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ScienceDirect
Food Chemistry 109 (2008) 630–637
FOOD CHEMISTRY
www.elsevier.com/locate/foodchem

Analytical Methods

Antioxidant and antiacetylcholinesterase activities of essential oils from *Cymbopogon schoenanthus* L. *Spreng.* Determination of chemical composition by GC–mass spectrometry and ^{13}C NMR

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Received 20 September 2007; received in revised form 20 December 2007; accepted 27 December 2007

Abstract

Cymbopogon schoenanthus L. *Spreng*, is an aromatic herb consumed in salads and used to prepare traditional meat recipes in Tunisia. The chemical composition, antioxidant activities and acetylcholinesterase inhibitory properties of the essential oils from fresh leaves, dried leaves and roots collected from three different locations in southern Tunisia, were evaluated. Essential oils were analysed by GC–mass spectrometry and ^{13}C NMR. The major components were limonene (10.5–27.3%), β-phellandrene (8.2–16.3%), δ-terpinene (4.3–21.2%) and α-terpineol (6.8–11.0%). Antioxidant activity was measured by DPPH assay. The results ranged from 36.0% to 73.8% (2 μl of essential oil per mL of test solution).

The antioxidant activity was also assayed using β-carotene–linoleic acid bleaching method. The best results ($IC_{50} = 0.47 \pm 0.04$ mg mL^{-1}) were obtained with the fresh leaves of plants collect in the desert region.

The greatest acetylcholinesterase inhibitory activity ($IC_{50} = 0.26 \pm 0.03$ mg mL^{-1}) was exhibited by the essential oil of the fresh leaves from the mountain region.
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Keywords: *Cymbopogon schoenanthus*; Essential oils; ^{13}C NMR; GC–MS; Antioxidant activity; Acetylcholinesterase activity

1. Introduction

Cymbopogon schoenanthus L. *Spreng*, is an aromatic herb known in Tunisia by the name of "El bekhirai". Fresh young leaves are consumed in salads and are used to prepare traditional meat recipes. Due to its pleasant aroma and taste it is used to prepare an aromatic "tea" that is much appreciated and largely consumed in the north of Africa (IUCN, 2005). Besides its use in culinary, *C. schoenanthus* is also used in folk medicine. Its medicinal properties are known from the antiquity, being already described by "Pliny the Eldey" in his book *Naturalis Historia* (Pline L'Ancien, 1848–1850). Le Floc'h (1983) reports its use for the treatment of rheumatism and fever. This author describes also its use as a diuretic, insecticide and a poultice to cure dromedary wounds. In the South of Tunisia, this plant is also used for the treatment of rheumatism, and to diminish fever. The plant is particularly appreciated for its medicinal action in North Africa and it is also used for the anorexia. In the Djanet area (Alger), it is well known for bringing back the appetite. The infusions are

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doi:10.1016/j.foodchem.2007.12.070

taken as a diuretic, it cures intestinal troubles and, in the form of decoction, it acts against food poisoning and helps also in the digestion.

Some of the diseases like rheumatism and fever, that this plant is used against, can be attributed to the formation of free radicals in the biological system (Bauerova & Bezek, 1999). Free radicals and other reactive oxygen species (ROS), (e.g. superoxide anion, hydroxyl radical, and hydrogen peroxide) are highly reactive molecules derived from the normal metabolism of oxygen or from exogenous factors and agents. ROS are reported to be a causative agent of various diseases such as arthritis, asthma, dementia, mongolism, carcinoma and Parkinson's disease (Perry et al., 2000). Lipid peroxidation is a complex process occurring in aerobic cells and reflects the interaction between molecular oxygen and polyunsaturated fatty acids. Radicals are known to take part in lipid peroxidation, which causes food deterioration, aging and cancer promotion (Ashok & Ali, 1999). Antioxidants act as radical-scavengers, and inhibit lipid peroxidation and other free radical-mediated processes: therefore, they are able to protect the human body from several diseases attributed to the reactions of radicals (Takao, Kiatani, Watanabe, Yagi, & Sakata, 1994). The use of synthetic antioxidants to prevent free radical damage has been reported to involve toxic side effects (Cornwell et al., 1998).

