



## ARCHIVIO ISTITUZIONALE DELLA RICERCA

### Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

Protective effects of chrysin against the neurotoxicity induced by aluminium: In vitro and in vivo studies

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Protective effects of chrysin against the neurotoxicity induced by aluminium: In vitro and in vivo studies / Campos H.M.; da Costa M.; da Silva Moreira L.K.; da Silva Neri H.F.; Branco da Silva C.R.; Pruccoli L.; dos Santos F.C.A.; Costa E.A.; Tarozzi A.; Ghedini P.C.. - In: TOXICOLOGY. - ISSN 0300-483X. - ELETTRONICO. - 465:(2022), pp. 153033.1-153033.13. [10.1016/j.tox.2021.153033]

This version is available at: <https://hdl.handle.net/11585/843655> since: 2023-10-10

*Published:*

DOI: <http://doi.org/10.1016/j.tox.2021.153033>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

(Article begins on next page)

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

This is the final peer-reviewed accepted manuscript of:

Campos, H.M., da Costa, M., da Silva Moreira, L.K., da Silva Neri, H.F., Branco da Silva, C.R., Pruccoli, L., dos Santos, F.C.A., Costa, E.A., Tarozzi, A., Ghedini, P.C., 2022. Protective effects of chrysin against the neurotoxicity induced by aluminium: In vitro and in vivo studies. *Toxicology* 465, 153033.

<https://doi.org/10.1016/j.tox.2021.153033>

The final published version is available online at:

<https://doi.org/10.1016/j.tox.2021.153033>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

*This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)*

***When citing, please refer to the published version.***

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Protective Effects of Chrysin against the Neurotoxicity Induced by Aluminium: In Vitro and In Vivo Studies

Hericles Mesquita Campos <sup>a</sup>, Michael da Costa <sup>a</sup>, Lorrane Kelle da Silva Moreira <sup>a</sup>, Hiasmin  
Franciely da Silva Neri <sup>a</sup>, Cinthia Rio Branco da Silva <sup>b</sup>, Letizia Pruccoli <sup>c</sup>, Fernanda Cristina  
Alcantara dos Santos <sup>b</sup>, Elson Alves Costa <sup>a</sup>, Andrea Tarozzi <sup>c</sup>, Paulo César Ghedini <sup>a, \*</sup>

<sup>a</sup> Department of Pharmacology, Institute of Biological Sciences, Federal University of Goiás,  
Goiania, GO, Brazil.

<sup>b</sup> Department of Histology, Embryology and Cell Biology, Institute of Biological Sciences,  
Federal University of Goiás, Goiania, GO, Brazil.

<sup>c</sup> Department for Life Quality Studies, University of Bologna, Rimini, Italy.

\* Corresponding author: Biochemical and Molecular Pharmacology Laboratory, Institute of  
Biological Sciences, Federal University of Goiás, Cep 74690-900, Goiania, GO, Brazil.

E-mail: pcghedini@gmail.com; phone +55 62 3521-1725

## Abstract

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Chronic exposure to aluminium (Al) can contribute to the progression of several neurological and neurodegenerative diseases. Al is a metal that promotes oxidative damage leading to neuronal death in different brain regions with behavior, cognition, and memory deficits. Chrysin is a flavonoid found mainly in honey, passion fruit, and propolis with antioxidant, anti-inflammatory, and cytoprotective properties. In this study, we used an integrated approach of *in vitro* and *in vivo* studies to evaluate the antioxidant and neuroprotective effects of chrysin against the neurotoxicity elicited by aluminium chloride (AlCl<sub>3</sub>). In *in vitro* studies, chrysin (5 µM) showed the ability to counteract the early oxidative stress elicited by tert-butyl hydroperoxide, an oxidant that mimics the lipid peroxidation and Fenton reaction in presence of AlCl<sub>3</sub> as well as the late necrotic death triggered by AlCl<sub>3</sub> in neuronal SH-SY5Y cells. *In vivo* studies in a mouse model of neurotoxicity induced by chronic exposure to AlCl<sub>3</sub> (100 mg/kg/day) for ninety days then corroborated the antioxidant and neuroprotective effect of chrysin (10, 30, and 100 mg/kg/day) using the oral route. In particular, chrysin reduced the cognitive impairment induced by AlCl<sub>3</sub> as well as normalized the acetylcholinesterase and butyrylcholinesterase activities in the hippocampus. In parallel, chrysin counteracted the oxidative damage, in terms of lipid peroxidation, protein carbonylation, catalase, and superoxide dismutase impairment, in the brain cortex and hippocampus. Lastly, necrotic cells frequency in the same brain regions was also decreased by chrysin. These results highlight the ability of chrysin to prevent the neurotoxic effects associated with chronic exposure to Al and suggest its potential use as a food supplement for brain health.

