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In-vitro Inhibition of Human Erythrocyte Acetylcholinesterase by *Salvia lavandulaefolia* Essential Oil and Constituent Terpenes

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Abstract

Sage (*Salvia* spp) is reputed in European herbal encyclopaedias to enhance memory, and current memory-enhancing/anti-dementia drugs are based on enhancing cholinergic activity by inhibiting cholinesterase. In this study the effects of *Salvia lavandulaefolia* Vahl. (Spanish sage) essential oil and some of its constituent terpenes on human erythrocyte acetylcholinesterase were examined in-vitro. The main constituents in the essential oil batch used for analysis of cholinesterase inhibition were camphor (27%), 1,8-cineole (13%), α- and β-pinene (10–15%) and bornyl acetate (10%) with other minor constituents (1% or less) including geraniol, limonene, linalool, terpineol and γ-terpinene.

Using the Ellman spectrophotometric method, kinetic analysis was conducted on the interaction of the essential oil and the main monoterpenoids, camphor, 1,8-cineole and α-pinene. IC50 values were obtained for the essential oil, 1,8-cineole and α-pinene and were 0·03 $\mu g\,mL^{-1}$, 0·67 mM and 0·63 mM, respectively. Camphor and other compounds tested (geraniol, linalool and γ-terpinene) were less potent (camphor IC50: >10 mM). The essential oil, α-pinene, 1,8-cineole and camphor were found to be uncompetitive reversible inhibitors.

These findings suggest that if the inhibitory activity of the essential oil is primarily due to the main inhibitory terpenoid constituents identified, there is a major synergistic effect among the constituents. Since no single constituent tested was particularly potent, it remains to be determined whether these in-vitro cholinesterase inhibitory activities are relevant to in-vivo effects of the ingestion of *S. lavandulaefolia* essential oil on brain acetylcholinesterase activity.

Inhibitors of acetylcholinesterase (EC 3.1.1.7) are the only symptomatic treatment of Alzheimer's disease available at present in Western medicine (Schneider 1996). Alzheimer's disease is a common neurodegenerative condition that affects the elderly population and the primary symptom is a loss of memory (Cummings et al 1996). A consistent neuropathological finding associated with cognitive dysfunction is a cholinergic deficit. This is characterised, in the post-mortem brain, by cholinergic neuronal and transmitter loss reflecting abnormalities in the basal forebrain nuclei projecting into the cortex (Agid et al 1990). The predominant marker of the cholinergic deficit is a significant decrease in the enzyme, choline acetyltransferase, involved in the synthesis of the neurotransmitter acetylcholine (Agid et al 1990; Court & Perry 1991).

The first licensed treatment for Alzheimer's disease, aimed at enhancing cholinergic activity, was based on inhibition of the enzyme acetylcholinesterase which hydrolyses acetylcholine, increasing acetylcholine available for transmission at the cholinergic synapse. Thus, synthetic drugs

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inhibiting acetylcholinesterase, (e.g. tacrine (Cognex) and more recently donepezil (Aricept) and rivastigmine (Excelon)) are used to treat cognitive dysfunction and memory loss associated with Alzheimer's disease. They are the only currently available approved drugs for treatment in the UK although they are limited in use due to their adverse side-effects such as gastrointestinal disturbance (Paton & Branford 1997; Small et al 1997; Melzer 1998).

Several plant species contain alkaloid cholinesterase inhibitors, for example galantamine, originally discovered in the bulbs of snowdrops (*Galanthus nivalis*), is currently in stage III clinical trials for Alzheimer's disease (Fulton & Benfield 1996). New naturally occurring anticholinesterases continue to be identified in a wide variety of plant species (Tang 1994; Park et al 1996).

Plants have been used to treat memory-related disorders for centuries. *Huperzia serata* and *Ginkgo biloba* have been used in Chinese medicine and *S. officinalis* and *S. lavandulaefolia* in Europe (Grieve 1980; Ryman 1991; Tyler 1993). Extracts and isolated constituents have pharmacological actions that may be relevant to the treatment of Alzheimer's disease (Bhattacharya et al 1995; Kanowski et al 1996; Perry et al 1996; Le Bars et al 1997; Schliebs et al 1997). Anticholinesterase activity of *S. lavandulaefolia* and *S. officinalis* oil in human brain tissue (post mortem) has recently been reported (Perry et al 1996) and this could account, at least in part, for its memory-enhancing reputation. The compounds responsible have not been identified, although there are reports that several monoterpenoids occurring in the oils of related plants (e.g. pulegone and menthone in *Mentha* species) inhibit acetylcholinesterase at relatively high concentrations (10^{-3}–10^{-4} M) (Gracza 1985; Grundy & Still 1985b; Ryan & Byrne 1988; Miyazawa et al 1997).

