European Journal of Medicinal Plants
4(7): 810-818, 2014

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Identification of a Novel $GABA_A$ Receptor Channel Ligand Derived from *Melissa officinalis* and *Lavandula angustifolia* Essential Oils

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Authors' contributions

This work was carried out in collaboration between all authors. Author PC designed the study, wrote the protocol and the first draft of the manuscript. Authors MM and RA conducted the experimental works. Author AE performed the statistical analysis. Authors SA MH managed the literature searches and the analyses of the study. All authors read and read and approved the final manuscript.

Original Research Article

Received 7th February 2014
Accepted 13th March 2014
Published 28th March 2014

ABSTRACT

Aims: *Melissa officinalis* (Mo) and *Lavandula angustifolia* (La) essential oils and their major constituents ((E) - caryophyllene, caryophyllene oxide, geranyl acetate, linalool, nerol, Oct-1-en-3-ol, 3-Octanone, myrcene, allo-ocimene, p-cymene and α- terpineol) assessed by GC-MS) which are shared by these two essential oils were probed in an attempt to identify the $GABA_AR$ ligand(s).
Study Design: [^{35}S] t-butylbicyclophosphorothionate (TBPS) radioligand binding assay to $GABA_A$ receptors. *In vitro* neuronal viability assay.
Place and Duration of Study: School of Biological and Biomedical Sciences, Durham University, United Kingdom (December 2012 and January 2013).
Results: One of the major component (s) of (Mo), trans-ocimene, inhibited [^{35}S] (TBPS) binding to native $GABA_A$ receptors in a concentration-dependent manner with an apparent

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IC_{50} of 40µM.
Concentrations (0.001 mg/ml) of whole (Mo) were shown to display modest beneficial effects upon neuronal viability while at a higher concentration (0.1 mg/ml) of (Mo) and (La) oils induced a neurotoxicity effect.
Conclusion: These data provide the first evidence that allo-ocimene is an neuroactive $GABA_A$ R inhibitory component found in both (Mo) and (La), and represents a novel $GABA_A$ receptor channel chemotype derived from a natural product.

Keywords: Melissa officinalis (Mo); Lavandula angustofolia (La); [^{35}S]-t-butylbicyclophosphorothionate ([^{35}S] TBPS); gamma-aminobutyric acid $(GABA)_A$ receptor.

1. INTRODUCTION

In the last 20 years, there has been a revival of interest in natural products and medicinal plants. *Melissa officinalis, Lavandula angustifolia, Ginkgo biloba, Salvia lavandulae folia* and *Rosmarinas officinalis* have been widely used in aromatherapy. This has involved the use of the whole plants, parts of plants, or the pure essential oils (EOs) with aims to treat health problems. Alzheimer's disease (AD) is a neurological disorder characterised by a progressive impairment of cognition and behavioural mood deficits that leads to a person unable to perform even simple daily life activities [1-6]. The management of behavioural and psychological symptoms of dementia (BPSD) such as agitation, psychosis and mood disorders remains a major problem for people with AD in clinical care [7,8]. BPSD develop in more than 90% of people with dementia over a five year period. *Melissa officinalis* (lemon balm) and *Lavandula augustifolia* (lavender), singly or in combination are the most well-known (EOs) that have been used for the treatment of agitation in controlled clinical trials. These (EOs) demonstrated significant effects in reduction of agitation, wandering, insomnia and social withdrawal [9,10,3]. There is mounting evidence that links BPSD to specific alteration in neurochemistry, which may underpin the basis of pharmacological manipulation. Dementia is associated with dysfunction in multiple neurotransmitter systems. Although the most well studied neuronal system dysfunction lies in the cholinergic system, there is also evidence supporting serotonergic, noradrenergic, dopaminergic and GABA systems [11]. These neurotransmitters are known to regulate behaviours and are amenable to pharmacological intervention. Several important classes of clinically used drugs such as benzodiazepines, barbiturates and anesthetics, have actions which are mediated by allosteric interaction at the $GABA_A$ receptor [12-14]. The great molecular diversity of the multi-subunit hetero-oligomeric $GABA_A$ receptor provides opportunities to develop novel drugs, for example for anxiety, sleep disorders, alcoholism and epilepsy by establishing the relevant molecular targets for receptor subtype specific action [15-17]. In this present report, we attempted to identify the component that probably accounts for $GABA_A$ receptor binding properties of the Melissa and Lavender essential oils [18,19].