**Keywords:** Aluminium; neurotoxicity, oxidative stress; antioxidant; chrysin.

## 1. Introduction

Aluminum (Al) is the third most abundant element in the earth's crust and occurs naturally in the environment, foodstuffs, and drinking water. According to the European Food Safety Authority (EFSA), the human tolerable weekly intake is 1 mg Al/kg body weight (BW) in a 60-kg adult. However, in some individuals this intake is exceeded as a result of estimated daily alimentary aluminum exposure of 1.6–13 mg (0.2–1.5 mg/kg BW/week) (Klotz et al., 2017).

The toxic effects of this metal occur through its accumulation over time and several sources contribute to Al exposure in humans, such as industrial areas (0.04 to 1.4 mg/m<sup>3</sup>/day), natural water (different cities around the world have reported concentrations as high as 0.4–2.7 mg/L), foods (3–11 mg/kg/day), drugs such as antacid (104–208 mg of Al per tablet) and aspirin (10–20 mg of Al per tablet), and cosmetics (10.98–694.5 mg/kg in lipsticks) (Krewski et al., 2007; Exley, 2013; Borowska & Brzóska, 2015).

Among the different forms of Al, the ionic form of Al<sup>3+</sup> can exert various cytotoxic effects through its ability to induce the formation of reactive oxygen species, oxidative damage, and neuronal death (Willhite et al., 2014). Al<sup>3+</sup> can also cross the blood-brain barrier and accumulate in various human brain regions, suggesting the contribution of Al to the progression of neurological and neurodegenerative diseases, including Alzheimer's disease (AD), dialysis dementia syndrome, autism spectrum disorder, multiple sclerosis, and epilepsy (Lukiw et al., 2018; Exley and Mold, 2019). In this regard, the chronic accumulation of Al in the brain induces deregulation of gene expression in neurons, gliosis, neuronal death, and functional decline resulting in deficits in cognition, memory, and behavior (Lukiw et al., 2018).

Although Al is a low redox metal, considerable evidence supports its pro-oxidant activity at the brain level (Maya et al., 2016; Garza-Lombó et al., 2018). Al can bind to brain phospholipids, which contain polyunsaturated fatty acids vulnerable to the oxidizing action of

1 reactive oxygen species (ROS), including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>), and  
2 hydroxyl (OH<sup>·</sup>) radical (Yuan et al., 2012). In particular, Al can strengthen the iron (Fe<sup>2+</sup>)-  
3 initiated lipid peroxidation in the Fenton reaction, which causes Fe<sup>3+</sup> formation and ROS  
4 production. Further, O<sub>2</sub><sup>-</sup> is neutralized by Al<sup>3+</sup> to form an Al-O<sub>2</sub><sup>-</sup> complex, which increases  
5 the oxidative capacity of O<sub>2</sub><sup>-</sup> (Ruipérez et al., 2012). In this context, the neurobehavioral  
6 deficits, such as defects in memory and motor functions, elicited by chronic exposure to Al  
7 also reflect the ability of this metal to promote oxidative stress and neurodegeneration in brain  
8 regions rich in polyunsaturated fatty acids, including the cortex and hippocampus (Drobyshev  
9 et al., 2018; Farhat et al., 2019). Recent studies also show a cross talk between oxidative stress  
10 and neuroinflammation in neurodegeneration elicited by Al chronic exposure (Maya et al.,  
11 2016). The neuroinflammatory response triggered by oxidative stress can contribute to  
12 cognitive impairments through neuropathological and neurochemical changes in the cortex and  
13 hippocampus (Fernandes et al., 2020).