A study of the kinetic properties of the major compounds present in *S. lavandulaefolia* essential oil using human erythrocyte acetylcholinesterase was undertaken to determine which of the known constituents of the essential oil is the most potent in inhibiting acetylcholinesterase and whether inhibition is competitive or non-competitive. Human erythrocyte acetylcholinesterase, instead of human brain cholinesterase, was used in this analysis as it is a pure form of cholinesterase without the potential hazard of brain tissue handling. Erythrocyte acetylcholinesterase is considered to have similar properties to brain acetylcholinesterase and is thus regarded as a suitable source of enzyme for kinetic studies relevant to the CNS (Al-Jafari & Kamal 1996).

Materials and Methods

Materials

Acetylcholinesterase from human erythrocytes, 5, 5-dithio-bis (2 nitrobenzoic acid) (DTNB), acetylthiocholine, (+)-α-pinene, terpineol (α and β), citronellal and γ-terpinene were purchased from Sigma (Poole, Dorset). *R*-(+)-limonene, 1,8-cineole, 1*R*-(+)camphor, linalool, 1*S*-(−)-β-pinene and geraniol were purchased from Aldrich Chemical Co. (Gillingham, Dorset). *S. lavandulaefolia* essential oil (BN 25) was purchased from G. Baldwins & Co. (London). The purity of the commercially-obtained compounds was checked by thin-layer chromatography and found to be >95% (solvent system: silica gel/toluene : ethylacetate (93 : 7) using 0·5% anisaldehyde in 5% sulphuric acid spray reagent) (Stahl 1969). By comparing their TLC and gas chromatography (GC) (Alltech fused silica capillary column Carbowax 20 M; 60–270°C at 2°C min^{-1}+80 min; injection and FID temperature 290°C; injection volume 0·1 μL) characteristics, the commercially-obtained monoterpenoids were confirmed to be the same as those present in the batch of *S. lavandulaefolia* essential oil.

Lipophilicity

TLC using normal, reverse phase and phosphatidylcholine-coated silica plates was carried out on the monoterpenoids to establish a lipophilicity (hydrophobicity) index to determine any relationship between the lipophilicity of the monoterpenoids and their anticholinesterase activity. Five microlitres of each monoterpenoid solution (10% in chloroform) were spotted onto the normal and reverse-phase TLC plates and treated with the solvent systems toluene : ethylacetate (93 : 7) and Na^+/K^+ phosphate buffer (pH 7·4) : acetonitrile (40 : 60), respectively. The hydrophobic character of the reverse-phase plates were made more analogous to physiological conditions by impregnation with the hydrophobic agent phosphatidylcholine as follows. The reverse-phase TLC plates were first scanned on a densitometer (Shimadzu Duel-Wavelength Chromato-Scanner model C5-930) at wavelengths 200–500 nm (phosphatidylcholine absorbs at 230 nm); the plates were coated by dipping into a tray (for 10 s) containing 5 mM phosphatidylcholine in acetone and drying at room temperature. They were rescanned at the same parameters to ensure coating had occurred evenly.

Molecular volume

The molecular volume of the monoterpenoids was calculated using the Hyperchem computer program (Iris Computers, Silicone Graphics) which calculates the volume according to the molecular structure.

Enzyme assay

The kinetic parameters of the acetylcholinesterase were measured over a concentration range of acetylcholine substrate from 0·03 to 0·50 mM and at concentrations of the monoterpenoids ranging from 0·09 to 9·4 mM and essential oil from 0·01 to 0·5 μL mL^{-1} with pre-incubation for 30 min (see below). Concentrations of essential oil and monoterpenoids were obtained by serial dilution with 96% ethanol. A combination of commercially-obtained monoterpenoids was made up to mimic the major constituents of the essential oil (30% camphor, 15% 1,8-cineole, 15% bornyl acetate, 5% α-pinene and 10% β-pinene in 96% ethanol). Inhibition of acetylcholinesterase was assessed by a modified version of the colorimetric method of Ellman et al (1961). Acetylcholinesterase (50 μL of 0·39 U mL^{-1} buffer pH 8; 1 unit hydrolysing 1 μmole acetylcholine min^{-1} at 37°C) and inhibitor solution (20 μL) were added to 2·0 mL phosphate buffer (pH 8) and incubated on ice (4°C) for 30 min. The reaction was started by adding DTNB (10 mM in phosphate buffer pH 7; 20 μL) and acetylcholine (0·03–0·5 mM in water; 20 μL) and the solution was placed in a spectrophotometer at 25°C. The thiocholine formed during hydrolysis of acetylcholine rapidly reacts with DTNB and releases a yellow 5-thio-2-nitrobenzoic acid anion. The production of this coloured anion was recorded for 6 min on a spectrophotometer at 412 nm. A blank, which included reagents with no enzyme, was used to control for non-enzymic hydrolysis of acetylcholine. The rate of acetylcholine hydrolysis was calculated according to the method of Ellman et al (1961). Initial velocities were measured as a function of inhibitor and substrate concentration.

