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# Pharmacological profile of essential oils derived from *Lavandula angustifolia* and *Melissa officinalis* with anti-agitation properties: focus on ligand-gated channels

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## Abstract

Both *Melissa officinalis* (Mo) and *Lavandula angustifolia* (La) essential oils have putative anti-agitation properties in humans, indicating common components with a depressant action in the central nervous system. A dual radioligand binding and electrophysiological study, focusing on a range of ligand-gated ion channels, was performed with a chemically validated essential oil derived from La, which has shown clinical benefit in treating agitation. La inhibited [$^{35}$S] TBPS binding to the rat forebrain gamma aminobutyric acid $(GABA)_A$ receptor channel (apparent IC50 = 0.040 ± 0.001 mg mL$^{-1}$), but had no effect on *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or nicotinic acetylcholine receptors. A 50:50 mixture of Mo and La essential oils inhibited [$^{3}$H] flunitrazepam binding, whereas the individual oils had no significant effect. Electrophysiological analyses with rat cortical primary cultures demonstrated that La reversibly inhibited GABA-induced currents in a concentration-dependent manner (0.01–1 mg mL$^{-1}$), whereas no inhibition of NMDA- or AMPA-induced currents was noted. La elicited a significant dose-dependent reduction in *both* inhibitory and excitatory transmission, with a net depressant effect on neurotransmission (in contrast to the classic $GABA_A$ antagonist picrotoxin which evoked profound epileptiform burst firing in these cells). These properties are similar to those recently reported for Mo. The anti-agitation effects in patients and the depressant effects of La we report in neural membranes in-vitro are unlikely to reflect a sedative interaction with any of the ionotropic receptors examined here. These data suggest that components common to the two oils are worthy of focus to identify the actives underlying the neuronal depressant and anti-agitation activities reported.

## Introduction

Agitation is a common and persistent feature of patients with severe dementia, usually characterized by a combination of aggression (verbal and/or physical), restlessness and shouting in the context of subjective anxiety (Akhondzadeh et al 2003). These symptoms can be highly distressing and impact profoundly on the quality of interaction with carers (Perry & Perry 2006).

The gamma aminobutyric acid (GABA), glutamate and cholinergic neuronal systems are particularly associated with neuropsychiatric symptoms that promote agitation and anxiety (Hardy et al 1987; Herrmann & Lanctot 1997; Garcia-Alloza et al 2005). Pharmacological treatment with neuroleptic agents is the first-line treatment for behavioural and psychological symptoms in dementia, despite only modest efficacy (Ballard et al 2002). In addition, these drugs have substantial adverse effects, including parkinsonism, akathisia, tardive dyskinesia, reduced well-being, risk of cardiac arrhythmias, social withdrawal, severe neuroleptic sensitivity reactions and possibly stroke (Brodaty et al 2003). Neuroleptic agents may also substantially accelerate cognitive decline and neuronal loss (specifically in the substantia nigra) (Brodaty et al 2003). Consequently, the Chief Medical Officer in the UK has recommended particular caution when prescribing neuroleptics to people with dementia, and in the US legislation has been introduced to regulate the prescription of neuroleptics to nursing home patients (Carson et al 2006).

Several new clinical trials have attested to the value of aromatherapy as an alternative to neuroleptics in people with dementia (Lee 2005; Perry & Perry 2006; Lin et al 2007). These include lemon balm (*Melissa officinalis;* Mo), lavender (*Lavandula angustifolia;* La), chamomile, bergamot, neroli and valerian (e.g. Viola et al 1995; Beabrun et al 2000; Brum et al 2001; Perry & Perry 2006). Despite the growing use of these essential oils, the mechanisms by which they exert their clinical effects are largely unknown. La is of particular interest on account of its known sedative and calming effects and relevant physiological actions (Viola et al 1995; Ballard et al 2002; Kennedy et al 2002, 2003, 2004, 2006; Akhondzadeh et al 2003; Perry & Perry 2006). The acute safety of these essential oils has also been well established in clinical populations and volunteer studies.

In order to maximize clinical benefit and develop new and more effective treatments in the future, it is important to select the most appropriate plant essential oil(s) and clarify their mechanism of action. Previously, we reported a pharmacological study of Mo essential oil (Abuhumdah et al 2008). Herein, we performed a detailed pharmacological study of the essential oils derived from La, which has similar but more potent anti-agitation properties than Mo in the clinic. Four batches of these oils, obtained from reputable European suppliers, were used in this study and a detailed chemical GC–MS analysis performed to validate the quality of the essential oils (Elliott et al 2007). La and Mo sourced from Baldwins and Fytosan gave qualitatively similar results (Elliott et al 2007) and were selected on the basis of GC–MS analysis that most closely resembled known published standard compositions (Elliott et al 2007; Abuhamdah et al 2008).

