques which may reduce the risk of biodegradation of terpenoids during the extraction and storage is therefore warranted.

4.1.1. Principle of supercritical fluid extraction

Alterations in temperature and pressure change the physical state of a substance between solid, liquid and gases; these states have marked differences in their densities. However, at a certain temperature and pressure (the critical point), there is no difference in density between the liquid and gaseous forms of the substance and it exists as a fluid that does not condense or evaporate. This is termed a supercritical fluid. The critical temperature and pressure above which supercritical fluids are formed vary with the substance and with its purity. For water the values are 374 ^{0}C and 220 atmospheres respectively, while for carbon dioxide the corresponding figures are 31 ^{0}C and 74

atmospheres respectively (Houghton and Raman, 1998). The use of carbon dioxide for the separation of compounds was suggested more than 60 years ago (Pilat and Godlewicz, 1940) but it was not applied before Stahl *et al.*, (1980) demonstrated that the supercritical stage of the gas can be used to extract natural products. Later Kusova and Vetrov (1987) used dichlorodifluoromethane (Freon 12), with the boiling point of -29 ^{0}C, in supercritical fluid extraction to obtain oil of *Elaeagnus angustifolia* L. with wound healing properties, while supercritical CO_2 was used to remove caffeine from tea and coffee (Corr, 2002).

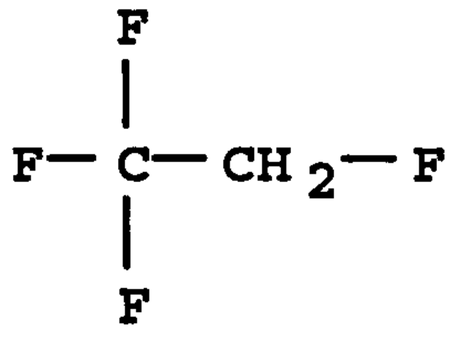


Figure 4.1. 1,1,1,2-Tetrafluoroethane.

Other names: 1,2,2,2-Tetrafluoroethane; AK 134a; Arcton 134a; Ecolo Ace 134a; F 134A; FC 134a; Forane 134a; Freon 134a; Fron 134a; HC 134a; HCFC 134a; HFA 134a; HFC 134a; Khladon 134a; Norflurane; R 134a; Refrigerant R 134a; SUVA 134a; TG 134a

1,1,1,2-tetrafluoroethane (Figure 4.1) has been developed as a substitute for fully and partially halogenated chlorofluorocarbons and hydrochlorofluorocarbons (trichlorofluoromethane Freon 11 and Freon 12), which have been showed to contribute to the stratospheric ozone layer depletion (Mayer *et al.*, 1995) and climate forcing. Degradation of 1,1,1,2-tetrafluoroethane occurs mainly in the troposphere by reaction with hydroxyl radicals leading to production of trifluoroacetic, formic, hydrofluoric acids and carbon dioxide. The gas has a global warming potential of 0.3 comparative to a reference value of 1.0 for Freon 11 (Mayer *et al.*, 1995). It is a non-flammable,

colourless, benign gas with a boiling point of -26.2 ^{0}C and a faint ethereal odour (Advanced Phytonics Ltd., chemical safety data sheet). Industrial and research applications of 1,1,1,2-tetrafluoroethane have recently been reviewed Corr (2002).

4.1.2. Extraction with Phytosol®

Wilder (1994) showed that extraction of botanicals with 1,1,1,2-tetrafluoroethane (Phytosol A) at ≤40 mbar (under pressure and at room temperature the gas liquefies and exhibits solvent properties) results in a higher yield of natural fragrant oils and a lower cost of production in comparison with steam distillation, solvent and carbon dioxide extractions. Thus, Rodrigues *et al.* (2003) reported that the best extraction rates which give a maximum yield of oil of *Pimpinella anisum* Linn., using supercritical carbon dioxide are 180 bar at 30 °C. From the economical point of view such differences in pressure requirements are in favour of Phytosol A. While supercritical CO_2 can produce high quality extracts with minimal solvent residues, the cost is prohibitive for many products due to the high capital cost of equipment rated for the high operating pressures (Corr, 2002).

Due to the relatively low polarity of Phytosol A a range of botanicals (*e.g.*, nitrogen containing molecules) extracted from a plant material may be restricted. Nevertheless, Nicola (1998) suggested that a combination of Phytosol A with a polar co-solvent enables extraction of at least one relatively polar component. A mixture of the hydrofluorocarbon and carbon dioxide may also increase the polarity of the supercritical medium (Abbot *et al.*, 2000). In addition, Advanced Phytonics Ltd., U.K., produces Phytosol A in a combination with butane/isobutene (Phytosol B) and dimethylether

(Phytosol D). Although, the three Phytosols® have some polarity differences, they are essentially designed for the extraction of non-polar materials. Extraction of *Salvia miltiorrhiza* with Phytosol A, B and D provided extracts with 0.031%, 0.029% and 0.033% of Tanshinone IIA (Appendix 1, database of active principals of *Salvia*) respectively and it was an equivalent to that obtained via supercritical carbon dioxide extraction, namely 0.031% (Dean *et al.*, 1998).

Moreover, Cerny (2000) reported that extract of *Rosmarinus officinalis* L. obtained using Phytosol A has a characteristic rosemary-like odour. The author also noted that plant waxes and triglycerides are not soluble in the solvent. In addition, in a six weeks pilot open-label study involving oral administration of *Salvia lavandulaefolia* oil, obtained by Phytosol A extraction, to patients with AD there was a statistically significant reduction in neuropsychiatric symptoms and an improvement in attention without adverse effects with the exception of increased blood pressure in two patients (out of eleven) with a history of hypertension (Perry *et al.*, 2003).

Evaporating Phytosol A as a solvent from a plant material at – 26.2 ^{0}C under a modest pressure may facilitate optimal extraction of molecules with cholinergic activities relevant to dementia therapy. In contrast the use of steam distillation may involve the loss of such molecules through hydrolysis or thermolysis and further photo-oxidation during storage (Misharina *et a*l., 2003). Furthermore, an anaerobic environment created by supercritical fluid extraction minimises the effects of oxidation and rancidity (Corr, 2002).

The purpose of this study was to investigate *in vitro* cholinergic activity of extracts of *Salvia* species (24 extracts) obtained via Phytosol A extraction and to analyse the bioactivity of the extracts in relation to their chemical compositions in order to identify a possible chemical fingerprint associated with maximum cholinergic activity. Monitoring changes in a chemical profile of the extracts during storage and comparing it with the activity of the latter may also help to identify such fingerprint which can be a useful marker in the quality control. In addition, the kinetic studies were conducted to describe a level of affinity and the biding mechanism between the extracts and both cholinesterases.

4.2. Results

4.2.1. Anti-cholinesterase activity and chemical composition of *Salvia* extracts

Extracts of twenty four species of sage were screened for anti-BuChE and anti–AChE activities and analysed for their chemical compositions. Quinidine, a selective inhibitor of BuChE, inhibited the enzyme with an IC50 value of 6.8×10^{-4} mg ml^{-1} (0.9μM), while physostigmine, a selective inhibitor of AChE, reached this value at 1.4×10^{-5} mg ml^{-1} (0.04μM). For the convenience of reading this Chapter contains information only on major constituents of the extracts, while Appendix 3 shows their whole chemical profiles.

4.2.1.1. Effects of storage time

Effects of storage time (Chapter 2, stored in sealed amber glass vials under oxygen free nitrogen at 5 ^{0}C) on the inhibitory activity of extracts of *S. apiana, S fruticosa, S. officinalis* var. *purpurea* and *S. corrugata* were also investigated. Table 4.1 shows that anti-BuChE activity of *S. apiana* oil increased 6 times, from IC_{50} values of 0.18±0.035

mg ml^{-1} to 0.03±0.002 mg ml^{-1}, during 70 days of storage, while anti-AChE activity did not significantly change. Increases in anti-BuChE activity of oils of *S. fruticosa* and *S. officinalis* var. *purpurea* were both 2.5 times but anti-AChE activity was reduced 2 times and 2.5 times respectively during the storage. In contrast, *S. corrugata* extract was a selective inhibitor for AChE with an IC_{50} value of 0.009 mg ml^{-1} in 30 days of extraction and 0.02 mg ml^{-1} in 100 days of extraction (Table 4.1). In the extract anti-AChE activity was two-fold reduced.

Table 4.1. Anti-cholinesterase activity of *Salvia* extracts in relation to storage time

Extract	Yield %	IC_{50}[c] mg ml^{-1} or Inhibition, %[d] mean±SD (n=4)[f]			
		30 days[g] BuChE	100 days[h] BuChE	30 days[g] AChE	100 days[h] AChE
S. apiana (light amber oil)[a]	1.4[b] (1.8)	0.18±0.035[†j]	0.03±0.002[†j]	0.04±0.005[†k]	0.03±0.01[†k]
S. fruticosa (amber oil)[a]	1.0[b] (1.25)	0.05±0.01[†j]	0.02±0.001[†j]	0.018±0.001[†]	0.043±0.008[†]
S. officinalis var. *purpurea* (amber oil with a tint of green)[a]	0.53[b] (0.57)	0.037±0.003[†j]	0.015±0.004[†j]	0.02±0.004[†]	0.05±0.004[†]
S. corrugata (reddish resin)[a]	0.5[b] (0.5)	16±2.4% [0.05 mg ml^{-1}][‡k]	20±3.6% [0.05 mg ml^{-1}][‡k]	0.009±0.004[†]	0.02±0.006[†]

[a]Appearance of extract (inhibitor). [b]Yield of extract based on one batch extraction. Total yield of the extraction, which is in some cases comprised oil and a precipitated residue, is shown in brackets. [c]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [d]Inhibitory activity of extracts which did not reach 50% enzyme inhibition. The percent activity corresponds to the solubility limit of oil. [f]Each oil was tested in triplicate. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of four sets of triplicate using a dose response equation of each triplicate. [g]30 days old extracts. [h]100 days old extracts. †-corresponded to [c]. ‡-corresponded to [d]. [†j]Statistically significant ($P<0.05$). [‡k, †k]Statistically insignificant ($P>0.05$).

An analysis of the chemical composition of oils of *S. apiana* and *S. fruticosa* revealed no significant changes between major constituents during the same storage time and conditions (Table 4.2), although changes were apparent among minor constituents (Appendix 3). For example, the amount of limonene in 30 days old oil of *S. apiana* was 5% but after the storage the compound became undetectable, while an unidentified minor constituent (2.2%) with the retention time of 16.4 minutes was present in 100 days old oil (Appendix 3). Moreover, β-pinene (0.7%) and caryophyllene oxide (1.1%) were only found in 100 days old oil of *S. fruticosa*, while benzene,1-methyl- 2-(1-methylethyl) (1%) was detected only in 30 days old oil.

Table 4.2. Major constituents[c] of extracts of *Salvia* species and effects of storage time

Compound[a]	RT, min[b]	*S. apiana*, % in oil[c]		*S. fruticosa*, % in oil[c]		*S. officinalis* var. *purpurea*, % in oil[c]	*S. corrugata* % in oil[c]
		30 day[d]	100 day[f]	30 day[d]	100 day[f]	30 day[d]	30 day[d]
1,8-Cineole	16.59	34.4	32.6	12.5	13.6		
Camphor	21.09	38.6	38.5	60.8	58.5		
α-caryophyllene	31.84					46.5	
Unidentified	71.15						20.6
Unidentified	72.28						66.7

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Constituents with no less than 10% in extracts as calculated from a total area of peaks on a basis of 1 % of a major peak

[d]Extracts with 30 days shelf-life

[f]Extracts with 100 days shelf-life

Freshly prepared *S. fruticosa* oil was used to analyse the effect of storage time after 7 and 37 days of extraction. Table 4.3 shows changes in anti-cholinesterase activity of oil and 70% EtOH extract of *S. fruticosa* during 30 days of storage time. After this period anti-BuChE activity of the oil increased 5 times, while the activity of ethanol extract did not change. The oil, after 30 days of storage, also showed a two times decrease in anti-AChE activity, whereas a change in the activity of ethanol extract was not significantly apparent. Anti-BuChE activity of the oil after 37 days of extraction, with an IC50 value of 0.02±0.03 mg ml^{-1}, was similar to that of 100 days, namely 0.02±0.001 mg ml^{-1} (Table 4.1). Anti-AChE activity of 37 days old *S. fruticosa* oil, with an IC50 value of 0.045±0.002 mg ml^{-1}, was also alike to that of 100 days, *i.e.*, 0.043±0.008 mg ml^{-1} (Table 4.1).

Table 4.3. Anti-cholinesterase activity of *S. fruticosa* extracts in relation to seven and thirty seven days of storage time

Method of extraction	IC_{50}[a] mg ml^{-1}, mean±SD (n=4)[b]			
	AChE		BuChE	
	7days	37 days	7 days	37 days
Phytosol A[c]	0.023±0.002[f]	0.045±0.002[f]	0.1±0.01[f]	0.02±0.03[f]
70% EtOH[d]	0.1±0.005[g]	0.09±0.01[g]	0.05±0.005[g]	0.054±0.002[g]

[a]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [b]Each oil was tested in triplicate. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of four sets of triplicate using a dose response equation of each triplicate. [c]Phytosol A extraction provided amber colour oil. [d]Ethanol extract, as a source of more polar compounds than the oil, was used for a comparison purpose. [f]Statistically significant ($P<0.05$). [g]Statistically insignificant ($P>0.05$)

4.2.1.2. Analyses of thirty days old extracts

Table 4.4 shows anti-cholinesterase activity of the oils of sage which reached 50% inhibition of both cholinesterases within their solubility limits. The chemical composition of constituents with no less than 10% in the oils is shown in Table 4.5, while the whole chemical profile of the oils is demonstrated in Appendix 3. Oils of *S. apiana*, *S. fruticosa*, *S. officinalis* var. *purpurea* (Table 4.1), *S. microphylla* var. *neurepia*, *S. africana-lutea*, *S. officinalis*, *S. involucrata*, *S. argentea* and *S. divinorum* (Table 4.4) had potent dual anti-cholinesterase activity, whereas anti-BuChE activity of *S. lavandulaefolia* oils was less apparent. In addition, Table 4.5 shows no obvious pattern between major compounds present in the oils and their dual activity.

Table 4.6 shows the extracts with less apparent dual anti-cholinesterase activity. Constituents with no less than 10% in these extracts are shown in Table 4.7, while the whole chemical profile is demonstrated in Appendix 3. *S. confertiflora* oil, with an IC_{50} value of 0.03±0.005 mg ml^{-1}, was a selective inhibitor for AChE. Oils of *S. glutinosa* and *S. napifolia* inhibited BuChE with IC_{50} values of 0.04±0.006 mg ml^{-1} and 0.08±0.01 mg ml^{-1} respectively, although a solubility limit of the oils did not allow preparation of higher concentrations which might result in 50% inhibition of human AChE. Thus, *S. glutinosa* oil showed 31±3.1% inhibition and *S. napifolia* 18±3.3% inhibition of AChE at final concentrations of 0.04 mg ml^{-1} and 0.008 mg ml^{-1} respectively. A solubility limit of *S. longistyla* oil also restricted preparation of its higher concentrations, which might result in 50% inhibition of BuChE.

Table 4.8 shows extracts of sage which did not reach 50% inhibition of ChEs. Oil of *S. discolo*r was an ineffective inhibitor for both enzymes, while oils of S. *atrocyanea*, *S.*

sclarea and *S. verbenaca* were ineffective only for BuChE. A solubility limit of the rest of extracts did not allow preparation of its higher concentrations, which might result in 50% inhibition of the enzymes. Table 4.9 shows constituents with no less than 10% in the extracts, while Appendix 3 demonstrates their whole chemical profiles.

Table 4.4. *Salvia* oils with apparent dual anti-cholinesterase activity

Oil	Yield %	IC_{50}[c] mg ml^{-1} mean±SD (n=4)[d]	
		BuChE	AChE
S. africana-lutea (yellow oil with a tint of green)[a]	0.62 [b] (0.66)	0.06±0.006	0.02±0.002
S. argentea (amber oil)[a]	0.15 [b] (0.26)	0.07±0.01	0.074±0.007
S. divinorum (dark, thick, brawn oil)[a]	0.2 [b] (0.2)	0.06±0.003	0.046±0.01
S. involucrata (thick amber oil)[a]	0.5 [b] (0.5)	0.07±0.003	0.03±0.003
S. keerlii (amber oil)[a]	0.46 [b] (0.46)	0.1±0.02	0.04±0.007
S. lavandulaefolia (amber oil with a tint of green)[a]	1.2 [b] (2.2)	0.16±0.03	0.04±0.001
S. microphylla var. *neurepia* (dark amber oil with a tint of green)[a]	0.22 [b] (0.3)	0.026±0.006	0.035±0.003
S. officinalis (amber oil)[a]	0.53 [b] (0.53)	0.06±0.003	0.015±0.001

[a]Appearance of the inhibitor. [b]Yield of the inhibitor based on one batch extraction. Total yield of the extraction, which is in some cases comprised oil and a precipitated residue, is shown in brackets. [c]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [d]Each oil was tested in triplicate. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of four sets of triplicate using a dose response equation of each triplicate.

Table 4.5. Major constituents of extracts of *Salvia* species with dual anti-cholinesterase activity

Compound[a]	RT, min[b]	% in oil[c]							
		S. africana-lutea	*S. argentea*	*S. divinorum*	*S. involucrata*	*S. keerlii*	*S.lavandulaefolia*	*S. microphylla* var. *neurepia*	*S. officinalis*
α-pinene	12.39						12.4		
1,8-cineole	16.59						16.6		
Camphor	21.09						37.7		16.0
(+)-4-carene	28.41						13.6		
Caryophyllene	30.76		22.6			10.4		12.7	
γ-elemene	31.17					10.0			
α-caryophyllene	31.84								30.4
1H-Cycloprop[e]azulene[†]	32.07	10.0							
β-elemenone	36.35					44.1			
Unidentified	45.49		37.1						
Unidentified	47.91					23.6			
Unidentified	49.28					49.0			
Squalene	63.26					22.1			
Unidentified	70.18					70.5			

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Constituents with no less than 10% in extracts as calculated from a total area of peaks on a basis of 1 % of a major peak.

[†]1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl -4-methylene-,(1aR-[1aα, 4aβ, 7α, 7aβ, 7bα)]

Table 4.6. *Salvia* extracts with less apparent dual anti-cholinesterase activity

Extract	Yield [b] %	IC_{50}[c] mg ml^{-1} or Inhibition, %[d] mean±SD (n=4)[f]	
		BuChE	AChE
S. confertiflora (dark amber oil)[a]	0.13 (0.2)	9±6.7 [0.05 mg ml^{-1}]‡	0.03±0.005†
S. haematodes (amber oil)[a]	0.13 (0.17)	29±1.5 [0.05 mg ml^{-1}]‡	0.09±0.03†
S. glutinosa (dark amber oil with a tint of red)[a]	0.2 (0.2)	0.04±0.006†	31±3.1 [0.04 mg ml^{-1}]‡
S. longistyla (brown resin with a tint of yellow)[a]	0.3 (0.3)	40±5.5% [0.04 mg ml^{-1}]‡	19±2.1% [0.01 mg ml^{-1}]‡
S. napifolia (amber oil)[a]	0.14 (0.18)	0.08±0.01†	18±3.3% [0.008 mg ml^{-1}]‡
S. verticilata (dark amber oil)[a]	0.12 (0.12)	38±2.1% [0.045mg ml^{-1}]‡	28±4.6 [0.01 mg ml^{-1}]‡

[a]Appearance of the inhibitor. [b]Yield of the inhibitor based on one batch extraction. Total yield of the extraction, which is in some cases comprised oil and a precipitated residue, is shown in brackets. [c]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [d]Inhibitory activity of extracts which did not reach 50% enzyme inhibition. The percent activity corresponds to the solubility limit of extract. [f]Each oil was tested in triplicate. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of four sets of triplicate using a dose response equation of each triplicate. †-corresponded to [c]. ‡-corresponded to [d]. [g]Extracts tested within 30 days of extraction. [h]Extracts tested after 100 days of extraction.

Table 4.7. Major constituents of extracts of *Salvia* species with less apparent dual anti-cholinesterase activity

Compound[a]	RT, min[b]	% in oil[c]					
		S. confertiflora	*S. haematodes*	*S. glutinosa*	*S. longistyla*	*S. napifolia*	*S. verticilata*
Caryophyllene	30.76	28.1	23.7	23.8	28.2	24.2	10.3
α-caryophyllene	31.84			22.8		10.2	
Germacrene D	32.69	19.0					10.7
1H-cyclopenta[1,3]-cyclopropa[1,2]benzene[†]	32.71					21.0	
Unidentified	34.51						10.7
Unidentified	68.64				17.9		

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Constituents with no less than 10% in extracts as calculated from a total area of peaks on a basis of 1 % of a major peak.

[†]1H-cyclopenta[1,3]-cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-, [3aS-(3aα,3bβ,4β,7α)]

Table 4.8. Anti-cholinesterase activity of *Salvia* extracts restricted by the solubility limit

Extract	Yield [b] %	Inhibition, %[c] mean±SD (n=4)[d]	
		BuChE	AChE
S. atrocyanea (dark amber oil with a tint of green)[a]	0.26 (0.34)	25±1.3% [0.08 mg ml^{-1}]	31±3.9% [0.02 mg ml^{-1}]
S. discolor (amber oil)[a]	0.7 (1.0)	12±3.0 [0.15 mg ml^{-1}]	11±8.5 [0.015 mg ml^{-1}]
S. Jamensis var. la luna (amber oil)[a]	0.2 (0.2)	40±2.9% [0.05 mg ml^{-1}]	34±2.6% [0.03 mg ml^{-1}]
S. sclarea (dark amber resin)	0.2 (0.2)	22±1.9% [0.1 mg ml^{-1}]	40±4.5% [0.02 mg ml^{-1}]
S. stenophylla (dark amber oil)[a]	0.12 (0.14)	33±2.5% [0.06 mg ml^{-1}]	38±2.8% [0.02 mg/ml^{-1}]
S. verbenaca (light amber oil)[a]	0.24 (0.24)	21±1.7% [0.05 mg ml^{-1}]	31±3.6% [0.02 mg/ml^{-1}]

[a]Appearance of the inhibitor. [b]Yield of the inhibitor based on one batch extraction. Total yield of the extraction, which is in some cases comprised oil and a precipitated residue, is shown in brackets. [c]Inhibitory activity of extracts which did not reach 50% enzyme inhibition. The percent activity corresponds to the solubility limit of extract. [d]Each oil was tested in triplicate. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of four sets of triplicate using a dose response equation of each triplicate.

Table 4.9. Major constituents of extracts of *Salvia* species with restricted solubility limit

Compound[a]	RT, min[b]	% in oil[c]					
		S. atrocyanea	*S. discolor*	*S. Jamensis* var. la luna	*S. sclarea*	*S. stenophylla*	*S. verbenaca*
Unidentified	30.747				10.9		
Caryophyllene	30.76			15.7			
Germacrene D	32.69				12.2		
Unidentified	46.54				12.3		
9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-	48.90					33.8	
Squalene	63.26	39.7		22.3			
Unidentified	67.60						13.0
Lup-20(29)-en-3-one	72.27		39.1				
Unidentified	73.24	18.0					
Unidentified	75.43			34.4			

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Constituents with no less than 10% in extracts as calculated from a total area of peaks on a basis of 1 % of a major peak.

4.2.2. Kinetic studies on inhibition of BuChE and AChE by extracts of *Salvia* species

Determination of kinetic constants for inhibition of both cholinesterases is shown in Appendix 2. For the convenience of reading, this Chapter summarises only processed data on the enzymes kinetics.

The Michaelis constant (Km) value and a limiting value of initial enzyme velocity (Vmax) for human BuChE were 0.037 mM and 1.3 nM min^{-1} respectively (Table 4.10; Appendix 2). Oils of *S. apiana*, *S. officinalis* var. *purpurea* and *S. fruticosa* showed non-competitive inhibition of BuChE. The inhibition was a combination of competitive and un-competitive type, with the competitive inhibitor constant (Kie) being less than the un-competitive one (Kies).

Table 4.10. Effect of BuChE inhibitors on Km, Vmax and Ki values

Inhibitor	Inhibitor concentration mg ml^{-1}	Km,[a] mM	Vmax,[a] nM min^{-1}	Kie,[b] mg ml^{-1}	Kies,[c] mg ml^{-1}	Type of inhibition[d]
Control	0	0.037	1.3			
S. apiana	0.08 0.1	0.068 0.08	0.7 0.5	0.025	0.05	non-competitive, mixed (Kies>Kie)
S. officinalis var. *purpurea*	0.0075 0.015	0.043 0.053	0.86 0.5	0.015	0.033	non-competitive, Mixed (Kies>Kie)
S. fruticosa	0.01 0.02	0.051 0.056	0.65 0.5	0.016	0.022	non-competitive, mixed (Kies>Kie)

[a]The apparent Km and Vmax values were calculated by their respective regression equations using a Lineweaver-Burk plot (Appendix 2). [b]The competitive inhibitor constant was determined using Dixon's plot (Appendix 2). [c]The un-competitive inhibitor constant was determined using Dixon's re-plot (Appendix 2). [d]Characterisation of a type of BuChE inhibition was made according to Engel (1981) (Appendix 2).

The Km and Vmax values for AChE were 0.1 mM and 0.97 nM min^{-1} respectively (Table 4.11; Appendix 2). Oil of *S. corrugata* showed a non-competitive, simple type of inhibition, with Kies being equal to Kie. In contrast to non-competitive inhibition of

BuChE, oil of *S. officinalis* var. *purpurea* demonstrated a competitive type of inhibition of AChE, *i.e.*, the oil binds only to the enzyme but not to the substrate. Oils of *S. apiana* and *S. fruticosa* showed non-competitive, mixed inhibition of the enzyme, *i.e.*, Kies>Kie (the oils binds both to the enzyme and the substrate).

Table 4.11. Effect of AChE inhibitors on Km, Vmax and Ki values

Inhibitor	Inhibitor concentration mg ml^{-1}	Km,[a] mM	Vmax,[a] nM min^{-1}	Kie,[b] mg ml^{-1}	Kies,[c] mg ml^{-1}	Type of inhibition
Control	0	0.1	0.97			
S. corrugata	0.0025	0.1	0.9	0.018	0.018	non-competitive, simple (Kies=Kie)
	0.01	0.1	0.78			
S. apiana	0.025	0.12	0.86	0.025	0.125	non-competitive, mixed (Kies>Kie)
	0.1	0.28	0.5			
S. officinalis var. *purpurea*	0.005	0.11	0.97	0.048		Competitive only Kie
	0.02	0.2	0.97			
S. fruticosa	0.02	0.12	0.78	0.043	0.093	non-competitive, mixed (Kies>Kie)
	0.08	0.14	0.51			

[a]The apparent Km and Vmax values were determined by their respective regression questions using a Lineweaver-Burk plot (Appendix 2). [b]The competitive inhibitor constant was determined using Dixon's plot (Appendix 2). [c]The un-competitive inhibitor constant was determined using Dixon's re-plot (Appendix 2). [d]Characterisation of different types of AChE inhibition was made according to Engel (1981) (Appendix 2).

4.2.3. Fractionation of residues from Phytosol A extraction

Extraction of sage species with Phytosol A in some cases (4.2.1.) provided extracts with two phases, *i.e.*, oil and a residue. The residue (hexane treated, Chapter 2) of three non-scented sage species were analysed for their chemical composition and screened for anti-ChE activity. The hexane treated residues of *S. argentea*, *S. napifolia* and *S. yunnanensis* comprised single peaks using GC/MS analysis. Figure 4.2 shows a total ion count of the residue of *S. yunnanensis*. On the figure an identified single peak represents 100% of a total area of peaks. The retention time of the peak was 36.94 minutes. The fraction inhibited human BuChE and human AChE with IC_{50} values of 0.07±0.005 mg ml^{-1} and 0.15±0.03 mg ml^{-1} respectively.

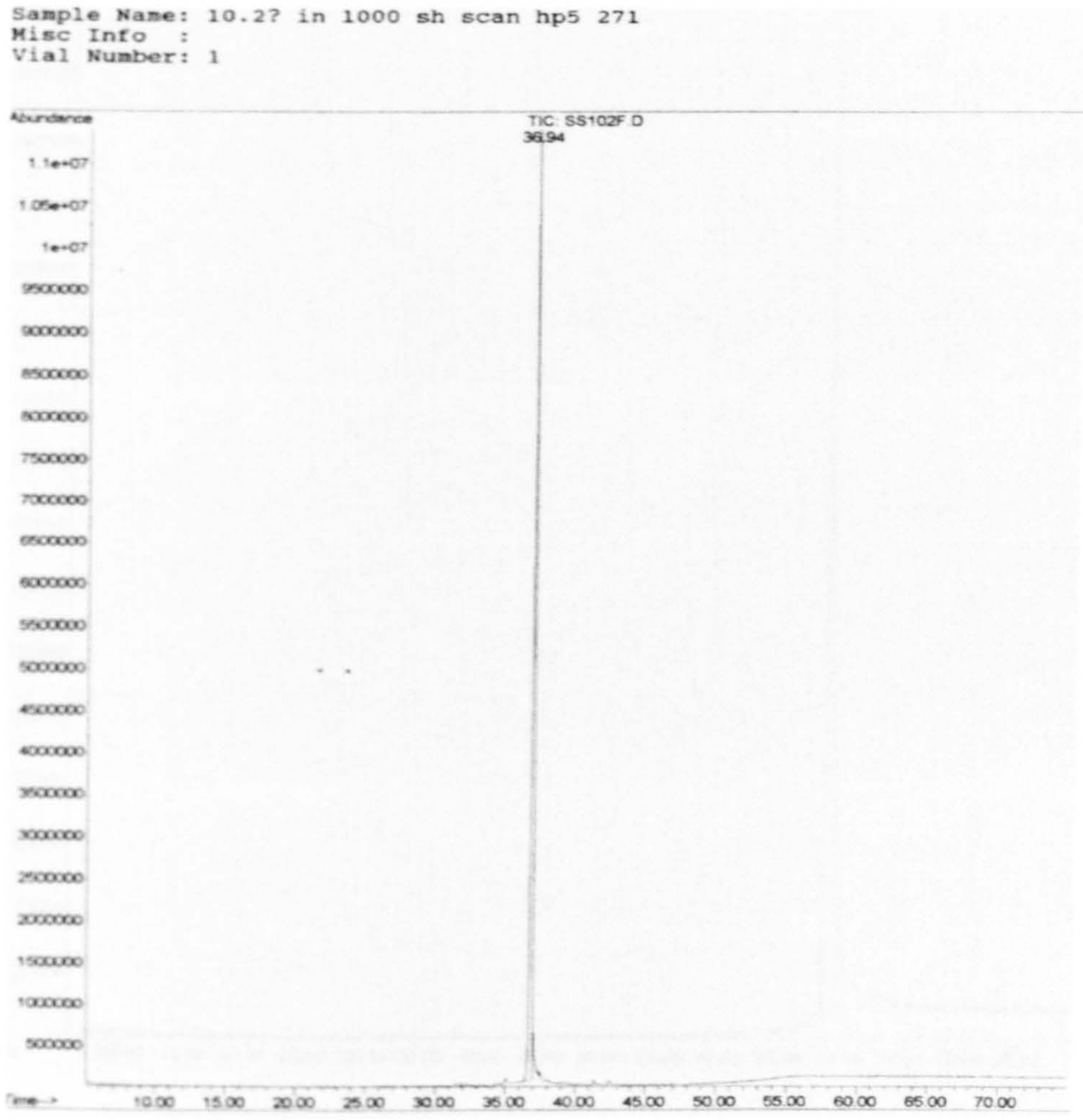


Figure 4.2. Total ion count of hexane treated residue of *S. yunnanensis*.

Figure 4.3 shows a total ion count of the hexane treasted residue of *S. argentea*. On the figure an identified peak, with the retention time of 68.50 minutes, represents 100% of a total area of peaks. This isolated fraction, at a final concentration of 0.1 mg ml^{-1}, gave no inhibition of both cholinesterases.

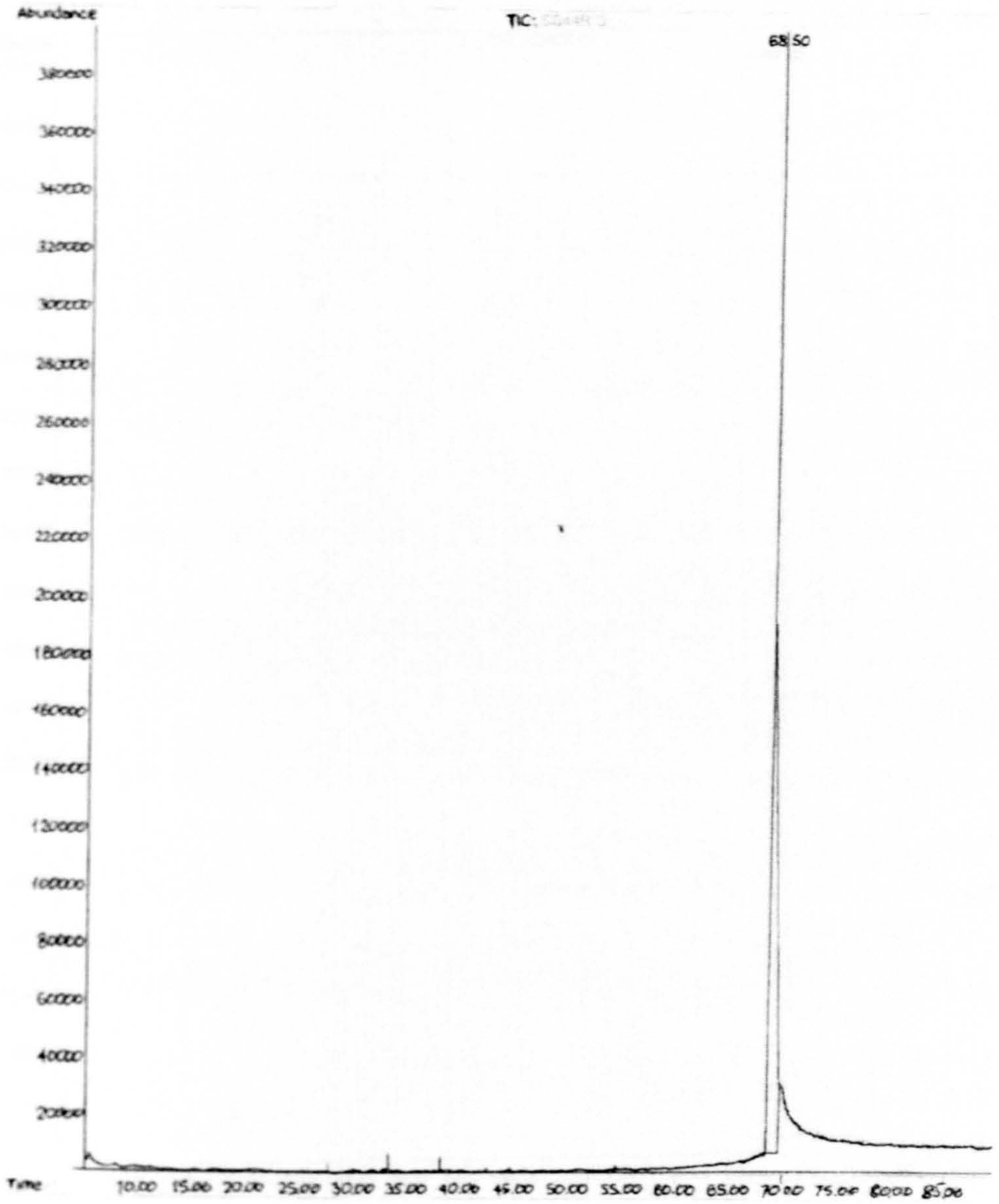


Figure 4.3. Total ion count of hexane treated residue of *S. argentea*.

