Non-toxic *Salvia sclareoides* Brot. extracts as a source of functional food ingredients: Phenolic profile, antioxidant activity and prion binding properties

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A R T I C L E   I N F O

A B S T R A C T

Salvia sclareoides is an aromatic herb native to Portugal, of which phenolic content (Folin–Ciocalteau method), chemical profile (HPLC/DAD), antioxidant activity (DPPH, β-carotene/linoleic acid assays), acute toxicity (MTT method, adapted for non-adherent cells), genotoxicity (short-term chromosomal aberration assay) and prion binding properties were evaluated in the acetone, water, ethanol, methanol and *n*-butanol extracts. The latter presented the highest phenolic content and antioxidant activity (DPPH assay), and was the single one with the flavonoids (+)-catechin, kaempferol *O*-glucoside and quercetin. Vanillic acid was the major component of all extracts but gallic, gentisic, caffeic, syringic, coumaric and ferulic acids were also found in some extracts. Only the *n*-butanol extract had components binding to the cellular form of human prion protein detected by NMR which showed specificity for two regions of the folded domain and for the unstructured N-terminal region. Extracts were not cytotoxic nor genotoxic, reinforcing the potential of *S. sclareoides* for nutraceutical purposes.

1. Introduction

Salvia is one of the largest genera of the Lamiaceae family and is widespread in the Mediterranean region, South-East Asia and Central America. *Salvia* species are reputed for their medicinal properties and they have been used in folk medicine to treat colds, wounds and skin infections, headache, cerebral ischaemia and memory disorders (Kivrak et al., 2009), as well as hepatitis (Jin, Quian, & Lu, 2011). Sage (*Salvia officinalis*) is cultivated in Europe and used as a culinary condiment and a food additive, and in traditional medicine for its antiseptic, astringent and spasmolytic effects. Spanish sage (*Salvia lavandulaefolia* Vahl.) and sage have also been used against memory loss in European folk medicine (Howes, Perry, & Houghton, 2003). In traditional Chinese medicine neurological pathologies, such as insomnia, are treated with *Salvia miltiorrhiza* Bge., which also decreases the intake of alcohol *in vivo* (Carai et al., 2000).

It is well established that oxidative stress, which results from the presence of excessive quantities of reactive oxygen species in biological systems, may cause cell membrane dysfunction and DNA damage. Many degenerative disorders, namely cardiovascular and brain diseases, arthritis, diabetes, cancer and immune system decline involve cellular damage possibly caused by free radicals. Antioxidant compounds play a crucial role in the treatment of these pathologies by acting as free radical scavengers, thus decreasing the extent of oxidative damage. Hence, evaluation of radical scavenging properties and antioxidant activity is relevant with plants claimed to have medicinal applications. A variety of *Salvia* species were reported to have promising radical scavenging activities, namely *S. officinalis* (Oboh & Hente, 2009), Turkish (Senol et al., 2010) and South African (Kamatou, Viljoen, & Steenkamp, 2010) species.

The antioxidant activity of *Salvia* species extracts has been related to their total phenolic content in a direct manner: extracts rich in polyphenols (mainly phenolic acids and flavonoids) usually have a higher antioxidant capacity (Tosun et al., 2009). Phenolic acids with antioxidant activity have been isolated from the extracts of the traditional Chinese herb *S. miltiorrhiza* (Zhao et al., 2011). These polyphenols acting as free radical scavengers have shown beneficial health-promoting effects in chronic and degenerative diseases. Some of them inhibit amyloid fibril formation and a new mechanism of action for these antioxidants has been proposed by Porat, Abramowitz, and Gazit (2006), based on structural constraints and specific aromatic interactions, which direct

polyphenol inhibitors to the amyloidogenic core. The polyphenols tannic acid, katacine and 2,2-bisepigallocatechin digallate were also studied against prion PrP^Sc (the infectious form of prion protein) and proved to inhibit the formation of fibrillar assemblies at the µM range (Porat et al., 2006).

More than 20 amyloid-related diseases, including type II diabetes, familial amyloidosis, Alzheimer's, Parkinson's, Huntington's and prion diseases are known, but therapeutical solutions for most of them are missing, which encourages chemists and biologists to further study polyphenols and their neuroprotective effects.