## Materials and Methods

### GC–MS

The GC–MS system consisted of an AutoSystem XL gas chromatogram coupled to a TurboMass quadrupole mass spectrometer (Perkin-Elmer, Waltham, MA, USA). Chromatography was performed on two different columns: a 30 m × 0.25 mm i.d. × 0.25 $\mu$m DB-5MS column (J. & W. Scientific, Folsom CA, USA) using a temperature programme of 40–300°C at a rate of 3°C $min^{-1}$, and a 30 m × 0.25 mm i.d. × 0.25 $\mu$m ZB-WAX column (Phenomenex, Macclesfield, UK) using a temperature programme of 40–240°C at a rate of 4°C $min^{-1}$. For both methods, the carrier gas was helium at a flow rate of 1 mL $min^{-1}$, and 1 $\mu$L injections (split 1:10) at 220°C were made by an autosampler. Detection was by MS, fitted with an electrospray ionization source operated at 70 eV, with a source temperature of 180°C; mass spectra were recorded in the range *m/z* 38–450 with a scan time of 0.50 s and an interscan delay of 0.20 s. The operating software was Turbomass, version 4.1.1 (Perkin-Elmer).

The La oil (Baldwins) was diluted to 1.0% (v/v) with diethyl ether (GLC pesticide residue grade, Fisher Scientific, Loughborough, UK) before analysis. Retention indices (RIs) were determined in relation to a series of *n*-alkanes (C10–C16, Supelco, Sigma-Aldrich, Poole, UK) and compounds were identified by comparing the RIs on the two solid phases (DB-5MS and ZB-WAX) and/or mass spectra with published data (Davies, 1990; Ausloos et al 1992; Adams 2001). Percentage compositions were calculated by integrating all peaks in total ion chromatograms (Table 1). For all experiments, dilutions of pure essential oil stock were performed fresh on the day of the assay.

[$^3$H] Flunitrazepam (specific activity 91.0 Ci $mmol^{-1}$) and [$^3$H] nicotine (specific activity 77 Ci $mmol^{-1}$) were obtained from Amersham Biotech (Little Chalfont, UK); [$^{35}$S]-*t*-butylbicyclophosphorothionate (TBPS; specific activity 80 Ci $mmol^{-1}$) was from Perkin Elmer Life Sciences, Waltham, MA, USA; [$^3$H] MK-801 (specific activity 25 Ci $mmol^{-1}$) and [$^3$H] muscimol (specific activity 36.5 Ci $mmol^{-1}$) were obtained from ARC, Melville, NY, USA; [$^3$H] alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; specific activity 40.8 Ci $mmol^{-1}$) was obtained from NEN Dupont. Picrotoxinin, diazepam, GABA, glutamate and ketamine were all obtained from Sigma (Poole, UK). All other chemicals were purchased from Sigma, unless stated otherwise.

GABA stocks ($10^{-2}$ M) were made in assay buffer. Diazepam stocks ($10^{-2}$ M) were prepared in absolute ethanol. Picrotoxinin stocks ($10^{-2}$ M) were prepared in DMSO. Ketamine stocks ($10^{-2}$ M) were prepared in assay buffer. No effect of solvents on radioligand binding assays was seen at concentrations below 0.1% (v/v) ethanol or DMSO (data not shown; Abuhamdah et al 2005).

### Tissue preparation

Adult male Wistar rats (200–300 g) were maintained under a 12 h light–dark cycle at 23°C and 65% humidity, with water and standard laboratory food available ad libitum. Animal treatment and husbandry were in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK (PPL 60/437). Animals were killed humanely using a Schedule 1 procedure. The brains were removed rapidly, and the required tissue (forebrain) dissected immediately and kept on ice. The tissue was homogenized in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 5 mM EGTA and 320 mM sucrose) using a dounce glass/glass homogenizer. The homogenate was centrifuged at 1000 *g* at 4°C for 10 min and the supernatant stored on ice. The pellet was re-homogenized in ice-cold buffer and centrifuged as above. The supernatants from the centrifugations were pooled and centrifuged at 12 000 *g* at 4°C for 30 min. The supernatant was discarded and the pellet resuspended in 50 mM Tris buffer containing 5 mM EDTA and 5 mM EGTA (5 mL $g^{-1}$ original tissue), processed using a five-step freeze–thaw protocol (Abuhamdah et al 2005, 2008), frozen and stored at −20°C.

### Radioligand competition binding assays

A series of dose–response competition binding experiments were performed using [$^{35}$S] TBPS, [$^3$H] muscimol, [$^3$H] flunitrazepam, [$^3$H] AMPA, [$^3$H] MK-801 and [$^3$H] nicotine, using well-washed adult rat forebrain membranes.