Figure 4.4 shows a total ion count of the hexane treated residue of *S. napifolia*. On the figure an identified peak, with the retention time of 68.525 minutes, also represents 100% of a total area of peaks. An amount of the isolated fraction was insufficient for carrying out anti-cholinesterase activity tests.

```
File        : M:\SS-OILS\SS46R.D
Operator    : p.d for s.s
Acquired    : 29 Jan 2003  11:11     using AcqMethod SSSCNF
Instrument :   GC/MSD 3
Sample Name: 4.6 res in 1000/1ul inj ss sh scan
Misc Info  :
Vial Number: 26
```

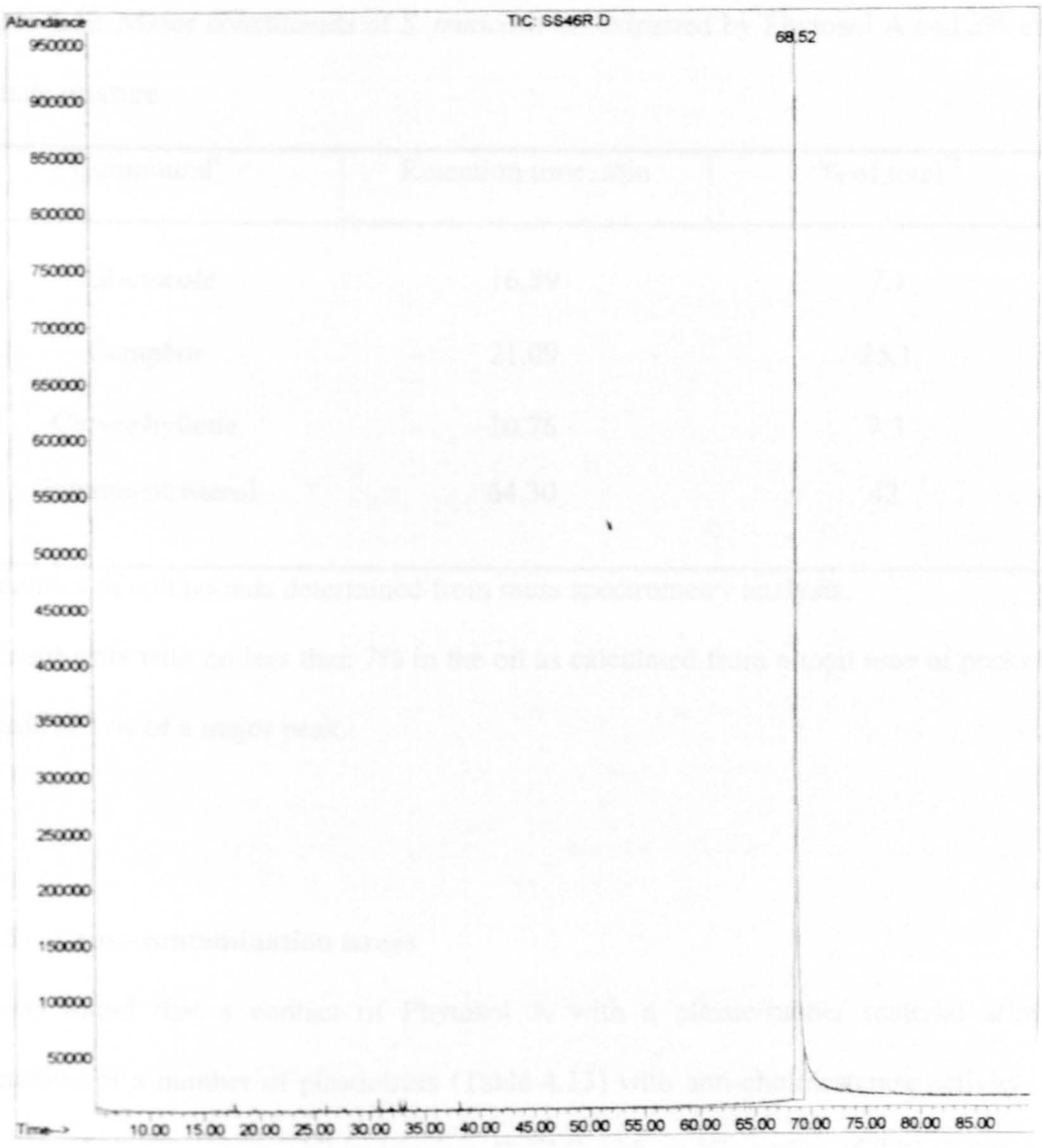


Figure 4.4. Total ion count of hexane treated residue of *S. napifolia*

4.2.4. Extraction of *S. fruticosa* with a mixture of Phytosol A and 3% ethyl acetate

A pilot test of extraction of aerial parts of *S. fruticosa* by a mixture of Phytosol A and 3% ethyl acetate provided a dark amber oil rich in gamma-sitosterol (42%) (Appendix 1 provides database of chemical constituents) and camphor (25.1%). A chemical composition of major constituents in the oil is shown in Table 4.12. It may be noted that gamma-sitosterol was not detected in the oil extracted only with Phytosol A (4.2.1).

Table 4.12. Major constituents of *S. fruticosa* oil extracted by Phytosol A and 3% ethyl acetate mixture

Compound[a]	Retention time, min	% of total [b]
1,8-cineole	16.59	7.1
Camphor	21.09	25.1
Caryophyllene	30.76	7.3
Gamma-sitosterol	64.30	42

[a]Identities of compounds determined from mass spectrometry analysis.

[b]Constituents with no less than 7% in the oil as calculated from a total area of peaks on a basis of 1 % of a major peak.

4.2.5. Cross-contamination issues

It was found that a contact of Phytosol A with a plastic/rubber material allows extraction of a number of plasticizers (Table 4.13) with anti-cholinesterase activity. A mixture of contaminants inhibited human BuChE with an IC_{50} value of 0.01±0.001 mg ml^{-1} and 32±4.2% of human AChE at a final assay concentration of 0.14 mg ml^{-1}.

Hence, any equipment with plastic or rubber surfaces used not only in the extraction process but also to contain the gas should thus be avoided. Chapter 2 (Materials and Methods) shows that the equipment used in the extraction process was plastic/rubber free as well as the cylinder containing the solvent. Thus, a hand set extraction kit (Chapter 2) supplied by Advanced Phytonics Ltd., was modified by replacing a rubber ring with polytetrafluoroethylene (PTEF) coated ring.

Table 4.13. Contaminants extracted by Phytosol A from a plastic/rubber material

Contaminant[a]	Retention time min	% of total [b]
Dibutyl phthalate	31.5	2.1
Benzyl butyl phthalate	38.293	25.9
1,2-benzenedicarboxylic acid, diisooctyl ester	41.5	71.8

[a]Identities of contaminants determined from mass spectrometry analysis.

[b]Contaminants with in the oil as calculated from a total area of peaks on a basis of 1 % of a major peak.

4.3. Discussion

4.3.1. Dual anti-cholinesterase activity of extracts

The main finding of the present study was that using Phytosol A as a solvent enables effective extraction of molecules with anti-BuChE and anti-AChE activities. 30 days old extracts of *S. africana-lutea, S. argentea*, *S. divinorum*, *S. fruticosa*, *S. involucrata*, *S. microphylla* var. *neurepia*, *S. officinalis*, and *S. officinalis* var. *purpurea* had marked dual anti-cholinesterase activity, *i.*e., range from 0.01 mg ml^{-1} to 0.1 mg ml^{-1}. This range of concentrations may be within theraputical doses since Tildesley *et al.*, (2003)

reported *in-vivo* that a dose of 50 µl of *Salvia lavandulaefolia* oil, which *in vitro* inhibits human (Perry *et al.*, 2000 b) and bovine (Savelev *et al.*, 2003) AChE with IC50 values of 0.03 mg ml^{-1} and 0.05-0.07 mg ml^{-1} respectively, significantly improved memory in a dose-dependent manner in healthy young volunteers. Thus, considering that an average human body contains five litres of blood and the oral administration of the oil (assume to reach the brain), 150 mg dose of Spanish sage which would give 50% inhibition of AChE is physiologically relevant. Hence, the single dose (50 µl) of steam distilled Spanish sage oil administrated by Tildesley *et al.* (2003) is approximately three times less than the one needed for 50% inhibition of AChE in the body. Moreover, an analysis of a chemical profile of these extracts revealed no apparent pattern between their constituents and anti-cholinesterase activity, suggesting that each extract has a unique combination/ratio of compounds which allows synergistic interactions (Chapter 6; Savelev *et al.*, 2003). This unique combination/ratio may also be a result of adaptation of plants to surrounding herbivores which can involve changes in concentrations of terpenes (Valladares *et al.*, 2002).

4.3.2. Storage effects

The activity of the extracts may vary during their storage. For instance, anti-BuChE activity of *S. fruticosa* oil was increased 5 times during 30 days of storage, *i.e.*, from 7 to 37 days. The profound changes in the activity of the oil which are due to chemical alterations during storage (Misharina *et al.*, 2003) were apparent during the first 37 days. After this period the activity of the oil reached its peak and remained unchanged for another 63 days. In contrast anti-cholinesterase activity of 70% EtOH extract of *S. fruticosa* did not significantly change during the first 37 days of storage. This may

indicate that non-polar compounds are subject to more rapid chemical transformations than polar.

Furthermore, anti-BuChE activity of 30 days old *S. apiana* oil may not be regarded as therapeutical with an IC_{50} value of 0.18±0.035 mg ml^{-1} but it may after 100 days of storage when the value increased six times. The peak of the maximum activity of the oil or its maturity may not necessary be reached in 100 days, hence, as in the case with *S. fruticosa* oil, the 37 days activity check may be warranted. Dual anti-cholinesterase activity *S. officinalis* var. *purpurea* oil remained within the theraputical range of concentrations during both check points (30 and 100 days) of the storage time however, less storage time activity check may be required.

An analysis of chemical composition of the oils revealed that changes in the activity of the oils during the storage are likely to be due to significant alterations in concentration of minor constituents (Appendix 3) interacting synergistically with almost unaltered concentrations of major constituents. For example, 30 days old *S. apiana* oil had, *inter alia*, 34.4% of 1,8-cineole, 38.6% of camphor, 5% of limonene and 0.8% of 4-carene, whereas after 70 days of storage the oil contained 32.6% of 1,8-cineole, 38.5% of camphor, no limonene or 4-carene, 2.2% and 1.7% of unidentified compounds with the retention time of 16.4 and 34.04 minutes respectively. Thus, six times increase in anti-BuChE activity of *S. apiana* oil may be attributed to a significant decrease in concentration of limonene from 5.0% in 30 days old oil to a non-detected level in 100 days. In addition, certain molecules of plant materials, extracted at the low boiling temperature, may undergo an extensive thermolysis during first days of storage, which could lead to metabolic production of active molecule species with a minor percent.

Finally, this data shows that the stability test on the claimed activities of essential oils is an important issue towards standardisation. It may have an important clinical application since Perry *et al.* (2003) reported a positive result of a clinical trial of Phytosol A extracted oil of *S. lavandulaefolia* on those with AD and Tildesley *et al.*, (2003) showed that steam distilled oil from the same plant enhanced memory in healthy young volunteers.

4.3.3. Kinetic studies

Kinetic studies using Lineweaver-Burk plot showed that *S. officinalis* var. *purpurea* oil, as galanthamine (Lilienfeld and Parys, 2000), is a competitive inhibitor of human AChE but non-competitive (mixed) for BuChE, *i.e.*, a combination of competitive and uncompetitive inhibition. To date, there is no report showing competitive inhibition of AChE by any steam distilled oils. This type of inhibition may be due to the abundant presence (46.5%) of α-caryophyllene and/or its synergistic interaction with thujone (6.1%), a non-competitive rapidly detoxified blocker of the $GABA_A$ receptor (Hold *et al.*, 2000), and/or other constituents (Chapter 6; Savelev *et al.*, 2003). The other extracts, with the dual activity showed a non-competitive (mixed) type of inhibition with Ki values corresponding to their IC_{50} values (Cortes *et al.*, 2001). The non-competitive (mixed) type of inhibition was also found in tacrine (Alhomida *et al.*, 2000), a licensed drug in the treatment of AD and physostigmine (Perola *et al.*, 1997).

4.3.4. Differential anti-cholinesterase activity of the extracts

Oils of *S. glutinosa* and *S. napifolia* showed a ratio preference towards inhibition of BuChE, as does rivastigmine (Giacobini, 2000). From a clinical point of view, this may

be used to treat more severe cases of AD, which show higher BuChE levels. Since BuChE is present in the neuritic plaques, together with AChE, a drug inhibiting BuChE could reduce the formation of Aβ. This also may produce significant increase in brain ACh without triggering side effects (Giacobini, 2000). A solubility limit of extracts *S. longistyla* and *S. Jamensis* var. la luna did not allow preparation of their higher concentrations which might result in obtaining 50% inhibition of BuChE within the theraputical doses, *i.e.*, less than 0.1 mg ml^{-1}.

In contrast *S. lavandulaefolia* oil was four times more selective inhibitor for AChE than BuChE. Six weeks treatment of subjects with mild to moderate AD with the oil, extracted using Phytosol A, resulted in reduction of neuropsychiatric symptoms and an improvement in attention (Perry *et al.*, 2003). This suggests that if the positive effect of the trial is attributed to inhibition of AChE, since the enzyme is more abundant at an early stage of the disease (Ballard, 2001; Greig *et al.*, 2001), then the IC_{50} value of the oil is clinically relevant. This study also suggests that active molecules of the oil reach the brain and inhibit AChE in select areas.

Extracts of *S. corrugata* and *S. confertiflora* selectively inhibited AChE with IC_{50} values of 0.009 mg ml^{-1} and 0.03 mg ml^{-1} respectively. Huperzine A and donepezil, licensed drugs in the treatment of AD, have also been reported (Giacobini, 2000) to be more selective towards inhibition of AChE. An analysis of a chemical composition of these extracts (Appendix 3) showed no obvious pattern between major constituents and acetylcholinesterase activity, suggesting that each extract evolves its own anti-herbivore defensive chemical profile (Schmeller *et al.*, 1997; Valladares *et al.*, 2002). The rest of

extracts, concentrations of which were also restricted by a solubility limit, had combinations of molecules with less apparent anti-BuChE activity.

Extracts with preferential cholinergic activity could potentially be used therapeutically according to severity of AD (Giacobini, 2000). For example, extracts with dual anti-ChE activity, may be appropriate to patients with those forms of the disease, where the level of AChE has not yet significantly declined (Davies *et al.*, 1999) but a possibility that BuChE could hydrolyse ACh exists (Mesulam *at al.*, 2002 a), *i.e.* a moderate stage. In addition, people with a family history of AD, possibly at their late forties, may benefit from using extracts with selective anti-AChE activity. For example, reversible inhibitors of AChE may not only postpone a decline in a level of ACh but also indirectly participate in the cholinergic anti-inflammatory pathway (Chapter 1) by increasing a level of ACh, which is in addition a functional agonist to the nAChR α7 subunit on a macrophage. The interaction of ACh with the subunit increases calcium influx in macrophage, which results in suppression of a pro-inflammatory cytokine, namely tumour necrosis factor (Wong *et al.*, 2003).

4.3.5. Extraction issues

Extraction of sage species with Phytosol A in most cases provided extracts with two phases, *i.e.*, oil and a residue. Despite being a non-polar solvent, Phytosol A may increase its polarity by taking (the solvent is lightly soluble in water, 0.08 %wt) some of the remaining water from the plant materials. The modest pressure during the extraction process may also be conducive for extraction of compounds which normally (considering only polarity of 1,2,2,2-tetrafluoroethane) do not dissolve in the solvent.

Hence, when Phytosol A evaporates it leaves an extract with constituents expressing different polarities, *i.e.*, the extract with two phases.

The hexane treated residues of *S. argentea*, *S. napifolia* and *S. yunnanensis* contained, in each case, an unidentified peak representing 100% of the isolated fraction. The isolated fraction of S. *yunnanensis* showed to be a potential source of isolation of single compounds with dual anti-cholinesterase activity. Thus, the residue of extracts should not be disregarded in a search for new biologically active compounds.

Phytosol A extracts of *S. atrocyanea*, *S. divinorum*, *S. Jamensis* var. la luna were rich in squalene (Figure 4.5.), a molecule with potential anti-cancer, anti-oxidant and detoxifying properties (Gregory and Kelly, 1999; Smith, 2000). The average intake of squalene is 30 mg per day in the United States, however, when consumption of olive oil containing 0.2-0.7% squalene is high the intake of it can reach 200 mg per day as observed in Mediterranean countries where a decreased risk for various cancers is associated with high olive oil consumption (Smith, 2000). To date, there is no report to demonstrate the presence of squalene in sage species.

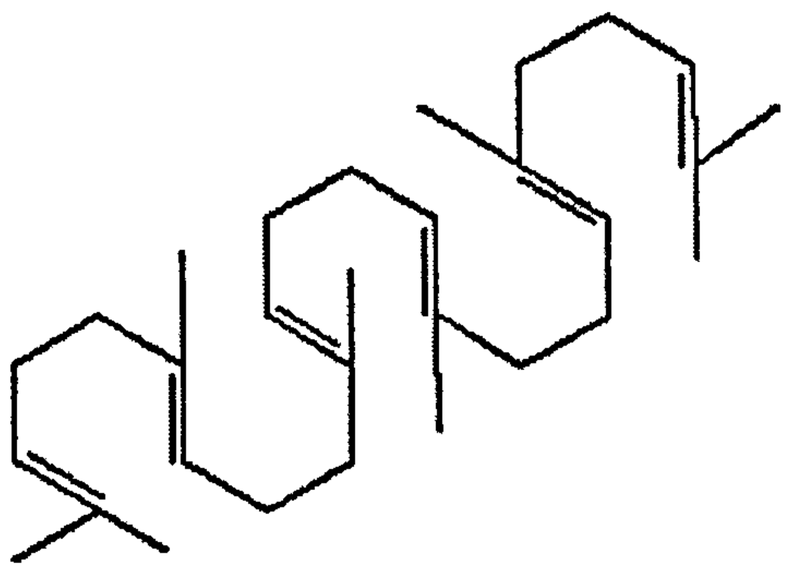


Figure 4.5. Squalene, $C_{30}H_{50,}$ (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene)

The use of Phytosol A as a solvent permitted extraction of vitamin E, a dietary compound that functions as an antioxidant scavenging toxic free radicals which may contribute to the pathological processes in AD. Sano *et al.* (1997) reported that in two years clinical trial 2000 IU a day of vitamin E slows the progression of AD in patients with moderate stage, while a combination of vitamin E and donepezil had also a positive effect in those patients (Klatte *et al.*, 2003). Thus, the abundant presence of vitamin E in extracts of S. *atrocyanea* (4.8%) and S. argentea (0.8%) may also provide an additional therapeutical value to their anti-cholinesterase activity.

Moreover, using Phytosol A in a combination with 3% ethyl acetate allowed extraction (42%) of γ-sitosterol (Figure 4.6.) in the *S. fruticosa* oil, which has not been reported in the oil obtained via Phytosol A alone. Such significant change in a chemical composition may have a profound effect on cholinergic activity of the oil and additional activity tests are therefore required. Nevertheless, the abundant presence of phytosterol may provide the oil with new theraputical values, namely cancer prevention (Li *et al.*, 2001) and blood lymphocyte proliferation (Bouic *et al.*, 1997). Due to its lipophilic nature γ-sitosterol can also act as a phytophore to other less lipophilic but active constituents. In addition, this is a first report of the presence of γ-sitosterol in *Salvia* species despite previous reports (Ulubelen *et al.*, 1994; Miura *et al.*, 2001; Wu, 2001) on isolation of β-sitosterol from the genus.

Finally, due to low viscosity, Phytosol A has good penetration properties into the materials to be extracted (Houghton and Raman, 1998). Any contact of the solvent with plastic/rubber objects increases a chance of extracting contaminants, such plasticizers/phthalates, with anti-ChEs activity. The presence of phthalates in plant

extracts may change a natural chain of chemical interactions (synergistic/antagonistic) within plant extracts and it also can rapidly increase production of reactive oxygen species *in vivo* (Rusyn *et al.*, 2001). To avoid this cross contamination plastic/rubber containing material, which are likely to be in a contact with the solvent, should be replaced with PTFE coated material (Chapter2).

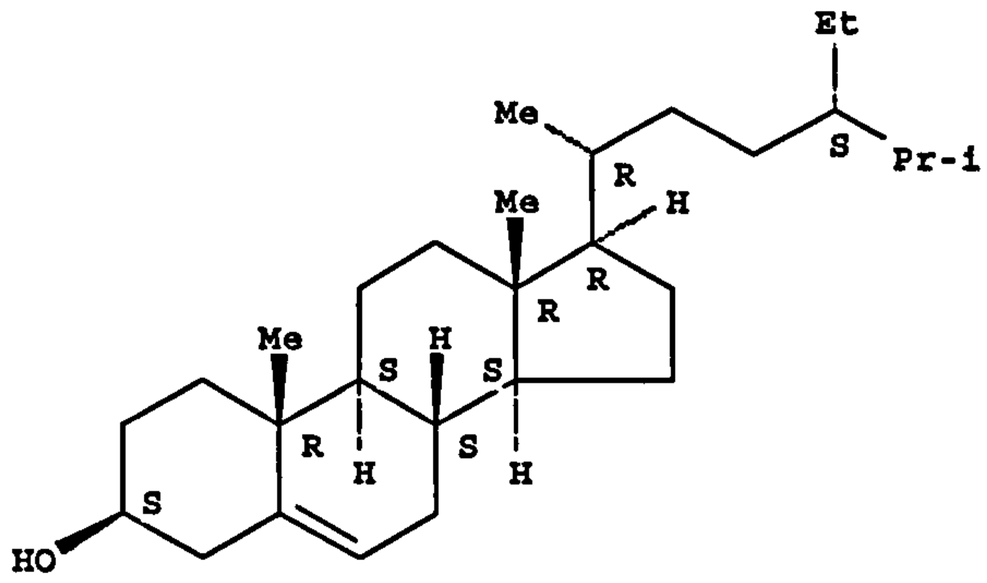


Figure 4.6. γ-Sitosterol. $C_{29}H_{50}O$, (Stigmast-5-en-3-ol, (3β,24S)- (9CI))

Chapter 5. Anti-cholinesterase activity of terpenoids present in extracts of *Salvia* species

5.1. Introduction

A diverse range of bioactivities of *Salvia* species (1.3.1.4.4.; Appendix 1) has warranted research into investigation of individual constituents that present in extracts of the plants. These constituents-products of metabolic pathways, known as secondary metabolites, are not directly involved in growth or development of plants but rather play a defensive role against predators and pathogens by having a toxic effect on the latter (Panagiotopoulos *et al.*, 2000).

According to Panagiotopoulos *et al.* (2000) there are three major groups of secondary products: terpenoids, phenolics and nitrogen containing compounds. Terpenoids, the most abundant compounds in sage species, are lipid synthesised from acety-CoA via the mevalonic acid pathway. The taxonomy is based on the number of five-carbon containing units of the terpenoids. Thus, the two five-carbon unit containing terpenoids are called monoterpenes (10 carbon atoms), the three five-carbon unit are sesquiterpenes (15 carbon atoms), four five-carbon unit are diterpenes (20carbon atoms), triterpenes (30 carbon atoms) and polyterpenes (more than 40 carbon atoms).

Phenolic compounds are aromatic substances mainly formed via the shikimic acid or the malonic acid pathway. This group has a hydroxyl group joined to an aromatic ring. In addition to its defensive role, phenolics act as attractants to insects at the flowering period. Rosmarinic, carnosic and salvianolic acids (Figure 1.15), carnosol and tanshinones (Figures 1.16 and 1.17) are representatives of this group of metabolites.

Nitrogen containing molecules, such as alkaloids and cyanogenic glycosides, are synthesised by common amino acids. In contrast to terpenes and phenolics, they are not abundant in sage.

A variety of terpenes have been found in essential oils of *Salvia* species (Giannouli and Kintzios, 2000; Appendix 1). Some of the major compounds were analysed for their bioactivity on the CNS relevant to treatment of dementia (Appendix 1). Perry *et at.*, (2000 b) reported that 1,8-cineole and α-pinene had human anti-AChE activity with IC_{50} values of 0.67 and 0.63 mM respectively, whereas camphor (IC50>10 mM), linalool, geraniol and γ terpinene were inactive. Camphor and borneol were also found (Park *et al.*, 2001; Park *et al.*, 2003) to inhibit nicotinic acetylcholine receptors in a non-competitive way. Stimpfl *et al.* (1995) reported that inhalation of 1,8-cineole increases the cerebral blood flow in the healthy humans and *in-vitro* it inhibits pro-inflammatory cytokines, TNF-α and IL1, production (Juergens *et al.*, 1998 a; Juergens *et al.*, 1998 b). Inhalation of linalool produced a dose-dependant sedative effect in mice (Jirovetz *et al.*, 1991). Re *et al.* (2000) demonstrated that linalool may inhibit ACh release via reduction of the influx of calcium in the presynaptic terminal or the inhibition of the voltage-gated sodium and potassium channels. In addition, Elisabetsky *et al.* (1995) reported an inhibitory effect of linalool on glutamate binding activity in rat cortex.

As there are no reports on anti-BuChE activity of compounds present in any species of *Salvia* the purpose of this study was to determine *in-vitro* whether the low molecular weight constituents of the genus contribute to inhibition of BuChE. Parallel AChE inhibitory activity was also investigated.

5.2. Results

Anti-BuChE activity of single chemicals is shown in Table 5.1. 3-carene was the only compound reaching 50% inhibition of the enzyme at a final concentration of 2 mM during the 5 minutes pre-incubation period, while the rest of the constituents showed little or no activity. Incubation time affected the inhibitory activity of 3-carene (Table 5.2) with the IC_{50} values being significantly decreased from 2 mM in 5 minutes to 0.7 mM in 60 minutes. The activity of β-pinene was also significantly increased from 24±4% inhibition at a final concentration of 3.8 mM for 5 minutes to an IC_{50} value of 2.3 mM in 60 minutes, whereas the activity of sabinene decreased from 28±5% in 5 minutes to 8±5% in 60 minutes at a final concentration of 1.8 mM. The potency of camphor did not change with time.

In contrast to inhibition of BuChE, human anti-AChE activity of β-caryophyllene, 3-carene, 1,8-cineole, α-pinene and β-pinene was apparent (Table 5.3). The assay solubility limit of β-caryophyllene did not allow further investigation of higher concentrations. β-caryophyllene, at a final concentration of 0.06 mM (0.0125 mg ml^{-1}), gave 32±3.0% inhibition of human AChE. To compare this activity with that of 3-carene, a concentration of 0.0125 mg ml^{-1} was substituted into the mean dose-response curve equation of 3-carene (y=20.8 Ln (X)+124.55) as X (concentration). Hence, Y (inhibition) was 33%. Thus, 3-carene at a final concentration of 0.0125 mg/ml (0.08mM) would give 33% inhibition of the human AChE, whereas β-caryophyllene at a final concentration of (0.06 mM) provided with 32±3% inhibition of the enzyme. This indicates that β-caryophyllene was a more potent inhibitor of human AChE than 3-carene. The remaining chemicals demonstrated modest or no activity towards inhibition of bovine or human AChEs.

Table 5.1. Anti-BuChE activity of compounds present in *Salvia* species

Compound	IC_{50}, mg ml^{-1} [a] mean±SD (n=4)[c]	Inhibition, %[b] mean±SD (n=4)[c]
α-pinene		0 [1.0 mg ml^{-1} (7.3 mM)]
β-pinene		24±4 [0.5 mg ml^{-1} (3.7 mM)]
Limonene		0 [0.5 mg ml^{-1} (3.6 mM)]
1,8-cineole		0 [1.5 mg ml^{-1} (9.8 mM)]
Linalool		13±2.0 [1 mg ml^{-1} (6.5 mM)]
3-carene	0.3±0.01 mg ml^{-1} (2 mM)	
α-thujone		10±2.0 [0.5 mg ml^{-1} (3.2 mM)]
Camphor		0 [1 mg ml^{-1} (6.6 mM)]
Borneol		0 [0.25 mg ml^{-1} (1.6 mM)]
α-terpineol		0 [0.5 mg ml^{-1} (3.2 mM)]
Bornyl acetate		0 [0.25 mg ml^{-1} (1.3 mM)]
α-copaene		0 [0.06 mg ml^{-1} (0.3 mM)]
Sabinene		28±5 [0.25 mg ml^{-1} (1.8 mM)]
Neryl acetate		0 [0.25 mg ml^{-1} (1.3 mM)]
Globulol		0 [0.05 mg ml^{-1} (0.2 mM)]
Guaiol		0 [0.0125 mg ml^{-1} (0.06mM)]
α-caryophyllene		18±3.6 [0.25 mg ml^{-1} (1.2 mM)]
β-caryophyllene		0 [0.5 mg ml^{-1} (2.5 mM)]
Caryophyllene oxide		12±4 [0.25 mg ml^{-1} (1.1 mM)]
Sclareol		0 [0.025 mg ml^{-1} (0.8 mM)]
Manool		0 [0.05 mg ml^{-1} (1 mM)]
Quinidine (control)	6.8×10^{-4} mg ml^{-1} (0.9μM)	

[a]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [b]Inhibitory activity of compounds which did not reach 50% enzyme inhibition. The percent activity corresponds to the values of solubility of each terpene. [c]Each chemical was tested in a set of four triplicates. Mean of

one triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of four sets of triplicates using a dose response equation of each triplicate. Incubation time for the constituents before adding the substrate was 5 minutes.

Table 5.2. Anti-BuChE activity of compounds over a course of incubation time

Compound	IC_{50}, mg ml^{-1}[a] or Inhibition, %[b] mean±SD (n=4)[c]		
	5 min	30 min	60 min
β-pinene	24±4 (0.5 mg ml^{-1})‡	43±7 (0.5 mg ml^{-1})‡	0.3±0.004†
3-carene	0.3±0.01†	0.16±0.03†	0.1±0.02†
Sabinene	28±5 (0.25 mg ml^{-1})‡	16±3 (0.25 mg ml^{-1})‡	8±5 (0.25 mg ml^{-1})‡
Camphor	0 (1 mg ml^{-1})‡	0 (1 mg/ml)‡	0 (1 mg ml^{-1})‡

[a]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [b]Inhibitory activity of compounds which did not reach 50% enzyme inhibition. The percent activity corresponds to the values of solubility of each terpene. [c]Each chemical was tested in a set of four triplicates. Mean of one triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of four sets of triplicates using a dose response equation of each triplicate. †-corresponds to [a]. ‡-corresponds to [b].

Table 5.3 Anti-AChE activity of compounds present in species of *Salvia*

Compound	IC_{50}, mg ml^{-1}[a] mean±SD (n=4)[d]	Inhibition, %[b] mean±SD (n=4)[d]
α-pinene	0.1±0.01 (0.7mM)[†]	
β-pinene	0.2±0.008 (1.5mM)	
Limonene		13±4.2 [0.07 mg ml^{-1} (0.5 mM)][†]
1,8-cineole	0.06±0.007 (0.4 mM)[†]	
Linalool		18±2.3 [0.5 mg ml^{-1} (3.25 mM)]
3-carene	0.03±0.005 (0.2 mM)[†]	
α-thujone		37±4 [0.5 mg ml^{-1} (3.2 mM)][†]
Camphor		13±3.2 [0.09 mg ml^{-1} (0.6 mM)[†]
Borneol		19±2.6 [0.25 mg ml^{-1} (1.6 mM)]
α-terpineol		22±5.5 [0.25 mg ml^{-1} (1.6 mM)][†]
Bornyl acetate		23±4.0 [0.25 mg ml^{-1} (1.3 mM)]
α-copaene		0 [0.06 mg ml^{-1} (0.3 mM)]
Sabinene		0 [0.125 mg ml^{-1} (0.6 mM)]
Neryl acetate		0 [0.125 mg ml^{-1} (0.65 mM)]
Globulol		0 [0.05 mg ml^{-1} (0.2 mM)]
Guaiol		0 [0.025 mg ml^{-1} (0.1 mM)]
α-caryophyllene		0 [0.25 mg ml^{-1} (1.2 mM)]
β-caryophyllene	0.03±0.003 (0.13 mM)	32±3 [0.0125 mg ml^{-1} (0.06 mM)][†]
Caryophyllene oxide		35±4.7 [0.25 mg ml^{-1} (1.1 mM)]
Sclareol		0 [0.025 mg ml^{-1} (0.8 mM)]
Manool		21±6.3 [0.05 mg ml^{-1} (1 mM)]
Physostigmine (control)	1.4×10^{-5} mg ml^{-1} (0.04μM)	

[a]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [b]Inhibitory activity of compounds which did not reach 50% enzyme inhibition. The percent activity corresponds to the values of solubility of each terpene. [d]Each chemical was tested in a set of four triplicates. Mean of one triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis

of four sets of triplicates using a dose response equation of each triplicate. †Compounds tested with the human enzyme otherwise with the bovine.

5.3. Discussion

The study showed lack of anti-BuChE activity in the low molecular weight compounds tested. Nevertheless, further tests on combinations comprising these molecules for the activity of the enzyme may be warranted (Savelev *et al.*, 2003). It also demonstrated slow onset of the inhibition of the enzyme during the incubation periods by single molecules such as 3-carene, which is abundant (16.5%) in oil of *Salvia aurea* L. (Serrato-Valenti *et al.*, 1997) and β-pinene, which is rich in oil (34.4%) of *Salvia candidissima* Vahl. (Bayrak and Attila, 1987). Volatility of the inhibitors restricted the course of incubation time at 30 ^{0}C, although Ogura *et al.* (2000) reported that rivastigmine, a non-volatile chemical, reached its maximum inhibition of the enzyme after six hours.

Bovine and human anti-AChE activity of terpenoids has been previously described (Miyazawa *et al.*, 1997; Miyazawa *et al.*, 1998; Perry *et al.*, 2000 b). This study confirmed that low molecular weight compounds readily inhibit AChE, in contrast to inhibition of BuChE. Anti-AChE activity of β-caryophyllene (Figure 5.1) and 3-carene (Figure 5.2) has never been previously reported and to date they are the most active non-oxygenated plant derived inhibitors with 15 and 10 carbons respectively. A lack of negative charge in these molecules may not allow their passage through the aromatic gorge of the enzyme but rather produce a quasi-steric hindrance for the substrate at an entrance of the gorge (Tai *et al.*, 2002). Moreover, their volatile nature may enable their

administration as inhaled vapour avoiding the alimentary system with its attendant denaturing of active molecular species.

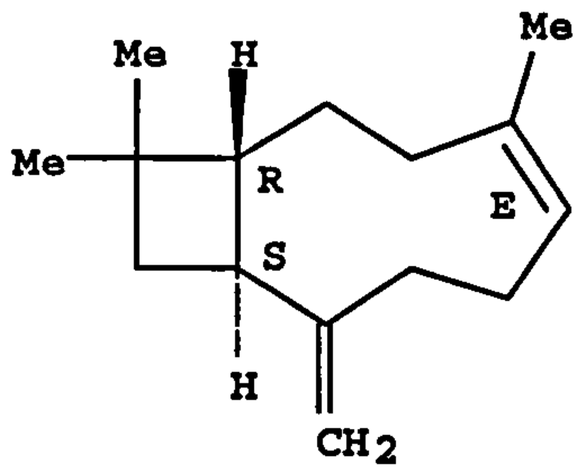


Figure 5.1. β-Caryophyllene C15 H24
Synonyms: Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, (1R,4E,9S)- (9CI)

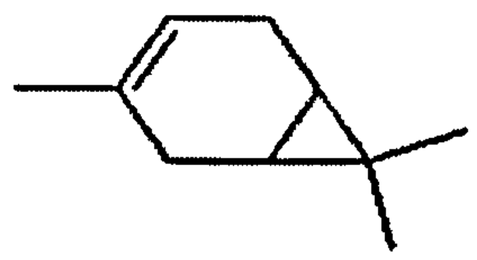


Figure 5.2. (+)-3-Carene; $C_{10}H_{16}$
Synonyms: 3,7,7-trimethyl bicyclohep-3-ene

The preferential inhibition of AChE, but not BuChE, by the secondary metabolites may be explained via the defensive mechanism of plants against herbivores (Schmeller *et al.*, 1997), as the latter contains cholinesterase structurally similar to the human AChE (Gnagey *et al.*, 1987; Marcel *et al.*, 1998). Selective anti-AChE activity of the single molecules may restrict their use to early stages of AD, where AChE is thought to be responsible for the hydrolysis of the neurotransmitter (Davies *et al.*, 1999). In addition, collecting *Salvia* species which are rich in β-caryophyllene, 3-carene and 1,8-cineole may provide oils with higher anti-AChE activity.

Chapter 6. Synergistic and antagonistic interactions of anticholinesterase terpenoids in *Salvia lavandulaefolia* essential oil

Synergy means behaviour of whole systems unpredicted by the behaviour of their parts taken separately.

Buckminster Fuller, 1975

6.1. Introduction

Study of interactions between biologically active agents in therapeutics, toxicology, environment and physiology is important for several reasons (Berenbaum, 1989). Those, as an example, include: increased rates of survival of patients with leukaemia and lymphomas when the latter were treated with combinations of four to six drug agents rather than with single ones; among elderly, non-hospitalised individuals, around 20% are taking combinations of drugs with the potential for aversive interactions; individuals exposed to more than one human carcinogen have a greater than expected incidence of cancer.

Traditionally used plants and their extracts comprise a number of chemical constituents whose mechanism of interactions within the human biological system is not yet fully understood. The presence of synergism in plant extracts was shown by Klohs *et al.* (1959) who used the root of the kawa plant (*Piper methysticum*). A crude oil extract and several isolated known sedative compounds were each tested for their effects on the central nervous system by determining their ability to antagonise clonic strychnine-induced convulsions and death in mice. The total extract was particular effective, and dihydromethysticin (DHM) was found to be the most potent individual compound. A mixture of kawain, dihydrokawain, methysticin, dihydromethysticin, yangonin and

dihydroyangonin, combined in the ratio they were found present in the crude extract, was assayed and its potency found to be similar to that of DHM. However, as DHM represented only 5% of the total extract, and the other constituents were known to be less potent than DHM, the mixture was deemed synergistic.

Liu *et al.* (1992) isolated a series of flavonoids which significantly enhanced *in-vitro* the anti-malarial activity of artemisinin, the most potent active constituents of *Artemisia annua*, against *Plasmodium falciparum*. Thus, the authors suggested that the whole plant extract may be more effective than the equivalent dose of the same active principle.

Chen *et al.* (1994) assayed the wound-healing properties of blood-red sap (*Croton lechleri*) from Ecuador. The authors concluded that there is no single compound for wound healing but that the whole sap contributes to the remedial process and synergy is therefore likely.

In a double blind placebo controlled clinical trial of Chinese medicinal plants (Sheehan & Atherton, 1992) 37 young patients with non-exudative atopic eczema showed significant improvement when they were treated orally with doses of an aqueous extract of ten Chinese herbs. A combination of pharmacological tests and clinical treatment with different combinations of herbs led to the conclusion (Phillipson *et al.*, 1994) that it was a ten herb mixture which was effective and that there was no single active herb or principle.

