ELSEVIER

Fitoterapia 78 (2007) 474–481

FITOTERAPIA

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A new lupene triterpenetriol and anticholinesterase activity of *Salvia sclareoides*

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Received 24 September 2006; accepted 15 February 2007
Available online 10 May 2007

Abstract

A new lupene triterpenetriol was isolated from the acetone extract of the aerial parts of *Salvia sclareoides* and characterised as (1β,3β)-lup-20(29)-ene-1,3,30-triol (**1**). In addition, nepetidin (**2**), nepeticin (**3**), lupendiol (**4**), (1β,11α)-dihydroxy-lup-20(29)-en-3-one (**5**), ursolic acid (**6**), sumaresinolic acid (**7**) and hederagenin (**8**), were identified in this *Salvia* sp. To the best of our knowledge, the compounds 2 and 7 are new constituents in *Salvia* spp. The acetone, ethanol, butanol and water extracts of the plant were screened for the in vitro inhibitory activity of acetylcholinesterase (AChE) and butyrilcholinesterase (BChE), enzymes which play a role in the Alzheimer disease. All extracts inhibited acetylcholinesterase activity at 10 μg/ml, a remarkable activity since the standard drug rivastigmine does not inhibit acetylcholinesterase at the same concentration. Regarding the butyrilcholinesterase, the acetone extract at 1000 μg/ml was able to inhibit completely the enzyme activity and the butanol and ethanol extracts, at this concentration, produced a potent inhibition of BchE.
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Keywords: *Salvia sclareoides*; Triterpenoid; (1β,3β)-Lup-20(29)-ene-1,3,30-triol; Acetylcholinesterase; Butyrilcholinesterase

1. Introduction

Salvia sclareoides belongs to the genus *Salvia*, which includes over 1400 species widespread throughout subtropical and temperate zones [1]. Recognised as a xerophyte native to the Mediterranean region, *Salvia* spp are pharmacologically active and used in folk medicine all around the world. Cardioactive and antibacterial terpenoids were isolated from the extracts of several plants [2–4].

Salvia spp. have also been associated with insecticidal [5] and antifungal activities [6–8]. *S. miltiorrhiza*, used in traditional Chinese medicine to treat several pathologies, including insomnia, has also been described as a source of

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0367-326X/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.fitote.2007.02.013

tanshinone, which proved to decrease alcohol consumption [9], and of neo-tanshinlactone, an active compound against human breast cancer cell lines [10]. Hydroxylated ursene derivatives isolated from *S. kronenburgii* were also found to be highly cytotoxic against renal, non-small cell lung and breast cancer cell lines [11]. The extracts of *S. fruticosa*, *S. hortensis* and *S. officinalis* are also known for their antioxidant activity [12,13]. With respect to *S. officinalis*, a medicinal plant with memory improving properties, it has been used in traditional Chinese medicine to treat Alzheimer patients [14]. The essential oil of some *Salvia* spp. was reported to have anticholinesterase activity [15]. A variety of acetylcholinesterase (AChE) inhibitors of plant origin has also been reported, which includes mono-, di- and triterpenoids [16]. Recently, the screening of plants used to treat memory disfunction for AChE inhibitory activity has also been described [17]. The AChE and BChE enzymes are involved in neurotransmission in the brain and their inhibition has an important role in the progression of the Alzheimer disease. The mode of action of commercially available drugs for treating this disease was initially based on the inhibition of the AChE enzyme. However, research has been made to better understand the role of BChE in Alzheimer's disease patients [18], and some drugs which inhibit both AChE and BChE have been commercialized. Therefore, the search for new substances, more efficient and less expensive than the currently used for patient's treatment, is urgently needed.

S. sclareoides, being an endemic plant to Portugal, was previously reported in Mendes et al. [19].

This paper describes the isolation and structure elucidation of the new compound **1** in addition to the known terpenoids **2–8** (Fig. 1). AChE and BChE inhibitory activity of *S. sclareoides* acetone extract is also reported. In addition, the anticholinesterase activity of its ethanol, butanol and water extracts was investigated.

