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Neuroprotective activity of isoquinoline alkaloids from of Chilean Amaryllidaceae plants against oxidative stress-induced cytotoxicity on human neuroblastoma SH-SY5Y cells and mouse hippocampal slice culture

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ABSTRACT

In this study we evaluate the chemical composition and neuroprotective effects of alkaloid fractions of the Amaryllidaceae species *Rhodophiala pratensis, Rhodolirium speciosum, Phycella australis* and *Phaedranassa lehmannii*. Gas chromatography-mass spectrometry (GC/MS) enable the identification of 41 known alkaloids. *Rhodolirium speciosum* and *Rhodophiala pratensis* were the most active extracts against acetylcholinesterase (AChE), with IC_{50} values of 35.22 and 38.13 μg/mL, respectively. The protective effect of these extracts on human neuroblastoma cells (SH-SY5Y) subjected to mitochondrial oxidative stress with rotenone/oligomycin A (R/O) and toxicity promoted by okadaic acid (OA) was evaluated. Only *Phycella australis* and *Rhodophiala pratensis* at 0.75 and 1.5 μg/mL, tend to reverse the cell death induced by R/O by around 12%. In OA assay, alkaloid fractions of *Phycella Australis* and *Phaedranassa lehmannii* displayed a concentration-dependent (0.375–3.0 μg/mL) effect with a maximum neuroprotective response of 78% and 84%, respectively. Afterwards, neuroprotective effects of *Phycella australis* (3 and 6 μg/mL) in mouse hippocampal slices stressed with oxygen glucose deprivation/reoxygenation (OGD/R), shown a protection greater than 14%. Finally, *Phycella Australis* (6 μg/mL) reverted the cell viability from 65% to 90% in slices treated with OA, representing a protection of 25% attributable to the alkaloids of this species.

1. Introduction

Alzheimer's disease (AD) is a chronic multifactorial progressive neurodegenerative disorder, characterized by irreversible deterioration of functions such as memory, language, and other cognitive functions (Kumar et al., 2015). Globally, the prevalence of AD was more than 35 million people in the year 2010, with projections of 65 million for the year 2030 and 115 million of people affected by the disease or related disorders for the year 2050 (Goure et al., 2014). Generally, the most accepted hypotheses driving AD are the cholinergic hypothesis, the so-called amyloid cascade and the aggregation of hyperphosphorylated tau oligomers inside neurons (X. Du et al., 2018a). Neuropathologically, AD is characterized by deposition of extracellular β-amyloid peptide (Aβ) aggregates (senile plaques) and abnormal intracellular deposits of the hyperphosphorylated tau protein, known as neurofibrillary tangles (NFTs), leading to neuronal and synaptic loss. However, none of these hypotheses have proved to be satisfactory to explain the pathology behind AD. Moreover, since a reductionist view of this disease has led to frequent therapeutic failures, constant revisions of these hypothesis have been necessary (Du et al., 2018b; Kametani and Hasegawa, 2018). For instance, mitochondrial dysfunction and mitochondrial cascades observed in AD-affected neurons have been suggested to explain the emergence of several symptoms of the disease. Moreover, there is increasing evidence that suggests that this phenomenon would even precede the aggregation of the Aβ peptide (Shardlow et al., 2018). However, some products of the processing of amyloid precursor protein

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(APP), as the C-terminal C99 segment appear associated with the portion of the endoplasmic reticulum that interacts with Mitochondria Associated Membranes (MAM), which regulates mitochondrial metabolism and perinuclear distribution of these organelles (Area-Gomez et al., 2018). An increase in MAM activity mediated by C-99 induces important bioenergetic changes that lead to neuronal dysfunction in AD. The brain is an organ particularly sensitive to oxidative stress and one of the consequences of mitochondrial dysfunction is the generation of free radicals, which in conjunction with an alteration in the homeostasis of metals such as copper, zinc and iron could overwhelm the cellular endogenous defenses aggravating the pathology (Huang et al., 2016). Among the drugs used for the treatment of AD, some acetylcholinesterase (AChE) inhibitors (AChEi), such as Tacrine (Cognex®), Donepezil (Aricept®) and Galanthamine (Razadyn®, Reminyl®), were the first to be approved by the Food and Drug Administration (FDA). Galanthamine, an isoquinoline alkaloid, is a natural product extracted from bulbs and flowers of *Galanthus, Narcissus* and *Lycoris* genera, which belong to Amaryllidaceae family (Torras-Claveria et al., 2013; Marco and do Carmo, 2006). The Amaryllidaceae family includes 75 genera and approximately 1100 species, widely distributed in tropical, subtropical and warm regions of the world, including South America, Mediterranean and Southern Africa. In previous reports about the chemistry of bulbs of *Rhodolirium andicola*, 13 alkaloids with AChEi activity have been identified by *in vitro* and *in silico* assays (Moraga-Nicolás et al., 2018). In five different species of *Rhodophiala,* 37 known alkaloids and 40 unknown compounds were identified using Gas chromatography-mass spectrometry (GC/MS). Also, in *vitro* enzymatic inhibition on acetylcholinesterase and butyryl cholinesterase (BuChE) was carried out (Tallini et al., 2018). This work also included the Colombian species *Phaedranassa lehmannii*, identifying 6 alkaloids by GC/MS. Additionally, the neuroprotective activity of *Phaedranassa lehmannii* alkaloids was assessed in a model of excitotoxicity induced by glutamate in rat cortical neurons (Cortes et al., 2018). Because of the importance of oxidative stress and mitochondrial dysfunction in neuronal death, in the present work we selected two classic models of cytotoxicity based on the production of reactive oxygen species (ROS). In the first one, cells are treated with a combination of rotenone/oligomycin A (R/O) in order to inhibit mitochondrial respiration blocking complex I and V (ATP synthase), respectively (Romero et al., 2010). In the second model, cerebral ischemia is reproduced using mice hippocampal slices subjected to oxygen and glucose deprivation/reoxygenation (OGD/R). Oxygen and glucose deprivation during cerebral ischemia triggers a cascade of events that includes the disruption of membrane potential due to the reduction in ATP production and mitochondrial membrane damage, which leads to a release of excitatory amino acids such as glutamate (Bonde et al., 2003). Increased intracellular calcium due to activation of glutamate receptors leads to mitochondrial function impairment via activation of protein kinase, phospholipase, protease, nitric oxide synthase and release of free radicals (Schulz et al., 1995). Excessive ROS production can induce an inflammatory response (Gloire et al., 2006). Oxidative stress results in macromolecular damage, reducing cell viability and is also implicated in various disease states. Nicotinamide adenine dinucleotide phosphate oxidase (NOX) is one of the main contributors to such excessive ROS production. Another contributor to ROS generation is nitric oxide (NO), generated by inducible NO synthetase (iNOS) (Egea et al., 2012; Buendia et al., 2015). On the other hand, okadaic acid (OA) was used as a selective and potent inhibitor of serine/threonine (Ser/Thr) phosphatase 1 and 2, promoting the hyperphosphorylation of the tau protein. The hyperphosphorylated tau protein is the main intraneuronal hallmark for the formation of neurofibrillary tangles, which are an integral component in the neuropathology of Alzheimer's disease. Experimental use of OA may have rapid metabolic consequences leading to cell death by alteration in phosphorylation-dephosphorylation rates *in vivo* of tau protein. Furthermore, it has been reported that the intracerebroventricular (i.c.v.) administration of OA in rats causes neurotoxicity associated with the intracellular increase in the levels of Ca^{2+}, oxidative stress and mitochondrial dysfunction in different areas of the brain (Kamat et al., 2013).

The knowledge of the chemical composition and pharmacology of the alkaloids present in Chilean Amaryllidaceae species belonging to the genera *Rhodophiala*, *Phycella* and *Rhodolirium* is limited. Regarding the latter, in the present work, we proposed to investigate the alkaloid composition of *Rhodophiala pratensis*, *Rhodolirium speciosum*, *Phycella australis* and *Phaedranassa lehmannii* using GC/MS. Furthermore, our study aimed to demonstrate the *in vitro* neuroprotective potential of these plants in Human Neuroblastoma cells (SH-SY5Y) subjected to mitochondrial oxidative stress with R/O. Also, in the same cell model we investigated the neuroprotection against OA-promoted tau hyperphosphorylation. Finally, the neuroprotective effect of the most promising specie was tested in hippocampal slices subjected to OA and OGD/R.

