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ORIGINAL ARTICLE

Acetylcholinesterase and butyrylcholinesterase inhibitory activity of *Pinus* species essential oils and their constituents

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Abstract

This study aimed to investigate the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity of the essential oils from *Pinus nigra* subsp. *nigra*, *P. nigra* var. *calabrica*, and *P. heldreichii* subsp. *leucodermis*. This activity is relevant to the treatment of Alzheimer's disease (AD), since cholinesterase drugs are currently the only drugs available to treat AD. *P. heldreichii* subsp. *leucodermis* exhibited the most promising activity, with IC_{50} values of 51.1 and 80.6 μg/mL against AChE and BChE, respectively. An interesting activity against AChE was also observed with *P. nigra* subsp. *nigra* essential oil, with an IC_{50} value of 94.4 μg/mL. Essential oils were analyzed by GC and GC-MS with the purpose of investigating their relationships with the observed activities. Among the identified constituents, terpinolene, β-phellandrene, linalyl acetate, *trans*-caryophyllene, and terpinen-4-ol were tested. *trans*-Caryophyllene and terpinen-4-ol inhibited BChE with IC_{50} values of 78.6 and 107.6 μg/mL, respectively. β-Phellandrene was selective against AChE (IC_{50} value of 120.2 μg/mL).

Keywords: Pinus *species; essential oil; GC-MS; acetylcholinesterase and butyrylcholinesterase inhibition*

Introduction

Neurodegenerative disease is a generic term applied to a variety of conditions arising from a chronic breakdown and deterioration of the central nervous system (CNS) neurons. Among the numerous variants of degenerative dementia, Alzheimer's disease (AD) is by far the most prevalent, affecting more than 20 million people worldwide. In spite of the multifactorial nature of AD, only one therapeutic approach is currently followed. This strategy is based on the so-called cholinergic hypothesis of cognitive dysfunction[1]. This hypothesis postulates that at least some of the cognitive decline experienced by patients of AD results from a deficiency in neurotransmitter acetylcholine (ACh) and thus in cholinergic neurotransmission in brain cortical or hippocampal regions, which seems to play a fundamental role in memory[2]. Moreover, in the late AD stage, levels of acetylcholinesterase (AChE) have declined by up to 85%, and butyrylcholinesterase (BChE) represents the predominant cholinesterase in the brain[3]. BChE, primarily associated with glial cells but also with specific neuronal pathways, cleaves ACh in a manner similar to AChE to terminate its physiological action[4,5]. Such studies have targeted BChE as a new approach to intercede in the progression of AD[6].

Thus, restoring the level of ACh through inhibition of both major forms of cholinesterase, AChE and BChE, continues to be a therapeutic useful approach to treat not only AD but also other forms of dementia.

The potential use of natural products has been successfully demonstrated in the field of AD[7,8]. Among natural sources, essential oils are attracting special attention. Indeed, the results of different recent studies indicate that several essential oils from food crops[9], medicinal plants[10–13], and tea tree oils[14,15] show significant anti-cholinesterase inhibitory activity. The effects of *Salvia lavandulaefolia* essential oil and some of its constituents

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on acetylcholinesterase have been reported *in vitro* and *in vivo*[16,17]. In our previous investigations, the inhibition of acetylcholinesterase and butyrylcholinesterase enzymes by *S. leriifolia*, *Origanum ehrenbergii*, and *O. syriacum* essential oils was reported[18–20]. Various essential oil components have been investigated for their effects on AChE. It has been found that the majority of AChE inhibitors identified in the essential oils are terpenoids[15,20–23]. Due to their small molecular size and lipophilicity, volatile constituents of essential oils are likely to readily cross the blood–brain barrier[24].

As a part of our efforts to find new anti-cholinesterase active compounds[18–20,25], in this study we aimed to evaluate the AChE and BChE inhibitory activity of essential oils from three *Pinus* species, i.e. *Pinus nigra* subsp. *nigra*, *P. nigra* var. *calabrica*, and *P. heldreichii* subsp. *Leucodermis*, and some identified components by screening them using the microplate assay method. The composition profiles of the essential oils were characterized by gas chromatography-mass spectrometry (GC-MS) analysis, and the relationships between the chemical components and the AChE and BChE inhibitory activity were outlined.

