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Natural Multi-Target Inhibitors of Cholinesterases and Monoamine Oxidase Enzymes with Antioxidant Potential from Skin Extracts of *Hypsiboas cordobae* and *Pseudis minuta* (Anura: Hylidae)

Roque Spinelli,[a] Ivan Sanchis,[a] Florencia M. Aimaretti,[a] Andres M. Attademo,[b, c] Madelon Portela,[d] Maria V. Humpola,[a] Georgina G. Tonarelli,[a] and Alvaro S. Siano*[a, c]

[a] Departamento de Química Orgánica, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, 3000 Santa Fe, Argentina, e-mail: asiano@fbcb.unl.edu.ar

[b] Laboratorio de Ecotoxicología, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, 3000 Santa Fe, Argentina

[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

[d] Analytical Biochemistry and Proteomics Unit, Faculty of Sciences, National University and Institut Pasteur de Montevideo, 11400 Montevideo, Uruguay

Alzheimer's disease (AD) is the most common cause of dementia, characterized by loss of selective neuronal and normal brain functions. Every year, ten million new cases are diagnosed worldwide. AD is a complex disease associated with all kind of different pathways, making their simultaneous modulation necessary. Nowadays anti-AD treatments are focused on enzymatic inhibitors. The study of the amphibians' skin had acquired great importance in the fields of biology and human health and represents an attractive and novel source for natural compounds with high potential in the development of new drugs. The present work exhibits the power of amphibian skins as a source of bioactive compounds. Herein we report the activity of extracts of two species from Hylidae family (*H. cordobae* and *P. minuta*) as reversible inhibitors of acetylcholinesterase and butyrylcholinesterase enzymes. Furthermore, the extracts inhibit MAO–B enzyme and showed antioxidant activities, acting on four important pathways of AD.

Keywords: amphibian skin, natural products, biological activity, cholinesterase, monoamine oxidase, antioxidants.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia, characterized by loss of selective neuronal and normal brain functions.[1] Every year, ten million new cases are diagnosed worldwide, especially in developed countries due to their higher life expectancy.[2] AD patients present deficit of the neurotransmitter acetylcholine (ACh), resulting in memory impairments.[3] ACh is hydrolyzed by acetylcholinesterase (AChE) (EC: 3.1.1.7) or butyrylcholinesterase (BChE) (EC: 3.1.1.8) enzymes.[4] Nowadays, cholinesterase inhibitors are the only symptomatic treatment indicated for AD, and the efforts in the development of new drugs continue focusing on these.[5]

Consequently, the AChE besides hydrolyzing the neurotransmitter ACh plays an essential role in the formation of the β-amyloid peptide (Aβ) deposits.[6] The deposits of Aβ plaques increase the cerebral damage in AD.[7] It is well documented that AChE possesses two binding sites involved in AD, the catalytic active site (CAS) responsible for ACh neurotransmitter hydrolysis, and the peripheral anionic site (PAS) located on the surface of the enzyme, responsible of non-cholinergic functions of AChE.[8] The PAS may not only stimulate the formation and aggregation

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Table 1. Data about the collection of amphibian specimens.

Species	Number of specimens[a]	Year of collection	Source	Average SVL [mm][b]	Average % of proteins[c]	Presence of Alkaloids[d]
H. cordobae	4	2016	Cordoba, (Cordoba) (30°51′47.72″S/ 64°29′36.21″W)	37–50	64.00	+
P. minuta	5	2016	Paraná, (Entre Ríos) (31°42′44.83″S/ 60°32′50.25″W)	45–65	41.18	−

[a]Total number of specimens used for the extraction of the biological samples. [b]SVL: snout-vent length. [c]% of proteins obtained by the bicinchoninic acid method. [d]Presence of alkaloids on TLC plate by Dragendorff's reagent. (+) Positive, (−) Negative; the number of + represents the number of spots detected by Dragendorff.

of Aβ but may also increase the neurotoxicity of amyloid fibrils.[9] Because of this, the cholinesterase inhibitors that interact with the peripheral anionic site are considered promising drugs for AD.[10–12] Also, Aβ aggregates lead to the production of reactive oxygen species (ROS), oxidative stress and eventually cell death.[13] Thus, the use of antioxidant agents that reduce the toxic effects of oxidative stress is useful for delaying or preventing these type of disorder.[14]

In addition to the cholinesterases, the monoamine oxidase enzymes (MAOs) have also received increasing attention in recent years due to its role in the treatment of AD.[15][16] Particularly, the MAO–B subtype is involved in the oxidative deamination of neurotransmitters, increasing the production of free radicals thus causing oxidative stress.[17] MAO–B increase with age and its activity is found exacerbated in AD patients, leading to enhanced metabolism of dopamine and to the production of large amounts of hydrogen peroxide, which ultimately give rise to neuronal damage.[15] Therefore, MAO–B inhibitors are useful in the treatment of neurodegenerative disorders, such as AD. Nowadays the pursuit of effective anti-Alzheimer treatments is focused on making simultaneous modulation of the different pathways associated with a complex disease as AD.

The skins of amphibian's anuran (frogs and toads) have proven to be a considerable source of compounds with a wide range of biological activity,[18] among which are the peptides that not only are the major constituent of it, but also the most reported about their biological activity.[19]

The frog host defense peptides are synthesized and stored in designated macroglands (called granular glands or poison gland) and are rapidly released in response to stress, environmental conditions or predators attack, acting like a defense weapon.[19,20] These peptides have shown a wide range of biological effects in mammals, like antimicrobial (antibacterial, antifungal, antiprotozoal, and antiviral), antitumor, immunomodulators, antidiabetics,[19] and most recently even as enzymatic inhibitors.[21,22]

The study of the amphibians' skin has acquired great importance in the biology and human health field and represents an attractive and novel source of natural compounds with high potential in the development of new drugs.[18,19]

The Litoral and central regions of Argentina have broad biodiversity of amphibians. The Litoral region exceed the fifty-one species,[23] while the central area has seven reported species.[24] The majority of them unexplored, making both a promising source of new bioactive natural products.

The aim of this work was the evaluation and characterization of two extracts of amphibian anuran skins as a potential source for new multi-target agents associated with different pathways of AD.

Results and Discussion

In this work skin extracts from two amphibian species belonging to the Hylidae family, *Hypsiboas cordobae* and *Pseudis minuta*, were tested.

P. minuta inhabits swamp, rivers, and freshwater lakes, while *H. cordobae* inhabit in arid and mountainous territories.[25] *Table 1* summarizes the information about the geographic regions from which they were collected, number of specimens used, snout-vent length (SVL), percentage of proteins and presence of alkaloids in the extracts.