2. Material and methods

2.1. Plant material

The region of collections of Chilean species was VIII Bio-Bio and XVI Ñuble between September–October 2017. *Rhodophiala pratensis* was collected in Hualpén, in the Campus of the University of Concepción, 60 m a.s.l., (36° 47 ′ S/73° 10 ′ W), Baeza 4340 (CONC). *Rhodolirium speciosum* was collected in Coronel, road to Santa Juana, 49 m a.s.l., (37° 10 ′ S/72° 58 ′ W), C. Baeza 4350 (CONC). *Phycella australis* was collected in the province of Itata, Cobquecura, 5 m a.s.l., (36° 05 ′ S/72° 08 ′ W). J. Alarcón S.N. (CONC). Colombian specie *Phaedranassa lehmannii* was collected in the Department of Cauca, municipality of Rosas in July 2016, 1900 m a.s.l., (2° 15 ′ S/76° 36 ′ W), Risers 5106. Voucher species are found in the herbarium of the University of Concepción and the University of Antioquia.

2.2. Alkaloids extraction

Bulbs of each species were washed with tap water, cut into approximately 3 cm pieces and lyophilized. The lyophilized plant material (500 g) was extracted with methanol until the material was exhausted, evaporating the solvent under vacuum. The residue was dissolved in 10 mL of 2% H_2SO_4, removing the neutral compounds with ethyl ether (3 × 10 mL) by adjusting the pH of the aqueous fraction in a range of 9.5–10.0 with 25% ammonium hydroxide. The alkaloids were extracted from the aqueous fraction with chloroform (3 × 50 mL). Organic layers were gathered and evaporated at reduced pressure, obtaining the fraction of alkaloids (FA).

2.3. Analysis by gas chromatography-mass spectrometry (GC/MS)

Analysis by GC/MS was carried out in an Agilent 7890 a GC (Agilent, Palo Alto, CA) with multimodal injector, Agilent triple Quad 7000 GC/MS detector (scan analysis by a quadrupole). A HP-5 MS Capillary column (30m × 0.250 mm x 0.25 μm, Agilent J&W, Palo Alto, CA) was used. The temperature used was: 100–180 °C at 15 °C/minute, 180–300 °C at 5 °C/minute and 10 min hot at 300 °C. The injection was carried out at 250 °C. The flow of the carrier gas (Helium) was 0.8 mL/min. The volume of injection was 1 μL. Kovats retention indexes (RI) of the compounds were recorded with a standard calibration n-hydrocarbon mixture (C8–C32). The percentage of the total ion current (TIC) for each compound is given in Table 1. The abundance of each compound was calculated using codeine as internal standard. The compounds were identified by comparing their mass spectral fragmentation with standard reference spectral using mass library (NIST 2.0). Also, alkaloid identification was performed considering the fragmentation pattern and characteristic molecular ions founded in the Amaryllidaceae family plants. Moreover, data obtained from the literature

Table 1
GC-MS experimental data, retention indexes (RI), M^+ and main fragments m/z (relative intensity) for alkaloids identified in bulbs of four species of Amarillidaceae.

Peak No	Compound	RI	M^+	m/z (relative intensity %)	Alkaloids identified in bulbs							
					R. pratensis		*R. speciosum*		*P. australis*		*P. lehmannii*	
					% relative[d]	μg/mg	% relative	μg/mg	% relative	μg/mg	% relative	μg/mg
	Lycorine type				**17,052**	**122,648**	**12,150**	**247,975**	**16,281**	**223,851**	**87,322**	**288,444**
1	11-12-dehydrolycorene	2359	253 (50)	252(100), 224(13), 166(12), 152(8), 139(11)	0,113	0812	0,180	3674	nd	nd	nd	nd
2	11-12-dehydrolycorene Derivative	2377	253 (51)	252(100), 224(16), 166(16), 152(13), 139(15)	0,057	0411	nd	nd	nd	nd	nd	nd
3	Anhydrolycorine	2495	251 (43)	250(100), 220(2), 192(14), 191(13), 165(4), 124(19)	1259	9055	2190	44,704	5396	74,196	2869	9476
4	O-Acetylpluviine	2555	329 (40)	328(−), 286(−), 268(64), 254(15), 242(100), 228(8), 198(−), 182(8), 151(11)	nd	Nd	nd	nd	nd	nd	0,086	0285
5	Caranine	2583	271 (41)	270(100), 252(46), 227(14), 226(20), 194(7), 154(8)	nd	Nd	0,024	0482	nd	nd	nd	nd
6	11,12-Didehydroanhydrolycorine	2602	249 (61)	248(100), 190(29), 163(9), 123(18), 95(49)	0,392	2820	0,115	2344	0,312	4291	nd	nd
7	Sternbergine	2731	331 (15)	270(18), 252(8), 229(72), 228(100)	nd	Nd	1114	22,737	nd	nd	nd	nd
8	Lycorine	2757	287 (16)	268(14), 250(8), 227(68), 226(100), 211(5), 147(15)	4013	28,867	6433	131,290	8367	115,036	32,684	107,964
9	Dihydrolycorine	2792	289 (36)	288(100), 272(7), 254(4), 214(6), 200(2), 187(6), 162(7), 147(16)	10,858	78,097	1407	28,713	0,772	10,618	nd	nd
10	Pseudolycorine derivative (1)	2806	331 (15)	289(−), 270(16), 252(7), 242(7), 229(69), 228(100), 147(5), 111(7)	nd	Nd	nd	nd	nd	nd	20,090	66,361
11	Pseudolycorine derivative (2)	2823	289 (1)	270(1), 229(5), 228(7), 147(2), 111(2), 109(100)	nd	Nd	nd	nd	1019	14,009	nd	nd
12	Pseudolycorine	2831	289 (17)	270 (13), 229(72), 228(100), 147(13), 111(19)	nd	nd	nd	nd	nd	nd	31,593	104,358
13	Assoanine	2837	267 (52)	266(100), 250(25), 222(17), 193(14), 180(23)	nd	nd	nd	nd	0,415	5701	nd	nd
14	2-O-Acetyllycorine	2844	329 (17)	328(14), 270(42), 269(68), 268(100), 252(43), 250(68), 227(44), 226(94)	nd	nd	0,687	14,031	nd	nd	nd	nd
15	Acetyllycorine derivative	2893	331 (45)	330(100), 270(65), 149(39)	0,360	2586	nd	nd	nd	nd	nd	nd
	Haemanthamine type				**24,305**	**174,816**	**0,077**	**1574**	**34,360**	**472,424**	**8148**	**26,915**
16	Vittatine	2466	271 (82)	272(13), 252(8), 199(100), 187(91), 173(32), 115(42)	0,751	5399	0,018	0374	2439	33,533	0,211	0699
17	8-O-Demethylmaritidine	2505	273 (100)	230(5), 202(29), 201(74), 189(10), 175(12), 174(31), 129(13), 128(21), 115(46), 56(13)	0,684	4921	0,056	1139	nd	nd	2239	7395
18	Maritidine	2510	287 (57)	270(8), 268(5), 258(7), 244(28), 215(100), 203(56), 196(7), 167(5), 128(25), 115(28), 91(14), 77(15)	0,250	1799	nd	nd	0,452	6217	nd	nd
19	Deacetylcantabricine	2539	275 (100)	276(19), 274(26), 258(24), 250(80), 246(27), 204(88), 203(67), 202(51), 188(27), 187(54),	nd	nd	0,002	0032	nd	nd	0,715	2362
20	Crinane-3-one	2579	271 (64)	270(28), 240(11), 238(18), 226(14), 211(38), 181(100), 153(33), 152(35), 115(22)	nd	nd	nd	nd	0,083	1141	nd	nd
21	Aulicine	2612	304 (18)	288(39), 246(25), 233(100), 218(34), 206(48), 163(17)	nd	nd	0,001	0029	nd	nd	nd	nd
22	Haemanthamine isomer	2637	301 (70)	272(100), 257(39), 240(25), 225(31), 211(31), 181(47), 153(29)	nd	nd	nd	nd	5078	69,816	nd	nd
23	Haemanthamine	2637	301 (11)	272(100), 257(11), 240(20), 225(9), 211(22), 181(44), 153(18)	15,650	112,563	nd	nd	22,363	307,467	nd	nd
24	Hamayne	2728	287 (5)	258(100), 242(9), 212(11), 211(16), 186(22), 181(23), 153(12), 128(21)	6962	50,077	nd	nd	1979	27,216	4983	16,459
25	6-hydroxyhaemanthamine	2732	317 (35)	284(48), 258(100), 233(67), 211(47), 201(89), 199(79), 181(71), 173(66), 115(89), 56(44)	0,008	0057	nd	nd	1966	27,034	nd	nd
	Galanthamine type				**0,273**	**1961**	**0,095**	**1942**	**0,046**	**0626**	**4530**	**14,964**
26	Galanthamine	2401	287 (77)	286(100), 244(30), 230(17), 216(53), 174(59), 128(18), 115(36)	nd	nd	0,002	0050	nd	nd	0,433	1430
27	Lycoramine	2417	289 (50)	288(100), 232(14), 202(23), 187(18), 159(12), 115(40)	nd	nd	nd	nd	0,046	0626	nd	nd
28	Sanguinine	2420	273 (100)	272(78), 256(21), 212 (18), 202(61), 160 (95)	nd	nd	nd	nd	nd	nd	4023	13,290
29	Norlycoramine	2459	275 (57)	274(100), 202(10), 188(12), 178(5)	0,273	1961	0,093	1892	nd	nd	nd	nd
30	Narwedine	2475	285 (66)	284(100), 216(36), 199(32), 174(67)	nd	nd	nd	nd	nd	nd	0,074	0244
	Homolycorine type				**41,310**	**297,133**			**39,424**	**542,044**		
31	O-Methyllycorenine	2529	331 (1)	300(−), 191(< 1), 109(100), 110(8), 108(11), 94(2), 82(2), 42(1)	41,310	297,133	nd	nd	39,424	542,044	nd	nd
	Montanine type				**1395**	**10,032**			**0,329**	**4525**		
32	Montanine	2621	301 (97)	270(100), 257(45), 252(28), 223(41), 185(57), 115(50)	1072	7709	nd	nd	nd	nd	nd	nd