Acetylcholine (ACh) is one of the most important neurotransmitters in animal systems. Increasing the levels of acetylcholine by inhibition of acetylcholinesterase (AChE), the enzyme responsible for its hydrolysis at the cholinergic synapses, serves as a strategy for the treatment of Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Atta-ur-Rahman & Choudhary, 2001). There are a few synthetic medicines, e.g. tacrine, donepezil, and the natural based-product rivastigmine for treatment of cognitive dysfunction and memory loss associated with AD (Oh, Houghton, Whang, & Cho, 2004). These compounds have been reported to have adverse effects including gastrointestinal disturbances and problems associated with bioavailability (Schulz, 2003), which reinforces the interest in finding better AChE inhibitors from natural resources. Recently, new therapeutic perspectives point out to the prevention of AD in the general population or those perceived as patients with an increased risk for this disease. This may be accomplished with drugs that can be able to neutralize ROS involved as mediator agents in the inflammatory process that ends in neuronal death. This strategy will demand very safe and not expensive drugs (Tschäpe & Hartmann, 2006).

Therefore, the aim of this work was to evaluate the antioxidant activities and acetylcholinesterase inhibitory properties of the essential oils obtained from dried and fresh plant material (leaves and roots) of *C. schoenanthus*, collected during flowering phase from three different locations in southern Tunisia. We also determined the chemical composition of the essential oils by capillary gas chromatography coupled to mass spectrometry (GC–MS) and nuclear magnetic resonance of carbon 13 (^{13}C NMR). It would also be noteworthy to point out that the composition of essential oils is influenced by the presence of several factors, such as local, climatic, seasonal and experimental conditions (Daferera, Ziogas, & Polissiou, 2000), thereby altering their biological activity.

2. Materials and methods

2.1. Plant material

Cymbopogon schoenanthus (L.) *Spreng*. ssp. *laniger* (Hook) Maire et Weill, was collected during the flowering phase (June–July 2006) from three locations in southern Tunisia: Echareb-mountains region (34°04.723°N, 009°04.177°E); Dhibat-desert region (32°10.309°N, 010°59.497°E) and Gourdhab-experimental plot (33°08.564°N, 010°49.531°E). In the latter case, the plants cultivated in Gourdhab were brought from Dhibat. Botanical identification was made by Dr. Mohamed Neffati (Director of the Ecology Laboratory of the "Institut des Regions Arides" Tunisia (I.R.A.)), according to the "Flora of Tunisia" (Cuénod, 1954). Voucher specimens were deposited at the herbarium of the I.R.A.

2.2. Chemicals

All chemicals were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, β-carotene, 2,6-di-tert-butyl-4-hydroxytoluene (BHT), acetylcholinesterase (AChE) type VI-S, from electric eel 349 U/mg solid, 411 U/mg protein, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), acetylthiocholine iodide (AChI), tris[hydroxymethyl]aminomethane (tris buffer), dimethylsulfoxide (DMSO) and Tween 40, were supplied from Sigma.

2.3. Essential oil extraction

The essential oils from a total eight samples were obtained by hydrodistillation during 3 h using a Clevenger-type apparatus (European Pharmacopeia, 1996). The yield of each essential oil was determined on average over the three replicates. These oils were dried over anhydrous sodium sulphate and kept at 4 °C until analysis.

2.4. Analysis of the essential oils

2.4.1. GC–mass spectrometry

GC–mass spectrometry (GC–MS) analyses were performed on an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 *N* mass selective detector (Agilent Technologies, Little Falls, DE, USA). A vaporization injector operating at 250 °C in the split mode (1:100) was used. A fused silica capillary column, 30 m × 0.25 mm ID × 0.25 μm film thickness (TRB-5MS; 5% diphenyl 95% dimethyl polydimethylsiloxane, Teknokroma, Spain) was used. The oven temperature was programmed from

45 °C for 1 min and then increased at 5 °C min^{-1} to 240 °C, and held isothermally for 5 min. High purity helium was used as carrier gas at 30 cm s^{-1}.

Electron ionisation mass spectra in the range 35–550 Da were recorded at 70 eV electron energy with an ionization current of 39.6 μA. The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 °C, respectively. A solvent delay of 5 min and a turbo molecular pump (10^{-5} Torr) were used. All data was recorded using a MS ChemStation (G1701CA; Rev C.00.00; Agilent Technologies). The identity of each compound was determined by comparison of its retention index (RI) relative to C_{10}–C_{24} *n*-alkanes (Adams, 2001), as well as of its spectra with the Wiley library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies). For semi-quantification purposes of the samples studied (1 μL), the normalised peak area of each compound was used without any correction factors to establish abundances. For each essential oil, the RI and the peak area percentages were calculated as mean values of three injections.

