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Pharmacology, Biochemistry and Behavior 75 (2003) 661–668
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Synergistic and antagonistic interactions of anticholinesterase terpenoids in *Salvia lavandulaefolia* essential oil

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Received 11 November 2002; received in revised form 9 April 2003; accepted 11 April 2003

Abstract

In vitro anticholinesterase activities of eight commercially available terpenoid constituents of *Salvia lavandulaefolia* have been investigated. These included 1,8-cineole, camphor, α-pinene, β-pinene, borneol, caryophyllene oxide, linalool and bornyl acetate. Dose-dependent inhibition of acetylcholinesterase (AChE) by these chemical constituents was determined using the method of Ellman [Biochem. Pharmacol. 7 (1961) 88]. The IC50 value of 1,8-cineole was 0.06 ± 0.01 mg/ml similar to that of the essential oil (0.05 ± 0.01 mg/ml). Analyses of the expected inhibitions based on the prediction of a zero interactive response of a combination at its naturally occurring ratios were carried out in comparison with observed inhibition. Minor synergy was apparent in 1,8-cineole/α-pinene and 1,8-cineole/caryophyllene oxide combinations, with interaction indexes not exceeding 0.5. In contrast, a combination of camphor and 1,8-cineole was antagonistic with an interaction index of 2. A combination of all eight compounds was zero interactive. A combination of six constituents, excluding 1,8-cineole and camphor, was used to compare the method of expected response of a combination with a method of summation. These findings reveal that the inhibitory activity of the oil results from a complex interaction between its constituents, which produce both synergistic and antagonistic responses between the component terpenes. Understanding such interactions is important in comparing species on the basis of chemical composition.

Keywords: Salvia; Synergism; Antagonism; Acetylcholinesterase; Terpenes; IC50 inhibition

1. Introduction

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 (AChE) (Levy et al., 1999; Jann, 2000; Coyle and Kershaw, 2001; Gruntzendler and Morris, 2001).

Perry et al. (2000a) suggested that Spanish sage (*Salvia lavandulaefolia*, Vahl) may be relevant in the treatment of dementia of the Alzheimer's type and also reported (Perry et al., 2000b) in vitro inhibition of 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. Other authors (Klohs et al., 1959; Miyazawa et al., 1998, 2001) have also presumed that the therapeutic effects of whole plant extracts may be superior to individual compounds from the plant.

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).

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doi:10.1016/S0091-3057(03)00125-4

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 AChE 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; Davis 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.

2. Materials and methods

2.1. Chemicals

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, (1*R*)-(+) camphor, (±)-linalool, (−)-borneol, (-)-bornyl acetate, caryophyllene oxide were purchased from Sigma, Fancy Road, Poole, Dorset, UK.

S. lavandulaefolia (fresh leaves) steam distilled oil was purchased from Baldwins, London.

2.2. AChE activity

Assessment of AChE inhibition was carried out using a method of Ellman et al. (1961) as modified by Nostrandt et al. (1993). A typical run consisted of 5 μl of the enzyme suspension at a final concentration of 0.008 U/ml; 200 μl of 0.1 M phosphate buffer pH 8.0; 5 μl of DTNB at a final concentration of 0.3 mM prepared in 0.1 M phosphate buffer pH 7.0 with 0.12 M of sodium bicarbonate; and 5 μl of the test solution in 86% ethanol. The reactants were mixed in a 96 well U-bottom polystyrene microtiter plate. The mixture was incubated for 30 min at 30 °C. The reaction was initiated by adding 5 μl of ATChI to give a final concentration of 0.5 mM. Each sample was assayed in triplicate and it also included a control in which 86% ethanol replaced the test inhibitor solution. Eighty-six percent EtOH was used in preparation of inhibitor stock solutions. Its final concentration of 2% did not inhibit the enzyme. A blank for each run consisted of 205 μl buffer (pH 8.0), 5 μl substrate, 5 μl DTNB and 5 μl ethanol but no enzyme. The blank was also in triplicate. The maximum concentrations of the inhibitors were limited by their solubility in the aqueous system (approximately 2% ethanol) used. Absorbance at 412 nm was measured on Titetric Multiskan Mcc/340 for a period of 6 min at 30 °C with Genesis-Lite Windows microplate software (Labsystem International). The computer software programme provided an automatic deduction of nonenzymatic hydrolysis of the substrate.

2.3. Dose–response curves and equations

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.

A concentration of an inhibitor corresponding to 50% inhibition of AChE (IC50 value) was calculated using the dose–response curve equations.

2.4. 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 $i=1,2,3,\ldots,n$; 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.

