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Pharmacological and neuroprotective profile of an essential oil derived from leaves of *Aloysia citrodora* Palau

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Keywords
Aloysia; Alzheimer's disease; antioxidant activity; essential oils; neuroprotection

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Abstract

Objectives The Jordanian 'Melissa', (*Aloysia citrodora*) has been poorly studied both pharmacologically and in the clinic. Essential oils (EO) derived from leaves of *A. citrodora* were obtained by hydrodistillation, analysed by gas chromatography-mass spectrometry (GC-MS) and were investigated for a range of neurobiological and pharmacological properties, as a basis for potential future use in drug discovery.

Methods A selection of central nervous system (CNS) receptor-binding profiles was carried out. Antioxidant activity and ferrous iron-chelating assays were adopted, and the neuroprotective properties of *A. citrodora* EO assessed using hydrogen peroxide-induced and β-amyloid-induced neurotoxicity with the CAD (Cath.-a-differentiated) neuroblastoma cell line.

Key findings The major chemical components detected in the *A. citrodora* EOs, derived from dried and fresh leaves, included limonene, geranial, neral, 1, 8-cineole, curcumene, spathulenol and caryophyllene oxide, respectively. *A. citrodora* leaf EO inhibited [^{3}H] nicotine binding to well washed rat forebrain membranes, and increased iron-chelation *in vitro*. *A. citrodora* EO displays effective antioxidant, radical-scavenging activities and significant protective properties vs both hydrogen peroxide- and β-amyloid-induced neurotoxicity.

Conclusions *A. citrodora* EO displays a range of pharmacological properties worthy of further investigation to isolate the compounds responsible for the observed neuroactivities, to further analyse their mode of action and determine their clinical potential in neurodegenerative diseases.

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder with characteristic clinical and pathological features associated with loss of neurons in certain brain areas, including cholinergic and glutamatergic systems, leading to impairment of memory, pyscho-behavioural disturbances and deficits in activities of daily living, which eventually leads to death.[1–3] In 2010, approximately 35 million people worldwide were suffering from AD, and this number is believed to increase to 66 million by 2030.[2] To date, limited therapeutics are available, which target the associated neuronal systems, namely acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) glutamate channel blockers.[4]

The interest in medicinal plant research and the aroma-therapeutic effects of essential oils (EOs) in humans has increased in recent years, especially for the treatment of neuropathologies with profound social impact such as AD. Oxidative stress plays a central role in the initiation and progression of AD. The brain is particularly vulnerable to oxidative damage because of its elevated oxygen utilisation rate; high content of polyunsaturated lipids, which are susceptible to lipid peroxidation; accumulation of transition

metals, which are capable of catalysing the formation of reactive oxygen species (ROS) and relative paucity of cellular antioxidants.[5–7] Intracellular and extracellular amyloid beta-protein (Aβ) accumulation and deposition are major features of AD.[4] This neurotoxic protein inhibits the electron transport chain in mitochondria, decreases the respiratory rate, induces the release of ROS and may also cause neurotoxicity through the direct production of ROS via its interaction with transition metals and lipid membranes.[7] To combat the cytotoxicity of ROS, cells are endowed with a variety of antioxidant defence mechanisms, including respective protective enzymes, namely catalase, superoxide dismutase, glutathione and glutathione peroxidase, as well as free-radical scavenger, such as ascorbate and vitamin E.[4,6] Natural antioxidants derived from a multitude of plants display cytoprotective properties *in vitro* and have a long history of use for human health benefits.[8]

Several plant species are used in medical herbalism for their effects on anxiety, restlessness, excitability and depression. These include lemon balm (*Melissa officinalis*), lavender (*Lavandula angustifolia*), chamomile (*Matricaria chamomilla*), bergamot (*Monarda* species), neroli (*Citrus* × *aurnatium*) and valerian (*Valeriana officinalis*).[9,10] Several recent clinical trials have concurred with the value of aromatherapy in people with dementia.[9,11] The safety of these EO-based approaches has also been established in clinical populations. Despite this, however, the central mechanisms by which the EOs exert their effects are largely unknown. *Aloysia citrodora* Palau Family (Verbenaceae), also known as lemon verbena, is a perennial plant that grows widely in South and Central America and in various parts of the Middle East, including Jordan. The plant has long been used in traditional medicine to prepare a tea for its calming effects, sedative action and to counter depression.[12–14] There is a large variety of phytopharmaceutical preparations containing this plant or its extracts used as an antipyretic, antispasmodic and diuretic agents.[12,15] Furthermore, this plant is used in the food industry to flavour different products.

