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Original Article

Selective BuChE inhibitory activity, chemical composition, and enantiomer content of the volatile oil from the Ecuadorian plant *Clinopodium brownei*

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ABSTRACT

This paper describes the chemical composition and the enantiomer content of the volatile oil hydrodistilled from *Clinopodium brownei* (Sw.) Kuntze, Lamiaceae. The plant was collected in the South of Ecuador. Thirty one components were identified by GC-MS, which accounted for the 96.15% of the volatile oil. The major components were pulegone (48.44%), menthone (34.55%) and β-acorenol (3.41%). Oxygenated monoterpenes (86.06%), followed by oxygenated sesquiterpenes (5.36%) constituted the most abundant fractions. The enantiomeric compositions of β-pinene, sabinene, 3-octanol, menthone, pulegone and menthyl acetate were determined by enantioselective GC-MS. (-)-Menthone showed the highest enantiomeric excess (*ee* = 83.4%). In *in vitro* tests, the volatile oil showed high selective inhibitory activity for butyrylcholinesterase with an IC_{50}, 13.4 ± 1.8 μg/ml. In contrast, it was weakly active against acetylcholinesterase with an IC_{50} >250 μg/ml.

Introduction

The Lamiaceae family comprises 150 genera, with about 2.800 species distributed worldwide. About 27 genera and 219 species have been registered to grow in Ecuador, 29 of which, representing 13.24% of the total number, are endemic. Most species grow in the Andean forests, moorlands and inter-Andean valleys, at an altitude above 1000 m.a.s.l, less frequently in the dry forests of the coast, Galapagos Islands, and Amazon jungle (León-Yánez et al., 2011; De la Torre et al., 2008).

Clinopodium brownei (Sw.) Kuntze, Lamiaceae, is a small semi creeper grass, with stems up to 40 cm long that trail along the ground or are sometimes more erect (Rojas and Usubillaga, 2000; Jaramillo et al., 2010; Aguirre et al., 2014). The plant grows in Ecuador at elevations between 2500 and 3600 m, in the provinces of Azuay, Chimborazo, Loja, Pichincha, and Tungurahua (Jorgensen and Léon-Yánez, 1999; Aguirre et al., 2014)

For centuries, plants have provided indispensable resources to Ecuadorian rural and indigenous communities (Tene et al., 2007). The use and trade of medicinal plants are actively practiced even today especially by the Saraguro community that lives in the south of Ecuador. At least 273 species of medicinal herbs are still retailed for the treatment of more than seventy different diseases (Andrade et al., 2017). Several plants are aromatic and are consumed alone or mixed with other plants, depending on the effects desired or the disease cured. Various *Clinopodium* species, especially *C. nubigenum* (Ruiz et al., 2010; Gilardoni et al., 2011) and *C. brownei*, are popular traditional herbs. *C. brownei* is known with the names of "*poleo chico, poleo pequeño* or *warmi poleo*" in the local *kichwa* language.

A tea of "poleo chico" is used by the Saraguros as a digestive and to relieve the discomfort of menstrual colic. It is also considered an effective expectorant agent, and a remedy to cure colds, flu, cough, bronchitis and asthma (Andrade et al., 2017). Allergic symptoms are treated with a poultice of the herb. In the Ecuadorian province of Chimborazo, an infusion of plant mixture that includes "poleo chico" (*C. brownei*), anise (*Pimpinella anisum*) and lemon juice (*Citrus limon*), is employed to relieve respiratory problems related to flu.

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The plant is also known with the synonym of *Satureja brownei* (Sw.) Briq. in Colombia, Cuba, Venezuela and Argentina, where it is used as a seasoning food, especially for meat. In folk medicine it is employed to cure influenza and as a carminative, digestive, cholagogue, spasmolytic, expectorant, diuretic, antiseptic remedy. It is also considered an insect repellent (Pino et al., 1997; Rojas and Usubillaga, 2000; Jaramillo et al., 2010). Furthermore, in Guatemala the maceration in water of the aerial parts of this plant is used to bath children with the disease called Chaquiq'yaj, whose symptoms are; diarrhea, vomiting, fever, lack of appetite, and thirst could be associated with a gastrointestinal (Vargas and Andrade-Cetto, 2018).