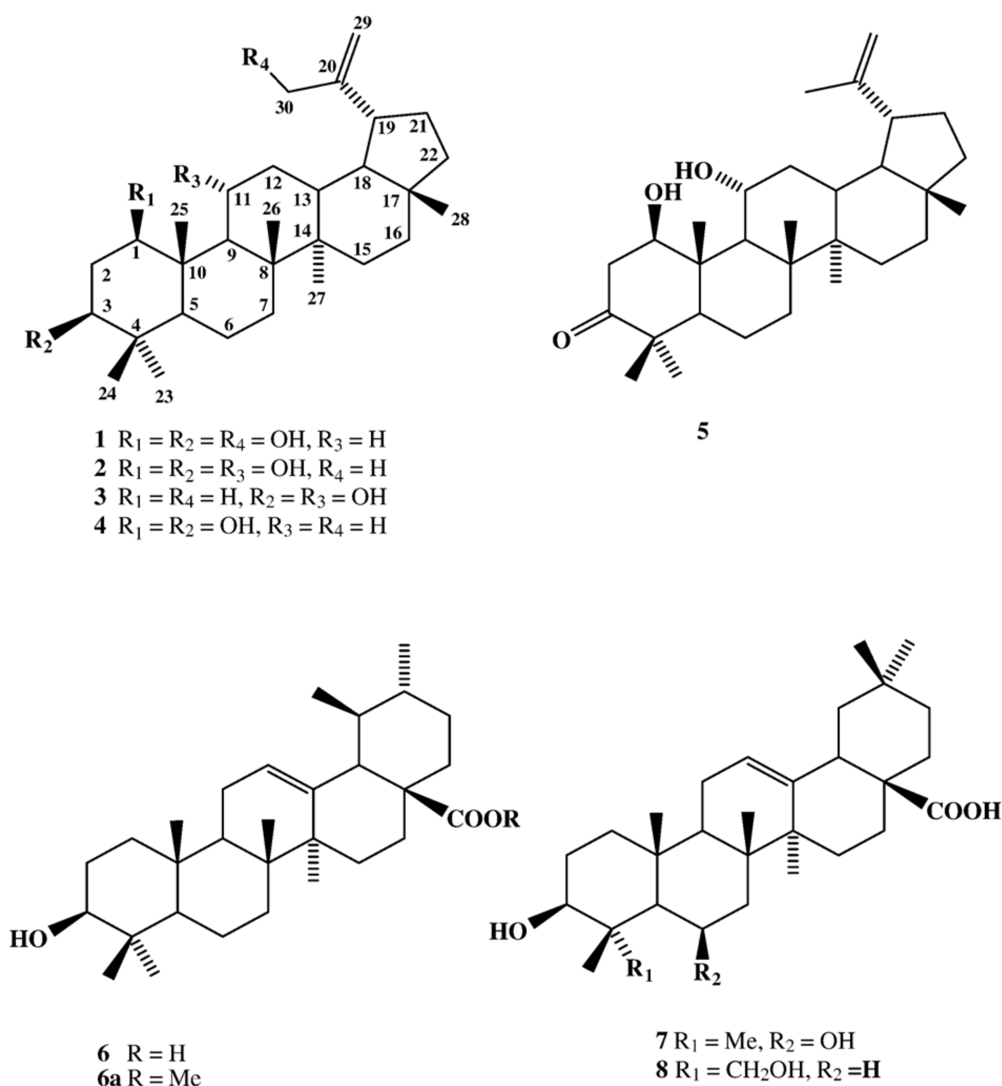

Fig. 1. Structure of compounds isolated from *S. sclareoides*.

2. Experimental

2.1. General

Melting points: digital melting point apparatus (Electrothermal). Optical rotations: Perkin-Elmer 343. ^{1}H NMR (400.1 MHz) and ^{13}C NMR (100.6 MHz) spectra, DEPT, HMQC, HMBC, COSY and NOESY spectra: Bruker ARX 400 in $CDCl_3$. IR: Mattson Satellite FT-IR spectrophotometer. EIMS 70 eV: Hewlett-Packard 5930.

2.2. Plant

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.

2.3. Extraction

The air-dried and finely powdered aerial parts (280 g) were extracted with acetone at r.t. Removal of the solvent gave an acetonic extract (11 g), which was then fractionated.

The EtOH extract (3 g) was prepared by extraction of powdered aerial parts (280 g) with EtOH.