Materials and methods

General

Methanol and dichloromethane were purchased from VWR International (Milan, Italy). Acetylcholinesterase (AChE) from *Electrophorus electricus* (EC 3.1.1.7, Type VI-S) and butyrylcholinesterase (BChE) from equine serum (EC 3.1.1.8), physostigmine, galanthamine hydrobromide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), α-pinene, β-pinene, limonene, sabinene, α-terpinene, γ-terpinene, terpinolene, linalool, linalyl acetate, *trans*-caryophyllene, α-humulene, δ-cadinene, palmitic acid, myristic acid, palmitic acid methyl ester, tricosane, heneicosane, and pentacosane were purchased from Sigma-Aldrich (Milan, Italy).

Plant material

The needles of *Pinus nigra* Arnold subsp. *nigra*, *P. nigra* Arnold var. *calabrica* C. K. Scheid, and *P. heldreichii* Christ subsp. *leucodermis* (Antoine) E. Murray were collected at the full flowering stage from plants growing in Calabria, Italy (*P. heldreichii* subsp. *leucodermis* and *P. nigra* subsp. *nigra* on the Parco Nazionale del Pollino, *P. nigra* var. *calabrica* on Sila) and authenticated by Dr. N. G. Passalacqua of the Botany Department at the University of Calabria (Italy). A voucher specimen has been retained at the Herbarium of the University of Calabria (CLU).

Essential oil isolation

The essential oils from the needles of *P. nigra* subsp. *nigra*, *P. nigra* var. *calabrica*, and *P. heldreichii* subsp. *leucodermis* were obtained by hydrodistillation for 3 h, using a Clevenger-type apparatus[26]. The oils were dried (anh. Na_2SO_4) and stored under N_2 at +4°C in brown bottles until analyzed and tested. The yield of the oils was 0.3% (v/w) for *P. nigra* subsp. *nigra*, 0.2% (v/w) for *P. nigra* var. *calabrica*, and 0.2% (v/w) for *P. heldreichii* subsp. *leucodermis*.

Gas chromatography-mass spectrometry (GC-MS) analysis

Analytical gas chromatography was carried out on a Hewlett-Packard 6890 gas chromatograph equipped with an SE30 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) and interfaced with a Hewlett-Packard 5973 mass selective detector. Ionization of the sample components was performed in electron impact mode (EI, 70 eV), with helium as the carrier gas. The analytical conditions were: oven temperature 5 min isothermal at 50°C, then 50–250°C at a rate of 13°C/min, then held isothermal for 10 min. Injector and detector were maintained at 250°C and 280°C, respectively. Analyses were also run by a HP-Innowax capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness). Gas chromatographic conditions were as given.

Gas chromatography (GC) analyses

Essential oils were analyzed by a Shimadzu GC17A gas chromatograph system. An SE30 capillary column (30 m with an internal diameter of 0.25 mm and a film thickness of 0.25 μm) was used with nitrogen as the carrier gas. GC oven temperature and conditions were as described above. Quantification of the components was performed on the basis of their GC peak areas and percentages of the characterized essential oil components were as given in Table 1.

Qualitative and quantitative analyses

Identification of the compounds was achieved through retention indices (*I*), against those from the literature[27,28] or those of authentic compounds available in our laboratory. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8–C_{24}) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with those stored in Wiley 138, Wiley 275, or NIST 98 libraries or with mass spectra from the literature[28]. Component relative concentrations were calculated based on GC peak areas without using correction factors.

Microtiter cholinesterase inhibition assay

Acetylcholinesterase and butyrylcholinesterase inhibiting activities were measured by slightly modifying the spectrophotometric method previously developed by Ellman *et al.*[29], which is based on the reaction of released thiocholine to give a colored product with a chromogenic reagent. *Electrophorus electricus* (EC 3.1.1.7, Type VI-S) AChE and equine serum (EC 3.1.1.8) BChE were used, while acetylthiocholine iodide and butyrylthiocholine iodide, respectively, were used as substrates of the reaction. 5,5′-Dithio-bis(2-nitrobenzoic-acid) (DTNB) was used for measurement of the cholinesterase activity. In

Table 1. Chemical composition of the essential oils from *P. nigra* var. *calabrica* (P1), *P. heldreichii* subsp. *leucodermis* (P2), and *P. nigra* subsp. *nigra* (P3).