2.4.3. ^{13}C NMR spectroscopy

All the ^{13}C NMR spectral data were recorded on a 100 MHz Bruker Avance. The samples were dissolved in $CDCl_3$. ^{13}C NMR analyses were performed on the whole samples, without any previous fractionation, following the pioneering work done by Formacek and Kubeczka (1982) and a methodology developed by Corticchiato and Casanova (1992) and Tomi, Bradesi, Bighelli, and Casanova (1995). The identification was based on comparison of the signals in the oil spectrum with those of reference spectra compiled in the laboratory spectral library, with the help of laboratory-made database (SDBS, ChemDraw and OriginLab). Each compound was identified taking into account (i) the number of identified carbons, (ii) the number of overlaying of signals, and (iii) the difference of chemical shift of each resonance in the mixture and in the reference spectra.

2.5. Antioxidant activity

2.5.1. General

The antioxidant activity was measured by the 1,1-diphenyl-2-pierylhydrazul (DPPH) assay described by Yamaguchi, Takamura, Matoba, and Terao (1998) and by β-carotene–linoleic acid method described by Tepe, Daferera, Sokmen, Sokmen, and Polissiou (2005).

2.5.2. DPPH assay

The antioxidant activity of *C. schoenanthus* essential oils was determined by a modification of the DPPH radical-scavenging method of Yamaguchi et al. (1998). Each sample (20 μL) was mixed with 900 μL of 100 mM Tris–HCl buffer (pH 7.4) and added to a mixture of 30 μL of ethanol and 50 μL of 0.5% (w/w) Tween 40 solution. This mixture was then added to 1 mL of 0.5 mM DPPH in ethanol (250 μM in the reaction mixture). Tween 40 was used as oil in water emulsifiers. The mixture was shaken with a mechanical shaker and left to stand for 30 min at room temperature in the dark room. DPPH is a stable free radical and has a dark violet colour. The absorbance was measured at 517 nm against the corresponding blank:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (a reaction with all the reagents except the test extract), and A_{sample} is the absorbance of the sample extract. Tests were carried out in triplicate.

2.5.3. β-Carotene–linoleic acid assay

The method described by Tepe et al. (2005) was used with a slight modification. A stock solution of β-carotene and linoleic acid was prepared with 0.5 mg of β-carotene in 1 mL chloroform, 25 μL of linoleic acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 mL of aerated distilled water were then added to the residue. 300 μL of each extract were added to 2.5 mL of the previous mixture. The essential oil was dissolved in DMSO. The test tubes were incubated in hot water (50 °C) for 2 h, together with two blanks, one containing the antioxidant BHT as a positive control and the other with the same volume of distilled water instead of the extracts. In the test tube with BHT, the yellow colour is maintained during the incubation period. The absorbance was measured at 470 nm. Antioxidant capacities (AA) of the tested solutions were calculated using the following equation (Shon, Kim, & Sung, 2003):

$$AA\% = (\beta\text{-carotene content after 2 h assay/initial} - \beta\text{-carotene content}) \times 100.$$

Tests were carried out in triplicate. Extract concentration providing 50% inhibition (IC_{50}) was obtained plotting inhibition percentage versus extract solutions concentrations.

2.6. Acetylcholinesterase inhibition

Acetylcholinesterase enzymatic activity was measured, using an adaptation of the method described by Ingkaninan, Temkittawon, Chuenchon, Yuyaem, and Thongnoi (2003). 98 μL (50 mM) Tris–HCl buffer pH 8, 30 μL sample and 7.5 μL acetylcholinesterase solutions containing 0.26 U mL^{-1} were mixed in an ELISA plate well and left to incubate for 15 min. Subsequently, 22.5 μL of AchI 0.023 mg mL^{-1} and 142 μL of (3 mM) DTNB were added. The absorbance at 405 nm was read when the reaction reached the equilibrium. A control reaction was carried out using water instead of extract. The absorbance value obtained was considered 100% activity. Inhibition (%) was calculated in the following way:

$$I\% = 100 - (A_{\text{sample}}/A_{\text{control}}) \times 100,$$

where A_{sample} is the absorbance of the reaction containing the extract and A_{control} the absorbance of the reaction control. Tests were carried out in triplicate and a blank with

Tris–HCl buffer instead of enzyme solution was done. Extract concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against extract solution concentrations.