Some plant material described as *M. officinalis* (known in Arabic as 'melissa') was identified as *A. citrodora* (also known locally as melissa); both species have similar traditional uses and produces a yellow to light green EO with special aroma similar to that of lemon.[16] *M. officinalis* plant has been of particular interest on account of its sedative, cognitive-enhancing and anxiolytic-like effects, all relevant pathophysiological actions seen in AD.[9,17–19] Very limited data are available that explore the effects of *A. citrodora* oils pertinent to AD, in comparison with *M. officinalis.* Detailed pharmacological research is required to verify its potential effectiveness. In this study, the EO derived from the leaves of *A. citrodora* was validated, prepared, analysed and investigated for the first time for a range of neurological properties in cell-free systems, including radioligand binding, radical-scavenging and iron-chelation properties. The potential neuroprotective properties of this EO against oxidative stress and Aβ-induced neurotoxicity were further investigated using an in-vitro neuronal cell line system to evaluate the potential of this EO as sources of potent multitargeted therapeutics for treatment of neurodegenerative diseases.

Material and Methods

Plant material

Fresh leaves of *A. citrodora* were collected from plants growing in the gardens of the Agricultural College of the University of Jordan in spring 2011, when growth rates were maximal. The plant was authenticated by Professor Suleiman Al-Olimat from Department of Pharmaceutical Sciences, Faculty of Pharmacy, The University of Jordan, Amman (deposited herbarium voucher specimen reference: AC-V1). A portion of collected leaves was dried carefully under shade at room temperature and then homogenised to fine powder and was stored in air within tight bottles.

Essential oils preparation

Each 500 g of fresh and dried leaves of *A. citrodora* was subjected to hydrodistillation with 1 l of water using a Clevenger-type apparatus (JSOW, Haryana, India) for 3 h. The oils obtained for each specimen were dried with anhydrous sodium sulphate and stored at 4°C in amber glass vials until analysis. For all experiments, dilutions of pure EOs stock were performed fresh on the day of the assay.

Gas chromatography–mass spectrometry

The GC–MS analyses were performed using an AutoSystem XL GC coupled to a TurboMass quadrupole mass spectrometer (Perkin-Elmer, Shelton, Connecticut, USA). Chromatography was performed on a 30 m × 0.25 mm ID × 0.25 μm DB-5 MS column (J & W Scientific Inc., Rancho Cordova, CA, USA) using a temperature programme of 40–300°C at a rate of 3°C/min. The carrier gas was helium at a flow rate of 1 ml/min and the injection volume was 1 μl (split 1:10) at 220°C, via an autosampler. Detection was by MS, fitted with electrospray ionisation source operated at 70 eV, with a source temperature of 180°C; mass spectra were recorded in the range *m*/*z* 38–600. The operating software was Turbomass, version 4.1.1 (Perkin-Elmer). The *A. citrodora* oils were diluted to 1.0% (v/v) with diethyl ether (GLC pesticide

residue grade, Fisher Scientific, Ltd. Loughborough, UK) prior to analysis. Retention indices (RIs) were determined in relation to a series of *n*-alkanes (C10–C16, Supelco, Poole, UK) and compounds were identified by comparing the RIs and mass spectra with published data. Percentage compositions of detected compounds were calculated by integrating all peaks in total ion chromatograms.

Radioligand binding profile

A series of concentration-dependent competition binding assays were performed as described previously[20] using [^{35}S] t-butylbicyclophosphorothionate (TBPS), [^{3}H] flunitrazepam, [^{3}H] MK801 and [^{3}H] nicotine binding assays.

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

Radical-scavenging capacity was determined according to the technique reported by,[6] using the EO at concentrations ranging from 0.0001 to 1 mg/ml. The mixture was shaken vigorously and allowed to reach a steady-state at room temperature for 30 min. Decolourisation of DPPH was determined by measuring the absorbance at $\lambda = 517$ nm with a SpectroScan 80D spectrophotometer (Biotech Engineering Management Co. Ltd. Nicosia – Cyprus). Butylated hydroxyanisole (BHA) was employed as the positive control.

Ferrous ion-chelating activity

The ferrous ion-chelating activity of EOs was assessed by the ferrozine assay as previously described.[21] The reaction was initiated by adding ferrozine (0.01 M), the mixture shaken vigorously and left aside at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. EDTA (0.1%) was used as a positive control.

CAD cell culture

(Cath.-a-differentiated) cultures were grown at 37°C and in 5% CO2 in 75 cm^2 tissue culture flasks (Sarstedt, Newton, NC, USA) in Dulbecco's modified eagles' medium DMEM/F-12 Media – GlutaMAX-I (GIBCO, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Sigma, St Louis, MO, USA) Cells were passaged every 6–7 days at a 1:4 dilution.