For a combination of the two agents, Eq. (1) can be written as:

$$\mathrm{da}/\mathrm{Da} + \mathrm{db}/\mathrm{Db} = 1 \qquad (2)$$

Where da and db are known and represent values of final concentrations of two agents in a combination. On a graph

Table 1
Anticholinesterase activity of essential oil and its constituents

Inhibitor	(i) IC50 (mg/ml)[a] $\bar{X}\pm$S.D. ($n=4$)	(ii) Activity[b] (%) $\bar{X}\pm$S.D. ($n=4$)
Spanish sage oil	0.05 ± 0.01	
1,8-Cineole	0.06 ± 0.01	
α-Pinene	0.09 ± 0.005	
β-Pinene	0.2 ± 0.004	
Camphor		39 ± 4.0 (0.5 mg/ml)
Linalool		18 ± 2.3 (0.5 mg/ml)
Bornyl acetate		23 ± 4.0 (0.25 mg/ml)
Caryophyllene oxide		35 ± 4.7 (0.25 mg/ml)
Borneol		19 ± 2.6 (0.25 mg/ml)

[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.

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 coordinates). Da, Db and the combination (da,db) are isoeffective (Eq. (2)). Therefore, a solution is to find the horizontal isoeffective straight line, which would determine doses of A (Da) and B (Db) to satisfy Eq. (2). 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 that 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 Eq. (2). 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 noninteractive 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 (Eq. (1)).

2.5. 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 Eq. (2). If the equation for zero interaction response resulted in a value significantly less than one, synergism was inferred; if more, then antagonism.

2.6. Statistics

The data were presented as mean ± S.D. of the mean. Group comparisons were analysed using a two-sample Student's t test with a probability value of $<.05$ as the level of statistical significance.

3. Results

3.1. IC50 values

Spanish sage oil and component terpenoids were tested for their anticholinesterase activity within their solubility limits. The compounds were divided into (i) those that reached 50% inhibition of AChE, and (ii) those that were less potent (Table 1).

The IC50 value of the oil did not significantly differ from that of 1,8-cineole and was marginally less than the IC50 value of α-pinene. Other constituents showed significantly less anticholinesterase activity.

3.2. Interactions

The terpenoids were assessed in combinations for their AChE inhibitory activity on a basis of their naturally occurring concentrations in the oil (Table 2). The anticholinesterase contribution of 1,8-cineole accounted for half the activity of the natural plant extract (Table 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., 2000b), and for this reason the combinations of the terpenoids, investigated in the present

Table 2
Inhibition of AChE by the essential oil and its constituents at concentrations based on ratios occurring in the oil

Compound	Percentage in oil[a]	Concentration (mg/ml)[b]	Percent Inhibition[c] $\bar{X}\pm$S.D. ($n=4$)
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 IC50 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.

Table 3
Inhibition of AChE by combinations of terpenoids

Combination	Concentration (mg/ml)[a]	Total (mg/ml)[b]	Inhibition (%) $\bar{X}\pm$S.D. ($n=4$)		Interaction index of combination[f] $\bar{X}\pm$S.D. ($n=4$)
			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	0.25	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	$0.009/8\times10^{-4}$	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	2.4±0.21 antagonism
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	0.007/0.01				
α-Pinene/β-pinene	$6\times10^{-4}/5\times10^{-4}$	0.02	19±2.8	18±2.3	0.96±0.7 zero interaction
Caryophyllene oxide	5.0×10^{-4}				
Borneol	0.0013				
Bornyl acetate	2.5×10^{-4}				
Linalool	4.0×10^{-4}				

[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 ($P<.05$) than expected, with the interaction index less than 1, synergy is the result. If the observed inhibition is significantly less ($P<.05$) than expected, with the interaction index more than 1, antagonism is inferred. If not significant ($P>.05$), with the interaction index of 1, zero interaction.

study, reflected the average naturally occurring composition in the plant extract.

In all combinations, it was considered that the expected inhibition would be within the response scale of individual constituents so that the isoeffective 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 1) and this allowed five concentrations of this combination based on the typical ratio to be tested (Table 3) (the number of concentrations of a combination

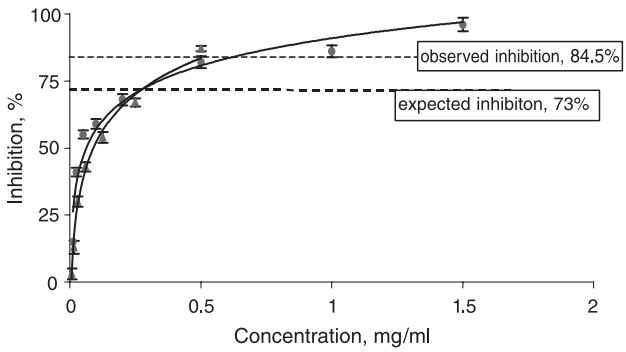

Fig. 1. Calculation of expected response of 1,8-cineole (da; 0.225 mg/ml) and α-pinene (db; 0.02 mg/ml) combination. (●) Dose–response curve of 1,8-cineole; (▲) dose–response curve for α-pinene. A horizontal line of the expected inhibition of 73±2.1% intersects the two dose–response curves at points where concentrations of 1,8-cineole (Da) and α-pinene (Db) on the *x*-axis are isoeffective with the combination (da;db), and therefore satisfies Eq. (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.