**Table 1** Composition (%) of essential oil from *Lavandula angustifolia* obtained from the GC–MS total ion chromatogram (based on all peaks). Compounds were identified by comparing retention indices (RI; calculated against an *n*-alkane series) and by comparing mass spectra with published data (Davies 1990; Adams 2001; Ausloos et al 1992) except for [c]compounds identified by comparing mass spectra with published data (Ausloos et al 1992)

| Compound | RI[a] | RI[b] | Composition (%)[a] | Composition (%)[b] |
|---|---|---|---|---|
| $\alpha$-Thujene | 921 | 1035 | trace | trace |
| $\alpha$-Pinene | 928 | 1031 | 0.1 | 0.1 |
| Camphene | 944 | 1057 | 0.1 | 0.1 |
| Sabinene | 967 | 1098 | trace | trace |
| $\beta$-Pinene | 971 | 1086 | 0.1 | trace |
| Oct-1-en-3-ol | 975 | 1444[c] | 0.2 | 0.2 |
| 3-Octanone | 980 | 1226[c] | 0.3 | trace |
| Myrcene | 984 | 1138 | 0.5 | 0.4 |
| Hexyl acetate | 1008 | 1246[c] | 0.3 | 0.3 |
| *o*-/*p*-Cymene | 1020 | 1239[c] | 0.2 | 0.3 |
| Limonene | 1025 | 1166 | 0.3 | 0.2 |
| 1,8-Cineole | 1028 | 1172 | 0.6 | 0.6 |
| (*Z*)-$\beta$-Ocimene | 1033 | 1207 | 3.3 | 3.4 |
| (*E*)-$\beta$-Ocimene | 1044 | 1224 | 1.3 | 1.6 |
| *trans*-Linalool oxide (furanoid) | 1069 | 1460 | 0.3 | 0.1 |
| *cis*-Linalool oxide (furanoid) | 1086 | 1428 | 0.2 | 0.2 |
| Linalool | 1105 | 1555 | 30.8 | 31.1 |
| 1-Octen-3-yl acetate | 1110 | 1362[c] | 1.2 | 1.0 |
| Camphor | 1150 | 1498 | 0.4 | 0.3 |
| Lavandulol | 1169 | 1685 | 1.0 | 1.1 |
| Borneol | 1178 | 1705 | 0.8 | 0.8 |
| Terpinen-4-ol | 1187 | 1603 | 3.7 | 4.0 |
| Cryptone | 1192 | 1662[c] | 0.2 | trace |
| $\alpha$-Terpineol | 1202 | 1702 | 1.3 | 1.7[d] |
| Nerol | 1232 | 1807 | 0.2 | 0.3 |
| Linalyl acetate | 1262 | 1563 | 36.7 | 37.2 |
| Lavandulyl acetate | 1294 | 1611 | 5.5 | 5.4 |
| Neryl acetate | 1368 | 1730 | 0.5 | 0.6 |
| Geranyl acetate | 1387 | 1761 | 1.0 | 1.1 |
| 7-*epi*-Sesquithujene | 1410 | 1613[c] | trace | trace |
| (*E*)-Caryophyllene | 1425 | 1590 | 3.6 | 3.6 |
| (*E*)-$\beta$-Farnesene | 1457 | 1669 | 1.7 | 1.9 |
| Germacrene D | 1483 | 1702 | 0.2 | 1.7[d] |
| $\beta$-Bisabolene | 1508 | 1724 | trace | trace |
| $\gamma$-Cadinene | 1512 | 1751 | 0.1 | 0.2 |
| Caryophyllene oxide | 1572 | 1959 | 0.6 | 0.7 |

[a]Chromatography performed on a DB-5MS phase; [b]chromatography performed on a ZB-WAX phase; trace: < 0.1%; [d]co-elution of $\alpha$-terpineol and germacrene D.

## $GABA_A$ receptors

### *[$^{35}$S]-t-butylbicyclophosphorothionate (TBPS) binding assay*

[$^{35}$S] TBPS binding was measured as described by Abuhamdah et al (2005, 2008). Briefly, well-washed rat membranes were incubated in 50 mM Tris buffer containing 0.2 M NaCl, pH 7.4, using approx. 20 nM [$^{35}$S] TBPS for 90 min at 25°C with a range of test concentrations ($10^{-11}$–$10^{-4}$ M). Non-specific binding was defined in the presence of 100 $\mu$M picrotoxinin.

### *[$^3$H] Muscimol binding assay*

[$^3$H] Muscimol binding assays were performed as described previously (Abuhamdah et al 2005, 2008). Briefly, well-washed rat forebrain membranes were incubated in 50 mM Tris buffer, pH 7.4, using approx. 10 nM [$^3$H] muscimol for 1 h at 4°C with a range of test concentrations ($10^{-11}$–$10^{-4}$ M). Non-specific binding was defined in the presence of 100 $\mu$M GABA.

### *[$^3$H] Flunitrazepam binding assay*

[$^3$H] Flunitrazepam binding assays were performed as described previously (Abuhamdah et al 2005, 2008). Well-washed rat forebrain membranes were incubated in 50 mM Tris buffer containing 5 mM EDTA and 5 mM EGTA using approx. 1 nM [$^3$H] flunitrazepam for 1 h at 4°C with a range of test concentrations ($10^{-11}$–$10^{-4}$ M). Non-specific binding was defined in the presence of 100 $\mu$M diazepam.

