An extract of Salvia (sage) with anticholinesterase properties improves memory and attention in healthy older volunteers

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Monoterpenoid extract of sage (*Salvia lavandulaefolia*) with cholinesterase inhibiting properties improves cognitive performance and mood in healthy adults

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Abstract

Extracts of sage (*Salvia officinalis/lavandulaefolia*) with terpenoid constituents have previously been shown to inhibit cholinesterase and improve cognitive function. The current study combined an *in vitro* investigation of the cholinesterase inhibitory properties and phytochemical constituents of a *S. lavandulaefolia* essential oil, with a double-blind, placebo-controlled, balanced crossover study assessing the effects of a single dose on cognitive performance and mood. In this latter investigation 36 healthy participants received capsules containing either 50 μL of the essential oil or placebo on separate occasions, 7 days apart. Cognitive function was assessed using a selection of computerized memory and attention tasks and the Cognitive Demand Battery before the treatment and 1-h and 4-h post-dose. The essential oil was a potent inhibitor of human acetylcholinesterase (AChE) and consisted almost exclusively of monoterpenoids. Oral consumption lead to improved performance of secondary memory and attention tasks, most notably at the 1-h post-dose testing session, and reduced mental fatigue and increased alertness which were more pronounced 4-h post-dose. These results extend previous observations of improved cognitive performance and mood following AChE inhibitory sage extracts and suggest that the ability of well-tolerated terpenoid-containing extracts to beneficially modulate cholinergic function and cognitive performance deserves further attention.

Keywords

Acetylcholinesterase, attention, memory, monoterpenoid, mood, sage, salvia, *Salvia lavandulaefolia*

Introduction

The ability of any plant extract to modulate central nervous system (CNS) function in humans is almost exclusively due to the presence of 'secondary metabolites': constitutive or induced compounds that are not involved in the immediate physiological survival of the plant, but which fill ecological roles that increase the general 'survivability' of the plant over the longer term (Harborne, 1993; Macias et al., 2007). For instance, one of the largest structural groups of phytochemicals, the alkaloids, predominantly function as feeding and allelopathic deterrents, and their biotic interactions include modulating neurotransmitter systems within their intended target phytophages (Harborne, 1993; Wink, 2003; Wink et al., 1998). These effects include interactions with the cholinergic system, and either directly or indirectly with the dopaminergic system, in herbivores (Hagen et al., 2009). Whilst alkaloids are always toxic, to mammals, when used at lower doses their neuro-toxic properties have the unintended attribute of making them useful as psychotropic agents in humans. Plant alkaloids, and derivates thereof, constitute the active components in many of our social drugs (Hagen et al., 2009) and have provided a multitude of medicinal compounds with CNS activity (Samuelsson, 2004). These include a number of potentially toxic cholinesterase inhibitors initially derived from alkaloid phytochemicals and used to treat dementia, including galantamine, huperzine, physostigmine and rivastigmine (Mukherjee et al., 2007).

In contrast to the alkaloids, the terpenoid family of phytochemicals has a spectrum of toxicity extending from highly toxic to benign. In line with this, they exert a more complex pattern of ecological effects for the synthesizing plant.

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These include toxic deterrence, but also attraction via taste, scent and visual cues, and direct modulation of animals' CNS function. As an example, terpenoids can play a direct defensive role as constitutive toxic deterrents (Wittstock and Gershenzon, 2002). However, they also function as attractants for insects, both for pollination (Raguso et al., 2006), and also as indirect defence agents. In this role they are synthesized and released by the plant in response to the presence of a herbivore in order to attract its natural predators (Maffei et al., 2007). Terpenoids are amongst the putative active components of many herbal extracts with low levels of side effects that have been shown to affect CNS function, including *Valeriana officinalis* (Valerian), *Bacopa monniera*, *Panax ginseng* and *Ginkgo biloba* (Kennedy, 2009).

Extracts or leaves of several edible members of the Salvia (sage) genus, including *Salvia officinalis* (SO) and *Salvia lavandulaefolia* (SL) have been used by humans for more than two millennia to improve cognitive function and attenuate cognitive decline (Kennedy and Scholey, 2006). Their putative active components encompass a broad range of terpenoids, including a-pinene, b-pinene, 1,8-cineole, thujone (SO only), camphor and geraniol (Perry et al., 2001). The secondary metabolite environmental roles of these phytochemicals may well underlie demonstrations of both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition seen *in vitro* in human brain homogenates and erythrocytes (Kennedy et al., 2006; Perry et al., 1996, 2000, 2001; Savelev et al., 2003, 2004; Scholey et al., 2008; Tildesley et al., 2003, 2005) and AChE inhibition seen *in vivo* in the rodent brain (Perry et al., 2002).

These cholinergic effects are matched by findings of improved memory performance in rats (Eidi et al., 2006) and demonstrations of improved cognitive function in humans following administration of single doses of SO and SL with cholinesterase-inhibiting properties. In the first of a series of double-blind, placebo-controlled, balanced cross-over studies, Tildesley et al. (2003) demonstrated improvements to immediate and delayed recall tasks in healthy young humans at 1-h and 2.5-h following a single dose of 50 µL SL essential oil and at 2.5-h following 100 µL. Similar mnemonic effects were subsequently confirmed following 25 µL and 50 µL of the same essential oil, along with improved serial subtraction task performance and improved levels of subjective alertness, calmness and contentment (Tildesley et al., 2005). A similar pattern of mood effects was seen in comparison with placebo following 300 mg and 600 mg of encapsulated dried SO leaf, with reduced anxiety also evident following the lower dose, with these mood effects abolished by completion of a laboratory psychological stressor (Kennedy et al., 2006). Recently, Scholey et al. (2008) also confirmed the memory-improving effects of the two lowest of four single doses (167, 333, 666, 1333 mg) of an ethanolic extract of SO administered to healthy elderly volunteers (>65 years). The 333 mg dose also improved attention task performance across all but one (2.5-h) of the four post-dose assessments (1-, 2.5-, 4- and 6-h).

Whilst pro-cholinergic effects may well underlie the improved mood and cognition-enhancing effects evident in the above studies, it is notable that secondary metabolites from sage extracts also exhibit anti-oxidant, anti-inflammatory and oestrogenic properties (Perry et al., 2003), all of which may be relevant to brain function both in healthy populations and in sufferers of age-associated cognitive decline and dementia (Kennedy and Scholey, 2006). Unlike alkaloid phytochemicals, the terpenoid constituents of sage extracts are also well tolerated and exhibit few unwanted side effects at the levels likely to be consumed by humans.