The subject of the present study is *Salvia sclareoides*, a species native to Portugal which exhibits a potent cholinesterase inhibitory activity (Rauter et al., 2007) of relevance to memory dysfunction and Alzheimer's disease. Extracts induced a significant inhibition of acetylcholinesterase, even at the concentration of 10 µg/ml, while the standard drug rivastigmine did not inhibit the enzyme at the same concentration. In addition, extracts from *S. sclareoides* were found to retard the rate of prion propagation in a cell-based screen (Martins et al., 2009). Herein, extracts from *S. sclareoides* are investigated with the following objectives:

(i) To search for functional food ingredients from *S. sclareoides*;
(ii) To determine the phenolic profile of *S. sclareoides* extracts obtained with solvents of different polarity;
(iii) To evaluate the antioxidant activity;
(iv) To evaluate the direct cytotoxicity and genotoxicity;
(v) To evaluate whether the extracts interact with human prion protein;
(vi) To demonstrate *S. sclareoides* benefits for nutraceutical and medicinal purposes.

2. Experimental

2.1. Plant material and extracts preparation

Aerial parts of *S. sclareoides* Brot. (Labiatae), collected at the Lizandro Estuary, Mafra, Portugal in May, were identified in the Herbarium João Carvalho Vasconcelos (LISI), Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal, where a voucher specimen was deposited. The air-dried and finely powdered aerial parts (280 g) of the plant were macerated overnight, in acetone, at room temperature (rt). The macerate was then filtered and concentrated to dryness, at 40 °C, under reduced pressure to give the acetone extract (11 g). The same procedure was performed to obtain the ethanol extract (3 g). The *n*-butanol and water extracts were prepared as follows: the aerial parts (100 g) were extracted firstly with dichloromethane at rt and solvent evaporation under reduced pressure, at 40 °C, gave the dichloromethane extract (2 g). The plant was then extracted with methanol to give, after evaporation of the solvent, the methanol extract (5 g). The last extraction was made with a 50% methanol aqueous solution. Methanol was evaporated and the aqueous phase was extracted with *n*-butanol, to give the butanolic extract (3 g). Finally, the aqueous phase was concentrated to dryness to give the water extract (8 g).

2.2. Determination of total phenolics

The content of phenolic compounds in *S. sclareoides* extracts was determined using Folin–Ciocalteau reagent (Miliauskas, Venskutonis, & Van Beek, 2004). For the preparation of the calibration curve, 20 µl aliquots of 0.024, 0.075, 0.105, 0.15 and 0.18 mg/ml gallic acid solution in water were mixed with 100 µl of tenfold water diluted Folin–Ciocalteau reagent and 80 µl of 7.5% (w/v) sodium carbonate solution. After 30 min at rt, the absorbance was measured at 765 nm on a UV/visible light spectrophotometer Shimadzu® and the calibration curve was drawn. For the

determination of plant extracts phenolic content, 20 µl of extract solution was mixed with the same reagents as described above, and after 1 h the absorption was measured. The experiment was carried out in triplicate. Total content of phenolic compounds in *S. sclareoides* extracts, expressed as gallic acid equivalents (GAE), was calculated as follows:

$$C = (c \times V)/m,$$

where C – total phenol content (mg GAE/g plant extract); c – concentration of gallic acid established from the calibration curve (mg GA/ml); V – volume of gallic acid/*Salvia* extract solution (=0.02 ml); m – weight of pure plant dry extract (g).

2.3. HPLC–DAD analysis

HPLC grade methanol (MeOH, 99.9%) was purchased from Merck (Darmstadt, Germany) and acetic acid (99.8%) from Riedel-de Haën (Seelze, Germany). Ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid, 99%), caffeic acid (3,4-dihydroxycinnamic acid, 97% predominantly *trans*), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid, 98%), gallic acid (3,4,5-trihydroxybenzoic acid, 97%), gentisic acid (2,5-dihydroxybenzoic acid, 98%), coumaric acid (*trans*-4-hydroxycinnamic acid, 98%), vanillic acid (4-hydroxy-3-methoxybenzoic acid, 97%), (+)-catechin hydrate [(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol, 98%], rutin hydrate (95%) and quercetin dehydrate (98%) were purchased from Sigma–Aldrich (Buchs, Germany). Chlorogenic acid [1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate), ⩾95%], and kaempferol 3-*O*-glucoside (⩾99%) were purchased from Extrasynthèse (Genay, France). Ultra-pure water was obtained from Milli-Q water purification systems (Millipore, Palm Springs, CA, USA). The stock solutions of individual standards (1000 mg l^{-1}) and samples were prepared in HPLC grade MeOH and used to prepare the working standard mixtures at the desired concentration. After experimental procedures, the stock solutions and working standards were wrapped in aluminium foil and stored at −4 °C to prevent photodegradation.