Inhibition kinetics

In many of the reported studies the enzyme is pre-incubated with the inhibitor for 5 min (Miyazawa et al 1988; Al-Jafari 1997), although the analysis by Grundy & Still (1985a) of pulegone-1,2-epoxide indicates that total inhibition occurred after a period of 30 min. Therefore, a standard 30-min preincubation was considered to provide a more accurate impression of the extent of inhibition by the test compounds.

There are a number of different methods used in the literature for analysis of the kinetics of enzyme activity and inhibition (Engel 1981). A common method of transformation, the Lineweaver-Burk plot has been considered to be statistically unsound (Hofstee 1952; Price 1989). Hofstee plots (v vs v/[s]) are considered to be a more accurate method of analysis and were used to determine the K_m and V_{max} values (Hofstee 1952). $K_{i(app)}$ (equilibrium constant for inhibition) was established by plotting $(V_o/V_i)-1$ against inhibitor concentration according to Salvesen & Nagase (1989).

Results

Dose-dependent inhibition

The percentage acetylcholinesterase activity of the commercially available constituents tested and their percentage in the oil are shown in Table 1.

Table 1. Inhibition of human erythrocyte acetylcholinesterase by constituents present in *S. lavandulaefolia* essential oil (n = 3).

Constituent	% in oil[a]	Molecular volume (Å)[b]	IC50	% Inhibition at 4·7 mM	
Camphor (**1**)	27	161	> 10 mM	27[c]	
1,8-Cineole (**2**)	17	167	0·67 mM	100	
Geraniol (**3**)	0·4	177	> 5 mM	10[c]	
(±)-Linalool (**4**)	< 1	161	> 5 mM	12[c]	
α-Pinene (**5**)	5	152	0·63 mM	100	
β-Pinene (**6**)	12	152	NE	68	
Terpineol (**7**)	Trace	172		~ 5 mM	48
γ-Terpinene (**8**)	Trace	157		> 5 mM	25[c]
S.lavandulaefolia oil	–	–	0·03 μL mL^{-1}	–	
Physostigmine	–	–	$4{\cdot}5\times10^{-8}$ M		

[a]Percentage determined from retention times of gas chromatography (mean of 2) of essential oil (batch number 25) and commercial constituents used for the cholinesterase inhibition. [b]Molecular volume determined using Hyperchem, Iris Computers, Silicon Graphics. [c]Relatively weak acetylcholinesterase inhibition. –, Not assessed.

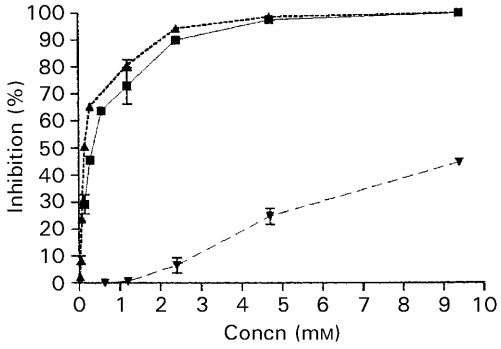

Figure 1. Inhibition of human erythrocyte acetylcholinesterase by commercially obtained constituents (■, cineole; ▲, α-pinene; ▼, camphor) of *S. lavandulaefolia* essential oil (n = 3 or 4 ± s.d.).

Dose-dependent inhibition of acetylcholinesterase was obtained for *S. lavandulaefolia* essential oil, camphor (**1**), 1,8-cineole (**2**) and α-pinene (**3**) (Figure 1). The IC50 values obtained were 0·03 μL mL^{-1}, >10 mM, 0·67 mM and 0·63 mM, respectively. No concentration-dependent inhibition could be obtained with β-pinene, borneol or limonene despite vigorous experimental control and repeated serial dilution of the oil.

Kinetics of inhibition

In the absence of a test substance the mean K_m was 0·17±0·01 mM and the mean V_{max} was 5·77±0·21 μmol 0·02 U^{-1} min^{-1} (n = 15). The kinetic results in the presence of the monoterpenoids and essential oil are shown in Table 2. All monoterpenoids showed uncompetitive, reversible inhibition of erythrocyte acetylcholinesterase (by the intersection on the y-axis of the Hofstee plot). An example of the transformed data to determine K_m and V_{max} values, in the form a Hofstee plot (v as a functions of v/[s]) is shown in Figure 2 and to determine K_i values, $(V_o - V_i) - 1$ against inhibitor concentration, is shown in Figure 3 for a concentration range of camphor.