The current, exploratory study assessed both the phytochemical components and behavioural effects of an SL essential oil that has been found in a pilot investigation to have particularly potent AChE inhibitory properties (IC_{50} at one-tenth the concentration of any previous extract), with a view to informing the choice of treatment for a future chronic dosage, randomized controlled trial. The study therefore confirmed the AChE inhibitory properties of the extract and sought to address several further issues: the characteristics of the extract in terms of components that might contribute to its potent cholinesterase inhibiting properties; the extent to which any cognition and mood effects of the extract are comparable with those seen previously with other, less potent sage extracts; and whether further cognitive/mood effects could be demonstrated during extended completion of mentally fatiguing, cognitively demanding 'executive' tasks that should be sensitive to cholinergic modulation.

The study therefore comprised an initial *in vitro* investigation. This was followed by a double-blind, placebo-controlled, balanced cross-over study in healthy young adults that assessed the cognitive/mood effects of a single dose of the extract, in a quantity of essential oil previously found to be optimal for improving memory performance (50 µL; Tildesley et al., 2003, 2005). In order to provide some comparison with previous studies investigating extracts with considerably lower AChE inhibitory properties, the assessment included a number of memory/attention tasks previously shown to be sensitive to cholinesterase-inhibiting extracts, and these were augmented by an assessment of subjective mood and cognition during 60 min of performing executive function/working memory and attention tasks.

Methods and materials

In vitro properties of the essential oil

Cholinesterase inhibition. The method employed was based on that developed by Savelev et al. (2004). The ability of the SL essential oil to inhibit human AChE and BuChE was assessed *in vitro* using the colorimetric method of Ellman (1961), as described previously (Okello et al., 2008). The essential oil was diluted in 53% ethanol prior to assay. Serial dilutions were prepared to give final assay concentrations of 0.1–0.001 mg/mL. A typical run consisted of 5 µL of AChE or BuChE at final assay concentrations of 0.03 U/mL; 220 µL of 0.1 M phosphate buffer pH 8; 5 µL of DTNB (prepared in 0.1 M phosphate buffer pH 7 with 0.12 M sodium bicarbonate) at a final assay concentration of 0.3 mM; and 5 µL of test solution. The reactants were mixed in 96-well plates, covered with the transparent plate sealers to prevent loss of the volatile oil. The reactants were pre-incubated at 30°C for 30 min. The reaction was initiated by adding 5 µL of substrate (1 mM ATChI or 5 mM BTChI final

assay concentrations). Change in absorbance kinetic mode was measured at 405 nm at 30°C for 6 min (with shaking every 20 s) using a Thermo Labsystems Multiskan Ascent™ plate reader with Ascent™ software. There were two controls to monitor for non-enzymatic hydrolysis in the reaction mixture; control 1 consisted of wells with no substrate (replace substrate with buffer) and control 2 consisted of wells with no essential oil (replace oil with 53% ethanol). Assays were performed in triplicate, repeated ×2.

Composition. Gas chromatography/Mass spectrometry (GC/MS) analysis of the essential oil was performed on a Hewlett-Packard 6890 GC with a split/splitless injector (280°C) linked to a Hewlett-Packard 5973 mass selective detector (electron voltage 70 eV, filament current 220 μA, source temperature 230°C, quad temperature 150°C, multiplier voltage 2400 V, interface temperature 300°C). Data acquisition was controlled by a HP Kayak XA PC chemstation computer in full scan mode (35–550 amu/s). A sample (1 μL) in dichloromethane was injected by an HP6890 auto sampler and the split opened after 1 min. Separation was performed on CB8 (50 m, 0.25 mm ID, 0.25 (mdf)) capillary column. The GC was temperature programmed from 40–300°C at 4°C/min and held at a final temperature for 20 min with helium as the carrier gas at a flow rate of 1 mL/min, initial pressure 11.5 psi, split 60 mL/min.

Design

The study employed a randomized, double-blind, placebo-controlled, balanced cross-over design.

Participants

In total, 36 young adult participants (male 10/female 26, mean age 23.8 years, SD 4.38) took part in the study. Participants self-reported that they were in good health and that they were taking no medication (other than the contraceptive pill), social drugs, or food supplements. Habitual smokers were excluded from the study, as was anyone who was pregnant (or seeking to become so). Prior to study days all participants abstained from caffeine and alcohol for a minimum of 12-h prior to the first testing session of the morning and throughout the testing session. Participants were also asked to have the same light breakfast (e.g. toast/cereal) and lunch (e.g. sandwich) at the same time on each study day, and to consume nothing else, with the exception of water. Participants' food intake on each study day was recorded on a diary card.

The study received ethical approval from the Northumbria University School of Psychology and Sport Sciences Ethics Committee and was conducted according to the Declaration of Helsinki (1964). All participants gave their informed consent prior to their inclusion in the study.

Treatments

The treatments were supplied as identical capsules by Pharmaton SA (Lugano) and were only identified by a code when delivered to the site. A disinterested third party arranged the allocation of treatments as per the counterbalancing schedule.

Depending on the condition to which they were allocated on each day, the participants received a single soft gel capsule containing either 50 μL of SL essential oil plus olive oil, or a placebo capsule containing olive oil.

Cognitive and mood measures

All tasks were delivered within the Computerized Mental Performance Assessment System (COMPASS), a purpose-designed software application for the flexible delivery of randomly generated parallel versions of standard and novel cognitive assessment tasks. With the exception of the paper and pencil tasks (word recall), all responses were made using the computer keyboard and mouse. In this case the assessment comprised a selection of standard psychometric tasks with stimuli chosen to possess good face validity in an 'everyday' context. The elements of the cognitive assessment are described below, and presented in Figure 1 in the order in which they took place.

Memory and attention tasks

Word list presentation. Participants were presented with lists of 15 nouns describing groceries and household items. Words were presented for 1 s with a 1-s inter-stimulus duration.

Picture presentation. Participants were presented with 15 pictures depicting man-made objects, buildings and scenes with stimulus duration of 2 s and a 1-s inter-stimulus duration.

Names–face presentation. Twelve faces were presented with a first name and surname underneath for 2 s with an inter-stimulus interval of 1 s.

Immediate word recall. Participants were given 60 s to write down as many of the previously presented words as possible. The outcome was the number of items correctly recalled.

Immediate name–face recognition. Participants were re-presented with the faces that were shown earlier, along with four possible first names and four possible surnames, including the correct first name and surname that was originally associated with the face. The task was repeated at the end of the test session. The outcomes were the number of items correct (first name and surname) and speed of response (ms).