HPLC–DAD analyses were carried out on an Agilent 1100 Series LC system (Agilent Technologies, Waldbronn, Germany), composed by the following modules: vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostatted column compartment (G1316A) and the diode array detector (G1315B). The data acquisition and instrumental control were performed by the software LC3D ChemStation (version Rev.A.10.0 [1757], Agilent Technologies). Analyses were performed on a Mediterranean Sea 18 column, 150 × 2.1 mm, 5 µm particle size (Teknokroma, Barcelona, Spain). The mobile phase consisted on a mixture of MeOH (solvent A) and 2.5% acetic acid aqueous solution (solvent B). The applied gradient was 0–50 min: 30–80% A, 50–55 min: 80–30% A and hold at 30% A for 5 min and the flow rate was 0.5 ml min^{-1}. The analyses were performed at 25 °C and the injection volume was 10 µl with a draw speed of 200 µl min^{-1}. The detector was set at 280 nm. For identification purposes, the retention parameters of each extract were compared with the standard controls and the peak purity with the UV–visible spectral reference data.

2.4. Antioxidant activity

The antioxidant potential of *S. sclareoides* extracts (*n*-butanol, ethanol, methanol, acetone and water), was assessed by two spectrophotometric methods: 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging activity and β-carotene/linoleic acid bleaching assay. Tests were carried out with extracts and reference antioxidants: the synthetic butylated hydroxytoluene (BHT) and the natural antioxidant, ascorbic acid (AA), in concentrations ranging from 1 to 500 µg/ml. A blank sample consisting of the

same reagent mixture and experimental procedure but without extract, BHT or ascorbic acid was also carried out. The reagents (p.a.) DPPH, β-carotene, linoleic acid, Tween 40, DMSO, methanol, chloroform, ascorbic acid and BHT were purchased from Sigma–Aldrich (Buchs, Switzerland). After performing the activity experiments, each extract/control concentration causing 50% antioxidant activity (IC$_{50}$) for both methods used, was estimated by mathematical regression from a dose–response curve.

2.4.1. DPPH free radical scavenging assay

The DPPH radical scavenging activity of *S. sclareoides* extracts was measured according to Özgen et al (2006), with slight modifications. The DPPH test is based on the ability of the extracts to donate a radical hydrogen to scavenge the stable DPPH radical. When this radical reacts with the antioxidant compound, it is reduced with the loss of the deep violet colour to light-yellow. The absorbance was measured at 517 nm on a visible light spectrophotometer Shimadzu®. Each *S. sclareoides* extract (20 mg) was dissolved in methanol (1 ml), with the exception of the water extract, which was dissolved in ultra-pure water. DPPH solution in methanol (0.27 mM) was prepared daily, before absorbance measurements, and to this solution (185 μl), kept in appropriate disposable plastic cuvettes (Plastibrand®), the extract solution (15 μl) was added and the mixture shaken vigorously. Final extract concentration ranged from 1 to 500 μg/ml, whereas DPPH solution was 0.25 mM. After 30 min incubation in the dark at rt, absorbance at 517 nm was measured. The experiment was carried out in triplicate. Reference synthetic antioxidant BHT (dissolved in methanol) and natural antioxidant ascorbic acid (dissolved in water) were also tested in the same concentrations of *S. sclareoides* extracts for comparison purposes. A blank sample consisting of the same reagent mixture and experimental procedure but without extract, BHT or ascorbic acid was always carried out. The %DPPH radical scavenging activity was calculated as follows:

DPPH radical scavenging effect (%)

$$= [(Abs_{blank} - Abs_{extract})/Abs_{blank}] \times 100,$$

where Abs_{blank} is the absorbance of the blank sample (time = 30 min) and $Abs_{extract}$ is the absorbance of the sample with the plant extract (time = 30 min).