2.7. Statistical analysis

The percentage composition of the essential oil samples was used to determine the relationship between the different samples of *C. schoenanthus* by cluster analysis using the NTSYS-pc software (version 2.02, Exeter Software, Setauket, New York) (Rohlf, 1998) Correlation coefficients were selected as a measure of similarity among the eight accessions, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro (2000) in: very high (if correlation ranged between 0.90 and 1.00), high (between 0.70 and 0.89), moderate (between 0.40 and 0.69), low (between 0.20 and 0.30) and very low (if <0.20). The cophenetic correlation values were determined to test the goodness of the fit of the data clustering by the Mantel test (Rohlf, 1998).

3. Results and discussion

3.1. Extraction yields

The essential oil yield obtained by the conventional hydrodistillation from fresh leaves, dried leaves and dried roots of the *C. schoenanthus*, collected during flowering phase in three different locations in southern Tunisia, desert, mountain and an experimental plot, ranged from 1.1% to 2.6% (w/w). The higher content was obtained in the dried leaves from the desert region (Table 1).

3.2. Chemical composition of the essential oils

In this work, the chemical composition of the eight essential oil samples from *C. schoenanthus* L. *Spreng* was analysed by GC–MS and/or ^{13}C NMR spectroscopy (Table 2). From the data obtained, the essential oils showed to be complex mixtures of several components, predominating monoterpenes and sesquiterpenes. It must be emphasised that the crossing data achieved from the RI, MS and ^{13}C NMR, allowed the identification of almost all individual compounds detected, even those that presented very similar mass spectra but different ^{13}C NMR spectra could be distinguished. This approach also helped to identify compounds that presented very similar RI. More than 30 constituents were identified representing 95.6%, 95.0%, 91.0%, 95.3%, 93.0%, 93.3%, 96.8% and 92.3% of the total oils of the fresh leaves form Dhibat (FLD), fresh leaves form Gourdhab (FLG) and fresh leaves from Echareb (FLE), dried leaves from Dhibat (DLD), dried leaves from Gourdhab (DLG), dried leaves from Echareb (DLE), dried roots from Dhibat (DRD) and dried roots from Echareb (DRE). Table 2 depicts the compounds identification and their percentages, as well as the RI values. These values are listed in order of their elution from TRB-5MS capillary column. The essential oils from *C. schoenanthus* were characterised by high percentages of monoterpene hydrocarbons (25.7–66.3%), for which the four major constituents were limonene (10.5–27.3%), β-phellandrene (8.2–16.3%), δ-terpinene (4.3–21.2%) and α-terpineol (6.8–11.7%). Some of these constituents, i.e. limonene and α-terpineol, have already been reported in the essential oils of *C. schoenanthus* from Algeria (de Sousa et al., 2005). The sesquiterpene fraction, ranging from 12.8% to 30.8%, consisted mostly of sesquiterpene hydrocarbons. Oxygenated sesquiterpenes were also found but in moderate amounts (6.3–27.3%). Other components, i.e. fatty acids methyl esters, were also detected at trace levels.

Although some species showed a quite stable oil composition, independently of the plant part studied or location, these species were collected from very different altitudes and climatic conditions and therefore, variability found in the chemical composition of the essential oils can be a consequence of the climate or other environmental factors (Fig. 1).

3.3. Antioxidant activity

The essential oils were subjected to a screening for the possible antioxidant activity by two complementary tests, namely DPPH free radical scavenging and β-carotene/linoleic acid systems.

3.3.1. DPPH radical-scavenging activity

Free radical scavenging capacities of the corresponding oil measured by DPPH assay are shown in Table 3. The oils

Table 1
Essential oil yields from fresh, dried leaves and dried roots of *C. schoenanthus* L. *Spreng* collected from three different locations in southern Tunisia (Dhibat, Echareb and Gourdhab) during flowering phases (June–July)

Location	Yield[a] (%) (w/w)		
	Dried leaves	Fresh leaves	Dried roots
Desert (Dhibat)	2.6 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
Mountain (Echareb)	1.2 ± 0.2	2.1 ± 0.2	1.1 ± 0.1
Experimental plot (Gourdhab)	2.1 ± 0.1	1.6 ± 0.1	Nd

Nd: Not determined.
[a] Values represent mean ± standard deviation of three replicates.

