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Original article

Carnosol from *Lepechinia mutica* and tiliroside from *Vallea stipularis*: Two promising inhibitors of BuChE

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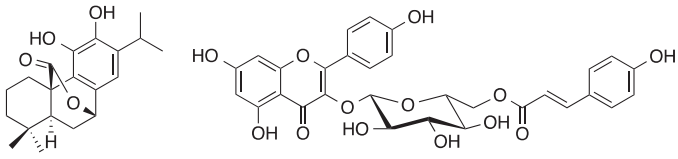
ABSTRACT

Lepechinia mutica (Benth.) Epling, Lamiaceae, and *Vallea stipularis* L.f., Elaeocarpaceae, are the object of the present study. These plants are endemic to the Andean region and have attracted our attention on the basis of interesting results obtained in a preliminary anticholinesterase screening. Actually, carnosol and tiliroside, isolated from *L. mutica* and *V. stipularis*, respectively, have shown a promising selective inhibitory activity against butyrylcholinesterase. Specifically, the anti-butyrylcholinesterase activity of carnosol was 5.15 μM and that of tiliroside was 52.9 μM, compared to 8.568 ± 0.570 μM of the positive control Donepezil. Carnosol and tiliroside were purified chromatographically from the ethyl acetate extract of *L. mutica* and *V. stipularis*, respectively. Spectrophotometric methods were used for enzymatic studies.

Introduction

The Alzheimer's disease (AD) affects more than 46.8 million people and is the most common neurodegenerative disease, requiring special cures amounting to an estimated worldwide cost of US $818 billion (Prince et al., 2016). The treatment or prevention strategies for neurodegenerative diseases have thus a great social and an economic importance. One of the factors known in the etiology of AD is the lesser availability of acetylcholine in the neuronal synaptic compartment that is essential for the maintenance of cognitive functions. Indeed, signs of a major cognitive decline are a clear evidence of biochemical brain changes consistent with AD (Francis, 2005). Consequently, acetylcholinesterase (AChE) inhibitors are widely used for the symptomatic treatment of AD and other dementias such as myasthenia gravis. Butyrylcholinesterase (BuChE) is one of the two major forms of cholinesterase enzymes widely expressed in humans, and it differs from acetylcholinesterase by the substrate specificity. However, both forms possess similar catalytic properties and are able to catalyze the degradation of the neurotransmitter acetylcholine (Darvesh et al., 2003). It has been noted that in AD brain the AChE activity is maintained or repressed, while the BuChE activity tends to increase (Mushtaq et al., 2014). This last finding has raised the interest in the discovery of drugs inhibiting both AChE and BuChE as well as that of selective BuChE inhibitors. Therefore, many efforts are being made to discover new and innovative lead compounds from natural sources (Williams et al., 2011) and, in this context, this paper is part of our ongoing project on the isolation and identification of new cholinesterase inhibitors from plants growing in the forests of South Ecuador (Armijos et al., 2016; Calva et al., 2017). Herein, we report the isolation of two compounds, named carnosol (**1**) and tiliroside (**2**), isolated from *Lepechinia mutica* and *Vallea stipularis*, respectively, and the evaluation of their inhibitory activity against the enzymes AChE and BuChE:



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Carnosol (**1**) is a well-known catechol diterpenoid which is typically extracted from different *Rosmarinus* species. *Romarinus* extracts show, among many other properties, beneficial effects on cognitive functions (Greig et al., 2005; Ozarowski et al., 2013) as well as high esterase inhibitory activities (Darvesh et al., 2003; Orhan et al., 2008; Dong et al., 2016). However, to the best of our knowledge, the colinesterase inhibitory activity of carnosol itself is poorly documented and controversial results have been reported (Fowler et al., 2005; Sallam et al., 2016). On the other hand, tiliroside (**2**), isolated from *Agrimonia pilosa*, was essayed only against AChE (IC_{50} 23.5 μM) (Jung and Park, 2007); however, it was found to inhibit neuroinflammation, which is one of the main factors causing neurodegenerative disorders (Velagapudi et al., 2014; Li et al., 2017a).

Lepechinia mutica (Benth.) Epling, Lamiaceae, is an endemic plant to Ecuador, growing only in the province of Loja at an altitude between 2200 and 3400 m a.s.l. (Missouri Botanical Garden, 2017). The phytochemical investigation about this plant has been limited so far to the contents of volatile fractions (Malagón et al., 2003; Ramírez et al., 2017), while in this work we have isolated a non-volatile secondary metabolite from the leaves.