Treatment of cell cultures with H_2O_2 and *Aloysia citrodora* essential oil

The hydrogen peroxide insult was performed at different concentrations to determine the dose-dependent effects on CAD cell survival. A stock solution of H_2O_2 (2500 μM) in PBS was freshly prepared. A series of concentrations were then prepared using Dulbecco's modified eagles' medium DMEM/F-12 Media – GlutaMAX-I supplemented with 10% FBS to achieve final concentrations of 250, 200, 150, 100 and 50 μM, respectively. Subconfluent cultures (70–80%) was dislodged by gentle pipetting; transferred and centrifuged at $200 \times g$ for 5 min at 4 °C. The pellet was then resuspended in DMEM/F-12 Media GlutaMAX-I supplemented with 10% FBS. The cell suspension was immediately plated in 24-well plates and incubated at 37°C and 5% CO_2. After 24 h, the media were replaced with media containing the different H_2O_2 concentrations prepared earlier and grown in culture for further 24 h after which an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. The effect of the EO was initially investigated by applying different concentrations of the oil (0.0001, 0.001, 0.01, 0.1 mg/ml) for 24 h to CAD cell cultures. Viability of neurons was assayed using a standard MTT assay.

Preconditioning and neuroprotection study

Survival of CAD cells was assessed after 24 h pretreatment with *A. citrodora* oil (0.001 and 0.01 mg/ml (w/v)) followed by 24 h post-treatment with 250 μM H_2O_2 (i.e. *A. citrodora*-containing media removed before addition of H_2O_2 – preconditioning protocol). A second set of experiments were performed when the *Aloysia*-containing media was not removed prior to exposure to the H_2O_2 – neuroprotection method). $N = 12$ well replicates from three independent cultures were carried out ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

MTT cell viability assay

Phosphate buffered saline (PBS) (136.9 mM 2.68 mM KCl, 4.3 mM Na_2HP0_4, 1.4 mM KH_2P0_4, pH 7.4) containing 5 mg/ml MTT was added to the cultures and incubated at 37°C and in 5% CO2 for 2.5 h. Then MTT-containing medium was removed, the surface of the wells was rinsed with PBS before the application of isopropanol. The optical density of sample was spectrophotometrically read at 595 nm (Thermo Labsystems Multiskan Ascent, V1.3, Basingstoke, UK).

Treatment of cell cultures with β-Amyloid

Human β-amyloid peptide, Ascent Scientific, UK, was dissolved in DMSO (dimethyl sulphoxide) at 1 mg/ml and stored at −80°C until use. The peptide was then incubated for 72 h at 37°C, 5% CO_2, without agitation. The peptide was then diluted to the required concentration using DMEM/F12 plus GlutaMAX +10% Fetal Calf Serum (FCS), inverted and gently added to neuronal cultures. DMSO in DMEM/F12 plus GlutaMAX +10% FCS was used as a control.

CytoTox 96 nonradioactive assay

CytoTox 96 assay kit (Promega Corporation, Madison, WI, USA) quantifies LDH (lactate dehydrogenase) levels as a measure of cell lysis. Media were removed from cells and centrifuged at 13 000 × *g* for 2 min. The supernatant containing the LDH was retained and the pellet resuspended in PBS, using the same volume removed from the cells, and placed back into the respective well of the 24-well plate. The plate was then frozen for 2 h at −20°C to lyse all remaining cells. Cells were subsequently thawed and centrifuged at 13 000 × *g* for 2 min; again, the supernatant was retained as a measure of total remaining LDH. Dilutions, 1:10 and 1:20, were made of each cell culture supernatant using PBS as the diluent with DMEM/F12 plus 10% FCS used to calibrate the assay. Equal volumes of substrate mix was added to test sample and incubated for 30 min at room temperature in the dark. Reactions were halted with acid stop solutions and the absorbance measured at 490 nm on a Multiskan Ascent Plate Reader, version 2.6 (Basingstoke, UK). Cell lysis in the media supernatant was calculated as a percentage of the sum of total LDH absorbance, combining the absorbance of positive control freeze–thawed cells and absorbance of media containing LDH.

Statistical analysis

Repeated measures ANOVA were performed in triplicate using GraphPad Prism 5.0 (Graphpad, La Jolla, CA, USA) program. *P* values below 0.05 were considered significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Results

The chemical compositions of the EOs from *A. citrodora* collected from Jordan are summarised in Table 1 (in the order of elution). A total of 63 compounds were identified representing 93% of the total oil. The major components for dried and fresh oils included limonene (13.6–20.1%), geranial (6.3–20.1%), neral (3.7–15.1%), 1,8-cineole (9.2–9.4%), curcumene (3.5–6.3%), spathulenol (3.1–5.0%) and caryophyllene oxide (2.2–8.4%), some quantitative variation in detected terpinoids and phenylpropanoid derivatives was observed between the dried and fresh leaves.

Radioligand binding profile

A series of concentration-dependent (0.0001–0.1 mg/ml) competition binding assays were performed as described previously,[20] using [^{35}S] TBPS, [^{3}H] flunitrazepam, [^{3}H] MK801 and [^{3}H] nicotine binding assays. No effects were observed for [^{35}S] TBPS, [^{3}H] flunitrazepam, [^{3}H] MK801 binding over the concentration range tested. In contrast, *A. citrodora* EO elicited a concentration-dependent inhibition of [^{3}H] nicotine binding (apparent $IC_{50} = 0.0018 \pm 0.0008$ mg/ml ($n = 3$ individual experiments).