Table 2
Chemical composition (%) of the essential oils of *C. schoenanthus* L. *Spreng* collected from three different locations in southern Tunisia (desert-Dhibat (D), mountain-Echareb (E) and experimental plot-Gourdhab (G))

Components	RI[a]	Fresh leaves			Dried leaves			Dried roots		Identification technique
		D	G	E	D	G	E	D	E	
4-Carene	907	1.6	2.1	2.7	1.7	1.4	2.5	1.9	1.3	GC–MS, ^{13}C NMR
l-Phellandrene	941	0.1	0.1	0.1	0.2	0.1	0.1	t	t	GC–MS, ^{13}C NMR
Sabinene	975	0.7	0.7	0.8	0.7	0.4	0.6	0.7	0.5	GC–MS
Limonene	1096	24.2	27.3	24.6	24.6	23.5	22.1	26.0	10.5	GC–MS, ^{13}C NMR
β-Phellandrene	1117	13.4	13.5	16.0	15.7	14.1	16.3	15.9	8.2	GC–MS, ^{13}C NMR
Δ-3-Carene	1184	1.2	1.4	1.7	1.3	1.2	1.4	1.4	0.9	GC–MS
δ-Terpinene	1192	9.6	21.2	8.4	8.9	11.3	7.4	9.7	4.3	GC–MS, ^{13}C NMR
α-Terpineol	1204	9.1	9.1	11.7	9.6	10.1	11.0	9.4	6.8	GC–MS, ^{13}C NMR
Piperitol *cis*	1263	0.1	0.1	0.3	1.2	0.5	0.2	0.5	1.2	GC–MS, ^{13}C NMR
Piperitol *trans*	1426	0.4	0.4	0.6	0.2	0.4	0.4	0.4	0.5	GC–MS, ^{13}C NMR
β-Bourbonene	1468	0.4	1.8	0.5	0.7	0.9	0.5	0.5	0.8	GC–MS
β-Elemene	1481	0.3	0.2	0.2	0.6	0.1	0.1	0.1	0.4	GC–MS, ^{13}C NMR
trans Caryophyllene	1506	0.4	0.3	0.6	0.2	0.2	0.3	0.6	0.8	GC–MS
Calarene	1539	0.4	0.4	0.6	0.4	0.8	0.6	0.4	0.9	GC–MS
β-Humulene	1543	0.5	0.5	0.3	0.9	0.6	0.5	0.2	0.5	GC–MS
β-Selinene	1548	0.5	0.1	0.5	0.6	0.2	0.6	0.3	1.2	GC–MS, ^{13}C NMR
Δ-Selinene	1560	0.3	0.3	0.5	0.2	0.6	1.3	0.5	1.5	GC–MS
δ-Amorphene	1568	0.3	0.2	0.1	0.4	2.1	0.7	t	t	GC–MS
Valencene	1581	0.4	0.2	0.2	0.4	0.2	0.2	4.1	7.2	GC–MS, ^{13}C NMR
α-Selinene	1606	0.2	2.2	2.4	4.1	2.4	1.9	0.5	0.6	GC–MS, ^{13}C NMR,
δ-Patchoulene	1619	5.3	0.5	0.2	0.8	0.4	0.2	0.2	0.3	GC–MS
β-Guaiene	1627	0.2	0.3	0.4	0.1	1.1	0.4	0.6	0.5	GC–MS
Germacrene A	1652	0.7	0.6	0.8	0.8	0.2	0.3	0.8	1.3	GC–MS, ^{13}C NMR
δ-Cadinene	1686	0.6	0.2	4.5	0.3	0.4	5.4	4.9	6.1	GC–MS
Δ-Cadinene	1702	0.7	3.8	1.5	0.7	1.2	2.0	2.1	4.2	GC–MS, ^{13}C NMR
α-Cadinene	1711	6.0	0.8	0.9	4.2	5.5	1.3	t	t	GC–MS
Elemol	1719	2.0	0.3	1.2	1.7	1.2	1.5	4.3	4.6	GC–MS
Germacrene B	1723	2.9	0.4	0.4	3.2	2.6	1.6	1.1	4.5	GC–MS, ^{13}C NMR
β-Eudesmol	1728	0.2	1.4	3.1	3.1	5.3	5.1	2.1	14.2	GC–MS, ^{13}C NMR
α-Eudesmol	1732	5.5	1.9	2.0	5.1	0.4	3.0	4.3	0.3	GC–MS
Junipercamphor	1761	4.1	2.7	3.3	2.8	3.5	3.7	3.3	8.2	GC–MS, ^{13}C NMR
Hexadecanoic acid, methyl ester	1866	0.4	t	t	t	t	t	t	t	GC–MS
Heptadecanoic acid, methyl ester	1978	2.4	t	t	t	t	t	t	t	GC–MS
9-Octadecanoic acid, methyl ester	2050	0.5	t	t	t	t	t	t	t	GC–MS
Monoterpenes hydrocarbons		50.8	66.3	54.2	53.1	52.1	50.3	55.6	25.7	
Oxygenated monoterpenes		9.6	9.6	12.6	11.0	11.0	11.7	10.3	8.5	
Sesquiterpenes hydrocarbons		20.1	12.8	14.6	18.6	19.5	17.9	16.9	30.8	
Oxygenated sesquiterpenes		11.8	6.3	10.8	12.7	10.4	13.3	14.0	27.3	
Others		3.3	t	t	t	t	t	t	t	
Total identified		95.6	95.0	91.0	95.3	93.0	93.3	96.8	92.3	