14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31 The use of natural antioxidants as potential therapeutics is a challenging area of  
32 neuroscience research (Tal et al., 2016). Among natural antioxidants, the chrysin is a flavonoid  
33 (5,7-dihydroxyflavone) found mainly in mushrooms (*Lactarius deliciosus* - 0.17 mg/kg, *Suillus*  
34 *bellinii* - 0.34 mg/kg), passion fruit (*Passiflora ocreata* - 40 mg/mL, *Passiflora edulis*, and  
35 *Passiflora caerulea* L. - 0.012 - 0.25 mg/mL), propolis (28 mg/mL), and in honey (e.g. honey  
36 of honeydew - 0.10 mg/kg, and forest honeys- 5.3 mg/kg) (Hadjmohammadi et al., 2010;  
37 Kalogeropoulos et al., 2013; Nabavi et al., 2015; El-Askary et al., 2017; Mani and Natesan,  
38 2018). In addition, marketed tablets/capsules may include 300-800 mg of chrysin (Nabavi et  
39 al., 2015; Manzolli et al., 2015; Mohos et al., 2018; Mohos et al., 2020).

40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
66  
67  
68  
69  
70  
71  
72  
73  
74  
75  
76  
77  
78  
79  
80  
81  
82  
83  
84  
85  
86  
87  
88  
89  
90  
91  
92  
93  
94  
95  
96  
97  
98  
99  
100  
101  
102  
103  
104  
105  
106  
107  
108  
109  
110  
111  
112  
113  
114  
115  
116  
117  
118  
119  
120  
121  
122  
123  
124  
125  
126  
127  
128  
129  
130  
131  
132  
133  
134  
135  
136  
137  
138  
139  
140  
141  
142  
143  
144  
145  
146  
147  
148  
149  
150  
151  
152  
153  
154  
155  
156  
157  
158  
159  
160  
161  
162  
163  
164  
165  
166  
167  
168  
169  
170  
171  
172  
173  
174  
175  
176  
177  
178  
179  
180  
181  
182  
183  
184  
185  
186  
187  
188  
189  
190  
191  
192  
193  
194  
195  
196  
197  
198  
199  
200  
201  
202  
203  
204  
205  
206  
207  
208  
209  
210  
211  
212  
213  
214  
215  
216  
217  
218  
219  
220  
221  
222  
223  
224  
225  
226  
227  
228  
229  
230  
231  
232  
233  
234  
235  
236  
237  
238  
239  
240  
241  
242  
243  
244  
245  
246  
247  
248  
249  
250  
251  
252  
253  
254  
255  
256  
257  
258  
259  
260  
261  
262  
263  
264  
265  
266  
267  
268  
269  
270  
271  
272  
273  
274  
275  
276  
277  
278  
279  
280  
281  
282  
283  
284  
285  
286  
287  
288  
289  
290  
291  
292  
293  
294  
295  
296  
297  
298  
299  
300  
301  
302  
303  
304  
305  
306  
307  
308  
309  
310  
311  
312  
313  
314  
315  
316  
317  
318  
319  
320  
321  
322  
323  
324  
325  
326  
327  
328  
329  
330  
331  
332  
333  
334  
335  
336  
337  
338  
339  
340  
341  
342  
343  
344  
345  
346  
347  
348  
349  
350  
351  
352  
353  
354  
355  
356  
357  
358  
359  
360  
361  
362  
363  
364  
365  
366  
367  
368  
369  
370  
371  
372  
373  
374  
375  
376  
377  
378  
379  
380  
381  
382  
383  
384  
385  
386  
387  
388  
389  
390  
391  
392  
393  
394  
395  
396  
397  
398  
399  
400  
401  
402  
403  
404  
405  
406  
407  
408  
409  
410  
411  
412  
413  
414  
415  
416  
417  
418  
419  
420  
421  
422  
423  
424  
425  
426  
427  
428  
429  
430  
431  
432  
433  
434  
435  
436  
437  
438  
439  
440  
441  
442  
443  
444  
445  
446  
447  
448  
449  
450  
451  
452  
453  