Table 1
^{1}H and ^{13}C NMR data for compound **1** (400 and 100 MHz, $CDCl_3$, J in Hz, δ in ppm) and relevant HMBC correlations

C	δ_C	δ_H	HMBC correlations
1	79.02	3.41 dd ($J_{1\alpha,2\alpha}$ 4.6, $J_{1\alpha,2b}$ 11.2) (H-1α)	Me-25
2	38.07	1.83 td ($J_{2\alpha,2\beta}$ 12.7) (H-2α), 1.66–1.24 m (H-2β)	–
3	75.70	3.24 dd ($J_{2\alpha,3\alpha}$ 4.4; $J_{2\beta,3\alpha}$ 12.1) (H-3α)	Me-23, Me-24
4	38.86	–	Me-23, Me-24
5	54.70	0.58 dd ($J_{5,6\alpha}$ 2.3; $J_{5,6\beta}$, 11.1)	Me-23, Me-24
6	17.94	1.66–1.24 m	H-5
7	34.10	1.66–1.24 m	Me-26
8	41.35	–	Me-26
9	51.39	1.66–1.24 m	Me-25, Me-26
10	43.40	–	Me-25
11	23.84	1.66–1.24 m	–
12	26.64	0.97–1.03 m (H-12α), 1.66–1.24 m ((H-12β)	–
13	37.54	1.66–1.24 m	–
14	42.92	–	Me-27
15	27.46	1.66–1.24 m	Me-27
16	35.48	1.18–1.14 m (H-16α), 1.66–1.24 m (H-16β)	Me-28
17	42.92	–	Me-28
18	48.98	1.66–1.24 m	Me-28
19	43.82	2.29 td ($J_{19,21\alpha}$ $J_{18,19}$ 11.52; $J_{19,21\beta}$ 5.56)	H-30a,b
20	154.73	–	H-18, H-22, H-29a,b
21	29.68	1.99–2.11 m (H-21β), 2.23–2.15 m (H-21α)	–
22	39.83	1.66–1.24 m	Me-28
23	27.84	0.93 s	H-3, H-5, Me-24
24	14.93	0.73 s	H-3, H-5, Me-23
25	11.88	0.89 s	–
26	16.23	1.04 s	–
27	14.42	0.93 s	–
28	17.73	0.77 s	–
29	107.02	4.90 s (H-29a), 4.92 d (J 0.7) (H-29b)	H-30a,b
30	65.04	4.06, 4.10, 4.11, 4.15 ABX system (J_{AB}14.6) (H-30a,b)	H-29a,b

The butanol and water extracts were prepared as follows: the aerial parts (100 g) were extracted first with CH_2Cl_2 at r.t., then with MeOH and finally with 50% MeOH aqueous solution. MeOH was evaporated and the aqueous phase was extracted with BuOH, to give a triterpene rich extract (3 g). The aqueous phase was concentrated to dryness to give the water extract (7.8 g).

The acetone extract was Si-gel CC eluting with EtOAc/benzene mixtures of increasing polarity giving fractions A (145 mg), B (340 mg), C(300 mg), D (460 mg) and E (203 mg), which were further Si-gel CC.

Fraction A was eluted with EtOAc/*n*-hexane (1:4) to give **6** and a mixture which was methylated and subjected to preparative TLC (CH_2Cl_2/EtOH, 40:1) giving ursolic acid methyl ester (**6a**) (3 mg) and **3** (8 mg).

Fraction B was first methylated and then eluted with EtOAc/*n*-hexane (1:4) affording **6a** (13 mg), **4** (20 mg) and two different mixtures which were separated by preparative TLC (CH_2Cl_2/MeOH, 20:1) giving **5** (11 mg), **4** (35 mg) and the new triterpenetriol **1** (5 mg).

Fraction C was eluted with EtOAc/toluene (1:4) to give **4** (7 mg) and **6** (15 mg). Fraction D was eluted with EtOAc/hexane (5:2) followed by PTLC eluting with CH_2Cl_2/EtOH (20:1) to give **7** (1 mg) and **8** (13 mg).

Fraction F was eluted with CH_2Cl_2/EtOH (10:1) to give **2** (13 mg).