Analytical Characterization

The percentages of proteins of the extracts were of 64.00 and 41.18% for *H. cordobae* and *P. minuta*,

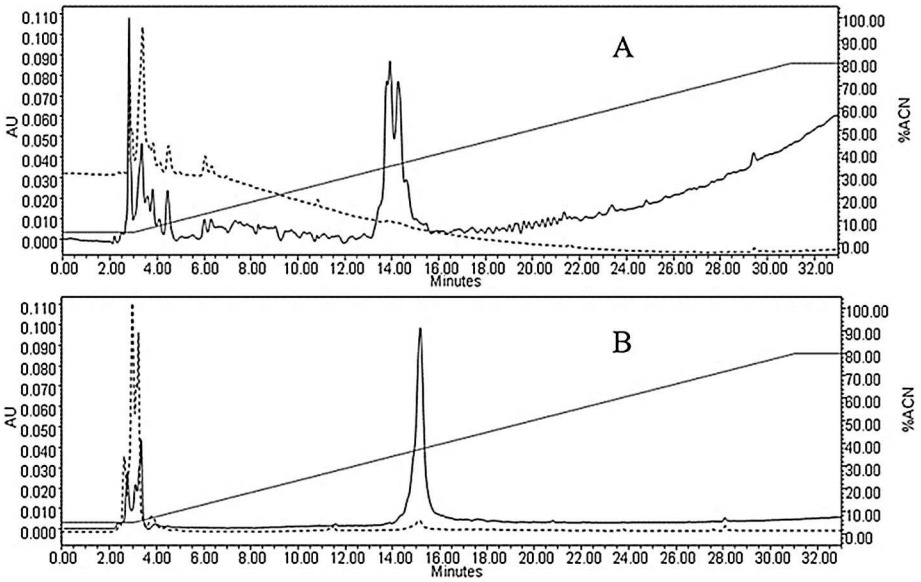

Figure 1. RP-HPLC Profile of the amphibian extracts. C_{18} analytical column. Linear gradient (5 to 80 %) from solution *A* (0.1 % TFA in water) to solution *B* (0.1 % TFA in MeCN) for 33 min. Flow rate of 0.8 ml min^{-1}. Detection: 220 nm (—) and 280 nm (·····). (A) *H. cordobae* extract. (B) *P. minuta* extract.

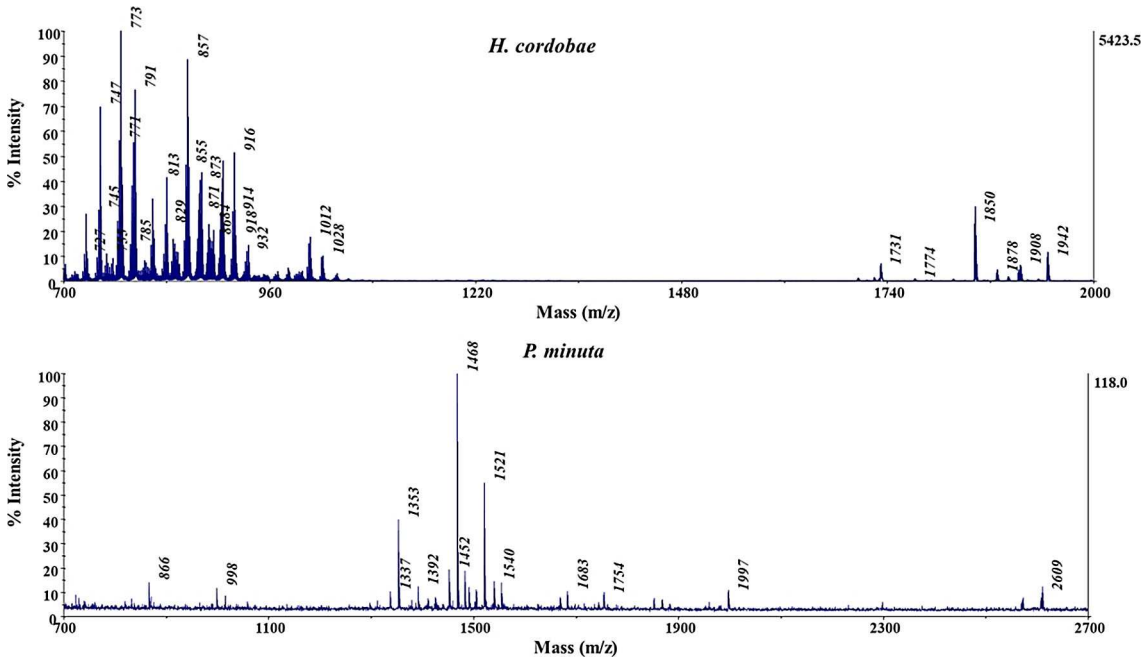

Figure 2. MALDI-TOF MS analysis of both extracts. Reflector positive method. Laser power of 4200.

respectively. The presence of alkaloids was observed by Dragendorff's reagent only in *H. cordobae* (*Table 1*).

The extracts were analyzed by RP-HPLC (*Figure 1*) and by MALDI-TOF MS spectrometry (*Figure 2*). The chromatograms evidenced the presence of hydrophilic and hydrophobic compounds in both species at 220 nm, while at 280 nm only hydrophilic compounds were showed.

Several reports demonstrated that the skins of amphibians are constituted by a considerable amount and variety of peptides and proteins,[26][27] and this fact was observed by the presence of compounds with a wide range of masses in the studied extracts by

MALDI-TOF MS analysis. The spectrum of *H. cordobae* was more complex than that of *P. minuta*. The MS for *H. cordobae* showed compounds with *m/z* ratio between 800 and 2000 Da, while for *P. minuta,* compounds with relative abundance with *m/z* between 1300 and 1600 were observed.

Furthermore, the presence of peptides and small proteins in the extracts were evidenced by Tricine-SDS-PAGE (*Figure 3*). *H. cordobae* extract showed the

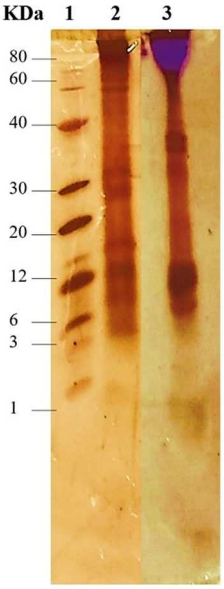

Figure 3. Tricine-SDS-PAGE silver staining. (1) Molecular weight markers (1 to 80 kDa). (2) *P. minuta* extract. (3) *H. cordobae* extract.

presence of bands of lower molecular weight than *P. minuta*; as MS also confirmed it (see *Figure 2*).

