Bioactive secondary metabolites from *Lippia salsa* Griseb. (Verbenaceae)

Florencia Antonella Musso, Valeria Cavallaro and Ana Paula Murray

INQUISUR-CONICET, Departamento de Química, Universidad Nacional del Sur, Avda. Alem 1253, Bahía Blanca, Argentina. e-mail: apmurray@uns.edu.ar

Abstract

The genus *Lippia* (Verbenaceae) is represented in Argentina by 31 species. Many of these species are traditionally used as medicinal plants or for culinary purposes. A wide range of biological activities has been reported for plants belonging to this genus. *Lippia salsa* Griseb. is an endemic species from Argentina that has been scarcely studied. As part of our continuing investigation of natural products as leads for inhibiting acetylcholinesterase (AChE), an enzyme relevant for the treatment of Alzheimer's disease (AD), *L. salsa* was selected based on the results of a screening of AChE inhibition. The ethanolic extract obtained from the aerial parts elicited an interesting enzymatic activity with an IC_{50} value of 0.89 mg/ml, determined by Ellman's method. A bioassay-guided fractionation of this extract led to a semi-purified fraction with higher AChE inhibition (79.2% at 0.45 mg/ml). From this active fraction two known flavones, apigenin and luteolin, have been isolated and identified, so far. Since the ability of these flavones to inhibit cholinesterase has already been proven, it is reasonable to think that the AChE inhibition observed for *L. salsa* can be, at least in part, attributed to these metabolites. Apigenin and luteolin are reported here for the first time in this species. Apigenin has gained particular interests in recent years as a beneficial and health promoting agent due to its low intrinsic toxicity. On the other hand, luteolin has been identified as a potent inhibitor of Aβ fibrils, strongly implicated in AD pathology and the neurotoxicity observed with this disease. These observations suggest that *L. salsa* can be a source of potential multi-targets agents against AD.

Keywords: Secondary metabolites, Cholinesterase inhibitors, Lippia, Verbenaceae

Introduction

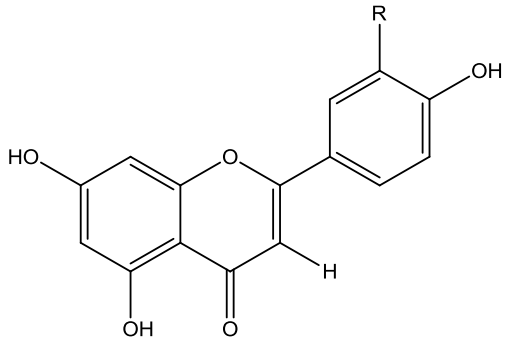
Alzheimer´s disease (AD) is a progressive neurodegenerative disorder associated with progressive memory loss, a decline in language skills, and other cognitive impairments. AD is characterized by neuronal loss and atrophy in crucial memory structures of the brain and causes functional deterioration of neurotransmitter systems, particularly a deficiency of acetylcholine (ACh) in the basal forebrain, which contributes to the cognitive deficits [1,2]. Acetylcholinesterase (AChE) plays a vital role in the regulation of cholinergic transmission. The inhibition of AChE increases the ACh level in the brain and has thus been implicated in the treatment of AD. Currently approved AD medications mainly comprise AChE inhibitors that

offer symptomatic treatment, but are unable to prevent disease progression and alter the outcome of the disease [1-4].

As part of our ongoing investigations of natural AChE inhibitors obtained from endemic plants [5], significant AChE inhibition was observed for the ethanolic extract of *L. salsa* (IC_{50} = 0.89 mg/mL). This result prompted us to isolate the active constituents.

Results and discussion

Aerial parts of *L salsa* were collected during the flowering period. Fresh material was dried and macerated in EtOH. It was observed that the ethanolic extract showed significant AChE inhibitory activity (IC_{50} = 0.89 mg/mL) evaluated by Ellman's method [6]. This extract was partitioned with solvents of different polarity. The ethylacetate sub-extract elicited the best AChE inhibition, so it was submitted to chromatographic separation. A bioassay-guided fractionation of this sub-extract led to a semi-purified fraction with higher AChE inhibition (79.2% at 0.45 mg/ml). From this active fraction two known flavones, luteolin (**1**) and apigenin (**2**), have been isolated and identified, so far (Figure 1). The presence of two other active flavonoids in this fraction has been confirmed by the bioactivity assay but their identification is limited by the scarce mass available of each one.