The chemical compositions of the volatile oils from *S. brownei* (Sw.) Briq., samples collected in Colombia (Jaramillo et al., 2010), Venezuela (Rojas and Usubillaga, 2000) and Cuba (Pino et al., 1997) have been investigated. An enantioselective analysis and a potential anticholinesterase activity of the oils have not been reported.

In continuation of our studies of the aromatic plants growing in Southern Ecuador, we describe, for the first time, the composition of the volatile oil (VO) distilled from aerial parts of *C. brownei* collected in Ecuador. In fact, it was interesting to compare the volatile content of this oil with those of the plant collected in other countries (Pino et al., 1997; Rojas and Usubillaga, 2000; Jaramillo et al., 2010). It is well known that the site of plant collection may affect the VO composition. Moreover, we consider it to be interesting to analyze the enantiomer distribution of some chiral components and to evaluate the *in vitro* acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities of the oil.

The enantiomeric distribution of some chiral components can strongly impact the bioactivity and fragrance of volatile oils, thereby affecting the usage criteria for the preparation of drugs and fragrances.

The enzyme acetylcholinesterase (AChE) predominates in the healthy brain, with butyrylcholinesterase (BuChE) considered to play a minor role in regulating brain acetylcholine (ACh) levels. Recent evidence suggests that both AChE and BuChE may have roles in the aetiology and progression of Alzheimer disease (AD) beyond regulation of synaptic ACh levels. It has been found that BuChE activity progressively increases in patients with Alzheimer's disease (AD), while AChE activity remains unchanged or declines. Both enzymes therefore represent legitimate therapeutic targets for ameliorating the cholinergic deficit considered to be responsible for the declines in cognitive, behavioral and global functioning characteristic of AD (McGleenon et al., 1999; Greig et al., 2002). Indeed, clinical studies have demonstrated that, with increased inhibition of ChEs, there is a linear improvement in cognitive functions as well as improvements in verbal and spatial memory tests and reaction times.

The development of specific BuChE inhibitors and further experience with dual enzyme inhibitors will, therefore, improve understanding of the etiology of AD and should lead to a wider variety of potent treatment options (Greig et al., 2002; Jones et al., 2016; Bosak et al., 2018). Moreover it was proposed that the dual inhibition of AChE and BuChE is beneficial for AD patient especially since BuChE replaces AChE in the acetyl choline catabolism in AD advanced patients (Giacobini, 2004).

In this context, structure based virtual screening (Dighe et al., 2016) and many natural products have tested for anticholinesterase activity and their use for Alzheimerś disease therapy has been proposed (Dos Santos et al., 2018). Desirable properties of botanical extracts or natural products include a comparatively better penetration of the blood–brain barrier than the pharmaceutical options and better specificity for human type cholinesterases (ChE). *In vitro* assays testing the anti-ChE of volatile oils and extracts from tropical plants are limited (see, as typical examples, references Kitphati et al., 2012; Owokotomo et al., 2015), and even rarer are the studies investigating the selective inhibitory activity of BuChE (see, for example, Calva et al., 2017). The aim of this study was to investigate the anticholinesterase activity of the volatile oil from *C. brownei* so to foster further researches in this important field of phytopharmacology.