The addition of tricine to the SDS-PAGE offers high power of resolution, covering molecular weight range of 1–80 kDa, being a useful tool for the detection of small protein and peptide.

TLC Analysis at 254 and 365 nm (*Figures 4A* and *4B*) showed the complex composition of the extracts and their variable polarity. These results are in concordance with RP-HPLC and MALDI-TOF MS analyses (see *Figures 1* and *2*).

Bioautography Assay

A preliminary analysis of the amphibian extracts was performed by TLC bioautography. This methodology allows the evaluation of inhibitory properties of a complex sample spotted onto a TLC plate covered with a gel that contains enzyme, substrate, and

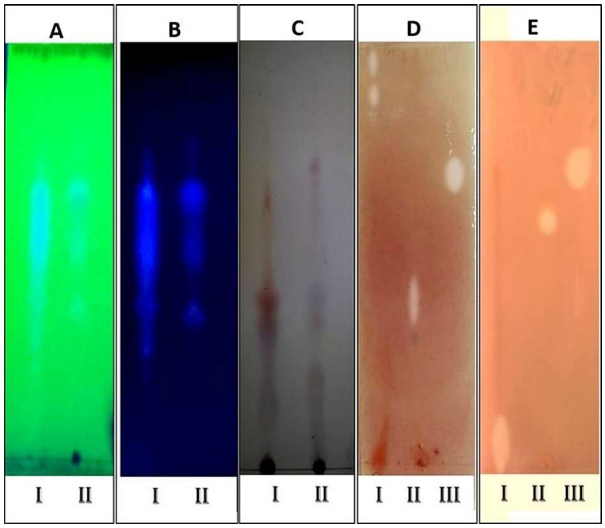

Figure 4. TLC Bioautography for detection of cholinesterase inhibitors. From left to right: (A) 254 nm UV light detection; (B) 365 nm UV light detection; (C) ninhydrin reagent; (D) bioautography for AChE inhibitor detection; (E) bioautography for BChE inhibitor detection. *P. minuta* extract (I), *H. cordobae* extract (II), Positive control (Caffeine, III). Elution was carried out with n-butanol/acetic acid/water (55.6:22.2:22.2).

chromogenic reagent.[28] Using this methodology, several authors have reported enzyme inhibitors involved in different pathological processes.[29,30]

In this case, we studied the inhibitory activity of two extracts of amphibian skin against AChE and BChE.[31] The cholinesterase's TLC bioautography relies on the enzyme cleavage of 1-naphthyl acetate to form 1-naphthol. The 1-naphthol reacts with Fast Blue B salt to give a purple colored diazonium dye. Regions of the TLC plate which contain cholinesterase inhibitors show up as white spots against the purple background.

H. cordobae presented inhibitory spots for AChE with $R_f = 0.4$ (*Figure 4D*) and for BChE with $R_f = 0.59$ (*Figure 4E*). These inhibition areas were reactive to ninhydrin reagent (*Figure 4C*) indicating the presence of peptidic compounds. For *P. minuta* two notable inhibition spots are showed for AChE with $R_{f1} = 0.86$ and $R_{f2} = 0.95$ (*Figure 4D*), of which only the spot with $R_f = 0.86$ reacted with ninhydrin reagent (*Figure 4C*), whereas for BChE a potent inhibition zone was presented with $R_f = 0.12$ (*Figure 4E*). The positive control showed inhibitory activity for both enzymes (*Figures 4D* and *4E*).

Easily to perform and reproducible, bioautography is a useful tool that offers valuable information for the

preliminary characterization of bioactive compounds from natural sources.

In vitro AChE and BChE Inhibitory Assays

The in vitro bioassay for the evaluation of the extracts as cholinesterase inhibitors was performed following Ellman methodology.[32]

Table 2. *In vitro* AChE and BChE inhibition data for the extracts.

Species	Enzymes	IC_{50} [μg ml^{-1}][a]	Selectivity for AChE[b]
H. cordobae	AChE	375 ± 27	1.04
	BChE	392 ± 14	–
P. minuta	AChE	507 ± 39	0.74
	BChE	377 ± 01	–

[a]IC_{50} values were determined by regression analyses of three replicate determinations. [b]Selectivity for AChE is defined as IC_{50} BChE/IC_{50} AChE. All values were expressed with a confidence of 95%.

Table 2 shows IC_{50} values for AChE and BChE and their selectivity. IC_{50} is defined as the concentration of sample that produces the 50% of enzymatic inhibition. The tested extracts showed inhibitory activity against AChE and BChE, reaching inhibition values that surpass the half of enzymatic activities. *H. cordobae* extract was the most active one with IC_{50} values of 375 μg ml^{-1} (AChE) and 392 μg ml^{-1} (BChE), showing similar potency for both enzymes.

Furthermore, *P. minuta* was more active against BChE (377 μg ml^{-1}) than against AChE (507 μg ml^{-1}), showing more affinity for the first (*Table 2*).

In all cases, the in vitro studies indicate that the extracts have inhibitory activities against both enzymes (see the dose-response curves in *Figure S1*, *Supplementary Material*), in agreement with the results obtained by TLC bioautography assay.

The kinetic parameters are useful for acquiring knowledge about the characterization of the enzymatic inhibitors. The values of K_m and V_{max} are critical to the identification of reversible competitive and non-competitive inhibition because these phenomena are defined in terms of significant effects upon K_m and/or V_{max} of an inhibitor.[33]

The Kinetic analysis (for AChE and BChE) was performed by Michaelis-Menten and Lineweaver-Burk plots, at IC_{50} concentrations (*Figures 5* and *6*). For *H. cordobae*, the V_{max} values of were significantly different ($P < 0.05$) whereas the K_m values were not significantly different ($P > 0.05$) with and without inhibitors, evidencing that the compounds act as non-competitive inhibitors, for both enzymes (*Table 3*).

On the other hand, *P. minuta* showed a competitive inhibition type for AChE and BChE, where the V_{max} values were not significantly different ($P > 0.05$) and K_m values were significantly different ($P < 0.05$), with and without inhibitors (*Table 3*).

For cholinesterases, several works report that competitive inhibitors act by blocking the entry of the substrate by interacting with CAS of the enzyme, whereas the non-competitive inhibitors act binding with PAS.[22,34]

The PAS site lies mainly on the surface of AChE, approximately 20 Å from the active site.[35] It binds

Table 3. Type of inhibition and enzymes kinetic parameters obtained for AChE and BChE with and without extracts presence.