[a] Relative to C_{10}–C_{24} *n*-alkanes determined using the TRB-5MS capillary column; t: traces (<0.05%).

are mainly composed by monoterpenes and sesquiterpenes hydrocarbons (Table 2) so, due to the low solubility of the essential oil in the test solution, in this test the IC_{50} value was not determined. The study was carried out using always the same quantity (20 μL) of all essential oils. The inhibition value obtained when using 2 μl of essential oil per mL of test solution ranged from $36.0 \pm 9.5\%$ to $73.8 \pm 2.1\%$. Similar values were obtained when analysing the antioxidant activity of known antioxidant standards, with the same dilution value (Table 3). The best results were obtained with essential oil from fresh and dried leaves from the plant cultivated in the experimental plot ($73.8 \pm 2.1\%$ and $67.3 \pm 17.5\%$, respectively) and dried roots from the mountain region plant ($61.0 \pm 5.6\%$). These values were of the same magnitude of those found with the standards: carvacrol $87.4 \pm 1.5\%$; verbenone $77.1 \pm 0.5\%$ and butyl hidroxytoluene BHT $100 \pm 0.0\%$. It may be interesting to notice that the best results were obtained with a plant collected from the desert (Dhibat) but cultivated in a parcel of land with daily care (Gourdhab). These results reinforce the knowledge that the environmental conditions strongly influence the chemical and the resulting activities in the plant extracts. In general, the drying process of this plant material improved the radical scavenging activity.

The antioxidant activity may be ascribed to the presence of the several chemical components. Monoterpenes found in these essential oils may act as radical scavenging agents. It seems to be a general trend that the essential oils, which contain monoterpene hydrocarbons, oxygenated monoterpenes and/or sesquiterpenes, have high antioxidative