Materials and methods

General experimental procedures

Solvents were reagent grade or HPLC grade and were purchased from Sigma-Aldrich. Optical rotation was measured on a Hanon P 810 polarimeter according to the standard ISO 192 guidelines. Refractive indices were measured on an ABBE refractometer according to the AFNOR NF 75–112 international standard method. Relative density was determined at 20 °C according to AFNOR NF T75-111. GC-MS and GC-FID analyses were carried out on an Agilent gas chromatograph (model 6890 N) coupled to an Agilent mass spectrometer (model 5973) and to a flame ionization detector. In qualitative and quantitative GC analyses a non-polar capillary column (DB-5MS, 5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 μm of film thickness) and a polar capillary column (HP-INNOWax, 30 m × 0.25 mm i.d., 0.25 μm of film thickness) purchased from Agilent Techinologies, were used. Enantioselective GC-MS analysis was performed on an Agilent Technologies 6890 N gas chromatograph previously described, using a chiral capillary column based on 30% 2,3-dietyl-6-*tert*-butyldimethylsilyl-β-CDX (25 m × 0.25 mm i.d. × 0.25 μm of thickness) from Mega (Legnano, MI, Italy)].

Plant collection

Clinopodium brownei (Sw.) Kuntze, Lamiaceae, was collected in the Pichic sector (9589878N-17692229E) of the Cantón Loja, located in the Loja province (Ecuador), at an altitude of 2656 m.a.s.l., in August 2017. The collection was permitted by the Ministry of Environment of Ecuador (MAE) with authorization No. 001-IC-FLO-DBAP-vs-DRLZCH-MA. The plant was identified by Bolivar Merino, curator of the Herbarium Loja at the Universidad Nacional de Loja, by comparison with reference samples stored in the Herbarium. The scientific name of the plant is based on the taxonomic description given in the catalog of vascular plants of Ecuador (Jorgensen and Léon-Yánez, 1999). A voucher sample of *C. brownei* (No. PPN-la-024) is deposited in the Herbarium of the Universidad Técnica Particular de Loja.

Extraction of the volatile oil

The VO of *C. brownei* was obtained by steam distillation of fresh aerial parts for 4 h at atmospheric pressure using a Clevenger-type apparatus. The distilled VO was separated from the aqueous phase and dried over anhydrous sodium sulfate, filtered and stored in a brown vial at 4 °C until analysis.

Chemical characterization of the volatile oil

GC-MS analysis

The qualitative analysis of the volatile oil was performed by GC-MS, 1 μl of a solution of the VO in CH_2Cl_2 (1: 100 v/v) was injected. The injector and the detector temperatures were set at 250 °C. The injection operated in split mode (split ratio 40:1). The carrier gas was helium at a flow rate of 1 ml/min in constant flow mode. The analysis was performed in thermal gradient conditions, with the following conditions: the oven temperature was kept at 60 °C for

Table 1
Chemical composition of the volatile oil from *Clinopodium brownie*.