Structure–activity relationship

No structure–activity relationship was found between monoterpenoid anticholinesterase activity and lipophilicity ($P < 0.05$) (Figure 4) (as measured by TLC R_F values) or molecular volume ($P < 0.05$) (as measured using Hyperchem, Iris Computers, Silicon Graphics) (Table 1).

The inhibitory activity of the major terpene constituents do not readily account for the inhibitory effect of the essential oil. A combination of the major constituents to mimic the whole essential oil gave an IC50 value of 0·3 mg mL^{-1} whereas the IC50 of the whole oil was 0·03 μL mL^{-1}. Thus, if it is assumed that the average molecular weight of the constituent terpenes is around 160, and that their IC50 values are on average 1 mM, then 50% enzyme inhibition would occur as a result of constituent terpenes at approximately 160 mg L^{-1} — approximately 5000 times the concentration of essential oil (0·03 mg L^{-1}) providing 50% inhibition. This suggests either that there is present in the oil an as yet unidentified minor constituent of high potency or that there is a high degree of synergy in the combined action of the terpenes.

Discussion

Of the constituents of *S. lavandulaefolia* tested, four showed weak erythrocyte acetylcholinesterase inhibition compared with the standard anticholinesterases such as physostigmine (IC50: 5×10^{-8} M) and tacrine (IC50: 2×10^{-8} mM) (Perry et al 1997). The most potent monoterpenoid inhibitors tested were α-pinene and 1,8-cineole which both gave total inhibition at 4·7 mM. Since 1,8-cineole and camphor were shown by gas chromatography to be major constituents of the *S. lavandulaefolia* essential oil (Table 1), the anticholinesterase activity of these compounds, and the most potent inhibitor (α-pinene), were examined further (Figure 1).

In the absence of a test substance the mean K_m was consistent with that found in other studies

Table 2. IC50, K_m, V_{max} and $K_{i(app)}$ values[a] for acetylcholinesterase inhibition by 3 monoterpenoids in *S. lavandulaefolia* essential oil.

Inhibitor	K_m (mM)	V_{max} (μmol L^{-1} min^{-1})	K_i at 0·25 mM acetylcholine	IC50
None (n = 4)	0·17	5·77	–	–
Essential oil (0·02 μL mL^{-1})	0·14	2·20	0·02 μg mL^{-1}	0·03 μg mL^{-1}
Camphor (1·2 mM)	0·22	4·14	4·39 mM	4·7 mM
1,8-Cineole (1·2 mM)	0·38	1·34	0·18 mM	0·67 mM
α-Pinene (1·2 mM)	0·21	2·0	0·79 mM	0·63 mM

[a]Calculated from Eadie-Hofstee plot (Hofstee 1952), and Salvesen & Nagase (1989) replot.

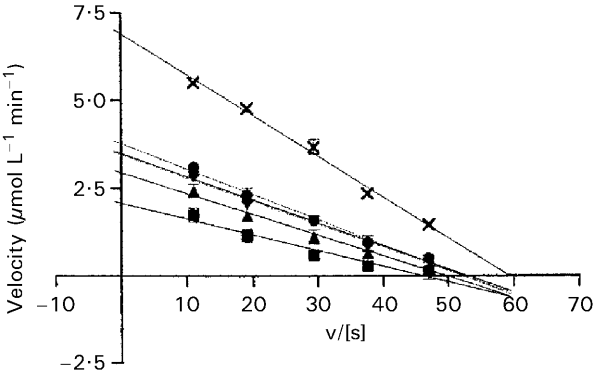

Figure 2. Eadie-Hoftstee plot of acetylcholinesterase velocity vs acetylcholine concentration in the absence and presence of camphor (■, 9·4; ▲, 4·7; ▼, 2·4; ◆, 1·2; ●, 0·59; ×, 0).

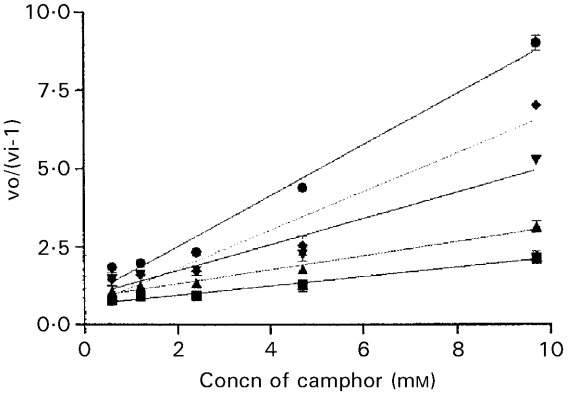

Figure 3. Salvesen & Nagase (1989) replot of acetylcholinesterase velocity in the absence or presence (−1) of camphor to determine the K_i (where slope $= 1/K_i$). Concn of acetylcholine: ■, 0·5; ▲, 0·25; ▼, 0·125; ◆, 0·0625; ●, 0·03125.

