South African Journal of Botany 119 (2018) 318–324

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

# Neuroprotective effects of *Flaveria bidentis* and *Lippia salsa* extracts on SH-SY5Y cells

V. Cavallaro [a], C.J. Baier [b], M.G. Murray [c], A. Estévez-Braun [d], A.P. Murray [a,*]

[a] INQUISUR-CONICET, Departamento de Química, Universidad Nacional del Sur, Avenida Alem 1253, B8000CPB Bahía Blanca, Argentina
[b] Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), - Departamento de Biología, Bioquímica y Farmacia (DBByF), Universidad Nacional del Sur (UNS), Bahía Blanca, Argentina
[c] INBIOSUR-CONICET, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, B8000CPB Bahía Blanca, Argentina
[d] Instituto Universitario de Bio-Orgánica "Antonio González", (CIBICAN), Departamento de Química Orgánica, Universidad de La Laguna, Avda. Astrofísico Fco. Sánchez N°2. La Laguna, Tenerife, Spain

ARTICLE INFO

*Article history:*
Received 7 March 2018
Received in revised form 21 September 2018
Accepted 2 October 2018
Available online xxxx

Edited by D Lišková

*Keywords:*
Neuroprotection
SH-SY5Y cells
Acetylcholinesterase inhibition
Halophytes

ABSTRACT

Halophyte plants have to survive in a hostile environment by developing adaptive responses. One of these strategies is the production of several protective molecules which make these plants an interesting source of bioactive compounds. Significant acetylcholinesterase inhibition was observed for ethanolic extracts obtained from *Lippia salsa* Griseb. (Verbenaceae) and *Flaveria bidentis* (L.) Kuntze (Asteraceae), two plants that are widely distributed in the salt marsh Salitral de la Vidriera. These results encouraged us to further study the active constituents and the potential neuroprotective properties of these plants. The flavonoids luteolin (**1**) and apigenin (**2**) were identified as the active components of *L. salsa*, while 6-methoxykaempferol-3-sulfate (**3**) was obtained from *F. bidentis*. In addition, we investigated the cytotoxicity, cellular protection against $K^+$-depolarization and antioxidant activity in SH-SY5Y neuroblastoma cells for these extracts and compound **3**. Results demonstrated that beyond acting as acetylcholinesterase inhibitors, they also exhibited neuroprotective effects against KCl-induced-$Ca^{2+}$ overload and oxidative stress.



## 1. Introduction

Salt-tolerant plants living in extreme environments have to deal with stressful conditions by developing adaptive responses like the production of several protective molecules (Ksouri et al. 2012; Lokhande et al. 2013). Halophytes have a powerful antioxidant system that protects them from cellular damage, metabolic disorders, and senescence processes caused by the production and accumulation of reactive oxygen species (ROS), due to unfavorable growing conditions. Secondary metabolites produced by halophyte plants have important biological activities, making these plants interesting due to their potential nutraceutical/pharmaceutical applications. Several salt marsh plants have traditionally been used for their medicinal or nutritional properties (Cybulska et al. 2014; Ksouri et al. 2012; Lokhande et al. 2013; Patel 2016).

Salitral de la Vidriera is a saline habitat located in the southwest of the Buenos Aires province, Argentina, in the inner part of Bahía Blanca's estuary (Latitude 38.76° S; Longitude: 62.5° W). Flora and fauna of this area have been thoroughly studied over the last decades (Nebbia and Zalba 2007). In particular, wild plants growing there have attracted our attention as a potential source of bioactive metabolites (Cavallaro et al. 2014; Gurovic et al. 2010; Murray et al. 2004; Rodriguez and Murray 2010). Continuing our search of natural compounds with anticholinesterase activity (Alza et al. 2014; Alza and Murray 2016), we have focused our research in endemic plants that are abundant in this saline environment.

The enzyme acetylcholinesterase (AChE) plays a vital role in the regulation of cholinergic transmission, which in turn, is relevant in Alzheimer's disease (AD) treatment. AD is a progressive neurodegenerative disorder associated with neuronal loss and atrophy in crucial memory structures of the brain, that cause functional deterioration of neurotransmitter systems, particularly a deficiency of acetylcholine in the basal forebrain, which contributes to cognitive deficits (León et al. 2013; Selkoe 2012). The inhibition of AChE increases the acetylcholine level in the brain and has thus been implicated in the treatment of AD.

*Abbreviations:* AChE, acetylcholinesterase; AD, Alzheimer's disease; ATCI, acetylthiocholine iodide; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid; EE-FLABI, *F. bidentis* ethanolic extract; EE-LISA, *L. salsa* ethanolic extract; EtOAc, ethyl acetate; EtOH, etanol; HRMS, High Resolution Mass Spectrometry; IC50, 50% inhibitory concentration; LDH, lactate dehydrogenase; MeOH, metanol; NMR, Nuclear Magnetic Resonance; ROS, reactive oxygen species.

* Corresponding author.
*E-mail address:* apmurray@uns.edu.ar (A.P. Murray).

https://doi.org/10.1016/j.sajb.2018.10.006
0254-6299/

During the aging process, the progressive damage of the endogenous antioxidant protection system is another described phenomenon in AD. Moreover, increasing evidence supports the significant impact of oxidative stress in the pathogenesis and progression of AD (Ansari and Scheff 2010). Studies have indicated that preventing the formation of free radicals could be useful for AD treatment (Muche et al. 2017; Poprac et al. 2017).

As part of our research project significant AChE inhibition was observed for the ethanolic extracts obtained from *Lippia salsa* Griseb. (Verbenaceae) and *Flaveria bidentis* (L.) Kuntze (Asteraceae), two plants that are widely distributed in the salt marsh Salitral de la Vidriera. In this paper we describe the extraction and bioassay-guided fractionation of *L. salsa* and *F. bidentis*, and the neuroprotective properties of these extracts and 6-methoxykaempferol-3-sulfate (**3**), one of the isolated metabolites.