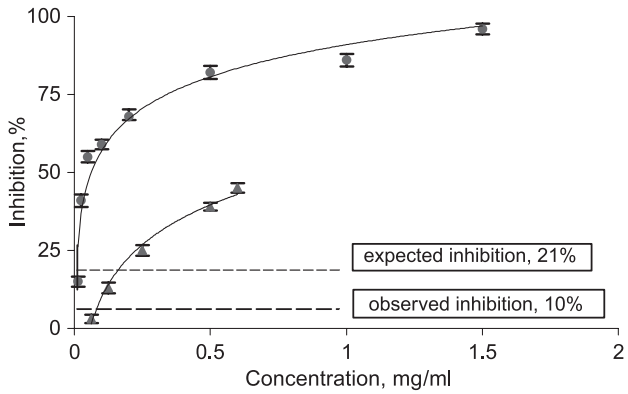

Fig. 2. Calculation of the expected response of 1,8-cineole (da; 0.009 mg/ml) and camphor (db; 0.013 mg/ml) combination. (●) Dose–response curve of 1,8-cineole; (▲) dose–response curve of camphor. A horizontal line of the expected inhibition of 21±2.0% intersects the two dose–response curves at points where concentrations of 1,8-cineole (Da) and camphor (Db) on the *x*-axis are isoeffective with the combination (da;db), and therefore satisfies Eq. (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.

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 3). In a combination of eight terpenes (Table 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 3) was calculated to estimate the significance of these chemical interactions.

3.3. 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.

Table 4
Assessment of inhibition of AChE by combinations of six terpenoids on the basis of two methods

Combination	Concentration[a] (mg/ml)	Inhibition (%)[c] $\bar{X}\pm$S.D. ($n=4$)	Inhibition (%) $\bar{X}\pm$S.D. ($n=4$)		
			Observed[d]	Expected[e]	Additive[f]
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 ($P>.05$) than expected, with the interaction index of 1 indicating the zero interactive response.
[e] Calculated zero interactive response of the combination.
[f] Consists of a sum of the expected inhibitions of individual compounds. The additive inhibition was significantly less ($P<.05$) than observed indicating synergism.

Table 5
Assessment of inhibition of AChE by combinations of terpenoids on a basis of IC50 values

Combination	Mass ratio	IC50 (mg/ml)[a] $\bar{X}\pm$S.D. ($n=4$)
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.

Antagonism was found in 1,8-cineole/camphor combinations with the interaction index of 2.

Fig. 1 illustrates synergism in the combination of 1,8-cineole and α-pinene, whereas Fig. 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.

3.4. Combinations of more than two compounds

The eight compound mixture was zero interactive (Table 3) and its inhibitory activity did not exceed that of the oil (Table 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 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 (1985).

The inhibitory activity of the tested combinations of terpenes was summarised on a basis of IC50 values (Table 5). The presence of camphor significantly decreased the inhibitory activity of 1,8-cineole raising the IC50 value of 0.06±0.01 (Table 1) to 0.18±0.2 mg/ml. 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 1).

4. Discussion

4.1. Inhibition of AChE

The anticholinesterase activity of 1,8-cineole was almost the same as the natural plant oil (Table 1). A similar

result was reported by Miyazawa et al. (1998) who found that an IC50 value of 0.026 mg/ml of *Mentha aquatica* (water mint) oil, for inhibition of bovine AChE at a final concentration of 0.0065 U/ml, was equivalent to that of 0.025 mg/ml of viridiflorol, a major terpene constituent of the oil. Their IC50 value of 1,8-cineole of 0.04 mg/ml was close to that obtained in the present study namely 0.06 ± 0.01 mg/ml.

It was also reported (Perry et al., 2002) that the 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 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, was similar to the one obtained in vitro with human AChE, i.e., 0.03 μl/ml (Perry et al., 2000b). The oil and its constituents showed uncompetitive type of inhibition.

This was more potent than IC50 values of 0.09 mg/ml for α-pinene, 0.1 mg/ml for 1,8-cineole and 0.72 mg/ml for camphor and also for a combination of major constituents as a "mimic oil" of 0.3 mg/ml. 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.