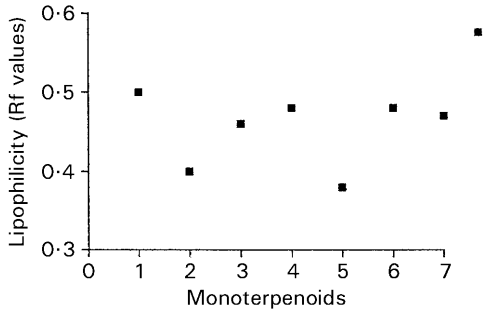

Figure 4. The relationship between the lipophilicity of monoterpenoids (as determined by Rf values on RP-8 TLC coated with phosphatidylcholine) and their strength of acetylcholinesterase inhibition. 1, α-Pinene; 2, 1,8-cineole; 3, β-pinene; 4, (±)-terpineol; 5, (+)-camphor; 6, (±)-linalool; 7, geraniol (1 = strongest, 7 = weakest anticholinesterase).

(Price & Stevens 1989; Chemnitius et al 1996; Al-Jafari 1997). From the IC50 values (Table 2), α-pinene was the most potent inhibitor, followed by cineole and camphor, and camphor is the major constituent (27%) of the oil. The K_i values gave a different order of potency, although camphor was consistently a weaker inhibitor. This incongruity may be expected as the graphical method of analysis for determining K_i values is different to that for determining IC50 values, which are considered statistically more sound. It is worth noting that IC50 and K_i values are not considered indicators of in-vivo action, where pharmacokinetics plays a major role. This is exemplified by a lack of correlation between the in-vitro K_i and in-vivo insecticidal properties of several monoterpenoids, for example pulegone (Grundy & Still 1985).

In comparison with other studies, Gracza (1985) reported an IC50 of 0·2 mM for cineole with electric eel acetylcholinesterase, comparable with the IC50 of 0·67 mM obtained in the present study in human erythrocyte acetylcholinesterase. In contrast Ryan & Byrne (1988) obtained a K_i value of 0·025 mM for cineole (0·025 mM) in electric eel acetylcholinesterase which is 10-fold less than the K_i of 0·18 mM determined here. This apparent variation in inhibition of acetylcholinesterase by compounds tested in separate studies (Table 3) could be due to differing inhibition of isoenzymes, the various assay methods or kinetic analysis. Inter- and intralaboratory variation in the assessment of anticholinesterase compounds (and cholinesterase activity) has been previously reported (Christenson et al 1994).

S. lavandulaefolia essential oil and the 3 monoterpenoids analysed in more detail, 1,8-cineole, camphor and α-pinene, showed graphically (by the intersection on the Hofstee plot) an uncompetitive type of inhibition when analysed by this method (Fersht 1977). A common graphical interpretation assumes that increasing inhibition associated with a decreasing substrate concentration is indicative of a competitive inhibitor (Miyazawa et al 1997). This competitive inhibition profile is also evident in the inhibition by the monoterpenoids analysed in our study (Figures 2 and 3). Other methods of plotting data may give different inhibition profiles. However, Hofstee (1952) is considered statistically sound (Price & Stevens 1989). In previously reported inhibition of acetylcholinesterase (from various sources) by monoterpenoids, analysis has shown competitive reversible action when analysed using different graphical methods (Gracza 1985; Miyazawa et al 1988; Ryan & Byrne 1988). One exception is pulegone-1,2-epoxide which has shown irreversible inhibition of electric eel acetylcholinesterase (Grundy & Still 1985b). The authors suggested the presence of both an epoxy and keto group are required for irreversible inhibition.

A number of studies have proposed structure–activity relationships for monoterpenoids and

Table 3. Variations in reported inhibition from studies (1–5) showing inhibition of acetylcholinesterase by monoterpenoids.