## 2. Material and methods

### 2.1. General experimental procedures

Nuclear Magnetic Resonance (NMR) measurements, including COSY, HSQC, HMBC and NOESY experiments, were carried out from deuterated dimethyl sulfoxide (DMSO-$d_6$) solutions, on a Bruker ARX300 spectrometer (300 and 75 MHz for hydrogen and carbon, respectively), a Bruker Avance 400 spectrometer (400 and 100 MHz for hydrogen and carbon, respectively) and a Bruker Avance 600 spectrometer (600 and 150 MHz for hydrogen and carbon, respectively). Chemical shifts are given in ppm ($\delta$) with TMS as an internal standard. High resolution mass spectrometry (HRMS) data were recorded with a LCT Premier XE (Waters) spectrometer. 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). Precoated TLC plates SIL G-100 UV254 were used for preparative TLC purification. *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) and eserine were purchased from Sigma.

### 2.2. Plant material

Aerial parts of *L. salsa* were collected during flowering period (November 2013) in Salitral de la Vidriera, near Bahía Blanca city, Buenos Aires, Argentina. Roots and aerial parts of *F. bidentis* were collected at the same location during flowering period (November 2012). Voucher specimens were deposited in the Herbarium of Universidad Nacional del Sur (BBB) in Bahía Blanca, Argentina, under the numbers Murray, M.G.544 (*F. bidentis*) and Murray, M.G.567 (*L. salsa*).

### 2.3. Extraction and bioassay-guided fractionation of L. salsa

Fresh material was dried at room temperature for 2 weeks (516 g), cut in small pieces and then, macerated with ethanol (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 ethyl acetate (EtOAc). The EtOAc sub-extract (1.25 g), that showed significant AChE inhibition (48% 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 to 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 Sephadex LH-20 column chromatography using methanol (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 1D and 2D NMR spectroscopic studies and by comparison of their spectroscopic data with those reported in the literature.

### 2.4. Extraction and bioassay-guided fractionation of F. bidentis

Fresh roots and aerial parts of *F. bidentis* (100 g) were extracted with ethanol (96% v/v) at room temperature for two weeks. Ethanolic extract was evaporated under reduced pressure and the residue was extracted with hexane:$H_2O$ (1:1) several times. The aqueous layer was concentrated to minimum volume and refrigerated for 24 h to obtain a yellow precipitate. Once filtered, the solid was recrystallized in water to obtain 200 mg of **3**. Its structure was elucidated by HRMS and mono- and bidimensional NMR. 6-methoxykaempferol-3-sulfate: HRMS (ESI) Calcd for $C_{16}H_{11}O_{10}NaS$ $[M + Na]^+$ 440.9868, found 440.9864. $^1H$ and $^{13}C$ NMR data are presented in Table 1.

### 2.5. Hydrolysis of compound 3

0.096 mmol (40 mg) of 6-methoxykaempferol-3-sulfate (**3**) were dissolved in 3 mL of HCl (0.1 M). The solution was stirred for 12 h under reflux and cooled to room temperature. Next, the reaction mixture was extracted with AcOEt (3 × 5 mL) and dried over $MgSO_4$. Pure desulfated 6-methoxykaemferol (**3a**) was obtained as bright yellow solid in 95% yield. HRMS (ESI) Calcd for $C_{16}H_{12}O_7$ $[M + Na]^+$ 339.0481, found 339.0478. $^1H$ and $^{13}C$ NMR data are presented in Table 1.

### 2.6. Methylation of compound 3

0.096 mmol (40 mg) of 6-methoxykaempferol-3-sulfate (**3**) was dissolved in 1 mL of ethyl ether. Then 0.384 mmol (0.2 mL) of trimethylsilyldiazomethane ($C_4H_{10}N_2Si$) and 37 mmol (1.5 mL) of MeOH were added. The solution was stirred at room temperature for 24 h. The reaction mixture was concentrated to dryness and purified by preparative TLC (Silica Gel F254, $CH_2Cl_2$: MeOH (4:1)) affording the permethylated derivative (**3b**) in 66% yield.

**Table 1**
$^1H$ and $^{13}C$ NMR data of compounds **3**, **3a** and **3b** in DMSO-$d_6$.

| Position | 3 | | 3a | | 3b | |
|---|---|---|---|---|---|---|
| | $\delta_C$[a] | $\delta_H$ ($J$ in Hz)[b] | $\delta_C$[a] | $\delta_H$ ($J$ in Hz)q[b] | $\delta_C$[c] | $\delta_H$ ($J$ in Hz)[d] |
| 1′ | 121,3 s | | 121,7 s | | 123,3 s | |
| 2′y 6′ | 130,8 d | 8,08 d (8.9) | 129,6 d | 8,04 d (12) | 130,2 d | 8,22 d (18) |
| 3′y 5′ | 115,2 d | 6,85 d (8.9) | 115,5 d | 6,92 d (12) | 113,5 d | 7,04 d (18) |
| 4′ | 159,9 s | | 159,3 s | | 160,6 s | |
| 2 | 151,4 s | | 147,0 s | | 151,4 s | |
| 3 | 132,0 s | | 135,4 s | | 139,3 s | |
| 4 | 178,1 s | | 176,2 s | | 178,7 s | |
| 5 | 156,5 s | | 151,8 s | | 152,7 s | |
| 6 | 131,2 s | | 130,9 s | | 134,4 s | |
| 7 | 157,2 s | | 157,2 s | | 157,1 s | |
| 8 | 93,8 d | 6,53 s | 93,8 d | 6,54 s | 96,8 d | 7,13 s |
| 9 | 152,6 s | | 151,5 s | | 152,9 s | |
| 10 | 104,6 s | | 103,5 s | | 112,4 s | |
| 6-$OCH_3$ | 60,1 q | 3,74 s | 60,0 q | 3,75 s | 61,0 q | 3,77 s |
| 7-$OCH_3$ | | | | | 56,4 q | 3,94 s |
| 5-$OCH_3$ | | | | | 61,9 q | 3,85 s |
| 4′-$OCH_3$ | | | | | 55,3 q | 3,81 s |

[a] Recorded at 150 MHz, multiplicity by DEPT.
[b] Recorded at 600 MHz.
[c] Recorded at 150 MHz, multiplicity by DEPT.
[d] Recorded at 400 MHz

### 2.7. 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 (Ellman et al. 1961). 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 (the inhibitor concentration required for 50% inhibition of the enzyme) were determined with GraphPad Prism 5. Eserine (99%) was used as the reference AChE inhibitor.