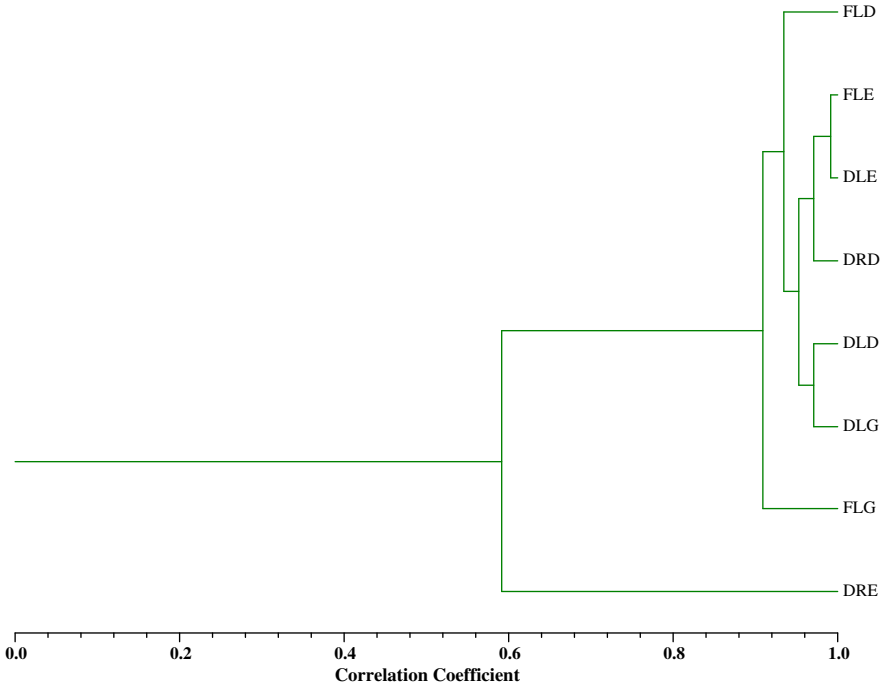

Fig. 1. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the *Cymbopogon schoenanthus* samples examined, based on correlation and using the unweighed pair-group method with arithmetic average (UPGMA). Fresh leaves (FLD, FLE and FLG), dried leaves (DLD, DLE and DLG) and dried roots (DRD and DRE) from Dhibat, Echareb and Gourdhab, respectively.

Table 3
Antioxidant activity, measured by the DPPH test, represented by percentage (%) of inhibition of essential oil from *C. schoenanthus* and some standards when using 2 μl of essential oil per mL of test solution

Location	% of inhibition DPPH test (2 μl mL^{-1})[a]					
	Essential oil			Carvacrol	Verbenone	BHT
	Fresh leaves	Dried leaves	Dried roots	87.4 ± 1.5	77.1 ± 0.5	100 ± 0.0
Desert (Dhibat)	36.0 ± 9.5	58.9 ± 1.0	49.7 ± 35.7[B]	–	–	–
Mountain (Echareb)	40.0 ± 0.4	61.0 ± 5.6	64.4 ± 6.0	–	–	–
Experimental plot (Gourdhab)	73.8 ± 2.1	67.3 ± 17.5	Nd	–	–	–

Nd: Not determined.
[a] Values represent mean ± standard deviation of three replicates.
[B] Turbid solution due to low solubility.

properties (Tepe et al., 2005). Antioxidant properties of essential oils from many plants have also been of great interest to the food processing industry, since their possible use as natural additives has emerged from a growing tendency to replace synthetic antioxidants with natural ones.

3.3.2. β-Carotene–linoleic acid assay

In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant, which results in a reduction in absorbance of the test solution with reaction time. This is due to the oxidation of linoleic acid that generates free radicals that attacks the highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. When this reaction occurs the β-carotene molecule looses its conjugation and, as a consequence, the characteristic orange colour disappears. The presence of antioxidant avoids the destruction of the β-carotene conjugate system and the orange colour is maintained. Essential oils were tested and all exhibited antioxidant activity (Table 4), the best results being obtained with the fresh leaves from the desert region, $IC_{50} = 0.47 \pm 0.04$ mg mL^{-1}. The plants from desert showed the highest activity. The values found are similar to the ones reported for *Mentha spicta* and *Mentha pulegium* (Mata et al., 2007). Comparing the activities of essential oils before and after drying, one can say that fresh material of wild *C. schoenanthus* collected either in the desert or in the mountain, affords essential oils with better antioxidant activities, while drying the cultivated plant material improves its activity. The antioxidant activity of the main compounds found in these extracts, limonene, α-terpineol and γ-terpinene was determined in a different study by Ruberto and Baratta (2000). Their results indicated that the antioxidant activity of these monoterpenes was similar to that found with the standard α-tocopherol.

Table 4
Antioxidant activity represented by IC_{50} (mg mL^{-1}) of essential oil of *C. schoenanthus* measured by the inhibitory effect on lipid peroxidation

Location	β-carotene/linoleic acid test IC_{50} (mg mL^{-1})[a]		
	Fresh leaves	Dried leaves	Dried roots
Desert (Dhibat)	0.47 ± 0.04	0.71 ± 0.03	1.07 ± 0.05
Mountain (Echareb)	0.64 ± 0.08	1.16 ± 0.05	0.85 ± 0.05
Experimental plot (Gourdhab)	1.19 ± 0.01	0.57 ± 0.06	Nd

Nd: Not determined.
[a] Values represent mean ± standard deviation of three replicates.