Compound	1 (IC50)	2 (K_i)	3 (K_i)	4 (% at 1·2 mM)	5 (IC50, K_i or % at 4·7 mM)
	Acetylcholinesterase source				
	Electric eel	Electric eel	Electric eel	Bovine erythrocyte	Human erythrocyte
1,8-Cineole	0·2 mM	–	$2{\cdot}5\times10^{-2}$ mM		IC50 0·8 mM K_i 0·18 mM
ρ-Cymene	–	–	–	39·8%	20%
Limonene	–	–	–	0·012 mM (22%)	29%
Linalool	–	–	5·5 mM		12·7%
Pulegone	–	0·39 mM	0·85 mM		50·3%
γ-Terpinene	–	–	–	26·6%	29·6%

(1 = Gracza 1985; 2 = Grundy & Still 1985; 3 = Ryan & Byrne 1988; 4 = Miyazawa et al 1997, 5 = this study).

anticholinesterase activity (Grundy & Still 1985b; Miyazawa et al 1997). Ryan & Byrne (1988) for example suggested, from the position of functional groups, that the interactions of the hydrocarbon skeletal terpenoids is with the acetylcholinesterase hydrophobic active centre. Since the degree of hydrophobicity of the active site(s) on the acetylcholinesterase molecule is reported to be different among the globular forms of acetylcholinesterase, this may further explain the variation in inhibition seen between studies. Compounds can show different types of inhibition (competitive, non-competitive or uncompetitive) not only according to the molecular type of acetylcholinesterase and the different modes and sites for inhibitors to bind to acetylcholinesterase, but also according to the concentration of the substrate (Chemnitius et al 1996; Al-Jafari 1997).

Miyazawa et al (1997) reported competitive inhibition of bovine erythrocyte acetylcholinesterase by 17 monoterpenoids with a *p*-menthane skeleton. The authors suggested that terpene ketones generally showed stronger inhibition than terpene alcohols and hydrocarbons. It was concluded that the presence of an isopropyl group increased the strength of inhibition as compared with isopropenyl groups and that the presence of conjugated double bonds were considered to give more potent inhibition. Further, when comparing enantiomers, (−)-carvone was found to be slightly more potent than (+)-carvone, whereas enantiomers of other terpenes, including limonene, menthol and terpinen-4-ol, showed little difference. The data obtained in the present study are consistent with this finding in that the (−) isomer was more potent than the (+) isomer of pinene. In contrast to the relations between structure and activity proposed by Miyazawa et al (1997), in our study a comparison of the structures (Figure 5) shows that the ketone camphor showed the weakest activity compared with the ether cineole and the alkene hydrocarbons α- and *β*-pinene. However not all these monoterpenoids contain a *p*-menthane skeleton.

In our analysis, possible structure–activity relationships were examined based on lipophilicity and molecular volume. No relationship was found between monoterpenoid anticholinesterase activity and lipophilicity or molecular volume (Table 1). There may however be a correlation between anticholinesterase activity and the presence of a cyclic ring structure. More studies are necessary to establish a structure–activity relationship between monoterpenoids and anticholinesterase activity.

It is assumed that the anticholinesterase activity of the major monoterpenoids constituents (camphor and 1,8-cineole) are responsible for the anticholinesterase activity of the whole essential oil. However, Miyazawa et al (1998) report the anticholinesterase activity of essential oils to be more potent than their major constituents and report that a mixture of the major constituents does not account for the whole activity of the oils. The inhibition of acetylcholinesterase by *S. lavandulaefolia* essential oil may be more potent than that of the primary monoterpenoid constituents (the combination of major constituents gave an IC50 of $0{\cdot}3\ \mathrm{mg\ mL^{-1}}$ whereas the IC50 of the whole oil was $0{\cdot}03\ \mu\mathrm{g\ mL^{-1}}$). Though it can be proposed that the monoterpenoids act synergistically to inhibit acetylcholinesterase, it cannot be excluded that a minor, as yet unidentified constituent of the essential oil is much more potent.

In conclusion the inhibition of acetylcholinesterase by *S. lavandulaefolia* and *S. officinalis* originally reported by Perry et al (1996) is likely to be due to the presence of more than one monoterpenoid present in the essential oil, the chief compounds