Simple reaction time. The participant was instructed to press a response button as quickly as possible every time a single stimulus (upwards pointing arrow) was presented on

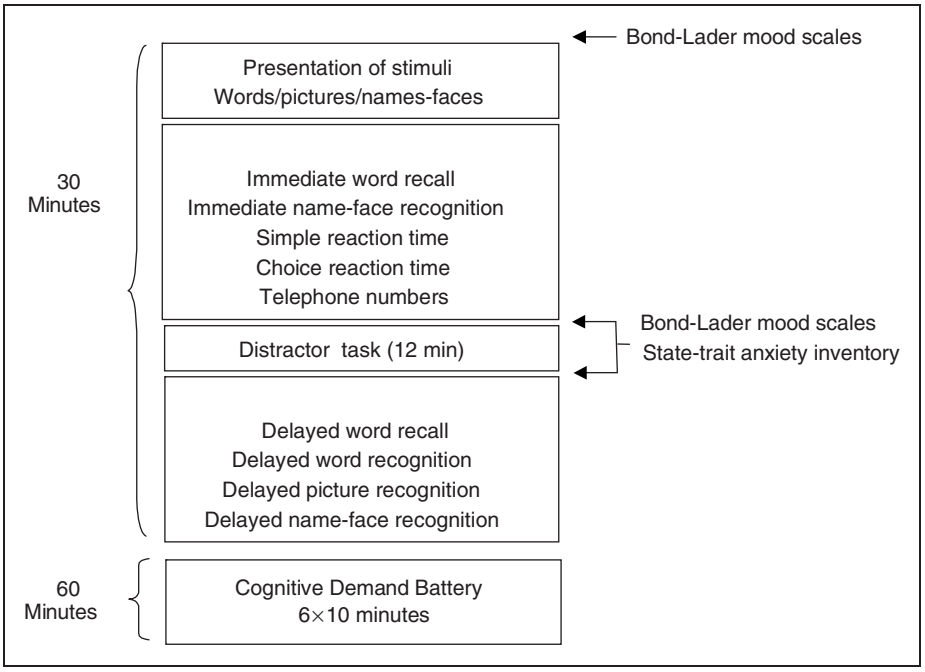


Figure 1. Timelines of each cognitive/mood assessment. The pre-dose baseline assessment differed from the post-dose assessments (represented here) in that it only included a single repetition of the tasks from the Cognitive Demand Battery.

the monitor. In total, 50 stimuli were presented with an inter-stimulus interval that varied randomly between 1 and 3.5 s. The outcome was the average reaction time (ms) to the stimuli.

Choice reaction time. The participant was instructed to press either the left or right arrow button as quickly as possible in response to stimuli that comprised an arrow pointing left or right. In total, 50 stimuli were presented with an inter-stimulus interval that varied randomly between 1 and 3.5 s. The outcomes were the number correct and average reaction time (ms) to the stimuli.

Telephone numbers working memory task. A nine-digit 'telephone number' was presented on screen for 5 s. Participants were instructed to remember the phone number for 10 s, after which they could input the number via the linear number keys on the keyboard. A total of eight 'telephone numbers' were presented, with the task outcome derived from a score for each number incorporating correct digits and correct order.

Distractor task – multi-tasking framework (Wetherell and Sidgreaves, 2005). A 12-min distractor task was interposed between the immediate and delayed elements of the memory and attention assessment. Previous research has shown that performance of the multi-tasking framework (MTF) engenders increases in self-ratings of negative mood, anxiety and stress-related physiological responses (Kennedy and Scholey, 2004; Kennedy et al., 2006). Mood was therefore assessed with Bond–Lader mood scales and the State Trait Anxiety Inventory (STAI) state subscale (see 'mood scales' below) immediately before and after the 12-min distractor task. A score representing the change in mood during the MTF was then calculated by subtracting the pre-task from the post-task mood scores.

Delayed word recall. Participants were given 60 s to write down as many of the previously presented words as possible. The outcome was the number of items correctly recalled.

Delayed word recognition. Participants were presented with the 15 original words from the list, plus 15 novel distractor words. They responded yes/no as to whether each word was in the original word list. The outcomes were number of words correct and average speed of response (ms).

Delayed picture recognition task. Participants were presented with the 15 original pictures, plus 15 distractors. They responded yes/no as to whether each picture was one of those presented earlier. The task was scored for number correct and average speed of response (ms).

Delayed names-face association task. Participants were re-presented with the faces that were shown earlier, along with four possible first names and four possible surnames, including the correct first name and surname that was originally associated with the face. The outcomes were the number of items correct (first name and surname) and average speed of response (ms).

Cognitive Demand Battery. The objective of the Cognitive Demand Battery (CDB) is to assess the impact of treatment on speed/accuracy and mental fatigue during continuous performance of cognitively demanding tasks. The working hypothesis underlying this approach is that any psychoactive properties of a test substance are liable to be more readily apparent during a period of intense cognitive demand and the 'mental fatigue' state elicited by this prolonged task performance.

Participants complete the 10-min battery of tasks six times in immediate succession (i.e. for a continuous period of 60 min). Application of this battery has been shown to reliably increase self-ratings of 'mental fatigue' and to be sensitive to a number of herbal and natural interventions (Kennedy and Scholey, 2004; Kennedy et al., 2008; Reay et al., 2005, 2006). The 10-min battery comprises the following tasks.

Serial threes subtraction task (2 min). Computerized versions of the serial subtraction tasks were implemented using tests of 2-min duration. Participants were required to count backwards in threes from a given number as quickly and as accurately as possible using the number keys to enter each response. A random starting number between 800 and 999 was presented on the computer screen, which was cleared by the entry of the first response. The task was scored for number of correct responses and number of errors. In the case of incorrect responses, subsequent responses were scored as positive if they were scored as correct in relation to the new number.

Serial sevens subtraction task (2 min). This was identical to the serial threes task with the exception that it involved serial subtraction of sevens.

Rapid Visual Information Processing task (RVIP – 5 min). The participant was required to monitor a continuous series of digits for targets of three consecutive odd or three consecutive even digits. The digits were presented at the rate of 100/min, and the participant responded to the detection of a target string by pressing the 'space bar' as quickly as possible. The task was continuous and lasted for 5 min, with eight correct target strings being presented in each minute. The task was scored for percentage of target strings correctly detected, average reaction time for correct detections, and number of false alarms.

'Mental fatigue' visual analogue scale. Participants rated their current subjective 'mental fatigue' state by making a mark on a 100 mm line with the end points labelled 'not at all' (left-hand end) and 'very much so' (right-hand end).

Mood/well-being measures. Mood was assessed with Bond–Lader mood scales (Bond and Lader, 1974) immediately prior to the cognitive assessment, and with Bond–Lader mood scales and the STAI state subscale before and after the MTF distractor task.

Bond–Lader mood scales. These scales have been utilized in numerous pharmacological, psychopharmacological and medical trials. These scales comprise a total of 16 100-mm lines anchored at either end by antonyms (e.g. 'alert–drowsy', 'calm–excited'). Participants indicate their current subjective position between the antonyms on the line. Outcomes comprise three factor analysis derived scores: 'Alertness', 'Calmness' and 'Contentment'.

STAI – 'state' subscale. The STAI state subscale (Speilberger et al., 1969) was administered as a paper and pencil questionnaire. The scale contains 20 items assessing the presence (e.g. 'I am tense') and absence (e.g. 'I feel at ease') of symptoms of anxiety at the present moment. Each item is scored from 1–4 with a total scale score between 20 and 80. A lower score represents lower anxiety.