(1β,3β)-Lup-20(29)-ene-1,3,30-triol (**1**): Colourless crystals, mp 276–278 °C; $[\alpha]_D^{25}$ −7° (*c* 0.5, $CHCl_3$); IR bands ($CHCl_3$): 3600, 1618 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1; EIMS *m/z* (rel. int.): 440 $[M–H_2O]^+$ (4), 422 $[M–2H_2O]^+$ (5), 406 $[M–2H_2O–Me]^+$ (2), 219 (7), 203 (9), 207 (6), 189 (6), 187 (9), 175 (13), 149 (26), 55 (100).

2.4. Anticholinesterase assays

The Ellman's assay [20] was used to screen in vitro anticholinesterase activities of *S. sclareoides* acetone, ethanol, butanol and water extracts. The activity of the enzymes produces a yellow compound that is detected with a spectrophotometer along the reaction time. The enzyme activity (%) and the enzyme inhibition (%) were calculated from the rate of absorbance change with time ($V = \Delta Abs/\Delta t$) data as follows:

$$\text{Enzyme Inhibition } (\%) = 100 - \text{ Enzyme Activity } (\%)$$

$$\text{Enzyme Activity } (\%) = 100 \times V/V_{max}.$$

Maximum rates (V_{max}) are obtained when no inhibitor is used. V is the rate obtained in the presence of the inhibitor compound.

2.4.1. Spectrophotometer and chemicals

A double beam spectrophotometer Shimadzu® equipped with thermostatic cell holders was used on visible range and operated on the kinetic mode. The absorbance data were acquired in a computer by means of UV Probe software. Appropriate disposable plastic cuvettes Plastibrand® were used in the kinetic experiments.

The following materials were purchased from Sigma-Aldrich: enzymes AChE from human erythrocytes and BChE from human serum; substrates acetylthiocholine iodide (ATchI) and *S*-butyrilthiocholine iodide (BTchI) and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). Other Sigma-Aldrich P.A. reagents used for preparing buffers and solutions were KH_2PO_4, KOH, $NaHCO_3$. Deionised/sterilized water was used to prepare the buffer pH 8.0, the substrate and DTNB solutions.

2.4.2. Solutions preparation

Preparation of 0.1 M phosphate buffer pH 8.0: potassium dihydrogen phosphate (136.1 mg) was dissolved in water (10 ml) and adjusted with potassium hydroxide to a pH of 8.0±0.1. Buffer was freshly prepared and stored in the refrigerator.

AChE solution 1.32 U/ml: the enzyme (1.02041 U, 10 µl) was diluted in freshly prepared buffer pH 8.0 until a final volume of 0.773 ml.

BChE solution 0.44 U/ml: the enzyme (2.9762 U, 1.0 mg) was dissolved in freshly prepared buffer pH 8.0 (6.764 ml).

DTNB solution 0.01 M: DTNB (3.96 mg) was dissolved in water (1 ml) containing sodium hydrogen carbonate (1.5 mg).

ATchI solution 0.022 M: ATchI (6.4 mg) was dissolved in water (1 ml).

BTchI solution 0.022 M: BTchI (7.0 mg) was dissolved in water (1 ml).

All solutions were stored in Eppendorf caps (100 μl aliquots) in the refrigerator.

The solid extracts of *S. sclareoides* and rivastigmine were dissolved in freshly prepared buffer pH 8.0 in concentrations ranging between 10.73 and 1073 μg/ml to yield the final concentration for the enzymatic test between 10 and 1000 μg/ml.

2.4.3. AChE and BChE activity assay

The enzyme solution (5 μl) was added to the cuvette, then the plant extract solution (205 μl) was added, and finally the DTNB reagent (5 μl). The mixture was kept for 15 min at 30 °C in a heated water bath, and then the substrate reagent (5 μl) was added. The cuvette absorbance data along reaction time was taken for 4 min at 30 °C. At least three replicates were made. Several assays without the plant extract were carried out in order to determine the average V_{max}. Also white assays without the enzyme and plant extract were carried out to check for any non-enzymatic hydrolysis of the substrate. The final concentrations of chemicals in the test were as follows: [AChE] = 0.03 U/ml, [BChE] = 0.01 U/ml, [*Salvia* Extract] = 10 to 1000 μg/ml, [DTNB] = 0.0002273 M, [ATchI] = [BTchI] = 0.0005 M.