			%[c]		
I^a/I^b	Compound	ID method[d]	P1	P2	P3
936/1032	α-Pinene	*I*, MS, Co-GC	24.6	24.2	25.3
953/1076	Camphene	*I*, MS	1.1	0.4	2.2
973/1138	Sabinene	*I*, MS, Co-GC	0.2	0.1	12.8
978/1118	β-Pinene	*I*, MS, Co-GC	10.9	8.4	4.8
1005/1186	α-Phellandrene	*I*, MS	0.4	—	0.3
1012/1159	δ-3-Carene	*I*, MS	1.7	0.2	—
1016/1188	α-Terpinene	*I*, MS, Co-GC	0.2	1.5	0.4
1025/1280	*p*-Cymene	*I*, MS	—	0.2	—
1030/1218	β-Phellandrene	*I*, MS, Co-GC	6.3	tr	tr
1032/1203	Limonene	*I*, MS, Co-GC	tr	7.8	22.6
1040/1246	(*Z*)-β-Ocimene	*I*, MS	4.0	—	—
1047/1266	(*E*)-β-Ocimene	*I*, MS	—	3.7	1.7
1059/1255	γ-Terpinene	*I*, MS, Co-GC	0.3	1.0	0.8
1089/1290	Terpinolene	*I*, MS, Co-GC	1.1	5.9	4.5
1098/1553	Linalool	*I*, MS, Co-GC	0.1	0.5	—
1100/1400	*n*-Nonanal	*I*, MS	0.1	0.1	tr
1113/	endo-Fenchol	*I*, MS	tr	0.1	—
1128/1499	α-Campholene aldehyde	*I*, MS	0.1	0.3	0.1
1138/1665	*trans*-Pinocarveol	*I*, MS	tr	0.2	—
1178/1611	Terpinen-4-ol	*I*, MS, Co-GC	0.1	0.8	0.4
1189/1683	α-Terpineol	*I*, MS	0.3	1.7	8.3
1228/	Myrtenyl acetate	*I*, MS	0.2	0.6	0.1
1240/	Linalyl acetate	*I*, MS, Co-GC	tr	3.6	tr
1265/1604	Thymol methyl ether	*I*, MS	0.4	0.2	—
1289/1597	Bornyl acetate	*I*, MS	0.7	2.7	0.2
1350/1466	α-Cubebene	*I*, MS	1.0	7.6	—
1370/1733	Neryl acetate	*I*, MS	0.3	—	—
1388/1765	Geranyl acetate	*I*, MS	0.4	0.4	—
1373/1192	α-Ylangene	*I*, MS	—	—	0.6
1377/1497	α-Copaene	*I*, MS	0.3	0.4	tr
1385/1535	β-Bourbonene	*I*, MS	—	0.5	—
1403/	α-Bergamotene	*I*, MS	1.0	—	—
1408/2030	Methyl eugenol	*I*, MS	0.5	0.3	—
1418/1612	*trans*-Caryophyllene	*I*, MS, Co-GC	4.2	4.5	3.0
1439/1628	Aromadendrene	*I*, MS	0.4	—	—
1441/1662	*trans*-β-Farnesene	*I*, MS	—	0.9	—
1454/1690	α-Humulene	*I*, MS, Co-GC	1.2	1.0	0.5
1478/1725	Germacrene D	*I*, MS	0.8	0.7	0.5
1483/	γ-Muurolene	*I*, MS	0.9	0.2	0.1
1495/1731	Epi-bicyclosesquiphellandrene	*I*, MS	—	—	0.7
1499/1742	α-Muurolene	*I*, MS	—	—	0.8
1512/	Bicyclo[4.4.0]dec-1-en, 2-isopropyl-5-methyl-9-methylene	*I*, MS	0.7	0.3	0.2
1515/1765	γ-Cadinene	*I*, MS	9.9	1.0	0.7
1524/1772	δ-Cadinene	*I*, MS, Co-GC	1.9	1.8	0.5
1534/2496	Ethyl laurate	*I*, MS	0.7	0.3	0.4
1567/2502	Lauric acid	*I*, MS	1.7	0.8	0.5
1612/	Tetradecanal	*I*, MS	0.3	0.5	0.2
1624/	Junipene	*I*, MS	—	—	0.1
1722/	Farnesol	*I*, MS	0.4	—	tr
1810/	Hexadecanal	*I*, MS	1.5	0.3	0.1
1881/	1-Octadecene	*I*, MS	0.3	0.2	0.3
1893/	1-Nonadecene	*I*, MS	0.9	—	—
1934/	Methyl palmitate	*I*, MS, Co-GC	—	0.8	—