### 2.8. SH-SY5Y human neuroblastoma cells

For standard growth conditions, SH-SY5Y cells were grown in medium consisting in DMEM/Ham F12 nutrient (Gibco) mixture (1:1) supplemented with 10% foetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco) at 37 °C in a 5% $CO_2$. For biochemical determinations, $1 \times 10^5$ SH-SY5Y cells were seeded in 24 well culture plates (Gibco) and maintained in the conditions described above.

### 2.9. Cytotoxicity assay and measurement of lactate dehydrogenase (LDH) activity

Cellular damage/death was spectrophotometrically measured evaluating the activity of the cytoplasmic enzyme LDH, which is released by cells with damaged plasma membranes. These data were compared with those obtained after a complete lysis of cells (see below). SH-SY5Y cells were grown as described above and treated with EE-FLABI (5 μg/mL), EE-LISA (5 μg/mL) and **3** (4.4 μg/mL) in growth medium. 48 h after drug treatment, 100 μL of medium was collected and LDH activity was determined using a kinetic assay according to manufacturer's instructions (Wiener LDH-P UV, http://www.wiener-lab.com.ar). LDH catalyzes a redox reaction, in which pyruvate is reduced to lactate through the oxidation of NADH to NAD+. Both NAD+ and NADH strongly absorb at 259 nm, but NADH also absorbs at 340 nm. These differences in the UV absorption allow quantifying the conversion of NADH to NAD+ measuring spectrophotometrically the UV absorption at 340 nm. Briefly, 100 μL of sample was incubated with 1 mL of commercial reactive (piruvate 1,6 mM; NADH 0,2 mM; NaCl 200 mM; Tris 80 mM, pH 7,2) 3 min and 30 s at 37 °C, measuring spectrophotometrically the absorption at 340 nm. The level of LDH in the sample is proportional to the decrease in absorbance at 340 nm. LDH release was quantified by comparison with 100% LDH release (complete lysis) obtained by treating the cells with 0.1% Triton X-100 in phosphate-buffered saline (PBS). To calculate cellular protection, LDH release en each sample was relativized to the LDH release obtained in culture medium (100%) and the LDH released upon 70 mM K+ or 60 μM $H_2O_2$ treatments, respectively (0%).

### 2.10. Statistical analysis

All results are presented as mean ± SEM from at least four independent experiments. LDH activity determinations were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. The observed differences were considered to be statistically significant when $p < .05$. Analysis of data was performed using Origin version 8 OriginLab Graphing Software.

## 3. Results and discussion

### 3.1. Botanical description of the studied plants

The genus *Flaveria* is represented by 22 species, most of them are native to North America. Three species also appear in South America and two of them are found in Argentina. *Flaveria bidentis* is a native species that inhabits loose and fertile soils in Argentina (Zuloaga et al. 2008). This species is naturalized in many places (Mexico, Central America, the West Indies, the southeastern United States, Europe, Africa, Asia) and is considered as an invasive species in South Africa where is known as "smelter's bush" (Gibbs Russell et al. 1987). It is an annual herb, typically glabrous, with branching stems and leaves that are opposite and decussate. The flowers are yellow, dimorphic and grouped in synflorescences.

The genus *Lippia* is comprised of 140 species, mostly distributed in warm and temperate America. In Argentina the genus is represented by 26 species, several of them are used in folk medicine. *Lippia salsa* is an endemic species of Argentina that grows frequently in saline environments. Its distribution covers the center and northwest of the country (Múlgura et al., 2012; Zuloaga et al. 2008). This survey records a new appointment for the Province of Buenos Aires. *Lippia salsa* is described as woody erect shrubs or undershrubs, with tetragone branches, and simple sessile leaves entire to variously toothed. The inflorescence as a capituliform spike is small. Calyx persistent and corolla gamopetalous, white or pink. The fruit is dry, ovoid, and divides into 2 mericarps or "nutlets" at maturity (Múlgura et al., 2012).

### 3.2. Isolation and identification of active metabolites

*Flaveria bidentis* and *L. salsa* were selected due to the AChE inhibition observed for their ethanolic extracts in a preliminary assay, 83% and 35% of inhibition at 0.5 mg/mL, respectively ($IC_{50}$ values of 0.12 and 0.89 mg/mL, respectively). For both plants, a bioactivity-guided fractionation approach was conducted in order to isolate the active metabolites.

The ethanolic extract obtained from *L. salsa* (EE-LISA) was partitioned with solvents of different polarity. The ethylacetate sub-extract elicited the best AChE inhibition (48% inhibition at 0.45 mg/mL). Then, it was submitted for chromatographic separation. The 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**), were isolated and identified (Fig. 1). Until now, phytochemical knowledge about *L. salsa* was limited to some volatile components identified from its essential oil (Juliani et al. 2004).

Considering that the presence of sulfated flavonoids is known for *F. bidentis* (Cabrera and Juliani 1976, 1979, 1977; Pereyra de Santiago and Juliani 1972; Teles et al. 2018), we decided to apply a separation method that was successfully used in the past for that kind of metabolite (Guglielmone et al. 2005). Briefly, the ethanolic extract of *F. bidentis* (EE-FLABI) was evaporated and the residue was suspended in hexane:$H_2O$ (1:1). AChE inhibition was then evaluated for both sub-extracts. The aqueous sub-extract proved to be 14.5 folds more active than the hexanoic one (83 *vs.* 5.7% enzymatic inhibition at 0.12 mg/mL). Then, the aqueous sub-extract was concentrated and cooled for 24 h. This activity guided fractionation led us to isolation by crystallization of the active compound **3** in good yield (4% w/w). The molecular formula $C_{16}H_{11}SO_{10}Na$ was deduced from the HRMS data of compound **3**. Analysis of the $^{13}C$ NMR spectrum revealed the presence of 10 quaternary carbons including a carbonyl group ($\delta_C$ 178.1 ppm), three methine and one methoxy group ($\delta_C$ 60.1 ppm) (Table 1). These data, together with the few signals observed in the $^1H$ NMR spectrum (Table 1),