Enzyme	*H. cordobae* V_{max}[a]			K_m[a]			Type of inhibition[c]
	Basal	Inhibitor	*P* value[b]	Basal	Inhibitor	*P* value[b]	
AChE	0.402 ± 0.019	0.254 ± 0.010	0.0003	0.098 ± 0.015	0.090 ± 0.012	0.5270	Non-competitive
BChE	0.282 ± 0.008	0.217 ± 0.008	0.0005	0.054 ± 0.007	0.050 ± 0.009	0.5966	Non-competitive
	P. minuta						
AChE	0.427 ± 0.017	0.430 ± 0.024	0.8792	0.106 ± 0.013	0.220 ± 0.029	0.0036	Competitive
BChE	0.387 ± 0.022	0.387 ± 0.033	0.6725	0.043 ± 0.012	0.207 ± 0.057	0.0085	Competitive

[a]V_{max} and K_m were determined using Michaelis-Menten and Lineweaver-Burk plots and expressed with a confidence of 95% at IC_{50} extract concentrations. [b]*P* value obtained by one-way ANOVA test, ($P < 0.05$) values significantly differences ($P > 0.05$) values not-significantly differences. [c]Type of inhibition calculated by *P* values analysis between V_{max} and K_m with and without presence of the extracts (inhibitor). Competitive inhibition: same V_{max} and different K_m values. Non-competitive inhibition: different V_{max} and same K_m values.

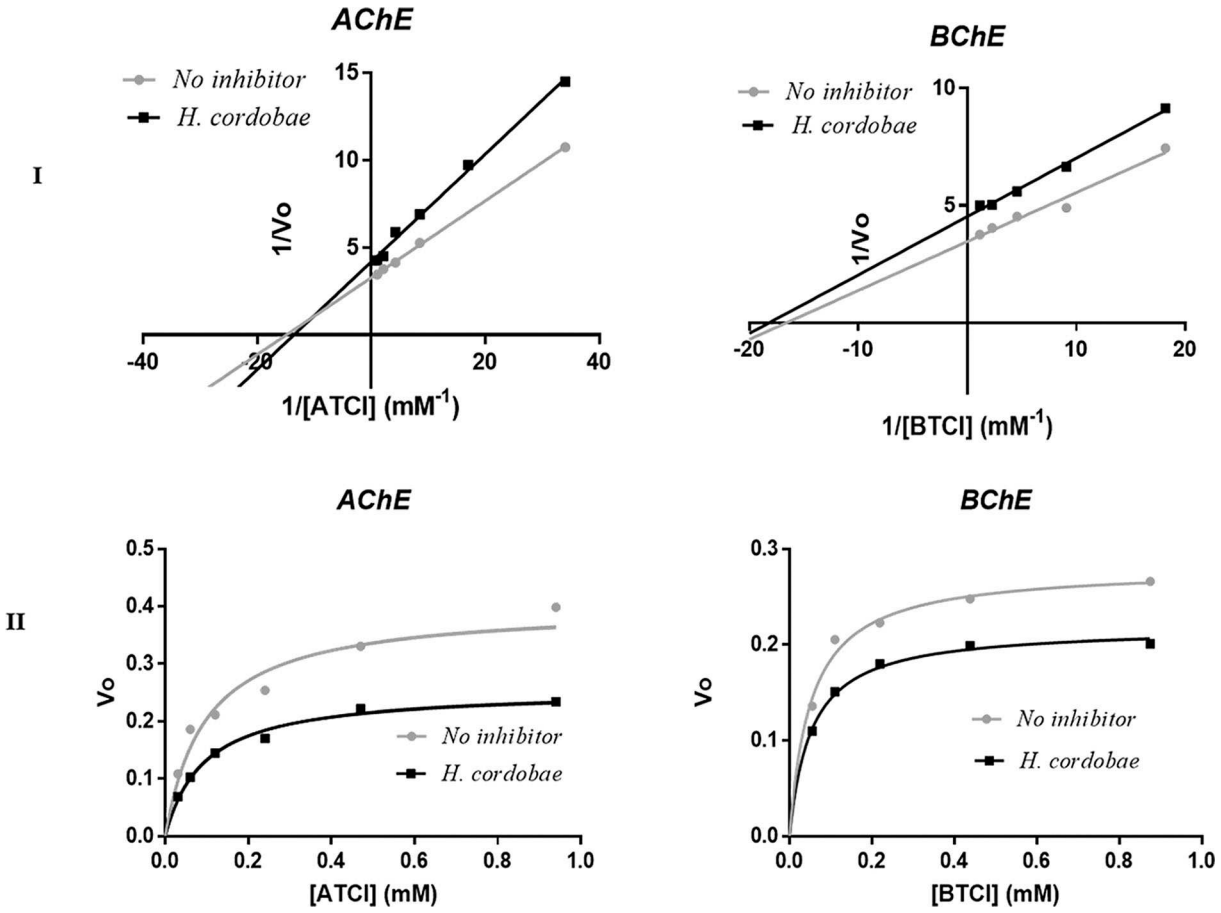

Figure 5. *H. cordobae* Lineweaver-Burk (double reciprocal) (I), and Michaelis-Menten (II) plots at IC_{50} doses (375 μg ml^{-1} for AChE and 392 μg ml^{-1} for BChE) and without inhibitors. These studies were performed with different doses of ATCI and BTCI substrates.

ACh as the first step in the catalytic pathway and allosterically modulates catalysis.[36,37] Moreover, it has also been identified as responsible for non-cholinergic functions of AChE, binding Aβ, stimulating its aggregation and increasing its neurotoxicity.[38] That is why the inhibition of PAS plays an essential role in AD.

The first reported natural peptides with a powerful inhibitory and selective function against the AChE were the fasciculins I, II, and III, isolated from the venom of the Eastern Green Mamba, *Dendroaspis angusticeps*.[39,40] Fasciculins have been shown a non-competitive inhibition of AChE through the interaction with the aromatic residues of PAS.[41]

In the last years, amphibians' skin peptides with inhibitory activity against cholinesterase enzymes were reported. In 2014[42] and 2017,[22] Siano et al. reported the peptide Hp-1971, isolated from *Hypsiboas pulchellus* (Anura: Hylidae), with dual inhibitory ability against AChE and BChE. In this work, the extracts of *H. cordobae* and *P. minuta* have shown to overcome the inhibition activities of the previously reported *H. pulchellus* extracts against the cholinesterases.[42] Because of this, the studies of both species are of great importance in search of new dual cholinesterase reversible inhibitors (AChE and BChE) with interaction with PAS.