Antioxidant activity in cell-free systems

DPPH radical-scavenging activity

DPPH is widely used to test the ability of compounds to act as free-radical scavengers or hydrogen donors and to evaluate antioxidant activity of plants. EOs of dried and fresh leaves exhibited a comparable radical-scavenging activity vs the DPPH radical as 0.1% BHA as reference standard (Figure 1).

Fe^{2+} chelating ability

A robust antioxidant capacity was observed with the *A. citrodora* EO using the iron-chelating test system. *A. citrodora* EOs derived from the fresh leaf displayed a significant dose-dependent iron-chelating property (between 0.01–1.0 mg/ml), compared with 0.1% EDTA as positive control (Figure 2).

Neuroprotective activity of *Aloysia citrodora* Palau vs H_2O_2- induced neurotoxicity in CAD cells

In the first instance, various concentrations of *A. citrodora* EO and H_2O_2 were assessed for neurotoxic activity. Concentrations above 0.1 mg/ml *A. citrodora* and 100 μM H_2O_2 elicited a neurotoxic effect (Figures 3–5).

200 μM and 250 μM H_2O_2 elicited a significant neurotoxic effect up to 62% loss of cell viability. Pre-exposure to *A. citrodora* EO and removal before insult had no effect upon levels of neurotoxicity with 250 μM H_2O_2, indicating that the oil has no preconditioning properties (results not shown).

In contrast to the preconditioning approach, in the presence of 0.01 and 0.001 mg/ml *A. citrodora* EO, 250 μM H_2O_2 (neuroprotection protocol) failed to elicit a neurotoxic effect, thus displaying a complete neuroprotection property at both concentrations.

Neuroprotective activity of *A. citrodora* oil vs amyloid-induced neurotoxicity in CAD cells

CAD cell cultures were subjected to a range of concentrations of synthetic β-amyloid, and cell viability assessed using an LDH release assay. Clear neurotoxicity was observed spanning the 2.5–25 μM β-amyloid concentration range, with up to 70% loss of cell viability (Figure 6a). *A. citrodora* (0.01 and 0.001 mg/ml) elicited a significant dose-dependent neuroprotection from 10 μM β-amyloid (up to approximately 50%) (Figure 6b).

Table 1 Percentage composition of the EOs from fresh and dried leaves of *Aloysia citrodora* grown in Jordan; data obtained from the gas chromatography-mass spectrometry total ion chromatograms (based on all peaks)

Compound	Fresh leaf oil percentage composition	Dried leaf oil percentage composition	Experimental RI	Published RI[a]
α-Thujene	0.1	0.1	923	930
α-Pinene	0.6	0.8	929	939
Sabinene	2.0	1.5	966	975
β-Pinene	0.1	0.1	969	979
1-Octen-3-ol	0.4	0.7	976	979
6-Methyl-5-hepten-2-one	1.5	3.4	980	986
Myrcene	0.3	0.3	983	991
Hexenyl acetate	0.1	Nd	1000	(3*E*)-: 1002 (3*Z*)-: 1005
2-δ-Carene	Tr	0.1	1009	1002
Cymene	0.1	0.3	1018	*o*-: 1026 *p*-: 1025
Limonene	**13.6**	**20.1**	1025	1029
1,8-Cineole	**9.2**	**9.4**	1027	1031
β-Ocimene	1.6	0.1	1041	(*Z*)-: 1037 (*E*)-: 1050
Bergamal	Nd	0.1	1049	1057
γ-Terpinene	0.1	0.3	1052	1060
cis-Sabinene hydrate	0.7	1.0	1066	1070
Perillene	Tr	0.1	1095	1101
Linalool	0.6	0.8	1098	1097
α-Pinene oxide	0.2	0.2	1102	1099
trans-Thujone	Nd	Tr	1115	1114
trans-*p*-Mentha-2,8-dien-1-ol	0.2	0.5	1121	1123
α-Campholenal	Tr	0.1	1125	1126
cis-Limonene oxide	0.1	0.2	1131	1137
cis-*p*-Mentha-2,8-dien-1-ol	0.4	0.8	1137	1138
Citronellal	0.2	0.1	1154	1153
Sabina ketone	Nd	0.1	1158	1159
Rosefuran epoxide	0.6	0.9	1172	1177
Terpinen-4-ol	Tr	0.9	1182	1177
trans-*p*-Mentha-1(7),8-dien-2-ol	1.6	0.1	1183	1189
α-Terpineol	3.6	3.6	1199	1189
Carveol	Tr	0.4	1224	*trans*-: 1217 *cis*-: 1229
Nerol	0.6	0.1	1231	1230
Neral	**15.1**	**3.7**	1246	1238
Carvone	Nd	1.0	1250	1243
Piperitone	0.6	0.3	1259	1253
Geranial	**20.1**	**6.3**	1278	1267
Thymol	Nd	0.1	1300	1290
Carvacrol	Nd	0.6	1308	1299
trans-Carvyl acetate	Nd	Tr	1340	1342
Piperitenone	Nd	Tr	1344	1343
α-Cubebene	Nd	Tr	1351	1351
Eugenol	Nd	0.1	1359	1359
α-Copaene	0.3	0.8	1379	1377
Geranyl acetate and β-bourbonene[b]	1.2	1.6	1385	Geranyl acetate: 1381 β-Bourbonene: 1388
α-Cedrene	0.2	0.6	1417	1412
(*E*)-Caryophyllene	2.6	2.1	1421	1419
β-Cedrene	0.1	0.3	1424	1421
β-Copaene	Tr	0.1	1430	1432
α-Humulene	0.2	0.3	1454	1455
Allo-aromadendrene	0.3	0.8	1458	1460
γ-Muurolene	1.5	0.6	1479	1480
Curcumene	**3.5**	**6.3**	1482	γ: 1483 ar: 1481
Bicyclogermacrene	1.9	0.4	1493	1500
Bisabolene	Nd	0.1	1506	B-: 1506 (*Z*)-α-: 1507
β-Curcumene	0.9	Tr	1507	1516
γ-Cadinene	0.2	0.4	1511	1514
δ-Cadinene	0.2	0.4	1514	1523
β-Sesquiphellandrene	Tr	Tr	1519	1523
trans-Calamenene	Nd	Tr	1525	1529
Nerolidol	0.5	0.7	1556	(*E*)-: 1563 (*Z*)-: 1533
Spathulenol	**3.1**	**5.0**	1568	1578
Caryophyllene oxide	**2.2**	**8.4**	1571	1583