Component	DB-5MS				HP-INNOWax			
	LRI[calc.a]	*LRI*[lit.b]	%[c]	SD[d]	*LRI*[calc.e]	*LRI*[lit.f]	%[c]	SD[d]
α-Pinene	925	932	0.23	0.14	1055	1039[(1)]	0.61	0.04
Sabinene	964	969	0.13	0.04	1119	1132[(2)]	0.17	0.00
β-Pinene	968	974	0.25	0.11	1108	1118[(2)]	0.37	0.01
3-Octanone	973	979	0.82	0.36	1255	1255[(3)]	1.37	0.02
p-Cymene	–	–	–	–	1270	1280[(4)]	0.02	0.00
Myrcene	976	988	0.17	0.05	1162	1165[(5)]	0.17	0.00
3-Octanol	991	988	0.11	0.07	1398	1394[(4)]	0.22	0.00
Limonene	1018	1024	0.20	0.12	1199	1198[(5)]	0.12	0.00
1,8-Cineole	1022	1026	0.05	–	1206	1204[(5)]	0.07	0.00
Linalool	1097	1095	0.28	0.01	1554	1553[(2)]	0.28	0.00
Isopulegone	–	–	–	–	1580	1583[(4)]	0.34	0.01
α-Caryophyllene	–	–	–	–	1585	1589[(6)]	0.97	0.01
Menthone	1149	1148	34.55	9.89	1459	1461[(7)]	44.98	0.10
Carvomenthone	–	–	–	–	1463	1446[(8)]	0.01	0.02
Benzyl acetate	1154	1157	0.06	–	–	–	–	–
Isomenthone	1157	1158	1.40	0.65	1484	1492[(9)]	1.90	0.01
Neomenthol	1163	1161	1.03	0.04	1598	1595[(10)]	1.13	0.00
cis-Verbenol	–	–	–	–	1630	1629[(11)]	0.01	0.01
Isomenthol	1173	1179	0.17	0.03	–	–	–	–
α-Terpineol	1190	1186	0.04	–	1697	1700[(5)]	0.07	0.00
Pulegone	1233	1233	48.44	5.77	1640	1637[(7)]	44.74	0.22
Piperitone	1247	1249	0.09	–	1717	1714[(12)]	0.08	0.00
trans-Carvyl acetate	–	–	–	–	1736	1734[(13)]	0.02	0.00
Piperitenone	–	–	–	–	1911	1927[(14)]	0.09	0.00
Neomenthyl acetate	1268	1271	0.56	0.09	–	–	–	–
Menthyl acetate	1286	1294	0.15	0	–	–	–	–
(*Z*)-Caryophyllene	1409	1408	1.25	0.26	–	–	–	–
α-Humulene	1445	1452	0.10	0.12	1657	1673[(3)]	0.21	0.02
6-Demethoxy-ageratochromene	1451	1461	0.16	–	–	–	–	–
ar-Curcumene	1472	1479	0.07	–	–	–	–	–
ϒ-Amorphene	1498	1495	0.10	–	–	–	–	–
trans-Calamenene	1505	1521	0.17	–	–	–	–	–
Caryophyllene oxide	1567	1582	1.05	–	1967	1964[(15)]	0.33	0.00
Guaiol	1607	1600	0.11	–	–	–	–	–
α-Acorenol	1620	1632	0.79	–	–	–	–	–
β-Acorenol	1634	1636	3.41	–	–	–	–	–
Ageratochromene	1649	1658	0.16	–	–	–	–	–
Methyl octadecanoate	2131	2124	0.05	–	–	–	–	–
Representative classes of compounds								
Monoterpene hydrocarbons			0.99			1.45 %		
Oxygenated monoterpenes			86.06			95.28		
Sesquiterpene hydrocarbons			1.68			1.18		
Oxygenated sesquiterpenes			5.36			0.33		
Others			2.06			0.03		
Total amount of identified compounds			96.15			98.27		

[a] Calculated linear retention indices on a DB-5MS capillary column.
[b] Linear retention indices on a DB-5MS column from reference (Adams, 2009).
[c] Average percentage from three GC-FID analyses.
[d] Relative standard deviation less than 10%.
[e] Calculated linear retention indices on a HP-INNOWax capillary column.
[f] Linear retention indices on a HP-INNOWax column from reference (Demirci et al., 2003; Cozzani et al, 2005; Ferretti et al., 2005; Sutour et al., 2008; Monforte et al., 2011; Yasa et al., 2011; Kang et al., 2012; De Falco et al., 2013; Padalia et al., 2013; Ansari et al., 2015; Kan et al., 2015; Schepetkin et al., 2015; Khan et al., 2016; Camacho and Castro, 2016; Rodríguez et al., 2018).

5 min, then programmed to 165 °C at a rate of 3 °C/min, subsequently to 250 °C at a rate of 15 °C/min, finally kept at 250 °C for 10 min.

The spectrometer, controlled by the data system MSD-Chemstation D.01.00 SP1, operated in electron-ionization mode at 70 eV; electron multiplier 1600 V; scan rate of 2 scan/s; mass range from *m/z* 40 to 350. The chemical components of the VO (Table 1) were identified by comparing their EIMS spectra with the spectra of compounds having close retention indices reported in the literature (Adams, 2009). The comparison of the indices is considered reasonable in a range of ±15 units. Linear retention indices (LRI) were determined by simultaneous injection of samples and a series of *n*-alkanes (C9-C25, TPH-6RPM of CHEM SERVICE), according to Van del Dool and Kratz (1963).