2.4.2. β-Carotene/linoleic acid bleaching assay

The β-carotene bleaching test estimates the capacity of a plant extract solution to inhibit β-carotene oxidation in the presence of linoleic acid in pro-oxidation conditions. The acetone, *n*-butanol, ethanol, methanol extracts of *S. sclareoides* and BHT were solubilised in DMSO, whereas plant water extract and ascorbic acid solutions were prepared with distilled water. The antioxidant activity of *S. sclareoides* extracts using the β-carotene/linoleic acid assay was measured according to Miller (1971) with minor modifications. Briefly, a stock solution of β-carotene/linoleic acid mixture was prepared as follows: β-carotene (0.2 mg) was dissolved in chloroform (1 ml) and then linoleic acid (22 μl) and Tween 40 (200 mg) were added. Chloroform was completely evaporated using a vacuum evaporator. Then, distilled water saturated with oxygen (40 ml) was added under vigorous shaking. The reaction mixture (2 ml) was dispensed into test tubes, and each plant extract solution (0.5 ml) was added. The mixture containing the plant extract or the control antioxidants was incubated at 50 °C for 2 h and absorbances were acquired along the incubation period at 470 nm. BHT and ascorbic acid were used as standards. The experiments were carried out in duplicate. The final concentrations of chemicals in the test were as follows:

[β-Carotene] = 4 μg/ml; [Linoleic acid] = 400 μg/ml; [*Salvia* extract] = 1–500 μg/ml. The antioxidant activity (AA) was calculated from the oxidation rates (R) as follows:

Ln (Abs) = ln (Abs$_0$) + $R \times t$, where R, the bleaching rate, is the slope of ln (Abs) vs. time line, which can be calculated by linear regression, being t the time in minutes.

AA (%) = [($R_{blank} - R_{extract}$)/R_{blank}] × 100, where R_{blank} and $R_{extract}$ are the oxidation rates of blank sample and sample containing the extract, respectively.

2.5. Toxicity studies

Acute toxicity assessment was performed by the MTT method adapted for non-adherent cells (Young, Phungtamdet, & Sanderson, 2005). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to quantify metabolically viable cells in all samples. Suspension cells (K562 human erythroblastoid cell line) were seeded onto 96-well plates, allowed to divide for 24 h and exposed to the test compounds for the following 24 h. Positive controls (hydrogen peroxide) and negative controls (pure solvent) were also included. After 48 h of culture, MTT was added to the cells at a final concentration of 0.5 mg/ml, followed by an incubation period of 3 h to allow the formazan crystals to form. After the incubation time, DMSO (100 μl) was added to each well. Solubilisation of formazan crystals was performed by agitation in a 96-well plate shaker for 20 min at rt. Absorbance of each well was quantified at 550 nm using 620 nm as reference wavelength on a scanning multiwell spectrophotometer (automated plate reader). Genotoxicity was assessed by the short-term *in vitro* chromosomal aberration assay according to Rueff et al. (1993). Briefly, cell cultures of peripheral blood lymphocytes from healthy donors were set up, and lymphoproliferation was induced with phytohemagglutinnin (2% v/v, 24 h incubation). Cells were exposed to the plant extracts for 24 h, and then colcemid, at a final concentration of 0.5 μg/ml, was added during the last 3 h of culture, to stop the dividing cells in the metaphase stage of mitosis. Metaphase spreads were obtained in standard glass microscopy slides, pre-washed and covered with a thin water film. Scoring was performed in a Zeiss optical microscope at 1250× magnification, by observing 100 complete metaphases (presenting 46 centromers) per case. Classification of chromosomal aberrations was done according to criteria described by Rueff et al. (1993). The mitotic index was also quantified by counting the number of metaphases per 1000 nuclei.