Tr: < 0.1%; Nd, not detected. Emboldened data accounts for the major components. All compounds identified by comparing experimental retention indices (RI; calculated against an *n*-alkane series with chromatography performed using a DB-5MS phase), and by comparing mass spectra with published data (Adams, 2001; NIST, 2008).[22,23].

[a]Published Retention Index. [b]Co-elution.

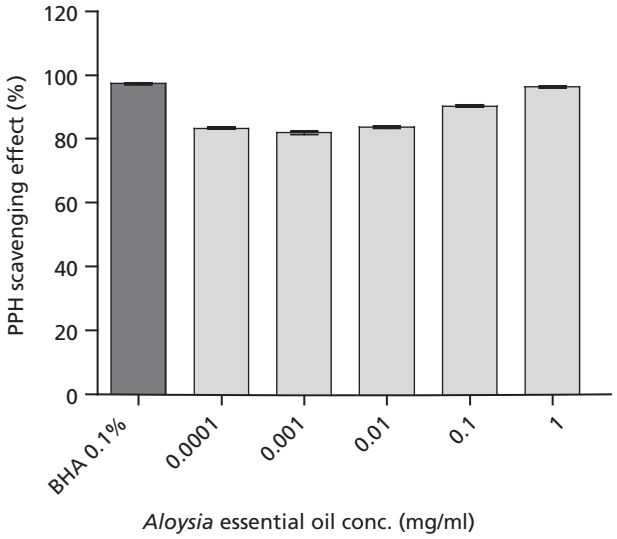

Figure 1 Antioxidant activity of *Aloysia citrodora* dried essential oil using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Values are mean ± Standard Error of the Mean (SEM), $n = 3$, $P < 0.001$.

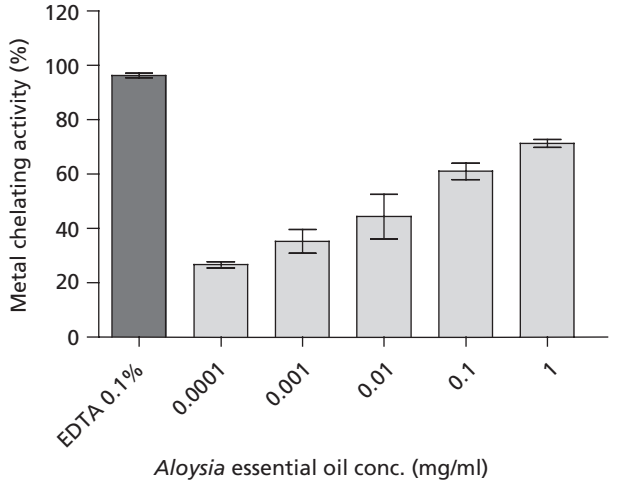

Figure 2 Metal chelation property of *Aloysia citrodora* essential oils using a metal iron-chelating assay. Data are compared with standard, EDTA (0.1%). Values are means ± standard deviation, from at least three separate experiments.