Procedure

Participants attended the Brain, Performance and Nutrition Research Centre on three separate occasions at 8.30–9.00 am. Testing took place in a suite of testing facilities with participants visually isolated from each other.

The first, introductory visit comprised obtaining of informed consent; training on the cognitive and mood measures; health screening; collection of demographic data, and random allocation to treatment counterbalancing order.

Following the introductory visit participants attended the laboratory on two further occasions 1 week apart. Participants attended having consumed no caffeine or alcohol for at least 12-h. On arrival on both occasions, participants undertook an initial cognitive/mood assessment comprising completion of the Bond–Lader mood scales, followed by the immediate elements of the memory and attention assessment. The distractor task (MTF) was then completed, with Bond–Lader mood scales and STAI completed before and after. This was followed by the delayed elements of the memory and attention assessment. Participants then made a single completion of the 10-min CDB tasks (see Figure 1). Once this baseline assessment was completed participants consumed their treatment for that day. At 1-h and 4-h after consuming their treatment, participants repeated the cognitive and mood assessment. These assessments were identical to the pre-dose assessment, with the exception that the full 60 min (i.e. six completions of the 10 minutes of tasks) of the CDB were undertaken. A standard light lunch was consumed immediately following the 1-h post-dose assessment. The task running order and timing for each of the post-dose assessments are shown in Figure 1.

Statistics

Data were baseline adjusted (calculated against pre-dose) prior to analysis.

The distribution of data for each measure was checked for symmetry and equivalence of variance (standard deviations) between conditions, with an Anderson–Darling test of normality carried out for those measures that departed from acceptable criteria.

Statistical analysis for the memory/attention and mood measures was by repeated measures analysis of variance (ANOVA) (condition × treatment order × post-dose assessment (1-h, 4-h)), with repetition included as a further factor for data from the three mood scale assessments. Planned comparisons were made between data from each treatment during each of the post-dose assessments (1-h, 4-h) utilizing Bonferonni corrected *t*-tests calculated using the Mean Squares Error from the ANOVA (Keppel, 1991). Planned comparisons are only reported for measures that showed a significant treatment effect or treatment × assessment effect on the ANOVA.

Analysis of the CDB data was by an initial repeated measures omnibus ANOVA (condition × treatment order × post-dose assessment (1-h, 4-h) × repetition (1–6)). Planned comparisons were then carried out between treatments for each repetition of the battery during each assessment as described above, with a Bonferonni correction applied for multiplicity during each assessment. To further protect against Type I errors only the planned comparisons for those measures that showed a significant treatment-related effect on a reduced model ANOVA (reported in results) are presented.

Power. Previous studies of sage extracts have demonstrated effect sizes ranging between Cohen's (Cohen, 1992) definition of medium to, in some cases, large effect sizes (e.g. Tildesley et al., 2003). Prior to this study the necessary sample size was therefore calculated assuming a medium effect size of $f = 0.25$ using G*Power (Erdfelder et al., 1996). This calculation suggested that for the proposed within-subjects design, with two post-dose repetitions of the tasks, 90% power at $\alpha = 0.05$ would be achieved with a sample size of 36.

Results

In vitro assays

Cholinesterase inhibition and essential oil components. The SL essential oil was a highly potent selective inhibitor of AChE, with an IC_{50} value of 0.003 mg/mL (3 µg/mL). The essential oil was therefore 10 times more active than the most potent SO/SL extract assessed previously, which had a maximum IC_{50} concentration of 0.03 mg/mL (Savelev et al., 2004). BuChE was weakly inhibited in this case, only achieving 22% inhibition at the highest concentration utilized (0.5 mg/mL). The inhibition curve for AChE, GC/MS chromatogram of the essential oil and the constituents of the essential oil are presented in Figure 2.

Behavioural assessment

Memory and attention. A number of memory and attention measures evinced significant differences between the treatment groups.

Simple reaction time. There was a main effect of treatment on the Simple Reaction Time task ($F(1,34) = 5.15$, $p < 0.05$) with a trend towards an interaction between treatment and session ($F(1,34) = 3.91$, $p < 0.1$). Planned comparisons of data from the individual assessments revealed that participants performed significantly faster in the essential oil condition during the 1-h post-dose assessment ($t(34) = 3.99$, $p < 0.01$).

Delayed word recall. Delayed word recall evinced a main effect of treatment ($F(1,34) = 5.49$, $p < 0.05$) with a strong trend towards an interaction between treatment and session ($F(1,34) = 3.87$, $p < 0.1$). The planned comparisons showed that following essential oil, participants outperformed placebo during both the 1-h ($t(34) = 5.3$, $p < 0.01$) and 4-h ($t(34) = 2.5$, $p < 0.05$) assessments.

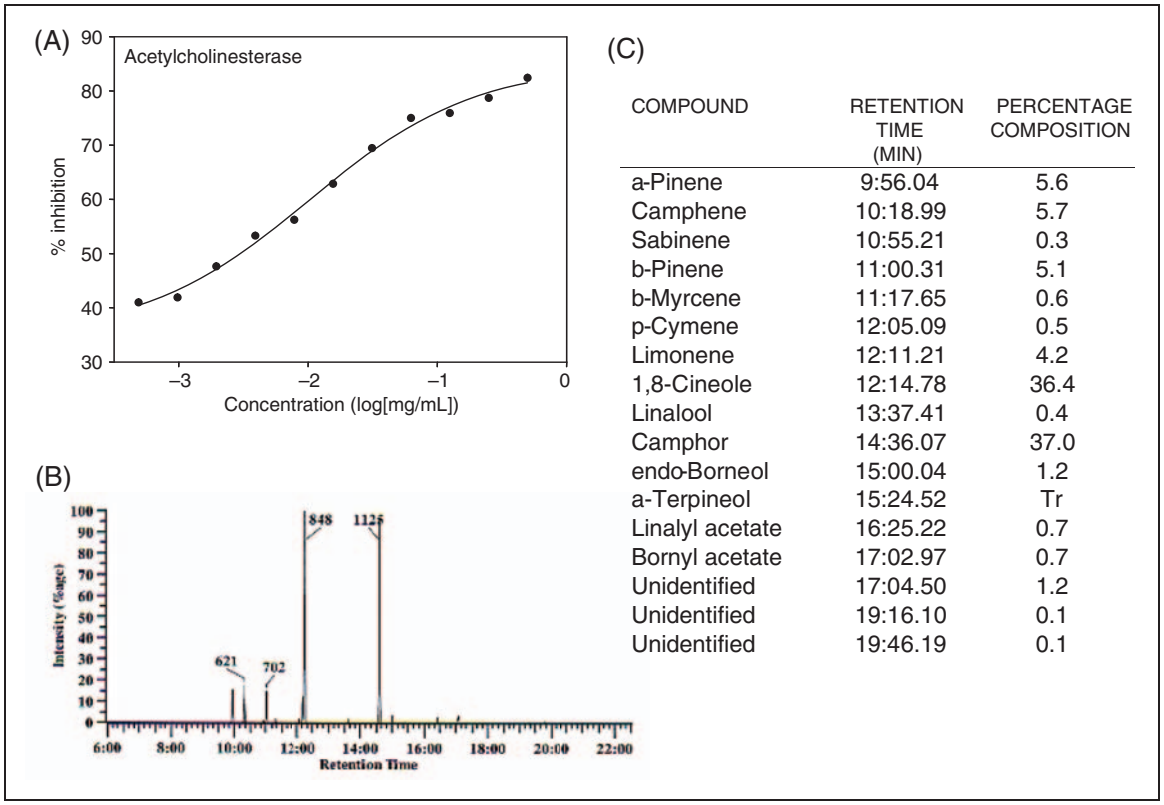
Word recognition task. There was also a significant main effect on the accuracy of performing (number correct) the Word Recognition task ($F(1,34) = 8.7$, $p < 0.01$), with a trend towards an interaction between treatment and assessment ($F(1,34) = 2.88$, $p < 0.1$). Planned comparisons showed that the essential oil outperformed placebo during the 1-h assessment ($t\ (34) = 3.28$, $p < 0.01$), with a trend towards the same effect at 4-h post-dose ($t\ (34) = 1.83$, $p < 0.1$).