2.6. Interaction with human prion protein

S. sclareoides acetone, methanol, ethanol, *n*-butanol and water extracts were dissolved in d$_6$-DMSO at approximately 100 mg/ml. NMR samples (0.5 ml) were prepared through the addition of the extract solutions (to a final concentration of approximately 1 mg/ml) to a solution containing 100 μM uniformly ^{15}N-labelled human prion protein (residues 91–231; huPrP$_{91-231}$) at pH 5.5 in 20 mM sodium acetate, 0.02% sodium azide, 10% D$_2$O and 10% d$_6$-DMSO. NMR spectra were acquired at 298 K on a Bruker DRX-800 spectrometer equipped with a 5 mm ^{13}C/^{15}N/^{1}H triple-resonance probe. Proton chemical shifts were referenced to 1 mM TSP added to the samples. ^{15}N chemical shifts were calculated relative to TSP, using the gyromagnetic ratios of ^{15}N and ^{1}H (^{15}N/^{1}H 0.101329118). NMR data were processed and analysed on Linux Workstations using Felix 2007 (Accelrys, San Diego, CA) software. ^{15}N-HSQC spectra of the huPrP$_{91-231}$–salvia extract samples were analysed for perturbation of huPrP$_{91-231}$ resonances relative to control samples, in which the extracts were omitted. Intensity changes of huPrP$_{91-231}$ resonances were displayed on the 3D structure of

huPrP$_{91-231}$ using spheres with radii corresponding to the percentage loss of resonance intensity.

3. Results and discussion

3.1. Chemical analysis

The total phenolic content (TPC) of each extract was first determined. The *n*-butanol extract had the highest TPC value (379 mg GAE/g dry extract), while the lowest one (22 mg GAE/g dry extract) was exhibited by the water extract (Table 1). Moreover, TPC of the *S. sclareoides* *n*-butanol extract was higher than those reported for South African *Salvia* species (45.6–212 mg GAE/g dry extract) (Kamatou et al., 2010) and for the Turkish plant *Salvia fruticosa*, where a TPC of 87.9 mg GAE/g dry extract was given for a dichloromethane extract (Senol et al., 2010). The TPC of *S. sclareoides* is, however, consistent with the one reported for the ethanolic extract of *Salvia juduica* (380.5 mg GAE/g dry extract) evaluated by the Folin Dennis method (Al-Ismail, Herzallah, & Rustom, 2007).

The chemical profile was determined by HPLC–DAD. Optimisation of the system was performed in order to achieve convenient instrumental conditions to analyse the standard phenolic compounds (Table 1). Preliminary assays indicated that all the 12 standards resolve in a suitable analytical time. Subsequently, acetone, ethanol, methanol and *n*-butanol extracts of *S. sclareoides* were analysed under the same instrumental conditions, but the water extract could not be studied under these conditions because its components were above the limit of detection at 280 nm. The relative percentages of phenolic compounds present in those extracts are depicted in Table 1. Amongst the analysed samples, the acetone and *n*-butanol extracts are those that showed the highest number of phenolic compounds, 8 and 9, respectively, out of the 12 standards used. Vanillic acid was the major component of all extracts. This phenolic acid has antisickling and anthelmintic activities and suppressed hepatic fibrosis in chronic liver injury (Itoh et al., 2010; Khadem & Marles, 2010). Syringic acid, only present in the acetone extract, is also known for its hepatoprotective effects (Itoh et al., 2010) while gentisic acid, mainly present in the same extract, has analgesic, anti-inflammatory, antirheumatic, and cytostatic properties, inhibiting also low density lipoprotein oxidation and the formation of cholesterol ester hydroperoxides in human plasma (Ashidate et al., 2005). Gallic acid, present in all extracts, has a variety of bioactivities, namely antineoplastic, bacteriostatic, antimelanomagenic, antioxidant properties, and has been proposed as candidate for the treatment of brain tumours (Lu et al., 2010). Chlorogenic acid was found in the methanol and *n*-butanol extracts and its protective effect against neurotoxocity reducing