Table 1. continued on next page

Table 1. Continued.

I^a/I^b	Compound	ID method[d]	%[c] P1	P2	P3
1942/	(*E*)-3-Cembrene A	*I*, MS	0.9	—	0.3
1960/	Sandaracopimaradiene	*I*, MS	—	0.8	—
1969/2931	Palmitic acid	*I*, MS, Co-GC	—	0.5	—
1987/2713	Myristic acid	*I*, MS, Co-GC	0.8	0.2	0.4
1996/	Methyl linoleate	*I*, MS	1.2	—	—
1989/2375	Manoyl oxide	*I*, MS	6.2	0.6	—
2000/2000	Eicosane	*I*, MS	0.7	—	—
2022/	Octadecanal	*I*, MS	0.2	—	—
2100/2100	Heneicosane	*I*, MS, Co-GC	—	0.5	0.3
2300/2300	Tricosane	*I*, MS, Co-GC	0.5	0.2	0.6
2365/	Methyl neoabietate	*I*, MS	tr	0.6	—
2500/2500	Pentacosane	*I*, MS, Co-GC	0.8	0.4	—
2900/2900	Nonacosane	*I*, MS, Co-GC	—	0.8	0.4
	Identified compounds		93.4	91.3	95.7

Note. Hydrodistillation (HD), cold-pressing (CP), supercritical fluid extraction (SFE).
[a]Retention index on SE30 MS column.
[b]Retention index on HP-Innowax column.
[c]Mean value ± standard error, *n* = three independent determinations. Compositional values less than 0.1% are denoted as traces (tr).
[d]*I*, Retention index; MS, mass spectrum; Co-GC: co-injection with authentic compound.

this procedure, AChE or BChE (0.20 U/mL in buffer, pH 8) plus *Pinus* essential oils at final concentrations in the test solution ranging from 20 to 500 µg/mL (20 µL) and pure compounds at final concentrations ranging from 10 to 200 µg/mL (20 µL) were added to 2 mL of buffer, pH 8, and pre-incubated in an ice bath at 4°C for 30 min. Tested essential oils and control were dissolved in 5% MeOH. Duplicate tubes were also treated this way, with 20 µL of positive control (0.1 mM), to allow interference of the test substances in the assay to be assessed, and to control for any hydrolysis of acetylcholine or butyrylcholine not due to enzyme activity. The reaction was started by adding DNTB solution (20 µL of 0.05 mM in buffer, pH 7) and acetylthiocholine iodide (ATCI) or butyrylthiocholine iodide (BTCI) (20 µL of 0.018 mM in buffer, pH 7) and tubes were kept in a water bath for 20 min at 37°C. The reaction was halted by placing the assay solution tubes in an ice bath and adding physostigmine (20 µL of 0.018 mM in buffer, pH 7). Blanks were used of reagents without essential oils, and the positive control physostigmine (20 µL of 0.018 mM in buffer, pH 7) was added. The hydrolysis of acetylthiocholine and butyrylthiocholine was monitored by formation of the yellow 5-thio-2-nitrobenzoate anion, as the result of the reaction of DTNB with thiocholine, released during enzymatic hydrolysis; this was immediately recorded on a spectrophotometer (Jenway 6300) at 405 nm and the percentage inhibition was calculated. The samples and positive controls were dissolved in 5% methanol, which was used for the control. All the reactions were performed in triplicate. The inhibition rate (%) was calculated by equation:

$$\text{Inhibition}\% = \frac{\left[\begin{array}{l}(\text{Blank} - \text{Blank positive control}) \\ -(\text{Experiment} - \text{Experiment control})\end{array}\right]}{(\text{Blank} - \text{Blank positive control})}$$