Picture recognition task. Similarly, following essential oil participants performed better than following placebo on the accuracy (number correct) of the Picture Recognition Task ($F\ (1,34) = 5.65$, $p < 0.05$). The planned comparisons revealed that this effect was apparent at both 1-h ($t\ (34) = 5.38$, $p < 0.01$) and 4-h ($t\ (34) = 3.3$, $p < 0.01$).

Mood measures. Data from the three Bond–Lader mood assessments (completed at the start of memory/attention assessment and before and after the MTF) showed that there was a significant interaction between treatment and assessment on the 'alert' factor ($F(1,34 = 10.16$, $p < 0.01$). The planned comparisons showed that whereas the treatment groups did not differ at the 1-h post-dose assessment, they rated themselves as significantly more 'alert' following essential oil during the 4-h post-dose assessment ($t\ (34) = 2.85$, $p < 0.01$). Following an Anderson–Darling test of normality, the data for this measure were normalized with a square root transformation, and the resulting ANOVA showed the same treatment × assessment interaction as had been evident when analysing the raw data ($F(1,34) = 5.71$, $p < 0.05$).

There was no significant effect on the STAI state subscale. There was no treatment-related modulation of the stress response during the MTF in terms of changes in the scores on the Bond–Lader mood scales and STAI (data not shown).

Measures from the memory and attention tasks and mood measures that evinced significant results are presented in Figure 3. Mean (plus SEM) baseline data and 'change from baseline' data from the post-dose assessments for the memory, attention and mood measures are presented in Table 1.



(C)

COMPOUND	RETENTION TIME (MIN)	PERCENTAGE COMPOSITION
a-Pinene	9:56.04	5.6
Camphene	10:18.99	5.7
Sabinene	10:55.21	0.3
b-Pinene	11:00.31	5.1
b-Myrcene	11:17.65	0.6
p-Cymene	12:05.09	0.5
Limonene	12:11.21	4.2
1,8-Cineole	12:14.78	36.4
Linalool	13:37.41	0.4
Camphor	14:36.07	37.0
endo-Borneol	15:00.04	1.2
a-Terpineol	15:24.52	Tr
Linalyl acetate	16:25.22	0.7
Bornyl acetate	17:02.97	0.7
Unidentified	17:04.50	1.2
Unidentified	19:16.10	0.1
Unidentified	19:46.19	0.1

Figure 2. (A) Inhibition of human erythrocyte acetylcholinesterase. (B) GC/MS total ion chromatogram of essential oil, with chromatography performed on a 50 m × 0.25 mm i.d. × 0.25 μm CB8–MS column. (C) Compounds in the *Salvia lavandulaefolia* essential oil (expressed as percentage composition calculated from areas of detected peaks).

Cognitive demand battery

Serial 3s. The only significant differences in performance of the tasks within the CDB were seen on the total number of Serial 3s subtractions performed. The ANOVA evinced a significant interaction between treatment and assessment ($F(1,748) = 6.13$, $p < 0.05$). Reference to the planned comparisons showed that this was manifested as significantly improved performance in the essential oil condition during the third ($t(748) = 2.58$, $p = 0.01$) and sixth ($t(748) = 2.76$, $p < 0.01$) repetitions of the battery during the 4-h post-dose assessment, with a strong trend towards the same effect during the second repetition ($t(748) = 1.93$, $p < 0.1$). There was no modulation during the earlier (1-h post-dose) assessment, and no significant differences in the number of errors committed.

Mental fatigue. There was also a trend towards a main effect of treatment ($F(1,748) = 3.67$, $p < 0.1$) and a significant interaction between treatment and assessment ($F(1, 748) = 5.9$, $p < 0.05$) on subjective ratings of mental fatigue during the 60 min of task performance. Planned comparisons of data from both assessments showed that during the 1-h post-dose assessment participants rated themselves as less fatigued following the fourth ($t(748) = 2.77$, $p < 0.05$), fifth ($t(748) = 2.86$, $p < 0.05$) and sixth ($t(748) = 3.01$, $p < 0.05$) repetitions of the battery following the essential oil. Similarly, at the 4-h post-dose assessment participants rated themselves as less fatigued following the second ($t(748) = 2.73$, $p < 0.05$), third ($t(748) = 4.79$, $p < 0.01$), fourth ($t(748) = 4.33$, $p < 0.01$), fifth ($t(748) = 4.28$, $p < 0.01$) and sixth ($t(748) = 4.56$, $p < 0.01$) repetitions of the battery while in the essential oil condition.

Mean (plus SEM) change from baseline data from the Serial 3s task (total subtractions) and mental fatigue ratings during each assessment are shown in Figure 4.