GC-FID analysis

Quantification (expressed as a percentage) of each identified compound, was done by comparing the GC peak area to the total area of the identified peaks (Table 1) without applying any correction factor. The gas-chromatographic conditions were almost identical to those used for the GC-MS analysis.

Enantioselective GC-MS analysis

The enantioselective analysis was performed by GC.MS, under the same conditions reported above except for the oven temperature that was set according to this program: The oven temperature was kept at 50 °C for 2 min; subsequently, it was increased to 220 °C at a rate of 2 °C/min, and then it was kept at 220 °C for 2 min.

Cholinesterase inhibition assay

Cholinesterase (ChE) inhibition was measured by a colorimetric procedure adapted from Ellman et al. (Ellman et al., 1961). The two types of cholinesterases, AChE and BuChE, are responsible for hydrolyzing acetylthiocholine (ATCh) (Bosak et al., 2018), the sulfur analog of the natural substrate of these enzymes. After hydrolysis, this substance produces an acetate ion and thiocholine. Thiocholine in the presence of the highly reactive ditiobisnitrobenzoate (DTNB) ion, produces a yellow color, which can be monitored quantitatively by spectrophotometric absorption measurement at 412 nm. All reagents used were obtained from Sigma-Aldrich. A typical inhibition assay volume of 200-μl contained a phosphate-buffered saline solution (pH 7.4), DTNB (1.5 mM), test sample in DMSO (1% v/v). Both AChE from Electrophorus electricus (type vs, lyophilized powder, 744 U/mg solid, 1272 U/mg protein) and BuChE from equine serum (lyophilized powder, ≥900 protein units/mg) were dissolved in phosphate buffer (PBS) pH 7,4 and were used at 25 mU/ml for the assay. After a preincubation of 10 min, ATCh iodide (1.5 mM) was added to start the reaction. During a 1 h incubation at 30 °C, reaction kinetics were read in 96-well microtiter plates on a PherastarFS (BMG Labtech) detection system. Enzymatic activities were tested in the presence of 0,05 to 250 μg/ml of VO dissolved in DMSO, whose concentration was kept constant. False-positives results (Rhee et al., 2001, 2003) can reasonably be excluded under these conditions. All measurements were performed in triplicate. IC_{50} values were calculated using the online GNUPLOT package (www.ic50.tk, www.gnuplot.info). Donepezil was used as the reference ChE inhibitor (McGleenon et al., 1999).

Results and discussion

Physical properties of the volatile oil

The average yield of the VO from the four distillations was 0.44% (v/w). The VO was pale yellow and had a strong minty smell. The results of the physical properties analyzed were: the relative density of the oil, d = 0.909 ± 0.005 g/l; refractive index, $n = 1.47 \pm 0.005$ and specific rotation, [α = −3.208 ± 0.17 (CH_2Cl_2, $c = 10.0$).

Chemical composition of the volatile oil

The chemical composition of the VO was determined by comparing the calculated linear retention indices (LRI^{calc}) on an apolar (DB-5MS) and polar (HP-INNOWax) GC capillary columns and the GC/MS spectrum of each compound with the corresponding data reported in the literature (Adams, 2009). The average percentage (%) and standard deviation (SD) of each oil component were calculated from the corresponding area peak (uncorrected) in the chromatograms of three consecutive GC-FID analyses carried out on the same column.

The analysis on the DB-5MS capillary column (Table 1) indicated that the VO of Ecuadorian *C. brownei* mainly consisted of oxygenated monoterpenes (86.06%), accompanied by less amounts of oxygenated sesquiterpenes (5.36%). Sesquiterpene hydrocarbons (1.68%) and monoterpene hydrocarbons (0.99%) were minor groups. Thirty-one compounds were identified; which represented 96.15% of the whole VO. The major constituents of the oil were pulegone (48.44%) and menthone (34.55%), which together accounted for about 83% of the whole sample. Among the minor components, β-acorenol (3.41%) and isomenthone (1.40%) were the most abundant ones. The results obtained from the analysis of the oil on the polar column were comparable.