## Glutamate receptors

### *[$^3$H] AMPA binding assay*

[$^3$H] AMPA binding assays were performed as described by Honoré & Nielsen (1985). The assay was performed using 5–10 nM [$^3$H] AMPA in 30 mM Tris-HCl, 2.5 mM $CaCl_2$, 100 mM KSCN, pH 7.1, at 4°C for 1 h. Non-specific binding was determined using 1 $\mu$M glutamate.

*[$^3$H] MK-801 binding assay*

[$^3$H] MK-801 binding assays were performed as described previously (Chazot et al 1993). Briefly, well-washed rat forebrain membranes were incubated in 25 mM sodium phosphate buffer with 10 $\mu$M glutamate, pH 7.4, using approx. 1 nM [$^3$H] MK-801 for 2 h at 22°C with a range of test concentrations ($10^{-11}$–$10^{-4}$ M). Non-specific binding was defined in the presence of 10 mM ketamine.

*[$^3$H] Nicotine binding assay*

[$^3$H] Nicotine binding was performed as described by Court et al (1997). The assay was performed in 50 mM Tris-HCl buffer, pH 7.8, containing 8 mM $CaCl_2$, using approx. 10 nM [$^3$H] nicotine at room temperature for 1 h. Non-specific binding was determined using 100 $\mu$M nicotine.

All six binding assays were terminated by rapid filtration through Whatman GF/B filters presoaked in phosphate buffer, which were washed (3 × 3 mL) using ice-cold 10 mM sodium phosphate buffer, pH 7.4, using a Brandel cell harvester (Alpha Biotech, London, UK). Filters were transferred into scintillation vials, liquid scintillation fluid added and the vials incubated for 16–24 h at room temperature. The bound radioactivity was quantified using a Beckman LS 500 CE scintillation spectrophotometer (Beckman Coulter, High Wycombe, UK) with a counting time of 4 min per vial.

### Primary cell culture

Neuronal cultures were prepared from 16–18-day-old rat embryos (Sprague–Dawley strain). Cortical cells were removed and separated by mechanical trituration without trypsin and plated onto poly-D-lysine coated coverslips (100 000 cells mL$^{-1}$) in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and 20 units mL$^{-1}$ penicillin and 20 $\mu$g mL$^{-1}$ streptomycin, and maintained at 37°C under 5% $CO_2$/95% $O_2$. After 24 h, the plating medium was replaced by a maintenance medium comprising neurobasal medium (Invitrogen, Paisley, UK), with 2% B27 supplement (Invitrogen), 1% glutamax (Invitrogen), 20 units mL$^{-1}$ penicillin and 20 $\mu$g mL$^{-1}$ streptomycin. Cells were used in experiments after 14–28 days in-vitro (Lees & Leach 1993; Lees et al 2000).

### Electrophysiology

Coverslips containing cultured neurones were placed in a 5 mm Perspex trench with continuous flow on the stage of an inverted microscope. The basal extracellular saline contained 142 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES and 30 mM D-glucose, pH 7.4 (NaOH). Intracellular saline consisted of 142 mM K-gluconate, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES and 11 mM EGTA, pH 7.4 (KOH). Electrodes were pulled from GC150T-10 borosilicate glass (Harvard Apparatus Ltd, Edinbridge, Kent, UK) with resistance of 3–5 MΩ. All potentials cited are those based on the pre-amplifier null potential and take no account of the liquid junction offset inherent in the use of the asymmetric solutions. Phase-bright pyramidal cells were identified and selected under ultra-long working distance phase-contrast optics (Nikon Diaphot, Segensworth, UK). At a holding potential of –40 mV, GABA (10 $\mu$M, 500 ms) was rapidly and quantitatively delivered to cultured cells using the Y-tube technique (Murase et al 1989). Whole-cell currents were filtered at 3 KHz prior to digitization at 6 KHz on a CED 1401 plus A/D D/A converter (Isis Electronics Ltd, Cheltenham, UK). The herbal oils were dissolved initially in DMSO, and diluted 1:1000 into test salines. DMSO was added routinely at 0.1% to drug-free salines and had no effect on any of the parameters reported here (Lees & Leach 1993; Lees et al 2000).

### Analysis

Data are presented as mean ± s.d. (Figures 1 and 2) or s.e.m. (Figure 3). Repeated measures one-way analysis of variance and Student's *t*-tests were used as appropriate using Prism 4 software (Graphpad, La Jolla, CA, USA). *P* values below 0.05 were considered significant.

## Results

Previous clinical evidence suggests that La elicits an anti-agitation or calming effect which would initially indicate a sedative neurosteroid-, benzodiazepine-, or barbiturate-like activity at the $GABA_A$ receptor. To test this hypothesis, the effects of La were initially investigated using a range of radioligands targeting the major binding sites of the $GABA_A$ receptor (Abuhamdah et al 2005, 2008).

### [$^3$H] Flunitrazepam binding assay

The effects of La on radioligand binding to the benzodiazepine site of the $GABA_A$ receptor were studied using the [$^3$H] flunitrazepam binding assay. Specific [$^3$H] flunitrazepam binding was defined using diazepam (100 $\mu$M). La alone did not alter the equilibrium binding of [$^3$H] flunitrazepam to $GABA_A$ receptors in adult rat forebrain membranes (Figure 1A).