Monoamine Oxidase Assay

Accumulated evidence indicates a solid relationship between several enzymes and AD. Cholinesterases and MAOs are closely associated with the disease symptomatology and progression. This strategy offers great chances to alter the course of AD, in addition to alleviation of the symptoms. More than 15 years of research has led to the identification of various dual cholinesterase/MAO inhibitors, while some are showing positive outcomes in clinical trials, thus giving rise to additional research efforts in the field.[16]

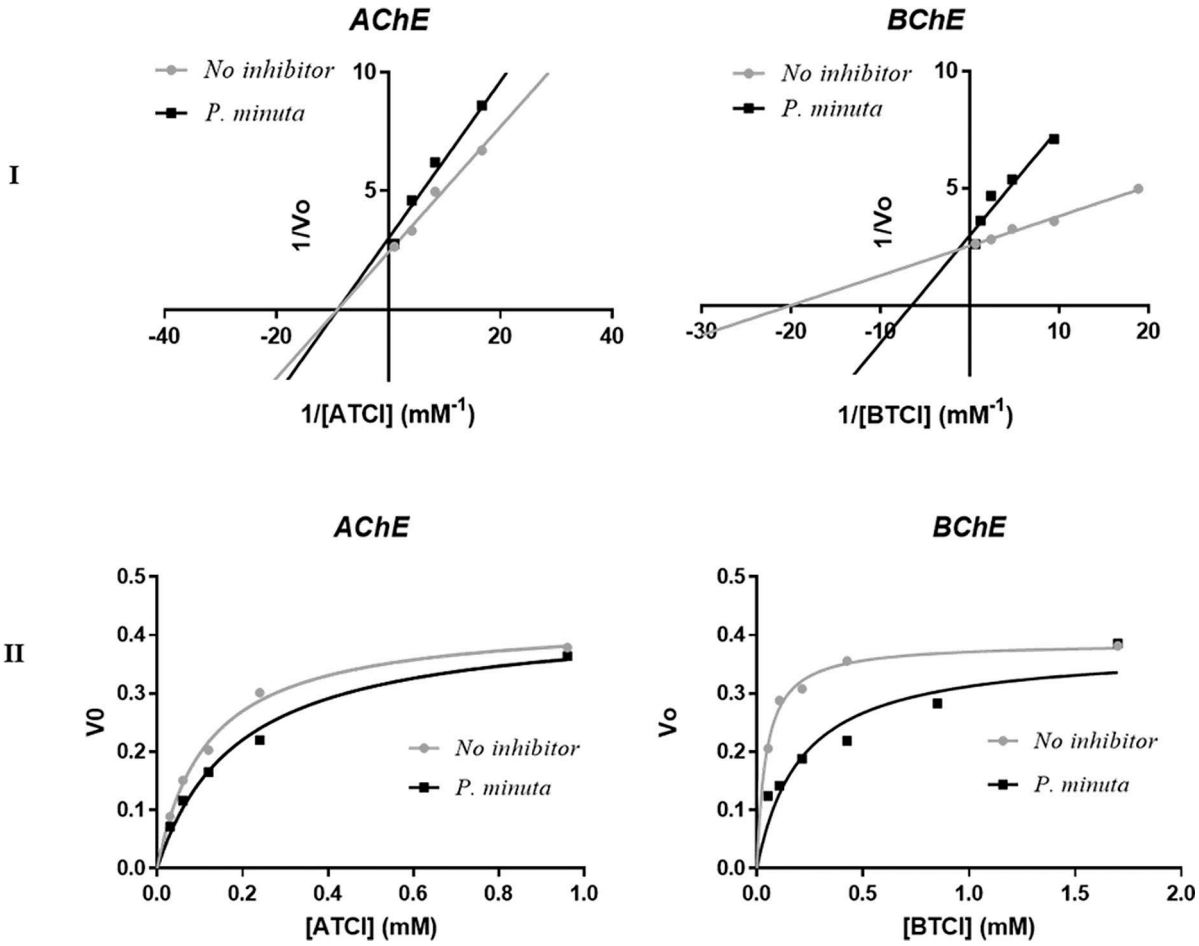

Figure 6. *P. minuta* Lineweaver-Burk (double reciprocal) (I), and Michaelis-Menten (II) plots at IC_{50} doses (507 μg ml^{-1} for AChE and 377 μg ml^{-1} for BChE) and without inhibitors. These studies were performed with different doses of ATCI and BTCI substrates.

In order to study the multi-target capability of the extracts, the inhibition of MAO–B was assayed (see the dose-response curves in *Figure S2*, *Supplementary Material*). In this sense, *P. minuta* showed inhibitory activity against MAO–B with an IC_{50} value of 610 μg ml^{-1} (*Table 4*). Notoriously, this extract resulted active against AChE (IC_{50} = 507 μg ml^{-1}) and BChE (IC_{50} = 377 μg ml^{-1}), being a promising candidate for the development of multi-target cholinesterase and MAO–B inhibitors. On the other hand, *H. cordobae* was not active against MAO–B at the tested concentrations (*Table 4*).

Table 4. In vitro MAO–B inhibition data for the extracts

Species	IC_{50} [μg ml^{-1}][a]
H. cordobae	> 800
P. minuta	610 ± 23

[a] IC_{50} values were determined by regression analyses of three replicate determinations. All values were expressed with a confidence of 95%.

Several reports demonstrated the potential of the natural products as MAO inhibitors, not only of isolated natural molecules but also of complete extracts from different sources such as plants, animals, fungal, and marine species.[43] The compounds isolated from these sources are of great molecular diversity, being flavonoids, coumarins, peptides, and alkaloids.[43–47] For example, the scorpion *Mesobuthus gibbosus* extract proved to have a peptidic venom capable of inhibiting MAOs.[44] Moreover, plan extracts of traditional Chinese medicine resulted active too, but in these cases, the alkaloids and flavonoids were the active compounds.[43] Interestingly, the analytical

characterization of the active extract of *P. minuta* showed the presences of peptides and proteins, and the lack of alkaloids, in its composition (see *Table 1*). This work is the first report that shows the capacity of amphibian skin extracts as MAO–B inhibitors.

Antioxidant Activity

The antioxidant activity of the extracts was evaluated by DPPH assay.[48] Both amphibians extract showed significate free radical scavenging ability at the IC_{50} concentration values of tested enzymes (*Figure 7*).