Berman *et al.* (2003 b) reported that a combination of tetrahydrocannabinol (THC), the psychoactive constituent of cannabis, and cannabidol (CBD), the inert constituent of

cannabis, was more affective in the treatment of patients with chronic neuropathic pain than THC on its own.

Miyazawa *et al.* (1998; 2001) have also presumed that the anti-acetylcholinesterase activity of essential oils of *Mentha* species may be superior to individual compounds from the plants.

Licensed drugs aimed at enhancing the cholinergic deficit associated with the cognitive dysfunction of Alzheimer's disease are at present based on the inhibition of the enzyme acetylcholinesterase (Levy *et al.*, 1999; Jann, 2000; Coyle and Kershaw, 2001; Grutzendler and Morris, 2001).

In a double blind, randomized and placebo-controlled trial Akhondzadeh *et al.* (2003) reported that ethanol extract of *S. officinalis* L. improved cognition and reduced agitation in patients with mild to moderate stage of Alzheimer's disease. Despite the positive outcome of the trial the medical application of the extract is likely to be restricted due to lack of standardisation, i.e., identification and control of its active constituents. The latter is important in order to ovoid an over dose or under dose prescriptions in the treatment.

Perry *et al.* (2000 a) suggested that Spanish sage (*Salvia lavandulaefolia*, Vahl) may also be relevant in the treatment of dementia of the Alzheimer's type and reported (Perry *et al.*, 2000 b) *in-vitro* inhibition of acetylcholinesterase (AChE) by Spanish sage oil and its constituents. It was proposed that the inhibitory activity of the natural plant extract is due to the synergistic nature of the oil rather than a single inhibitor.

An assessment of the enzyme inhibitory activity of a combination of chemicals in terms of zero interaction, synergy or antagonism depends on a definition of what the expected response of a mixture should be. The interactions of a defined combination of compounds can be generally described as having a zero interaction, in which the response of the combination is that expected from the individual dose-response curves; synergy, in which the response is greater than expected; and antagonism, in which it is less. There are a number of methods, which have been proposed to demonstrate synergistic interactions between agents (Gessner, 1988; Berenbaum, 1989). The critical point in selecting an appropriate method is an understanding of the nature of a combination and the shapes of the dose-response curves of the agents. A linear or close to linear relationship is a basic assumption of the approach based on summation (Berenbaum, 1989).

Interaction effects have also been studied using the isobole approach (Loewe, 1953; DeJongh, 1961), which is based on the necessity of agents to produce a specified response, such as death in 50% of the animals, known as LD50. A complication in analysing chemical interactions of natural constituents in inhibiting an enzyme such as acetylcholinesterase is that some constituents are less potent inhibitors and therefore, may not reach 50% inhibition of the enzyme on a dose-response scale over the range of concentrations examined. An essential oil comprises many constituents and to mimic combinations of such constituents regardless of number, in order to analyse the chemical interactions, is challenging.

The method of Berenbaum (1978; 1985) is based on an assumption of zero-interactivity of agents in a combination. This approach facilitates analysis of a combination of agents with different types of dose-response relation or dose-scale and permits combinations of

any number of agents. This method was adopted in this study to explore the chemical interactions of principal constituents of Spanish sage essential oil.

The aim of this work was to investigate the hypothesis that the activity of the natural plant extract is greater than the combined activity of individual components in their naturally occurring ratios. Because of the declining activity of AChE in AD (Davies and Maloney, 1976; Perry *et al.*, 1977; Davies *et al.*, 1999) a low enzyme concentration, relative to Ellman *et al.* (1961) was selected to ascertain the anticholinesterase activity of the natural plant oil and terpenoid combinations.

6.2. Results

IC50 values

The oil and component terpenoids were tested for their anticholinesterase activity within their solubility limits. The compounds were divided into (i) those which reached 50% inhibition of AChE, and (ii) those which were less potent (Table 6.1.).

The IC_{50} value of the oil did not significantly differ from that of 1,8-cineole and was marginally less than the IC_{50} value of α-pinene. Other constituents showed significantly less anticholinesterase activity.

Interactions

The terpenoids were assessed in combinations for their AChE inhibitory activity on a basis of their naturally occurring concentrations in the oil (Table 6.2.). The anticholinesterase contribution of 1,8-cineole accounted for half the activity of the natural plant extract (Table 6.2.) and therefore was considered as the main agent, which could show synergy with other constituents. The reported chemical composition of Spanish sage oil is variable (Giannouli and Kintzios, 2000; Perry *et al.*, 2000 b) and for

this reason the combinations of the terpenoids, investigated in the present study, reflected the average naturally occurring composition in the plant extract.

Table 6.1. Anticholinesterase activity of essential oil and its constituents

Inhibitor	(i) IC50 (mg ml^{-1})[a] $\overline{X}$ ±SD (n=4)	(ii) Activity[b], % $\overline{X}$ ±SD (n=4)
Spanish sage oil	0.05±0.01	
1,8-cineole	0.06±0.01	
α-pinene	0.09±0.01	
β-pinene	0.2±0.03	
Camphor		39±4.0 (0.5 mg ml^{-1})
Linalool		18±2.3 (0.5 mg ml^{-1})
Bornyl acetate		23±4.0 (0.25 mg ml^{-1})
Caryophyllene oxide		35±4.7 (0.25 mg ml^{-1})
Borneol		19±2.6 (0.25 mg ml^{-1})

[a]Final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations. [b]Inhibitory activity of compounds, which did not reach 50% enzyme inhibition, as calculated from the dose-response curves equations. The percent activity was obtained from the dose-response curve equations of the agents and corresponds to the values of solubility of each terpene. A concentration of each inhibitor was substituted into the dose-response curve equation of each inhibitor as X (concentration). Hence Y would represent inhibition, i.e., the activity of an inhibitor at its particular concentration.

Table 6.2. Inhibition of AChE by the essential oil and its constituents at concentrations based on ratios occurring in the oil

Compound	% in oil[a]	Concentration mg ml^{-1} [b]	% Inhibition[c] $\overline{X}$ ±SD (n=4)
Spanish Sage	100	0.05	50±1.7
1,8-cineole	26.8	0.013	26±1.6
camphor	24.7	0.012	0
α-pinene	6.6	0.003	0
β-pinene	5.4	0.0027	0
Borneol	3.2	0.0016	0
Caryophyllene oxide	1.2	6×10^{-4}	0
Linalool	0.8	4×10^{-4}	0
Bornyl acetate	0.7	3.5×10^{-4}	0

[a]Chemical composition of Spanish Sage obtained by GC-MS analysis. [b]Final assay concentration of compounds, which corresponds to their chemical composition in the oil and was calculated from the IC_{50} value of the whole oil. The whole oil of 0.05 mg/ml, which gave 50% inhibition of the enzyme (IC50 value of the oil) was taken as 100%, for the convenience of calculations. Hence, the concentration of the agents was calculated on a basis of their percentage composition in the oil. [c]Percentage inhibition was calculated from the dose-response curve equations of each chemical at the concentrations in column 3.

In all combinations it was considered that the expected inhibition would be within the response scale of individual constituents, so that the iso-effective line intersects the dose-response curves of all agents. The inhibitory activity of 1,8-cineole and α-pinene

exceeded 50% inhibition of AChE (Table 6.1.) and this allowed five concentrations of this combination, based on the typical ratio, to be tested (Table 6.3.) (the number of concentrations of a combination depended on the inhibitory activity of the compounds and of their aqueous solubility in that combination). Caryophyllene oxide and camphor did not exceed 50% inhibition of the enzyme. As the result, only three concentrations of 1,8-cineole/caryophyllene oxide and two of 1,8-cineole/camphor were analysed (Table 6.3.). In a combination of eight terpenes (Table 6.3.) there was only one concentration, which allowed the isoeffective line intersecting the dose-response curves of all agents within their response scales and solubility limits. The interaction index (Table 6.3.) was calculated to estimate the significance of these chemical interactions.

Combinations of two compounds

A minor synergy was apparent in 1,8-cineole/α-pinene and 1,8-cineole/caryophyllene oxide combinations at its higher concentrations, whereas at the lower concentration the interaction index expressed a zero-interactive response. Antagonism was found in 1,8-cineole/camphor combinations with the interaction index of 2.

Figure 6.1 illustrates synergism in the combination of 1,8-cineole and α-pinene, whereas Figure 6.2 shows antagonism in the combination of 1,8-cineole and camphor. The dose-response curves of the agents are the mean of four dose-response estimations.

Table 6.3. Inhibition of AChE by combinations of terpenoids

Combination	Concentration mg ml^{-1} [a]	Total mg ml^{-1} [b]	Inhibition, % $\overline{X}$ ± SD (n=4)		Interaction index of combination [f] $\overline{X}$ ± SD (n=4)
			observed [c]	expected [d]	
1,8-cineole/ α-pinene	0.45 0.04	0.5	92±1.4	84±2.7	0.59±0.11 synergy
1,8-cineole/ α-pinene	0.225 0.02	0.245	84.5±1.7	73±2.1	0.50±0.08 synergy
1,8-cineole/ α-pinene	0.09 0.008	0.1	63.5±0.6	58±1.4	0.71±0.06 synergy
1,8-cineole/ α-pinene	0.045 0.004	0.05	50±1.3	47±1.0	0.83±0.08 synergy
1,8-cineole/ α-pinene	0.009 8×10^{-4}	0.01	20±1.3	21±2.0	1.1±0.11 zero- interaction
1,8-cineole/ caryophyllene oxide	0.045 0.003	0.048	56±2.4	46±1.2	0.55±0.05 synergy
1,8-cineole/ caryophyllene oxide	0.0225 0.0015	0.024	40±1.6	35±1.3	0.73±0.04 synergy
1,8-cineole/ caryophyllene oxide	0.009 0.0006	0.01	23±4.0	21±2.5	0.91±0.1 zero-interaction
1,8-cineole/ camphor	0.045 0.065	0.1	33±3.4	47±1.5	antagonism 2.4±0.21
1,8-cineole/ camphor	0.009 0.013	0.02	10±4.8	21±2.0	2.08±0.39 antagonism
1,8-cineole camphor a-pinene b-pinene caryophyllene oxide borneol bornyl acetate linalool	0.007 0.01 6×10^{-4} 5×10^{-4} 5.0×10^{-4} 0.0013 2.5×10^{-4} 4.0×10^{-4}	0.02	19±2.8	18±2.3	0.96±0.7 zero interaction

[a]Final concentrations of individual compounds in the assay. [b]Final concentrations of the combinations. [c]Inhibition of the combination obtained experimentally. [d]Calculated zero-interactive response of the combinations. [f]If the observed inhibition is significantly more ($P<0.05$) than expected, with the interaction index less than 1, synergy is the result.

If the observed inhibition is significantly less ($P<0.05$) than expected, with the interaction index more than 1-antagonism is inferred, if not significant ($P>0.05$) with the interaction index of 1-zero-interaction.

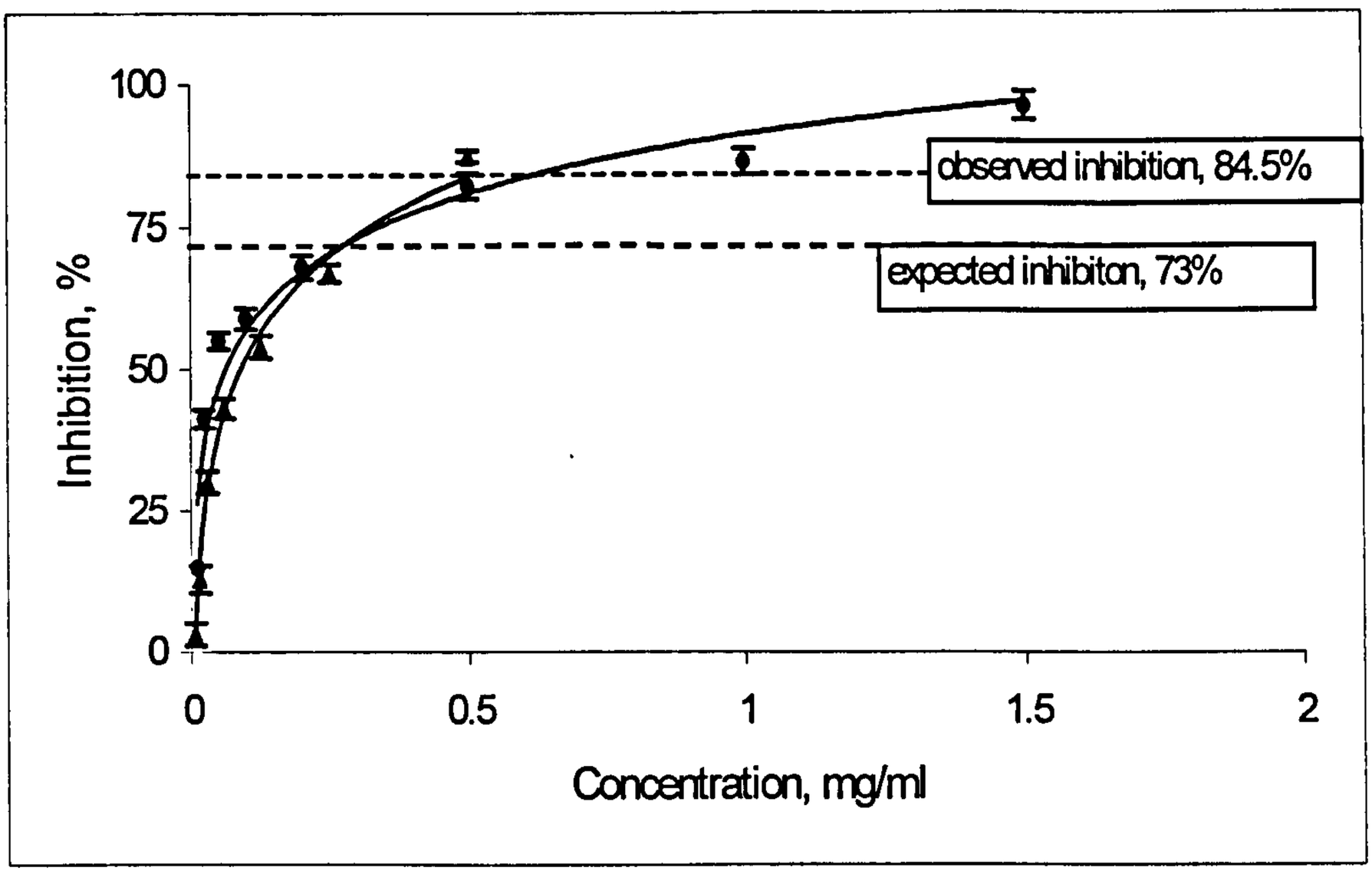


Figure 6.1. Calculation of expected response of 1,8-cineole (da; 0.225 mg ml^{-1}) and α-pinene (db; 0.02 mg ml^{-1}) combination. ● , dose-response curve of 1,8-cineole; ▲, dose-response curve for α-pinene. A horizontal line of the expected inhibition of 73±2.1% intersects the two dose-response curves at points where concentrations of 1,8-cineole (Da) and α-pinene (Db), on the X-axis, are iso-effective with the combination (da;db) and, therefore satisfies equation 2, namely 0.225/0.236+0.02/0.304=1. Both of these compounds would produce 73±2.1% inhibition, so this is the response of the combination to be expected from the concentration-response curves of the compounds.

The observed inhibition was 84.5±1.7%, i.e., more than expected, indicating synergism.

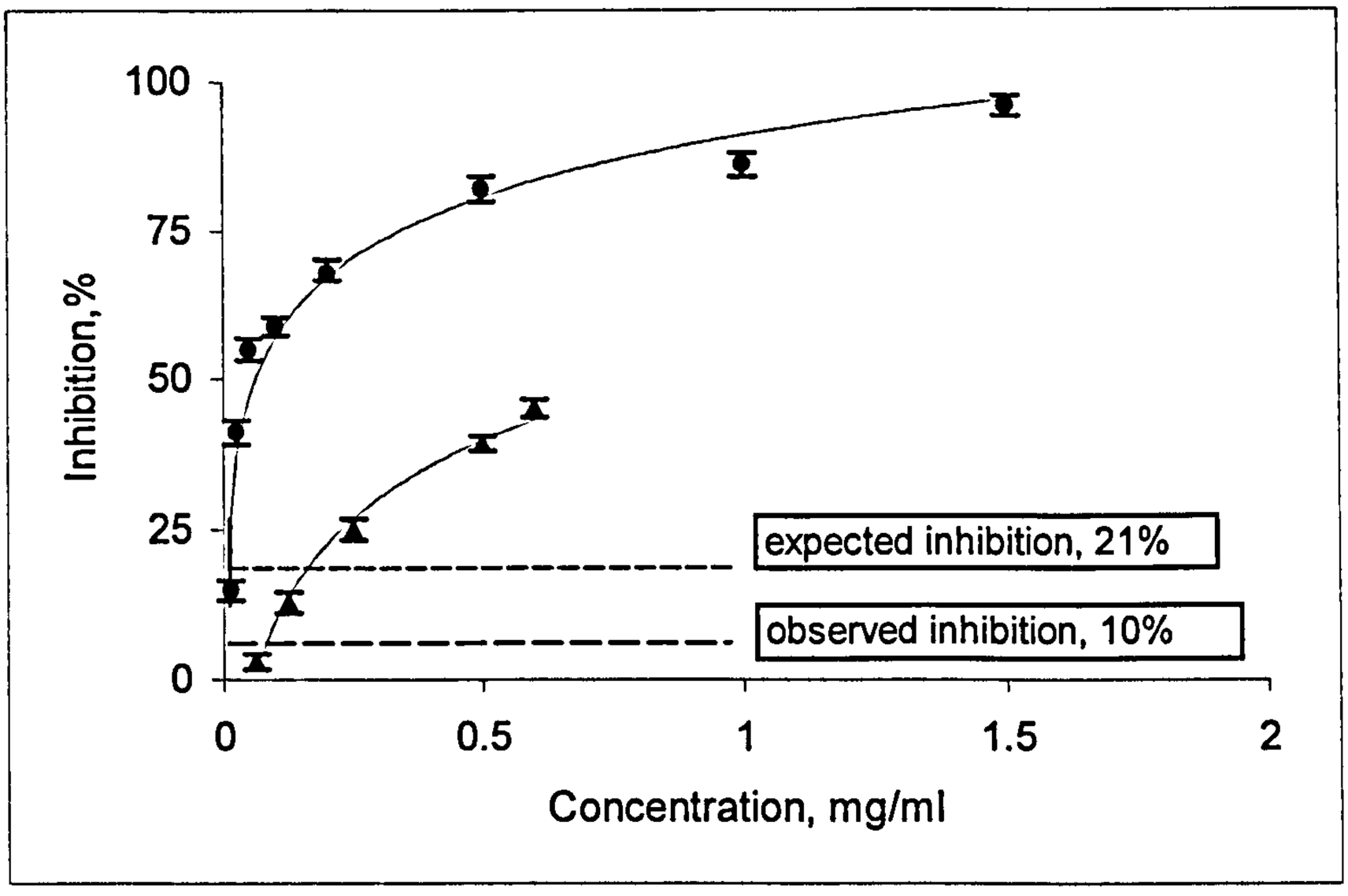


Figure 6.2. Calculation of the expected response of 1,8-cineole (da; 0.009 mg ml^{-1}) and camphor (db; 0.013 mg ml^{-1}) combination. ●, dose-response curve of 1,8-cineole; ▲, dose-response curve of camphor. A horizontal line of the expected inhibition of 21±2.0% intersects the two dose-response curves at points where concentrations of 1,8-cineole (Da) and camphor (Db), on the X-axis, are iso-effective with the combination (da;db) and, therefore satisfies equation 2, namely 0.009/0.0094+0.013/0.23=1. Both of these compounds would produce 21±2.0% inhibition, so this is the response of the combination to be expected from the concentration-response curves of the compounds. The observed inhibition was 10±4.8%, i.e., less than expected, indicating antagonism.

Combinations of more than two compounds

The eight compounds mixture was zero-interactive (Table 6.3.) and its inhibitory activity did not exceed that of the oil (Table 6.1.). The inhibitory activity of a combination, excluding 1,8-cineole, as the most potent inhibitor, and camphor, which is the antagonist to 1,8-cineole, comprising minor constituents was analysed. In the combinations of the remaining six terpenes (Table 6.4.) the additive inhibition was significantly less than observed, indicating synergism, when the method of summation was applied. In contrast there were zero-interaction responses according to the approach of Berenbaum.

Table 6.4 Assessment of inhibition of AChE by combinations of six terpenoids on the basis of two methods

Combination	Concentration	Inhibition,% [c]	Inhibition, %, $\overline{X}$ ±SD (n=4)		
	mg ml^{-1} [a]	$\overline{X}$ ±SD (n=4)	observed [d]	expected [f]	additive [g]
α-pinene	0.008	4±4.8			
β-pinene	0.007	0			
caryophyllene oxide	0.006	0			
borneol	0.016	0			
bornyl acetate	0.0034	0			
linalool	0.004	0			
TOTAL:	0.044 [b]		14±2.4 (1.22 ±0.3)	16±2.5	4±4.8
α-pinene	0.004	0			
β-pinene	0.0035	0			
caryophyllene oxide	0.003	0			
borneol	0.008	0			
bornyl acetate	0.0017	0			
linalool	0.002	0			
TOTAL:	0.022		6.5±2.6 (0.95±0.13)	5.5±1.6	0

[a]Final concentrations of individual compounds in the assay. [b]A sum of individual concentrations. [c]Expected inhibition of individual compounds, calculated from its dose-response curve equations accordingly to their final concentrations in the combination. [d]Inhibition obtained experimentally and compared with the expected one (column 5),

with the interaction index shown in the brackets. The inhibition was not significantly more ($P>0.05$) than expected, with the interaction index of 1, indicating the zero-interactive response. [f]Calculated zero-interactive response of the combination. [g]Consists of a sum of the expected inhibitions of individual compounds. The additive inhibition was significantly less ($P<0.05$) than observed, indicating synergism.

The inhibitory activity of the tested combinations of terpenes was summarised on a basis of IC_{50} values (Table 6.5.). The presence of camphor significantly decreased the inhibitory activity of 1,8-cineole raising the IC_{50} value of 0.06±0.01 mg ml^{-1} (Table 6.1.) to 0.18±0.2 mg ml^{-1}. When combined with α-pinene and caryophyllene oxide 1,8-cineole showed marginal synergistic responses. The inhibitory activity of eight and six compound combinations did not exceed that of the oil (Table 6.1.).

Table 6.5 Assessment of inhibition of AChE by combinations of terpenoids on a basis of IC50 values

Combination	Mass ratio	IC50, mg ml^{-1}[a] $\overline{X}$ ±SD (n=4)
1,8-cineole/α-pinene	0.7:0.06	0.05±0.005
1,8-cineole/caryophyllene oxide	0.7:0.05	0.043±0.003
1,8-cineole/camphor	0.7:1	0.18±0.02
Six compounds	[b]	0.18±0.01
Eight compounds	[c]	0.11±0.01

[a]Concentration of combinations required for 50% inhibition of enzyme AChE, as calculated from the dose-response curve equations of the combinations. [b]Combination

comprising α-pinene, β-pinene, caryophyllene oxide, borneol, bornyl acetate and linalool in the ratio as 0.06:0.05:0.05:0.1:0.03:0.03. [c]Combination comprising 1,8-cineole, camphor, α-pinene, β-pinene, caryophyllene oxide, borneol, bornyl acetate and linalool in the ratio as 0.7:1:0.06:0.05:0.05:0.1:0.03:0.03.

6.3. Discussion

6.3.1. Inhibition of AChE

The anticholinesterase activity of 1,8-cineole was almost the same as the natural plant oil (Table 6.1.). A similar result was reported by Miyazawa *et al.* (1998) who found that an IC_{50} value of 0.026 mg ml^{-1} of *Mentha aquatica* (water mint) oil, for inhibition of bovine AChE at a final concentration of 0.0065 U ml^{-1}, was equivalent to that of 0.025 mg ml^{-1} of viridiflorol, a major terpene constituent of the oil. Their IC_{50} value of 1,8-cineole of 0.04 mg ml^{-1} was close to that obtained in the present study, namely 0.06±0.01 mg ml^{-1}.

It was also reported (Perry *et al.*, 2002) that essential oil of *S. lavandulaefolia* inhibits the rat brain AChE *in–vivo*. There was a decrease in AChE activity in the striatum and hippocampus, though not in the cortex, at doses of 20 µl and 50 µl suggesting that constituents of the oil or their metabolites reach the brain and inhibit AChE in select areas. The anti-AChE activity of the oil for bovine AChE, namely 0.05±0.01 mg ml^{-1} was similar to the one obtained *in-vitro* with human AChE, i.e., 0.03 µl ml^{-1} (Perry *et al.*, 2000 b). The oil and its constituents showed uncompetitive type of inhibition.

This was more potent than IC_{50} values of 0.09 mg ml^{-1} for α-pinene, 0.1 mg ml^{-1} for 1,8-cineole and 0.72 mg ml^{-1} for camphor and also for a combination of major constituents as a "mimic oil" of 0.3 mg ml^{-1}. On a basis of these data synergy in the oil was proposed. Variables in the assays, such as final concentrations of reactants and total volume of the reaction mixture, which are surface related to adsorption of these sparingly-soluble compounds, are likely to produce a variation in the inhibitory activity in independent investigations.

6.3.2. Evidence of synergy

Evidence of synergy was apparent when the inhibitory activity of the individual terpenes, measured at the same concentrations as existed in the oil at its IC_{50} value, was not as great as the whole oil (Table 6.2.). In the original plant extract the potency of 1,8-cineole is likely to be different due to the presence of other constituents, which could interact with 1,8-cineole therefore, it may not account for the half of the activity of the oil.

The inhibitory activity of 1,8-cineole, with oxido-p-menthane structure, was the most potent amongst the constituents, containing in addition the (+), (-)-isomer hydrocarbons, ketone, deoxy-and-hydroxy hydrocarbon structures (Figure 6.3.). Synergistic interactions, which were found in the mixtures of 1,8-cineole/α-pinene and 1,8-cineole/caryophyllene oxide varied in their interaction indexes and gradually declined to the zero-interactive responses at the lower concentrations (Table 6.3.). This may be due to effects of dilution reducing an ability of the inhibitors (weak aqueous system), which may be responsible for inhibition of a peripheral site of the enzyme, to block an entrance for the substrate to pass through to the narrow aromatic gorge of the enzyme.

This demonstrates that interactions in a combination vary depending on the concentrations and ratios between the agents. It should also be noted that the isomeric forms of the commercially available compounds may also contribute to anti-AChE activity of their combinations in comparison with the activity of the extract. Potential effects of the iso-forms on the enzyme were not assessed in this study.

Results, obtained more recently (personal communication with Dr E. Okello, School of Biology), indicate that Spanish sage oil and 1,8-cineole are rapidly reversible inhibitors and there was a decrease in AChE inhibitory activity of approximately 40% during the incubation time of 30 minutes at 30 ^{0}C with the enzyme final concentration of 0.008 U ml^{-1}. In contrast Perola *et al.* (1997) reported that physostigmine, a slowly reversible inhibitor, increased its inhibitory activity within this period. The incubation time employed in this study could reduce the synergistic and antagonistic interactions of the terpenoid combinations. The period of incubation may be an important variable, considering that there is no incubation time *in-vivo*, in an investigation of chemical interactions of rapidly reversible inhibitors.

The possible clinical importance of relatively small deviations from zero-interaction, indicating the presence of minor synergy or antagonism in a combination has been previously discussed (Berenbaum, 1987; Hall *et al.*, 1983). It was observed (Atherton *et al.*, 1981; Berenbuam 1987), in antibacterial studies, that minor interactions *in-vitro* may not only result in significant synergism *in-vivo* but also make a difference to the duration of effective drug level *in-vivo*. Such interactions could also be relevant to patients with AD where a concentration of AChE in hippocampus and cerebral cortex decreases to 10 to 15 % of its normal values at advanced stages of the disease (Perry *et*

al., 1978 a). Further experiments *in-vivo* are needed to ascertain the effects of these interactions on the CNS.

Because such responses are likely to occur to the natural plant extract, it is important to select an active synergistic mixture with the optimum therapeutical properties. There is preliminary evidence, from our studies, indicating that 1,8-cineole is a selective inhibitor for AChE but not for butyrylcholinesterase, another therapeutical target in the treatment of Alzheimer's disease (Yu *et al.*, 1999) and if caryophyllene oxide or α-pinene, which showed synergistic properties in the combinations with 1,8-cineole, inhibit butyrylcholinesterase such mixtures may be called synergistically selective.

The further complexity of interactions in the natural plant extract was apparent from significant antagonism, with the interaction index of 2, which appeared in the combination of 1,8-cineole and camphor (Table 6.3.). This suggests that the "mimic oil" composed by Perry *et al.* (2000 b), as described above, may have had antagonistic properties because of the presence of camphor that would influence the degree of proposed synergy in *S. lavandulaefolia* oil.

6.3.3. Methodological issues

The present analysis has also demonstrated how two methods may be used in exploring the interactions between agents and how this may give different results when applied to the same set of data, so that a mixture may appear zero-interactive according to one and synergistic to another method (Table 6.4.).

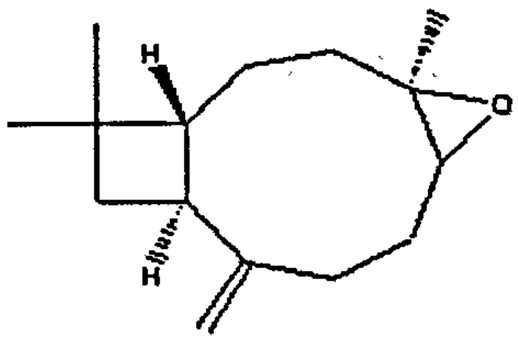
The method of summation was included as an example of how an inappropriately selected approach can lead to a misleading interpretation. The terpenes used in this study had non-linear dose-response curves and therefore a sum of their inhibitory

activity would not reflect a response of a zero-interactive combination (Berenbaum, 1989). Figures 6.1 and 6.2 illustrate examples of such non-linearity for 1,8-cineole in combinations with camphor and α-pinene. As the result, the synergistic response, evaluated by the method of summation, in the combination of six terpene compounds (Table 6.4.) was disregarded and the combination was deemed as zero-interactive according to the method of Berenbaum (1978; 1989).

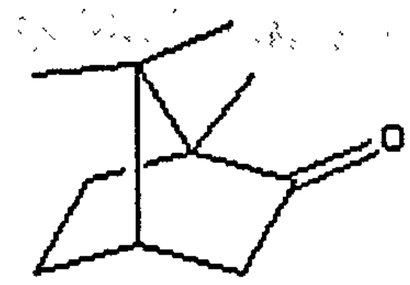
The comparative analysis of mixtures on a basis of IC_{50} values (Table 6.5.) cannot reveal a true interaction within the combinations. For example, in the combination of 1,8-cineole and camphor there are two roles, which could express either synergy or antagonism. It could be said, comparing Tables 6.1 and 6.5, that camphor is the antagonist to 1,8-cineole, because it increased the IC_{50} value of the latter from 0.06±0.01 mg ml^{-1} (Table 6.1.) to 0.18±0.02 mg ml^{-1} (Table 6.5.) but on the other hand, 1,8-cineole is synergistic to camphor, because it decreased the inhibitory activity of the latter from 39±4.0% at 0.5 mg ml^{-1} (Table 6.1.) to the IC_{50} value of 0.18±0.02 mg ml^{-1} (Table 6.5.). On the other hand, applying the method of expected inhibition, based on the zero-interactive response, allowed evaluation of antagonistic interactions of these combinations. The IC_{50} value of the whole oil (Table 6.1.) was similar to the combination of 1,8-cineole/α-pinene and less potent than the combination of 1,8-cineole/caryophyllene oxide (Table 6.5.). The IC_{50} values of eight and six compound combinations, based on the typical chemical composition of Spanish sage essential oil, were significantly less potent than that of the whole oil. The zero-interactive responses of these two combinations suggest that minor constituents are more likely involved in the anticholinesterase activity of the essential oil.

It has to be pointed out that, because of a decreased level of AChE in the brain during AD (Perry *et al.*, 1978), the analysis of the chemical interactions were carried out at the low concentration of the enzyme, compared to a typical of 0.08 U ml^{-1} (Ellman *et al.*, 1961). Therefore, minor synergy and antagonism in the natural plant extract may only occur under these particular experimental conditions.

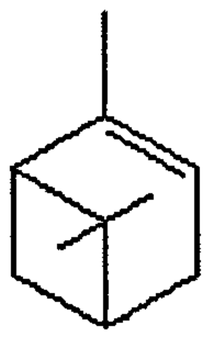
Chemical compositions of *S. lavandulaefolia* vary (Perry *et al.*, 1999; Giannouli and Kintzios, 2000; Karousou *et al.*, 2000) and as a result, identifying plants with desirable chemical contents may help to extract oils with maximum therapeutical properties. This study shows that high 1,8-cineole and low camphor contents in the oil may increase its anticholinesterase activity. *S. fruticosa* may be ideal for AChE inhibition with a high level of 1,8-cineole up to 75% and low camphor in a range of 0.8-30.3% (Byarak and Akgul, 1987; Karousou *et al.*, 1998; Karousou *et al.*, 2000; Länger *et al.*, 1996). Other properties of sage species, such as anxiolytic, antioxidant, oestrogenic, antidepressive and anti-inflammatory (Perry *et al.*, 2000 a) could equally be monitored for optimum in a chemical composition of oils by analysing the activity of chemical components and interactions between them.



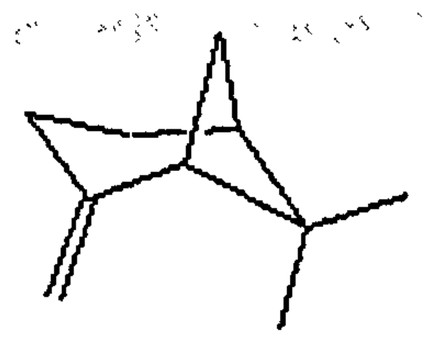
Caryophellene oxide: $C_{15}H_{24}O$ (220.4)



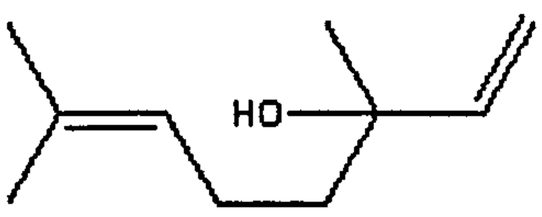
Camphor: $C_{10}H_{16}O$ (152.2)



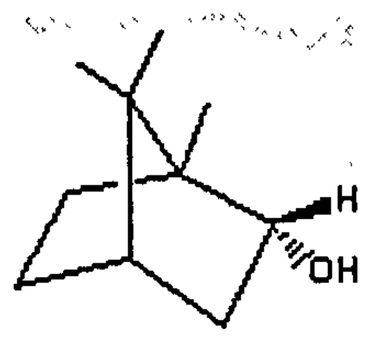
α-pinene: $C_{10}H_{16}$ (136.2)



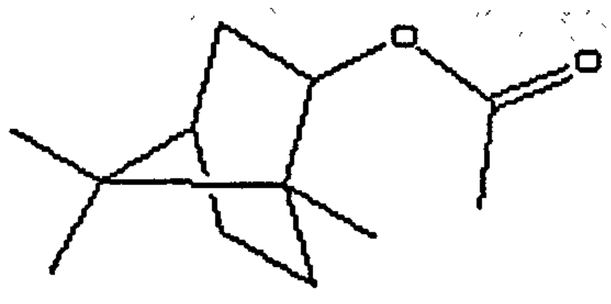
β-pinene: $C_{10}H_{16}$ (136.2)



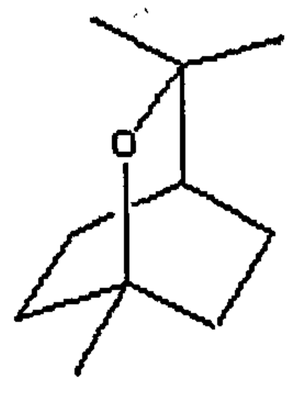
Linalool: $C_{10}H_{18}O$ (154.3)



Borneole: $C_{10}H_{18}O$ (154.3)



Bornyl acetate: $C_{12}H_{20}O_2$ (196.3)



1,8-cineole: $C_{10}H_{18}O$ (154.3)

Figure 6.3. Terpenoid components of *S. lavandulaefolia* essential oil. Molecular weights are shown in the brackets.

Chapter 7. Phytosol extracts of *Salvia* species in relation to β-secretase, interleukin 8 and cholinergic receptor activities

7.1. Introduction

Selkoe (2001) reviewed the principal steps in the AD cascade that lead to the loss of synapses and glia cell somata, the dentritic dystrophy, and the neurotransmitter deficits that are the basis for the dementia. In brief, these steps include mutation in APP (presenilin (PS) 1 and PS2 genes); increased production, accumulation and aggregation of $A\beta_{42}$ ($A\beta_{39-43}$) peptides that lead to a progressive neuritic injury within amyloid plaques followed by inflammatory responses (*i.e.*, microglial activation and cytokine release); disruption of neuronal metabolic and ionic homeostasis (oxidative injury); phosphatase activities (hyperphosphorylated tau, paired helical filaments), widespread neuronal and neuritic dysfunction and neurodegeneration in hippocampus and cerebral cortex with progressive neurotransmitter deficits. Thus, it is of interest to test an agent such as *Salvia* for some of these non-transmitter related activities.