### [$^3$H] Muscimol binding assay

The effects of La on radioligand binding to the agonist binding site of the $GABA_A$ receptor was studied using the [$^3$H] muscimol binding assay. Specific binding was defined using 100 $\mu$M GABA. La alone did not alter the equilibrium binding of [$^3$H] muscimol to $GABA_A$ receptors in adult rat forebrain membranes (Figure 1B). In contrast, Mo enhanced the specific binding of [$^3$H] muscimol in a concentration-dependent manner, with a maximum enhancement at a concentration of 1 mg mL$^{-1}$, and apparent EC50 of 0.099 ± 0.001 mg mL$^{-1}$ (Abuhamdah et al 2008).

### [$^{35}$S] TBPS binding assay

To investigate the effect of La on the channel site of the $GABA_A$ receptor, [$^{35}$S] TBPS binding activity was

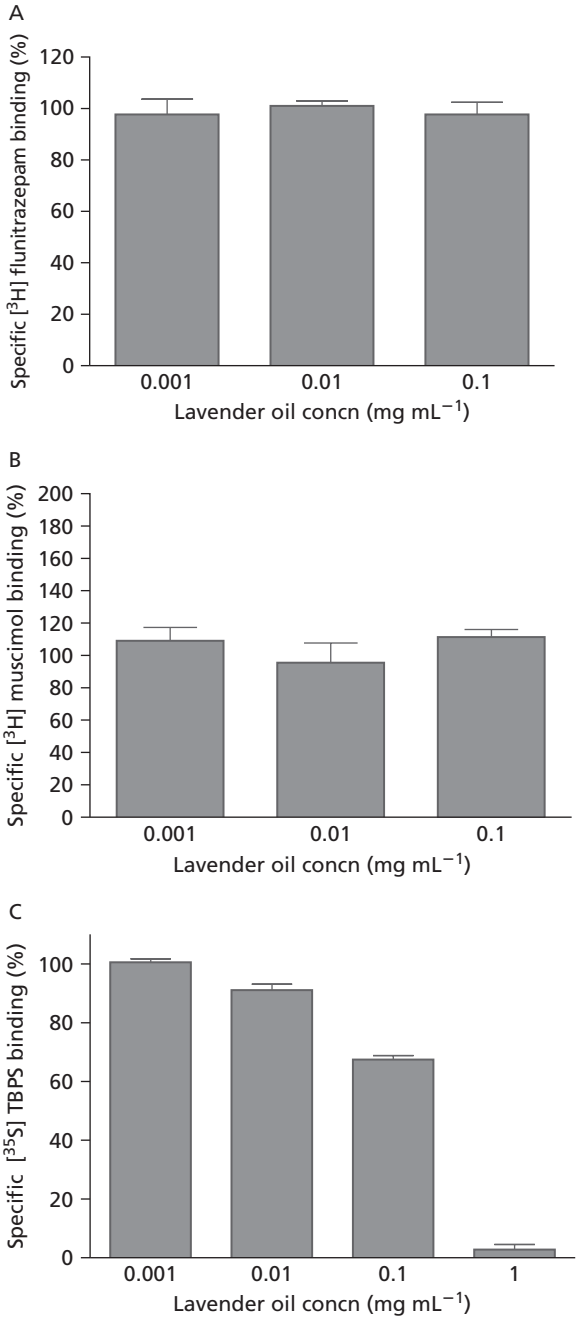


**Figure 1** Effect of *Lavandula angustifolia* essential oil on [$^3$H] flunitrazepam binding (A), [$^3$H] muscimol binding (B) and [$^{35}$S] TBPS binding (C). A series of competition binding experiments were performed using well-washed rat forebrain membranes. Data are mean ± s.d. from at least three separate experiments. The IC50 for [$^{35}$S] TBPS was 0.300 ± 0.001 mg mL$^{-1}$ (mean ± s.d. for at least three independent experiments).

determined using well-washed adult rat forebrain membranes. Specific binding was defined using 100 $\mu$M picrotoxinin (Figure 1C). A dose-dependent complete inhibition of [$^{35}$S] TBPS binding was observed (IC50 = 0.300 ± 0.001 mg mL$^{-1}$).