1: R = OH
2: R = H

Figure 1

Flavonoids are well-known natural compounds that attract increasing attention due to a wide range of pharmacological properties related to a variety of neurological disorders, like neuroprotective effect [7], AChE inhibitory activity [8] and free radical scavenging ability [9], among others. Thus, the isolation of active flavonoids from natural sources is a strategy for the research on anti-Alzheimer´s disease drugs. Flavonoids are also seen as beneficial and health promoting agents due to their low intrinsic toxicity, being regarded as multi-target botanical therapeutics or drugs.

Since the ability of these flavones to inhibit cholinesterase has already been proven, it is reasonable to think that the AChE inhibition observed for *L. salsa* can be, at least in part,

attributed to these metabolites. Apigenin and luteolin are reported here for the first time in this species. Apigenin has gained particular interests in recent years as a beneficial and health promoting agent due to its low intrinsic toxicity. On the other hand, luteolin has been identified as a potent inhibitor of Aβ fibrils, strongly implicated in AD pathology and the neurotoxicity observed with this disease. These observations suggest that *L. salsa* can be a source of potential multi-targets agents against AD.

Experimental

General: NMR measurements, including COSY, HSQC and HMBC experiments, were carried out from MeOD solutions, on a Bruker ARX300 spectrometer (300 and 75 MHz for hydrogen and carbon respectively). UV spectra were recorded on a JASCO V-630BIO spectrophotometer. Silica gel 60 (70–230 mesh, Merck) was used for column chromatography. Analytical TLC was performed on Silicagel 60 F254 sheets (0.2 mm thickness, Merck). p-Anisaldehyde-acetic acid spray reagent and UV light (254 and 366 nm) were used for detection. Acetylcholinesterase from electric eel (type VI-S), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), eserine and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma.

Plant Material: Aerial parts of *L. salsa* were collected during flowering period (November 2013) near Bahía Blanca city, Buenos Aires, Argentina. Voucher specimens were identified by Dra Maria Gabriela Murray from Universidad Nacional del Sur, Bahía Blanca, Argentina.

Extraction and bioassay-guided fractionation: Fresh material was dried at room temperature during 2 weeks (516 g), cut in small pieces and then, macerated with EtOH (3 L) for 2 weeks. The ethanolic extract (34 g) showed an IC_{50} value of 0.89 mg/mL in the *in vitro* AChE inhibition assay. This extract was suspended in H_2O and partitioned with Hexane and EtOAc. The ethyl acetate sub-extract (1.25 g), that showed significant AChE inhibition (40 % inhibition at 0.45 mg/mL), was submitted to a column chromatography with Silica gel 60 (60 g) and hexane:EtOAc as eluent. Chromatographic separation was monitored by TLC and the fractions were pooled according their chromatographic profile. Fractions 14-16 (110 mg) were the most active with 79.2 % of AChE inhibition at 0.45 mg/mL. These fractions were submitted to a column chromatography with Sephadex LH-20 and MeOH as eluent. Fractions were monitored by TLC (CH_2Cl_2:MeOH 95:5) and compound **1** (5.4 mg) was obtained pure from fractions 22-25. Compound **2** (2.4 mg) was purified by preparative TLC (CH_2Cl_2:MeOH 95:5) from fractions 17-21 (7.7 mg). Compounds **1** and **2** were identified by ^{1}H and ^{13}C NMR, mono and bidimensional experiments, by comparison of their spectroscopic data with the literature.

Cholinesterase inhibition assay: Electric eel (*Torpedo californica*) AChE was used as source of cholinesterase. AChE inhibitory activity was measured *in vitro* by the spectrophotometric method developed by Ellman with slight modification [6]. The lyophilized enzyme, 500 U AChE, was prepared in buffer A (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4) to obtain 5 U/mL stock solution. Further enzyme dilution was carried out with buffer B (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) to produce 0.126 U/mL enzyme solution. Samples were dissolved in buffer B. Compounds required 2.5 % of MeOH as cosolvent. Enzyme solution (300 µL) and 300 µL of sample solution were mixed in a test tube and incubated for 60 min at room

temperature. The reaction was started by adding 600 µL of the substrate solution (0.5 mM DTNB, 0.6 mM ATCI, 0.1 M Na_2HPO_4, pH 7.5). The absorbance was read at 405 nm for 180 s at 27°C. Enzyme activity was calculated by comparing reaction rates for the sample to the blank. All the reactions were performed in triplicate. IC_{50} values were determined with GraphPad Prism 5. Eserine (99%) was used as the reference AChE inhibitor.

Acknowledgements

This work was financially supported by the National Research Council of Argentina (CONICET), Universidad Nacional del Sur (Argentina) and ANPCYT. A.P.M. is Research Member of CONICET. V.C. is grateful to CONICET for her doctoral fellowship.

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