7.1.1. β-secretase as a target for the treatment of AD

APP is a single transmembrane polypeptide that is trans-located into the endoplasmic reticulum, a network of tubules and vesicles which is involved in the synthesis of proteins and proteins membrane insertion, via its signal peptide and then post-translationally modified through the secretory pathway (Selkoe, 2001). Its half-life is relatively brief, 45-60 minutes in most cells tested (Weidemann *et al.*, 1989). Reports by Haass *et al.* (1992), Seubert *et al.* (1992) and Shoji *et al.* (1992) demonstrated that Aβ is generated and secreted by all cells that express APP and it can be measured in the brain, plasma and cerebrospinal fluid (CSF). Games *et al.* (1995) showed that over expression

of APP in transgenic mice leads not only to increased Aβ levels but to development of the pathological hallmarks of AD, including numerous extracellular Aβ deposits, neuritic plaques, synaptic loss.

Both during and after the trafficking of APP through the secretory pathway, it can undergo a variety of proteolytic cleavages to release secreted derivatives into vesicle lumens and the extracellular space. There are three proteolytic activities, α, β and γ-secretases, involved in the cleavage of APP (Selkoe, 2001) (Figure 7.1.). The first proteolytic cleavage is made by α-secretase and it results in the release of the large soluble fragment (α-APPs) into the lumen/extracellular space and retention of an 83-residue COOH-terminal fragment (CFT or C83) in the membrane. Some APP molecules, not subjected to α-secretase cleavage, can be cleaved by β-secretase (a beta-site APP-cleaving enzyme, BACE1 or memapsin2) generating a smaller derivative (β-APPs) and retaining a 99-residue CFT (C99). The C83 and C99 fragments may be cleaved by a protease(s) called γ-secretase (also called presenilins) to release the Aβ-peptides. Presenilins, unstable holoproteins, produce fragments which are the principal biologically functional form of presenilins. However, Kume *et al.* (2003) suggested that γ-secretase may cleave APP independently of α-and β-secretase pre-cutting.

It could be argued that blocking Aβ generation would affect other metabolites that may have an important physiological function; however β-secretase inhibition would not affect the vast majority of APP molecules that are processed by the α-secretase pathway, and in contrast to γ-secretase inhibition, it would not interfere with the Notch signalling pathway (receptors that mediate cells interaction) (Selkoe, 2001, Citron, 2002).

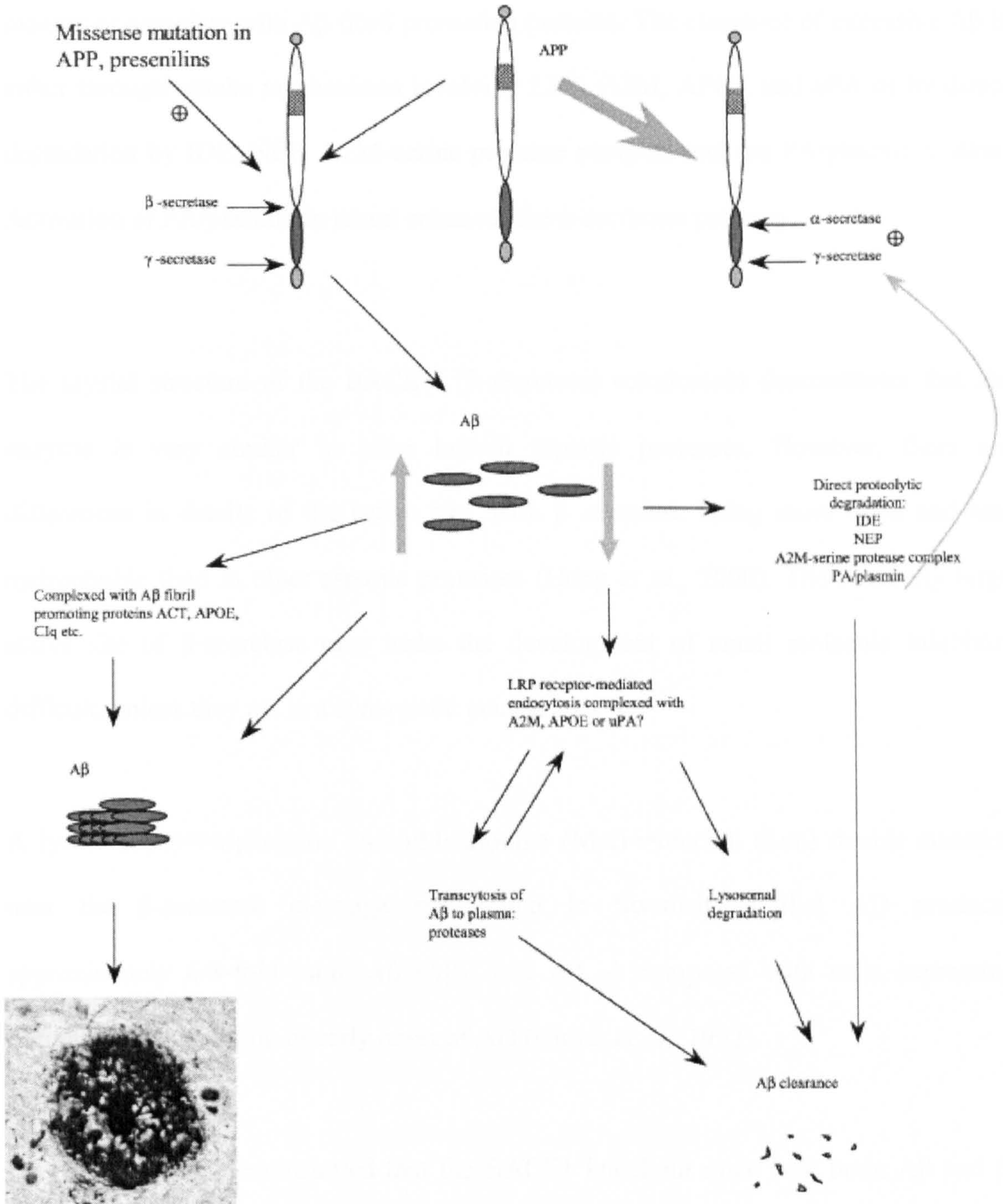


Figure 7.1. Overview of possible pathways for Aβ biogenesis, clearance and fibrillisation (Source: Ling *et al.*, 2003). The α-cleavage pathway is the major route for APP processing. Aβ is derived from APP through two sequential cleavages by β- and γ-secretases. The Swedish APP mutation enhances β-cleavage causing increased formation of Aβ. All other C-terminal Aβ mutations and PS mutations increase the ratio of $A\beta_{42/40}$ without increasing total Aβ. $A\beta_{42}$ can aggregate in a concentration-dependent

manner or associate with Aβ fibril promoting proteins. The clearance of excessive Aβ is either through uptake mechanisms involving LRP, A2M, APOE and uPA or by direct degradation by IDE, NEP, A2M–serine protease complex and the PA/plasmin system. Activation of PA/plasmin in return enhances the α-secretase pathway.

The crystal structure of the BACE-1 (β-secretase) ectodomain demonstrates that the enzyme is very similar to other known aspartic proteases. However, there are differences in details of the active site, with β secretase being more open and less hydrophobic than in other aspartic proteases (Hong *et al*., 2000). The relatively large active site of β-secretase may make the development of small molecule inhibitors difficult, unless they act in a synergistic manner.

A lysine (Lys)→asparagine (Asn)/Methionine (Met)→leucine (Leu) double mutation near the β-secretase cleavage site found in Swedish familial AD produces approximately 6-8-fold more Aβ ($A\beta_{40}$ and $A\beta_{42}$) compared with cells expressing normal β-APP resulting in early onset of AD (Citron *et al*., 1992).

Luo *et al*. (2001) demonstrated that the BACE1 knockout mice lack brain Aβ and β-secretase-cleaved APP CTFs. Moreover the mice were healthy and fertile. Cai *et al*. (2001) showed that the BACE1 knockout mice abolish cortical Aβ without observed side-effects and concluded that BACE-1 is the principal neuronal protease required for the cleavage of APP to generate Aβ peptides. Roberts *et al*. (2001) also reported that BACE1 knockout mice lack the major β-secretase in brain and have a significantly reduced level of Aβ peptide production, yet these mice have no deficiencies in physiology or basic behaviours. In addition, Hom *et al*. (2003) demonstrated *in-vitro*

that selective inhibition of BACE-1 by a synthetic compound suppresses Aβ secretion in human embryonic kidney cells.

The BACE1 knockout data published to date encourage the development of β-secretase inhibitors as a therapeutic strategy in the treatment of AD because it suggests that, at least in mice, other β-secretase substrates and products are not very important or are provided by compensatory mechanisms, whereas no compensatory mechanism allows Aβ production. Because the BACE1 knockout mice do not have a clinically relevant phenotype, they have not led to the identification of functional β-secretase substrates yet (Citron, 2002).

To date, there are no reports available on anti-secretase activity of any species of plants. Oils of *S. apiana, S. fruticosa and S. officinalis* var. *purpurea* had not only marked dual anti-ChEs activity but also high yield and thus were selected for the screening.

7.1.2. Neuroinflammation as a target in the treatment of AD

A role of inflammation in the development of AD has recently been reviewed (Aisen, 2000; Akiyma *et al.*, 2000; Cooper *et al.*, 2000; Selkoe, 2001; Weiner and Selkoe, 2002). In addition, Neurobiology of Aging (2001: volume 22, issue 6) provided a special issue dealing with inflammatory processes in AD. The cholinergic anti-inflammatory pathway involving the nAChR α7 subunit and pro-inflammatory cytokines has also been discussed (1.3.1.3.).

As noted in these reviews, the appropriate targets for anti-inflammatory therapy remain to be clarified. Cytokine activities in the brain appear to be complex, with both pro-

inflammatory and anti-inflammatory roles. Meda *et al.* (2001) and Bales *et al.* (2000) reviewed evidence suggesting that deposition of Aβ represent a triggering factor in glial activation leading to inflammatory responses in the brain. Thus, studying a mechanism of such responses may provide a significant insight into the pathophysiology of AD, and may also lead to the identification of new strategies for AD treatment.

Etanercept, a protein antagonist of the TNF-α receptor, is a newly approved treatment for arthritis (Aisen, 2000). Cooper *et al.* (2000) noted that inflammation in arthritis, like the inflammation in AD, is highly complex and involves multiple inflammatory responses. If treating a single inflammatory mediator such as TNF-α, is effective in arthritis, then there is a possibility that a similar strategy may also be beneficial in AD.

Chemokines belongs to a rapidly expanding family of cytokines with the primary function of recruitment of leukocytes to the site of inflammation (Selkoe, 2001). IL-8 is a 72-amino-acid peptide expressed in many cell types and is released in response to proinflammatory stimuli. It is a potent inflammatory mediator that belongs to a family of CXC (the first cysteine pair is separated by an intervening amino acid) proinflammatory chemotactic cytokines (Xiong *et al.*, 2003). Accumulating evidence (MacDermott, 1999; Frangogiannis *et al.*, 2002; Xiong *et al.*, 2003) implicates interleukin 8 (IL-8) as a major mediator of acute macrophage-mediated inflammation. Horuk *et al.* (1997) and Xia *et al.* (1997) demonstrated that one of the receptors (CXCR2) of IL-8 is expressed in the neuritic portion of plaques surrounding deposits of amyloid. The authors suggested that IL-8 may participate not only in reactive processes of normal neuronal function but also in the neurodegenerative process. Moreover, Lue *et al.* (2001) and Walker *et al.* (2001) demonstrated a significant dose dependent

increase (up to 11.7-fold) of secreted pro-inflammatory IL-8 in human *post-mortem* brain microglia when the latter was exposed to increasingt doses of Aβ.

Kelder *et al.* (1998) reported an increased level of IL-8 in patients with human immunodeficiency virus-associated dementia (HAD), whereas Zheng *et al.* (2001) and Xiong *et al.* (2003) showed that IL-8 inhibit long-term potentiation, a neural response linked closely to learning and memory via CXCR2 activation in the hippocampus and suggest this also may contribute to the cognitive dysfunction associated with HAD.

Plants reputed anti-inflammatory activities may be a source of therapeutics with anti-inflammatory properties. Mudanpi, the root cortex of *Paeonia suffruticosa* Andrews (Ranunculaceae), is an important crude drug used in Chinese traditional medicine as an anti-inflammatory agent (Oh *et al.*, 2003). Oh *et al.* (2003) demonstrated that *in-vitro* 95% methanol extract of root of *Paeonia suffruticosa* (mudanpi) suppresses IL-8 secretion. These authors also reported that dimethyl sulfoxide (DMSO) has been used in the preparation of plant extracts. Poliakova *et al.* (1993) showed that *in vivo* DMSO may reduce inflammation of external and middle ear, while Smirnova *et al.* (2002 a; 2002 b) described a meditative role of IL-8 in inflammation of the middle ear cleft. In addition, Nakamuta *et al.* (2001) demonstrated that *in vitro* DMSO inhibits TNF-α production, one of the primary pro-inflammatory cytokines. Thus, the use of DMSO may complicate the results reported by Oh *et al.* (2003).

Extracts of neem tree (*Azadirachta indica* Juss. Meliaceae), East-Indian globe-thistle (*Sphaeranthus indicus* Linn. Asteraceae) and also myrrh oil (*Commiphora molmol*) have been shown to inhibit secretion of IL-8. (Jain and Basal, 2003; Tipton *et al.*, 2003). Hart *et al.* (2000) reported that the main component of essential oil of *Melaleuca*

alternifolia (tea tree) terpinen-4-ol suppresses inflammatory activity of IL-8, while α-terpineol and 1,8-cineole are inactive.

Salvia apiana and *Salvia fruticosa* have historically been reputed as plants with anti-inflammatory properties (1.3.1.4.2.). To date, there is no data available on the suppression of IL-8 secretion by any species of *Salvia*.

7.1.3. Cholinergic receptors as a target in the treatment AD

A role of nicotinic and muscarinic receptors in development of AD has been described in Chapter 1 (1.2.1.3; 1.2.2.). Chapter 1 also describes interaction of current licensed ChEI drugs and the cholinergic receptors (1.3.1.1.).

Plant extracts have been shown to have both cholinergic nicotinic and muscarinic human receptor binding activities (Wake, 2001). Wake (2001) reported that one of 80% EtOH extracts of *Melissa officinalis*, exhausted of water-soluble quaternary ammonium compounds, has [^{3}H]-nicotinic displacement activity with an IC_{50} value of 0.7 mg ml^{-1}, whereas the crude EtOH extract of the same plant is less active, with an IC_{50} value of 3.6 mg ml^{-1}. In contrast an 80% EtOH extract of *Salvia verticillata* ssp. *verticillata*, also exhausted of water-soluble quaternary ammonium compounds, had less nicotinic activity with an IC_{50} 0.5 mg ml^{-1} compared with the crude EtOH extract with an IC_{50} 1.0 mg ml^{-1}. Wake noted that the extracts prepared from autumn-harvested leaves are relatively inactive compared to the summer-harvested leaves.

Wake (2001) also showed, using [N-methyl-^{3}H]-scopolamine displacement assay, that one of fresh leaf 80% EtOH extracts of *Melissa officinalis* has muscarinic activity with

an IC50 value of 0.06 mg ml^{-1}. In addition, the EtOH extract of *Salvia elegans* Vahl., `Scarlet Pineapple' exhibited a total displacement of [N-methyl-^{3}H]-scopolamine at a concentration of 1.4 mg of dried plant per ml (Wake *et al.*, 2000). Lewis *et al.* (1999) reported that 80% EtOH extract of *Panax ginseng*, CA Meyer, (Araliaceae) has relatively low affinity for nicotinic and even less for muscarinic receptors, with IC_{50} values of 2.12 mg ml^{-1} and 5.25 mg ml^{-1} respectively. There are also reports indicating that camphor and borneol specifically inhibit nAChRs in a non-competitive way (Park *et al.*, 2001; Park *et al.*, 2003).

The objective of the present section of this research project was to test extracts of *Salvia* species for β-secretase inhibition, suppressive activity of IL-8, and nicotinic and muscarinic receptor activities.

7.2. Results

7.2.1. Essential oils of sage and their chemical constituents in relation to β secretase

8% EtOH in the assay did not inhibit β secretase (Table 7.1.). Although, EtOH showed some tendency towards the inhibition of the enzyme over the course of the incubation time, the student t-test analysis indicated that such inhibition was insignificant with the confidence interval exceeding 5% ($P>0.05$).

Neither oils of *S. apiana*, S. *fruticosa*, S. *officinalis* var. *purpurea* nor their single compounds 1,8-cineole and 3-carene had anti-β secretase activity at tested concentrations (Table 7.2.). A tendency of 1,8-cineole to inhibit the protease was statistically insignificant, with the confidence interval exceeding 5% ($P>0.05$).

Table 7.1. Anti-β secretase activity of EtOH

Inhibitor	β-Secretase activity, mean±SD (n=3)[a]			
	30 min	60 min	90 min	120 min
8% EtOH	32615±3743 (P=0.09, P>0.05)[b]	36237±4685 (P=0.17, P>0.05)[b]	38221±4620 (P=0.32, P>0.05)[b]	42158±4870 (P=0.4, P>0.05)[b]
H_2O	39630±3336	42026±3128	42327±3690	45721±4076

[a]Activity is expressed in fluorescence units. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of three sets of triplicate.

[b]Statistical significance. If P value is more than 0.05, the inhibition is not significant. During the four intervals of incubation time P values exceeded 0.05.

Table 7.2. Anti-β secretase activity of oils and their single constituents

Inhibitor	Concentration mg ml^{-1} [b]	β-Secretase inhibition, % mean±SD (n=3)[a]			
		30 min	60 min	90 min	120 min
S. fruticosa	0.4	0	0	0	0
S. officinalis var *purpurea*	0.1	0	0	0	0
S. apiana	0.3	0	0	0	0
1,8-cineole	0.5	5.5±8.3	5±8.7	4±6.9	5±8.7
3-carene	0.2	0	0	0	0

[a]Agents were tested in triplicate. Mean of triplicate was designated as n=1. Standard deviation (SD) was calculated on a basis of three sets of triplicate.

[b]Concentration of test solutions were close to their solubility limit in the assay.

7.2.2. Oils of *S. apiana* and *S. fruticosa* in relation to interleukin 8

There was 18±2.1% inhibition of the IL-8 secretion by *S. apiana* oil in the HT29-MXT cells after 24h of incubation, while oil of *S. fruticosa* was inactive (Figure 7.2.). In both cases, the first four hours of incubation produced no inhibition.

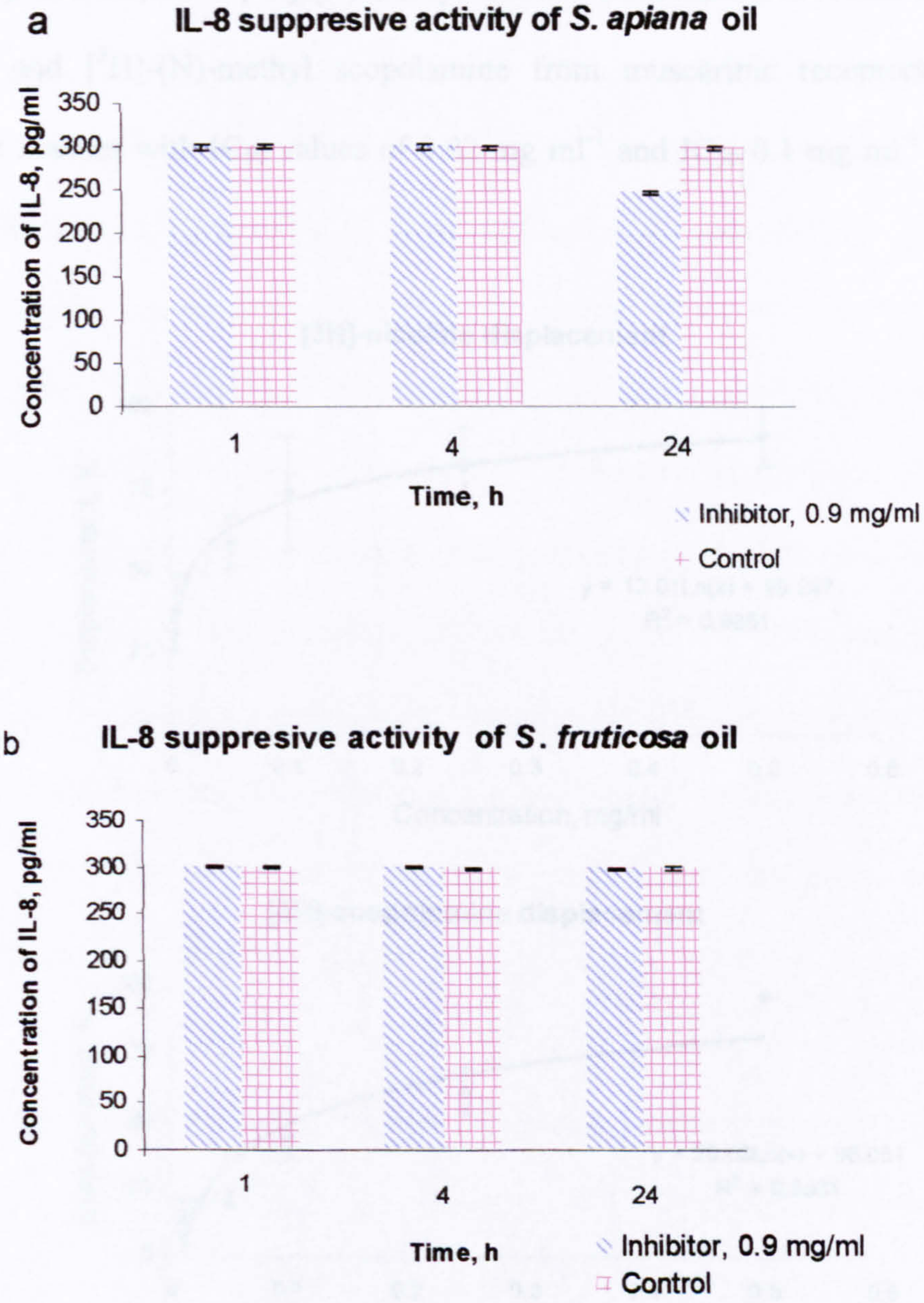


Figure 7.2. IL-8 suppressive activity of oils of *S. apiana* (a) and *S. fruticosa* (b)

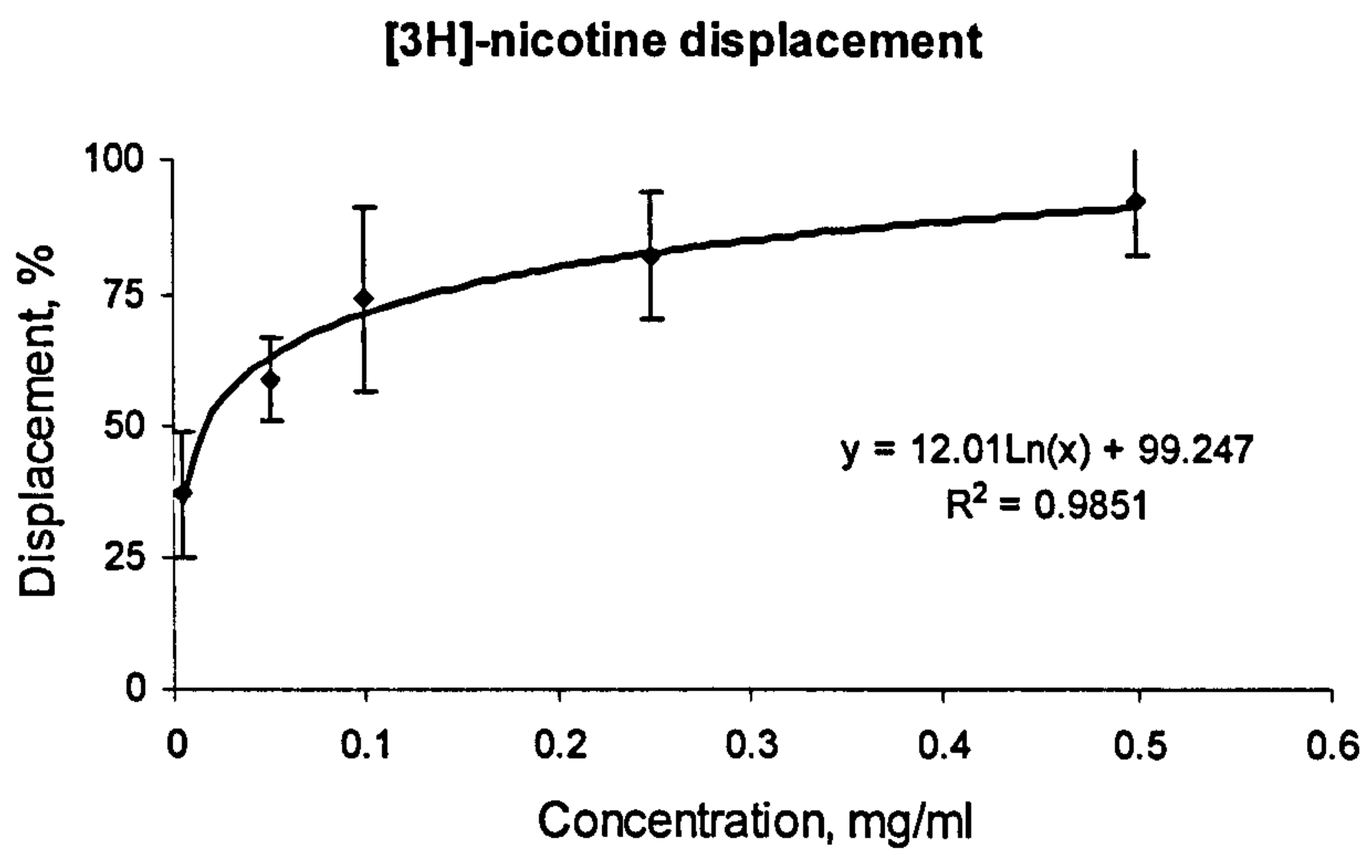
Each proposed inhibitor and control was tested in triplicate over the period of incubation time. Standard deviation was calculated on a basis of three sets of triplicate

The computer software allowed conversion of rates of secretion of IL-8 into values of concentrations, *i.e.*, pg ml^{-1}.

7.2.3. Oil of *Salvia apiana* in relation to nicotinic and muscarinic receptors

Oil of *S. apiana* displaced [^{3}H]-(N)-methyl nicotine from human nicotinic acetylcholine receptors and [^{3}H]-(N)-methyl scopolamine from muscarinic receptors in a dose dependent manner with IC_{50} values of 0.02 mg ml^{-1} and IC_{50} 0.1 mg ml^{-1} respectively (Figure 7.3.).

a



b

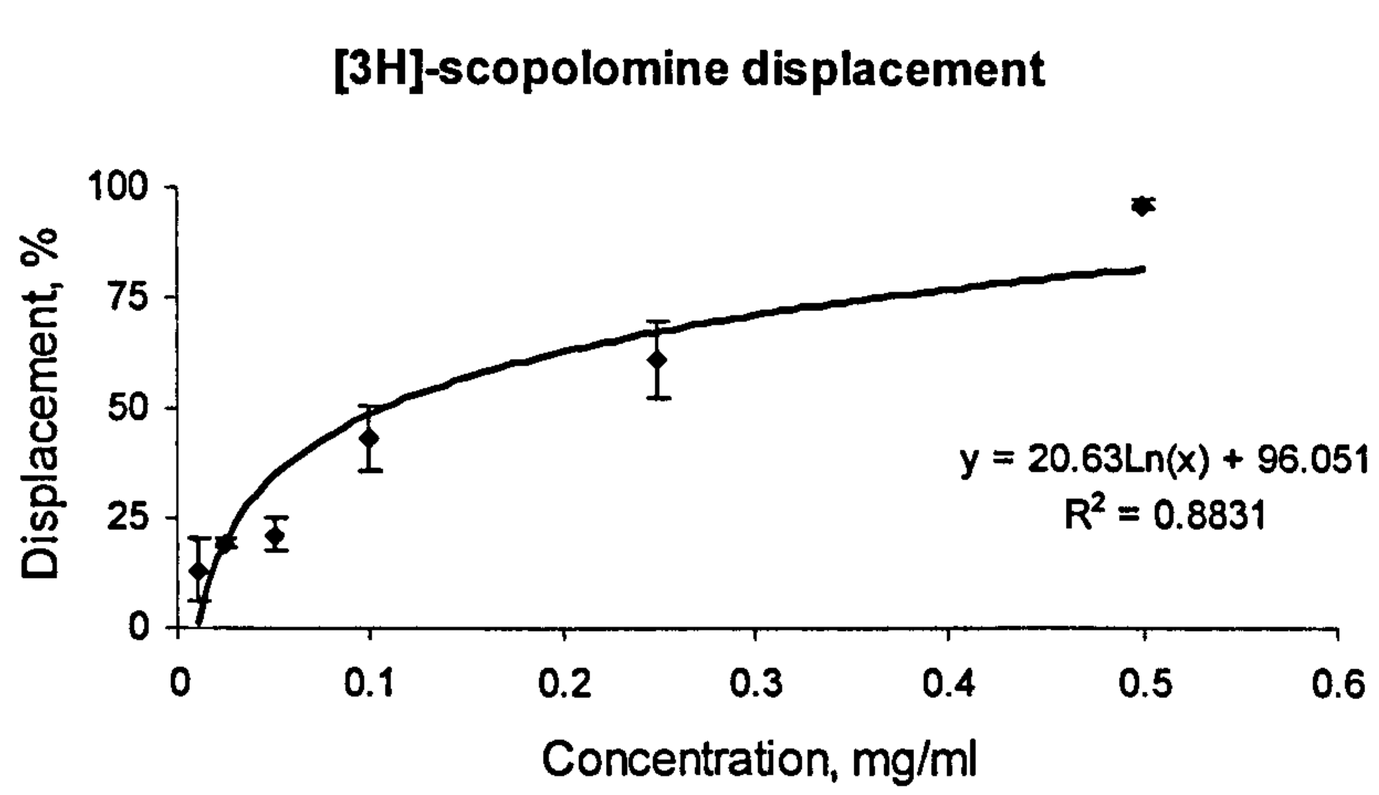


Figure 7.3. Nicotinic and muscarinic receptors binding activity of *S. apiana* oil

a. [^{3}H]-(N)-methyl nicotine displacement curve of *S. apiana* oil

b. [^{3}H]-(N)-methyl scopolamine displacement curve of *S. apiana* oil

In both cases there was more than 90% displacement of the radioligands by the oil at a concentration of 0.5 mg ml^{-1}. As calculated from the dose-response curve equations (Figure 7.3.) IC_{50} values of the oil for [^{3}H] nicotine and [^{3}H] scopolamine displacement were 0.02±0.024 mg ml^{-1} and 0.1±0.015 mg ml^{-1} respectively.

7.3. Discussion

7.3.1. Anti-β secretase activity of oils of sage and their constituents

The present study shows that neither oils of *S. apiana*, *S. fruticosa* or *S. officinalis* var *purpurea* nor 1,8-cineole and 3-carene inhibited β secretase over the course of incubation time. The relatively large active site of β-secretase (Hong *et al.*, 2000) may help to prevent its inhibition by small molecules. Even the essential oil, with their recognised synergistic mode of action, did not react with the active site, although this may not be a case for other plant extracts. Interestingly, 1,8-cineole showed a steady but an insignificant trend towards reduction of β secretase activity over the periods of incubation time. The fact that 8% EtOH does not inhibit the protease may permit analyses of other hydrophilic compounds.

Ghosh *et al.* (2001) reported syntheses of several human β-secretase (memapsin-2) inhibitors. OM99-2 (Figure 7.4) has a K_i value of 1.6 nM and it is more potent than inhibitor OM99-1 (Figure 7.4), K_i of 36 nM. The therapeutic potential of inhibitor OM99-2 may be limited because of its high molecular weight and numerous peptide bonds. However, its structure may serve as an important molecular template for structure-based design of memapsin-2 inhibitor drugs and may help to identify other plant derived inhibitors.

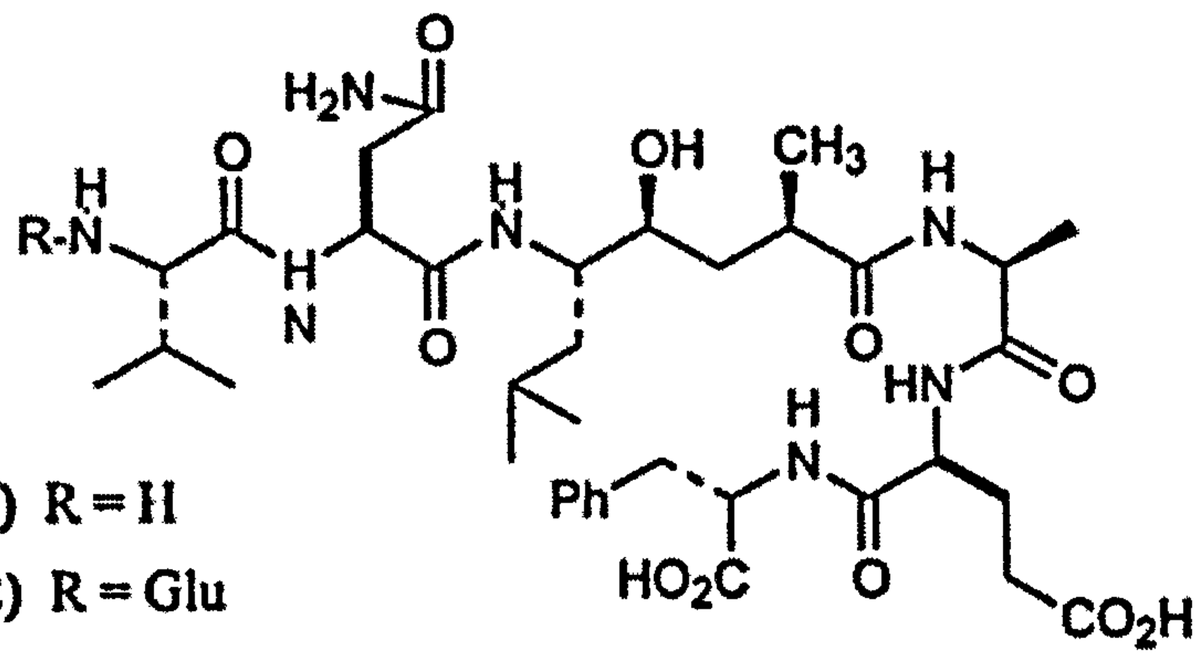


Figure 7.4. OM99-1 and OM99-2. Glu (Glutamate)

7.3.2. Oils of *S. apiana* and *S. fruticosa* in relation to interleukin 8

The present study demonstrates that the oil of *S. apiana* has a modest suppressive effect on IL-8 secretion in the human mucin- and IL-8 cytokine secreting cells HT29-MTX, while the oil of *S. fruticosa* has not. The suppressive effect of the *S. apiana* oil was not apparent before 24 hours of incubation and the longer time may be needed to observe a greater inhibitory effect. For instance, Hart *et al.* (2000) reported that terpinen-4-ol, a main constituent of tea tree oil, suppresses IL-8 production after 40 hours of incubation. Nevertheless, the inhibitory effect (18%) of *S. apiana* oil may be relevant therapeutically in the context of suppression of acute inflammation, as a certain level of expression of IL-8 is required in order to antagonise transmembrane G-protein-coupled receptors, a signalling inflammatory pathway of IL-8 (Wells *et al.*, 1996; personal communication with Dr Smirnova, department of Physiological Sciences, the Medical School, University of Newcastle), hence the profound inhibition of the cytokine may destabilise a natural defensive mechanism of the immune system towards inflammation. It should also be noted that the HT29-MTX culture cells contain more than 90% of goblet cells, a source of IL-8 secretion (Dr Smirnova, personal communication). There

are no reports suggesting presence of goblet cells in the brain. Production of IL-8 (other cytokines) in the brain is attributed mainly to macrophages. Further experiments, involving macrophage-containing cells line, are therefore warranted.

1,8-cineole, one of the main constituents of *S. fruticosa* oil (Chapter 4), has been reported to have no suppressive activity on IL-8 secretion (Hart *et al.*, 2000) and therefore may contribute to the lack of activity in whole oil. The modest activity of *S. apiana* oil may indicate that camphor, major constituent of the oil (Chapter 4), also does not have suppressive IL-8 activity. Thus, essential oils of sage with major constituents other than 1,8-cineole and camphor but terpineol-4-ol (Hart *et al.*, 2000) may worth further investigation for inhibition of IL-8 secretion.

7.3.3. Oil of *S. apiana* in relation to nicotinic and muscarinic receptors

The current study shows that oil of *S. apiana* contains a compound, or a combination of compounds acting synergistically, that displaces [^{3}H]-nicotine from human nAChR and [^{3}H]-scopolamine from human mAChR in a dose-dependent manner with IC_{50} values of 0.02±0.024 mg ml^{-1} and IC_{50} 0.1±0.015 mg ml^{-1} respectively. The shape of a dose-response curve for scopolamine displacement may indicate some degree of non-specific interference within the oil at its low concentration, although for nicotinic displacement such interference was less apparent. Further study on kinetics of these displacements may evaluate receptor-specific activities.