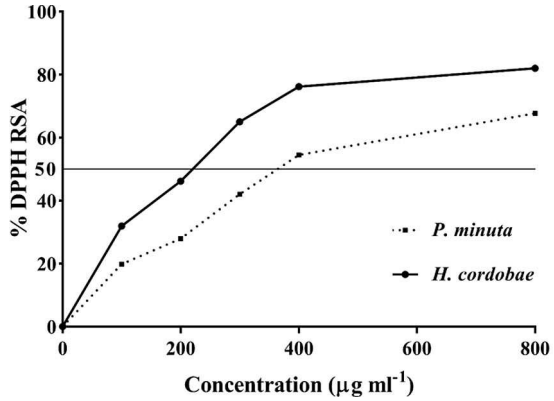

Figure 7. Antioxidant activity expressed as % of DPPH radical scavenging activity of amphibian's extract.

Between these, *H. cordobae* presented the higher one, with an EC_{50} value of 198 μg ml^{-1}. Instead, the EC_{50} value for *P. minuta* was 370 μg ml^{-1}.

A significant number of antioxidant compounds, including peptides,[49,50] were isolated from frogs' skin. The potential of the peptides as radical scavengers is focused on their amino acidic composition, sequence, structure, and hydrophobicity. Aromatic residues (Tyr, Trp, and Phe) can donate electrons while keeping their stability via resonance structure.[51] Furthermore, hydrophobic amino acids could be enhanced by the antioxidative properties, especially from naturally occurring peptides.[52] The analysis and characterization of both extracts studied in this work demonstrated to be constituted by compounds with these characteristics.

Toxicity Activity

To examine the cytotoxic effects of the bioactive extracts, the hemolytic activity and viability in CHO–K1 cell line by MTT assay were performed. The results are shown in *Figure 8*.

P. minuta showed to be non-cytotoxicity against CHO–K1 cell line at assayed concentrations, and against human erythrocytes, the fifty percent of hemolysis was reached at 660 μg ml^{-1}. Notably, the

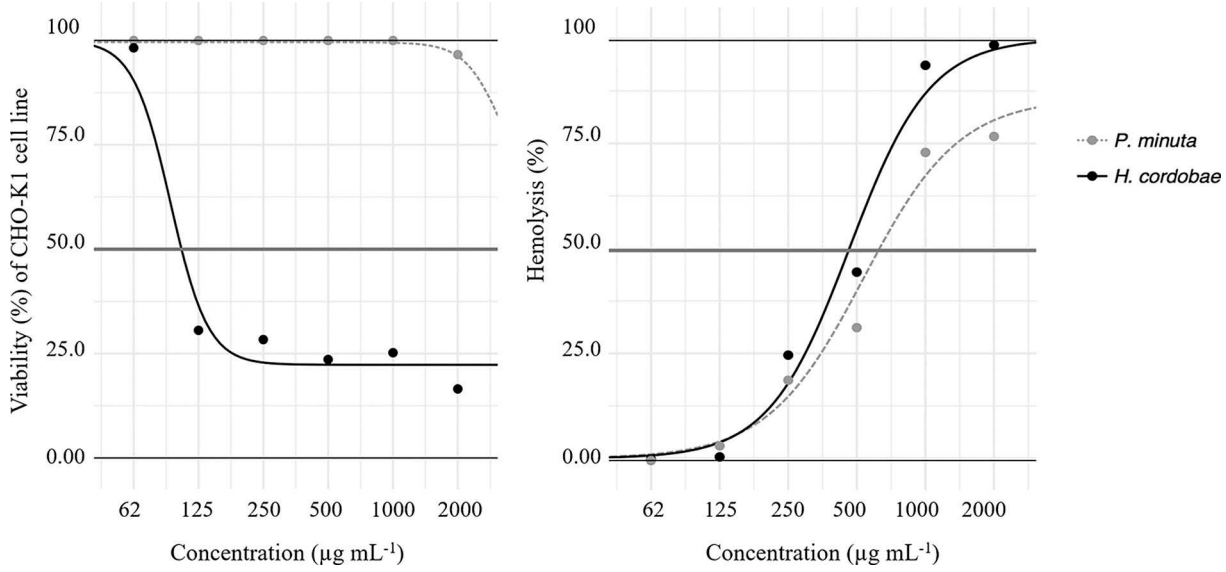

Figure 8. Toxicity analysis. % of viability of CHO–K1 cell line (left) and % of hemolysis of human erythrocytes (right) as a function of the concentration of the extract.

IC_{50} values for AChE, BChE, MAO–B and antioxidant activity were of 507 $\mu g\,ml^{-1}$, 377 $\mu g\,ml^{-1}$, 610 $\mu g\,ml^{-1}$ and 370 $\mu g\,ml^{-1}$, respectively. Being more active than cytotoxic, this fact that makes *P. minuta* of great interest for its study (see the comparative plot in *Figure S3*, *Supplementary Material*).

On the other hand, *H. cordobae* reached the fifty percent of cytotoxicity against CHO–K1 and human erythrocytes at 100 $\mu g\,ml^{-1}$ and 460 $\mu g\,ml^{-1}$, respectively. Showing IC_{50} values for AChE, BChE and antioxidant activity of 375 $\mu g\,ml^{-1}$, 392 $\mu g\,ml^{-1}$ and 198 $\mu g\,ml^{-1}$, respectively. This extract was toxic against CHO–K1 cell lines at bioactive concentrations (see the comparative plot in *Figure S4*, *Supplementary Material*).

The knowledge of the cytotoxic activity of a sample is of great importance because this is a limiting factor for its applicability. Previous studies have demonstrated that the potential of bioactive agents of the amphibians' skin extracts has their limitation in the cytotoxic activity that these present.[21] However, it is essential to remark that even though the extract presents certain toxicity, this is also an indicator of the presence of powerful bioactive compounds as several authors have demonstrated.[53,54]

Conclusion

The present work exhibits the potential of the amphibian extracts to act on four important pathways of Alzheimer's disease (AChE, BChE, MAO–B, and anti-free radical), being the first work that demonstrate these capabilities.

Herein we report the activity of extracts of two species from Hylidae family (*H. cordobae* and *P. minuta*) belonging to different habitat from Argentina as reversible inhibitors of acetylcholinesterase and butyrylcholinesterase enzymes. Currently, these types of inhibition are used as a pharmacological target in Alzheimer's disease.

On the other hand, both tested extracts showed high antioxidant activities. Furthermore, *P. minuta* extract showed activity against MAO–B enzyme, acting as a dual cholinesterase/MAO–B inhibitor.

AD is a complex disease associated with all kind of different pathways, making the simultaneous modulation necessary. The studies of the amphibian's skin exhibit a multi-target intervention, making them potential compounds in the pursuit of effective anti-Alzheimer treatments.