Statistical analysis

Differences were evaluated by one-way analysis of variance (ANOVA) test completed by a multicomparison Dunnett's test. Differences were considered significant at $p < 0.01$. The 50% inhibitory concentration (IC_{50}) was calculated from a dose–response curve obtained by plotting the percentage of inhibition versus concentration with the use of GraphPad Prism 4.0 software.

Results and discussion

Essential oils composition

In order to identify putative active compounds present within the essential oils, gas chromatography systems were employed. The chemical composition of the oils is reported in Table 1. *P. nigra* var. *calabrica* essential oil was characterized by 53 constituents (93.4% of the total oil), in which the dominant components were α-pinene (24.6%), β-pinene (10.9%), γ-cadinene (9.9%), β-phellandrene (6.3%), manoyl oxide (6.2%), and *trans*-caryophyllene (4.2%). *P. nigra* subsp. *nigra* essential oil was characterized by 41 components, representing 95.7% of the total oil. The main components were α-pinene (25.3%), limonene (22.6%), sabinene (12.8%), α-terpineol (8.3%), β-pinene (4.8%), and terpinolene (4.5%). Many terpenoid compounds are typical constituents of conifer resin, and have been reported from *P. nigra* foliage before[30–33]. Regardless of how the pine twigs were treated, α-pinene, β-pinene, β-myrcene, limonene, and β-phellandrene were the major monoterpenes. The components (*E*)-β-caryophyllene and germacrene D were the predominating sesquiterpenes, confirming former studies[30–33]. Macchioni *et al.*[30] analyzed essential oils from needles, branches without needles, and cones of *P. nigra* separately. They detected the components 1,8-cineole, pinocamphone, and myrtenal in branches and cones only,

but not in needle tissue. On the other hand, α-terpinyl acetate, β-cubebene, (*E*)-β-farnesene, and α-humulene were exclusively detected in needle tissue[30].

A total of 50 compounds (91.3% of the total oil) were identified in *P. heldreichii* subsp. *leucodermis* essential oil. α-Pinene (24.2%) and β-pinene (8.4%), limonene (7.8%), α-cubebene (7.6%), terpinolene (5.9%), and *trans*-caryophyllene (4.5%) were the major constituents. A recent study[34] reported the essential oil compositions of *P. heldreichii* from Montenegro and Serbia. In the pine-needle terpene profile from three populations from Montenegro, and one from Serbia, the dominant constituents were limonene (26.3%), α-pinene (17.5%), germacrene D (13.5%), and β-caryophyllene (10.4%), constituting ~67.7% of the essential oil. Medium-to-high contents (0.5–10%) of the following 16 additional components were found: β-pinene, β-myrcene, α-humulene, δ-cadinene, α-muurolene, (*E*)-hex-2-enal, β-gurjunene, γ-muurolene, isopimarol, camphene, γ-cadinene, aromadendrene, β-bisabolene, *trans*-β-farnesene, α-cadinene, and (*Z*)-hex-3-en-1-ol.

The analyzed essential oils showed marked differences, especially from the quantitative point of view of the most abundant components. In particular, in comparison to the other oils, *P. nigra* subsp. *nigra* essential oil was characterized by a high content of sabinene (12.8%), limonene (22.6%), and α-terpineol (8.3). Manoyl oxide (6.2%) and γ-cadinene (9.9%) were present in high quantity only in *P. nigra* var. *calabrica* essential oil. Interestingly, β-phellandrene (6.3%) and α-bergamotene (1.0%) were detected only in *P. nigra* var. *calabrica* oil, and linalyl acetate (3.6%) only in *P. heldreichii* subsp. *leucodermis* essential oil.