2. MATERIALS AND METHOD

2.1 Chemicals and Herb Samples

The sample of (Mo) was obtained from Fytosan (France) and the components of (Mo) essential oil were either obtained from Sigma (Poole, UK) or Acros (Loughborough, UK). This batch of (Mo) was selected and validated based on previous pharmacological and

chemical studies using batches from four common suppliers (Baldwins (London, UK), Pranarom (Lille, France), Quinessence (Coalville, UK), Fytosan (Die, France); the composition correlates with the natural products Herbal Pharmacopoeia standards and was confirmed to be stable throughout the research programme (Dr Melanie-Jayne Howes, Jodrell Laboratory, Kew Gardens). Furthermore, a batch was selected and used in a parallel clinical trial in late-stage of Alzheimer's disease patients [18,19]. The GC-MS profile carried out at the Royal Botanic Garden at Kew (London), displayed no major changes in (Mo) composition following long-term storage (18 months) at 4°C in the dark (composition reported previously in [18,19]). Dilutions of essential oil stocks or components were performed fresh on the day of assay. [^{35}S]-t-butylbicyclophosphorothionate (TBPS; specific activity 80 Ci mmol-1) was from Perkin Elmer Life Science (Waltham, MA, USA. Essential oil stock solutions or constituents were prepared in dimethylsulphoxide (DMSO) first then in the appropriate assay buffers. The solvents had no effect on radiolig and binding assays at concentrations below 0.1% (v/v) ethanol or DMSO [15].

2.2 Radiolig and Binding Assay

2.2.1 Tissue preparation and processing

Animal treatment and husbandry were in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK. Brains from adult male Wistar rats (200-300g) were sacrificed humanely by using a standard Schedule 1 procedure. The brains were removed rapidly, and the required tissue (forebrain) dissected immediately and kept cool on ice. The tissue was then homogenised using a Dounce glass/glass homogeniser in ice–cold homogenisation buffer containing: 50mM Tris HCl, pH 7.4, containing 5mM EDTA, 5mM EGTA and 320 mM sucrose. The homogenate was then centrifuged at 1000xg for 10min at 4°C.The supernatant was stored in ice and the pellet re-homogenised in ice-cold buffer, centrifuged at 1000g for 10min at 4°C.The supernatants from the first and second centrifugation steps were pooled and centrifuged at 15,000xg for 30min at 4°C.The supernatant was discarded and the pellet re-dissolved in 50mM Tris containing 5mM EDTA and 5mM EGTA, processed using a five-step freeze–thaw protocol [15], frozen and stored at -20°C. The protein concentration was then determined using the Lowry assay [17] using bovine serum albumin as the standard protein.

2.2.2 Radiolig and binding assay

[^{35}S] TBPS binding were determined as described by [15] using well-washed rat forebrain membranes. In order to measure [^{35}S] TBPS binding, membranes were incubated in 50mM Tris buffer containing 0.2MNaCl, pH 7.4; using approximately 20nM [^{35}S] TBPS for 90 min at 25°C with a range of test concentrations of (Mo) constituents (0.001-0.1mg/ml). Non-specific binding was defined in the presence of 10µM picrotoxinin.

2.3 Mixed Cortical Cultures

Mixed cortical were prepared as previously described [16]. Briefly, cultures were prepared from 16- to 18-day-old rat embryos (Sprague-Dawley strain) in B27neurobasal media and plated on poly-D-lysine coated 24-well plates at a cell density of 3000cells/mm^2. The cells were maintained by adding fresh neurobasal media at DIV3 (Days *In vitro* 3) and DIV7 (Days *In vitro* 7).