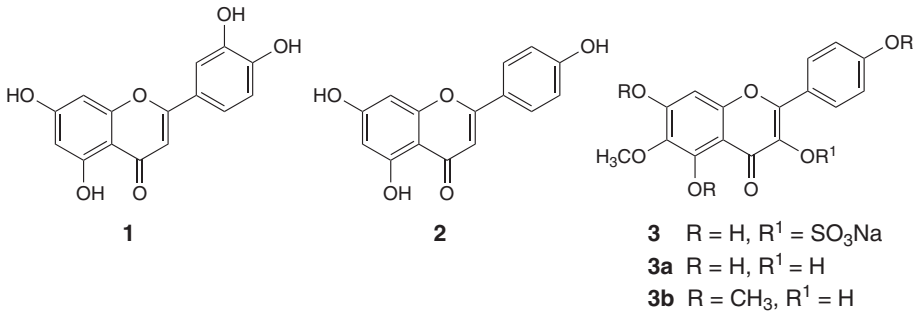


**Fig. 1.** Flavonoids isolated from *L. salsa* and *F. bidentis*.

suggest the presence of a highly substituted flavonoid bearing a sulfate group. The two doublets observed in the $^1$H NMR spectrum at $\delta_H$ 6.85 and 8.08 ppm, with a coupling constant of 8.9 Hz, together with the a singlet at $\delta_H$ 6.53 ppm lead us to propose a flavone skeleton with a 4′ substituted B ring and a trisubstituted A ring. The methoxy group ($\delta_H$ 3.74 ppm) was located at C-6 (ring A) with the aid of a HMBC experiment that showed a cross peak between those protons and C-6 (131.2 ppm).

Treatment of compound **3** with 0.1 M HCl gave the desulfated derivative (**3a**) with the molecular formula $C_{16}H_{12}O_7$ as determined by HRMS. The location of the sulfate group at C-3 was established by comparison of the chemical shifts values of C-2, C-3, C-4 and C-5 signals in the $^{13}$CNMR spectra (Table 1) for compound **3** and **3a**. Thus, the upfield shift ($\Delta = -3.4$ ppm) of the C-3 signal and the downfield shift of the C-2 ($\Delta = +4.4$ ppm), C-4 ($\Delta = +1.9$ ppm) and C-5 ($\Delta = +4.7$ ppm) signals in the spectrum of **3** compared to its desulfated analog indicated the attachment of one sulfate group at C-3 (Dueñas et al. 2012).

In order to verify the location of the three hydroxyl groups present in compound **3**, a permethylated derivative (**3b**) was prepared by treatment of **3** with an excess of trimethylsilyldiazomethane. This derivative showed three extra methoxy groups in the $^1$H and $^{13}$C NMR spectra ($\delta_H/\delta_C$ 3.81/55.3, 3.85/61.9 and 3.94/56.4) that were attached to C-4′, C-5, and C-7, on the basis of the long-range correlations detected in the HMBC spectrum. All these data lead us to identify compound **3** as 6-methoxykaempferol-3-sulfate. As far as we know, flavonoid **3** has only been isolated previously from *F. chloraefolia* and has been characterized only by UV spectroscopy (Barron and Ibrahim 1987). The present study is the first report of complete NMR data of **3** and the first investigation of its potential biological activity.

### 3.3. Biological activity

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, AChE inhibitory activity, and free radical scavenging ability, among others (Lou et al. 2011; Schroeter et al. 2001; Uriarte-Pueyo and Calvo 2011). Thus, isolation from natural sources and semi-synthesis of new effective flavonoid derivatives are interesting strategies for research on anti-AD drugs.

In order to analyze the multitarget activity of ethanolic extracts obtained from *L. salsa* and *F. bidentis* as well as compound **3**, we investigated the cytotoxicity, cellular protection against $K^+$-depolarization and antioxidant activity in SH-SY5Y neuroblastoma cells.

### 3.4. In vitro AChE inhibition

Since the ability of flavones **1** and **2** to inhibit cholinesterase has already been proven (Guo et al. 2010; Katalinić et al. 2010; Uriarte-Pueyo and Calvo 2011) it is reasonable to think that the AChE inhibition observed for *L. salsa* can be, at least in part, attributed to these metabolites. Compounds **1** and **2** are reported here for the first time in this species.

Luteolin (**1**) has been identified as a potent inhibitor of β-amyloid fibrils, strongly implicated in the AD pathology and neurotoxicity observed with this disease (Churches et al. 2014). Also, Zhou and co-workers demonstrated that **1** efficiently attenuates zinc-induced tau hyperphosphorylation through its antioxidant action, playing a key role in decreasing the clinical progression of AD (Zhou et al. 2012). On the other hand, apigenin (**2**) has gained particular interest in recent years as a beneficial and health promoting agent due to its low intrinsic toxicity. Furthermore, it has been proven that **2** has the ability to inhibit Beta-secretase 1, an enzyme target related to AD progression, and to protect rat cerebral endothelial cells against $A\beta_{25-35}$-induced toxicity (Shimmyo et al. 2008; Zhao et al. 2011). These observations suggest that *L. salsa* can be a source of potential multi-target agents against AD.

Once the sulfated flavonoid **3** was obtained pure, it was characterized as an AChE inhibitor by Ellman's method showing an $IC_{50}$ value of 94 μg/mL. Even if **3** is a better inhibitor than the ethanolic extract ($IC_{50}$ = 0.12 mg/mL), it should be considered as a moderate AChE inhibitor. On the other hand, the desulfated derivative (**3a**) resulted in being inactive against AChE, proving that the sulfate group is relevant for enzyme inhibition. Even though a few flavonoids have already been reported as AChE inhibitors(Murray et al. 2013), this is the first report of a sulfated flavonoid with the ability to inhibit AChE *in vitro*.