2.4.4. Statistical data analysis

The *t*-test (one sided) was carried out in order to evaluate if the average inhibition of the enzymes with *S. sclareoides* extracts (10, 100, 500, 1000 μg/ml) and the positive control rivastigmine are significantly higher than the average inhibition (0%) obtained in the assay without any inhibitor. The *t*-test gives a probability between 0.00 and 1.00. When the probability ≤0.05 or 5%, this means that the inhibition obtained with the extract at a certain concentration is significant. Then, for each extract, further comparison of the inhibitions obtained with the concentrations 10 and 1000 μg/ml was performed.

3. Results and discussion

The acetone extract of the aerial parts of *S. sclareoides* was subjected to column chromatography to give the new compound **1**. Its EIMS showed the loss of one and two water molecules at *m/z* 440 and 422, respectively. In the ^{1}H NMR spectrum (Table 1) the singlets observed at δ 0.73, 0.77, 0.89, 0.93 (6H) and 1.04 correspond to six methyl groups. The signals of H-30a,b appeared as an ABX system at ca. δ 4 ppm exhibiting the coupling constants $^{2}J_{AB}$ 14.6 Hz and $^{4}J_{29b,30}$ 0.7 Hz. The resonances observed for the methylene group (H-29a,b) appeared as a doublet at δ 4.92 (H-29b) and as a singlet at δ 4.90 (H-29a). Two double doublets at δ 3.24 and 3.43 were assigned to the methyne protons H-3 and H-1. Their chemical shift and coupling constants were in full agreement with those reported for other 1β,3β-dihydroxylupene derivatives [21]. These structural features were confirmed by the ^{13}C NMR spectrum, which showed the signals at δ 107.02 and 154.73 for the 20(29) double bond. The resonances at δ 65.04, 75.70 and 79.02 were assigned by HMQC to the hydroxymethyl group (C-30) and to the carbons bearing secondary hydroxyl groups,

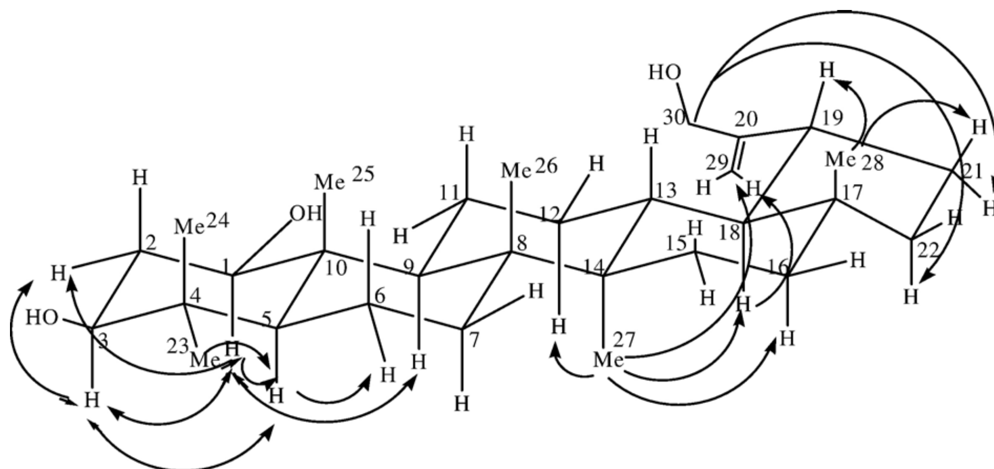

Fig. 2. NOESY correlations in the ^{1}H NMR spectrum of compound 1.

Table 2
Inhibition (%) of the *S. sclareoides* extracts on acetylcholinesterase

Extract	Concentration (μg/ml)	Inhibition (%)
Acetone	1000	100**
	500	61**
	10	44**
Ethanol	1000	77**
	500	56**
	10	39*
Butanol	1000	100**
	500	96**
	10	61**
Water	1000	67**
	100	62**
	10	65**
Rivastigmine [a]	100	95**
	10	0