apoptosis induced by beta-amyloid (A beta) was recently reported (Lee et al., 2011). In addition, it exhibits anticholinesterase and free radical scavenging activities and may exert anti-amnesic activity (Kwon et al., 2010). The antioxidant, anti-inflammatory and immunostimulatory ferulic acid (Chao & Lin, 2011) promotes degeneration of recombinant beta-amyloid peptide, as described by Picone et al. (2009). Also caffeic acid has neuroprotective effects against A beta-induced toxicity by the inhibition of calcium influx and tau phosphorylation (Sul et al., 2009), and protects neuronal cells against oxidative stress induced cytotoxicity (Jeong et al., 2011). Both caffeic and coumaric acids are capable of inducing neuroprotective effects in Parkinson's disease (Vauzour, Corona, & Spencer, 2010) to a similar extent to that seen with the flavonoids (+)-catechin and quercetin, also present in the *n*-butanol extract (Martin, Gonzalez-Burgos, Carretero, & Gomez-Serranillos, 2011). Not only quercetin but also rutin, present in the acetone extract alone, are known to revert cognitive deficits (Tongjaroenbuangam et al., 2011). Kaempferol *O*-glucoside also contributes to the nutraceutical relevance of this plant considering the wide range of pharmacological activities, including neuroprotective properties known for kaempferol glycosides (Calderon-Montano, Burgos-Moron, Perez-Guerrero, & Lopez-Lazaro, 2011).

Studies with animal models with a supplemented diet with polyphenols have produced neuroprotection and favourable effects concerning age-related cognitive and motor decline (Giacalone et al., 2011). The present results reinforce the importance of *S. sclareoides* for its traditional use as food condiment and medicinal plant, and as a source of functional food ingredients against neurodegenerative diseases.

3.2. Antioxidant activity

3.2.1. DPPH radical scavenging activity

The antioxidant activity of *S. sclareoides* extracts was determined using the DPPH radical scavenging method (Table 2). The *n*-butanol extract was the most bioactive with an IC$_{50}$ = 7.9 μg/ml. This extract was more active than the synthetic antioxidant BHT (IC$_{50}$ = 29.7 μg/ml) used as control, and showed an antioxidant strength of the same order of magnitude as ascorbic acid, a natural antioxidant (IC$_{50}$ = 4.9 μg/ml). Methanol and ethanol extracts had IC$_{50}$ values of 114 and 134 μg/ml, respectively, while the water extract was the least active one, with a DPPH radical scavenging activity (RSA) of 19.6% at 500 μg/ml (IC$_{50}$ > 500 μg/ml).

3.2.2. β-Carotene/linoleic acid bleaching assay

The antioxidant activity of the extracts was also assessed using the β-carotene/linoleic acid method (Table 2). In this assay, the acetone extract presented the highest activity (IC$_{50}$ = 38.4 μg/ml), which is of the same order of magnitude as that exhibited by ascorbic acid (IC$_{50}$ = 26.1 μg/ml). Similar values were obtained for the *n*-butanol, methanol, ethanol and water extracts. In contrast to the relatively high antioxidant activity observed with the *n*-butanol extract using the DPPH method, the capacity of this extract to inhibit β-carotene oxidation did not differ significantly from that of the other extracts.

3.3. Acute cytotoxicity and genotoxicity

Acute toxicity and genotoxicity were assessed *in vitro* using peripheral blood human lymphocytes and K562 human lymphoblast immortalised cells. Cells tested with a final extract concentration of up to 4 mg/ml showed no evidence of toxicity. The viability of the cells was not affected and no evidence of genotoxic risk was observed (Table 3).

Table 1
Relative percentage of phenolics in *Salvia sclareoides* extracts evaluated by HPLC–DAD at 280 nm.

Standards	Rt (min)	Extracts			
		MeOH	EtOH	BuOH	Acetone
Gallic acid	1.189	0.8	0.8	1.2	1.7
(+)-Catechin	3.421	–	–	0.5	–
Gentisic acid	4.085	–	1.0	–	3.6
Chlorogenic acid	4.346	1.1	–	1.3	–
Caffeic acid	5.085	–	0.8	1.4	2.4
Vanillic acid	5.551	2.5	1.3	2.8	6.9
Syringic acid	6.680	–	–	–	2.1
Coumaric acid	9.692	1.3	0.1	0.6	0.8
Ferulic acid	12.876	0.8	–	0.5	0.1
Rutin hydrate	25.104	–	–	–	1.6
Kaempferol *O*-glucoside	32.302	–	–	0.7	–
Quercetin dehydrate	36.658	–	–	1.4	–

Table 2
Antioxidant activity (%)[a], IC$_{50}$[b] and total phenolic content[c] of *Salvia sclareoides* extracts.