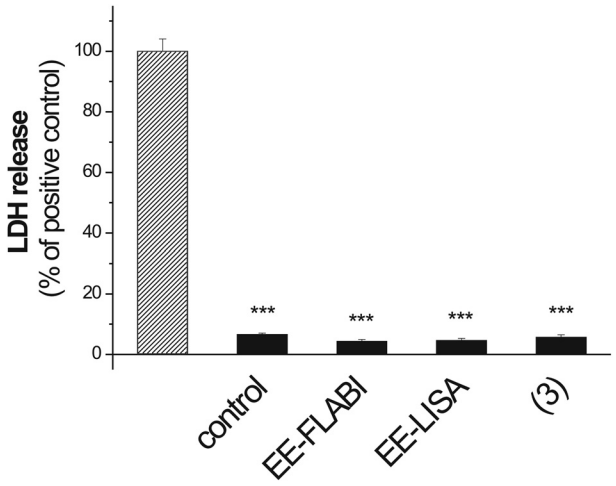


**Fig. 2.** *Cytotoxic effect of EE-FLABI, EE-LISA and 3 on SH-SY5Y cells.* Cell viability of SH-SY5Y cells treated for 48 h with vehicle (control) or EE-FLABI (5 μg/mL), EE-LISA (5 μg/mL) and **3** (4.4 μg/mL). Cell viability was determined by LDH assay and represented respect of 100% LDH release (bar with diagonal lines). Data are expressed as percentage of LDH released compared with the positive control. Data are expressed as the means ± SEM of at least four independent experiments. (***) denote $p < .001$ respect of 100% LDH release (bar with diagonal lines).

### 3.5. Cytotoxicity

We analyzed the cytotoxic effect of EE-FLABI (5 μg/mL), EE-LISA (5 μg/mL) and **3** (4.4 μg/mL) on SH-SY5Y cells as in the work by Alza et al. (2014). Lactate dehydrogenase (LDH) is a soluble enzyme localized in cellular cytoplasm and its release into the cell culture medium is an accepted marker of cell death. Treatment of SH-SY5Y cells with EE-FLABI, EE-LISA and **3** did not affect the cell viability within the time range used in the experiments (Fig. 2).

### 3.6. Cellular protection

We investigate the potential protective activity of EE-FLABI, EE-LISA, and **3** against $Ca^{2+}$ overload in SH-SY5Y cells as in the work by Marco-

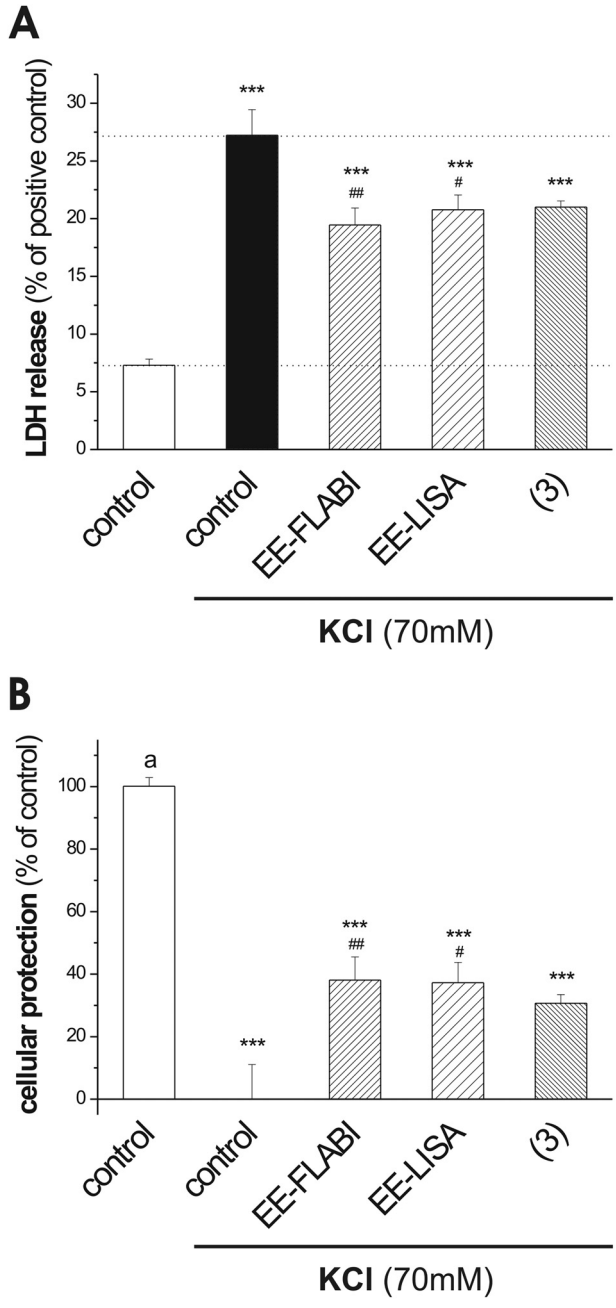


**Fig. 3.** *Evaluation of cellular protective effects of plant extracts against depolarizing concentration of KCl (70 mM) in SH-SY5Y cells.* SH-SY5Y cells were pretreated with EE-FLABI (5 μg/mL), EE-LISA (5 μg/mL) and **3** (4.4 μg/mL) for 24 h prior to the 70 mM KCl insult. Afterwards, a depolarizing concentration of KCl (70 mM) was added to the culture medium and the cells were incubated for 24 additional h, in the presence of EE-FLABI, EE-LISA and **3**. Cellular damage was analyzed by a LDH release assay as described in the "Materials and Methods" section. (A) Data are expressed as percentage of LDH released compared with the positive control. (B) Data are expressed as % of cellular protection. To calculate % protection, LDH released was calculated considering 100% of cellular protection the LDH released in the presence of drug vehicle and in absence of KCl. Data are expressed as the means ± SEM of at least four independent experiments. (***) denote $p < .001$ respect of control; (#) and (##) denote $p < .05$ and $p < .01$, respectively, respect of control + KCl.