Table 5
Acetylcholinesterase inhibition capacity represented by IC_{50} (mg mL^{-1}), of essential oil of *C. schoenanthus*

Location	Acetylcholinesterase inhibition IC_{50} (mg mL^{-1})[a]		
	Fresh leaves	Dried leaves	Dried roots
Desert (Dhibat)	0.67 ± 0.09	0.52 ± 0.07	0.32 ± 0.03
Mountain (Echareb)	0.26 ± 0.03	0.44 ± 0.09	0.27 ± 0.03
Experimental plot (Gourdhab)	0.61 ± 0.17	0.28 ± 0.03	Nd

Nd: Not determined.
[a] Values are expressed as means ± standard deviation of three replicates.

3.4. Acetylcholinesterase inhibitory activity

Acetylcholine is a compound liberated at the synaptic gap as a neurotransmitter. Neurotransmitter disturbances and insufficient cholinergic functions are identified among the pathological features in central nervous system disorders. The most important changes observed in the brain are a decrease in cortical levels of the neurotransmitter acetylcholine. Inhibition of acetylcholinesterase therefore can restore the level of acetylcholine in the brain (Howes & Houghton, 2003). Plants have been used traditionally to enhance cognitive function and to alleviate other symptoms associated nowadays with Alzheimer's disease (Howes & Houghton, 2003). Most of the drugs used in Alzheimer therapy are formed by an enzyme inhibitor, e.g. galantamine, isolated from the extract of snowdrop (Mukherjee, Kumar, Mal, & Houghton, 2007). Few reports exist for the inhibitor activity of acetylcholinesterase by essential oils. The AChE inhibitory activity of the essential oils of fresh leaves, dried leaves and dried roots of the *C. schoenanthus* has never been reported before. Essential oil of this plant was tested to determine their ability as acetycholinesterase inhibitors and the results are depicted in Table 5. The greatest inhibitory activity was exhibited by the essential oil of fresh leaves of the plant collected from the mountain region ($IC_{50} = 0.26 \pm 0.03$ mg mL^{-1}). Analysis of results shows that these oils are moderate AChE inhibitors. Galantamine, a compound used pharmacologically showed an IC_{50} value of 1 mg/mL (Orhan, Sener, Choudhary, & Khalid, 2004).

This inhibitory activity cannot be attributed to the monoterpenes compound because the extract with higher activity as acetylcholinesterase inhibitor has the lowest content in this kind of compounds. The inhibition activity found with *C. shoenanthus* essential oils collected from Echareb have similar values to the ones obtained with *Foeniculum vulgare*, *M. spicta* and *M. pulegium* (Mata et al., 2007).

Drying the plant material also affects the inhibitory properties of the essential oils obtained. The better activities were exhibited with the essential oils obtained from the fresh wild plant material coming from the mountain region and with the dried material of the cultivated plant. The essential oils obtained from roots have similar activity of acetylcholinesterase inhibiton. The value of dried roots from the mountain region has a value of $IC_{50} = 0.27 \pm 0.03$ mg mL^{-1} and dried roots from the desert a value of $IC_{50} = 0.32 \pm 0.03$ mg mL^{-1}.

4. Conclusions

This is the first report on the antioxidant activity and acetylcholinesterase inhibition properties of essential oil of *C. schoenanthus* obtained from fresh and dried plant material. Results indicates that *C. schoenanthus* may be considered a functional food and reinforce the notion that the place from where the plants are collected or the conditions of cultivation have a strong influence on the biochemical activity of the plants. Essential oils obtained from fresh plants collected in a mountainous region (Echareb) were the more active on what concerns AChE inhibition and deserve further study on the compounds responsible for this activity. Consume of the plant either fresh in salads or dried in aromatic teas may act as a nutritional supplement with antioxidant activity. The inhibition of lipidic peroxidation exhibited by essential oil from desert region (Dhibat), and simultaneously its pleasant odour, indicates that it can be added in the food preparation, increasing its nutritional value and improving its preservation. The moderate acetylcholinesterase inhibitor activity together with the antioxidant activity indicates that *C. schoenanthus*

essential oils can be used in the prevention of neurodegenerative ailments.

Acknowledgements

The authors wish to thank Prof. Ana Cristina Figueiredo (DBV, FCUL, Lisbon) for her kind help utilizing the NTSYSpc database. One of the authors, A. Khadri, would also like to acknowledge Dr. Pedro Falé (CBV, FCUL, Lisbon) for his guidance in experimental work.

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