Extract	Method	S. sclareoides extract concentration (µg/mL)							IC$_{50}$ (µg/mL)	TPC (mg GAE/g dry extract)
		1	3	5	10	50	100	500		
Acetone	DPPH	<5	<5	<5	<5	6.1 ± 0.8	13.9 ± 1.2	30.5 ± 2.1	>500	67.0 ± 2.7
	β-Carotene	6.9 ± 2.8	19.2 ± 4.4	39.6 ± 11.9	35.2 ± 3.4	51.6 ± 1.7	54.4 ± 2.3	80.8 ± 4.5	38.4	
Butanol	DPPH	<5	6.1 ± 2.0	32.9 ± 2.4	53.9 ± 0.7	56.9 ± 4.0	58.7 ± 3.5	59.5 ± 2.9	7.9	379.4 ± 2.7
	β-Carotene	<5	25.2 ± 1.7	29.4 ± 3.9	32.4 ± 4.0	40.0 ± 3.4	54.0 ± 0.6	76.4 ± 1.7	57.6	
Methanol	DPPH	<5	<5	<5	<5	5.3 ± 1.3	42.9 ± 3.9	47.7 ± 1.8	113.9	120.4 ± 2.7
	β-Carotene	<5	5.7 ± 11.1	19.3 ± 2.3	17.3 ± 0.6	53.9 ± 2.4	58.4 ± 5.7	76.1 ± 2.2	60.6	
Ethanol	DPPH	<5	<5	<5	<5	6.6 ± 4.8	37.2 ± 4.8	40.9 ± 2.5	133.7	140.8 ± 4.7
	β-Carotene	<5	<5	16.5 ± 2.8	26.0 ± 2.8	37.6 ± 7.9	47.6 ± 0.6	81.2 ± 9.6	78.6	
Water	DPPH	<5	<5	<5	<5	3.9 ± 6.2	13.7 ± 4.5	19.6 ± 4.1	>500	22.4 ± 0.7
	β-Carotene	<5	<5	9.7 ± 7.2	34.3 ± 0.7	52.4 ± 6.1	51.2 ± 4.2	65.1 ± 0.5	85.1	
AA	DPPH	3.0 ± 2.1	31.0 ± 2.9	48.5 ± 0.8	70.9 ± 1.5	72.8 ± 1.1	71.1 ± 2.1	73.1 ± 0.6	4.9	–
	β-Carotene	23.1 ± 6.8	26.1 ± 8.2	32.1 ± 3.3	39.8 ± 2.3	61.6 ± 1.2	68.3 ± 6.8	71.8 ± 5.2	26.1	
BHT	DPPH	<5	7.4 ± 1.8	9.6 ± 2.7	34.1 ± 2.7	59.8 ± 1.1	59.7 ± 0.7	61.3 ± 0.7	29.7	–
	β-Carotene	44.6 ± 10.9	68.8 ± 0.0	81.2 ± 1.7	72.2 ± 0.9	86.0 ± 1.7	84.0 ± 1.1	92.7 ± 2.1	0.27	

[a] Results of antioxidant activity in percentage are expressed as the average ± standard deviation of three replicates; AA (Ascorbic acid) and BHT (butylated hydroxytoluene) are antioxidants used as controls for comparison purposes.
[b] IC$_{50}$ is the concentration causing 50% antioxidant activity estimated by non-linear regression.
[c] Total phenol content, TPC, is expressed in mg of gallic acid equivalents (GAE) per g of dry *Salvia* extract.

Table 3
In vitro toxicity of *Salvia sclareoides* extracts: acute toxicity (4.0 mg/ml) in human immortalised leukaemia cells (K562) and genotoxicity study (1.9 mg/ml) in human peripheral blood lymphocytes.

S. sclareoides extract	Acute toxicity	Genotoxicity	
	Cellular viability (percentage of blank)	Mitotic index	Chromosomal aberrations
Acetone	–	10.50 ± 4.1	1.25 ± 1.3
Butanol	151.5 ± 52.6	10.25 ± 2.2	1.00 ± 1.2
Methanol	140.6 ± 68.2	n.d	n.d.
Ethanol	105.2 ± 46.9	9.25 ± 3.8	0.75 ± 1.0
Water	79.3 ± 28.0	14.00 ± 3.7	1.00 ± 0.8
Controls			
Blank	100 ± 37.5	12.75 ± 3.3	1.00 ± 0.8
H$_2$O$_2$ 2%	34 ± 17.6	1.75 ± 1.0	5.00 ± 2.2

n.d. – not determined.