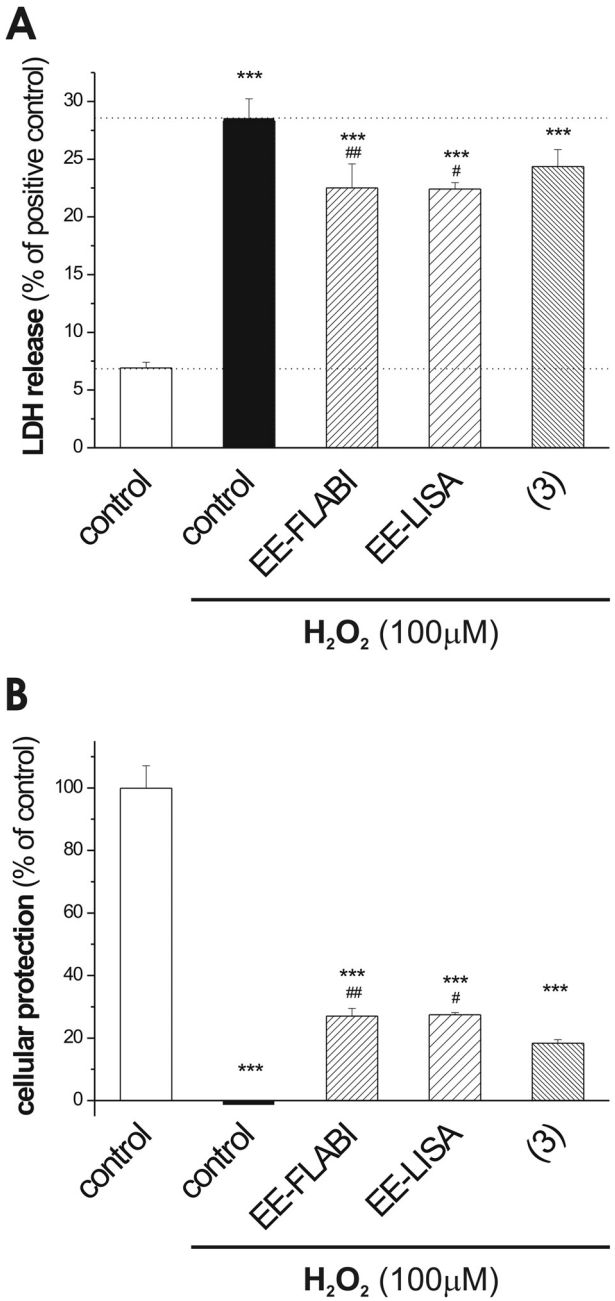


**Fig. 4.** *Evaluation of antioxidants properties of plant extracts against exposure of SH-SY5Y cells to 100 μM $H_2O_2$.* SH-SY5Y cells were pretreated with EE-FLABI (5 μg/mL), EE-LISA (5 μg/mL) and **3** (4.4 μg/mL) for 24 h prior to the oxidative insult. Afterwards, 100 μM $H_2O_2$ was added to the cell culture and incubated for 24 additional h, in the presence of EE-FLABI, EE-LISA and **3**. Cellular damage was analyzed by a LDH release assay as described in the "Materials and Methods" section. (A) Data are expressed as percentage of LDH released compared with the positive control. (B) Data are expressed as % of cellular protection. To calculate % protection, LDH released was calculated considering 100% of cellular protection the LDH released in the presence of drug vehicle and in absence of $H_2O_2$. Data are expressed as the means ± SEM of at least four independent experiments. (***) denote $p < .001$ respect of control; (#) and (##) denote $p < .05$ and $p < .01$, respectively, respect of control + $H_2O_2$.

Contelles et al. (2009). For this purpose, SH-SY5Y cells, previously treated with the aforementioned compounds, were subsequently exposed for 24 h to a depolarizing medium containing KCl 70 mM, which induced $Ca^{2+}$ overload and cell death. Plant extracts and **3**, at a concentration of 5 and 4.4 μg/mL, respectively, were administered 24 h before incubation of the cells with high $K^+$ and maintained during the entire experiment. Thereafter, release of LDH was measured as a marker of cell damage. As shown in Fig. 3A–B, the plant extracts EE-FLABI and EE-LISA showed a significant neuroprotection against high $K^+$ treatment compared to untreated cells. For **3** we found a trend (one-way ANOVA: $p < .06$ for control + KCl *vs.* **3** + KCl) towards cellular protection against $Ca^{2+}$ overload.

### 3.7. Antioxidant activity

The antioxidant activity of EE-FLABI (5 μg/mL), EE-LISA (5 μg/mL) and **3** (4.4 μg/mL) was evaluated on SH-SY5Y neuroblastoma cells exposed to 100 μM $H_2O_2$ for 24 h (Cañas et al. 2007; Marco-Contelles et al. 2009). Plant extracts and **3** were administered 24 h before incubation of the cells with $H_2O_2$ and maintained during the entire experiment. The results shown in Fig. 4A–B indicate that plant extracts EE-FLABI and EE-LISA showed protective activity against free radicals. Again, for **3** we found a trend (one-way ANOVA: $p < .06$ for control + $H_2O_2$ *vs.* **3** + $H_2O_2$) towards cellular protection against $H_2O_2$.

## 4. Conclusions

Prompted by the AChE inhibition observed for the ethanolic extracts of *L. salsa* and *F. bidentis*, we conducted a bioassay guided approach to identify active metabolites present in these two species. AChE inhibition could be explained by the presence of compounds **1**–**3**. Also, in the present work we described that the ethanolic extracts obtained from *L. salsa* and *F. bidentis*, as well as 6-methoxykaempferol-3-sulfate (**3**), beyond acting as AChE inhibitors, they exhibit neuroprotective effects against KCl induced-$Ca^{2+}$ overload and oxidative stress. The fact that they provide protection against calcium overload and free-radical generation denotes that they could exert their effects on the apoptotic cell death cascade beyond the particular mechanism of each toxic agent. Our findings prove that these two plants are interesting candidates as neuroprotective agents for further studies.