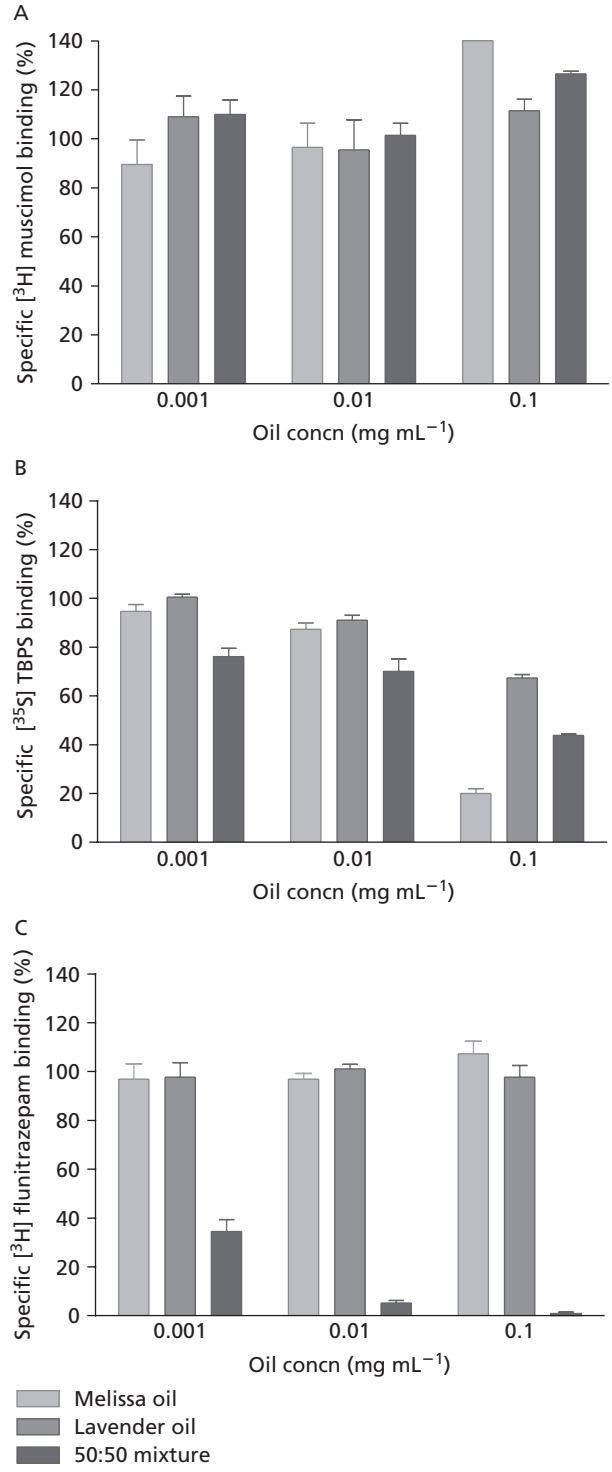


**Figure 2** Synergistic effects of *Lavandula angustifolia* and *Melissa officinalis* on [$^3$H] flunitrazepam binding to the $GABA_A$ receptor. A series of competition binding experiments were performed using well-washed rat forebrain membranes: (A) [$^3$H] muscimol; (B) [$^{35}$S] TBPS; (C) [$^3$H] flunitrazepam for *M. officinalis* alone, *L. angustifolia* alone and a 50:50 mixture of the two oils. Data are (mean ± s.d. from at least three separate experiments). IC50 for [$^3$H] flunitrazepam was 1.0 ± 0.1 $\mu$g mL$^{-1}$ (mean ± s.d. for at least three independent experiments).