The main traditional use of *L. mutica* is as an antiseptic remedy. Moreover, the leaves of *L. mutica* are employed to treat headache and nervous affections (Naranjo and Escaleras, 1995; Tene et al., 2007; Esteves et al., 2010; Drew and Sytsma, 2013). Other plants of this genus are used in folk medicine to treat uterine tumors and stomach ailments, to control diabetes mellitus and to cure diarrhea.

Vallea stipularis L.f. is a plant belonging to the family Elaeocarpaceae. This is a group of flowering plants, which contains approximately 615 species of trees and shrubs divided into 12 genera (Coode, 2004; Christenhusz and Byng, 2016). The genus *Vallea* consists of only two species, which are distributed throughout the Andean region from Venezuela to Bolivia. The plant is known with the traditional names of chuillur, achacapuli, cugur, crosckash, peralilo, rosa, cubillo, guisho, pera caspi, achiotico, campano, capulí, depending on the place of growth. Different medicinal uses are mentioned by the indigenous inhabitants of the Andes, that use this plant to treat scurvy, to heal gastritis and rheumatism, and as a purgative, anti-inflammatory, and analgesic remedy (de la Cruz et al., 2014). The biological activities *V. stipularis* have been evaluated as regards the antiulcerogenic, anti-inflammatory (Rafaile, 2014) and analgesic effects (Ortiz, 2016). Previous phytochemical analyses of this plant have showed the presence of known flavonoids and tannins (Amado and Choconta, 2016). In this study, tiliroside (**2**) has been isolated for the first time from a sample of *V. stipularis* collected in Ecuador.

Materials and methods

General experimental procedures

All solvents were reagent grade or HPLC grade and were purchased from Sigma-Aldrich. Preparative flash column chromatography was carried out on Merck LiChroprep RP-18 (25–40 m) or Merck silica gel (230–400 mesh), as reversed and normal phases, respectively. Medium-pressure flash-chromatography was carried out on a Biotage® Isolera™ Spektra instrument, equipped with a UV detector (positioned at 205 and 254 nm) and a Cartridge C-18, (KP-18-HS) 100 g, operating with an eluent flow of 30 ml/min. TLC was performed over Merck F_{254} glass plates (RP-18), or aluminum-supported (0.25 mm) sheets (silica gel). Spots were detected under UV light (254 and 366 nm) and, additionally, they were stained by exposure to a 0.5% solution of vanillin in H_2SO_4/EtOH (4:1), followed by heating at 100 °C. Discovery DSC-18, 60 ml, SPE columns were used for chlorophyll removal, containing 10 g of C-18 reversed phase. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, with concentration (*c*) expressed as g/100 ml. Melting points were measured with a Fisher–Johns hot-plate instrument. Mass spectra were obtained with a Thermo Scientific LTQ XL Linear ION Trap mass spectrometer, equipped with an ESI source. NMR spectra were recorded on an NMR Varian 400 MHz spectrometer, and on Bruker AC 300 and 200 MHz spectrometers.

Plant material

The collection of *Lepechinia mutica* (Benth.) Epling, Lamiaceae, leaves, authorized by the Ministry of Environment of Ecuador (MAE) with authorization n. 001-IC-FLO-DBAP-VS-DRLZCH-MA, was performed in the Quilanga region of Loja Province, Ecuador, in September 2009. The plant was identified by Dr. Bolivar Merino, of the Universidad Nacional de Loja. A voucher specimen, with number PPN-la-005, has been deposited at the Herbarium of the Universidad Técnica Particular de Loja. *Vallea stipularis* L.f., Elaeocarpaceae, leaves and flowers were collected at Oña, at the border between Azuay and Loja Provinces, Ecuador, under permission of the Ministry of Environment of Ecuador (MAE authorization no. 001-IC-FLO-DBAP-VS-DRLZCH-MA). The plant was identified by Dr. Nixon Cumbicus and a voucher specimen, with the number PPN-ELi-001, has been deposited at the Herbarium of the Universidad Técnica Particular de Loja.

Extraction and isolation

Dried leaves of *L. mutica* (250 g) were soaked in EtOAc (2500 ml). The extraction was carried out three times at room temperature, for 1 h each. Solvent evaporation yielded 24.1 g of a semisolid brown residue. Chlorophylls were removed from the extract by filtration through a C-18 SPE column. Elution was done with MeOH–H_2O 9:1 (v/v), followed by 100% Me_2CO. After solvent evaporation, 13.79 g of chlorophyll-free extract was obtained. A sample of this extract (1 g) was fractionated by medium-pressure flash chromatography (Isolera) on a C-18 cartridge (KP-18-HS), containing 100 g of stationary phase. An elution gradient from MeOH/H_2O (2:1) to 100% MeOH was applied, with eluent flow of 30 ml/min, affording 19 main fractions (1:19). Fraction 11 (511.54 mg) was subjected to silica gel column chromatography. Elution with a mixture of hexane/EtOAc (97:3) afforded pure carnosol (**1**) (38.47 mg) as a colorless crystalline solid, mp 233–237 °C, $[\alpha]_D^{20}$ −51.9 (*c* 0.05 in $CHCl_3$). The ^1H and ^{13}C NMR data were identical with the literature (Brieskorn et al., 1964; Inatani et al., 1982; Dimayuga et al., 1991; Abdelhalim et al., 2014).