Discussion

The SL essential oil under investigation was largely composed of monoterpenoids and exhibited considerably greater inhibition of human erythrocyte AChE than the most potent SO/SL extract previously investigated (Savelev et al., 2004). In terms of behaviour, administration of a single dose of 50 μL of essential oil resulted in improved cognitive performance. This was seen in an attenuation of the decreases seen in attention and memory task performance, most notably during the 1-h post-dose assessment, with this effect diminishing during the 4-h post-dose assessment. Performance of the CDB, in terms of the Serial 3s subtraction task, was also improved but only at the later (4-h) assessment. This pattern was also seen in the attenuation of mental fatigue during task performance, which was more pronounced during the 4-h post-dose

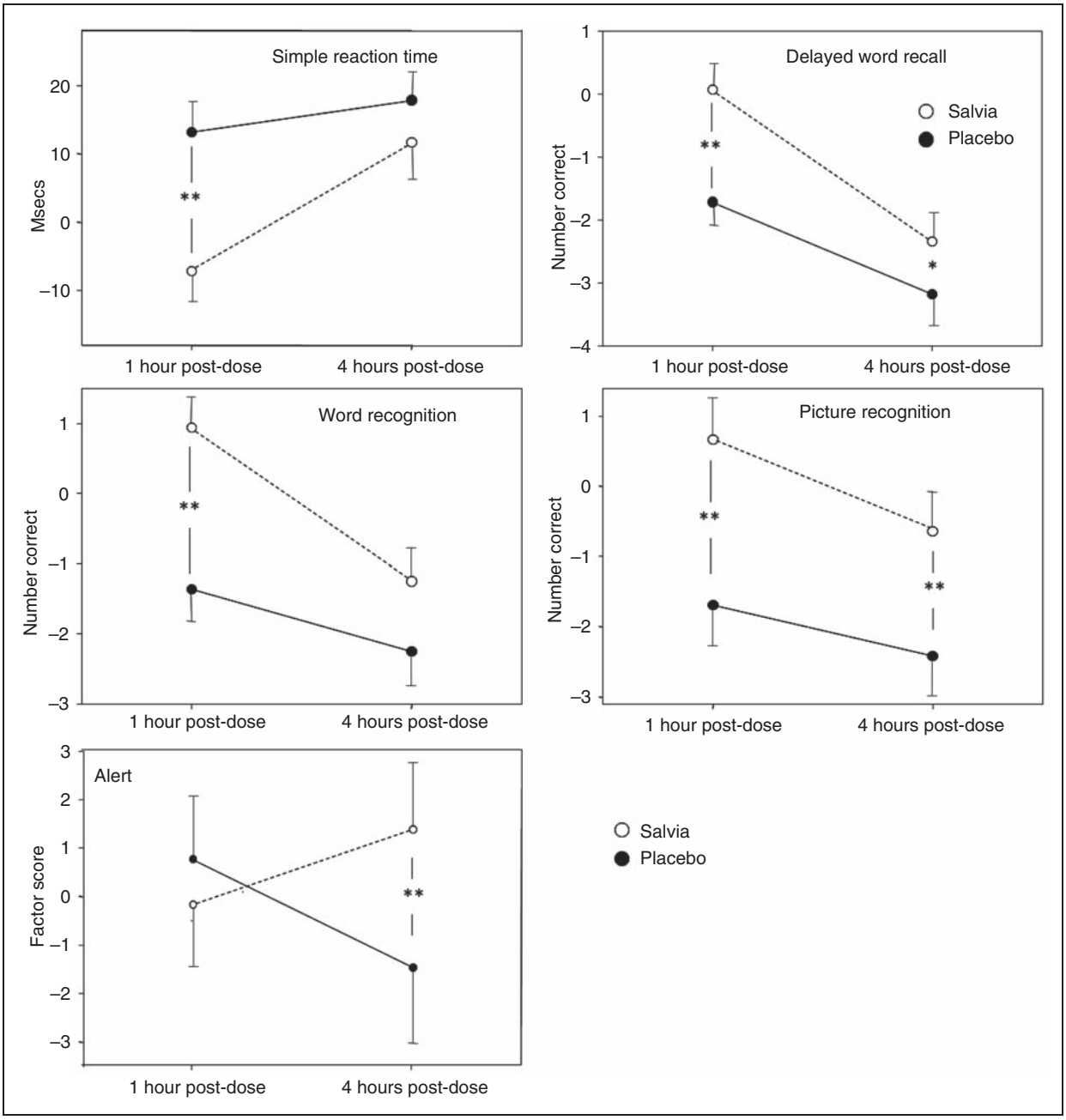


Figure 3. Mean (plus SEM) change from baseline data for the 1-h and 4-h post-treatment assessments on the memory, attention and mood measures that showed significant effects on the ANOVA. (*$p < 0.05$, **$p < 0.01$ from the planned comparisons, made using *t*-tests with a Bonferroni correction).

assessment, and subjective ratings of alertness, which were only significantly increased during this later assessment.

The improvements seen in cognitive performance replicate the improved secondary memory task performance seen previously in healthy younger and older adults (Scholey et al., 2008; Tildesley et al., 2003, 2005), and the improved attention task performance seen in an elderly cohort (Scholey et al., 2008) following single doses of sage extracts with less potent cholinesterase inhibiting properties. Increased 'alertness' has also been demonstrated previously (Kennedy et al., 2006; Tildesley et al., 2005). The effects of sage extracts on extended performance of mentally demanding tasks, and the resultant increase in mental fatigue, has not previously been investigated.

Naturally, these results might have been as a consequence of a statistical anomaly. In any experiment it is necessary to strike a balance between the possibility of 'false positive' Type I errors and the obscuring of genuine findings by being overly conservative and thereby increasing the likelihood of Type II errors (Keppel, 1991). Given that this was an exploratory study, we chose to control for the number of comparisons per task, but not the error rate across the entire experiment.

Table 1. Mean (plus SEM, in italics) baseline scores and change from baseline scores from the 1-h and 4-h assessments for the memory, attention and mood measures. Data for the mood measures are averages across the three repetitions carried out within each assessment. Data shown in bold are the measures that showed significant modulation