(*continued on next page*)

Table 1 (*continued*)

Peak No	Compound	RI	M^+	m/z (relative intensity %)	Alkaloids identified in bulbs							
					R. pratensis		*R. speciosum*		*P. australis*		*P. lehmannii*	
					% relative[d]	μg/mg	% relative	μg/mg	% relative	μg/mg	% relative	μg/mg
33	Pancracine	2689	287 (100)	270(25), 243(34), 223(33), 199(51), 185(67), 115(51)	0,323	2323	nd	nd	0,329	4525	nd	nd
	Narciclasine type				**1031**	**7412**	**0,036**	**0738**	**0,559**	**7677**		
34	Trisphaeridine	2279	223 (100)	222(38), 167(10), 165(11), 164(16), 138(30), 111(37)	1031	7412	0,036	0738	0,414	5688	nd	nd
35	5,6-Dihydrobicolorine	2326	239 (43)	238(100), 180(15), 139(9)	nd	nd	nd	nd	0,145	1989	nd	nd
	Tazettine type				**6482**	**46,619**			**6257**	**86,033**		
36	6-O-Methylpretazettine	2608	345 (17)	344(19), 330(12), 261(100), 239(24), 230(14), 201(22)	1116	8026	nd	nd	nd	nd	nd	nd
37	Tazettine	2659	331 (12)	316(7), 298(12), 247(100), 227(11), 211(12), 201(20), 181(17), 152(13), 115(23)	5243	37,710	nd	nd	5868	80,679	nd	nd
38	3-Epimacronine	2812	329 (11)	314(12), 245(100), 244(24), 201(78), 70(29)	0,123	0883	nd	nd	0,389	5354	nd	nd
	Miscellaneous				**1868**	**13,435**			**1372**	**18,868**		
39	Ismine	2274	257 (30)	238(100), 225(6), 211(7), 196(10), 180(9), 168(9), 154(5)	0,483	3473	nd	nd	0,085	1172	nd	nd
40	Demethylmesembrenol	2308	275 (2)	206(6), 205(45), 115(3), 70 (100)	nd	nd	nd	nd	1287	17,696	nd	nd
41	Galanthindole	2501	281 (100)	264(14), 263(18), 262(22), 252(16), 204(12), 191(21), 132(27), 107(27)	1385	9962	nd	nd	nd	nd	nd	nd
	Not identified				**6278**	**45,156**	**87,643**	**1788,693**	**1373**	**18,873**		
42	Not identified (1)	2396	281 (< 1)	259(2), 250(2), 225(2), 157(1), 147(18), 129(100), 112(23), 83(17), 70(30), 57(41)	0,577	4151	0,174	3542	nd	nd	nd	nd
43	Not identified (2)	2398	259 (45)	258 (65), 226 (53), 201(32), 200(100)	nd	nd	nd	nd	0,014	0198	nd	nd
44	Nerinine type alkaloid (1)	2477	281 (1)	271(3), 254(2), 238(1), 207(1), 128(2), 115(2), 109(100), 108(14)	5032	36,192	0,850	17,348	0,407	5591	nd	nd
45	Not identified (3)	2515	315 (42)	316(8), 270(3), 254(71), 250(23), 229(44), 228(100), 222(9), 124(6)	nd	nd	0,030	0603	nd	nd	nd	nd
46	Not identified (4)	2539	313 (9)	312(20), 289(21), 267(100), 266 (59), 252(37), 250(39), 238(25), 223(15), 202(16), 192(11), 185(39)	nd	nd	0,047	0958	nd	nd	nd	nd
47	Not identified (5)	2595	345 (38)	344(100), 319(7), 252 (7), 248(5), 226(10), 161(11), 147(5), 129(10), 115(9)	nd	nd	0,665	13,563	nd	nd	nd	nd
48	Homolycorine type alkaloid (1)	2626	345 (3)	256(52), 226(3), 207(3), 191(7), 175(3), 168(2), 151(2), 139(100), 124(41), 94(6)	nd	nd	0,203	4134	nd	nd	nd	nd
49	Nerinine type alkaloid (2)	2770	344 (2)	281(3), 250(2), 226(4), 207(7), 191(2), 155(2), 141(2), 127(3), 109(100)	0,669	4813	nd	nd	0,783	10,763	nd	nd
50	Homolycorine type alkaloid (2)	2851	286 (< 1)	281(1), 270(10), 221(1), 207(1), 178(1), 147(1), 139(100), 124(61)	nd	nd	0,303	6175	nd	nd	nd	nd
51	Homolycorine type alkaloid (3)	2903	331 (< 1)	330(1), 270(1), 254(< 1), 226(< 1), 174(< 1), 162 (1), 125(100), 96(40)	nd	nd	50,114	1022,788	nd	nd	nd	nd
52	Lycorine type alkaloid (1)	2905	357 (28)	356(63), 270(100), 254(43), 227(30), 147(15), 69(72)	nd	nd	nd	nd	0,084	1159	nd	nd
53	Not identified (6)	2948	388 (75)	284(32), 254(100), 226(18), 207(38), 147(24), 135(22)	nd	nd	nd	nd	0,068	0929	nd	nd
54	Homolycorine type alkaloid (4)	2977	206 (< 1)	178(1), 125(100), 115(1), 108(1), 96(37), 82 (3)	nd	nd	28,219	575,937	nd	nd	nd	nd
55	Lycorine type alkaloid (2)	3011	375 (46)	374(100), 330(5), 270(67), 254(67), 226(18), 214(8), 207(12), 177(10), 147 (25)	nd	nd	nd	nd	0,017	0233	nd	nd
56	Homolycorine type alkaloid (5)	3042	281 (6)	270(2), 253(3), 207(18), 177(3), 156(2), 139(6), 125(100), 108(1), 96(40), 73(7)	nd	nd	7038	143,645	nd	nd	nd	nd

[a]Peak number of signals in the chromatograms.
[b]IR: Kovats retention index.
[c]Quantitative values obtained by response factor of codeine internal standard (μg alkaloid per mg of alkaloidal fraction).[d] Percentages of relative peak area of compounds in the samples analyzed. nd: not detected. Values less than 0.01% are described as "traces" (tr).

were used for the identification of the compounds. The area of the GC/MS peaks depends not only on the concentration of the related compounds but also on the intensity of their mass spectral fragmentation. Therefore, values were expressed as a percentage of TIC.