In both cases, the oil, a final concentration of 0.5 mg ml^{-1}, displaced more than 90% of [^{3}H]-nicotine and [^{3}H]-scopolamine from human membrane. In contrast, Wake (2001)

reported that 80% EtOH extract of *Salvia elegans*, a final concentration of 1.2 mg ml^{-1}, displaced 85% of [^{3}H]-nicotine and 100% of [^{3}H]-scopolamine.

The preliminary results indicate that oil of *S. apiana* contains constituent(s) with nicotinic and muscarinic binding properties. Further fractionation of the oil therefore is warranted. Finally, if a positive effect of EtOH extract of *S. officinalis* (Akhondzadeh *et al.*, 2003) and Phytosol A extracted oil of *S. lavandulaefolia* (Perry *et al.*, 2003) on those with AD is attributed not only to inhibition of ChEs but also to its nicotinic and muscarinic binding activities than the IC_{50} values of the oil may become clinically relevant.

Chapter 8. General discussion

8.1. *Salvia* species as a source of agents with activities relevant to dementia treatment

8.1.1. Clinical relevance

The ethnobotanical reputation of sage as a plant with memory enhancing properties (Chapter 1) has recently been corroborated with positive results from *in vivo* studies. A single dose of 50µl of steam distilled oil of *S. lavandulaefolia* enhanced memory, compared to placebo, in a dose-dependent manner in healthy young adults (Tildesley *et al.*, 2003), while clinical administration of the plant oil obtained by Phytosol A extraction improved behaviour and attention in an open label trail in subjects with mild to moderate AD (Perry *et al.*, 2003). Further, Akhondzadeh *et al.* (2003) reported, in a double blind placebo controlled clinical trail, that administration of 60 drops a day (16 weeks) of a 45% EtOH extract of *S. officinalis* during four months significantly enhanced cognition and reduced agitation in patients with mild to moderate AD. An EtOH extract of *S. officinalis* inhibited human AChE with an IC_{50} value of 0.03 mg ml^{-1} (personal communication with Dr. Wake, medicinal plant research centre University of Newcastle). This IC50 value is similar to those obtained by most of the extracts in this study (Chapter 3 and 4). Thus, if the effect of the trial is attributed to inhibition of cholinesterases then the IC_{50} values of the active extracts may become clinically relevant. These positive results not only indicate that sage species are the source of molecules with activities relevant for dementia treatment, but also provide information to clinical significance of an IC_{50} value of extract.

Moreover, the positive outcome of the trials may indicate that active molecule species penetrate the blood brain barrier and interact, *inter alia*, with a cholinergic system within the brain which is responsible for cognition (Bartus *et al.*, 1982; Perry *et al.*, 1986 b). In addition, previous *in vitro* reports (Chapter 1) showed that extracts of sage species inhibit the major enzyme of human AChE and have affinity for human cholinergic acetylcholine receptors. The present study further contributes to improving the efficacy of non-polar extracts of *Salvia* species in the treatment of dementia.

8.1.2. Evidence of synergy

One of the novel findings of this study (Chapter 5) was that none of the twenty one low molecular weight compounds tested had marked anti-BuChE activity, although the whole extracts invoked slow onset of a non-competitive type of inhibition of the enzyme within a therapeutically relevant range of concentrations, *i.e.*, 0.01 mg ml^{-1}<IC_{50}<0.1 mg ml^{-1}. Non-competitive inhibition of BuChE was also reported (Chapter1) in tacrine and donepezil, licensed drugs in the treatment of AD, and physostigmine. An abundant presence, in some cases more than 50%, of the inactive constituents in the extracts provides no obvious explanation for variations in anti-BuChE activity of the species in terms of the chemical composition of their extracts, although major synergistic interactions among certain minor and major constituents within the latter may explain these differences.

In contrast inhibition of human AChE by those twenty one terpenoids was more apparent. Moreover, the screening of these compounds resulted in another new finding. Thus, β-caryophyllene (caryophyllene) and 3-carene (Chapter 5) were the most active non-oxygenated terpenoids ever reported with IC_{50} values of 0.1 and 0.2 mM

respectively, although the most active oxygenated terpene viridiflorol inhibited bovine AChE with the same value as β-caryophyllene (Miyazawa *et al.*, 1998). A lack of negative charges in β-caryophyllene and 3-carene molecules may not allow their passage to the active site via the aromatic gorge of the enzyme but rather produce a quasi-steric hindrance for the substrate at the entrance of the gorge (Tai *et al.*, 2002).

Interestingly, all extracts, such as *S. argentea*, *S. keerlii*, *S. microphylla* var. *neurepia*, S *S. confertiflora, S. haematodes, S. glutinosa, S. longistyla, S. napifolia, S. verticilata and S. Jamensis* var. la luna which are rich in β-caryophyllene (more than 10%) expressed anti-AChE activity (Chapter 4), although the solubility limit did not allow preparation of higher concentrations which might result in 50% inhibition of AChE within a range of theraputical concentrations. This may be explained via synergistic interactions of certain constituents with β-caryophyllene. Evidence of such interactions was apparent when the inhibitory activity of β-caryophyllene, the only major compound with more than 10% in *S. microphylla* var. *neurepia* oil, did not account for the activity of the whole oil (Table 8.1). It can be noted that the inhibitory contribution of β-caryophyllene alone is only 29% and the rest of the activity may be attributed to minor synergistic interactions between β-caryophyllene and other constituents of the oil. As another example, the amount of 1,8-cineole in steam distilled oil of *S. fruticosa* (8/02) (Chapter 3) is 21.5%. If the oil, which gave 50% inhibition of AChE at a final concentration of 0.04 mg ml^{-1} at incubation time of 5 minutes is taken as a mixture of 100% then a final concentration of 21.5% 1,8-cineole in the oil would be 0.0086 mg ml^{-1} or 0.05 mM. As calculated from a mean dose-response curve of 1,8-cineole a final concentration of 0.0086 mg ml^{-1} would give 20% inhibition of the enzyme, hence the compound alone would not also account for the IC_{50} value of the oil and nor would the other tested constituents.

Table 8.1. Inhibition of AChE by essential oil of *S. microphylla* var. *neurepia* and β-caryophyllene at the concentration occurring in the oil

Inhibitor	% in oil[a]	Concentration mg ml^{-1} [b]	% Inhibition[c] mean±SD (n=4)
S. microphylla var. *neurepia* oil	100	0.035	50±0.003
β-caryophyllene (caryophyllene)	12.7	0.006	29±1.6

[a]Percent inhibitor in the oil obtained by GC/MS analysis. [b]An IC_{50} value of the whole oil of 0.035 mg ml^{-1}, which gave 50% inhibition of the enzyme was taken as 100%, for the convenience of calculations. Hence, the concentration of β-caryophyllene was calculated on a basis of its percentage in the oil. [c]Percentage inhibition was calculated from the dose-response curve equations of each inhibitor. Thus, 0.035 mg ml^{-1} was substituted into the equations of the oil to calculate expected percent of AChE inhibition, while 0.006 mg ml^{-1} was substituted into the equations of β-caryophyllene to calculate expected percent of inhibition.

Anti-AChE activity of the extracts is mainly due to the activity of the principal chemical constituents, with a minor degree of synergy, whereas anti-BuChE activity cannot be accounted for in terms of the principle constituents indicating major synergy. Indeed, the presence of minor synergy in inhibition of bovine AChE between 1,8-cineole/a-pinene and 1,8-cineole/caryophyllene oxide (Savelev *et al.*, 2003; Chapter 6) may support this speculation. In addition, despite the abundant presence (46.5%) of inactive α-caryophyllene in *S. officinalis* var. *purpurea* oil inhibition of AChE was competitive. This shows that synergistic interactions of constitutes within *Salvia* extracts may result in two ways of inhibition of the enzyme, *i.e.*, competitive and non-competitive.

Further, regardless of differences between the supercritical extraction and steam distillation both methods allowed extraction of molecules with dual anti-cholinesterase activity. Most inhibitors presently used in AD therapy are not selective for AChE, however they all show significant clinical efficacy (Giacobini, 2000). Considering the pronounced decrease in AChE activity in certain brain areas (Chapter 1) of patients with advanced AD and the large pool of BuChE available in glia, neurons and neuritic plaques (Chapter 1), it may be an advantage for a ChE inhibitor to be non-selective rather than AChE selective. A balance between AChE and BuChE inhibition may result in optimal theraputical efficacy if a non-selective ChE inhibitor is used in mild to moderate forms of AD. An observation of Costa *et al.* (1999) showed that inhibition of BuChE activity in the cerebral spinal fluid correlates more strongly with cognitive improvement than inhibition of AChE during rivastigmine trail.

8.1.3. Antiinflammatory and neuroprotective roles of dual anti-cholinesterase inhibitors

Dual anti-cholinesterase activity of the extracts may also be therapeutically relevant for those with mild cognitive impairment, since Mesulam *et al.* (2002) reported that widely spread BuChE can hydrolyse ACh in the normal brain. Although this contradicts to a theory (Giacobini, 2000; Chapter 1), based on the differences in the enzymatic affinity constant (Km), that in the normal brain AChE is the main enzyme responsible for ACh hydrolysis with BuChE playing a supportive role and that selective AChE inhibitors may be more desirable for those with mild and moderate stages of AD (Davis *et al.*, 1999), familial AD and mild cognitive impairment.

Further, inhibitors with dual anti-cholinesterase activity may be involved in the cholinergic anti-inflammatory pathway (Chapter 1) by increasing a level of ACh, which is a functional ligand for the α7 nAChR on the macrophage (Wang *et al.*, 2003). Upon binding of ACh to the receptor subunit suppression of pro-inflammatory cytokine TNF synthesis occurs via an increase in Ca^{2+} influx within a macrophage (Wang *et al.*, 2003). Neumann *et al.* (1998) reported that glial-derived TNF interacts with neurons as part of an injury or inflammatory process, while later Zassler *et al.* (2003) showed *in-vivo* that such interaction induces axonal damage of cholinergic nerve terminals and nerve fibers, suggesting that this can lead to subsequent retrograde cell death of cholinergic neurons in the basal nucleus of Meynert. Thus, glial and neuronal inhibition of BuChE and AChE by the extracts with dual activity may increase the level of ACh which apart from neurotransmission can have an indirect neuroprotective role via controlling overexpression of TNF.

8.1.4. Extracts with multiple activities

Evidence (Court *et al.*, 2001; O'Neill *et al.*, 2002; Perry and Piggott, 2003; Piggott *et al.*, 2003; Chapter 1) of early stage nicotinic and muscarinic abnormalities in AD and DLB supports the use of cholinergic agonists in prospective treatment of the diseases. Galanthamine, one of the most successful plant-derived licensed drugs in the treatment of AD, apart from dual anti-cholinesterase activity, has affinity for nAChRs (Chapter 1). This study showed that using Phytosol A as a solvent allows extraction of *S. apiana* oil not only with dual anti-ChE activity but also with marked human nicotinic and muscarinic ligand binding properties. Development of non-toxic cholinergic ligands for therapeutical purposes may enable treatment of cholinergic receptor/transmitter related cognitive problems to be carried out with less risk of undesirable side-effects. In

addition, the oil expressed modest suppression of a pro-inflammatory cytokine IL-8. Thus, *in-vitro* multiple activity of the oil makes it a first choice candidate for a clinical trial on those with AD and DLB. A theory of a multiple drug therapy towards AD (Prasad *et al.*, 2002) may find one of its solutions in herbal standardised extracts.

Finally, *Salvia* extracts with dual anti-ChEs activity and/or cholinergic receptors binding properties may have a synergistic effect together with the licensed drugs currently used in the treatment of AD. Such an effect may reduce administrative doses of these drugs and consequently could result in reduction of undesirable side-effects caused by the latter. The lipophilic nature of the extracts, as a phytophore, may also enable faster delivery of the drugs to the brain.

8.2. Species variation

This study also demonstrated a lack of correlation between geographical distribution of the species (Appendix 1) and a chemical profile of their extracts, which may be due to adaptation of the plants to particular enviromental conditions. For example, Bellomaria *et al.* (1992) reported, as confirmed in this study, that oil of *S. fruticosa* may contain 42% camphor and 20% 1,8-cineole if collected in the Troodos area of Cyprus but 55% 1,8-cineole and 6% camphor if harvested in the Anglisidhes and Stavros areas. Oils of sage species from western Crete and northern parts of Greece also contain low amounts of camphor and high of 1,8-cineole (Karousou *et al.*, 2000).

In-vitro a naturally occurring combination of camphor and 1,8-cineole was antagonistic in inhibiting bovine AChE with an interaction index of 2 (Savelev *et al.*, 2003). Collecting *Salvia* species rich in 1,8-cineole but not camphor may therefore provide oils

with more potent cholinergic activities, as it may also plants which are abundant in β-caryophyllene and 3-carene.

It was reported (Park *et al.*, 2001; Park *et al.*, 2003) that camphor and borneol are specific, non-competitive antagonists to nicotinic acetylcholine receptors and as the level of ACh decreases with the progression of the disease (Chapter 1) these two molecules may become competitive with the neurotransmitter. It could also accentuate, especially to non-smokers, the loss of nicotinic receptors during ageing (Court *et al.*, 1997). Extracts with dual anti-cholinesterase activity and lower camphor and borneol content may thus be the most appropriate for clinical trials among the species so far investigated.

8.3. Methodological issues

A comparative analysis of oils of *S. fruticosa* (8/02) and *S. officinalis* obtained via Phytosol A extraction and steam distillation revealed not only differences in their chemical composition but also significant variations in the ratio of major and minor constituents, which resulted in favour of cold extracted oils in terms of their anti-ChEs activity. Thus, anti-AChE activity of Phytosol A oil of *S. officinalis*, with an IC_{50} value of 0.015±0.001 mg ml^{-1}, was 5 times more potent than the steam distilled oil, while anti-BuChE activity, with an IC_{50} value of 0.06±0.003mg ml^{-1}, was only apparent in the cold extracted oil.

In contrast, variations in a ratio of major and minor constituents of *S. fruticosa* (8/02) oil resulted once again in favour of Phytosol A oil. Thus, the steam distilled oil did not inhibit human BuChE, whereas the Phytosol extracted oil had an IC_{50} value within the

theraputical rage of concentrations. In addition, anti-AChE activity of the latter was two times more potent.

These examples demonstrate that the low boiling temperature of Phytosol A may prevent hydrolysis and thermolysis of active molecular species during the extraction process. It should also be pointed out that there is a possible artefact which can occur from the modification of labile materials undergoing chemical changes as a result of the drying or extraction processes (Tyler, 1994). These changes may be brought about by the mechanical disruption of the plant tissues freeing enzymes, normally existing within strictly defined, often membrane bound regions of the cytoplasm (Stryer, 1988), which can act upon various substrates in a random fashion. This may produce materials not normally present in the living plant. Furthermore, a slight change in the extraction process could result in an extract containing novel or batch-exclusive molecules which may alter the extract's behaviour when assayed for biological activity.

Extraction of *Salvia* species with Phytosol A provided extracts with major chemicals which have never been reported to be present in the genus (Chapter 4). The study also showed that these extracts are a potential source of isolation of single unknown compounds with activities relevant to treatment of dementia.

8.4. Recommendations for further research

Further research into *Salvia* species as a source of materials for dementia therapy would need to take the following points into consideration.

1. Prospective treatment of dementia is likely to be based on a multiple drug therapy, hence screening and standardisation of extracts with activities relevant to dementia therapy is vital. Standardised active fractions/extracts targeting both cholinesterases, cholinergic acetylcholine receptors, pro-inflammatory cytokines, secretases, protein phosphatase 1 and reactive oxygen species may be amalgamated in order to study a synergetic effect of its combination(s). For example, a standardised fraction with anti-BuChE activity, comprising a known number of synergistically interactive molecules, may be combined with a standardised fraction expressing nicotinic receptor ligand binding properties. If such combination remains synergistically interactive further fractionation (standardisation) of the latter may thus be warranted.

2. *In vitro* and *in vivo* experiments are needed to ascertain potential synergistic effect of extracts with anti-cholinesterase activity and the licensed drugs (cholinesterase inhibitors) used in the treatment of AD. A positive interaction within these combinations may reduce doses of the prescribed drugs needed and therefore their undesirable side-effects.

3. It would be interesting to conduct *in vivo* experiments designed to monitor TNF-α secreting activity after administration of extracts with dual anti-cholinesterase activity in healthy and demented subjects. A reduced level of the pro-inflammatory cytokine would indicate at a possible neuroprotective effect of such inhibitors. This may be achieved by

measuring a level of cytokines in the blood before and after administration of the extracts.

4. Phytosol A may be recommended for isolation of single compounds from non-scented plants. The solvent can also be used for isolation of polar plant constituents if combined with a polar co-solvent. The extraction of polar compounds (fractions) may proceed this way;

1. Plant material (PM) + Phytosol A >>> PM1 + extract (extract for analysis)

2. PM1 + Phytosol A in a combination with a polar solvent (PS) >>> PM2 + extract (extract for analysis)

3. PM2 + Phytosol A and PS >>> PM3 + extract (extract for analysis)

4. PM4 + extract

Changing physical parameters, such as temperature, percentage of co-solvent and the intensity of vortex, during the extraction process may also be recommended for the purpose of better process standardisation. A residue which appears to be a part of the most extracts may be used as a source of isolation of new single molecules.

6. Phytosol A extracts showed to be more superior to steam distilled oils for anti-ChEs activity, especially towards inhibition of BuChE. A comparative analysis of a chemical composition of these extracts and their anti-ChEs activity did not allow identification of a chemical fingerprint which would explain such superiority. Hence, more comparative analyses of these two methods are needed in order to evaluate a link between the bioactivity and a chemical profile of the extracts.

8. As a ratio of G_1 and G_4 forms of AChE changes during AD it is of an interest to analyse *Salvia* extracts for their selectivity. Preferentiality towards inhibition of the G1 form (remains unchanged in AD) thus would be more desirable, although galanthamine and donepezil are not selective for any forms.

9. The ethnopharmacological approach to screening plants for useful therapies for dementia is supported in this study and could be extended to other plant species.

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Appendices

Appendix 1.

Salvia database

The database is recorded on the attached CD disk. It includes botanical descriptions of the species, plant images, and abstracts/articles on their reported bioactivities and/or chemical compositions. (nss64; Velevas31) or visit www.ncl.ac.uk/medplant

Species	Image	Articles \ Abstracts
S. aethiopis Linn.1753 "African sage"	☑	chemical composition (S. hypoleuca /multicaulis) terpenoids (incl. S.recognita) chemical composition bioactivities +1 2
S. africana-lutea Linn. 1762 (syn. *S. aurea*) "Golden African Sage"	☑	analgesic chem. composition structural / chemical analysis chem. composition
S. apiana Jepson 1908 "White sage"	☑	antibacterial volatile inhibitors (incl. S.leucophylla)
S.argentea Linn. 1753 "Silver sage"	☑	chemical composition terpenoids + 1
S. atropatana *S. albimaculata* *S. anastomosans* *S. aytachii*		chemical composition constituents constituents chemical composition
S. aucheri Benth. 1836	☐	antibacterial chemical composition
S. brandegei Munz 1932 syn. *S. mellifera* var. *revoluta*	☑	chemical composition
S. cabulica Benth	☐	chemical composition
S. candelabrum	☑	antioxidant terpenoids

S. candidissima Vahl 1804	☐	chem. composition terpenoids constituents + **1** diterpenoids
S. cardiophylla	☐	constituents cardiophyllidin
S. clevelandii (Gray) Green Blue Sage	☑	chemical composition
S. coccinea Juss. ex Murr. 1778 syn. *S. pseudococcinea*	☑	chem.comp compounds neoclerodane
S. columbariae Benth 1833 "Chia"	☑	chemical composition
S. cryptantha Montbret et Aucher ex Benth	☐	chemical composition chemical composition
S. deserta	☐	constituents triterpenoids
S. desoleana Atzei & Picci	☑	permeation through mucosa chemical composition +**1** CNS (incl. *S. sclarea*) antimicrobial choleretic
S. dominica Linn. 1753 syn. *S. graveolens*	☑	chem. composition (incl. *S. fruticosa*) chem. composition
S. elegans Vahl. 1804 syn. *S. incarnata* ("Pineapple sage)	☑	aroma compounds CNS
S. euphratica Mont et Aucher ex Benth	☐	chemical composition constituents + **1**
S. farinacea Benth. 1833 "Mealy sage"	☑	constituents phytochemistry salvifarin

S. fruticosa Mill. 1768 (syn. *S. triloba* Linn. 1781) "Greek sage"	chem. composition (more 1 2 3 4) chem. composition (incl. *S. willeana*) CNS (incl. *S. officinalis / verbenaca*) antimicrobial and cytotoxic oil and yield components hypoglycemic constituents diterpenoids antioxidant toxicity
S. glutinosa Linn. 1753 "Jupiters Distaff"	major compound (oil bacter. test) antioxidant
S.guaranitica St. Hill. ex Benth. 1833 (syn. *S. caerulea, S. ambigens*)	sedative & hypnotic
S. hispanica Linn.1753 syn. S. neohispanica, S. chia, S.tetragona	chem. composition (insects deter) oil content
S. hydrangea DC. ex Benth	chem. composition (more 1 2)
S. hypargeia Fisch. & Mey. 1854	cytotoxic diterpenoids antibacterial diterpenoids
S. lavandulaefolia Vahl "Spanish Sage"	Trial in healthy volunteers Clinical trial — Synergy Brain rat in-vivo — CNS-bioactivity Dementia — pancreatic endocrineabortifacient + 1 spasmolytic + 1 — hypoglycemic composition — cytotoxic
S. leriifolia Benth	chem. composition anti-inflammatory antihyperglycemic morphine anti-ulcer
S. libanotica Boiss. et Gaill	chem. comp & toxicity chemopreventive

S. mellifera Greene 1892 "Black sage"	☑	constituents (more 1 2 3 4) chemical composition bioactive diterpenoids volatilization (camphor) volatilization (mechanism)
S. miltiorrhiza "Dan Shen"	☑	memory and cognition ischemic diseases CNS (cholinergic) CNS-neuronal CNS anticancer antioxidant cytotoxicity hepatic fibrosis
S. moorcroftiana		chemical composition
S.multicaulis Vahl. 1804 syn. *S.acetabulosa*	☑	chem. composition + 1 terpenoids + 1 salvimultine
S. munzii Epling 1935 "San Miguel sage"	☑	chemical composition
S. nemorosa Linn. 1762	☑	anti-nociceptive antioxidant terpenoids anticonvulsive chemical composition
S. officinalis Linn. 1753 1. 2 "Common Sage"	☑	Clinical trial for Alzheimer's disease chemical and seasonal variations parameter for *Salvia* sp chem. composition + 1 anti-inflammatory antioxidant ursolic acid + 1 constituents cholinergic dementia drying-effect cytotoxic toxicity
S. oppositiflora Ruiz. & Pav. 1798	☑	chemical composition
S. pratensis Linn. 1753 "Meadow Clary" (*S. haematodes)*	☑	CNS-cardiovascular ursolic acid (S. sclarea) chem. comp lupeol sexual

S.plebeia Brown 1810 (syn. *S. brachiata*)	☑	cytotoxic /antimicrobial antioxidant + **1** hepatitis
S. pomifera Linn. 1753 (syn. S. *calycina*)	☐	essential oils / distribution chem. composition + **1** **2** antioxidant
S. przewalskii Maxim. 1881	☑	constituents constituents przewalskinic acid
S. sclarea Linn. 1753 "Clary sage"	☑	chem.comp (more **1** **2** **3** **4** **5**) oil from healthy / infected plants anti-inflammatory and analgesic terpenoids / antibacterial quinones (tanshinone) cytotoxic effect metal content oil dynamics CNS spasmolytic sedative manool sclareol yield
S. schimperi Benth.	☐	chem. composition + **1** oil properties
S. syriaca L.		chem. composition + **1** cardioactive terpenoids salvisyriacolide alkaloids
S. tomentosa Mill. 1768	☑	chem. composition + **1** **2** (antibacterial) chem & pharm studies constituents + **1** **2** terpenoids antioxidant flavonoids
S. transsylvanica Schur ex Griseb syn. *S. baumgartentii*	☑	bioactivity
S. verticillata Linn. 1753	☑	CNS activity chemical composition
S. virgata Jacq. 1770 *syn. S. sibthorpii* "Meadow sage"	☑	chemical composition

Database of active principals of *Salvia* species

The database is recorded on the attached CD disk. It includes a chemical structure of molecules (or a link to a website for identification of a chemical structure) and abstracts/articles on their bioactivities.

NAME OF CHEMICAL	PROPERTIES
borneol	Antagonist for nAChr
camphor	bacterial bioluminescence blood catechol amine level CNS-convul/brain hydration no antiinflammatory CNS-sialic acid /gangliosides leaves drying effect CNS-spasms /benzimidazole adrenalin-synergism coronary CNS-spasms, free-NH3 circulation CNS-neuronal necrosis bromide in blood radiosensitizing CNS-LD50 (borneol) hepatotoxicity no anti-oxidant CNS-spasms in fish activ CNS-coenzyme A fetal growth antitussive CNS-toxicity case in hypoxia mutagenic CNS-cholinolytic toxicity tumour CNS-glycolytic cerebral circulation brain tissue CNS- nicotinic CNS-spasms respiration
Copaene	about flies attractant (more **1** **2**)
Carnosic acid & Carnosol	antioxidant (more **1** **2** **3** **4** **5** **6** **7** **8**) anti-mutagen /antioxidant chemopreventive (more **1**) nerve growth factor CNS anti-tumour leukemia HIV-1
Caryophyllene & Caryophyllene oxide	antimalarial antimutagenic anaesthetic anticarcinogenic histamine antiinflammatory (a-pinene) prostaglandins antiinflammatory (cytoprotective) CNS (Ca^{+}) toxicity (a,b-pinene) (more **1** **2** **3**) insecticidal spasmolytic (camphor)

1,8-cineole	about	CNS-spasm	CNS-spasm
	anti-inflammatory	CNS-AChE	CNS-
	bronchopulmonary	inhalation	skin
	cytokine inhibitor	anti-inflammatory /antinociceptive	
	liver (more 1)	gastro protective (antioxidation)	
	toxicity	toxicity (planaria, more terpenes)	
	antitussive	non estrogenic (thujone/ pinene)	
	mutagenicity	toxicity	toxicity antioxidant
	quantitative analysis	water-ethanol-cineole	
cirsiliol	sedative	CNS	
germacrene	receptor neuron	sex inducer (more 1 2)	
globulol	antifungal		
guaiol	toxicity		
alpha-humulene	anticarcinogenic (incl. beta-caryophyllene)		
linalool	anti-flea	antioxidant	anticonvulsant mechanism
	acaricidal	antibacterial	anticonvulsant (more 1)
	spasmolytic	nicotinic	effect of inhalation
	sedative	depressive	glutamate binding
	anaesthetic	insecticidal	parasitic otisis oil loss due to solubility
limonene	anticonvulsive (mycrene)	olfactory receptor (more 1 2 3 + 4)	
	anti tumour (more 1)	toxicity (more 1 2 3 - terpenes - 4 5)	
	antimicrobial	brain vapour absorption (camphor)	
	antioxidant	skin drugs penetration (cineole)	
	air control	anti-cancer (more 1 2)	
	metabolites	water-ethanol-limonene	
	skin	chemopreventive	

manool	antifungal	antimicrobial	extraction	
alpha / beta pinene	inhalation	inhalation	anti inflammatory	inhibited attraction
	intoxication	toxicity	chemopreventive	immunostimulant
	skin	spasmolytic	cytotoxicity	hexobarbital sleep
	not antioxidant	antioxidant	skin toxicity	spasmolityc
	non estrogenic			

Sabinyl acetate	contents in Spanish sage	fetotoxicity	anti-implantative
Sitosterols	cancer prevention toxicity	sitosterolemia T-cells	effect on cholinesterol
Salvianolic acid A B D F G I J	apoptosis neurotoxicity + **1** atherosclerosis anti-viral cataract		cerebral ischemia (more **1 2 3** memory) oxidative stress (more **1**) pharmacological activity lipid peroxidation (liver) adriamycin toxicity antioxidant (more **1**)
Squalene	potential clinical use (review) safety trial radioprotective		CNS (more **1**) chemopreventive (more **1**)
Tanshinone I (A) Tanshinone II (B)	anti-ischemia cancer cytotoxicity	anti-cancer leukemia	antioxidant (more **1 2 3 4 5 6**) learning & memory CNS-blocking Ca2+ neuroprotective neuroprotective
α-terpineol	about toxicity toxicity inhalation neurotoxicity pulmonary effect insecticidal (more **1 2**) toxicity (more terpenes **1 2 3**)		
α, β-thujone	neurotoxicity spasms in fish neurotoxic action cannabinoid receptors porphyrogenic (inc., camphor, a-pinene) spontaneous activity and conditioned behaviour no antiinflammatory /estrogenic low antioxidant		
Ursolic acid	CNS-AChE anti-HIV-1 anti-invasive COX-2 antioxidant anti-ulcer apoptosis nitric oxide energy release histamine antibacterial		anti-tumour (more **1 2 3 4 5**) anti-inflammatory (more **1 2 3**) hepatoprotective (more **1**) lipid peroxidation (more **1**) cytotoxic cytostatic anti-carcinogenic chemo preventive mast cell protection immuno-stimulant atherosclerosis anti-angiogenic liver injury
viridiflorol	CNS-acetylcholinesterase inhibition (incl., 1,8-cineole, linalool, limonene, borneol, linalyl acetate, ...)		

Appendix 2. Kinetic Studies

2.1. Determination of kinetic constants for the inhibition of butyrylcholinesterase by oils of sage

In order to determine a limiting value of initial enzyme velocity (Vmax) and the equilibrium constant of the reversible combination of BuChE with its substrate, Michaelis constant (Km), a Lineweaver-Burk plot (Lineweaver and Burk, 1934) was used (Figure 2.1).

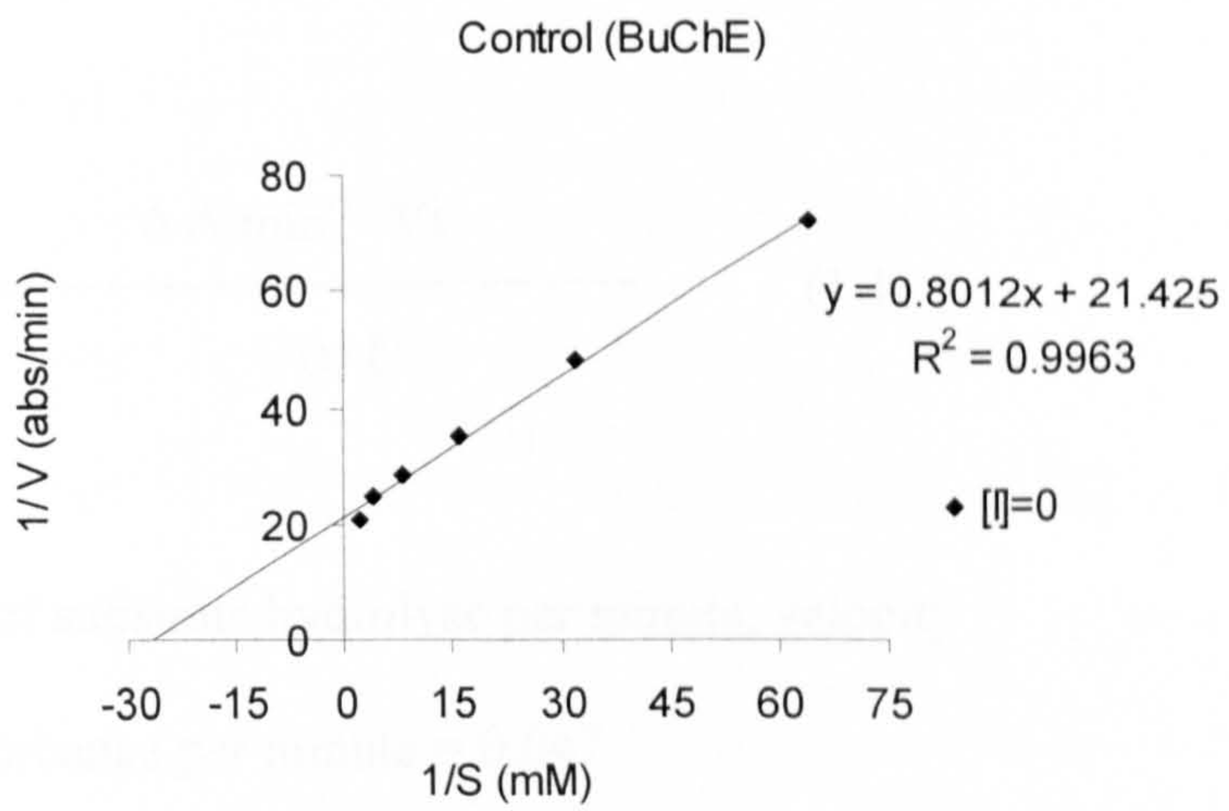


Figure 2.1. Lineweaver-Burk plot shows a reciprocal of the change in absorbance per minute versus acetylcholine concentration in the absence of inhibitor. Each point on the graph is a mean of eight experimental values (n=8).

To calculate Km and Vmax of the enzyme a following equation was used, *i.e.*, Y=0.8012X+21.425 (Figure 2.1.) where Y is 1/V and X is 1/S. The intercept on the ordinate (Y) is the reciprocal of 1/Vmax, whereas the intercept on the abscissa (X) gives the reciprocal of -1/Km. It may be noticed that the intercept on the ordinate would only take place when X=0 and the intercept on the abscissa when Y=0.

Hence,

if Y=0, X=-26.74 or X=-1/-26.74=0.037 or Km=0.037 mM.

if X=0, Y=21.425 or Y=1/21.425 or Vmax=0.047 abs min^{-1}.

Since the extinction coefficient of the yellow anion is known (Ellman, 1959), Vmax value of 0.047 abs min^{-1} may be converted into internationally recognised units of enzyme velocity, namely molar per minute (M min^{-1}) (Lowry and Passonneau, 1972). Equation 1.1, derived from the Beer-Lambert law, has been used for calculation of the rates of hydrolysis (Ellman *et al.*, 1961).

$$\text{Rates (M min}^{-1}) = \frac{\Delta \text{ A min}^{-1} \times Vt}{e \times \ell} \qquad (1.1)$$

Where

Rates = amount of substrate hydrolyse per minute, velocity

Δ A min^{-1} = absorbance per minute = 0.047

Vt = total volume of reaction mixture, L = 0.00022

e = extinction coefficient = 13600 M^{-1} cm^{-1} (Ellman, 1959)

ℓ = path length, cm = 0.6 (a distance that the light travel through the reaction mixture with a total volume of 0.00022 litre)

$$\text{Rates (M min}^{-1}) = \frac{0.047 \times 0.00022}{13600 \times 0.6} = 0.0000000013$$

1.3×10^{9} M min^{-1} = 1.3 nM min^{-1}

Thus, the dissociation constants Km and Vmax for BuChE are 0.037 mM and 1.3 nM min^{-1} respectively.

Lineweaver-Burk plot was also used to determine the dissociation constants (Km and Vmax) and a type of inhibition of oils of *S. apiana*, *S. officinalis* var. *purpurea* and *S. fruticosa*, while Dixon's plot (Dixon, 1953) and its re-plot described by Dixon and Webb (1979) were adopted for the determination of competitive and un-competitive enzyme inhibitor constants respectively.

Two concentrations of *S. apiana* oil were used to analyse changes in Km and Vmax values of the enzyme–substrate complex (Figure 2.1.). Table 2.1 demonstrates an increase in Km values but a decrease in Vmax values when a concentration of the inhibitor rises, while Figure 2.1 shows that convergence of the reciprocals is above abscissa. According to Engel (1981) and Cornish-Bowden and Wharton (1988) these facts characterise general non-competitive (mixed) type of inhibition of BuChE by the oil, which is simultaneous occurrence of competitive and un-competitive inhibition. A non-competitive inhibitor (i) can bind to both enzyme (e) and enzyme-substrate (es) complex. Figure 2.1 reflects a case when the inhibitor-enzyme-substrate constant (Kies) of un-competitive binding is more than the inhibitor-enzyme constant (Kie) of competitive binding, *i.e.*, Kies>Kie.

Figure 2.3 shows a graphical method for determination of Kie value (Dixon, 1953) of *S. apiana* oil. The value of 0.025 mg ml^{-1} was read off directly of the plot. In the case of mixed inhibition, Dixon's plot can not be used for determination of Kies, whereas a plot of S (substrate concentration)$\times V^{-1}$ (absorbance per minute) against inhibitor concentrations (I) is appropriate (Dixon and Webb, 1979). Figure 2.4 demonstrates a graphical method for determination of Kies value. The value of 0.05 mg ml^{-1} was obtained directly from the graph. Thus, the un-competitive inhibitor constant of the oil is more than the competitive one, *i.e.*, 0.05 mg ml^{-1} > 0.025 mg ml^{-1}.