Cholinesterase activity

In spite of the multifactorial nature of Alzheimer's disease, most current agents follow one therapeutic approach, based on the so-called cholinergic hypothesis of cognitive dysfunction. Currently, cholinesterase inhibition is the major treatment for the symptoms of AD, and inhibition of AChE and BChE are therapeutic targets for improving the cholinergic deficit.

The *in vitro* cholinesterase inhibitory activity of three *Pinus* species growing in Calabria (Italy) essential oils and some identified constituents was evaluated by Ellman's spectrophotometric method using an enzyme-linked immunosorbent assay (ELISA) microplate reader. All studied essential oils were able to inhibit *in vitro* enzymes in a concentration-dependent manner (Figure 1). IC_{50} values were calculated and are reported in Table 2. *P. heldreichii* subsp. *leucodermis* exhibited the most promising activity, with IC_{50} values of 51.1 and 80.6 μg/mL, against AChE and BChE, respectively. Interesting activity against AChE was observed also with *P. nigra* var. *calabrica* and *P. nigra* subsp. *nigra*, with IC_{50} values of 101.5 and 94.4 μg/mL, respectively. BChE appeared less sensitive to *P. nigra* var. *calabrica* and *P. nigra* subsp. *nigra* essential oil (IC_{50} values of 128.0 and 162.5 μg/mL, respectively). AChE plays an important role in the central nervous system. It is one of the fastest known enzymes, and catalyzes the cleavage of acetylcholine in the synaptic cleft after depolarization. Inhibitors of AChE, such as galanthamine, are used frequently in the pharmacotherapy of AD. The less specific BChE has recently been a focus of research, because BChE concentration stays the same, or is even up-regulated, while AChE is dramatically down-regulated in the brains of patients suffering from AD[36]. It was previously reported that essential oils rich in terpenes exhibit a strong inhibitory activity against both enzymes[35,37,38]. Among the *Pinus* essential oil components, five compounds, namely β-phellandrene, terpinolene, terpinen-4-ol, linalyl acetate, and *trans*-caryophyllene, were tested for the first time in this study to evaluate their potential activity (Table 2). The dose-dependent inhibitory activity of terpinolene and β-phellandrene against AChE is reported in Figure 2. β-Phellandrene showed a selective activity against AChE,