2.4 Oil Experiment

On DIV7, Sunflower oil (Control oil) or Melissa oil (0.001 or 0.01mg/ml both dissolved in DMSO<0.01%v/v) in fresh media was added to neuronal cultures (4wells) for 24h. The emulsifying agent which was used is DMSO, which showed no effect in control experiments and, therefore, this justified that it is the (Mo) that is having the effect on the neuronal cultures and not the emulsifying agent. Furthermore, the sham treatment was performed with Sunflower oil in the presence of DMSO.

2.5 Statistical Analysis

Data were expressed as the mean±SEM and analysed using one-way of variance and Student's *t*-test as appropriate using Prism 4 software (Graph Pad, CA.USA). P values below 0.05 were considered significant.

3. RESULTS AND DISCUSSION

3.1 Effects of Melissa Oil constituents on the Channel Binding Site of the $GABA_A$ receptor labelled by [^{35}S] TBPS

The effects of the twelve major oil constituents, common to both *Melissa officinalis* and *Lavandula angustifolia* essential oils, were probed on the channel site of $GABA_A$ receptor. [^{35}S] TBPS binding studies were carried out to a well washed adult forebrain, using three different concentrations (0.001,0.01,0.1mg/ml) of the constituents (linalool, limonene, (E)-caryophyllene, caryophyllene oxide, nerol, p-cymene, geranyl acetate, 3- octanone, oct-1-en-3-ol, myrcene, ocimene and α-terpineol). The inhibition of [^{35}S] TBPS binding by increasing concentrations of the oil constituents was dose-dependent, attaining 50% inhibition between 0.01-0.1mg/ml for many of the constituents Fig. 1, Table 1.

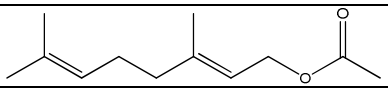
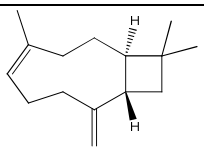
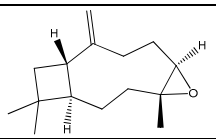
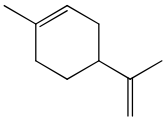
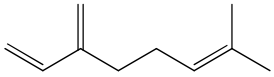
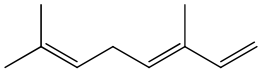
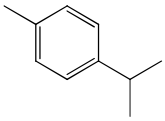
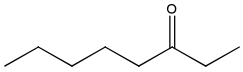
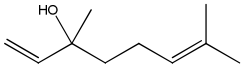
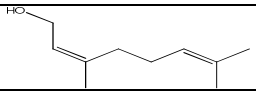
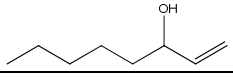
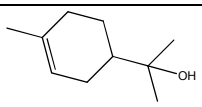
3.2 Effects of Melissa Essential oil (Mo) on Neuronal Viability

Previous studies [18,19] suggest that (Mo) may display neuro protective properties, via a sodium channel blockade mechanism. This potential was investigated using a primary neuronal culture system as described in [16]. At 0.001mg/ml, (Mo) elicited a modest preconditioning effect (increased viability versus saline control) (approx.10%, $p<0.05$), but at >0.01mg/ml (Mo) was shown to cause neurotoxicity (approx. 20%, $p<0.01$) Fig.2. the latter which is consistent with the $GABA_A$ receptor inhibitory pharmacology Fig.1. and [18].