2.4. Acethylcholinesterase (AChE) inhibition assay

The inhibitory activity upon AChE was determined spectrophotometrically in 96-well microplates by Ellman's method with slight modifications (Ellman et al., 1961; Alarcón et al., 2015). The buffer used was prepared as follows: Buffer A, 50 mM Tris-HCl, pH 8.0; Buffer B, 50 mM Tris-HCl, pH 8.0, containing 0.1% of bovine serum albumin in buffer A; and buffer C, 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and 0.02 M $MgCl_2$ x $6H_2O$ in buffer A. The fraction of alkaloids of each of the species under study was prepared in the range of concentrations from 15,625 μg/mL to 1000 μg/mL in dimethyl sulfoxide (DMSO) with serial dilutions in buffer A, adding 25 μL of the solution to the 96 wells plate. Successively, iodide of Acetylcholine iodide (ATCI) (25 μL, 15 mM) in distilled water, 5.5-dithiobis-2-nitrobenzoic acid (DTNB) in buffer C (125 μL, 3 mM) and buffer C (50 μL) was added sequentially. The absorbance was determined at 405 nm, using a microplate reader with readings every 45 s (three lectures). *Electrophorus electricus* acetylcholinesterase solution (25 μL, 0.2 U/mL) was added, again determining the absorbance every 45 s (30 min). Galanthamine hydrobromide was used as a positive control. The percentage of inhibition was calculated using the equation: inhibition (%) = ((1-(A_{sample}/$A_{Control}$)) x 100) where A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the blank (buffer A). Nonlinear regression parameters were plotted for each curve and the IC_{50} values were obtained using the GraphPad Prism 5.0 software.

2.5. Cell culture and treatments

The human neuroblastoma cell line SH-SY5Y (ATCC® CRL-2266™) was used between passages 4–13. The cells were maintained in a 1:1 ratio of a nutrient mixture F-12 (HAM12) (Sigma-Aldrich, Madrid, Spain) and minimum essential medium (MEM) supplemented with 15 non-essential amino acids, 1 mM sodium pyruvate, 10% inactivated fetal bovine serum (FBS), penicillin 100 U/mL, and streptomycin 100 M g/mL (Reagents of Invitrogen, Madrid, Spain) in T250 culture flasks and preserved at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For the assays, cells were subculture in 96-well plates (TPP, Zellkutur and Labortechnologie, Trasadingen, Switzerland). In each well, cells were cultured at a density of 50,000 cells/well. The concentrated solutions (5 mg/500 mL) of the alkaloid fraction of the four species studied and galanthamine (as control drug) were prepared in DMSO. The control group was incubated in 0.1% DMSO, maintaining the same concentrations of DMSO for all samples studied. The different concentrations of the alkaloid and galanthamine fractions were incubated 24 h before adding toxic stimuli (rotenone/oligomycin A, okadaic acid). The cells were co-incubated for an additional 24 h with extracts and galanthamine in the presence of toxics. When the drug and cytotoxic treatments were performed, MEM medium was used with 1% FBS.

2.6. MTT cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltriazoliumbromide assay (MTT) described previously by Mosmann (1983) was used for the determination of cell viability. This assay is based on the metabolic reduction of MTT, performed by the mitochondrial enzyme succinate-dehydrogenase, into a blue colored compound (formazan) determined by spectrophotometry. Galanthamine and the fraction of alkaloids were dissolved in DMSO, subsequently the solutions were diluted in culture medium giving six concentrations (6.0; 3.0; 1.5; 0.75; 0.375 mg/mL). Plates were incubated at 37 °C with 5% CO_2 for 24 h. After incubation time, 10 μL of MTT (5 mg/mL) were added and again incubated at 37 °C for 2 h. The formazan crystals formed after incubation were dissolved using 200 μL DMSO and the absorbance was determined at 540 nm in microplate reader. All assays were performed in triplicates of six different cell batches.

2.7. Neuroprotection assay against cell death induced by R/O and OA

Once the cells reach 75–80% confluence, they were pre-incubated with the alkaloids for 24 h. Subsequently, cells were treated with R/O (10 μM/3.3 μM) for 24 h. For OA-induced toxicity, cells (75–80% confluence), were pre-treated with the alkaloids for 24 h. Subsequently, 20 nM AO was added for 24 h. At the end of the experiments with R/O and OA stimuli, cell viability was determined by MTT assay (section 2.6.).

2.8. Animal care and treatments

Two to three old C57BL/6J mice were retained under temperature and controlled lighting conditions. The water and food were provided *ad libitum*. The animals used in the experiments were treated making the greatest effort to minimize the suffering of the animal, in addition to using the smallest number of animals possible. The protocol for the Care and use of laboratory animals was followed, considering that initially all the experiments were pre-approved by the Ethics Committee for the Care and use of research animals of the Autonomous University of Madrid with the Spanish Royal Decree of 1 February 2013 (53/2013) and in accordance with the European Union Directive of 22 September 2010 (2010/63/UE).

2.9. Hippocampal slices preparation

The experiments were carried out in hippocampal slices of 2–3 months old C57BL/6J mice of the animal colony of the bioterium of the Autonomous University of Madrid. The mice were quickly decapitated under anesthesia with intraperitoneal sodium pentobarbital (60 mg/kg). Forebrains were rapidly removed and placed in an ice-cooled Krebs bicarbonate dissection buffer solution (pH 7.4), containing: 120 mM NaCl, 2 mM KCl, 0.5 mm $CaCl_2$, 26 mM $NaHCO_3$, 10 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11 mM glucose and 200 mM sucrose. The hippocampi were quickly dissected and cut into transverse slices of 300 μm thick using a Mcllwain tissue Chopper. After this, they were introduced in a bath at 34 °C with continuous bubbling of a mixture 95% O_2/5% CO_2 for a stabilization period of 45 min.

2.10. Oxygen-glucose-deprivation/reoxygenation (OGD/R) model

For OGD/R experiments, after the initial pre-incubation period (30 min), the hippocampal slices corresponding to the control group were incubated for 15 min in a Krebs solution with the following composition: 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4 and glucose 11 mM (pH 7.2) balanced with 95% O_2/5% CO_2. OGD condition was induced by incubation of the slices in a glucose-free Krebs solution, balanced with a gas mixture of 95% N_2 and 5% CO_2. Glucose was replaced by 2-deoxyglucose. After the OGD period, the slices were returned to a normal Krebs oxygenated solution containing glucose (reoxygenation period) for 120 min. During the OGD and the period of reoxygenation, the selective agonist of α7 nicotinic receptors for acetylcholine PNU-282,987 (2.6 μg/mL) was used as control drug. In parallel, crude alkaloid extract of *Phycella australis* was added to the slices at different concentrations (3 and 6 μg/mL). The experiments were performed at 37 °C. Control experiments and OGD/R were performed using five slices of the same animal for each experimental group.

2.11. In vitro effect of OA on hippocampal slices

After an initial pre-incubation period (30 min), 1 μM of OA, 6 μg/mL was added to the mouse hippocampal slices. The extract of the crude of alkaloids of *Phycella australis* (3 and 6 mg/mL), 10 μM of melatonin (as positive control) and the Control group, were incubated for 360 min in a solution containing 50% of culture medium MEM enriched with pyruvate and glutamine, and 50% of a Krebs solution with the following composition: 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4 and 11 mM glucose (pH 7.2) balanced with 95% O_2/5% CO_2. The experiments were performed at 37 °C. Five slices of the same animal were used for each experimental group.