It is interesting to compare the composition of the volatile oil from the Ecuadorian *C. brownei* with those of the VO from the synonymous species *S. brownei* collected in Colombia (Jaramillo et al., 2010), Venezuela (Rojas and Usubillaga, 2000), and Cuba (Pino et al., 1997). Although hydro-distilled vegetative materials were not the same, all the four oils belong to the same chemotype of the type pulegone/menthone. The pulegone percentage varied from about 48 to 71% in the four oils, while the percentage of less abundant menthone was between 16 and 33%. The Cuban VO, containing 54.63% pulegone and 32.92% menthone (Pino et al., 1997), was the most similar to the Ecuadorian sample. The percentages of the minor components also varied significantly between the four oils. Only the oil from *C. brownei* contained a substantial quantity of oxygenated sesquiterpenes. These differences are not unexpected since the composition of an VO produced by a plant, can vary, depending on several factors such as plant collection site, yearly weather conditions, harvest date (Zouari-Bouassida et al., 2018), plant age, soil conditions (Farley and Howland, 1980).

The presence of high amounts of the two monoterpene ketones pulegone and menthone in the VO of *C. brownei*, together with other minor monoterpene components, well explains the peppermint-like flavour and fragrance of the plant. The biological properties of these oil components justify the uses of the plant in traditional medicines as a carminative, digestive, spasmolytic, anti-inflammatory and anti-allergic remedy (Almeida et al., 2012; Ku and Lin, 2013), and as a natural insect repellent (Iovinella et al., 2014; Kumar et al., 2014). Pulegone has also been reported to have pediculicidal (Gonzalez-Audino et al., 2011), antifungal (Ebadolli et al., 2017), and insecticidal (Franzios et al., 1997) activities. However, due to some hepatoxicity, pulegone should not be used in concentrations greater than 1% in cosmetology and pharmaceuticals (Essa et al., 2016)

In addition, the VO of *C. brownei* can also be considered a potential high source of pulegone and menthone for applications in flavoring, perfumes, and cosmetics. Therefore, an adequate sustainable management of the plant can become economically profitable.

Table 2
Enantiomeric analysis of the components of *Clinopodium brownei* volatile oil.

Enantiomer	*LRI*[a]	Enantiomeric distribution (%)	*ee* (%)
(+)-β-Pinene	970	56.42	12.84
(−)-β-Pinene	1002	43.58	
(+)-Sabinene	988	58.53	17.06
(−)-Sabinene	1000	41.47	
(+)-3-Octanol	1127	20.56	58.88
(−)-3-Octanol	1129	79.44	
(+)-Menthone (**1a**)	1176	4.17	91.66
(−)-Menthone (**1b**)	1186	95.83	
(+)-Pulegone (**2a**)	1207	49.52	0.96
(−)-Pulegone (**2b**)	1238	50.48	
(+)-Menthyl acetate	1257	79.69	59.38
(−)-Menthyl acetate	1282	20.31	

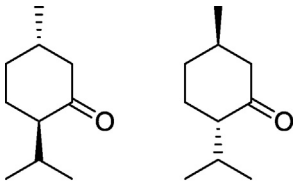
[a] Calculated linear retention indices on a 2,3-diethyl-6-*tert*-butyldimethylsilyl-β-CDX chiral capillary column.

Enantioselective GC/MS analysis

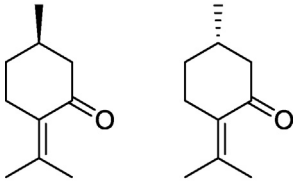
Enantiomer components and their enantiomeric excesses (*ee*) in *C. brownei* volatile oil were determined by-enantioselective GC-MS analysis. Six couples of enantiomers were detected (Table 2), which were baseline separated. The order of enantiomer elution was established by separated injections of enantiomerically pure standards.