454  
455  
456  
457  
458  
459  
460  
461  
462  
463  
464  
465  
466  
467  
468  
469  
470  
471  
472  
473  
474  
475  
476  
477  
478  
479  
480  
481  
482  
483  
484  
485  
486  
487  
488  
489  
490  
491  
492  
493  
494  
495  
496  
497  
498  
499  
500  
501  
502  
503  
504  
505  
506  
507  
508  
509  
510  
511  
512  
513  
514  
515  
516  
517  
518  
519  
520  
521  
522  
523  
524  
525  
526  
527  
528  
529  
530  
531  
532  
533  
534  
535  
536  
537  
538  
539  
540  
541  
542  
543  
544  
545  
546  
547  
548  
549  
550  
551  
552  
553  
554  
555  
556  
557  
558  
559  
560  
561  
562  
563  
564  
565  
566  
567  
568  
569  
570  
571  
572  
573  
574  
575  
576  
577  
578  
579  
580  
581  
582  
583  
584  
585  
586  
587  
588  
589  
590  
591  
592  
593  
594  
595  
596  
597  
598  
599  
600  
601  
602  
603  
604  
605  
606  
607  
608  
609  
610  
611  
612  
613  
614  
615  
616  
617  
618  
619  
620  
621  
622  
623  
624  
625  
626  
627  
628  
629  
630  
631  
632  
633  
634  
635  
636  
637  
638  
639  
640  
641  
642  
643  
644  
645  
646  
647  
648  
649  
650  
651  
652  
653  
654  
655  
656  
657  
658  
659  
660  
661  
662  
663  
664  
665  
666  
667  
668  
669  
670  
671  
672  
673  
674  
675  
676  
677  
678  
679  
680  
681  
682  
683  
684  
685  
686  
687  
688  
689  
690  
691  
692  
693  
694  
695  
696  
697  
698  
699  
700  
701  
702  
703  
704  
705  
706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718  
719  
720  
721  
722  
723  
724  
725  
726  
727  
728  
729  
730  
731  
732  
733  
734  
735  
736  
737  
738  
739  
740  
741  
742  
743  
744  
745  
746  
747  
748  
749  
750  
751  
752  
753  
754  
755  
756  
757  
758  
759  
760  
761  
762  
763  
764  
765  
766  
767  
768  
769  
770  
771  
772  
773  
774  
775  
776  
777  
778  
779  
780  
781  
782  
783  
784  
785  
786  
787  
788  
789  
790  
791  
792  
793  
794  
795  
796  
797  
798  
799  
800  
801  
802  
803  
804  
805  
806  
807  
808  
809  
810  
811  
812  
813  
814  
815  
816  
817  
818  
819  
820  
821  
822  
823  
824  
825  
826  
827  
828  
829  
830  
831  
832  
833  
834  
835  
836  
837  
838  
839  
840  
841  
842  
843  
844  
845  
846  
847  
848  
849  
850  
851  
852  
853  
854  
855  
856  
857  
858  
859  
860  
861  
862  
863  
864  
865  
866  
867  
868  
869  
870  
871  
872  
873  
874  
875  
876  
877  
878  
879  
880  
881  
882  
883  
884  
885  
886  
887  
888  
889  
890  
891  
892  
893  
894  
895  
896  
897  
898  
899  
900  
901  
902  
903  
904  
905  
906  
907  
908  
909  
910  
911  
912  
913  
914  
915  
916  
917  
918  
919  
920  
921  
922  
923  
924  
925  
926  
927  
928  
929  
930  
931  
932  
933  
934  
935  
936  
937  
938  
939  
940  
941  
942  
943  
944  
945  
946  
947  
948  
949  
950  
951  
952  
953  
954  
955  
956  
957  
958  
959  
960  
961  
962  
963  
964  
965  
966  
967  
968  
969  
970  
971  
972  
973  
974  
975  
976  
977  
978  
979  
980  
981  
982  
983  
984  
985  
986  
987  
988  
989  
990  
991  
992  
993  
994  
995  
996  
997  
998  
999  
1000