Edited by D Lišková.

## Acknowledgements

This work was financially supported by the National Research Council of Argentina(CONICET) and Universidad Nacional del Sur (Argentina). C.J.B. and A.P.M. are Research Members of CONICET. V.C. thanks to Universidad de La Laguna, Tenerife, for a short stay grant. We thank Prof. A. G. Ravelo for helping us in the characterization of compound **3.**

## References

Alza, N.P., Murray, A.P., 2016. Chemical constituents and acetylcholinesterase inhibition of *Senecio ventanensis* Cabrera (Asteraceae). Records of Natural Products 10.

Alza, N.P., Richmond, V., Baier, C.J., Freire, E., Baggio, R., Murray, A.P., 2014. Synthesis and cholinesterase inhibition of cativic acid derivatives. Bioorganic and Medicinal Chemistry 22. https://doi.org/10.1016/j.bmc.2014.06.030.

Ansari, M.A., Scheff, S.W., 2010. Oxidative stress in the progression of Alzheimer disease in the frontal cortex. Journal of Neuropathology and Experimental Neurology 69, 155–167. https://doi.org/10.1097/NEN.0b013e3181cb5af4.

Barron, D., Ibrahim, R.K., 1987. 6-Methoxyflavonol 3-monosulphates from *Flaveria chloraefolia*. Phytochemistry 26, 2085–2088. https://doi.org/10.1016/S0031-9422(00)81763-X.

Cabrera, J., Juliani, H., 1976. Quercetin-3-acetyl-7,3′-4′-trisulphate from *Flaveria bidentis*. Lloydia 39, 253–254.

Cabrera, J.L., Juliani, H.R., 1977. Isorhamnetin 3,7-disulphate from *Flaveria bidentis*. Phytochemistry 16, 400. https://doi.org/10.1016/0031-9422(77)80079-4.

Cabrera, J.L., Juliani, H.R., 1979. Two new quercetin sulphates from leaves of *Flaveria bidentis*. Phytochemistry 18, 510–511. https://doi.org/10.1016/S0031-9422(00)81907-X.

Cañas, N., Valero, T., Villarroya, M., Montell, E., Vergés, J., García, A.G., López, M.G., 2007. Chondroitin sulfate protects SH-SY5Y cells from oxidative stress by inducing heme oxygenase-1 via phosphatidylinositol 3-kinase/Akt. The Journal of Pharmacology and Experimental Therapeutics 323, 946 LP–953.

Cavallaro, V., Alza, N.P., Murray, M.G., Murray, A.P., 2014. Alkaloids from *Habranthus tubispathus* and *H. jamesonii*, two amaryllidaceae with acetyl- and butyrylcholinesterase inhibition activity. Natural Product Communications 9.

Churches, Q.I., Caine, J., Cavanagh, K., Epa, V.C., Waddington, L., Tranberg, C.E., Meyer, A.G., Varghese, J.N., Streltsov, V., Duggan, P.J., 2014. Naturally occurring polyphenolic inhibitors of amyloid beta aggregation. Bioorganic & Medicinal Chemistry Letters 24, 3108–3112. https://doi.org/10.1016/J.BMCL.2014.05.008.

Cybulska, I., Brudecki, G., Alassali, A., Thomsen, M., Jed Brown, J., 2014. Phytochemical composition of some common coastal halophytes of the United Arab Emirates. Emirates Journal of Food and Agriculture 26, 1046–1056. https://doi.org/10.9755/ejfa.v26i12.19104.

Dueñas, M., González-Manzano, S., Surco-Laos, F., González-Paramas, A., Santos-Buelga, C., 2012. Characterization of sulfated quercetin and epicatechin metabolites. Journal of Agricultural and Food Chemistry 60, 3592–3598. https://doi.org/10.1021/jf2050203.

Ellman, G., Courtney, K., Andres, V., Featherstone, R., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88–95.

Gibbs Russell, G.E., Welman, W.G.M., Retief, E., Immelman, K.L., Germishuizen, G., Pienaar, B.J., Van Wyk, M., Nicholas, A., 1987. List of species of southern African plants. Memoirs of the Botanical Survey of South Africa. Botanical Research Institute, Dept. of Agriculture, South Africa.

Guglielmone, H.A., Agnese, A.M., Núñez Montoya, S.C., Cabrera, J.L., 2005. Inhibitory effects of sulphated flavonoids isolated from *Flaveria bidentis* on platelet aggregation. Thrombosis Research 115, 495–502. https://doi.org/10.1016/J.THROMRES.2004.11.019.

Guo, A.J.Y., Xie, H.Q., Choi, R.C.Y., Zheng, K.Y.Z., Bi, C.W.C., Xu, S.L., Dong, T.T.X., Tsim, K.W.K., 2010. Galangin, a flavonol derived from Rhizoma *Alpiniae officinarum*, inhibits acetylcholinesterase activity in vitro. Chemico-Biological Interactions 187, 246–248. https://doi.org/10.1016/J.CBI.2010.05.002.

Gurovic, M.S.V., Castro, M.J., Richmond, V., Faraoni, M.B., Maier, M.S., Murray, A.P., 2010. Triterpenoids with acetylcholinesterase inhibition from *Chuquiraga erinacea* D. Don. subsp. *erinacea* (Asteraceae). Planta Medica 76, 607–610. https://doi.org/10.1055/s-0029-1240582.

Juliani, H.R., Koroch, A., Simon, J.E., Biurrun, F.N., Castellano, V., Zygadlo, J.A., 2004. Essential oils from Argentinean aromatic plants. Acta Horticulturae 491–498.

Katalinić, M., Rusak, G., Domaćinović Barović, J., Šinko, G., Jelić, D., Antolović, R., Kovarik, Z., 2010. Structural aspects of flavonoids as inhibitors of human butyrylcholinesterase. European Journal of Medicinal Chemistry 45, 186–192. https://doi.org/10.1016/J.EJMECH.2009.09.041.