2.12. MTT assay in hippocampal slices

Cell viability in hippocampal slices was determined by the ability of cells to reduce MTT as previously described (section 2.6) with slight modifications. Hippocampal slices were collected immediately after treatment and incubated with MTT (0.5 mg/mL) in Krebs bicarbonate solution for 30 min at 37 °C. Metabolic reduction of MTT is performed by the mitochondrial enzyme succinate-dehydrogenase generating a precipitate formazan. After incubation period, the formazan produced in the hippocampal slices was solubilized with the addition of 200 μL of DMSO. The slices were removed before reading the optical density in a microplate ELISA reader at 540 nm. The absorbance values obtained in the control slices were taken as 100% of viability.

2.13. Statistical analysis

Results of the *in vitro* experiments are presented as the mean ± standard error of the means (SEM) of three independent experiments, carried out in triplicate. The differences between the groups were determined by the application of a one-way ANOVA, followed by a post hoc analysis of Newman-Keuls, using the software GraphPad Prism version 5.0 (GraphPad software, La Jolla, CA, USA). Statistical significance level was considered with $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$. A Principal component multivariate (PCA) analysis was performed to determine the significance of the variability of factors in the results of the matrix, considering the following variables: species (*Rhodophiala pratensis, Rhodolirium speciosum, Phycella australis and Phaedranassa lehmannii*), chemical composition (alkaloids) and bioactivity (neuroprotection and inhibition of acetylcholinesterase). The data were processed by a computational analysis using a correlation matrix with the Info-Stat software.

3. Results and discussion

3.1. Analysis of alkaloid extracts by CG/MS

The alkaloids present in plants of the family Amaryllidaceae derive from phenylalanine and tyrosine amino acids via the precursor *O*-metilnorbelladine. Three types of Amaryllidaceae alkaloid skeletons are biosynthesized alternately by oxidative phenol coupling routes, being the basis in the formation of the diversity of alkaloids in Amaryllidaceae (Kilgore and Kutchan., 2016). Accordingly, Amaryllidaceae alkaloids have been classified into nine structural derivatives: norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, tazettine, montanine and galanthamine (Ghosal et al., 1985). In the present work, the alkaloid fraction obtained from bulbs of *Rhodophiala pratensis, Phycella australis, Rhodolirium speciosum* and *Phaedranassa lehmannii*, were analyzed by GC/MS. Overall, this analysis allowed the detection of 56 alkaloids, among them 41 correspond to known alkaloids, whereas 15 remain unidentified (Table 1). Lycorin and homolycorin-type alkaloids were the most ubiquitous compounds found in the four species analyzed in our study. These molecules derived from the phenol oxidative coupling *ortho-para* leading to the precursor *O*-metilnorbelladine. Relative percentage of lycorine and homolycorine-type alkaloids in *Rhodophiala pratensis* is represented by 17.05% and 41.31% while in *Phycella australis* corresponds to a16.28% and 39.42%, respectively (Table 1). In *Rhodolirium speciosum* the content of lycorine-type alkaloids reached 12.15%, while in *Phaedranassa lehmannii* the content reached up to 87.32%, being the plant with higher content of these class of compounds. The haemantamine-type alkaloids present in *Rhodophiala pratensis, Phycella australis, Rhodolirium speciosum* and *Phaedranassa lehmannii*, come from a secondary *para-para* cyclization (Berkov et al., 2010), representing 24.30%, 34.36%, 0.08% and 8.15% of the total content of alkaloids, respectively. Besides, the haemantamine-derived alkaloids montanine, tazettine and narciclasine were detected in traces. The highest content of haemantamine-type alkaloids is present in *Phycella australis* (34.36%), being haemantamine the main compound (Table 1).

Recently, a work carried out in the genus *Rhodophiala*, reported the presence of 37 known alkaloids and 40 unidentified peaks. This work was focalized in species collected in different geographic points of Chile (Tallini et al., 2018). In this study was reported that lycorine and haemantamine-type alkaloids predominate in the species of *Rhodophiala* (70%), thus confirming the results found in our work for *Rhodophiala pratensis*. However, it must be said that in the study of Tallini and coworkers, samples of *Rhodophiala* were collected in Arcos de Calan (Maule), Nevado de Chillán, Las Trancas, Malalcahuello, Lonquimay Volcano, and Sierra Nevada, which are zones exclusively located in the Andes Mountains. On the other hand, Lizama-Bizama and collaborators (2018), analyzed samples of different species of *Rhodophiala* collected in Atacama (Huasco), Antuco (Bio-Bio) and Colmuyao Beach (Bio-Bio). They reported the presence of alkaloids derived from crinine, galanthamine, homolycorine, tazettine and montanine. Among them, a predominance of crinine and montanine derivatives was observed, although the elucidation of the specific structure for such alkaloids was not included. The samples analyzed in our study were obtained from VIII and XVI regions in coastal areas at the same time of year. The structural type of main alkaloids coincides in both studies. The main difference observed is given by the presence of two alkaloids derived from haemantamine: maritidine and 6-hydroxyhaemantamine, which have not been reported in Chilean *Rhodophiala* so far.

On the other hand, Chilean plants of the genus *Rhodolirium* have been studied recently, determining the presence of 13 alkaloids typically reported in the Amaryllidaceae family (Moraga-Nicolás et al., 2018). In this latter, samples were collected in the Conguillio National Park (Andean Mountains), in December 2018. Regarding our study, the presence of galanthamine is common in all samples. However, we note that in *Rhodolirium speciosum* samples collected in Santa Juana, predominates those alkaloids derived from lycorine. We successfully identified 15 alkaloids of a total of 25; however, due to its low concentrations, 10 of them remain unidentified. In the work of Moraga-Nicolás, galanthamine, tazettine and crinine-type alkaloids predominate (Moraga-Nicolás et al., 2018). On the other hand, studies carried out with the Colombian plant *Phaedranassa lehmannii*, report the presence of 6 alkaloids, among them sanguinine and 8-*O*-demethylmaritidine (Cortes et al., 2018) stand out. In our study, the alkaloid fraction of *Phaedranassa lehmannii* has 12 compounds, predominating the presence of lycorin, pseudolycorine, hamayne and sanguinine.

3.2. Acethylcholinesterase inhibition assay

In Table 2, the IC_{50} values for the different plants studied in the present work are shown. *Rhodolirium speciosum* and *Rhodophiala pratensis* were the most potent AChE inhibitors displaying the lowest IC_{50} values of 35.22 and 38.13 μg/mL, respectively. Indeed, the potent *in vitro* inhibitory activity upon AChE enzyme reported recently in *Rhodolirium andicola* by Moraga-Nicolás and collaborators (2018), is related

Table 2
Plant origin and inhibitory activity against Acetylcholinesterase.

Species	Locality	Voucher number	Detected alkaloids	IC_{50} AchE (μg/mL) ± S.E.M.
Rhodophiala pratensis	VIII región del Bio-Bío (Chile)	C. Baeza 4340 (CONC)	27	38.13 ± 0.95
Rhodolirium speciosum	VIII región del Bio-Bío (Chile)	C. Baeza 4350 (CONC)	25	35.22 ± 0.86
Phycella australis	XVI región de Ñuble (Chile)	J. Alarcón s.n. (CONC)	27	80.12 ± 1.03
Phaedranassa lehmannii	Departamento del Cauca (Colombia)	Alzate 5106	12	67.73 ± 0.52

to the presence of galanthamine, lycoramine, 6-α-deoxy-tazettine and norpluvine diacetate. These compounds also showed a great *in silico* affinity for the *Electrophorus electricus* AChE (1C2B), which was demonstrated by molecular docking inside the catalytic site of its crystalline structure. As shown in Table 1, these alkaloids are also abundant in *Rhodolirium speciosum and Rhodophiala pratensis,* which could explain their greatest AChE inhibitory activities. In species of the genus *Phaedranassa*, as is the case of *Phaedranassa dubia,* seven known alkaloids and a new compound (phaedranamine) have been identified. These authors also reported *in vitro* antiprotozoal activity for such extracts (Osorio et al., 2010). In the present study, *Phycella australis* has an IC_{50} of 80.12 μg/mL and *Phaedranassa lehmannii* an IC_{50} of 67.73, suggesting that alkaloids contained in these last species have a lower affinity for the enzyme, most likely because the crude extract has a variety of undefined compounds that could affect the interaction (competitively or uncompetitive) of the alkaloids with the active site of the enzyme. Galanthamine presents the lowest IC_{50} value (1.08 ± 0.05 μg/mL), confirming the specific chemical affinity of this compound with the active site of the enzyme. Indeed, the high binding affinity of galanthamine for AChE is related with the hydroxyl group at C-3 present in its structure (Bores et al., 1996). Importantly, galanthamine is 53-fold more selective for human AChE than butyrylcholinesterase (BChE). Harvey (1995) reported that the IC_{50} of galantamine for AChE and BChE are 0.35 and 18.6 mM, respectively.