1 Sairazi et al., 2017). These evidences suggest the ability of natural antioxidants present in  
2 honey and propolis, such as chrysin, to cross the blood-brain barrier (BBB) and affect the  
3 oxidative stress on the central nervous system (CNS) level. In this regard, an *in silico* approach  
4 to predict the pharmacokinetics of chrysin by the SwissADME web tool reveal its good overall  
5 drug-likeness profile including high gastrointestinal absorption and BBB permeant (Daiana  
6 and Zoete, 2016; Daiana et al., 2016).  
7  
8  
9  
10  
11  
12  
13

14 Chrysin showed antioxidant and neuroprotective effects against cognitive decline in a  
15 rat model of diabetes and neurodegeneration induced by 6-hydroxydopamine in a mouse model  
16 of Parkinson's disease (Li et al., 2014; Manzolli et al., 2015; Nabavi et al., 2015). However, the  
17 neuroprotective effects of chrysin against the neurotoxicity evoked by Al remain unknown.  
18  
19  
20  
21  
22  
23

24 In this study, we used an *in vitro* experimental approach to evaluate: 1) the antioxidant  
25 effects of chrysin against the oxidative stress induced by various oxidants and Fenton reaction  
26 promoted by aluminium chloride (AlCl<sub>3</sub>) in neuronal SH-SY5Y cells, as well as the ability of  
27 chrysin to counteract both the oxidative stress and inflammation evoked by lipopolysaccharides  
28 (LPS) in microglial THP-1 cells; 2) the neuroprotective effects of chrysin against the  
29 neurotoxicity in terms of cytostatic effects and necrosis elicited by AlCl<sub>3</sub> in neuronal SH-SY5Y  
30 cells; 3) we, then, assessed the antioxidant and neuroprotective activity of chrysin in a mouse  
31 model of neurotoxicity induced by chronic exposure to AlCl<sub>3</sub>. Chrysin was administered to  
32 mice using the oral route to explore its potential use as a food supplement for brain health.  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48

## 49 **2. Materials and Methods**

### 50 **2.1 Chemicals**

51 AlCl<sub>3</sub>, iron sulfate (FeSO<sub>4</sub>), chrysin (97% purity - Fig. 1), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>),  
52 tert-butyl hydroperoxide (t-BuOOH), acetylthiocholine (ATCh), 2,2'-azinobis-(3-  
53 ethylbenzothiazoline-6-sulfonic acid) (ABTS•+), butyrylthiocholine (BTCh), 2-7-

1 dichlorodihydrofluorescein diacetate (H2DCF-DA), 5,5'-dithiobis-(2-nitrobenzoic acid)  
2 (DTNB), malondialdehyde (MDA), epinephrine bitartrate, 2,4-dinitrophenylhydrazine  
3 (DNPH), sodium dodecyl sulfate (SDS), eosin, hematoxylin, and serum albumin (BSA) were  
4 obtained from Sigma Chemical (St. Louis, MO, USA). All other chemicals were obtained in  
5 an analytical grade or from standard commercial suppliers.  
6  
7  
8  
9  
10  
11  
12  
13

## 14 **2.2 In vitro tests**

### 15 **2.2.1 Cell culture**

16  
17  
18  
19 Human neuronal SH-SY5Y cells were purchased from Lombardy and Emilia Romagna  
20 Experimental Zootechnic Institute (Italy). SH-SY5Y cells were routinely grown in Dulbecco's  
21 Modified Eagle Medium whit phenol red supplemented with 10% fetal bovine serum, 2 mM  
22 L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37 °C in a humidified  
23 incubator with 5% CO<sub>2</sub>. For all experiments the SH-SY5Y cells were used below passage 12  
24 to avoid phenotype changes and cellular senescence.  
25  
26  
27  
28  
29  
30  
31  
32

33  
34 Human monocyte THP-1 cells were purchased from Cell bank Interlab Cell Line  
35 Collection (Italy). THP-1 cells were routinely grown in Roswell Park Memorial Institute  
36 (RPMI) 1640 Medium with phenol red supplemented with 10% fetal bovine serum, 2 mM L-  
37 glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37 °C in a humidified incubator  
38 with 5% CO<sub>2</sub>. THP-1 cells were differentiated into microglia-like cells with phorbol 12-  
39 myristate 13-acetate (PMA, 10 µg/ml) for 24 h at 37°C in 5% CO<sub>2</sub>.  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50

### 51 **2.2.2 Neuronal viability**

52  
53 The neuronal viability was assessed using the tetrazolium salt colorimetric assay as  
54 previously described by Ortiz et al., 2020. Briefly, SH-SY5Y cells were seeded in a 96 well  
55 plate at  $2 \times 10^4$  cells/well, incubated for 24 h, and subsequently treated with various  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



1 concentrations of chrysin (1.25 – 40  $\mu$ M) for 24 h at 37°C in 5% CO<sub>2</sub>. The cell viability, in  
2 terms of mitochondrial metabolic function, was evaluated by the reduction of 3-(4,5-dimethyl-  
3 2-thiazolyl)-2,5-diphenyl2H-tetrazolium bromide (MTT) to its insoluble formazan. The  
4 treatment was replaced with MTT in HBSS [0.5 mg/mL] for 1 h at 37°C in 5% CO<sub>2</sub>. After  
5 washing with HBSS, formazan crystals were dissolved in isopropanol. The amount of formazan  
6 was measured (570 nm, reference filter 690 nm) using a multilabel plate reader (VICTOR™  
7 X3, PerkinElmer, Waltham, MA, USA). The quantity of formazan was directly proportional to  
8 the number of living cells. Data are expressed as percentages of neuronal viability with control  
9 taken as 100% viability.  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23