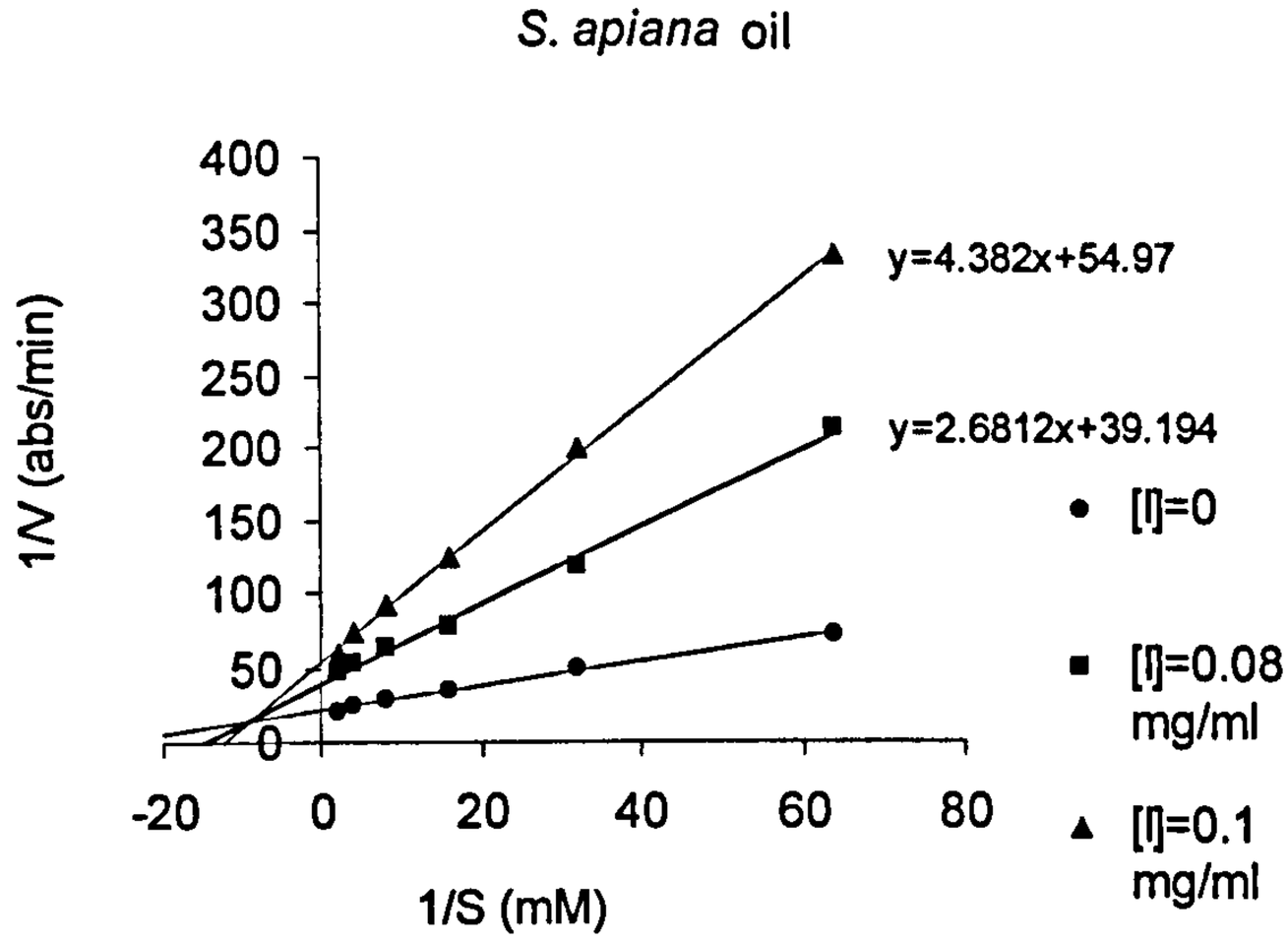


Figure 2.2. Lineweaver-Burk plot shows reciprocals of the change in absorbance per minute versus butyrylcholine concentration in the absence and presence of two inhibitor concentrations of *S. apiana* oil. Each point on the graph is a mean of eight experimental values (n=8).

Table 2.1. Effect of *S. apiana* oil on the dissociation constants

Inhibitor, mg ml^{-1}	Dissociation constants [a]	
	Km, mM	Vmax, nM min^{-1}
[I]=0 (control)	0.037	1.3
[I]=0.08	0.068	0.7
[I]=0.1	0.08	0.5

[a]The apparent constants were calculated using linear equations of reciprocals from Figure 2.1 Km values increase but Vmax decrease when the inhibitor concentrations rise.

Determination of the kinetic constants of oils of *S. officinalis* var. *purpurea* (Figure 2.5; Table 2.2) and *S. fruticosa* (Figure 2.6; Table 2.3) were carried out in the same way as described in the example of *S. apiana* oil.

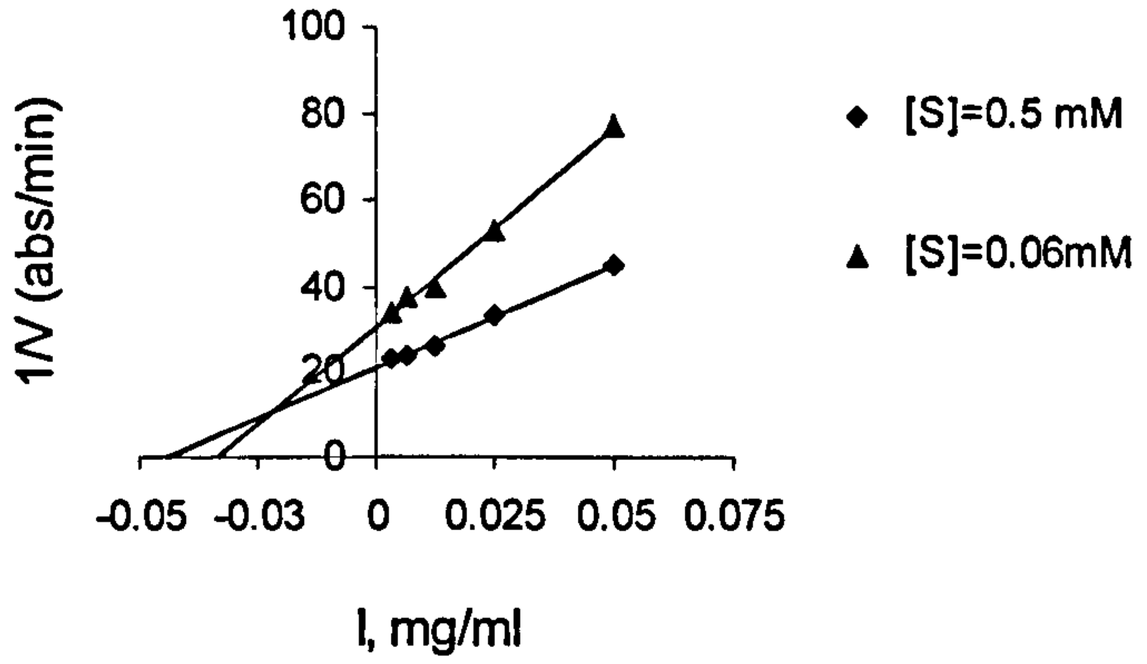


Figure 2.3. Dixon's plot. Determination of competitive inhibition constant for *S. apiana* oil. If the absorbance per minute^{-1} is plotted against inhibitor concentrations, at two constant substrate concentrations, two straight lines are obtained with a point of intersection on the left of the vertical axis above abscissa. Projection of this point on the horizontal axis provides Kie value, which can therefore be read off directly. Each point on the graph is a mean of eight experimental values (n=8).

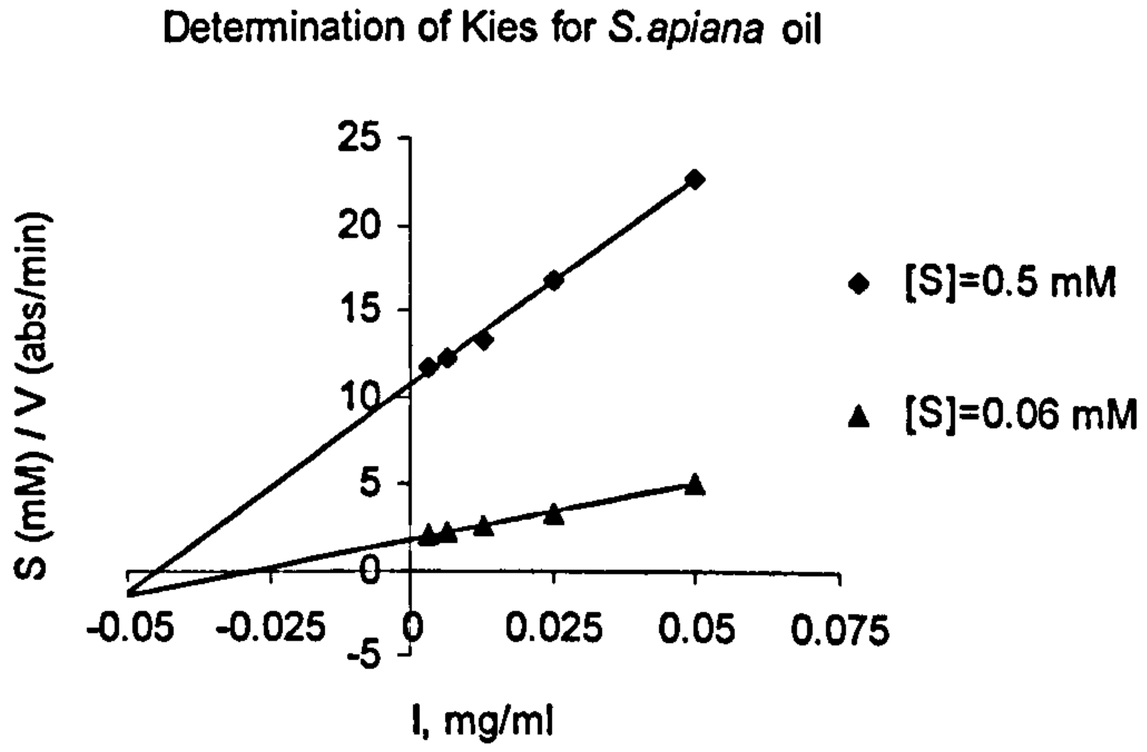
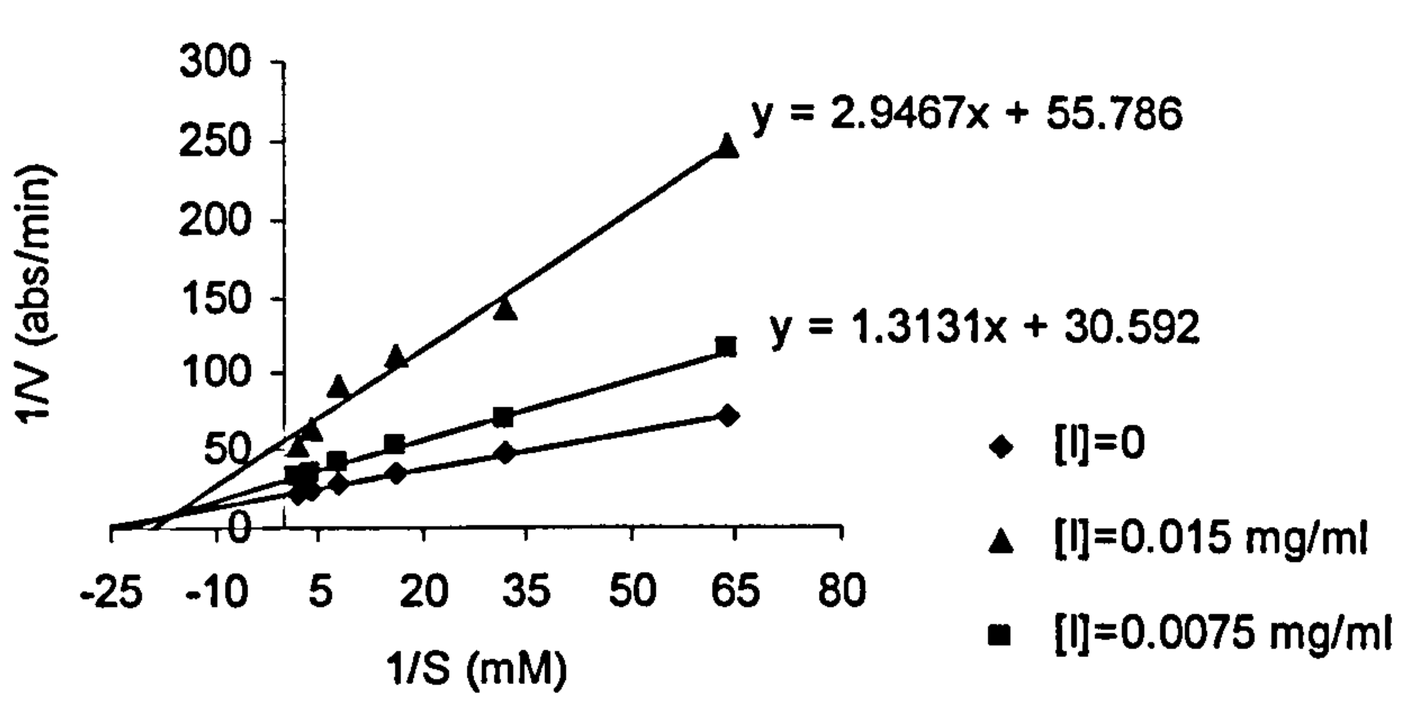
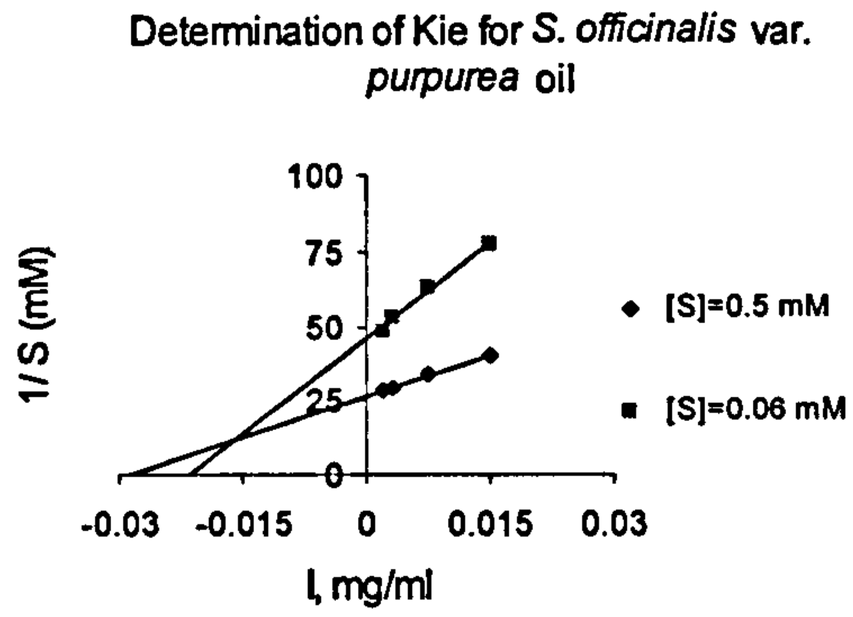


Figure 2.4. Determination of un-competitive inhibition constant for *S. apiana* oil. At two constant substrate concentrations, two straight lines intersects at a point below the

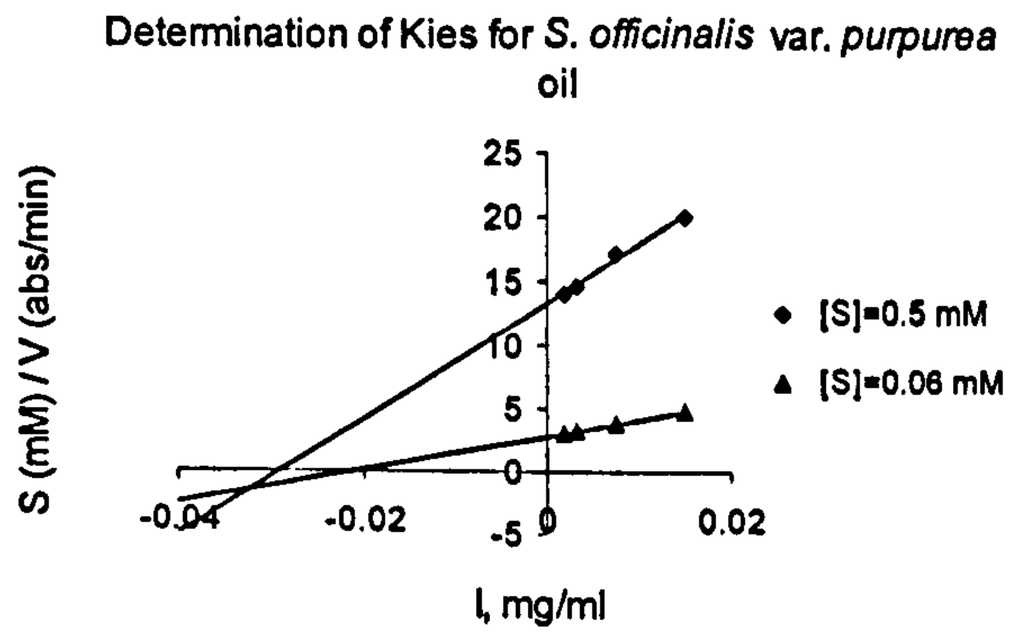
abscissa, if Kies>Kie. Projection of this point on the abscissa provides Kies value, which can therefore be read off directly. Each point on the graph is a mean of eight experimental values (n=8).



a. Lineweaver-Burk plot shows reciprocals of the change in absorbance per minute versus butyrylcholine concentration in the absence and presence of two inhibitor concentrations.



b. Determination of a competitive inhibitor constant



c. Determination of an un-competitive inhibitor constant

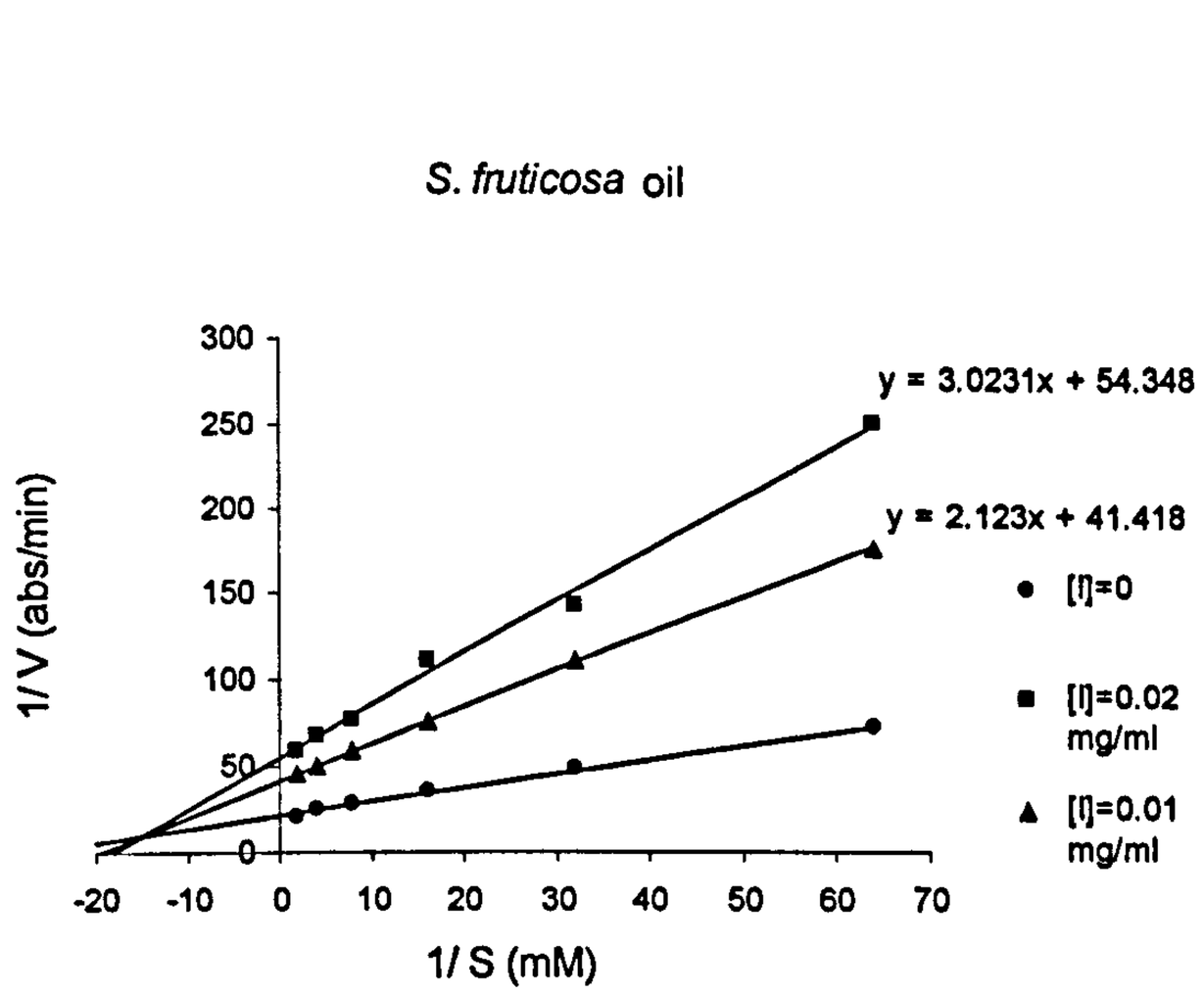
Figure 2.5. Determination of the dissociation and inhibitor constants for *S. officinalis* var. *purpurea* oil. Each point on the graphs is a mean of eight experimental values (n=8). **a**). Lineweaver-Burk plot shows a non-competitive type of inhibition of BuChE by the oil, *i.e.*, Kies>Kie. Km values increase but Vmax values decrease when the inhibitor concentrations rise (Table 2.2). Intersection of the reciprocals is above abscissa. **b**). The competitive inhibitor constant (Kie) of 0.015 mg ml^{-1} was read directly off the graph. **c**).

The un-competitive inhibitor constant (Kies) of 0.033 mg ml^{-1} was also obtained directly off the graph.

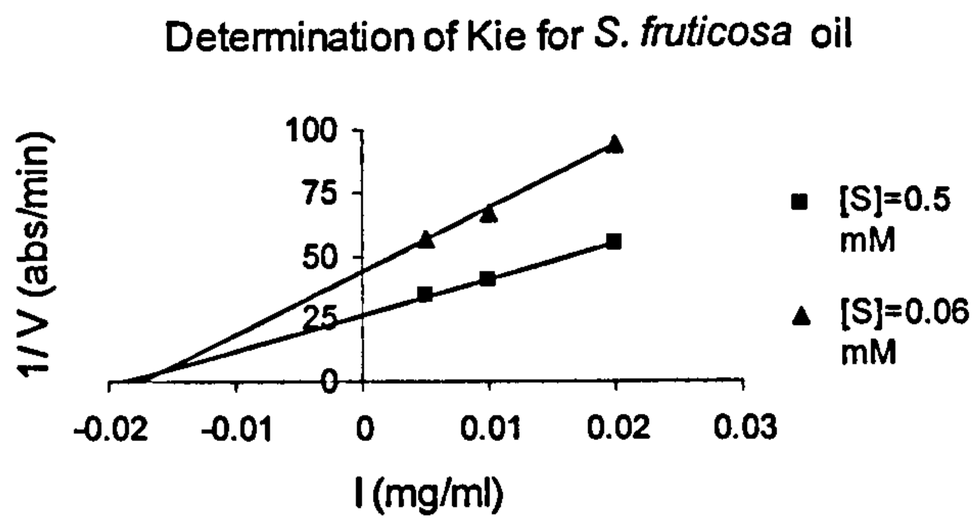
Table 2.2. Effect of *S. officinalis* var. *purpurea* oil on the dissociation constants

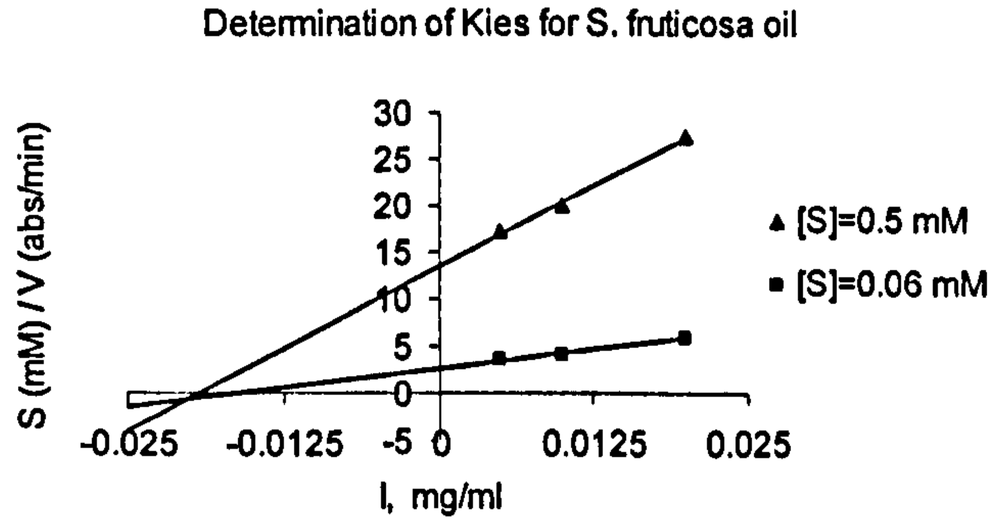
Inhibitor, mg ml^{-1}	Dissociation constants [a]	
	Km, mM	Vmax, nM min^{-1}
[I]=0 (control)	0.037	1.3
[I]=0.0075	0.043	0.86
[I]=0.015	0.053	0.5

[a]The apparent constants were calculated using linear equations of reciprocals from Figure 2.5(a) and Equation 1.1. Km values increase but Vmax decrease when the inhibitor concentrations rise.



a. Lineweaver-Burk plot shows reciprocals of the change in absorbance per minute versus butyrylcholine concentration in the absence and presence of two inhibitor concentrations





b. Determination of a competitive inhibitor constant

c. Determination of an un-competitive inhibitor constant

Figure 2.6. Determination of the dissociation and inhibitor constants for *S. fruticosa* oil. Each point on the graphs is a mean of eight experimental values (n=8). **a**). Lineweaver-Burk plot shows a non-competitive type of inhibition of BuChE by the oil, *i.e.*, Kies>Kie. Km values increase but Vmax values decrease when the inhibitor concentrations rise (Table 2.3). Intersection of reciprocals is above abscissa. **b**). The competitive inhibitor constant (Kie) of 0.016 mg ml^{-1} was read directly off the graph. **c**). The un-competitive inhibitor constant (Kies) of 0.022 mg ml^{-1} was also obtained directly off the graph.

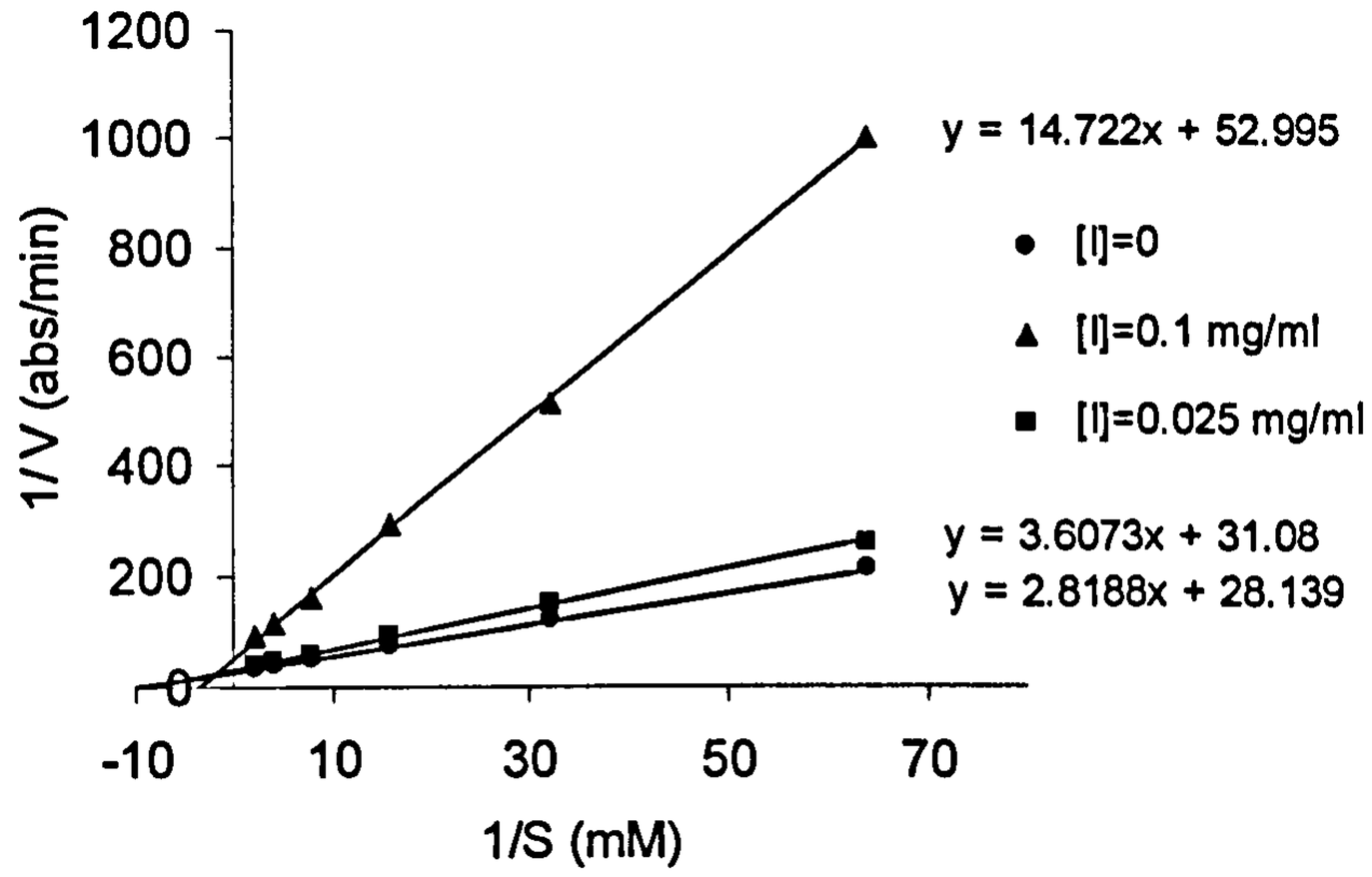
Table 2.3. Effect of *S. fruticosa* oil on the dissociation constants

Inhibitor, mg ml^{-1}	Dissociation constants [a]	
	Km, mM	Vmax, nM min^{-1}
[I]=0 (control)	0.037	1.3
[I]=0.01	0.051	0.65
[I]=0.02	0.056	0.5

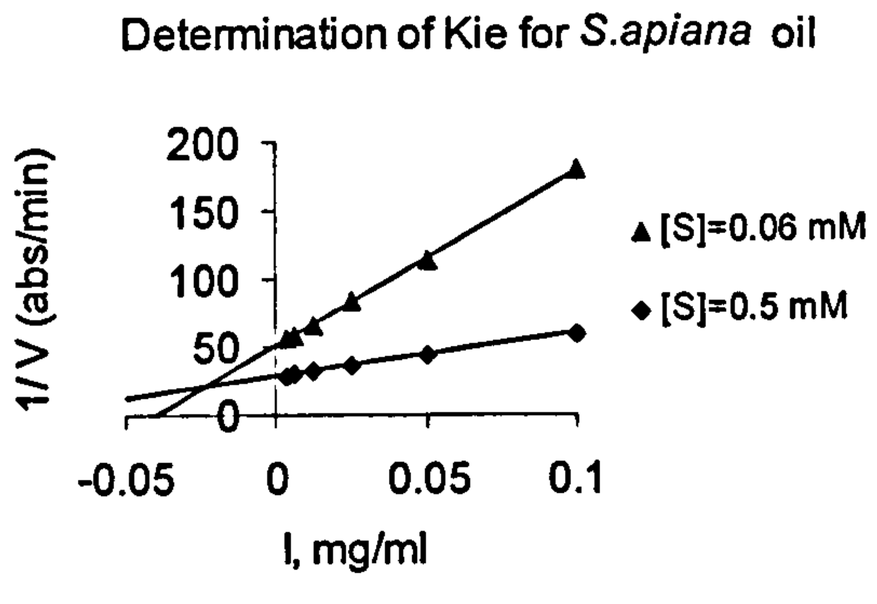
[a]The apparent constants were calculated using linear equations of reciprocals from Figure 2.6(a) and Equation 1.1. Km values increase but Vmax decrease when the inhibitor concentrations rise.

2.2. Determination of kinetic constants for the inhibition of acetylcholinesterase by oils of sage

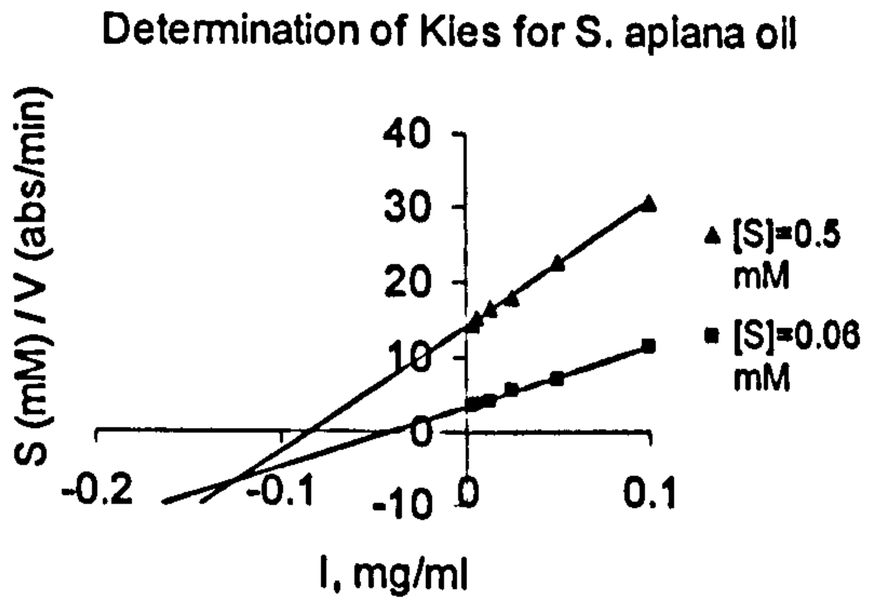
Determination of the kinetic constants of oils of *S. apiana*, *S. corrugate*, *S. officinalis* var. *purpurea* and *S. fruticosa* were carried out as described above, section 2.1.



a. Lineweaver-Burk plot shows reciprocals of the change in absorbance per minute versus acetylcholine concentration in the absence and presence of two inhibitor concentrations



b. Determination of a competitive inhibitor constant



c. Determination of an un-competitive inhibitor constant

Figure 2.7. Determination of the dissociation and inhibitor constants for *S. apiana* oil.

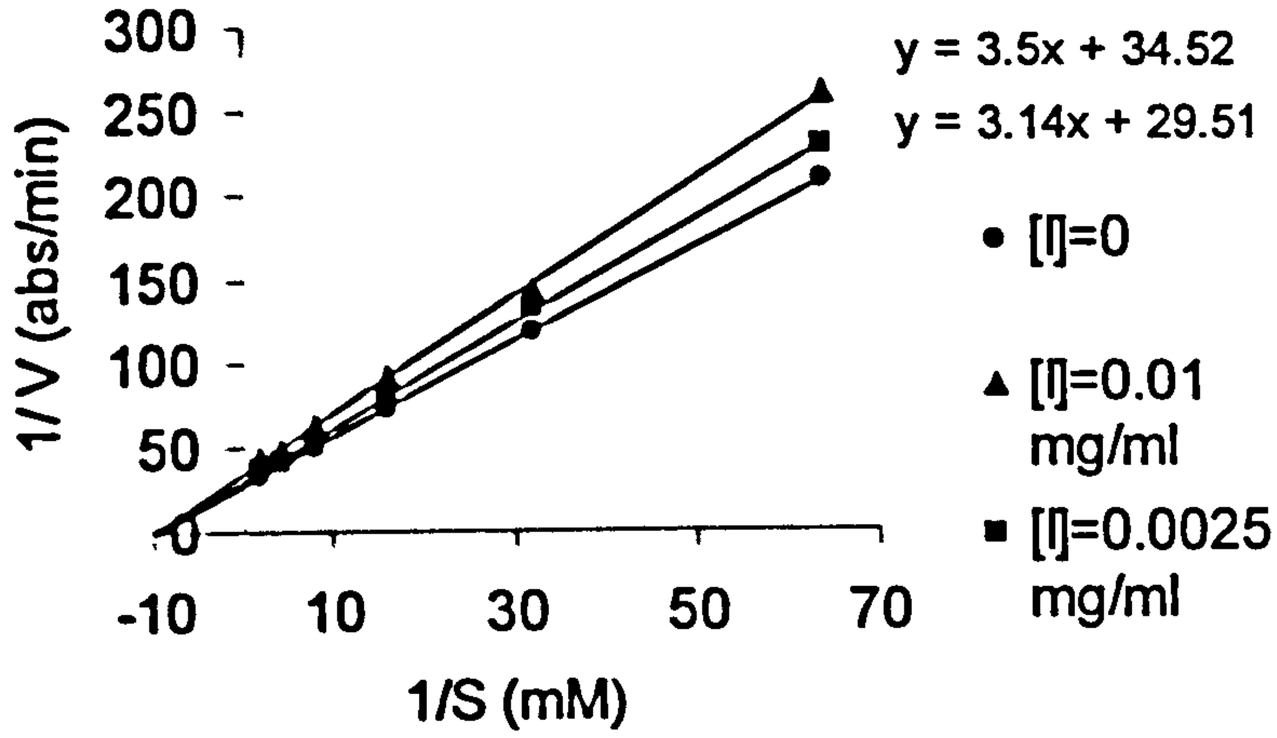
Each point on the graphs is a mean of eight experimental values (n=8).

a). Lineweaver-Burk plot shows a non-competitive type of inhibition of AChE by the oil, *i.e.*, Kies>Kie. Km values increase but Vmax values decrease when the inhibitor concentrations rise (Table 2.4.). Intersection of the reciprocals is above abscissa. **b**). The competitive inhibitor constant (Kie) of 0.025 mg ml^{-1} was read directly off the graph. **c**). The un-competitive inhibitor constant (Kies) of 0.125 mg ml^{-1} was also obtained directly off the graph.