The leaves of *V. stipularis* were dried in the darkness, selected and milled. Subsequently, the dry material (156 g) was defatted with CH_2Cl_2 and extracted with EtOAc, in the same way as *L. mutica*. Solvent evaporation under reduced pressure yielded 1.33 g of EtOAc dry extract. A portion of the extract (1 g) was subjected to silica gel column chromatography (CC) and eluted according to an increasing polarity gradient, with a mixture of hexane and EtOAc from 99:1 to 90:10, to afford 10 fractions of 100 ml each. Combined fractions 3–6 contained tiliroside (**2**) as a yellow powder, $[\alpha]_D^{20}$ −75.0 (*c* 1.00 in MeOH). The ^1H and ^{13}C NMR data were identical with the literature (Luhata et al., 2016).

Enzymatic inhibition activity

The cholinesterase (ChE) activities were assayed following a colorimetric protocol adapted from Ellman et al. (1961). Cholinesterases efficiently catalyze the hydrolysis of acetylthiocholine (ATCh), the sulfur analog of the natural substrate of these enzymes. Upon hydrolysis, this substrate analog produces acetate ion and thiocholine. Thiocholine, in the presence of the

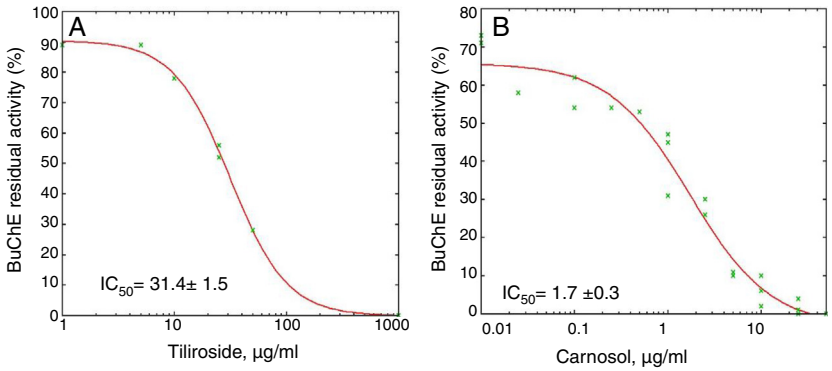

Figure 1. Determination of the IC_{50} values of tiliroside (curve A) and carnosol (curve B) against butyrylcholinesterase (BuChE). IC_{50} is the half maximal inhibitory concentration.

highly reactive dithiobisnitrobenzoate (DTNB) ions, generates a yellow color, which can be quantitatively monitored by spectrophotometric absorption at 412 nm. A typical 200 µl inhibition assay volume contained phosphate-buffered saline solution (pH 7.4), DTNB (1.5 mM), test sample in DMSO (1%, v/v, final). Both acetylcholinesterase from *Electrophorus electricus* (Type V-S, lyophilized powder, 744 U/mg solid, 1272 U/mg protein) and butyrylcholinesterase from equine serum (lyophilized powder, ≥900 units/mg protein) were dissolved in PBS pH 7.4 and used at 25 mU/ml for the assay. After 10 min of pre-incubation, the substrate acetylthiocholine iodide (1.5 mM) was added to start the reaction. During 1 h of incubation, 96-well microtiter multiplates were read on a PherastarFS (BMG Labtech) detection system. Donepezil was used as the positive reference ChE inhibitor. In this assay, we did not exclude the possibility of false-positive inhibition results previously described for high concentration (>100 µg/ml) of amino or carbonyl compounds (Rhee et al., 2001, 2003).

For all enzymatic assays, all reagents were obtained from Sigma-Aldrich. Stock samples were dissolved in DMSO at 100 mg/ml. AChE and BuChE activities were tested in the presence of 1000 to 0.01 µg/ml of the products dissolved in DMSO. For the assay, DMSO concentration was kept constant. The enzymatic activity assays were performed at 30 °C. All measurements were made in triplicate. When possible, the IC_{50} values were calculated using the Gnuplot package on line (Very Simple IC_{50} Tool Kit, 2018; Gnuplot, 2018).