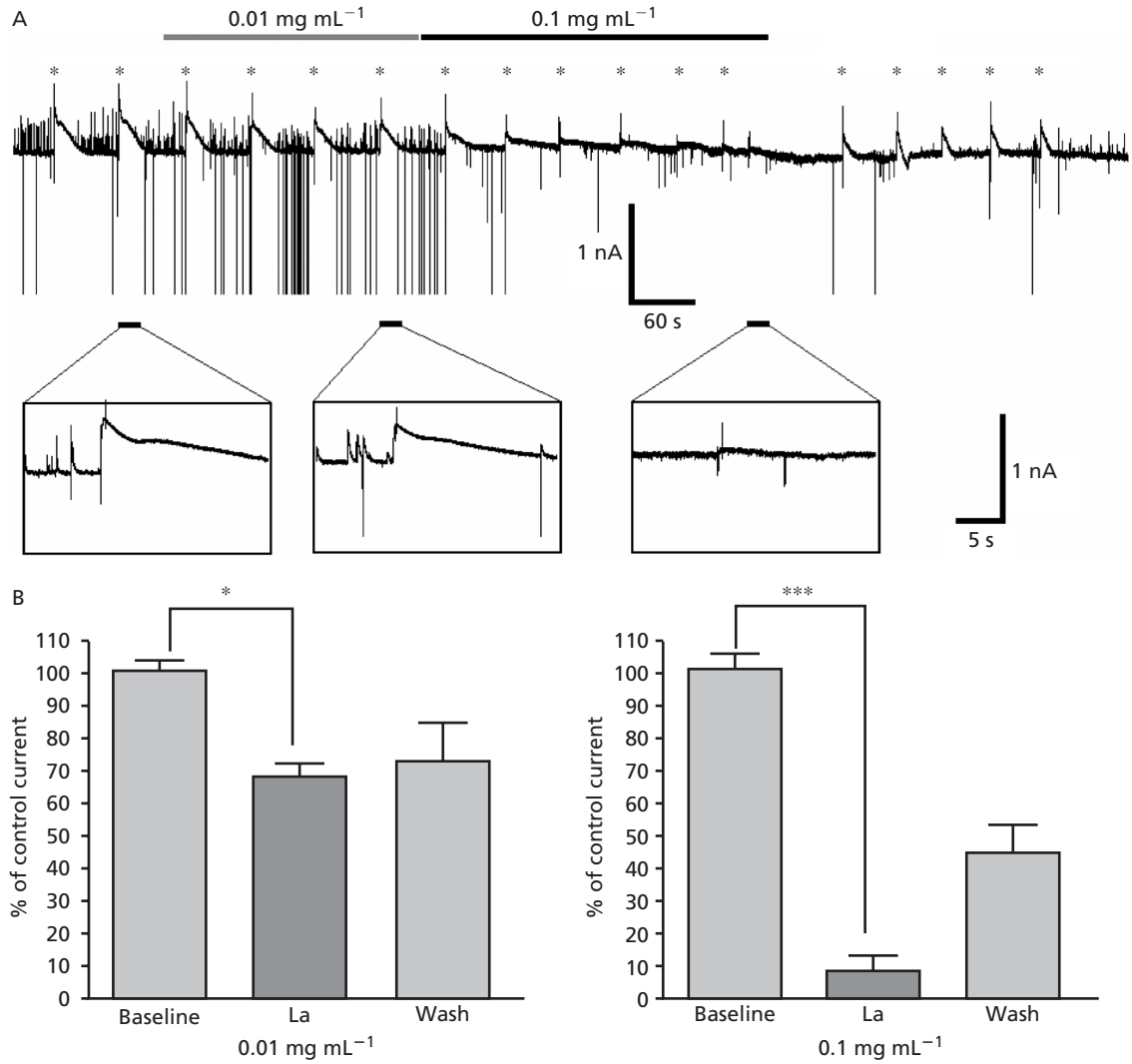


**Figure 3** Effect of *Lavandula angustifolia* (La) essential oil on GABA-mediated currents in primary cortical neurones. (A) La oil at 0.01 mg $mL^{-1}$ produced a rapid and partially reversible block of spontaneous inhibitory post-synaptic currents (fast upward deflections in the trace, shown in detail in the expanded insets below the compressed trace). The asterisks indicate application of 500 ms pulses of exogenous GABA (10 $\mu$M). Note these events are blocked to a similar extent as the spontaneous synaptic inhibitory events and that the excitatory input to the cell (downward deflections indicate excitatory post-synaptic currents and spontaneous action currents) is also reduced during exposure to La. (B) Compounded data on the effects of La on evoked responses to exogenous GABA applications. La at 0.01 mg $mL^{-1}$ reduced GABA currents (control, 101.90 ± 2.20%; La, 68.81 ± 4.07%; mean ± s.e.m. for n = 3; $*P < 0.05$) with partial washout in some replicates (73.37 ± 11.91%, n = 2). At 0.1 mg $mL^{-1}$ La almost completely blocked the evoked GABA currents (control, 102.00 ± 4.47%; La, 8.87 ± 3.25%; mean ± s.e.m. for n = 4; $***P < 0.001$), again with reversibility in some cells (wash 44.54 ± 9.03, n = 2).

### Effects of oil mixture

The effects of a 50:50 mixture of Mo and La essential oils were determined to assess the potential combination effects often claimed with these types of agents. For [$^3$H] muscimol and [$^3$H] TBPS binding, there was no evidence of synergism with the mixture (Figures 2B and 2C). In contrast, for [$^3$H] flunitrazepam binding, while the individual oils had no effect up to 1 mg $mL^{-1}$, the oil mixture inhibited [$^3$H] fluntrazepam binding with an IC50 of less than 0.001 mg $mL^{-1}$, indicating a profound synergistic interaction between components in the two oils (Figure 2C).

### Selectivity of the action of lavender oil on $GABA_A$ receptors

In order to assess the selective pharmacology of La in more detail, the effects of the oil were determined on a number of other common neuronal ligand-gated ion channels, including the excitatory ligand-gated ion channels gated by NMDA, using a [$^3$H] MK-801 binding assay, and by AMPA using [$^3$H] AMPA. In addition, the effect of Mo was also determined on neuronal nicotinic receptors ($\alpha4\beta2$, $\alpha7$ nAChRs) using a [$^3$H] nicotine binding assay (Court et al 1997). La had no effect (positive or negative) on [$^3$H] MK-801, [$^3$H] AMPA or

[$^3$H] nicotine binding up to a concentration of 1 mg mL$^{-1}$ (data not shown).

The effects of a 50:50 mixture of Mo and La essential oils were determined to assess the potential combination effects often seen with these types of agents, as seen with [$^3$H] flunitrazepam. There was no evidence for synergistic effects on [$^3$H] AMPA or [$^3$H] nicotine binding, and only a minor low-affinity effect on [$^3$H] MK-801 binding at 1 mg mL$^{-1}$ (inhibition approx. 30%).