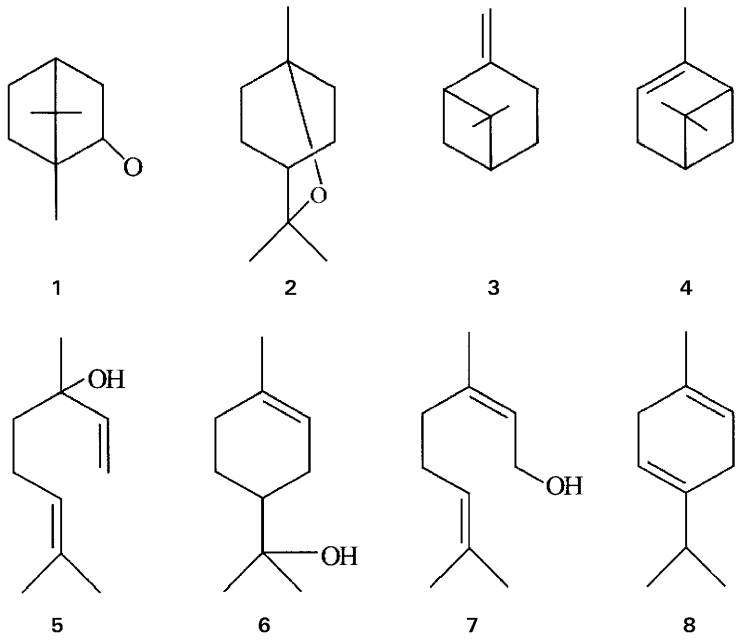

Figure 5. Monoterpenoid components of *S. lavandulaefolia* essential oil. Percentage of oil: **1**, (+)-camphor, 30%; **2**, 1,8-cineole, 15%; **3**, β-pinene, 10%; **4**, α-pinene, 5%; **5**, (+)-linalool, 1–2%; **6**, (±)- terpineol, $< 1\%$; **7**, geraniol, 0·4%; **8**, γ-terpinene, 1–2%.

responsible being pinene, 1,8-cineole and camphor. The probable synergistic action of the constituents of *S. lavandulaefolia* essential oil is currently being investigated.

In the light of these findings, *S. lavandulaefolia* essential oil or a defined combination of its constituents, could be considered for further studies in the symptomatic treatment of Alzheimer's disease, although it remains to be determined whether the essential oil monoterpenoids (or metabolites) inhibit brain acetylcholinesterase in-vivo with relevant potency.

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References

Agid, Y., Graybiel, A., Ruberg, M., Hirsch, E., Brandel, J.-P., Cervera, J. J., Lehericy, S., Malessa, S., Ransmayr, G., Javoy-Agid, F. (1990) Cholinergic systems in Alzheimer's disease, Parkinson's disease and progressive supranuclear palsy. In: Nagatsu, T. (ed.) Basic, Clinical, and Therapeutic Aspects of Alzheimer's and Parkinson's Disease. Volume 1, Plenum Press, New York, pp 427–430

Al-Jafari, A. A. (1997) The inhibitory effect of the neuromuscular blocking agent gallamine triethiodide, on camel retina acetylcholinesterase activity. Toxicol. Lett. 90: 45–51

Al-Jafari, A. A., Kamal, M. A. (1996) Optimization and kinetic studies of human erythrocyte membrane-bound acetylcholinesterase. Biochem. Mol. Biol. Int. 38: 577–586

Bhattacharya, S. K., Kumar, A., Ghosal, S. (1995) Effects of glycowithanolides from *Withania somnifera* on an animal model of Alzheimer's disease and perturbed central cholinergic markers in rats. Phytother. Res. 9: 110–113

Chemnitius, J.-M., Haselmeyer, K.-H., Gonska, B.-D., Kreuzer, H., Zech, R. (1996) Indirect parasympathomimetic activity of metoclopramide: reversible inhibition of cholinesterases from human central nervous system and blood. Pharmacol. Res. 34: 65–72

Christenson, W. R., Van Goethem, D. L., Schroeder, R. S., Wahle, B. S., Dass, P. D., Sangha, G. K., Thyssen, J. H. (1994) Interlaboratory cholinesterase determinations and the effect on the results of statistical evaluation of cholinesterase inhibition. Toxicol. Lett. 71: 139–150

Court, J. A., Perry, E. K. (1991) Dementia: the neurochemical basis of putative transmitter orientated therapy. Pharmacol. Ther. 52: 423–443

Cummings, B. J., Pike, C. J., Shankle, R., Cotman, C. W. (1996) β-Amyloid deposition and other measures of neuropathology predict cognitive status in Alzheimer's disease. Neurobiol. Aging 17: 921–933

Ellman, G. L., Courtney, K. D., Andres, V., Featherstone, R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88–95

Engel, P. C. (1981) Enzyme Kinetics: The Steady-State Approach. Chapman & Hall, London, pp 14–36

Fersht, A. (1977) Enzyme Structure and Mechanism. W. H. Freeman & Co. Ltd, pp 84–94

Fulton, B., Benfield, P. (1996) Galanthamine. Drugs Aging 1: 60–65

Gracza, L. (1985) Molecular and pharmacological investigation of medicinal plant substances II. Inhibition of acetylcholinesterase by monoterpene derivatives *in vitro*. Z. Naturforsch. 40: 151–153

Grieve, M. (1980) A Modern Herbal. Penguin Books, UK, pp 700–705

Grundy, D. L., Still, C. C. (1985a) Inhibition of acetylcholinesterase by pulegone1-,2 epoxide. Pest. Biochem. Phys. 23: 383–388