** $P<0.001$; * $P<0.01$.
[a] Rivastigmine is the standard drug.

namely C-3 and C-1, respectively. The presence of the hydroxymethyl at C-30 was confirmed on the basis of two-dimensional correlations (HMBC, Table 1) by detection of the correlations for H-30a,b with C-29 at δ 107.02 and for H-29a and H-29b with C-30 at δ 65.04. H-30a,b showed also a correlation with C-19 at δ 43.82, which appears usually in lupene derivatives around δ 48.0 [21]. Assignment of the signals of C-25, C-23 and C-24 was easily accomplished by the observed correlations with H-1 (C-25) and H-3 (C-23 and C-24). The resonances of the quaternary carbons C-4, C-8, C-10, C-14 and C-17 were also assigned by the correlations with the methyl groups bonded to these carbons (Table 1). NOESY, COSY and HMBC experiments were run in order to assign the ^{1}H NMR signals of the molecule, being the most relevant NOESY correlations depicted in Fig. 2. They allowed to determine either the resonances of the methyne protons H-5, H-9, H-13, H-18, H-19 or those of the methylene protons, including their α or β orientations, in most cases.

Nepetidin (**2**) appears in *Salvia* species for the first time, being previously isolated from *Nepeta hindostana* [22], while nepeticin (**3**), reported as a constituent of *N. hindostana* [23], was described as a secondary metabolite of *Salvia pinnata* [24] and of *S. candelabrum* [25]. The triterpene ketone **5** has been first isolated from *S. deserta* [21]. Physical

Table 3
Inhibition (%) of the *S. sclareoides* extracts on butyrilcholinesterase

Extract	Final concentration (μg/ml)	Inhibition (%)
Acetone	1000	100**
	500	72**
	100	19*
	10	9
Ethanol	1000	79**
	500	15*
	100	11
	10	0
Butanol	1000	94*
	500	86*
	100	0
	10	9
Water	1000	22
	100	22
	10	0
Rivastigmine [a]	100	100*
	10	100*

** $P<0.001$; * $P<0.01$.
[a] Rivastigmine is standard drug.

and spectroscopic data for compounds **2–5** were in full agreement with the literature. Along with lupendiol (**4**) and ursolic acid (**6**) previously identified in this plant [19], we have characterised the oleanene derivative sumaresinolic acid (**7**), reported in *Salvia* species for the first time and previously described in *Enkianthus campanulatus* [26] and in *Miconia stenostachya* [27]. Also hederagenin (**8**) was identified in this plant. This triterpenoid is known to possess in vitro antitrichomonas activity [28] and its activity as an anti-platelet agent was also reported [29].

The maximum rates (V_{max}) of 32 mAbs/min for AChE and 75 mAbs/min for BChE were obtained by the kinetic assays conducted without inhibitor. Given the variability of the inhibition results obtained with both enzymes, statistical *t*-tests were carried out, being the final conclusions based upon them. The inhibition results as well as *t*-test probabilities are shown in Tables 2 and 3. All the extracts inhibited significantly the enzyme AChE at 10 μg/ml, a promising result when compared to that of other plants reported in the literature [30] and of rivastigmine, a drug used for medication of Alzheimer's disease patients, that were unable to inhibit AChE at this concentration. The BChE was significantly inhibited by the acetone, ethanol and butanol extracts at the concentrations ≥ 500 μg/ml (acetone or butanol extracts) or 1000 μg/ml (ethanol extract), while rivastigmine inhibited BChE already at the concentration of 10 μg/ml. The selectivity for BChE detected for rivastigmine is in agreement with data previously reported for this compound [31]. The water extract did not show any significant inhibition of the enzyme at the concentrations tested. Regarding the degree of inhibition, the acetone extract at 1000 μg/ml was able to inhibit completely AChE and BChE. The butanol extract inhibited totality the activity of AChE also at 1000 μg/ml. AChE inhibition values higher than 50% were obtained with the butanol and water extracts at concentrations ≥ 10 μg/ml, as well as with the acetone and ethanol extracts at concentrations ≥ 500 μg/ml. With respect to BChE inhibition, more than 50% inhibition was achieved with the acetone and butanol extracts at concentrations ≥ 500 μg/ml and with the ethanol extract at 1000 μg/ml. In conclusion, the *S. sclareoides* acetone extract, whose phytochemical study indicated that it is a triterpene rich extract, and the butanol extract, displayed both a highly potent inhibition of both enzymes at 1000 μg/ml, being significant the inhibition of AChE by all the extracts tested at the concentration of 10 μg/ml. Considering these findings, the further study of the plant appears to be promising for the search of new agents against the Alzheimer's disease.

Acknowledgements

The authors thank Fundação para a Ciência e Tecnologia for the Post-Doctoral Grant BPD 14408/2003.

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