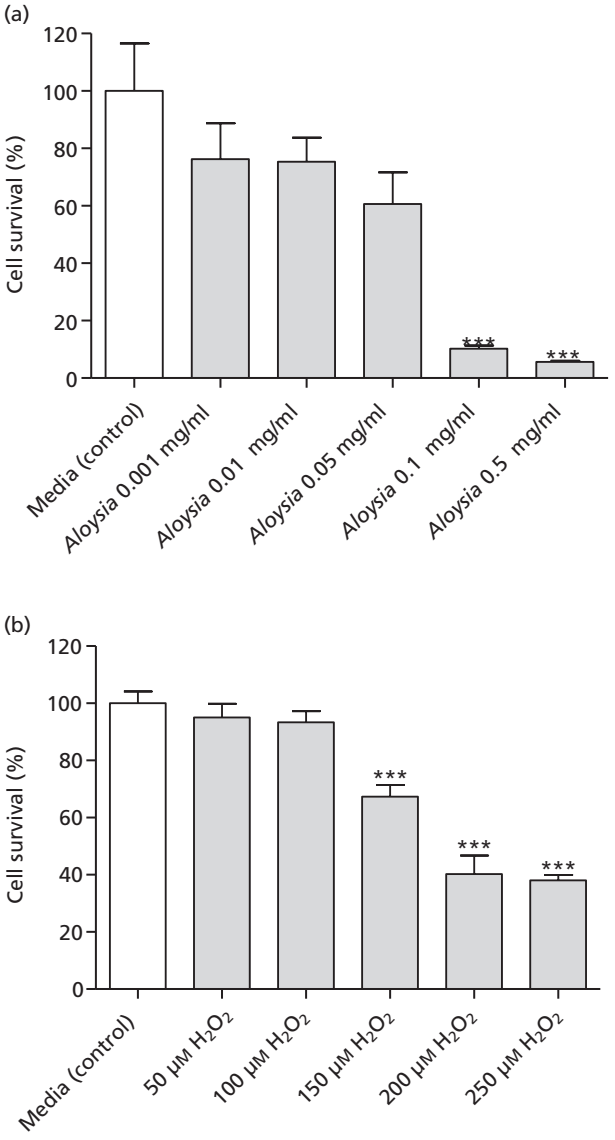

Figure 3 (a) Treatment of CAD (Cath.-a-differentiated; 24 h) cell cultures with *Aloysia citrodora* (***$P < 0.001$ compared with media control). Concentrations 0.001, 0.01 and 0.05 mg/ml elicited no significant neurotoxic effect upon CAD cells. (b) Insult of CAD (24 h) cell cultures with hydrogen peroxide (***$P < 0.001$ compared with control).

Discussion

More than 60 components, comprising of mainly mono- and sesquiterpenoids were detected in the EOs obtained from Jordanian *A. citrodora* dried or fresh leaves (major components listed in Table 1). Irrespective of whether derived from dried or fresh leaves, limonene, geranial, neral and 1,8-cineole were the dominating monoterpenoids. The qualitative composition of the EOs from fresh and dried *A. citrodora* leaves was similar, although some quantitative differences were observed; notably, the EO from fresh leaves contained a higher percentage composition of geranial and neral (isomeric monoterpenoids collectively known as citral) compared with the EO from dried leaves, whereas dried leaves EO contained a higher percentage of limonene, compared with the fresh leaves EO. The monoterpenoids limonene, geranial, neral and 1,8-cineole have previously

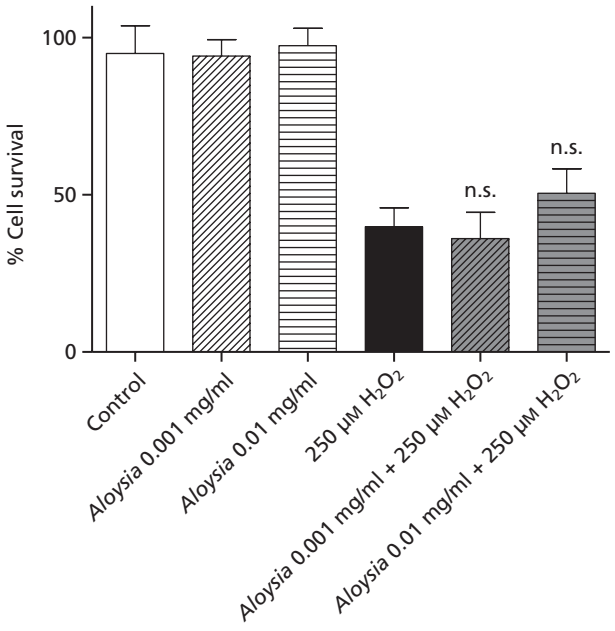

Figure 4 Preconditioning of CAD (Cath.-a-differentiated) cell cultures with *Aloysia citrodora* before 24 h insult with 250 μM hydrogen peroxide. No significant difference in comparison with 250 μM H_2O_2-treated cells.