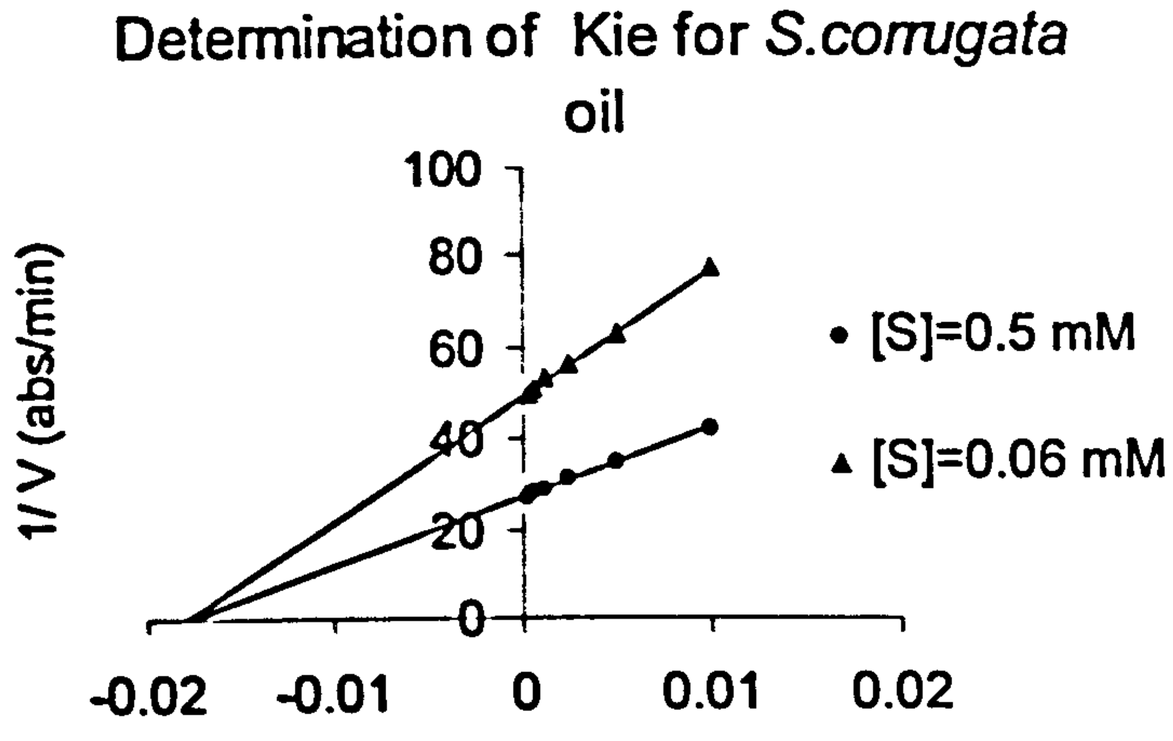
Table 2.4. Effect of *S. apiana* oil on the dissociation constants

Inhibitor, mg ml^{-1}	Dissociation constants [a]	
	Km, mM	Vmax, nM min^{-1}
[I]=0 (control)	0.1	0.97
[I]=0.025	0.12	0.86
[I]=0.1	0.28	0.5

[a]The apparent constants were calculated using linear equations of reciprocals from Figure 2.7(a) and Equation 1.1. Km values increase but Vmax decrease when the inhibitor concentrations rise.



a



b

Figure 2.8. Determination of the dissociation and inhibitor constants for *S. corrugata* oil. Each point on the graphs is a mean of eight experimental values (n=8).

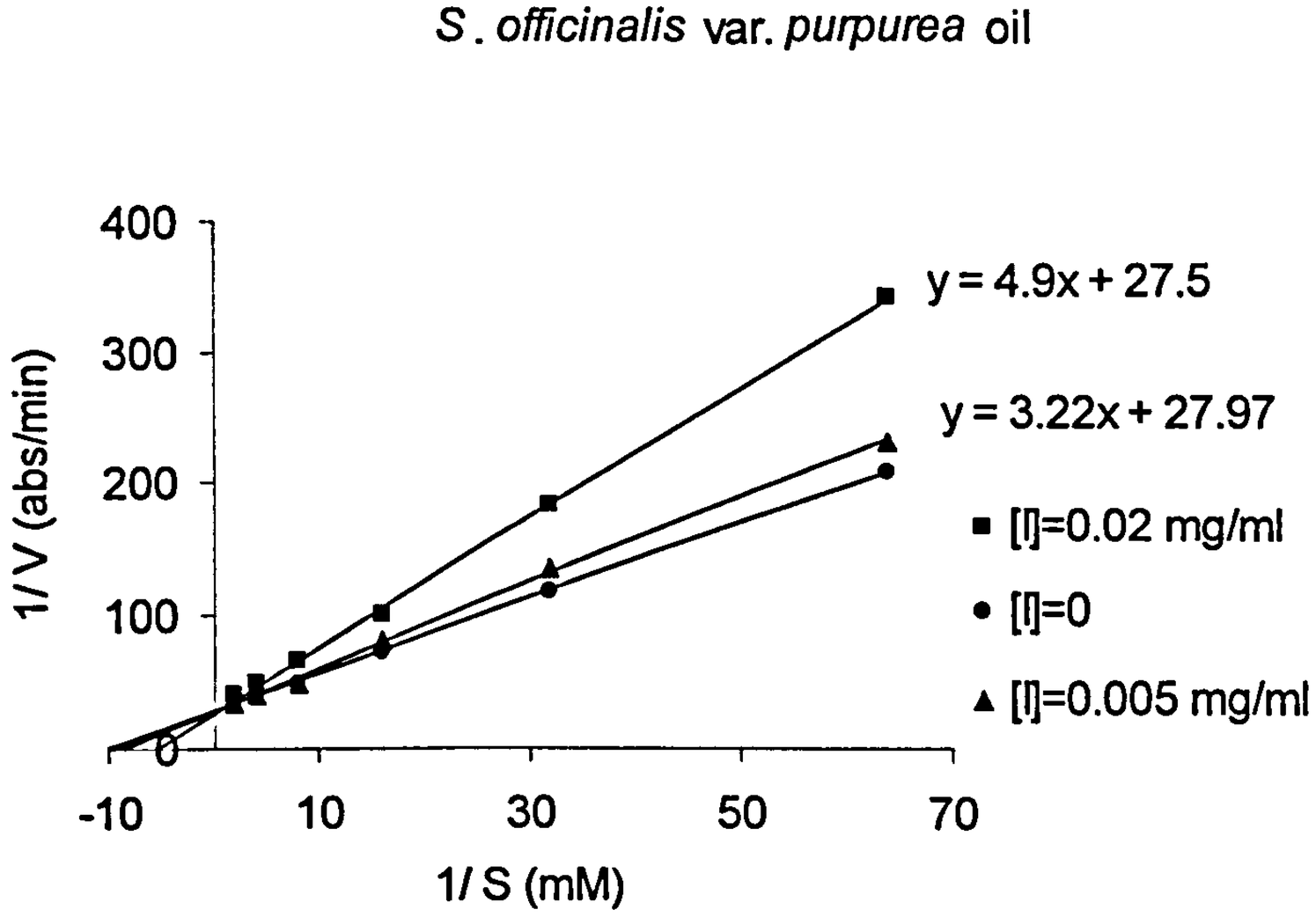
a). Lineweaver-Burk plot shows a non-competitive (simple) type of inhibition of AChE by the oil, *i.e.*, Kies=Kie (Engel, 1981). Km values are unchanged but Vmax values decrease when the inhibitor concentrations rise (Table 2.5). Convergence of the reciprocals is on abscissa. b). The competitive inhibitor constant (Kie) of 0.018 mg ml^{-1} was read directly off the graph.

Table 2.5. Effect of *S. corrugata* oil on the dissociation constants

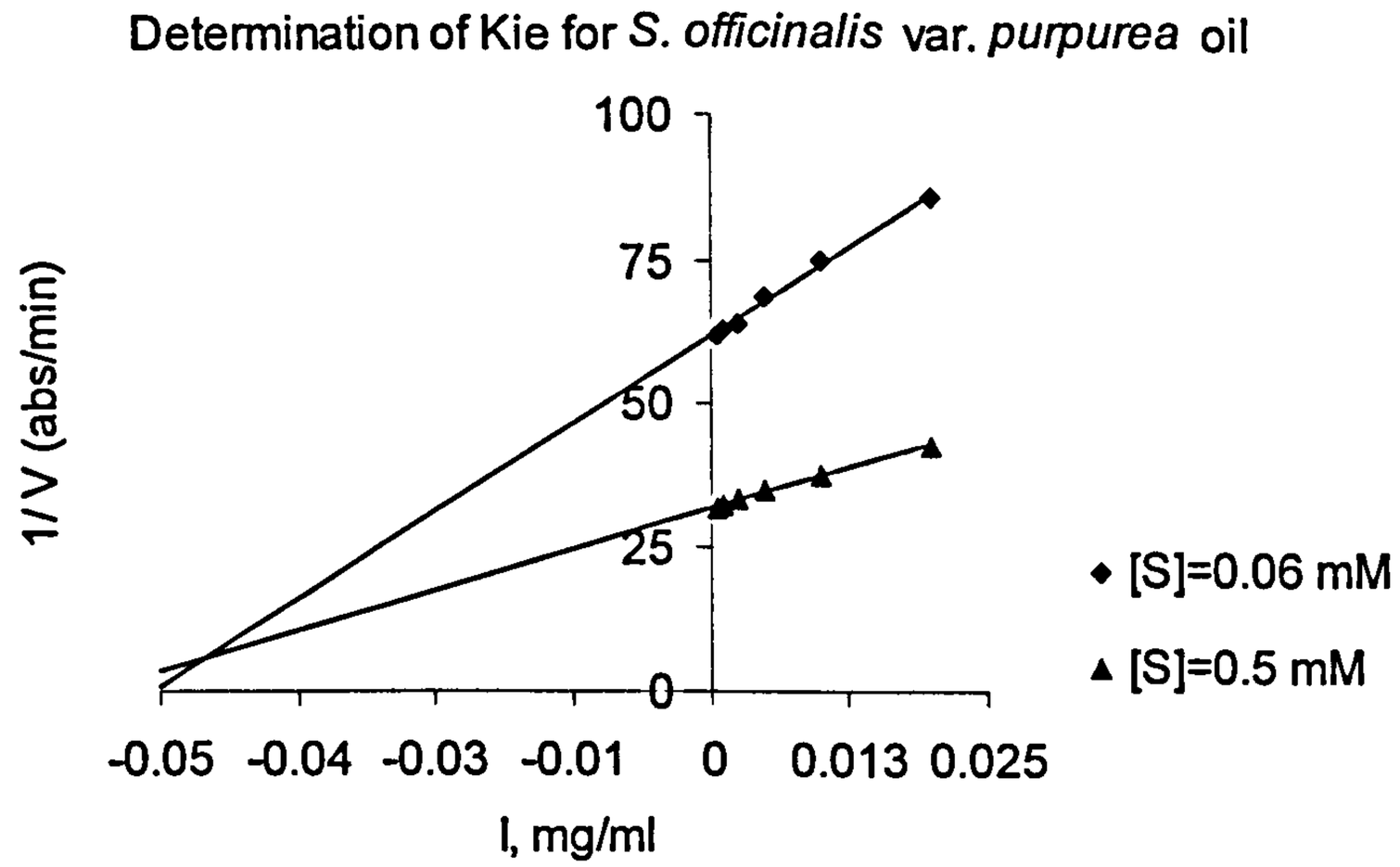
Inhibitor, mg ml^{-1}	Dissociation constants [a]	
	Km, mM	Vmax, nM min^{-1}
[I]=0 (control)	0.1	0.97
[I]=0.0025	0.1	0.9
[I]=0.01	0.1	0.78

[a]The apparent constants were calculated using linear equations of reciprocals from Figure 2.8(a) and Equation 1.1. Km values are unchanged but Vmax values decrease when the inhibitor concentrations rise.

According to Engel (1981) oil of *S. corrugate* showed a non-competitive (simple) type of inhibition of AChE, namely the competitive inhibitor constant is equal to the un-competitive one (Figure 2.8.). Oil of *S. officinalis* var. *purpurea* showed a competitive type of inhibition of AChE, *i.e.*, the inhibitor combines only with the enzyme but not with the enzyme-substrate complex (Figure 2.9.). Oils of *S. apiana* (Figure 2.7.) and *S. fruticosa* (Figure 2.10.) had a non-competitive (mixed) type of inhibition, *i.e.*, Kies>Kie.



a



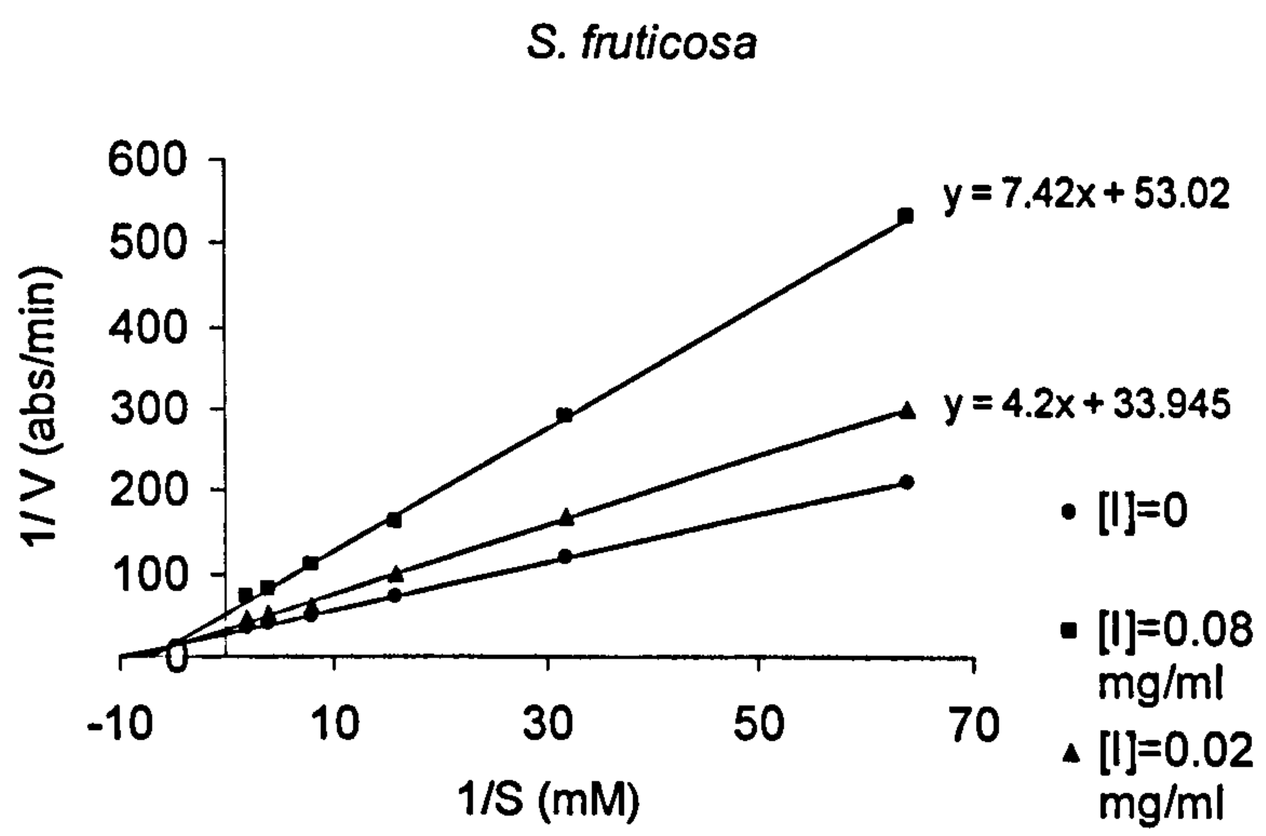
b

Figure 2.9. Determination of the dissociation and inhibitor constants for *S. officinalis* var. *purpurea* oil. Each point on the graphs is a mean of eight experimental values (n=8). **a**). Lineweaver-Burk plot shows a competitive type of inhibition of AChE by the oil (Engel, 1981). Km values increase but Vmax values are unchanged when the inhibitor concentrations rise (Table 2.6.). Convergence of the reciprocals is on ordinate axis. **b**). The competitive inhibitor constant (Kie) of 0.048 mg ml^{-1} was read directly off the graph.

Table 2.6. Effect of *S. officinalis* var. *purpurea* oil on the dissociation constants

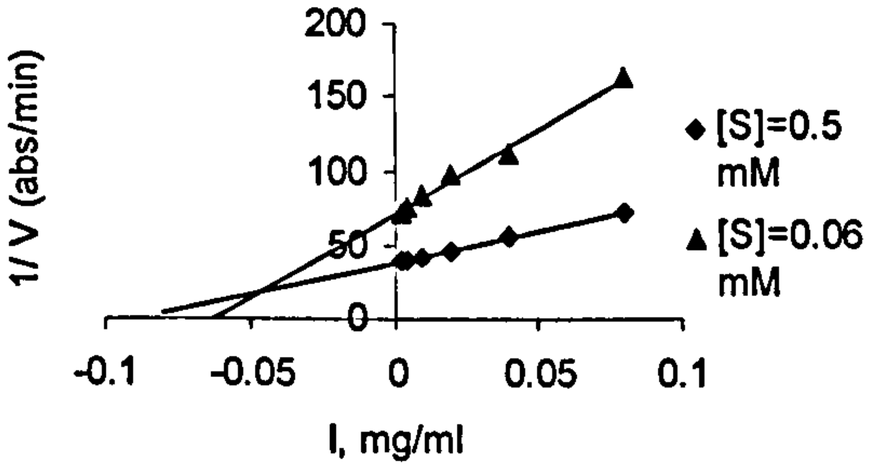
Inhibitor, mg ml^{-1}	Dissociation constants [a]	
	Km, mM	Vmax, nM min^{-1}
[I]=0 (control)	0.1	0.97
[I]=0.005	0.11	0.97
[I]=0.02	0.2	0.97

[a]The apparent constants were calculated using linear equations of reciprocals from Figure 2.9(a) and Equation 1.1. Km values increase but Vmax values are unchanged when the inhibitor concentrations rise.



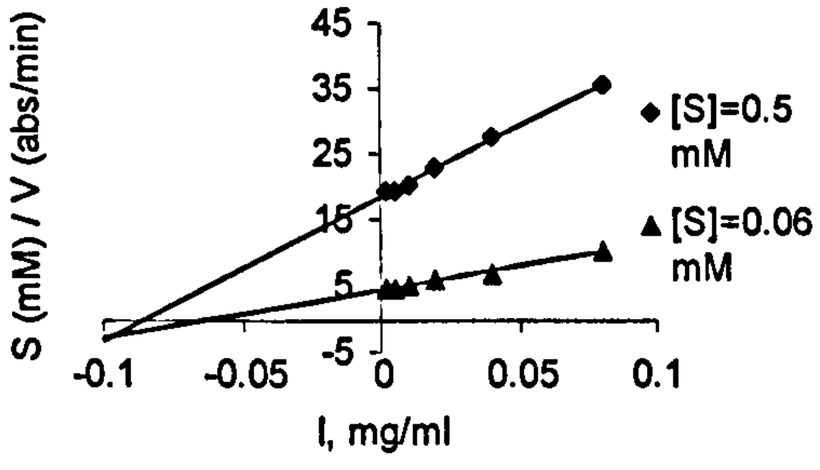
a. Lineweaver-Burk plot shows reciprocals of the change in absorbance per minute versus acetylcholine concentration in the absence and presence of two inhibitor concentrations

Determination of Kie for S. fruticosa



b Determination of a competitive inhibitor constant

Determination of Kies for *S. fruticosa* oil



c Determination of an un-competitive inhibitor constant

Figure 2.10. Determination of the dissociation and inhibitor constants for *S. fruticosa* oil. Each point on the graphs is a mean of eight experimental values (n=8).

a). Lineweaver-Burk plot shows a non-competitive type of inhibition of AChE by the oil, *i.e.*, Kies>Kie. Km values increase but Vmax values decrease when the inhibitor concentrations rise (Table 2.7.). Intersection of the reciprocals is above abscissa. **b**). The competitive inhibitor constant (Kie) of 0.043 mg ml^{-1} was read directly off the graph. **c**). The un-competitive inhibitor constant (Kies) of 0.093 mg ml^{-1} was also obtained directly off the graph.

Table 2.7. Effect of *S. fruticosa* oil on the dissociation constants

Inhibitor, mg ml^{-1}	Dissociation constants [a]	
	Km, mM	Vmax, nM min^{-1}
[I]=0 (control)	0.1	0.97
[I]=0.02	0.12	0.78
[I]=0.08	0.14	0.51

[a]The apparent constants were calculated using linear equations of reciprocals from Figure 2.10(a) and Equation 1.1. Km values increase but Vmax values decrease when the inhibitor concentrations rise.

Appendix 3. Chemical composition of *Salvia* species extracted via Phytosol A

Appendix 3 consists of a chemical composition of extracts of *Salvia* species obtained via the supercritical cold extraction process using Phytosol A.

Table 3.1. Chemical composition of extracts of *Salvia* species within thirty and one hundred days of storage time

Compound[a]	RT, min[b]	*S. apiana*, % in oil[c]		*S. fruticosa*, % in oil[c]		*S. officinalis* var. *purpurea*, % in oil[c]	*S. corrugata* % in oil[c]
		30 days[d]	100days[f]	30 days[d]	100days[f]	30 days[d]	30 days[d]
α-pinene	12.39	2.7	0.8	0.8	0.8	---	---
Camphene	13.01	2.2	1.5	3.4	2.2	1.8	---
β-pinene	14.24	3.5	2.7	---	0.7	4.2	---
3-carene	15.63	2.3	1.9	---	---	---	---
Benzene,1-methyl- 2-(1-methylethyl)	16.32	1.4	0.4	1.0	---	---	---
Unidentified	16.4	---	2.2	---	---	---	---
D-Limonene	16.46	5.0	---	3.8	2.5	---	---
1,8-Cineole	16.59	34.4	32.6	12.5	13.6	---	---
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)	17.73	0.6	0.5	---	---	---	---
Terpineol, 2 beta	18.04	---	0.5	---	---	---	---
(+)-4-carene	18.92	0.8	---	---	---	---	---
Cyclohexene, 1-methyl-4-(1-methylethylidene)	18.95	---	0.5	1.5	1.4	---	---
Thujone	19.23	---	---	---	---	6.1	---
Camphor	21.09	38.6	38.5	60.8	58.5	4.4	---
Bicyclo[3.3.1]heptan-3-one, 2,6,6-trimethyl	21.74	---	---	---	---	1.6	---
Borneol	21.93	0.6	2.3	1.6	1.8	---	---

Bicyclo[2.2.1]heptan-2-ol 1,7,7-trimethyl, acetate (1S-endo)	26.27	0.9	1.2	1.9	2.3	2.0	---
Copaene	29.33	---	---	---	---	1.4	---
1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7b-octa-hydro-1,1,4,7-tetramethyl, [1aR-(1aα, 4α, 4aβ, 7bα)]	30.44	---	---	---	---	1.2	---
Caryophyllene	30.76	0.5	0.7	7.4	9.8	4.1	---
[+]-Epi-bicyclosesqui-phellandrene	31.1	---	---	---	---	1.7	---
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl -4-methylene-, (1a,a)	31.39	---	---	1.5	1.8	2.8	---
α-caryophyllene	31.84	---	---	---	---	46.5	---
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl -4-methylene-,(1aR-[1aα, 4aβ, 7α, 7aβ, 7bα)]	32.07	---	---	---	---	1.1	---
Naphthalene, 1,2,3,4,4a,5, 6,8a-octahydro-7-methyl-4-methylene-1-(1-methyl-ethyl)-,(1α, 4aα, 8aα)-	32.54	---	---	---	---	3.6	---
1H-Cycloprop[e]azulene, 1a2,3,5,6,7,7a,7b octa-hydro -1,1,4,7-tetramethyl [1aR-(1a.α,7α,7aβ,7b.α)]	33.13	---	---	0.8	1.0	3.2	---
α-muurolene	33.3	---	---	---	---	2.0	---
Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-, (S)	33.45	0.6	0.8	---	---	---	---
Naphthalene, 1,2,3,4,4a,5, 6,8a,octahydro-7-methyl-4-methylene-1-(1-methyl ethyl)-, (1α,4aβ,8aα)	33.69	---	---	---	---	1.6	---
Cyclohexanol, 3-ethenyl-3-methyl-2-(1-methyleth-enyl)-6-(1-methylethyl)-, [1R-(1α, 2α, 3β, 6α)]	33.86	2.1	2.3	---	---	---	---
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(methylethyl)-,(1S-cis)	33.96	0.8	0.6	0.6	0.8	5.1	---
Unidentified	34.04	---	1.7	---	---	---	---

Caryophyllene oxide	35.78	---	---	---	1.1	---	---
Guaiol	36.16	---	---	1.0	1.2	---	---
α-bisabolol	38.61	---	0.9	---	---	---	---
Unidentified	38.82	---	3.8	---	---	---	---
Unidentified	54.89	---	---	---	---	2.9	---
Squalene	63.26	---	---	---	---	---	2.3
Unidentified	63.97	---	---	---	---	---	0.8
Unidentified	71.15	---	---	---	---	---	20.6
Unidentified	71.32	---	---	---	---	---	4.1
4,4,6a,6b,8a,11,11,14a,-octamethyl-14,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,1,14a14b,-octadecahydro 2H-picen-3-one	71.44	---	---	---	---	---	1.7
Unidentified	71.64	---	---	---	---	---	1.0
Unidentified	71.93	---	---	---	---	---	0.7
Unidentified	72.28	---	---	---	---	---	66.7
Unidentified	73.97	---	---	---	---	---	1.0
Unidentified	74.2	---	---	---	---	---	1.0

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Relative percentage as calculated from a total area of peaks on a basis of 1 % of a major peak

[d]Extracts with no more than 30 days shelf-life.

[f]Extracts with 100 days shelf-life

--- not detected

Table 3.2. Chemical composition of extracts of *Salvia* species with dual anti-cholinesterase activity

Compound[a]	RT, min[b]	% in oil[c]							
		S. africana-lutea	*S. argentea*	*S. divinorum*	*S. involucrata*	*S. keerlii*	*S. lavandulaefolia*	*S. microphylla* var. *neurepia*	*S. officinalis*
α-pinene	12.39	2.2	---	---	---	---	12.4	1.1	2.3
Camphene	13.01	---	---	---	---	---	2.5	3.2	4.2
β-pinene	14.24	---	---	---	---	---	1.0	---	7.8
Unidentified	14.27	---	---	---	---	---	---	0.7	---
β-myrcene	14.97	17.6	---	---	---	---	1.2	---	---
Benzene,1-methyl- 2-(1-methylethyl)	16.32	---	---	---	---	---	3.3	---	---
D-limonene	16.46	---	---	---	---	---	6.3	---	1.6
Unidentified	16.48	---	---	---	---	---	---	1.9	---
1,8-cineole	16.59	---	---	---	---	---	16.6	4.6	4.0
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)	17.73	---	---	---	---	---	0.7	---	---
Terpineol, Z-beta	18.1	---	---	---	---	---	0.6	---	---
Cyclohexene, 1-methyl-4-(1-methylethylidene)	18.95	---	---	---	---	---	0.7	---	---
Thujone	19.23	---	---	---	---	---	---	---	8.0
Unidentified	20.94	---	---	---	---	---	3.5	---	---
Camphor	21.09	---	---	---	---	---	37.7	8.9	16.0
Bicyclo[3.3.1]heptan-3-one, 2,6,6-trimethyl	21.74	---	---	---	---	---	---	---	1.1
Borneol	21.93	---	---	---	---	---	1.1	---	---
Bicyclo[2.2.1]heptan-2-ol 1,7,7-trimethyl, acetate (1S-endo)	26.27	---	---	---	---	1.0	---	7.4	---
Bornyl acetate	26.28	---	---	---	---	---	1.1	---	2.1

Unidentified	26.49	---	---	---	---	---	5.0	---	---
1,5,5-trimethyl-6-methylene-cyclohexene	28.02	---	---	---	---	3.2	---	1.0	---
(+)-4-carene	28.41	---	---	---	---	---	13.6	---	---
α-cubebene	29.32	4.3	4.5	---	---	---	---	---	---
Copaene	29.33	---	---	---	---	---	---	3.5	---
Cyclohexane, 1-ethenyl-1-methyl-2, 4-bis(1-methyl ethenyl)-,[1S-(1α,2β,4β)]	29.85	---	1.3	---	---	2.3	---	1.5	---
1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7b-octa-hydro-1,1,4,7-tetramethyl, [1aR-(1aα, 4α, 4aβ, 7bα)]	30.44	---	---	---	---	---	---	---	1.1
Caryophyllene	30.76	6.6	22.6	---	---	10.4	1.5	12.7	2.9
Unidentified	31.00	---	---	---	---	2.5	---	---	---
1H-cyclopenta(1,3)cyclo-propa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-,[3aS-(3aα,3bβ,4β,7α	31.06	---	2.2	---	---	---	---	0.3	1.3
γ-elemene	31.17	---	---	---	---	10.0	---	4.7	---
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl -4-methylene-, (1a,a)	31.39	4.6	---	---	---	---	---	2.0	2.7
α-caryophyllene	31.84	2.9	5.1	---	---	2.9	0.6	1.2	30.4
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl -4-methylene-,(1aR-[1aα, 4aβ, 7α, 7aβ, 7bα)]	32.07	9.7	---	---	---	---	---	0.2	---
Naphthalene, 1,2,3,4,4a,5, 6,8a-octahydro-7-methyl-4-methylene-1-(1-methyl-ethyl)-,(1α, 4aα, 8aα)-	32.54	1.4	---	---	---	---	---	1.4	2.8
Germacrene D	32.69	---	---	---	---	---	---	0.4	---
1H-cyclopenta[1,3]-cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-, [3aS-(3aα,3bβ,4β,7α	32.71	---	6.4	---	---	---	---	---	---

Eudesma-4(14), 11 diene	32.87	2.4	---	---	---	---	---	3.1	---
1H-Cycloprop[e]azulene, 1a2,3,5,6,7,7a,7b octa-hydro -1,1,4,7-tetramethyl [1aR-(1a.α,7α,7aβ,7b.α)]	33.13	---	---	---	---	---	---	---	2.4
Naphthalene, 1,2,3,4,4a,5, 6,8a,octahydro-4a, 8-di methyl-2-(1methylethenyl [2R-(2α,4aα, 8aβ)]	33.14	5.8	---	---	---	---	---	5.7	---
Unidentified	33.17	---	---	---	---	4.6	---	---	---
Unidentified	33.23	---	1.4	---	---	---	---	---	---
Azulene, 1,2,3,5,6,7,8,8a,-octahydro-1 ,4-dimethyl-7-(1-methylethenyl)-, [1S-(1α,7α,8aβ)]	33.44	---	---	---	---	---	---	0.5	---
Naphthalene, 1,2,3,4,4a,5, 6,8a,octahydro-7-methyl-4-methylene-1-(1-methyl ethyl)-, (1α,4aβ,8aα)	33.69	6.0		---	---	---	---	0.9	1.1
Cyclohexanol, 3-ethenyl-3-methyl-2-(1-methyleth-enyl)-6-(1-methylethyl)-, [1R-(1α, 2α, 3β, 6α)]	33.87	4.6	---	---	---	---	---	---	---
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(methylethyl)-,(1S-cis)	33.96	7.6	6.7	---	---	1.4	---	---	3.7
Unidentified	34.04	2.1	---	---	---	---	---	---	---
Unidentified	34.33	---	---	---	---	---	---	0.8	---
Naphthalene, 1,2,4a,5,6, 8a-hexahydro-4,7-dimeth-yl-1-(1-methylethyl)-, (1α,4a,α,8a α)	34.39	2.0	---	---	---	---	---	---	---
Unidentified	34.54	---	---	---	---	---	---	0.8	---
Cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-,[1R-(1α, 3α,4]]-β	34.72	---	---	---	---	---	---	1.3	---
Germacrene B	35.0	---	---	---	---	0.8	---	1.0	---
Unidentified	35.77	---	---	---	---	---	1.2	0.8	---
Guaiol	36.16	---	---	---	---	---	---	5.5	---
Unidentified	36.22	---	---	---	---	3.0	---	---	---
β-elemenone	36.35	---	---	---	---	44.1	---	---	---
2-naphthalenemethanol,	37.16	1.7	---	---	---	---	---	---	---

1,2,3,4,4a,5,6,7-octahydro α,α,4a,8-tetramethyl-, (2R-cis)									
Unidentified	37.4	8.2	---	---	---	---	---	---	---
2-naphthalenemethanol, decahydro-α,α,4a-trimethyl-8-methylene, [2R-(2α,4aα,8aβ)]	37.69	3.7	---	---	---	---	---	---	---
2-naphthalenemethanol, 1,2,3,4,4a,5,6,8a,-octahydro-α,α,4a,8-tetramethyl-, [2R-(2α,4aα,8aβ)]	37.78	4.6	---	---	---	---	---	5.8	---
Unidentified	38.14	---	---	---	---	0.8	---	0.6	---
3,7-cyclodecadien-1-one, 3,7-dimethyl-10-(1-methylethylidene)-, (EE)-	38.96	---	---	---	---	9.1	---	---	---
Unidentified	41.86	---	---	---	---	---	---	---	1.6
Unidentified	42.59	---	1.3	1.0	---	---	---	0.3	---
Unidentified	43.55	---	---	---	---	---	---	0.5	---
Unidentified	44.14	---	---	---	---	---	---	0.2	---
Unidentified	44.46	---	---	---	---	---	---	0.3	---
10S, 11S-Himachala-3(12), 4-diene	44.59	---	---	---	---	---	---	0.3	---
(E,E)-7,11,15,-trimethyl-3-methylene-hexadeca-1, 6,10,14-tetraene	44.61	---	1.2	---	---	---	---	---	---
Unidentified	45.49	---	37.1	---	---	---	---	9.0	---
Unidentified	45.85	---	2.4	---	---	---	---	---	---
Unidentified	46.21	---	2.1	---	---	---	---	---	---
Unidentified	47.46	---	---	---	4.3	---	---	---	---
Unidentified	47.69	---	---	---	0.6	---	---	---	---
Unidentified	47.91	---	---	---	23.6	---	---	---	---
Unidentified	48.01	---	---	---	---	---	---	0.2	---
Unidentified	48.29	---	---	---	7.0	---	---	---	---
Unidentified	48.37	---	---	---	5.9	---	---	---	---
Unidentified	48.62	---	---	---	2.8	---	---	---	---

Unidentified	49.28	---	---	---	49.0	---	---	---	---
Unidentified	50.45	---	---	---	---	---	---	0.2	---
Unidentified	53.84	---	---	---	---	---	---	0.3	---
Unidentified	54.89	---	---	---	---	---	---	---	2.8
Unidentified	56.46	---	---	---	3.1	---	---	---	---
Heptacosane	60.96	---	---	2.4	---	---	---	---	---
Unidentified	63.25	2.0	--0.8-		---	---	---	0.6	---
Squalene	63.26	---	---	22.1	---	---	---	---	---
2,6,10,14,18,22-tetracosa-Hexaene, 2,6,10,15,19,23-Hexamethyl-, (all-E)-	63.27	---	---	---	---	2.7	---	---	---
Unidentified	63.30	---	---	---	1.9	---	---	---	---
Vitamin E	68.32	---	0.8	---	---	---	---	---	---
Unidentified	70.18	---	---	70.5	---	---	---	---	---
Unidentified	70.9	---	---	1.1	---	---	---	---	---
Unidentified	71.47	---	---	3.1	---	---	---	---	---

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Relative percentage as calculated from a total area of peaks on a basis of 1 % of a major peak

--- not detected

Table 3.3. Chemical composition of extracts of *Salvia* species with less apparent dual anti-cholinesterase activity

Compound[a]	RT, min[b]	% in oil[c]					
		S. confertiflora	*S. haematodes*	*S. glutinosa*	*S. longistyla*	*S. napifolia*	*S. verticilata*
Camphene	13.01	---	---	---	2.0	---	---
Unidentified	15.50	---	---	0.3	---	---	0.3
Unidentified	16.51	---	---	0.4	---	---	0.3
Unidentified	16.62	---	---	0.5	---	---	---
Unidentified	16.92	---	---	0.3	---	---	---
Unidentified	19.36	---	---	---	---	1.0	---
Unidentified	21.157	---	---	0.4	---	---	---
Unidentified	21.648	---	---	0.3	---	---	---
Bicyclo[2.2.1]heptan-2-ol 1,7,7-trimethyl, acetate (1S-endo)	26.27	---	---	---	6.4	---	---
1,5,5-trimethyl-6-methylene-cyclohexene	28.02	---	---	---	0.7	---	---
Unidentified	27.71	0.8	3.4	---	---	---	---
Unidentified	27.73	---	---	0.7	---	0.3	0.5
1,5,5-trimethyl-6-methylene-cyclohexene	28.02	5.3	---	---	---	1.3	0.8
α-cubebene	29.32	3.2	---	---	0.3	---	---
Copaene	29.33	---	---	---	---	0.5	---
Unidentified	29.62	0.4	---	---	---	---	1.6
Unidentified	29.63	---	---	1.3	---	0.7	---
Cyclohexane, 1-ethenyl-1-methyl-2, 4-bis(1-methyl ethenyl)-,[1S-(1α,2β,4β)]	29.85	3.3	---	1.8	0.6	1.2	1.0
Caryophyllene	30.76	28.1	23.7	23.8	28.2	24.2	10.3