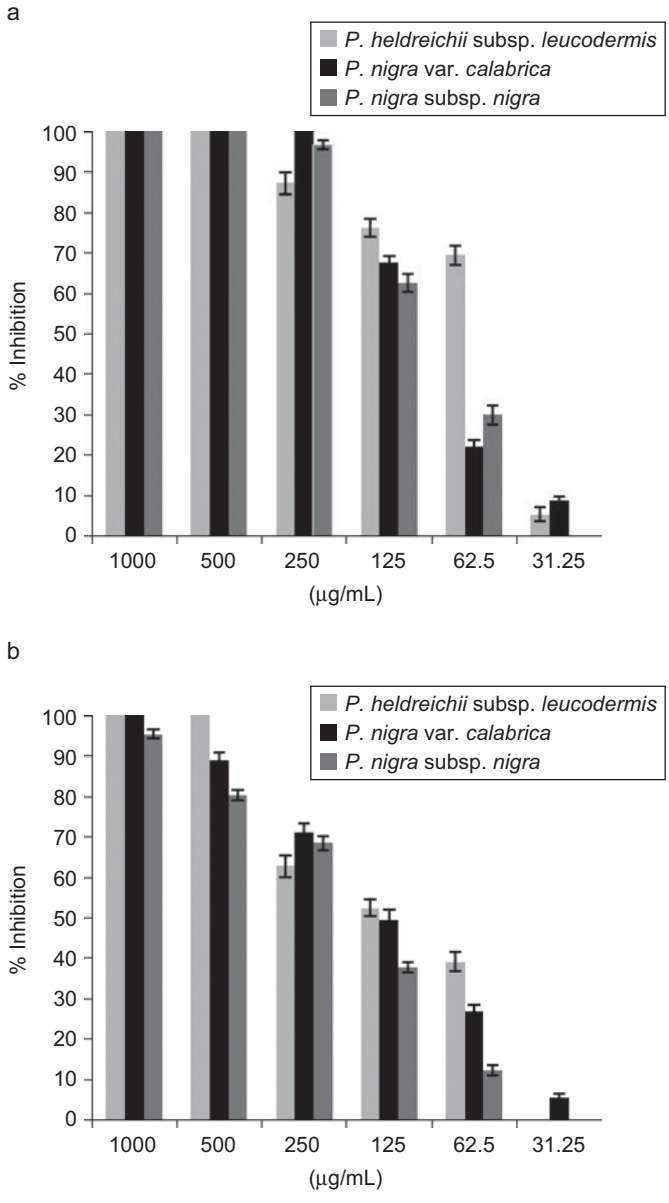

Figure 1. Dose-dependent inhibitory activity of *Pinus* essential oils against AChE (a) and BChE (b). Data are given as mean ± SD ($n = 3$).

Table 2. Cholinesterase inhibitory activity of essential oils and identified constituents from *Pinus* species (IC_{50}, μg/mL).

	AChE	BChE	SI (BChE/AChE)
Essential oils			
P. heldreichii subsp. *leucodermis*	51.1 ± 1.8*	80.6 ± 2.3*	1.6
P. nigra var. *calabrica*	101.5 ± 2.4*	128.0 ± 2.4*	1.3
P. nigra subsp. *nigra*	94.4 ± 1.8*	162.5 ± 1.4*	1.7
Terpenes			
β-Phellandrene	120.2 ± 2.0*	> 50	
Terpinolene	156.4 ± 2.8*	147.1 ± 2.5*	0.9
Terpinen-4-ol	21.4% (1.2 mM)[21]	107.6 ± 1.2*	
Linalyl acetate	38% (82 μg/mL)[35]	168.7 ± 2.1*	
trans-Caryophyllene	32% (0.06 mM)[22]	78.6 ± 1.3*	
Positive controls			
Physostigmine	0.1 ± 0.01	0.2 ± 0.01	2
Galanthamine hydrobromide	0.3 ± 0.04	3.0 ± 0.04	10

Note. IC_{50} values are mean ± SD (n = 3). One-way ANOVA analysis: ***$p < 0.0001$; Dunnett's test: *$p < 0.01$.

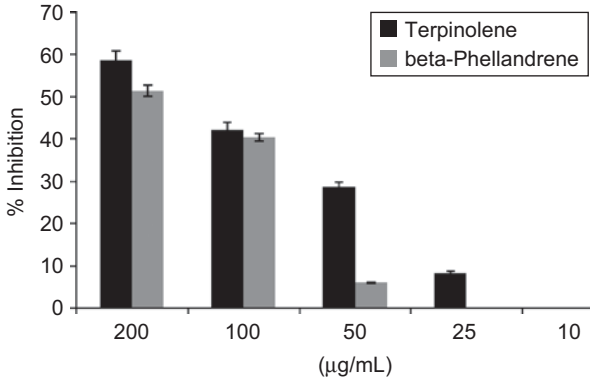

Figure 2. Dose-dependent inhibitory activity of terpinolene and β-phellandrene against AChE. Data are given as mean ± SD (n = 3).

with an IC_{50} value of 120.2 μg/mL, while terpinolene inhibited both AChE and BChE enzymes with IC_{50} values of 156.4 μg/mL and 147.1 μg/mL, respectively. Except β-phellandrene, all tested terpenes showed BChE inhibitory activity (Table 2, Figure 3), with IC_{50} values ranging from 78.6 μg/mL to 168.7 μg/mL for *trans*-caryophyllene and linalyl acetate, respectively. Perusal of the literature revealed a promising agreement between our results and the reported activities of a number of terpenes. Indeed, an impressive body of information exists on the anti-cholinesterase activity of plant terpenes, and in particular of monoterpenes. Many works have concentrated on the action of compounds identified in *Pinus* essential oils and on the synergistic effect of a mixture of some terpenes. Perry *et al.*[16] reported the AChE inhibitory activity of α-pinene with an IC_{50} of 0.63 mM. The AChE inhibitory property was also reported for several monoterpene hydrocarbons such as α-terpinene and limonene[21]. In contrast a weak AChE inhibitory activity was found for linalyl acetate that inhibited the enzyme with a percentage of 38% at 82 μg/mL[39]. β-Pinene inhibited AChE with a percentage