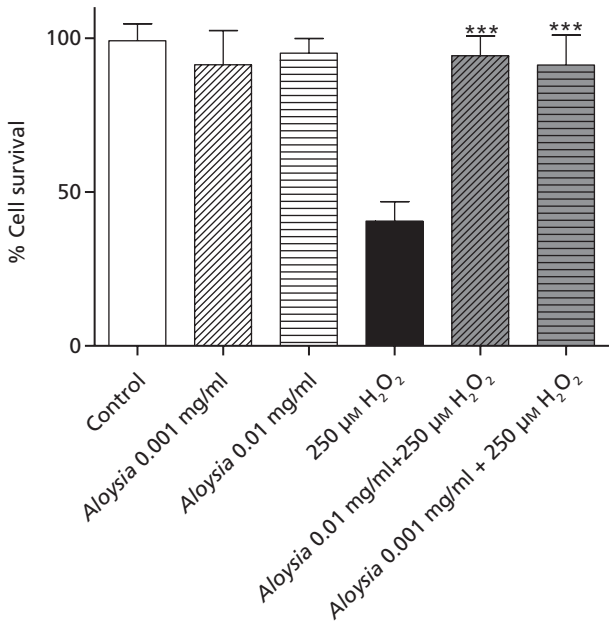

Figure 5 Protection of CAD (Cath.-a-differentiated) neuronal cells with *Aloysia citrodora* from 24 h 250 μM hydrogen peroxide insult (***$P < 0.001$ compared with 250 μM H_2O_2-treated cells)

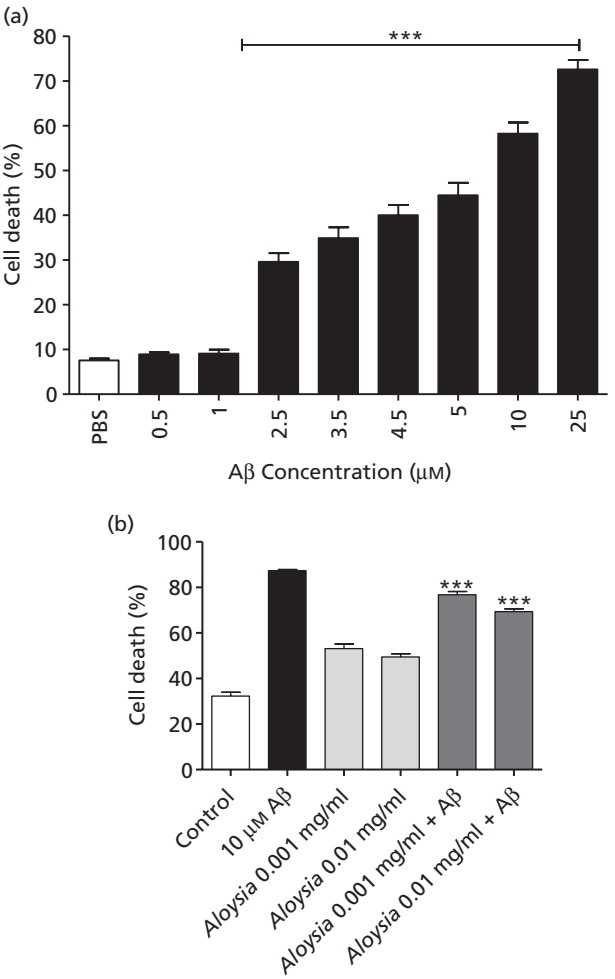

Figure 6 (a) Insult of CAD (Cath.-a-differentiated; 24 h) cell cultures with β-amyloid (***$P < 0.001$ compared with PBS control). (b) Protection of CAD cells with *Aloysia citrodora* from 10 μM β-amyloid insult (***$P < 0.001$ compared with 10 μM Aβ treated cells). $N = 6$ independent experiments.

been reported as some of the main constituents of EOs from *A. citrodora* (including EOs documented from species described as synonyms for *A. citrodora*.[14,24–27] It is essential to characterise EOs investigated pharmacologically, as oil composition from a particular species may vary considerably due to various factors, including genetic and environment influences.[28–34]

The EOs obtained from dried or fresh *A. citriodora* leaves shared the terpenic components and were characterised by high contents of monoterpenes and sesquiterpenes. The fraction of monoterpene was enriched, mainly due to an increase in the contribution of limonene with the dried leaf oil. All other components remained more or less unchanged both qualitatively and quantitatively. According to the literature, limonene is the component found to occur in

highest quantities in EOs of the genus *Lippia*, followed by p-cymene, α-pinene, camphor, β-caryophyllene, linalool and thymol in a decreasing order.[18,19,24–34] In our study, α-pinene, α-terpinene, sabinene, linalool and caryophyllene were also identified in low amounts. However, our results did not show the presence of carvacrol, camphor and thymol, which have been mentioned in other studies regarding *A. citrodora*.[31,35] These monoterpenoids have previously been reported as some of the main constituents of EOs from *A. citrodora* from different parts of the world.[18,19,24–27] The literature emphasises that a variety of geographical and ecological factors can lead to qualitative and quantitative differences in the EO produced. At the same time, a number of other factors can influence its composition, such as the developmental stage of the plant, its physiology, the age of leaves and growing conditions. Furthermore, the chemical composition of the EO is also affected by the isolation procedure and analysis conditions.[18,19,24–36]