Also, we propose the use of TLC bioautography assay as a rapid and effective technique for the screening of natural products.

Experimental Section

Materials and Reagents

Members of the Ecotoxicology department collected the amphibian's specimens (*H. cordobae* and *P. minuta*) under the supervision of members of the bioethical committee of the Facultad de Bioquímica y Ciencias Biológicas – Universidad Nacional del Litoral (Argentina). All the frogs used in this work were manipulated following the recommendations of the American Society of Ichthyologists & Herpetologists.[55] Ethanol, acetic acid, butanol, and methanol analytical grade, acetonitrile and trifluoroacetic acid (TFA) HPLC grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Milli-Q water was obtained using a water purification system (Millipore, Bedford, MA, USA), Fast Blue B salt, α-naphthyl acetate, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), *S*-butyrylthiocholine iodide (BTCI), 2,2-diphenyl-1-picrylhydrazyl (DPPH), α-cyano-4-hydroxycinnamic acid (HCCA), *o*-dianisidine, benzylamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), tricine, polyacrylamide, bicinchoninic acid salt, bovine serum albumin (BSA), RPMI-1640 medium, ninhydrin and Dragendorff reagents were purchased from Sigma-Aldrich (Argentina). Acetylcholinesterase (AChE) from *Electrophorus electricus* (EC: 3.1.1.7) were purchased from Sigma–Aldrich (Argentina). The Chinese hamster ovary K1 (CHO–K1) cellular line were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA).

Extracts Isolation

The extracts of both species were obtained by the previously reported solvent extraction technique.[27] Briefly, each specimen was euthanized following the recommendations of the American Society of Ichthyologists & Herpetologists (ASIH).[55] The skin was removed and triturated, followed by the addition of a solution of ethanol/water (60:40). Aliquots of acetic acid were added to help the extraction and solubilization. The solution was kept in constant agitation during 2 h at 0 °C, followed by centrifugation at 10.000 rpm, at 4 °C, the process was repeated twice.

The supernatant was separated and concentrated under reduced pressure and lyophilized.

Protein Quantification

The bicinchoninic acid assay (BCA) was used for protein quantification.[56] The extracts were dissolved in Milli-Q water, and 100 μl of each extract were incubated with 2 ml of the BCA reagent at 60 °C for 30 min. After cooling, absorbance was measured at 562 nm in a Metrolab 1700 UV/VIS spectrophotometer. A calibration curve with Bovine Serum Albumin (BSA) in the concentration range from 0.1 to 1.5 mg ml^{-1} was performed.

Peptides and Alkaloids Determination

The extracts were developed by thin layer chromatography (TLC) with a solvent mixture of butanol/acetic acid/water (55.6:22.2:22.2). Ninhydrin[57] (for peptides) and Dragendorff[58] (for alkaloids) reagents were used as developing agent.

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)

The samples obtained by SE were dissolved in Milli-Q water containing 0.1 % of TFA, filtered with a 0.45 μm membrane disc (Merck, Millipore) and analyzed by RP-HPLC on a Waters equipment (USA) using an Atlantis (Waters) C_{18} analytical column (5 μm, 4.6 mm × 150 mm). The run was performed using a linear gradient of 5–80 % MeCN/H_2O for 33 min and 0.1 % of TFA was added to each solvent; flow rate: 0.8 ml min^{-1}. The eluates were monitored by their absorbance at 220 and 280 nm; 20 μl of a 10 mg ml^{-1} solution of each sample was injected.

Mass Spectrometry Analysis

Samples were analyzed in MALDI TOF/TOF Abi Sciex 4800 equipment, used MS reflector positive acquisition method in the mass range of 700–4200 *m/z*. The laser power was of 4200. The samples were desalted with C_{18} PolyLC tips using 0.1 % of TFA. For tip elution MeCN/H_2O (70:30) with 0.1 % of TFA were used. MALDI plate was loaded with extracts and the matrix. The matrix used was α-cyano-4-hydroxycinnamic acid (HCCA) in MeCN/H_2O (70:30) with 0.1 % of TFA.

Tricine-SDS-PAGE

Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was performed on discontinuous polyacrylamide gels (stacking gel: 1 %, separation gel: 16 %) under reducing conditions, following the Schägger methodology.[59] Small proteins and peptides bands were visualized by silver staining.[60] Concentrations of 10 mg ml^{-1} of extracts were tested. Low-range molecular weight between 1 to 80 kDa was used as molecular weight markers.

Bioautography Cholinesterase Assay

The bioautography assay was used as screening method of the inhibitory activity of the extracts.[61] Briefly, agar (1 % P/V) was dissolved at 80 °C in Tris-HCl buffer (50 mM, pH = 7.8). The solution was cool to 50 °C and AChE solution (60 U ml^{-1}) from *Electrophorus electricus* was added. The extracts were developed in a TLC plate (Merck; silica gel 60 F_{254}) of 50 cm^2 with n-butanol/acetic acid/water (55.6:22.2:22.2). TLC plate was sprayed with a mixture of α-naphthyl acetate (6.70 mM) and Fast Blue B (2.60 mM) solution. Then the plate was dried at room temperature, and AChE-agar solution was spread over the TLC plate. Once gelled, the plate was incubated at 37 °C for 20 min in the absence of light. To corroborate the efficiency of the bioautography, Caffeine solution was used as positive control.[62] The R_f values were calculated as $R_f = Dc/Ds$, Dc and Ds represent the distance traveled by compounds and distance moved by the solvent front in the TLC plate, respectively.

In vitro Cholinesterase Inhibition Assay

The inhibitory potential of the extracts against the cholinesterase enzymes was determined following Ellman's method with slight modifications. The principle of the method based on the measurement of the rate of thiocholine production by the hydrolysis of substrates (acetylthiocholine or butyrylthiocholine). This is accomplished by the continuous reaction of the thiol with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to produce the yellow anion of 5-thio-2-nitrobenzoic acid.[32] Briefly, increasing concentration (50, 100, 200, 400, and 800 μg ml^{-1}) of the samples were prepared in phosphate buffer. For AChE inhibitory assay, 50 μl of extract samples were added to a 96 wells microplate followed by 50 μl of AChE (0.25 U ml^{-1}) in phosphate buffer (pH 7.5). After 30 min of incubation at 25 °C, 100 μl of a solution of DTNB (0.2 mM) and acetylth-

iocholine iodide (ATCI) (0.24 mM) was added into each well. The absorbance of the colored product was measured at 405 nm using a Thermo Scientific FC.