Results and discussion

*Phytochemical characterization of carnosol (**1**) and tiliroside (**2**)*

The identification of compound carnosol (**1**) was supported by NMR and ESI-MS data and by single-crystal X-ray analysis. The ^{13}C NMR spectrum indicated the presence of four methyls, four methylenes, two methines, one oxymethine, six aromatic carbon atoms, two quaternary alifatic carbon atoms and one six-membered lactone carbonyl group (Brieskorn et al., 1964; Inatani et al., 1982). According to the ^{1}H NMR spectrum, the aromatic ring must be penta-substituted, and one substituent was an isopropyl group. The data nicely corresponded with those of carnosol reported in the literature (Dimayuga et al., 1991; Abdelhalim et al., 2014). Moreover, the sign of the rotatory power indicated that the absolute configuration corresponded to the enantiomer (−)-(5*S*,7*S*,10*R*)-carnosol, the only known in literature.

Analogously, the chemical structure of tiliroside (**2**) was determined by 1D and 2D NMR experiments and by comparison of the data with the literature (Luhata et al., 2016).

Esterase inhibitory activity of isolated compounds

Fig. 1 shows the inhibitory activity curves of compounds (**1**) and (**2**), respectively, against BuChE. According to their maximum stability and relative activity, carnosol (1) and tiliroside (2) were assayed at concentrations between 0.01 and 50 µg/ml in DMSO. The point at 1000 µg/ml for tiliroside was mathematically added to the curve due to the precipitation of this compound at concentrations higher than 50 µg/ml. In our conditions, Donepezil, the positive reference ChE inhibitor, showed an IC_{50} 95.5 nM for AChE and 8568 nM for BuChE.

From this data, we can conclude that both compounds are highly selective inhibitors against BuChE, while AChE was not inhibited under the same conditions. The calculated IC_{50} values of carnosol (**1**) and tiliroside (**2**) against butyryl cholinesterase were 1.7 µg/ml (5.15 µM) and 31.4 µg/ml (52.9 µM), respectively. Strikingly, we could not confirm the IC_{50} value of 23.5 µM reported in the literature for tiliroside against AChE (Jung and Park, 2007). On the other hand, carnosol exhibits an interesting selectivity against BuChE and the corresponding IC_{50} value is even lower than those of the reference compounds donepezil and allicin (Kumar, 2015). The high differences in the IC50 observed for the reference and the tested compounds in their inhibitory potential reflect the selectivity of donepezil versus AChE and versus BuChE for tiliroside (**2**) and carnosol (**1**). Thus, carnosol (**1**) can be added to the list of natural and/or synthetic chemicals with highly promising potential for the treatment and/or the prevention of AD (Li et al., 2017b). The activity data are summarized in Table 1.

The selective inhibitory activity of carnosol and tiliroside against BuChE has not been previously described. The high inhibition of BuChE makes carnosol a very promising lead compound for further studies on the relationship between structure and bioactivity, and for the possible development of more bioactive derivatives against neurodegenerative diseases. Furthermore, the high content (about 4%) of carnosol indicates that *L. mutica* is one of the richest sources of this natural product. Our data complete the experimental knowledge of the many biological properties reported for carnosol (Johnson, 2011; Birtić et al., 2015) and tiliroside (Velagapudi et al., 2014; Li et al., 2017a) and their extracts. In particular, the high BuChE of carnosol well correlates with the well-known effects of

Table 1
Inhibitory power (IC_{50}) of carnosol (**1**) and tiliroside (**2**) toward acetyl- (AChE) and butyryl-(BuChE) esterases, respectively.

IC_{50}	AChE, 25 mU/ml	BuChE, 25 mU/ml
Donepezil	0.096 ± 0.012 µM	8.568 ± 0.570 µM
Carnosol	>50 µg/ml (>151 µM)	1.7 µg/ml (5.1 µM)
Tiliroside	>50 µg/ml (>84 µM)	31.4 µg/ml (52.9 µM)

widely used dietary plants, such as *Romarinus* species on cognitive functions (Greig et al., 2005; Ozarowski et al., 2013). Therefore, we envision the possibility to prepare standardized extracts of *L. mutica* and related species for the development of effective and safe phytomedicines and botanical dietary supplements against neurodegenerative disorders. Moreover, we plan to further investigate different extracts of *L. mutica* and *V. stipularis* in search of metabolites that may corroborate the traditional uses of these plants on the basis of scientific evidences or that may function in synergy with carnosol and tiliroside, increasing their activities.

Author contributions

JR and AIS isolated and characterized the metabolites; CA, GG and GV conceived and designed the experiments; NB and CL performed the enzymatic experiments and analyzed the data. All the authors contributed writing the paper.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of Interest

The authors declare no conflicts of interest.

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