				Post-dose change from baseline			
Task	Treatment	Pre-dose Baseline		1-h		4-h	
Telephone number (number)	Salvia	3.19	*0.31*	0.00	*0.36*	−0.25	*0.37*
	Placebo	3.19	*0.39*	0.19	*0.36*	0.36	*0.28*
Simple RT (ms)	Salvia	288.47	*5.05*	**−7.18**	*4.43*	**11.70**	*5.49*
	Placebo	281.29	*4.63*	**13.19**	*4.40*	**17.83**	*4.10*
Choice RT (% accuracy)	Salvia	96.28	*0.56*	−1.00	*0.51*	−2.17	*0.64*
	Placebo	95.28	*0.63*	0.67	*0.52*	−0.89	*0.61*
Choice RT (ms)	Salvia	405.82	*9.44*	−4.75	*5.87*	−1.03	*5.67*
	Placebo	401.07	*7.16*	−0.56	*3.58*	5.26	*6.23*
Imm name/face (number correct)	Salvia	6.00	*0.41*	−0.56	*0.42*	−0.39	*0.40*
	Placebo	5.44	*0.41*	−0.06	*0.45*	−0.36	*0.33*
Del name/face (number correct)	Salvia	5.72	*0.39*	−0.47	*0.36*	−0.89	*0.41*
	Placebo	5.25	*0.52*	−0.06	*0.47*	−0.56	*0.37*
Immediate word Recall (number)	Salvia	8.22	*0.37*	0.15	*0.37*	−0.94	*0.41*
	Placebo	8.38	*0.36*	−0.39	*0.33*	−0.78	*0.39*
Delayed word Recall (number)	Salvia	7.75	*0.37*	**0.08**	*0.42*	**−2.33**	*0.46*
	Placebo	7.83	*0.38*	**−1.71**	*0.36*	**−3.18**	*0.49*
Word Recognition (number correct)	Salvia	25.83	*0.48*	**0.94**	*0.44*	**−1.25**	*0.47*
	Placebo	26.78	*0.42*	**−1.36**	*0.46*	**−2.25**	*0.49*
Word Recognition RT (ms)	Salvia	1226.11	*44.18*	−23.17	*36.42*	−26.25	*31.43*
	Placebo	1202.94	*40.17*	−7.14	*37.41*	−36.08	*34.37*
Picture Recognition (number correct)	Salvia	23.64	*0.57*	**0.67**	*0.60*	**−0.64**	*0.57*
	Placebo	24.31	*0.57*	**−1.69**	*0.57*	**−2.42**	*0.56*
Picture Recognition RT (ms)	Salvia	1386.06	*53.18*	−45.00	*35.47*	−58.28	*33.50*
	Placebo	1341.06	*40.82*	−44.47	*30.58*	−27.47	*32.20*
Alert[1] (factor score)	Salvia	54.68	*2.36*	**−0.17**	*1.27*	**1.39**	*1.37*
	Placebo	57.17	*1.91*	**0.76**	*1.31*	**−1.47**	*1.55*
Content[1] (factor score)	Salvia	61.80	*2.11*	0.13	*0.92*	0.63	*0.81*
	Placebo	63.90	*1.75*	−0.68	*1.00*	−0.86	*1.04*
Calm[1] (factor score)	Salvia	58.62	*2.06*	0.54	*1.15*	−2.51	*1.58*
	Placebo	59.68	*1.93*	−3.31	*1.20*	−3.68	*1.97*

[1]Data averaged across the three repetitions per assessment.

This raises the possibility of treatment effects arising by chance. However, of the 25 mood and cognitive performance measures reported here we found improvements on seven measures (1.25 Type I errors would be predicted by chance at $\alpha = 0.05$). With regards to these effects they were all in a positive direction, and the findings of improved memory, attention and alertness replicate the findings in previous similar studies (Kennedy et al., 2006; Scholey et al., 2008; Tildesley et al., 2003, 2005). It therefore seems likely that the results here represent genuine treatment-related benefits.

The essential oil here was shown to be a considerably more potent AChE inhibitor than previous extracts, with an IC_{50} which represented a concentration a tenth of that of the most potent extract investigated previously. Increased arousal/alertness, and improved performance on attention, memory and 'executive' tasks can all be accommodated within a simple 'cholinergic' explanation of the results seen here (Sarter et al., 2003). However, the observation that beneficial effects decrease with increased doses of essential oil (Tildesley et al., 2003), and similar behavioural improvement from extracts with varying anticholinesterase properties across studies would tend to suggest that cholinesterase inhibition only has to exceed a certain threshold to modulate brain function (Kennedy et al., 2006; Scholey et al., 2008; Tildesley et al., 2003, 2005).

In terms of the essential oil itself, the constituents were almost exclusively monoterpenoids, including camphor (37%), 1,8-cineole (36.4%), camphene, α-pinene, β-pinene, limonene and endo-borneol. While high levels of camphor have been identified across SL essential oils (Langa et al., 2009) the levels of 1,8-cineole were higher than those reported previously (Langa et al., 2009; Perry et al., 2003). Research suggests that 1,8-cineole is the most potent AChE inhibitor among the individual constituent chemicals of SL, and acts in this respect both synergistically with other constituents and antagonistically with camphor (Perry et al., 2003; Savelev et al., 2003, 2004). It may well be the case that the unusually high ratio of 1,8-cineole in comparison with other monoterpenes, and camphor in particular, underlies the increased AChE inhibition seen here.

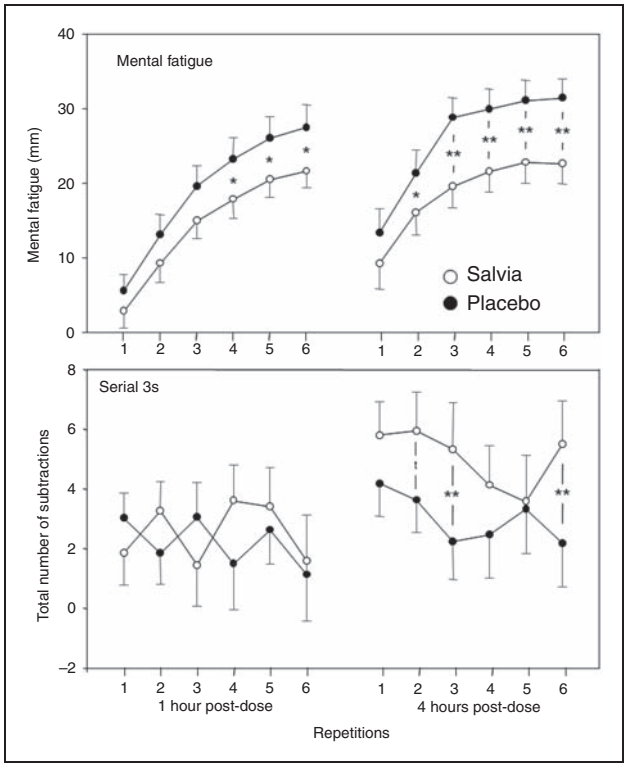


Figure 4. Mean (plus SEM) change from baseline data during each repetition at the 1-h and 4-h post-treatment assessments on the measures from the Cognitive Demand Battery that reached significance on the ANOVA (assessment × treatment in both cases). (t, $p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$ from the planned comparisons, made using t-tests with a Bonferroni correction).