3.3. Cytotoxicity for Chilean Amaryllidaceae alkaloid fractions upon SH-SY5Y cells

We first determined the cytotoxicity *per se* of the alkaloid fractions under study in SH-SY5Y cells. When we tested concentrations higher than 6.0 μg/mL, the alkaloids present in the samples exerted some toxicity (> 20%), which could generate interference in the further neuroprotection experiments. However, it was observed that at concentrations between 0.375 and 3.0 μg/mL, toxicity was less than 20% for all samples (Fig. 1); therefore, this range of concentration was chosen to perform the neuroprotection experiments. Galanthamine did not presented toxicity at the assayed concentration.

3.4. Neuroprotection assay against cell death induced by (R/O)

R/O produced mitochondrial disruption blocking the mitochondrial complex I and V of the respiratory chain, respectively (Romero et al., 2010; Hoglinger et al., 2005). In Fig. 2 it is shown that *Phycella australis* and *Rhodophiala pratensis* achieve cell viabilities of 85% and 83% at concentrations of 0.75 and 1.5 μg/mL, respectively. These results show that, with respect to R/O (73% viability), these alkaloid fractions tend to reverse the cell death induced by R/O by around 12%. Moreover, some extracts even decreased cell viability. Taken together, none of the alkaloids tested nor galantamine, provided significant neuroprotection in the R/O toxicity model. The synergistic effect between galanthamine and melatonin was previously reported in SH-SY5Y cells where toxicity was induced by R/O (30 μM/10 μM) (Romero et al., 2010). In this study, the authors reported that the protective effects of galantamine (10–300 nM) and melatonin (0.3–10 nM) were concentration-dependent when assessed individually. The maximum protection of galanthamine (56%) and melatonin (50%) was observed at concentrations of 300 nM and 10 nM, respectively. The combination of galanthamine (30 nM) and melatonin (0.3 nM) resulted in a similar protection found for these compounds assessed individually. In previous studies, cytotoxicity and neuroprotection assays in SH-SY5Y cells have been performed for different species of Amaryllidaceae (Castillo et al., 2018; Li et al., 2013). In such studies, oxidative stress was induced in SH-SY5Y cells using cobalt chloride ($CoCl_2$), hydrogen peroxide (H_2O_2) and β-amyloid peptide ($A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}42}$). In this latter study, they found that *Caliphruria subedentata* extract decreased cell death, DNA damage and modulation of alterations in the morphology of mitochondria induced by $A\beta_{1\text{-}42}$. This study was the first reporting the anti-genotoxic activity of a member of this family of plants (Castillo et al., 2018). However, in this report, the contribution of other non-alkaloid compounds of the extract was not considered, therefore the effect cannot be attributed exclusively to such molecules. On the contrary, in the present study the tested extracts correspond to an alkaloid fraction where the concentration of interfering compounds is low. Therefore, the comparison of results between both studies can only be done partially.

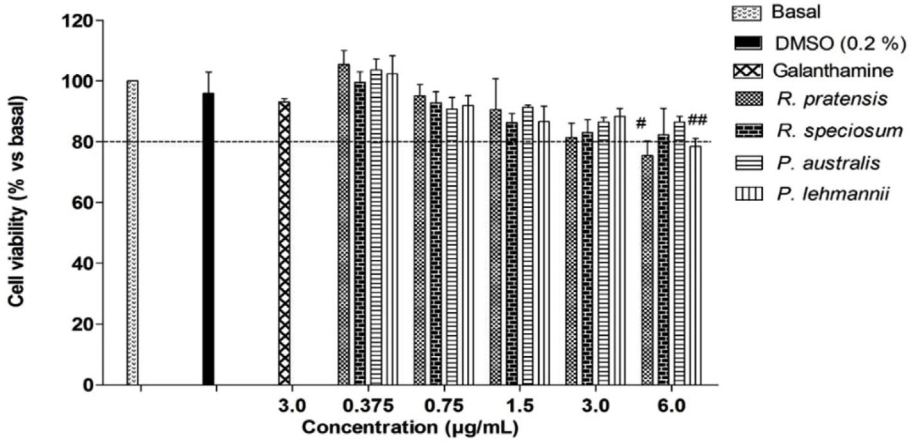

Fig. 1. Cytotoxicity of different concentrations of alkaloid fractions of *Rhodophiala pratensis*, *Rhodolirium speciosum*, *Phycella australis* and *Phaedranassa lehmannii* on SH-SY5Y cells, assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltriazoliumbromide (MTT assay), after treatment for 24 h. The toxic level was set at 80% of cell viability (dotted line). Toxic concentrations were considered at values lower than 80% of cell viability. Concentration of galanthamine was 3.0 μg/mL. The values are expressed as means ± SEM calculated for three individual experiments, each one carried out in triplicate. ##p < 0.01, #p < 0.05 versus 0.2% dimethyl sulfoxide (DMSO) as vehicle.

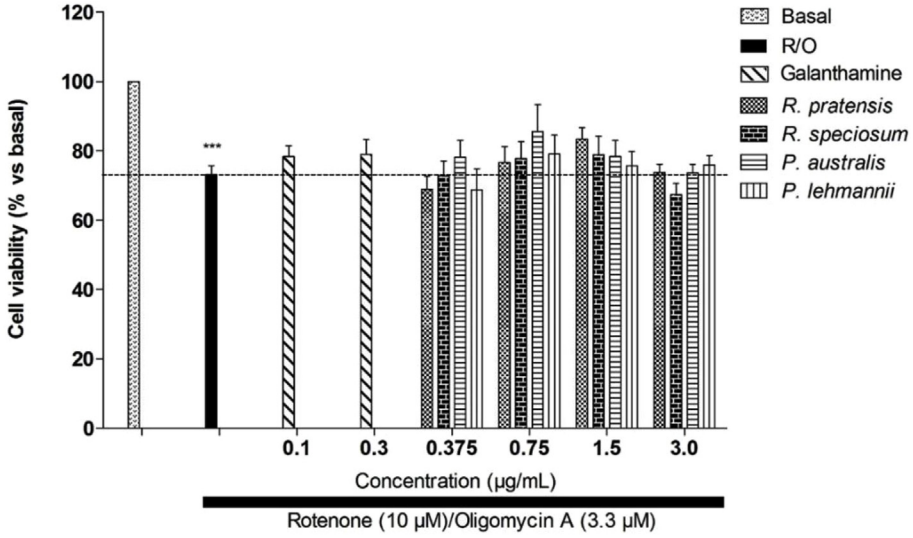

Fig. 2. Protective effect of the alkaloid fractions of *Rhodophiala pratensis, Rhodolirium speciosum, Phycella australis* and *Phaedranassa lehmannii* against the cytotoxicity induced by 10 µM rotenone/3.3 µM oligomycin A (R/O) in SH-SY5Y cells, assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltriazoliumbromide (MTT assay). Basal condition corresponds to cells treated with 0.1% dimethyl sulfoxide (DMSO) as vehicle. Concentrations of galanthamine were 0.1 and 0.3 µg/mL. The values are expressed as means ± SEM of six different cell batches. ***p < 0.001 versus Basal.

3.5. Neuroprotection assay against OA-induced cytotoxicity

In order to evaluate the effect of the alkaloid fractions of the plants under study in a model of neuronal death associated with Alzheimer's disease, we chose the model of toxicity induced by OA in the human neuroblastoma SH-SY5Y cell line. In this experiment, OA replicates the pathological characteristics of Alzheimer's disease related to the hyperphosphorylation of the tau protein through the inhibition of Ser/Thr phosphatases. This model has been previously used to evaluate the neuroprotective properties of galanthamine, donepezil and rivastigmine, all AChE inhibitors currently used for the treatment of patients with Alzheimer's disease (Arias et al., 2005). In the latter study, galanthamine presented the maximum neuroprotective activity at a concentration of 0.3 µM, while donepezil and rivastigmine showed a neuroprotective effect at concentrations of 1 and 3 µM, respectively (Arias et al., 2005).