Unidentified	31.05	---	1.8	1.2	---	---	---
1H-cyclopenta(1,3)cyclo-propa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-,[3aS-(3aα,3bβ,4β,7α	31.06	5.8	---	---	2.4	7.3	3.6
Unidentified	31.16	---	---	1.0	---	---	---
γ-elemene	31.17	---	---	---	1.8	---	---
Unidentified	31.23	---	---	---	---	0.8	---
Unidentified	31.54	---	---	0.5	---	---	---
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl -5-methyl-9-methylene	31.546	---	---	---	0.9	3.0	---
Unidentified	31.552	2.4	---	---	---	---	1.4
Humulen-(v1)	31.71	---	---	---	0.3	---	---
α-caryophyllene	31.84	6.5	---	22.8	7.5	10.2	3.7
Unidentified	31.843	---	3.2	---	---	---	---
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl -4-methylene-,(1aR-[1aα, 4aβ, 7α, 7aβ, 7bα)]	32.07	1.1	---	---	---	---	---
Unidentified	32.13	---	---	---	---	---	0.7
Unidentified	32.14	1.1	---	---	0.5	1.6	---
Unidentified	32.322	---	---	---	---	1.1	
Unidentified	32.56	1.1	---	---	---	0.4	---
Germacrene D	32.69	19.0	---	2.2	---	---	10.7
Unidentified	32.699	---	3.8	---	---	---	---
1H-cyclopenta[1,3]-cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-, [3aS-(3aα,3bβ,4β,7α	32.71	---	---	---	6.3	21.0	---
Unidentified	33.04	---	---	---	0.5	0.9	0.8
Unidentified	33.18	7.1	---	---	---	---	---
Unidentified	33.213	---	8.7	2.3	1.8	5.2	3.4
Unidentified	33.442	---	---	1.6		---	---

Unidentified	33.50	---	---	---	---	1.1	3.9
Unidentified	33.603	---	---	---	---	---	0.5
Unidentified	33.683	---	---	---	0.4	---	0.6
Naphthalene, 1,2,3,4,4a,5, 6,8a,octahydro-7-methyl-4-methylene-1-(1-methyl ethyl)-, (1α,4aβ,8aα)	33.69	0.5	---	---	0.4	0.6	---
Unidentified	33.943	---	---	0.4	---	---	---
Unidentified	33.957	---	---	--	---	---	1.6
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(methylethyl)-,(1S-cis)	33.96	6.8	---	---	---	---	---
Naphthalene, 1,2,3,4-tetrahydro-1, 6-dimethyl-4-(1-methylethyl)-, (1S-cis)	33.963	---	---	---	1.1	5.0	---
Unidentified	34.51			0.5	1.3	2.1	10.7
Unidentified	34.54	---	---	---	---	0.7	---
Unidentified	34.96	---	---	---	0.7	---	0.6
Unidentified	35.0	---	---	0.3	---	---	---
Unidentified	35.79	---	---	1.9	0.4	---	1.0
Unidentified	36.09	---	1.7	0.5	0.6	0.9	1.1
Unidentified	36.535	---	---	1.7	---	---	---
Unidentified	37.01	---	---	---	---	---	0.3
Unidentified	37.29	---	---	---	0.9	---	0.9
Unidentified	37.55	---	---	---	---	---	0.3
Unidentified	37.99	---	---	---	0.3	---	0.4
Unidentified	38.133	---	6.6	1.1	---	---	1.4
Unidentified	38.14	0.5	---	---	---	---	---
Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-	38.36	1.6	---	---	0.6	---	0.6
Unidentified	38.365	---	---	---	---	1.6	---
Unidentified	39.424	---	---	0.6	---	---	---

Unidentified	39.75	---	---	---	---	---	0.3
Unidentified	41.182	---	---	0.3	---	---	---
Unidentified	41.513	---	---	0.4	---	---	---
Unidentified	42.40	---	---	0.4	---	---	---
Unidentified	42.42	---	2.1	---	---	---	0.7
Unidentified	42.59	---	8.2	1.7	---	1.2	---
Unidentified	42.734	---	1.3	---	---	---	---
Unidentified	43.20	---	---	0.7	---	---	---
Unidentified	43.214	---	3.2	---	---	0.4	---
Unidentified	43.66	---	---	1.0	---	---	---
Unidentified	43.671	---	5.6	---	---	0.7	---
Unidentified	44.655	---	---	0.4	0.4	---	---
Unidentified	44.78	---	---	0.7	---	---	---
Unidentified	45.89	---	---	0.4	---	---	---
Unidentified	46.23	---	---	0.4	---	---	---
Unidentified	47.130	---	1.6	---	---	---	---
Unidentified	48.023	---	---	0.3	---	---	---
Unidentified	48.751	---	---	0.4	---	---	---
Unidentified	48.911	---	5.9	2.5	---	---	---
Unidentified	48.92	0.6	---	---	0.3	0.4	---
Unidentified	51.745	---	---	5.3	---	---	---
Unidentified	51.96	---	---	2.3	---	---	---
Unidentified	54.69	---	---	0.5	---	---	---
Unidentified	55.78	---	---	0.9	---	---	---
Unidentified	57.211	---	2.1	---	---	---	---
Unidentified	58.24	---	---	---	---	0.3	---
Unidentified	58.638	---	1.9	---	---	---	---
Unidentified	60.956	---	3.0	---	---	---	---
Unidentified	63.250	---	4.1	1.9	---	---	---

Squalene	63.26	---	---	---	8.8	---	---
Unidentified	63.264	4.8	---	---	---	---	---
Unidentified	66.265	---	---	---	0.3	---	---
Unidentified	67.71	---	---	7.2	---	---	---
Unidentified	68.19	---	---	---	0.5	---	---
Unidentified	68.308	---	---	---	0.4	---	---
Unidentified	68.64	---	---	---	17.9	---	---
Unidentified	70.355	---		---	3.3	---	---
Unidentified	72.258	---	8.0	---	---	---	---

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Relative percentage as calculated from a total area of peaks on a basis of 1 % of a major peak

--- not detected

Table 3.4. Chemical composition of extracts of *Salvia* species which did not reach* 50% inhibition of cholinesterases

Compound[a]	RT, min[b]	% in oil[c]					
		S. atrocyanea	*S. discolor*	*S. Jamensis* var. la luna	*S. sclarea*	*S. stenophylla*	*S. verbenaca*
Unidentified	12.39	---	---	0.3	---	---	---
Unidentified	14.09	---	---	---	---	---	2.6
Unidentified	15.484	---	---	---	---	---	1.1
Unidentified	15.53	0.5	---	---	---	---	---
Unidentified	16.3	---	---	0.3	---	---	---

Unidentified	16.04	---	---	---	---	---	0.6
D-limonene	16.46	---	---	1.0	---	---	0.7
Unidentified	16.604	---	---	0.8	---	---	---
Unidentified	19.115	---	---	---	---	---	0.5
Unidentified	20.68	---	---	---	---	---	0.7
Unidentified	21.14	---	---	1.4	---	---	---
Unidentified	21.16	0.4	---	1.4	---	---	---
Unidentified	21.18	---	---	---	---	0.4	2.2
Unidentified	21.65	---	---	---	---	---	2.0
Unidentified	22.972	---	---	---	---	---	0.7
Unidentified	25.187	---	---	---	7.9	---	---
Unidentified	27.23	---	---	---	---	---	1.5
Unidentified	27.72	2.0	---	0.4	---	0.5	2.8
Unidentified	28.032	---	---	1.1	---	0.8	---
Unidentified	28.17	---	---	---	---	---	0.8
Copaene	29.33	---	---	3.2	2.1	---	---
Cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl)-,[1S-(1α.3aα,3bβ, 6aβ,6b.a)	29.63	---	---	3.4	---	0.4	---
Cyclohexane, 1-ethenyl-1-methyl-2, 4-bis(1-methyl ethenyl)-,[1S-(1α,2β,4β)]	29.85	---	4.5	---	---	2.0	---
Unidentified	30.74	---	---	---	---	7.5	---
Unidentified	30.747	---	---	---	10.9	---	---
Caryophyllene	30.76	---	3.4	15.7	---	---	2.5
1H-cyclopenta(1,3)cyclo-propa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-,[3aS-(3aα,3bβ,4β,7α	31.06	---	---	1.3	4.0	3.0	---
γ-elemene	31.17	---	0.4	---	---		---
Unidentified	31.55	---	---	0.9	---	1.3	---
Unidentified	31.83	---	---	---	---	1.9	---

Unidentified	31.838	---	---	1.3	2.1	---	---
α-caryophyllene	31.84	---	0.7	---	---	---	---
Unidentified	32.07	0.6	---	---	---	---	---
1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl -4-methylene-,(1aR-[1aα, 4aβ, 7α, 7aβ, 7bα)]	32.072	---	---	0.9	---	---	---
Unidentified	32.13	---	---	---	---	0.7	---
Naphthalene, 1,2,3,4,4a,5, 6,8a-octahydro-7-methyl-4-methylene-1-(1-methyl-ethyl)-,(1α, 4aα, 8aα)-	32.54	---	---	4.8	---	---	---
Germacrene D	32.69	---	0.6	2.8	12.2	---	---
1H-cyclopenta[1,3]-cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-methylethyl)-, [3aS-(3aα,3bβ,4β,7α	32.71	---	---	---	---	7.9	---
Unidentified	33.10	---	---	---	---	0.8	---
Unidentified	31.854	---	---	---	---	---	2.4
Unidentified	33.213	3.9	0.8	---	4.6	---	4.7
Unidentified	33.227	---	---	3.0	---	3.1	---
Unidentified	33.501	---	---	0.3	---	---	---
Unidentified	33.61	---	---	---	---	---	2.4
Unidentified	33.695	---	---	0.3	---	---	---
Unidentified	33.958	---	---	1.5	1.7	---	---
Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(methylethyl)-,(1S-cis)	33.96	---	---	---	---	1.1	---
Unidentified	35.556	---	---	0.3	---	---	---
Unidentified	35.784	---	---	1.8	--	---	---
Unidentified	36.09	---	---	---	---	0.7	4.4
Unidentified	36.64	---	---	---	---	---	1.3
Unidentified	38.134	3.3	0.4	0.5	---	---	4.5
Unidentified	38.145	---	---	---	---	0.7	---

Unidentified	38.29	---	---	---	---	---	0.8
Unidentified	38.373	---	---	---	---	0.5	---
Unidentified	41.39	---	----	---	---	---	0.7
Unidentified	42.40	2.0	---	---	---	---	1.5
Unidentified	42.415	---	---	---	---	0.4	---
Unidentified	42.59	1.2	---	1.5	---	5.3	6.4
Unidentified	42.75	1.0	---	---	---	0.7	---
Unidentified	43.09	---	---	---	---	---	0.5
Unidentified	43.217	---	---	0.5	---	1.8	---
Unidentified	43.225	---	---	---	---	---	2.4
Unidentified	43.362	---	---	---	---	---	0.6
Unidentified	43.673	---	---	0.7	---	3.1	2.6
1H-naphthol[2,1-b]pyran, 4a,5,6, 6a,7,8,9,10,10a,10b-decahydro-3,4a,7,7,10a-pentamethyl-, [4aR (4aα,6aβ,10aα,10bβ)]	43.88	---	---	---	2.0	---	---
Hexadecanoic acid, methyl ester	44.78	---	---	---	---	2.6	0.9
Unidentified	44.815	---	---	0.8	---	---	---
Unidentified	45.03	---	---	---	5.7	---	---
Unidentified	45.212	---	---	---	3.8	---	---
Unidentified	45.489	---	---	0.3	---	---	---
Unidentified	45.54	---	---	---	3.1	---	---
Unidentified	45.89	---	---	---	3.4	---	---
Unidentified	46.05	---	---	---	4.6	---	---
Unidentified	46.217	0.9	---	---	---	---	---
Unidentified	46.38	---	---	---	5.3	---	---
Unidentified	46.54	---	---	---	12.3	---	---
Unidentified	46.61	---	---	---	---	---	0.7
Unidentified	46.99	---	---	---	4.2	---	---
Unidentified	47.062	---	---	---	5.4	---	---

Unidentified	47.393	---	---	---	2.8	---	---
Unidentified	47.64	1.1	---	---	---	---	---
Unidentified	47.815	---	---	---	---	---	0.5
Unidentified	48.12	---	---	---	---	---	0.5
Unidentified	48.23	---	---	---	---	---	0.6
Unidentified	48.75	1.1	---	0.9	---	---	---
9,15,-octadecadienoic acid, methyl ester, (Z,Z)-	48.752	---	---	---	---	4.4	---
Unidentified	48.79	---	---	---	---	---	1.5
9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-	48.90	6.4	---	7.1	---	33.8	---
Unidentified	48.934	---	---	---	---	---	7.4
Unidentified	49.482	---	---	---	---	0.7	---
Unidentified	49.73	0.5	---	---	---	---	---
Unidentified	49.85					0.4	---
Unidentified	50.26	---	---	---	---	0.4	---
Unidentified	50.41	1.5	---	---	---	---	---
Unidentified	50.43	---	---	---	---	3.1	---
Unidentified	50.48	---	---	1.8	---	---	---
Unidentified	51.33	---	---	0.3	---	---	---
Unidentified	52.92	0.5	---	---	---	---	---
Unidentified	53.22	0.5	---	0.5	----	---	0.7
Unidentified	53.82	---	---	---	---	0.4	---
Unidentified	54.69	---	---	0.8	---	---	---
Unidentified	56.52	---	---	---	---	---	1.0
Unidentified	57.22	1.0	---	0.9	---	0.9	2.5
Unidentified	57.611	---	---	---	1.7	---	---
Unidentified	58.13	0.4	---	---	---	---	---
Unidentified	58.170	---	---	---	---	0.5	0.7
Unidentified	58.62	---	---	1.2	---	---	2.4

Unidentified	60.27	---	---	---	---	0.4	1.7
Unidentified	60.944	---	---	---	---	1.4	4.9
Heptacosane	60.96	3.9	---	1.0	---	---	---
Unidentified	61.79	1.5	---	---	---	---	---
Unidentified	62.26	---	---	2.4	---	---	---
Unidentified	63.06	---	---	5.8	---	---	---
Squalene	63.26	39.7	---	22.3	---	---	4.6
Unidentified	63.264	---	2.0	---	---	3.7	---
Unidentified	65.64	0.7	---	0.3	---	---	---
Unidentified	67.41	---	3.4	---	---	---	---
Unidentified	67.60	---	---	---	---	---	13.0
Vitamin E	68.32	4.8	---	---	---	---	---
Unidentified	68.37	---	0.7	---	---	---	---
Lup-20(29)-en-3-one	72.27	---	39.1	---	---	---	---
Unidentified	73.24	18.0	---	---	---	---	---
Unidentified	75.43	---	34.4	---	---	---	---

[a]Identities of compounds determined from mass spectrometry analysis

[b]RT-retention time of the compounds

[c]Relative percentage as calculated from a total area of peaks on a basis of 1 % of a major peak

--- not detected

*-due to the solubility limit of the extracts

Appendix 4. Calculations of concentrations

1.1. Preparation of inhibitor solutions with any number of agents in a combination

1,8-cineole and camphor combination was taken as an example of how final concentrations of individual compounds in a combination may be calculated. The calculation was made on a basis of an IC50 value for the whole oil, *i.e.*, 0.05 mg ml^{-1} and percentage contents of each compound in the whole oil (Chapter 6, Table 6.2.)

There are 26.8% of 1,8-cineole and 24.7% of camphor in the *S. lavandulaefolia* essential oil (Chapter 6, Table 6.2.)

0.05 mg ml^{-1} (whole oil IC_{50} value) – 100% (as a mixture containing all compounds)

X mg ml^{-1} (camphor) – 24.7%
Y mg ml^{-1} (cineole) – 26.8%
⇨ 0.05×24.7/100=0.012
0.05×26.8/100=0.013

X=0.012 mg ml^{-1} (camphor final concentration)

Y=0.013 mg ml^{-1} (1,8-cineole final concentration)

Thus, final concentrations of 0.012 mg ml^{-1} camphor and 0.013 mg ml^{-1} 1,8-cineole are in the naturally occurring ratio. The total final concentration of camphor/cineole mixture is 0.025 mg ml^{-1}. To prepare a mixture with an assay final concentration of 0.025 mg ml^{-1} each of the individual compound stock solutions is needed to be calculated.

1.1.1. Calculation of a stock solution for 1,8-cineole (example)

1 0.013 mg (Wf) – 1000µl (1,8-cineole final concentration in 1ml, Vs)

X mg (Wm) – 220 µl (total volume of the assay mixture, Vt)

X=0.00286 mg (amount of 1,8-cineole in the assay mixture, Wm)

Where,

Wf- amount of inhibitor in the assay final concentration, mg

Vs-volume of 1 ml to express the amount of inhibitor in the assay final concentration, µl

Wm-amount of one inhibitor in the reaction mixture, mg

Vt-total volume of the reaction mixture, µl

2 0.00286 mg (Wm) – 5 µl (volume of 1,8-cineole transferring into the reaction mixture, Vp)

X mg (Wfs) – 300 µl (150 µl of 1,8-cineole and 150 µl of camphor to make the final stock solution, V1, n)

X=0.1716 mg (amount of 1,8-cineole in 150 µl when taken from its stock solution; or amount of 1,8-cineole in the final stock solution of 300 µl, Ws)

Where,

Vp- amount of inhibitor transferring into the reaction mixture, µl

Wfs-amount of inhibitor in the final stock solution of 300 µl, or in 150 µl taken from cineole stock solution, mg

V1-volume of both inhibitors in the final stock solution, µl

Ws-amount of cineole required to prepare the stock solution in 1 ml, mg

3 0.172 mg (Wfs) - 150 µl (volume taken from cineole stock, V1)

X mg (Ws) – 1000 µl (volume of cineole to make a stock solution, Vs)

X = 1.14 mg (amount of 1,8-cineole needed to make a stock solution of 1 ml)

Thus, 1.14 mg of 1,8-cineole need to be dissolved within 1 ml 86% EtOH to make a stock solution of 1.13 mg ml^{-1}. This would give 1,8-cineole assay final concentration of 0.013 mg ml^{-1}. This has been calculated for 300 µl of 1,8-cineole/campor stock solution.

The same principal of calculations may be applied to camphor. Alternatively, the calculations of 1,8-cineole final concentration and its stock solution may be simplified into mathematical equations;

(i) $Wm=Wf\times Vt/Vs$

Where,

Wm-amount of 1,8-cineole in the reaction mixture, mg

Wf- amount of cineole in the assay final concentration, mg

Vt-total volume of the reaction mixture, µl

Vs-volume of 1 ml to express the amount of cineole in the assay final concentration, µl

(ii) $Wfs=Wm\times V1\times n/Vp$

Where,

Wfs-amount of cineole in the final stock solution of 300 µl, or in 150 µl taken from cineole stock solution, mg

V1-volume of both inhibitors in the final stock solution, µl

n-amount of inhibitors in the final stock solution

Vp-volume of the final stock solution (cineole/camphor) going into the reaction mixture, µl

(iii) $Ws=Wfs\times Vs/V1$

Where,

Ws-amount of cineole required to prepare the stock solution in 1 ml, mg

Vs-volume of 1 ml to prepare cineole stock solution, µl

V1-volume of one inhibitor taken from the stock into the final stock solution, µl

Since, Wfs (ii) and Wm (i) are known, amount of 1,8-cineole to make the stock solution (Ws) and the final assay concentration (Wf) may be calculated as;

(iv) $Ws=Wf\times Vt/Vs\times V1\times n/Vp\times Vs/V1$

or

(v) $Ws=Wf\times Vt\times n/Vp$ (for stock)

From (v) Wf is

(vi) $Wf=Ws\times Vp/Vt\times n$ (for final concentration)

To calculate a stock or a final concentration of a combination comprising any number of inhibitors equation (v) and (vi) may be applied respectively.

Example: cineole final concentration is 0.013 mg ml^{-1}, Wf

The assay total volume is 220 ml, Vt

n=2, two chemicals are needed to make the final stock solution.

5 µl from the final stock solution (cineole/camphor) were pipetted into the assay reaction mixture, Vp

The amount of cineole (Ws) required for the stock of 1 ml solution may be calculated as in (v):

$Ws=Wf\times Vt\times n / Vp$,

$Ws=0.013\times 220\times 2 / 5=1.14$ mg

1.14 mg of 1,8-cineole is needed to make the stock solution of 1 ml, which would give a final assay concentration of 0.013 mg ml^{-1}.

The same calculations can be applied to camphor.

Appendix 5. Calculation of expected inhibition

An IC50 value of the individual compounds and their standard deviations were calculated on a basis of equations (Table V.1.) obtained from the dose-response curves of these compounds.

Table V.1. Dose-response curve equations for the monoterpenoids

Compound /	n 1[a]	n 2[b]	n 3[c]	n 4[d]
Cineole	y=17.429Ln(x)+99.755	y=14.823Ln(x)+90.668	y=15.481Ln(x)+95.398	y=15.911Ln(x) 95.366
Camphor	y=12.310Ln(x)+36.115	y=15.448Ln(x)+48.176	y=13.313Ln(x)+39.71	y=12.407Ln(x) 39.282
α-pinene	y=20.756Ln(x)+94.952	y=19.891Ln(x)+96.783	y=17.562Ln(x)+93.965	y=18.254Ln(x)+96.282
β-pinene	y=19.675Ln(x)+82.108	y=20.976Ln(x)+86.61	y=18.383Ln(x)+76.531	y=19.457Ln(x)+81.564
Car. oxide	y=11.777Ln(x)+56.846	y=11.573Ln(x)+55.552	y=11.8Ln(x)+53.7	y=12.31Ln(x)+61.8
Borneole	y=6.285Ln(x)+25.251	y=7.31Ln(x)+23.574	y=8.05Ln(x)+22.544	y=7.822Ln(x)+26.170
Bornyl acetate	y=9.444Ln(x)+36.354	y=10.202Ln(x)+34.911	y=9.353Ln(x)+35.112	y=8.234Ln(x)+30.781
Linalool	y=8.3895Ln(x)+21.125	y=7.561Ln(x)+18.73	y=8.425Ln(x)+19.172	y=8.117Ln(x)+18.983

[a] Equation obtained from a dose response curve produced in triplicate test 1

[b] Equation obtained from a dose response curve produced in triplicate test 2

[c] Equation obtained from a dose response curve produced in triplicate test 3

[d] Equation obtained from a dose response curve produced in triplicate in test 4.

The calculation of the expected and observed inhibitions is shown, as an example, using the data of 1,8-cineole and α-pinene mixture of 0.245 mg ml^{-1} with 1,8-cineole (da) of 0.225 mg ml^{-1} and α-pinene (db) of 0.02 mg ml^{-1} (Chapter 6, Table 6.3.). Figure V.1 shows a dose-response curve, as a mean of four (Table V.1), of each of the compounds.

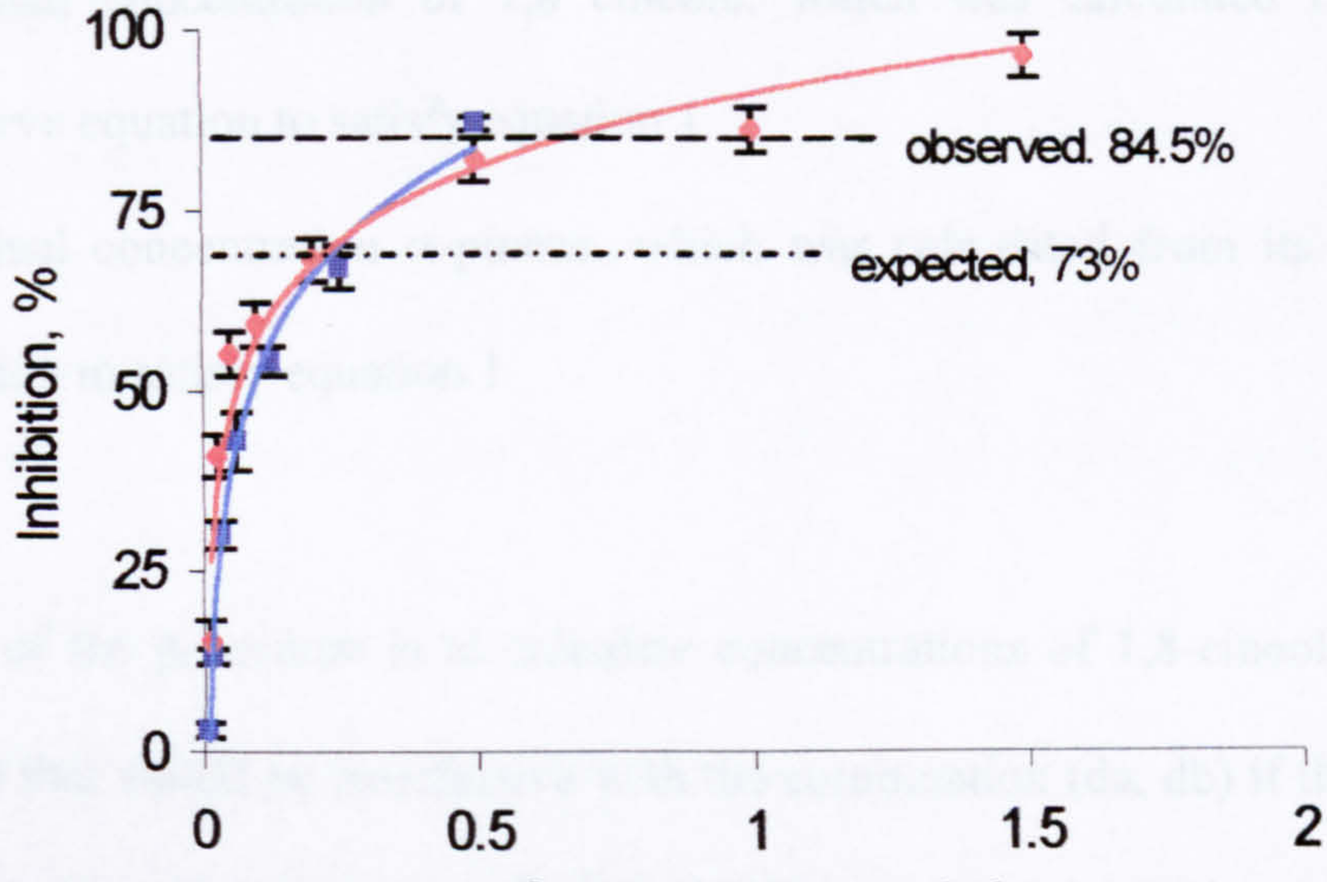


Figure V.1. Dose-response curves of 1,8-cineole and α-pinene

◊-1,8 cineole dose-response curve, □-α-pinene dose-response curve.

Expected inhibition of the mixture 0.245 mg ml^{-1} (Synergy 6, Table 6.3.) was 73±2.1%; observed 84.5±1.7%, *i.e.*, more than expected, indicating synergy.

The expected inhibition of AChE by the combination has been calculated as follow;

For one set of triplicate

Equations obtained from the dose-response curves of 1,8-cineole and α-pinene are Y=15.481Ln(X)+95.398 and Y=17.562Ln(X)+93.96 respectively (Table V.1, n3).

Equation 1 (Chapter 2) stipulates the zero-interactive respond of the mixture

$$da/Da + db/Db = 1 \qquad (1)$$

where,

da- final concentration of 1,8-cineole in the mixture (as one of the compounds of the mixture)

db- final concentration of α-pinene in the mixture (as one of the compounds of the mixture)

Da- individual concentration of 1,8 cineole, which was calculated from its dose-response curve equation to satisfy equation 1

Db- individual concentration α-pinene, which was calculated from its dose-response curve equation to satisfy equation 1

The object of the procedure is to calculate concentrations of 1,8-cineole (Da) and α-pinene (Db) that would be isoeffective with the combination (da, db) if the combination is zero-interactive. To approximately estimate the expected inhibition of AChE by the mixture, 0.225 mg ml^{-1} (da) was substituted as X (concentration) into the cineole equation to find Y (inhibition)

Y=15.481 Ln(0.225)+95.398

Y=72, or 72% inhibition

72% inhibition is a rough estimate of what the combination would be expected to produce. This is based on the data (Chapter 6, Table 6.1.) that camphor showed little anti-AChe activity in comparison with 1,8-cineole.

From now, it is a matter of time to find the expected inhibition of the mixture which would satisfy equation 1.

Expected inhibition of 73% was randomly chosen as a close figure to 72. Because the expected inhibition has an isoeffective quality (Equation 1) it has to intersect both of the dose-response inhibition curves (Figure V.1).

73% was substituted as Y (inhibition) into 1,8-cineole dose-response equation to find corresponding to it 1,8-cineole individual concentration (Da);

$73=15.481Ln(X)+95.398$

$X=0.24$ (concentration) or 0.24 mg ml^{-1}

Now, 73% of expected inhibition was substituted as Y (inhibition) into α-pinene dose-response equation to find corresponding to it α-pinene individual concentration (Db);

$73=17.562Ln(x)+93.965$

$X=0.303$ (concentration) or 0.303 mg ml^{-1}

For 73% of preliminary expected inhibition Da and Db were 0.24 and 0.303 mg ml^{-1} respectively. If Da and Db are isoeffective (Equation 1), intersecting the two dose-response curves with the same horizontal line (Figure V.1), they would satisfy equation (1) namely,

$0.225/Da + 0.02/Db = 1$

$0.225/0.24 + 0.02/0.303 = 0.937 + 0.066=1$

As Da and Db satisfy equation 1, 73% inhibition is accepted as the expected response of the combination.

For the mixture 0.245 mg ml^{-1} the expected inhibition is 73% and corresponds to one set of triplicates (Table V.1, n3).

In the same manner, as described above, equations of three remaining set of triplicates n1, n2 and n4 (Table V.1.) were used to calculated the mean ±SD of the expected

inhibition. For the four sets of triplicates the expected inhibition is 73±2.1% (Chapter 6, Table 6.3.). The observed inhibition of the combination was 84.5±1.7% (Chapter 6, Table 6.3.), *i.e.*, more than we expected (synergy). To calculate Da and Db for the observed inhibition in order to show the interaction index of the mixture, 84.5% was substituted as Y (inhibition) into 1,8-cineole and α-pinene dose-response curve equations.

Cineole	α-pinene
Y=15.481Ln(X)+95.398	Y=17.562Ln(X)+93.96
84.5=15.481Ln(X)+95.398	84.5=17.562Ln(X)+93.96
X=0.49 or 0.49 mg/ml	X=0.58 or 0.58 mg/ml
Da=0.49 mg/ml	Db=0.58 mg/ml

The interaction index for the observed effect of the combination was calculated as;

$da/Db + db/Db < 1$

$0.225/Da + 0.02/Db < 1$ (synergy effect)

$0.225/0.49 + 0.02/0.58 = 0.46 + 0.034 = 0.5$

$0.5 < 1$ (indicating synergy)

The interaction index of 0.5 was calculated only for one set of triplicates (n3). For all of n's (n=4) the interaction index of the combination was

$0.225/0.52+0.02/0.56=0.47$ *i.e.*, less than 1, indicating synergy.

The same approach of calculation of the expected inhibition was applied to the rest of the combinations used in this study.

The calculation of expected inhibition along with results of the observed inhibition may be expressed graphically.

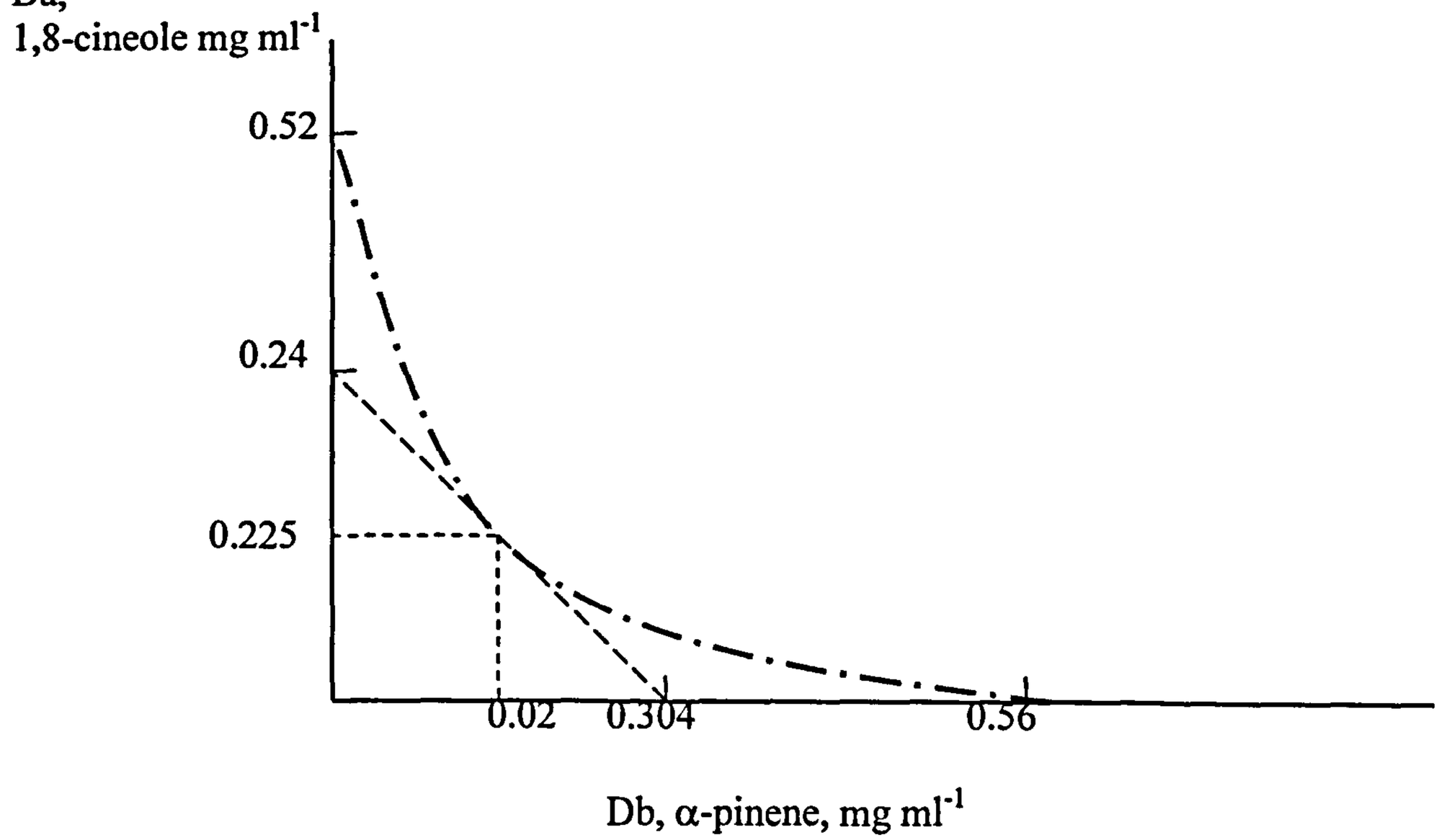


Figure V.2 The isobole for the observed effect of 1,8-cineole/α-pinene combination

The iso-effective linear of the mixture of 0.245 mg ml^{-1} (cineole 0.225 mg ml^{-1} (da) and α-pinene 0.02 mg ml^{-1} (db)) is drawn through the point representing the combination and it ends on the individual concentration axes, which satisfied Equation 1 for the expected inhibition of 73±2.1% (n=4) with Da of 0.24±0.005 mg ml^{-1} and Db of 0.304±0.05 mg ml^{-1}, namely

0.225/0.240+0.02/0.304=1

Thus, 0.24 and 0.304 are the points for the iso-effective linear.

The isobole is drawn through the point representing the combination of 0.245 mg ml^{-1} and its ends on the individual concentration axes. The individual concentrations for both

ends were calculated using 1,8-cineole and α-pinene dose-response curve equations (Table V.1), where observed inhibition of 84.5±1.7% (n=4,) was assumed as Y and substituted into the equations of both chemicals at 0.245 mg ml^{-1} mixture combination. Hence, for cineole X (Da) was 0.52±0.1 mg ml^{-1} (n=4) and α-pinene (Db) 0.56±0.04 mg ml^{-1} (n=4).

The isobole for the observed effect of inhibition is concave-up and the interaction index for the mixture is 0.225/0.52+0.02/0.56=0.47, *i.e.*, less than 1, indicating synergy.

The values of 0.304±0.05 (representing the iso-effective linear) and 0.56±0.04 (representing one of the ends of the isobole) locating on camphor concentration axes are significantly different ($P<0.05$), without overlapping itself. The same is refer to the values locating on 1,8-cineole concentration axes, namely 0.52±0.1 and0.24±0.005 ($P<0.05$).

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