Melissa officinalis is highly valued in herbal medicine and is native to Europe and Western Asia. *A. citrodora* is a common plant in Mediterranean region. Both species have been described by the same common name ('Melissa') and have been used in traditional herbal medicine as calming agents, raise attention and help to counter depression with a good safety profile.[27] However, confusion over plant names and their identity emphasises why it is essential to authenticate and chemically characterise plants used for medicinal applications, as performed for the *A. citrodora* investigated in this present study. Our studies investigating the composition of *M. officinalis* EOs with mechanistic effects relevant to the CNS report the major components to be carophyllene (10–26%), geranial (15–31%) neral (10–22%);[23] the latter two terpinoids are also major components in the *A. citrodora* EOs. However, limonene and 1,8-cineole were not major components of *M. officinalis* EOs,[23] in contrast to their high percentage compositions in the *A. citrodora* EOs.

Pharmacological targets for the reduction of anxiety, agitation and aggression include the neurotransmitter systems, acetylcholine, glutamate and Gamma-Aminobutyric acid (GABA). In this work, we have characterised the effect of *A. citrodora* EOs on two major binding sites of the $GABA_A$ receptor (the benzodiazepine site and the ion channel site) to detect any $GABA_A$ receptor modulatory activity. To confirm selectivity, interactions with other common ligand gated ion channel receptors (NMDA) and neuronal nicotinic receptor were also investigated. *A. citrodora* EOs inhibited [^{3}H] nicotine binding in a concentration-dependent manner with no significant effects on other major ligand gated ion channels expressed in the central nervous system. This is in contrast to the European lemon balm oil, where no significant effect on [^{3}H] nicotine binding activity was detected (up to 0.1 mg/ml), but displayed a clear inhibitory effect on [^{35}S] TBPS binding to the $GABA_A$ receptor channel site.[23] Neuronal nicotinic acetylcholine receptors represent novel targets for CNS therapeutics and may have substantial roles in mediating antinociception and modulating cognitive performance. The oil constituents responsible for this activity require identification. The potential for developing nAChR ligands (particularly agonists) for use in AD, Parkinson's disease, smoking cessation, anxiety, depression and schizophrenia remains high.

Natural antioxidants that are present in medicinal herbs are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. These antioxidants, which can scavenge free-radicals include many flavonoids and other phenolic compounds. In the present study, we have evaluated the free-radical scavenger activity of *A. citrodora* EO. The *A. citrodora* oil exhibited a radical-scavenging activity at all tested concentrations. This activity has been reported earlier in several studies for related plants.[36–39]

Oxidative cellular damage is a multiphase process involving free-radical chain initiation and propagation steps. An important mechanism of antioxidative action is the chelation of transition metals, thus preventing catalysis of hydrogen peroxide decomposition via the Fenton-type reaction[31] Therefore, the ability of *A. citrodora* EO to chelate iron (II) was investigated. It has been reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilising the oxidised form of the metal ion.[40] As can be seen, the oil derived from fresh leaves was effectively capable of chelating iron (II) and did so in a concentration-dependent manner to approximately 70% of the level of the standard chelator, EDTA. The iron (II) chelating activity of the *A. citrodora* EO is of significance, because it has been proposed that the transition metal ions contributes to the oxidative damage in many neurodegenerative disorders and one of the lines of treatments currently under investigation in the field is selective, low-affinity binding of transition metals.[40,41] Interestingly, this property is not shared by the European *M. officinalis* EO. This suggests that a comparison of compositions is worthy in an attempt to identify the neuroactive components underlying the pharmacological differences between the two EOs.

The pharmacological properties displayed by *A. citrodora* EO is consistent with a neuroprotective profile. Indeed, our studies showed, for the first time, that *A. citrodora* EO provides complete and partial protection vs H_2O_2- and β-amyloid-induced neurotoxicity, respectively, both relevant to oxidative stress-induced damage in major neurodegenerative disease.

Conclusions

In conclusion, the chemical analysis of the EOs from leaves of *A. citrodora* cultivated in Jordan showed the presence of limonene, geranial, neral, 1,8-cineole, curcumene, spathulenol and caryophyllene oxide as the main components. This study demonstrated for the first time that the *A. citrodora* EO possess nicotinic cholinergic, antioxidative and significant neuroprotective activities, all of which are of relevance to potential AD therapy, as well as other neurodegenerative diseases. We are currently attempting to delineate the neuroactive elements in this oil, using functional group-based fractionation, responsible for the biological activity reported herein, and to confirm its utility *in vivo*.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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