Given the potentially pivotal role played here by 1,8-cineole, its secondary metabolite roles as an individual monoterpenoid phytochemical are of some interest. Evidence suggests that one role that 1,8-cineole plays is as a feeding deterrent which is expressed within the plant as a consequence of damage by the herbivore (Vuorinen et al., 2004). Similar synergies as those seen with regards to AChE inhibition exist in terms of these feeding deterrence properties, with 1,8-cineole being the most potent constituent of essential oils in terms of deterrence, but with the full monoterpenoid extract exhibiting properties in excess of the constituent chemicals (González-Coloma et al., 2006). 1,8-cineole also exhibits direct toxicity in larval and adult beetles (Kordali et al., 2007; Stamopoulos et al., 2007), but not as a consequence of AChE inhibition (Picollo et al., 2008). In general, monoterpenoid combinations that include 1,8-cineole have been shown to be toxic to some taxa, for instance ectoparasitic mites and house flies (Damiani et al., 2009; Palacios et al., 2009) but harmless to others, for instance bees (Damiani et al., 2009). It may be relevant that 1,8-cineole is also a fragrant attractant for insect pollination (Raguso et al., 2006) and may well function as one constituent of the bouquet of volatile components released by a plant during herbivorous attack in order to attract the natural insect predators of the attacking herbivores (Rohloff and Bones, 2005). These non-lethal and benign roles of monoterpenoids in plant–insect interactions are particularly pertinent here, as acetylcholine plays a major role in insect behaviour and memory. Both nicotinic and muscarinic receptors are expressed within insect nervous systems, and cholinergic agonists and antagonists have been shown to up-regulate and down-regulate behavioural parameters, respectively (Dacher and Gauthier, 2008; Ismail et al., 2008). While insects deliberately attracted to a plant for pollination or to function as agents of indirect defence are unlikely to directly consume plant tissue, and therefore may avoid elements of toxicity, emission of a volatile that had severe negative effects on the cholinergic systems of symbiotes would be disadvantageous. It therefore seems possible that the interactions of monoterpenoids, including 1,8-cineole, with the cholinergic nervous system of symbiotic insects must be at least neutral in nature and may be potentially beneficial to the insect. While the possibility has yet to be explored, it may well be that AChE inhibition by secondary metabolites, other than toxic alkaloids, can be advantageous in behavioural terms to insects that have a symbiotic relationship with the emitting plant. Similar cholinergic effects may then extend to other consuming animals, including humans, due to the similarities in CNS neurotransmitter systems and functions between species.

Of course, it is also possible that an unmeasured, non-cholinergic parameter is responsible for the behavioural effects evinced here. The results showed a biphasic pattern, with the simple memory and attention tasks being preferentially improved at the earlier (1-h) assessment, and the more difficult serial subtraction task, mental fatigue and alertness being preferentially improved at the later (4-h) assessment. This pattern could be predicated on an interaction between the treatment and fatigue/alertness, with participants being generally less fatigued and more alert, and therefore less susceptible to improvement in both of these parameters (and therefore difficult tasks), during the first assessment. The pattern could also be as a consequence of the differing absorption profiles of the constituent monoterpenoids. For instance, whilst there are few data on the absorption of complex mixtures of monoterpenoids, the single constituent 1,8-cineole has been shown to peak in the plasma of possums at approximately 60 min post-dose (McLean et al., 2008), whereas limonene peaks in human plasma at 2.5-h post-dose (Wang et al., 2007). The effects here may therefore be underpinned by more than one mechanism of action with differing time profiles of activity.

In the current study we assessed cholinesterase inhibition by the extract for several specific reasons: previous evidence shows that sage extracts generally possess this property; pro-cholinergic treatments are currently prescribed for several dementias; and this property is potentially relevant to cognitive function, and declining cognitive function as a consequence of both age and dementia. However, we have not explored other potential mechanisms that might be equally relevant. The possibility of these extracts possessing further, complementary, properties requires further attention.

Plant-derived alkaloids, by function and chemical nature, are toxic to mammals. However, taken in appropriate doses this broad class of phytochemicals has provided a rich source

of drug discovery for medicines, and constitute the majority of our social drugs (Samuelsson, 2004). Of particular relevance, a number of current treatments for Alzheimer's disease are derived from alkaloid secondary metabolites that perform the function for the plant of interfering with the cholinergic system of herbivores. These include galantamine, huperzine, physostigmine and rivastigmine (Mukherjee et al., 2007). In the UK the National Institute for Clinical Excellence (NICE) recently recommended against the existing, prescribed cholinesterase inhibiting treatments, except for patients suffering moderate levels of dementia, on the basis of modest efficacy, high levels of side effects and high cost (NICE, 2009). Terpenoids, on the other hand, perform a much wider range of functions for the plant, which can include the symbiotic attraction of animals, all of which have cholinergic components to their CNS. Therefore they exhibit a broad spectrum of toxicity for mammals, from highly toxic to being entirely edible. In this respect, sage extracts have a history spanning several millennia as medicinal treatments for cognitive deterioration and are associated with very few side effects (Kennedy and Scholey, 2006), as are a number of other putatively psychoactive herbal extracts with terpenoid active constituents, including *Ginkgo biolba*, *Valeriana officinalis* and *Panax ginseng* (Kennedy, 2009). Unfortunately, little methodologically adequate research has been directed towards these more complex plant extracts, and the question of efficacy in terms of brain function is still an open question. This comparative lack of research interest is partly due to current gaps in our understanding of the relative contributions of the complex constituents of plant extracts to any efficacy, and an inability to adequately standardize the levels of the appropriate phytochemicals in any final product. The results seen here, both in terms of potent *in vitro* AChE inhibition and enhancement of mood and cognitive performance, certainly argue against the suggestion that the structural diversity and relatively weak anticholinesterase activity of terpenoids render them unlikely to constitute treatments for cognitive disorders (Houghton and Seth, 2003). However, they do suggest that more research should be directed towards understanding the relative contributions and synergies within complex chemical cocktails in order to move towards extracts with more potent medicinal properties. Certainly the overall and comparative levels of active phytochemicals in plant material depend on a wide range of parameters that are capable of manipulation, including climate, soil composition, light levels and time and season of harvest (Santos-Gomes et al., 2002), geographic location (Putievsky et al., 1986; Santos-Gomes et al., 2002), habitat (Ben Farhat et al., 2009), salinity (Ben Taarit et al., 2009), exposure to phytophages (Dicke et al., 2009; Vuorinen et al., 2004), pathogens, and ultraviolet light (Langcake and Pryce, 1976), and hydration status (Bettaieb et al., 2009). Given the sophistication of contemporary agricultural techniques, it would seem feasible to grow well-standardized plants that benefit from augmented and constrained levels of beneficial and detrimental components, respectively, while retaining the positive synergistic properties of whole extracts. As a single example, increasing the salinity of a hydroponic medium has been shown to preferentially augment the concentration of 1,8-cineole, but not camphor, within SO essential oil (Ben Taarit et al., 2009), and such growing conditions may provide replicable extracts with the high AChE inhibitory properties seen here. However, the possibility of tailor-made natural extracts rich in the most advantageous constituents requires a fuller understanding of the interaction and synergy between active components, their secondary metabolite roles in the plant, and their physiological activity and CNS/behavioural effects in mammals.

In conclusion, the extract under investigation was a comparatively potent AChE inhibitor that improved cognitive performance and mood in healthy young humans following a single dose. The currently prescribed cholinesterase inhibiting treatments for Alzheimer's disease, a number of which are based on alkaloid phytochemicals, have been shown to have limited efficacy and, at more effective doses, engender high levels of negative side effects. Any potential treatment for both the symptoms of dementia and the natural decrements in cognitive performance that come with age, that is well tolerated due to the chemical structure of its active components, deserves further investigation. It is intended that an extract with a similar monoterpenoid profile and cholinergic properties as that investigated here will be taken forward into a chronic study assessing cognitive and mood effects in the elderly.

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