### Electrophysiology

The apparent inhibitory effects of La on [$^{35}$S] TBPS binding would predict a pro-convulsant effect, which is obviously not apparent in the use of this essential oil. We therefore investigated the effects of La using an electrophysiological approach. Patch-clamp experiments on primary cultures from rat cortex demonstrated that La reversibly reduced currents through the $GABA_A$ channel in a concentration-dependent (0.01–1 mg mL$^{-1}$) manner, consistent with the binding profile (Figure 3). The blocking action at both 0.01 and 0.1 mg mL$^{-1}$ was statistically significant compared with control currents (data not shown). Note that at 0.01 mg mL$^{-1}$, spontaneous inhibitory post-synaptic currents were almost completely blocked by La and recovered relatively quickly compared with the partial blocking effect noted on the evoked GABA responses (Figure 3A). La concurrently reduced the incidence of both excitatory and inhibitory spontaneous synaptic traffic in the cultured networks (see inset traces in Figure 3A), which is not a feature of selective $GABA_A$ antagonists in these cells.

In contrast, previous studies have shown that the selective $GABA_A$ antagonist picrotoxin blocks both evoked outward currents and spontaneous inhibitory post-synaptic currents. This disinhibition resulted in paroxysmal depolarizing shifts and epileptiform burst firing in all four of the picrotoxin-treated pyramidal cells (Abuhamdah et al 2008).

## Discussion

Pharmacological targets for the reduction of agitation (and accompanying or underlying aggression or anxiety) include the neurotransmitter systems serotonin (5HT), dopamine, acetylcholine (via nicotinic and muscarinic receptors) and GABA. In this study, we have characterized the effect of La on the three major binding sites of the $GABA_A$ receptor: the benzodiazepine site, the GABA site and the ion channel site, to detect any $GABA_A$ modulatory activity. To confirm selectivity, interactions with other common ligand-gated ion channel receptors – NMDA, AMPA and neuronal nicotinic receptors – were also investigated.

We have shown that La inhibited [$^{35}$S] TBPS binding in a concentration-dependent manner in native $GABA_A$ receptors. Interestingly, the oil alone showed no significant effects on the agonist and benzodiazepine sites bindings. This is in agreement with a recently published study on another anti-agitation essential oil, Mo, which showed a similar pharmacological profile to La (Abuhmadah et al 2008), although Mo elicited an increase in muscimol binding as well as inhibition of [$^{35}$S] TBPS binding. Interestingly, as was reported for Mo, some degree of specificity towards the $GABA_A$ receptor was evident, with no effects on other major ligand-gated channels expressed in the central nervous system. Another interesting novel finding reported in the current study was the observation that a 50:50 mixture of La and Mo produced a high-affinity binding profile for [$^3$H] flunitrazepam. This shows a profound synergistic relationship between component(s) in the two oils. Synergistic properties have been previously claimed for other oils or natural product medicaments or 'nutraceuticals'. The identification of chemical components underpinning these effects is worthy of further study.

To confirm our radioligand binding findings, an electrophysiological study was performed using cultured rat cortical neurones. La at 0.01–0.1 mg mL$^{-1}$ significantly reduced GABA-evoked currents in cultured neurones and, surprisingly, silenced both inhibitory and excitatory traffic in neuronal networks. Our comparative experiment with the selective $GABA_A$ receptor antagonist picrotoxin unequivocally confirms that selective block of the $GABA_A$ channel enhances synaptic drive, elicits full-blown action potentials and evokes epileptiform activity in the form of paroxysmal depolarizing shifts and concomitant high-frequency firing (Abuhamdah et al 2008). La at a concentration of 0.01 mg mL$^{-1}$ profoundly inhibited GABA-induced current and spontaneous synaptic activity; both excitatory and inhibitory synaptic activities were almost completely blocked in neuronal networks at this concentration (note that full block of post-synaptic currents evoked by exogenous GABA was achieved at 0.01 mg mL$^{-1}$). These net depressant effects are possibly mediated by a relatively high-affinity pre-synaptic interaction, as reported recently for Mo (Abuhamdah et al 2008). Similar depressant actions are seen with clinically useful drugs that target voltage-gated ion channels and depress membrane excitability via a pre-synaptic interaction (Lees & Leach 1993; Errington et al 2006; Errington et al 2008). Notably, La displays significantly more intrinsic activity in its depressant functional effect than Mo in the same concentration range (0.01–0.1 mg mL$^{-1}$) (Abuhamdah et al 2008), which may reflect its more profound sedative properties.

### Conclusion

The pharmacological activities of La and Mo appear to reflect their uses in traditional medicine as sedative and/or anti-agitation agents (Perry & Perry 2006; Abuhamdah et al 2008). We conclude that La does exert depressant effects on neural activity, but that this is clearly not a reflection of its interaction with the $GABA_A$ complex. At least one component in the oil does appear to bind to the $GABA_A$ receptor, but functionally this reduces inhibitory synaptic currents and responses to exogenous GABA application. We are currently attempting to delineate the active elements in both oils using functional group-based fractionation, to identify the depressant component(s) and assess the behavioural effects of these essential oils. The oils share a number of components (e.g. geranyl acetate, (*E*)-caryophyllene), but also have distinct components (e.g. linalool, lavandulol) (Elliott et al 2007).

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