In previous studies, both Melissa and Lavender essential oils have been shown to possess $GABA_AR$ inhibitory activity using [^{35}S] TBPS binding and electrophysiological methodologies [18,19]. Results presented in this study show that, in concentration dependent manner, most of the (Mo) constituents demonstrated only modest antagonistic effects on [^{35}S] TBPS binding with the exception of trans-ocimene Fig. 1. Which inhibited [^{35}S] TBPS binding at 0.01mg/ml compared to the control. In fact, trans-ocimene inhibited [^{35}S] TBPS binding with an apparent IC_{50} of 0.006 mg/ml (40μM) Table 1, while nerol and linalool demonstrated inhibition only at the highest concentration tested, which was 0.1 mg/ml. Concentrations (0.001 mg/ml) of whole (Mo) were shown to display modest beneficial effects upon neuronal viability, consistent with our previous studies indicating effects of both oils upon voltage-gated sodium channels at low concentrations [18,19,20 and unpublished studies], while at a higher concentration (0.1mg/ml) of (Mo) Fig. 2 and La (not shown) induced a neurotoxicity

effect. Interestingly, this study has revealed a new $GABA_A$ receptor ligand, namely trans-ocimene.

Table 1. Effects of Melissa and Lavender oil common constituents on the channel binding site of the $GABA_AR$ labelled by [^{35}S] TBPS

Chemical name	Structure	IC_{50}(mg/ml)	IC_{50}(µM)
Melissa essential oil		0.019	-
Geranyl acetate		17.0	87200
(E)-Caryophyllene		0.028	138
Caryophyllene oxide		0.584	2650
Limonene		0.256	1878
Myrcene		6.840	50205
Ocimene*		0.006	40
p-Cymene		0.035	262
3-Octanone		0.038	299
Linalool		0.900	5837
Nerol		0.079	513
Oct-1-en-3-ol		1.140	8891
α-Terpineol		0.211	1369