3.4. Interaction with human prion protein

In order to probe the relationship between *S. sclareoides* extracts and prion propagation, we have investigated whether the extracts characterised above display any specific interactions with the cellular form of human prion protein (PrP^C). PrP^C is the substrate for formation of the pathological form of the protein (PrP^{Sc}), and is a candidate for anti-prion therapeutics (Mallucci & Collinge, 2005; Nicoll, 2009). The stabilisation of the PrP^C resulting from ligand binding has the potential to inhibit or delay the pathological cascade that is initiated by conformational change of the protein.

The effects of individual extracts on the NMR spectra of recombinant human prion protein (huPrP$_{91-231}$) were probed using a ^{15}N-HSQC-perturbation assay (Nicoll et al., 2010). Only the *n*-butanol extract caused any perturbation of the protein resonances, which manifested as an attenuation of specific resonances in the spectrum (Fig. 1). The residues with affected resonances reside in three separate regions of the protein: residues 93–112 (in the unstructured N-terminal region of PrP^C), 130–187 (comprising the two strands of the beta sheet, helix 1 and the beginning of helix 2), and 205–225 in helix 3 at the C-terminus of the structured domain. Such attenuation of resonances is normally associated with ligand exchange dynamics, and is commonly observed under non-saturating conditions for ligands with dissociation constants in the µM range. While the exact location of ligand binding is not unambiguously determined by the measurements, there is
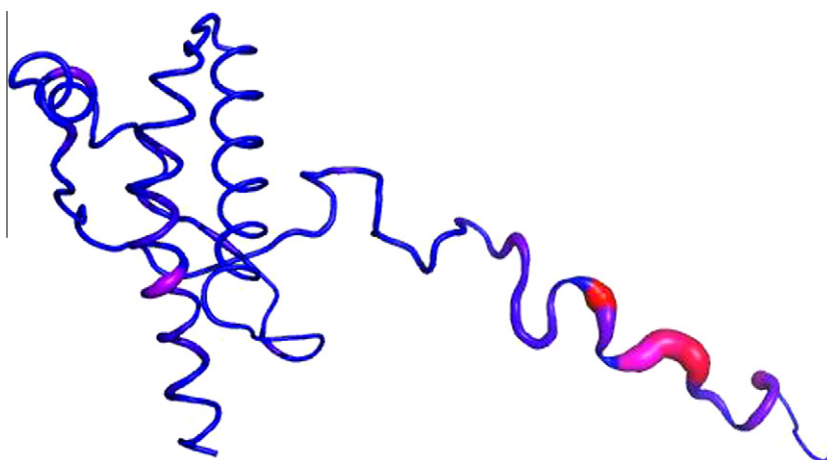

Fig. 1. Structure of huPrP$_{91-231}$ indicating residues affected by the butanol extract, where the colour and thickness of the ribbon indicates increasing degree of signal attenuation, hence the magnitude of the effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clearly some specific binding of components within the *n*-butanol extract to PrP^C and, most likely, associated changes in conformation in the folded domain and conformational restriction in the N-terminal region.

4. Conclusion

The present study establishes *S. sclareoides* as a viable source of functional food ingredients, due to its high antioxidant and radical scavenging activities, as well as prion binding properties. The NMR measurements showed that components extracted from *S. sclareoides* had the capability to bind to the cellular form of human prion protein in a specific manner, and hence provide the potential to compete against the recruitment of prion protein into its pathological cascade. The active component(s) were present in the *n*-butanol extract alone and binding caused conformational perturbation in three separate regions of the protein, including the highly dynamic N-terminal region. The *n*-butanol extract is also the richest one in phenolics, which are increasingly of interest in the food industry, and shows promise for neurodegenerative diseases. Furthermore, the tested extracts are neither cytotoxic nor genotoxic, endorsing *S. sclareoides* as a promising plant for nutraceutical and medicinal purposes.

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