Grundy, D. L., Still, C. C. (1985b) Isolation and identification of the major insecticidal compound of poleo (*Lippia stoechadifolia*). Pest. Biochem. Physiol. 23: 378–382

Hofstee, B. H. J. (1952) On the evaluation of the constants V_{max} and K_m in enzyme reactions. Sci. 116: 329–331

Kanowski, S., Herrmann, W. M., Stephan, K., Wierich, W., Hörr, R. (1996) Proof of efficacy of the Ginkgo Biloba special extract Egb 761 in outpatients suffering from mild to moderate primary degenerative dementia of the Alzheimer type or multi-infarct dementia. Pharmacopsychiatry 29: 47–56

Le Bars, P. L., Katz, M. M., Berman, N., Itil, T. M., Freedman, A. M., Schatzberg, A. F. (1997) A placebo-controlled, double-blind, randomised trial of an extract of *Ginkgo biloba* for dementia. J. Am. Med. Assoc. 278: 1325–1331

Melzer, D. (1998) New drug treatment for Alzheimer's disease: lessons for healthcare policy. Br. Med. J. 316: 762–764

Miyazawa, M., Watanabe, H., Kameoka, H. (1997) Inhibition of acetylcholinesterase activity by monoterpenoids with a *p*-menthane skeleton. J. Agric. Food. Chem. 45: 677–679

Miyazawa, M., Watanabe, H., Umemoto, K., Kameoka, H. (1998) Inhibition of acetylcholinesterase activity by essential oils of *Mentha* species. J. Agric. Food. Chem. 46: 3431–3434

Park, C. H., Kim, S.-H., Choi, W., Lee, Y.-J., Kim, J. S., Kang, S. S., Suh, Y. H. (1996) Novel anticholinesterase and antiamnesic activities of dehydroevodiamine, a constituent of *Evodia rutaecarpa*. Planta Medica 62: 405–409

Paton, C., Branford, D. B. (1997) Advances in the treatment of Alzheimer's disease. Pharm. J. 259: 693–696

Perry, N., Court, G., Bidet, N., Court, J., Perry, E. K. (1996) Cholinergic activities of European herbs and potential for dementia therapy. J. Ger. Psychiatr. 11: 1063–1069

Perry, N., Houghton, P. J., Jenner, P. (1997) Inhibition of erythrocyte acetylcholinesterase by droplet counter-current chromatography fractions of *Salvia lavandulaefolia* oil. J. Pharm. Pharmacol. 49 (Suppl.4): 34

Price, N. C., Stevens, L. (1989) Fundamentals of Enzymology. Oxford University Press, UK, pp 143–150

Ryan, M. F., Byrne, O. (1988) Plant-insect coevolution and inhibition of acetylcholinesterase. J. Chem. Ecol. 14: 1965–1975

Ryman, D. (1991) Aromatherapy. Judy Piakus Publishers, UK, pp 190–194

Salvesen, G., Nagase, H. (1989) Inhibition of proteolytic enzymes.In: Beynon, R. J., Bond, J. S. (eds) Proteolytic Enzymes: A Practical Approach. IRL Press, Oxford University, UK, pp 83–87

Schliebs, R., Liebmann, A., Bhattacharya, S. K., Kumar, A., Ghosal, S., Bigl, V. (1997) Systemic administration of defined extracts from *Withania somnifera* (Indian Ginseng) and Shilajit differentially affects cholinergic but not glutamatergic and gabaergic markers in rat brain. Neurochem. Int. 30: 181–190

Schneider, L. S. (1996) New therapeutic approaches to Alzheimer's disease. J. Clin. Psychiatry 57 (Suppl. 14): 30–36

Small, G. W., Rabins, R. V., Barry, P. P., Buckholtz, N. S., DeKosky, S. T., Ferris, S. H., Finkel, S. I., Gwyther, L. P., Khachaturian, Z. S., Lebowitz, B. D., McRae, T. D., Morris, J. C., Oakley, F., Schneider, L. S., Streim, J. E., Sunderland, T., Teri, L. A., Tune, L. E. (1997) Diagnosis and treatment of Alzheimer disease and related disorders. J. Am. Med. Assoc. 278: 1363–1371

Stahl, E. (1969) Thin-Layer Chromatography: A Laboratory Handbook. 2nd edn, Springer-Verlag, Berlin

Tang, X. C. (1994) Neuropharmacological effect of active principle isolated from Chinese medicinal herbs. Neuropschychopharmacology 10 (3S/2): 691S

Tyler, V. E. (1993) The Honest Herbal. 3rd edn, Pharmaceutical Products Press, US, pp 271–273