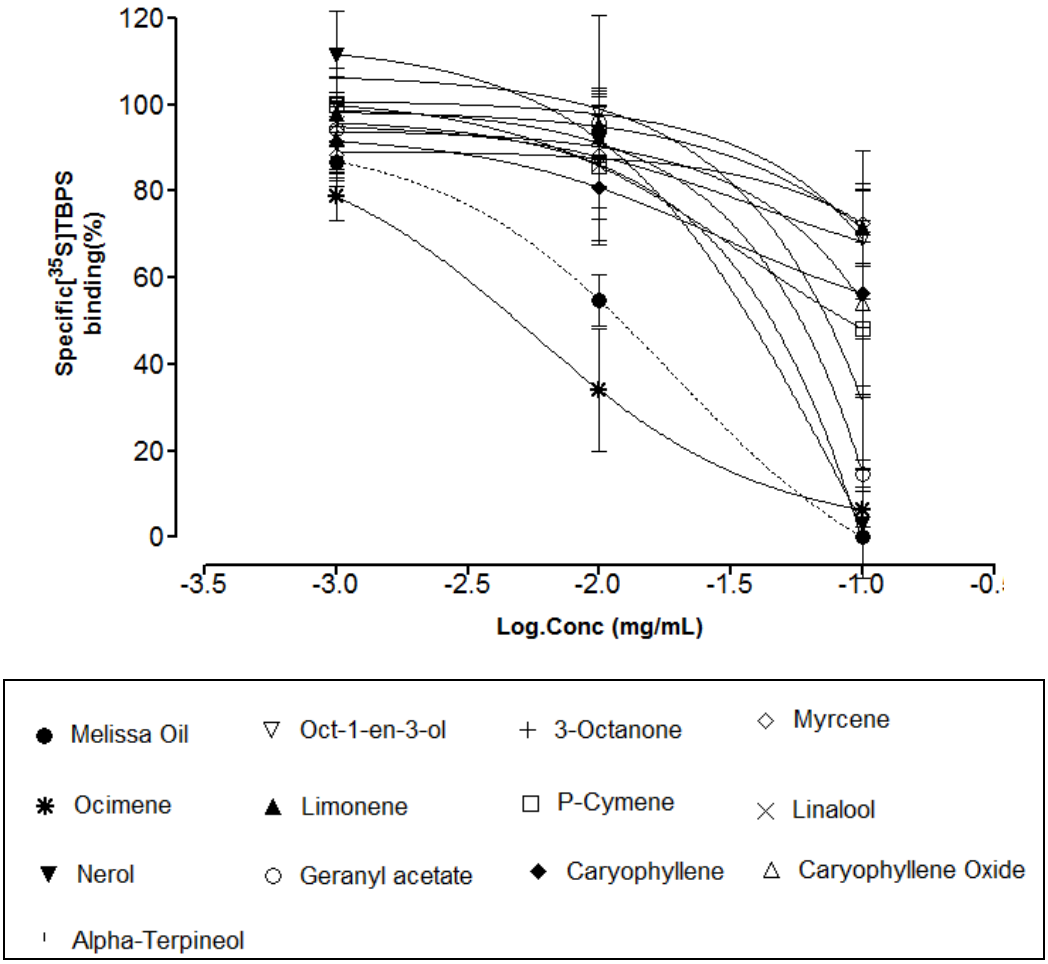


Fig. 1. The effects of the shared Melissa and Lavender essential oil constituents on [^{35}S] TBPS binding to well-washed rat forebrain membranes. Data are expressed as the mean±SD from at least three separate experiments

Although the (Mo) constituent(s) demonstrated antagonistic effects, potential therapeutic effects of (Mo) and its constituent(s) need to be investigated further. Bilobalide, a single natural product sesquiterpene trilactone derived from *Ginkgo biloba*, which has been used for the treatment of AD displayed a similar inhibitory effect on $GABA_A$ receptor as seen herein, and also neuroprotective effects, presumably through other mechanisms [21], again similar to that observed for Melissa essential oil. Electrophysiological studies have shown that (Mo) depress membrane excitability at low concentrations [18], despite this effect upon $GABA_A$ receptor at higher concentrations. Hence, it can be hypothesized that the potential therapeutic effects of (Mo) on the $GABA_A$ receptor is dominated by neuronal depression (via voltage-dependent sodium channel blockade, demonstrated by previous published electrophysiology studies [18,19]).

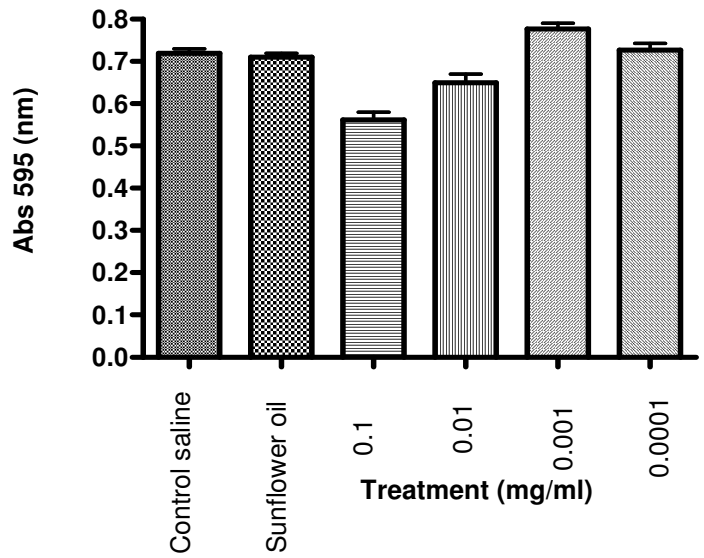


Fig. 2. Dose-dependent effects of Melissa oil (Mo) on primary neuronal culture viability. Results are mean absorbance ± SEM for n = 6 individual replicates. Mild toxicity at high concentrations with low affinity to $GABA_A$ receptor inhibitory properties/ modest protection at 0.001 mg/ml

4. CONCLUSION

The present study has identified one compound (trans-ocimene) which likely represents the $GABA_A$ receptor inhibitory component previously identified in Mo and La. Therefore, the results of this study provided a better understanding of the pharmacology of the Melissa essential oil and its constituents related to BPSD and in other central nervous system disorders such as refractory epilepsy and chronic pain states.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

This study was partly funded by the Alzheimer's Society (UK).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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