The same procedures were applied for BChE inhibitory assay, with modification of the enzyme and substrate used, which were BChE from human serum and *S*-butyrylthiocholine iodide (BTCI; 7 mM), respectively. In two cases, galantamine was used as positive control. The percentage of inhibitions and IC_{50} were calculated. The IC_{50} were determined by plotting the percentage inhibition as a function of the concentration of tested compounds. Percentage of inhibition was calculated as $(1 - EAS/EAB) \times 100$, EAS and EAB represent the absorbance enzymatic activity of the sample and basal, respectively.

Kinetic Assays

The kinetic inhibition for AChE and BChE against the extracts was studied following Ellman's methods.[32] For this, 50 µl of extracts sample at IC_{50} concentrations were incubated in a 96 wells microplate with 50 µl of each enzyme in phosphate buffer (pH 7.5) at 25 °C for 30 min. After that, increasing concentrations of the respective substrates (0.03 to 0.94 mM for ATCI and 0.05 to 1.70 mM for BTCI) were added with DTNB solution (0.2 M). The kinetic was measured for 2.28 min with a total of 75 reads at 405 nm. The data analysis was carried out by GraphPad Prism 7 Software. The kinetic parameters (K_m and V_{max}) with and without inhibitors were determined by Michaelis-Menten and Lineweaver-Burk plots.

In vitro MAO-B Inhibition Assay

The inhibitory potential of the extracts against the MAO–B enzyme (from a mitochondrial fraction of rat brain) was determined by the method described by Soto-Otero et al., with slight modification.[62] Briefly, 20 µl of the extracts with increasing concentrations (50, 100, 200, 400, and 800 µg ml^{-1}) in phosphate buffer were mixed with 45 µl of MAO–B. After 30 min of incubation at 30 °C, 10 µl of Horse Radish Peroxidase (5 U ml^{-1}), 20 µl of a solution of benzylamine (20 mM) and 20 µl of a solution of *o*-dianisidine (5 mM) were added and incubated at 30 °C for 60 min. The absorbance of the colored product was measured at 405 nm using a Thermo Scientific FC.

DPPH Radical Scavenging Activity

The antioxidant activity was determined by DPPH radical scavenging assay according to the method of Memarpoor-Yazdi with minor modifications.[48] 0.4 ml of the extracts with increasing concentrations (100, 200, 400, and 800 µg ml^{-1}) were mixed with 1.2 ml of methanol and 0.4 ml of DPPH (0.15 mM in methanol). The mixture was incubated for 30 min in the dark. The absorbance of the resulting solution was measured at 517 nm. The control sample contained 1.6 ml of methanol and 0.4 ml of DPPH, and ascorbic acid was used as positive controls. Percentage of DPPH radical scavenging activity (RSA) and EC_{50} (effective concentration of compound that decreases the initial concentration of DPPH by 50%) were calculated. Percentage of DPPH RSA was calculated as $(1 - As/Ac) \times 100$, *As* and *Ac* represent the absorbance of the sample and control, respectively.

Cell Culture and MTT Assay

The Chinese hamster ovary K1 (CHO–K1) cellular line were purchased from ATCC and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} of penicillin and 100 g ml^{-1} of streptomycin at 37 °C in 5% CO_2 in a humidified atmosphere.

The cytotoxicity was determined by MTT assay. The cells were plated in 96 well microplates at a density of 2×10^4 cells per well. After 24 h, the cells were incubated with various concentrations of amphibian extracts (62, 130, 250, 500, 1000, and 2000 µg ml^{-1}) to evaluate the effects and calculate the viability values. 24 h later, the medium of each well was removed, and 10 µl of MTT (5 mg ml^{-1}) was added to each well, and the plates were newly incubated for 4 h at 37 °C. The formazan crystal was dissolved by adding 100 µl of dimethyl sulfoxide (DMSO) to each well.[63] Afterward, the absorbance was measured at 495 nm, and the viability percentage were calculated (viability percentage = (treated cells OD/untreated cells OD) × 100). Doxorubicin was used as positive control.

Hemolytic Activity (HA)

Human erythrocytes were isolated from heparinized blood by centrifugation (1000 g for 10 min) after washing three times with saline solution. Erythrocyte solutions were prepared to a concentration of 0.4% (v/v) in isotonic saline solution. Test tubes containing

200 µl of erythrocyte solution were incubated at 37 °C for 60 min with increasing concentrations of the extracts (62, 125, 250, 500, 1000, and 2000 µg ml^{-1}). After centrifugation at 1000 g for 5 min, the supernatant absorbance was measured at 405 nm. Lysis induced by 1 % Triton X-100 was taken as 100 %.[64]

Data Analysis

All experiments were assayed in triplicates. The statistical analysis was performed using the computing environment R.[65] A confidence interval of 95 % was considered. Furthermore, the package GRmetrics.[66] from the Bioconductor project was used[67] for the creation of the dose-response sigmoidal models and the calculations of the IC_{50} of the extracts. The plots were created with the package ggplot2.[68] Finally, statistically significant differences were studied by one-way analysis of variance (ANOVA) at $p \leq 0.05$.

Abbreviations

AD, Alzheimer's disease; ACh, acetylcholine; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; MAO, monoamine oxidase; Aβ, β-amyloid peptide; CAS, catalytic active site; PAS, peripheral anionic site; BCA, bicinchoninic acid assay; RP-HPLC, Reverse phase High Performance Liquid Chromatography; ACN, acetonitrile; TFA, trifluoroacetic acid; SDS-PAGE, sodium dodecyl sulphate polyacrylamide; TLC, thin layer chromatography; RSA, radical scavenging activity; IC_{50}, inhibitory concentration 50; EC_{50}, effective concentration 50.

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Author Contribution Statement

Roque Spinelli (Ph.D. student): performance of research work and bioassays and manuscript writing. Ivan Sanchis (Ph.D. student): collaboration in kinetics bioassays. Florencia María Aimaretti (biotechnologist): optimization and performance of MAO–B bioassays. Andres Maximiliano Attademo (Ph.D. biologist, member of the Universidad Nacional del Litoral Ethics Committee): collection, information, and identification of amphibian species. Prof. Madelon Portela: performance and analysis of proteomic assays by MALDI-TOF. Maria Verónica Húmpola (Ph.D.): performance and analysis of cytotoxicity bioassays. Georgina Guadalupe Tonarelli (Ph.D.): head of Departamento de Química Orgánica (FBCB-UNL). Alvaro Sebastián Siano (Ph.D.): head of the research group, Ph.D. advisor of Roque Spinelli, manuscript writing and supervision.

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