Ksouri, R., Ksouri, W.M., Jallali, I., Debez, A., Magné, C., Hiroko, I., Abdelly, C., 2012. Medicinal halophytes: Potent source of health promoting biomolecules with medical, nutraceutical and food applications. Critical Reviews in Biotechnology 32, 289–326. https://doi.org/10.3109/07388551.2011.630647.

León, R., Garcia, A.G., Marco-Contelles, J., 2013. Recent advances in the multitarget-directed ligands approach for the treatment of Alzheimer's disease. Medicinal Research Reviews 33, 139–189. https://doi.org/10.1002/med.20248.

Lokhande, V.H., Gor, B.K., Desai, N.S., Nikam, T.D., Suprasanna, P., 2013. *Sesuvium portulacastrum*, a plant for drought, salt stress, sand fixation, food and phytoremediation. A review. Agronomy for Sustainable Development 33, 329–348. https://doi.org/10.1007/s13593-012-0113-x.

Lou, H., Fan, P., Perez, R.G., Lou, H., 2011. Neuroprotective effects of linarin through activation of the PI3K/Akt pathway in amyloid-β-induced neuronal cell death. Bioorganic & Medicinal Chemistry 19, 4021–4027. https://doi.org/10.1016/J.BMC.2011.05.021.

Marco-Contelles, J., León, R., de los Ríos, C., Samadi, A., Bartolini, M., Andrisano, V., Huertas, O., Barril, X., Luque, F.J., Rodríguez-Franco, M.I., López, B., López, M.G., García, A.G., do Carmo Carreiras, M., Villarroya, M., 2009. Tacripyrines, the first tacrine—dihydropyridine hybrids, as multitarget-directed ligands for the treatment of Alzheimer's disease. Journal of Medicinal Chemistry 52, 2724–2732. https://doi.org/10.1021/jm801292b.

Muche, A., Arendt, T., Schliebs, R., Piccini, A., Borghi, R., Guglielmotto, M., 2017. Oxidative stress affects processing of amyloid precursor protein in vascular endothelial cells. PLoS One 12, e0178127. https://doi.org/10.1371/journal.pone.0178127.

Múlgura, M., O'Leary, N., Rotman, A., 2012. Dicotyledoneae. Verbenaceae. In: Ramona Anton, A.M., Zuloaga, F.O. (Eds.), Flora Argentina Vol. 14. Estudio Sigma SRL, Buenos Aires, Argentina, pp. 1–220.

Murray, A.P., Rodriguez, S., Frontera, M.A., Tomas, M.A., Mulet, M.C., 2004. Antioxidant metabolites from *Limonium brasiliense* (Boiss.) kuntze. Zeitschrift für Naturforschung C: A Journal of Biosciences 59.

Murray, A.P., Faraoni, M.B., Castro, M.J., Alza, N.P., Cavallaro, V., 2013. Natural AChE inhibitors from plants and their contribution to Alzheimer's disease therapy. Current Neuropharmacology 11.

Nebbia, A.J., Zalba, S.M., 2007. Comunidades Halófilas de la costa de la Bahía Blanca (Argentina): Caracterización, mapeo y cambios durante los últimos cincuenta años. Boletín de la Sociedad Argentina de Botánica 42, 261–271.

Patel, S., 2016. Salicornia: Evaluating the halophytic extremophile as a food and a pharmaceutical candidate. 3 Biotech 6, 1–10. https://doi.org/10.1007/s13205-016-0418-6.

Pereyra De Santiago, O., Juliani, H., 1972. Isolation of quercetin 3,7,3′,4′-tetrasulphate from *Flaveria bidentis* L. Otto Kuntze. Experientia 28, 380–381.

Poprac, P., Jomova, K., Simunkova, M., Kollar, V., Rhodes, C.J., Valko, M., 2017. Targeting free radicals in oxidative stress-related human diseases. Trends in Pharmacological Sciences 38, 592–607. https://doi.org/10.1016/j.tips.2017.04.005.

Rodriguez, S.A., Murray, A.P., 2010. Antioxidant activity and chemical composition of essential oil from *Atriplex undulata*. Natural Product Communications 5.

Schroeter, H., Spencer, J.P., Rice-Evans, C., Williams, R.J., 2001. Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. The Biochemical Journal 358, 547–557.

Selkoe, D.J., 2012. Preventing Alzheimer's disease. Science 337, 1488–1492.

Shimmyo, Y., Kihara, T., Akaike, A., Niidome, T., Sugimoto, H., 2008. Flavonols and flavones as BACE-1 inhibitors: Structure–activity relationship in cell-free, cell-based and in silico studies reveal novel pharmacophore features. Biochimica et Biophysica Acta - General Subjects 1780, 819–825. https://doi.org/10.1016/J.BBAGEN.2008.01.017.

Teles, Y., Souza, M., Souza, M., 2018. Sulphated flavonoids: Biosynthesis, structures, and biological activities. Molecules 23, 480. https://doi.org/10.3390/molecules23020480.

Uriarte-Pueyo, I., Calvo, M., 2011. Flavonoids as acetylcholinesterase inhibitors. Current Medicinal Chemistry https://doi.org/10.2174/092986711798184325.

Zhao, L., Hou, L., Sun, H., Yan, X., Sun, X., Li, J., Bian, Y., Chu, Y., Liu, Q., 2011. Apigenin isolated from the medicinal plant *Elsholtzia rugulosa* prevents β-amyloid 25–35-induces toxicity in rat cerebral microvascular endothelial cells. Molecules https://doi.org/10.3390/molecules16054005.

Zhou, F., Chen, S., Xiong, J., Li, Y., Qu, L., 2012. Luteolin reduces zinc-induced tau phosphorylation at Ser262/356 in an ROS-dependent manner in SH-SY5Y cells. Biological Trace Element Research 149, 273–279. https://doi.org/10.1007/s12011-012-9411-z.

Zuloaga, F., Morrone, O., Belgrano, M.J., 2008. Catalogue of the vascular plants of the Southern Cone (Argentina, Southern Brazil, Chile, Paraguay, and Uruguay). Monogr. Syst. Bot. Missouri Bot. Gard., Missouri, USA.