(−)-Menthone had a high *ee*, whereas the enantiomeric excesses of (+)-menthyl acetate and (−)-3-octanol were moderate. In contrast, the enantiomeric excesses of (+)-sabinene, and (+)-β-pinene were low, and pulegone was almost racemic. These results fur-

ther confirm that chiral secondary metabolites are often present in plants as enantiomeric mixtures.



1a **1b**



2a **2b**

The chemical composition and some aspects of the biological activity of the volatile oil from *C. brownei* collected in Ecuador have been described for the first time. The plant is widely used in the traditional medicine of the peoples living on the Andean region to prepare healing infusions. The volatile oil is characterized by high concentration of oxygenated monoterpenes among which menthone (**1a/b**) and pulegone (**2a/b**) are the most abundant. These ketones confer the characteristic minty flavor of the plant and its leaf infusion.

The enantiomeric distribution of some chiral components has also been determined by enantioselective GS-MS analysis. Thus, the enantiomeric composition of *C. brownei* VO may be used as a powerful tool for determining the plant authenticity.

Anti-cholinesterase activity

The volatile oil of *C. brownei* showed weak (IC_{50} > 250 μg/ml) inhibitory activity for AChE; in contrast, the VO exhibited high (IC_{50} 13.4 ± 1.8 μg/ml) inhibitory activity for BuChE. The ChE reference inhibitor donepezil showed IC_{50} 0.040 +/−0.005 μg/ml against AChE and IC_{50} 3.6 +/− 0.2 μg/ml against BuChE.

The *in vitro* cholinesterase inhibitory assay, the VO of *C. brownei* exhibited high selective activity against BuChE, which was higher than against AChE. This bioactivity makes the volatile oil of *C. brownei* a promising source of lead compounds for further studies on the relationship between structure and anti-BuChE activity for the possible development of drugs against neurodegenerative diseases. In this context, it is interesting to note that, in previous studies, both menthone and pulegone have shown an interesting AChE inhibitory activity and pulegone was the most potent compound among a series of monoterpenoids with a *p*-menthane skeleton (Miyazawa et al., 1997). Moreover, the volatile oil from *C. niveum* containing respectively 19.7% pulegone and 56.2% isomenthol does not inhibit AChE up to 2 mg/ml (Orhan et al., 2009). This is consistent with the relative specificity of pulegone IC_{50} values for BuChE (IC_{50} 10.64 μg/ml, 70 μM) over AChE (IC_{50} 1368 μg/ml, 9 mM) extracted from data in Marçal et al. (2012). Other selective BuChE inhibitors are described such as the well described rivastigmine (IC^{50} 30 nM, 7.5 ng/ml) R-enantiomer of ZINC12613047 with a >4700-fold selectivity for BChE (IC_{50} 21.3 nM) over AChE (102.1 μM) (Orhan et al., 2017).

The studied VO from *C. brownei* has an IC_{50} of 13.4 μg/ml versus the BuChE. Pulegone is one of the two major compounds identified in this VO and could be partially responsible for this BuChE inhibitory potential. However, we must consider that another compound (or a mixture) present at a lower concentration completes or synergizes the VO effect.

The AChE inhibitory effect of *C. brownei* VO (IC_{50} > 250) turned out to be lower than the one observed for the *C. nubigenum* (IC_{50} 67.450) (Bedini et al., 2019), the reason of the difference in AChE inhibitory activity can be explained by the diferents main compounds between both VO, the *C. nubigenum* (carvacrol, pulegone) and *C. brownei* (pulegone, menthone).

Author contributions

AM, JC, JR, GV and CA share contributions to data analysis and preparation of the manuscript; JMA and CA collected the plant; NB and CL were responsible for the biological tests. All the authors have revised and approved the manuscript.

Conflicts of interest

All the authors declare no conflict of interest.

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