In our study, the alkaloid fractions showed neuroprotective effect in a concentration-dependent manner (0.375–3.0 µg/mL) (Fig. 3). *Rhodophiala pratensis* and *Rhodolirium speciosum* did not show significant differences compared with the control treated with OA. On the other hand, concentrations of 3.0 µg/mL of *Phycella Australis* and *Phaedranassa lehmannii* displayed a maximum neuroprotective response of 78% and 84%, respectively. These differences in the percentage of cellular viability were statistically significant compared with the control of 15 nM OA (viability of 61%), indicating that the alkaloid fraction of these species preclude neurotoxicity induced by OA in SH-SY5Y cells (Fig. 3). However, galanthamine did not present a protective effect in this excitotoxicity model. Considering the results found for *Phycella australis*, the alkaloid fraction of this species was chosen for its evaluation in mouse hippocampal slices, a more complex and physiological model, in the OA and deprivation of oxygen and glucose toxicity models.

3.6. Effect of Phycella australis on OGD/R model

The mouse model of neurotoxicity induced by OGD/R in hippocampal slices, was used as a preliminary assay of neuroprotection. Neuronal death is caused by release of glutamate, which is generated by the disruption of the membrane potential associated with the reduction of ATP and the damage to mitochondrial membrane (Buendia et al., 2015). The results shown in Fig. 4 suggest that the alkaloid fraction of *Phycella australis* alleviated the process of neurotoxicity generated in mouse hippocampal slices stressed with OGD/R. The percentage of cell viability (75% and 79%) at the tested concentrations of 3 and 6 µg/mL shows a protection greater that 14% ($P < 0.05$, Fig. 4) compared to the one observed for the control with OGD/R condition alone (viability of

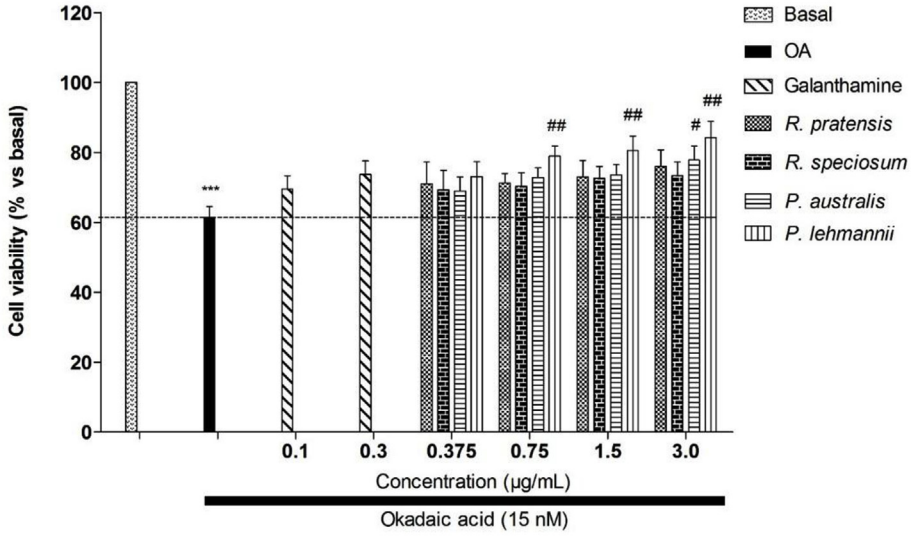

Fig. 3. Protective effect of the alkaloid fractions of *Rhodophiala pratensis, Rhodolirium speciosum, Phycella australis* and *Phaedranassa lehmannii* against the cytotoxicity induced by 20 nM okadaic acid (OA) in SH-SY5Y cells, assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltriazoliumbromide (MTT assay). The basal was treated with 0.1% dimethyl sulfoxide (DMSO) as vehicle. Concentrations of galanthamine were 0.1 and 0.3 µg/mL. The values are expressed as means ± SEM of six different cell batches. ***p < 0.001 versus Basal; # #p < 0.01, #p < 0.05 versus OA. .

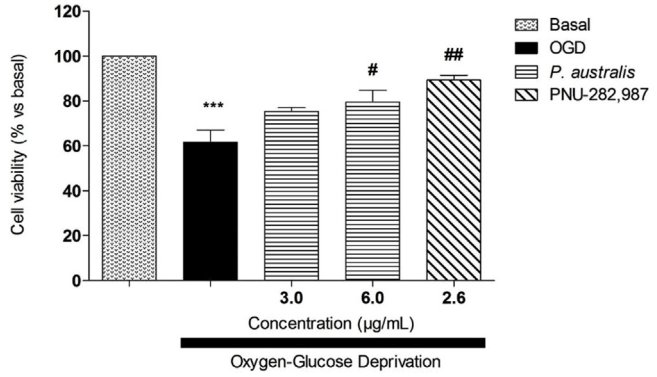

Fig. 4. Neuroprotective effect of the alkaloid fraction of *Phycella australis* bulbs (3 and 6 µg/mL), assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltriazoliumbromide (MTT assay), after neurotoxicity induced by oxygen glucose and deprivation/reoxygenation (OGD/R) in hippocampal slices. PNU-282,987 (2.6 µg/mL) was used as a positive control. The data are reported as mean means ± SEM of 2 animals (n = 2). ***p < 0.001 versus Basal; ##p < 0.01 #p < 0.05 versus OGD/R. One-way ANOVA followed by Newman-Keuls was performed. Data were normalized versus control values (100% cell viability).

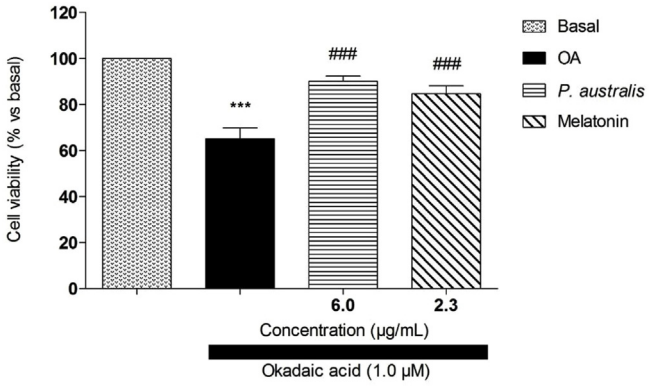

Fig. 5. Neuroprotective effect of the alkaloid fraction of *Phycella australis* bulbs (6 µg/mL), against toxicity induced by 1.0 µM okadaic acid (OA) in hippocampal slices. Melatonin (2.3 µg/mL) was used as positive control drug. Cell viability was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay). Data correspond to the means ± SEM. of 6 animals (n = 6). ***p < 0.001 versus Basal; ###p < 0.001 versus OA. One-way ANOVA followed by Newman-keuls was performed. Data were normalized to control values that were taken as 100% of cell viability.

61%). These results suggest that alkaloids present in this plant could prevent the mitochondrial damage generated by the stimulation of glutamate receptors. The drug PNU-282,987 was used as positive control. During several years it was accepted that galanthamine is an allosteric enhancer of human α4β2 and α7 neuronal nicotinic receptors (Texidó et al., 2005). However, such positive allosteric modulatory effect in neuronal nicotinic ACh (nACh) receptors function has been refuted in a recent work of Kowal et al. (2018). Therefore, we cannot assume that the neuroprotective effect of alkaloid fraction of *Phycella australis* is ascribed to allosteric modulation of these class of nicotinic receptors rather than AChE inhibition and antioxidants effects. Considering the results obtained in the model of neurotoxicity with OGD/R condition, it was determined that the greatest neuroprotective activity of the alkaloid fraction of *Phycella australis* appears at a concentration of 6 µg/mL. Therefore, this concentration was chosen for the evaluation of the neuroprotective activity of *Phycella australis* against the neurotoxicity induced by OA in mouse hippocampal slices.