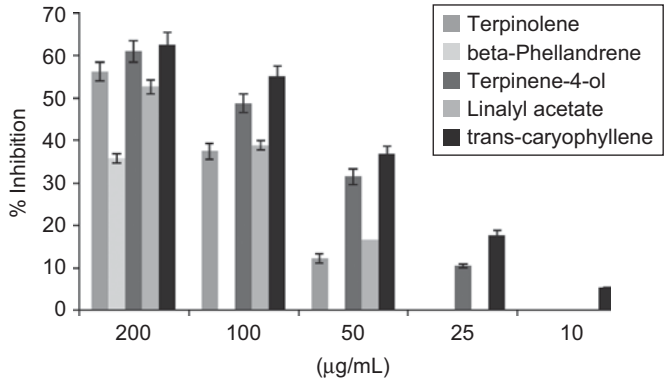

Figure 3. Dose-dependent inhibitory activity of terpinolene, β-phellandrene, terpinen-4-ol, linalyl acetate, and *trans*-caryophyllene against BChE. Data are given as mean ± SD (n = 3).

of 48.5% at 1 mM[22]. Cholinesterase inhibitory activity was described also for 3-carene that reached 50% inhibition of BChE at a final concentration of 2 mM during the 5 min pre-incubation period. Incubation time affected the inhibitory activity; in fact the IC_{50} values were significantly decreased from 2 mM in 5 min to 0.7 mM in 60 min. In contrast to the inhibition of BChE, the human anti-AChE activity of β-caryophyllene, 3-carene, α-pinene, and β-pinene was apparent. β-Caryophyllene, at a final concentration of 0.06 mM, gave 32% of AChE inhibition, whereas 3-carene, at a final concentration of 0.08 mM, gave 33% of AChE inhibition. Limonene and sabinene were recently tested to evaluate their potential anti-cholinesterase activity[20]. Sabinene exhibited the highest activity against AChE and BchE, with IC_{50} values of 176.5 μg/mL and 218.6 μg/mL, respectively.

The synergistic effect of different mixtures of terpenes has also been evaluated[15,23]. Generally, the findings reveal that the inhibitory activity of the essential oil results from a complex interaction between the constituents, which produce both synergistic and antagonistic responses between the component terpenes.

Various previous studies have demonstrated the biological activity of essential oils and their components after oral administration[40–42]. Moreover, Perry *et al.*[43] reviewed the pharmacological activity of *S. lavandulaefolia* essential oil for the treatment of Alzheimer's disease in a study involving oral administration. After 6 weeks of treatment a significant reduction in neuropsychiatric symptoms and an improvement in attention were observed. Recently, focus has also been on the nasal mucosa as an alternative administration route. A number of drugs including lipophilic compounds are administered intranasally, and can be rapidly absorbed into the brain[44,45]. Therefore, following oral or nasal administration, *Pinus* essential oils and/or identified compounds could be tested *in vivo* to evaluate their potential beneficial therapeutic effects in AD treatment.

Conclusions

In summary, the essential oils from three *Pinus* species, namely *P. nigra* subsp. *nigra*, *P. nigra* var. *calabrica*, and

P. heldreichii subsp. *leucodermis*, were evaluated for their chemical composition and acetylcholinesterase and butyrylcholinesterase inhibitory activity. Our results demonstrated that Pinus essential oils and some constituents have properties for the treatment of Alzheimer's disease and provide further data supporting the value of carrying out clinical studies using these species. In fact, further studies of the essential oils and/or the identified compounds on the pharmacokinetics, particularly on administrative routes or mode of action, are warranted.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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