4.2. 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 IC50 value, was not as great as the whole oil (Table 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 (Fig. 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 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

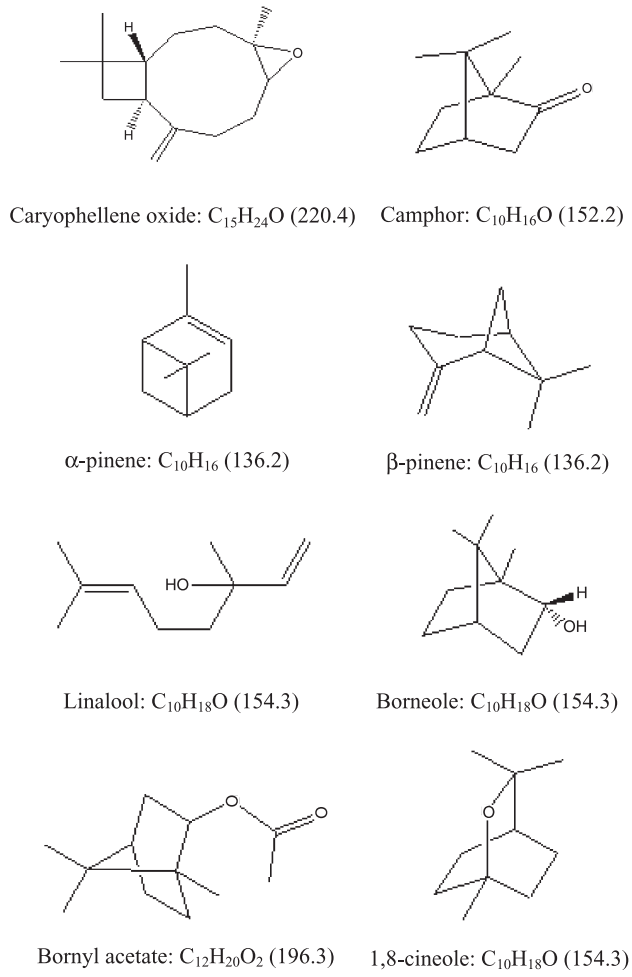

Fig. 3. Terpenoid components of *S. lavandulaefolia* essential oil. Molecular weights are shown in the brackets.

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 isoforms on the enzyme were not assessed in this study.

Results obtained more recently (unpublished data) 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 min at 30 °C with the enzyme final concentration of 0.008 U/ml. 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; Berenbaum, 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% from 15% of its normal values at advanced stages of the disease (Perry et al., 1978). 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 3). This suggests that the "mimic oil" composed by Perry et al. (2000b), as described above, may had antagonistic properties because of the presence of camphor that would influence the degree of proposed synergy in *S. lavandulaefolia* oil.

4.3. 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 4).

The method of summation was included as an example of how an inappropriately selected approach can lead to the misleading interpretation. The terpenes used in this study had nonlinear dose–response curves, and therefore a sum of their inhibitory activity would not reflect a response of a zero interactive combination (Berenbaum, 1989). Figs. 1 and 2 illustrate examples of such nonlinearity 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 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 IC50 values (Table 5) cannot reveal a true interaction within the combinations. For example, in the combination of 1,8-cineole and camphor, there are two roles that could express either synergy or antagonism. It could be said, comparing Tables 1 and 5, that camphor is the antagonist to 1,8-cineole because it increased the IC50 value of the latter from 0.06 ± 0.01 (Table 1) to 0.18 ± 0.02 mg/ml (Table 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 (Table 1) to the IC50 value of 0.18 ± 0.02 mg/ml (Table 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 IC50 value of the whole oil (Table 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 5). The IC50 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 was carried out at the low concentration of the enzyme compared to a typical of 0.08 U/ml (Ellman et al., 1961).Therefore, minor synergy and antagonism in the natural plant extract may only occur under these particular experimental conditions.

4.4. Therapeutic value of essential oil

Chemical compositions of *S. lavandulaefolia* vary (Perry et al., 1999; Giannouli and Kintzios, 2000; Karousou et al., 2000), and as the 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. *Salvia 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, 2000; Länger et al., 1996). Other properties of sage species, such as anxiolytic, antioxidant, oestrogenic, antidepressive and antiinflammatory (Perry et al., 2000a) could equally be monitored for optimum in a chemical composition of oils by analysing the activity of chemical components and interactions between them.

Acknowledgements

Funding was provided by Advanced Phytonics, and the School of Biology of the University of Newcastle for studentship (S. Savelev) is gratefully appreciated.

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