3.7. *In vitro effect Phycella australis on OA-induced cytotoxicity on hippocampal slices*

In this experiment, cell viability reached a 90% at a concentration of 6 µg/mL of *Phycella australis* extract (Fig. 5). In contrast, cell viability in OA-treated slices was 65%, which represents a protection of 25% ascribed to *Phycella australis* alkaloids. This increase in cellular viability shows that alkaloid fraction of *Phycella australis* could prevent hyperphosphorylation of the tau protein. Studies regarding inhibition of tau hyperphosphorylation by Amaryllidaceae alkaloids are scarce. Recently, Hulcová and coworkers (2018) studied 28 different Amarillydaceae alkaloids assessing their glycogen synthase kinase-3β (GSK-3β) inhibition activity. GSK-3β is one of the serine/threonine protein kinases responsible for abnormal tau phosphorylation. In the study of Hulcová, caranine (lycorine type alkaloid) and 9-O-demethylhomolycorine and masonine (homolycorine type alkaloids), shown GSK-3β inhibitions over 60% being the IC_{50} of the three alkaloids around 30 µM. However, in our study only traces of caranine were detected in *Rhodolyrium speciosum* but not in *Phycella australis*. As was discussed in section 3.1. (see Table 1), this specie contains mainly derivatives of lycorine (16.28%); haemanthamine (34.36%); homolycorine (39.42%) and tazettine (6.26%). Importantly, Hulcová reported that haemanthamine, tazettine, homolycorine and acetyllycorine inhibit GSK-3β between 49 and 54%. Thereby, we could say that *Phycella australis* protects OA damage to hippocampal slices due to the presence of these type of alkaloids. These compounds account for 95% of total alkaloids found in *Phycella australis*. In conjunction, these results confirm the potent neuroprotective activity of the alkaloid fraction of *Phycella australis* against toxic stimulus with OA, because the response of neuroprotection is in line with the observed in SH-SY5Y cells. As a positive control in this assay we use melatonin, since this drug has positive effect on cognitive functions, and it is used to manage sleep disorders in patients with Alzheimer's disease (Fig. 5). Recent publications regarding synergistic activity of galanthamine, reported that when this compound is used in conjunction with melatonin, provides beneficial properties in an *in vitro* model of Alzheimer's disease where toxicity was induced by a combination of β-amyloid (0.5 µM) and OA (1 nM) in organotypic hippocampal slice cultures (Buendia et al., 2016). These authors reported that this stimulation increases cell death in 95%, promoting the hyperphosphorylation of tau protein, oxidative stress and neuroinflammation. Considering these experimental conditions, galanthamine and melatonin presented a maximum neuroprotective effect at a concentration of 1 µM when it was tested individually. On the contrary, when galanthamine (10 nM) plus melatonin (1 nM) were studied, it was observed that this combination of compounds reduced the neurotoxicity induced by a double toxic stimulus with β-amyloid/OA, suggesting the possibility of a protection linked to synergistic effects. Accordingly, *Phycella austalis* alkaloids most likely could act through synergistic mechanisms leading to reduce neuroinflammation and oxidative stress generated by OA.

Finally, the PCA analysis shows that the alkaloids in the alkaloid fraction of *Phycella australis* and *Rhodolirium speciosum*, have quantitative differences in the chemical composition (Fig. 6), while the compounds haemantamine and O-methyllycorenine present in the alkaloid fraction of *Rhodophiala pratensis* coincide with those in *Phycella australis*, although with different bioactivity. The first two main components explained 68.3% (PC1) and 18.8% (PC2) of the total variance in the dataset, respectively (Fig. 6). In this PCA analysis, *Phaedranassa lehmannii* is distinguished by the presence of the alkaloids lycorine and pseudolycorine. Importantly, PCA analysis allow to observe a clear arrangement of the type of alkaloid (lycorine, pseudolycorine and haemanthamine), and its close relation with the neuroprotection against OA, which is in line with the results discussed above.

Complex pathologies like Alzheimer's disease alter different routes and are rarely caused by dysfunction in a single gene or signaling pathway (Zhang et al., 2015). Despite the fact that over the past 15–20

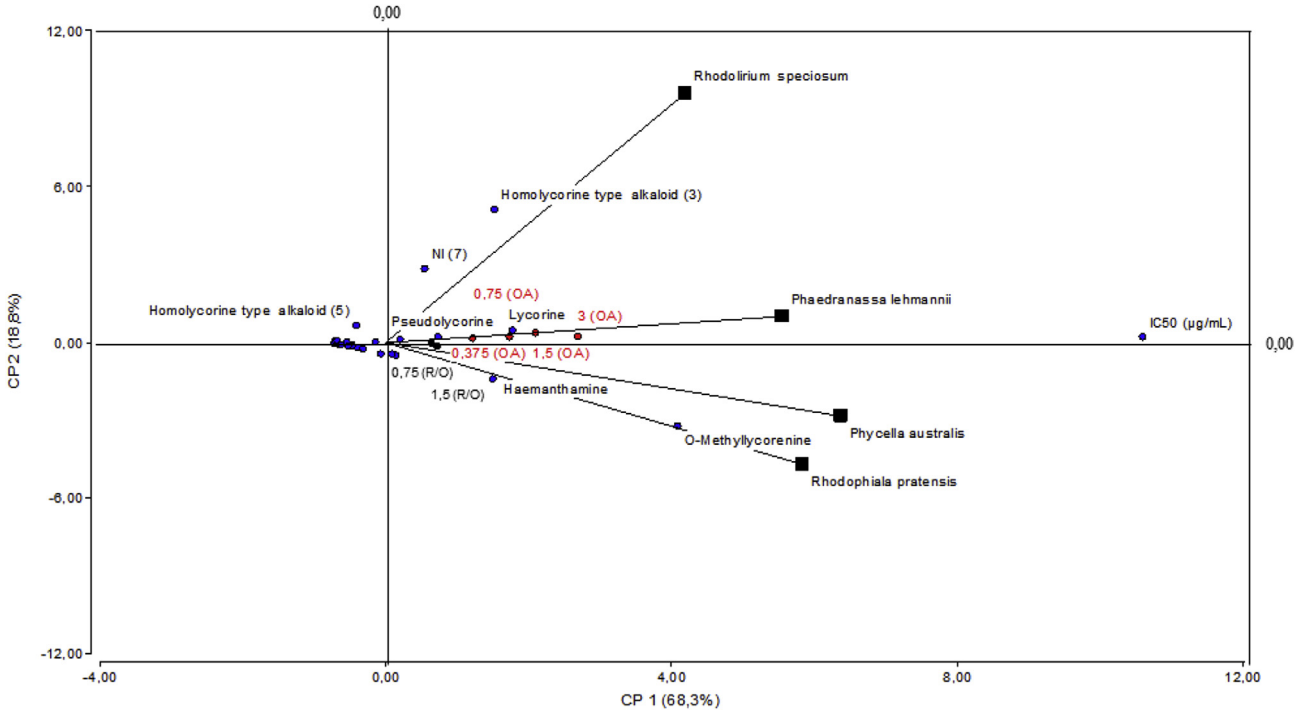

Fig. 6. Principal Component Analysis of the alkaloid fractions from Amaryllidaceae species. The factors assessed were species, chemical composition and bioactivity (neuroprotection and acetylcholinesterase inhibition). Red circle: okadaic acid (OA) assay, black circle: rotenone/oligomycin A (R/O) assay, blue circle: alkaloid type, black square: specie. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

years great scientific and economic efforts have been made to find new drugs to treat this devastating disease, the truth is that since memantine was marketed in 2003 no new drug has been approved for the treatment of AD. Taken together, in this work we report the phytochemical profile of three Chilean and one Colombian Amarillidaceae plants using GC-MS. Some of the compounds identified here are reported for the first time for these species. Although, these protective activities could be related to the interaction of the different isoquinolinic alkaloids, other compounds could act as modulators of the observed activity. The remarkable neuroprotective activity of *Phycella australis* observed in our study, suggests that alkaloids present in this Amaryllidaceae, could indeed be a source of secondary metabolites with potent neuropharmacological activities. Our findings also support the idea that the neuroprotective properties of these type of extracts could be exerted through multi target synergistic mechanisms.

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