### 24 **2.2.3 Antioxidant activity in neuronal SH-SY5Y cells**

25 The antioxidant activity of chrysin was evaluated in neuronal SH-SY5Y cells as  
26 previously described by Tarozzi et al., 2012. Briefly, cells were seeded in a 96 well plate at 3  
27  $\times 10^4$  cells/well, incubated for 24 h, and subsequently loaded with the fluorescent probe  
28 H<sub>2</sub>DCF-DA (10  $\mu$ g/mL) for 30 min at room temperature. At the end of incubation, cells were  
29 treated with various concentrations of chrysin (1.25 – 5  $\mu$ M) and H<sub>2</sub>O<sub>2</sub> or *t*-BuOOH (100  $\mu$ M)  
30 for 30 min. The intracellular ROS formation was measured (excitation at 485 nm and emission  
31 at 535 nm) using a VICTOR™ X3 multilabel plate reader. Data are expressed as Arbitrary Unit  
32 of Fluorescence (AUF).  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45

46 The total antioxidant activity (TAA) was measured on both the cytosolic and membrane  
47 enriched fractions as previously reported by Tarozzi et al., 2014. Briefly, SH-SY5Y cells were  
48 seeded in cultures dishes at 4  $\times 10^6$  cells/dish for 24 h at 37°C in 5% CO<sub>2</sub>. At the end of  
49 incubation, cells were treated for 2 h with chrysin (5  $\mu$ M). After washing with cold phosphate  
50 buffer saline (PBS), cells were collected in 1 ml of PBS and centrifuged for 10 min at 10,000  
51 g at 4 °C. The supernatant was removed and after washing with PBS, the pellet was finally  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 reconstituted in 600  $\mu$ l of 0.05 % Triton X-100. Cells were then homogenized and allowed to  
2 stand at 4 °C for 30 min. Cytosolic and membrane enriched fractions were subsequently  
3  
4 separated by centrifugation at 15,000 g for 15 min at 4 °C. TAA in cell fractions was  
5 determined by the decoloration of the radical cation of ABTS<sup>•+</sup>, in terms of quenching of  
6  
7 absorbance at 740 nm. Data for each sample were compared with the concentration-response  
8  
9 curve of a standard antioxidant, such as Trolox (a water-soluble vitamin E analog) and  
10  
11 expressed as  $\mu$ mol of Trolox Equivalent Antioxidant Activity per mg of protein ( $\mu$ molTE/mg  
12  
13 protein).  
14  
15  
16  
17  
18

19 The antioxidant activity of chrysin against the oxidative stress induced by the synergic  
20 effect of Fenton reaction and AlCl<sub>3</sub> was evaluated in neuronal SH-SY5Y cells. Briefly, cells  
21  
22 were seeded in a 96 well plate at  $3 \times 10^4$  cells/well, incubated for 24 h, and subsequently loaded  
23  
24 with the fluorescent probe H<sub>2</sub>DCF-DA (10  $\mu$ g/mL) for 30 min at room temperature. At the end  
25  
26 of incubation, cells were first treated for 15 min with FeSO<sub>4</sub>/H<sub>2</sub>O<sub>2</sub>/AlCl<sub>3</sub> (50  $\mu$ M/200  $\mu$ M/50  
27  
28  $\mu$ M) and then treated for 15 min with chrysin (5  $\mu$ M). The ROS formation was measured  
29  
30 (excitation at 485 nm and emission at 535 nm) using a VICTOR™ X3 multilabel plate reader.  
31  
32  
33  
34  
35  
36 Data are expressed as AUF.  
37  
38  
39  
40

#### 41 **2.2.4 Antioxidant activity in microglial THP-1 cells**

42  
43 The antioxidant activity of chrysin against the oxidative stress induced by *t*-BuOOH  
44 was evaluated in microglial THP-1 cells. Briefly, THP-1 cells were seeded in cultures dishes  
45  
46 at  $2.5 \times 10^6$  cells/dish and incubated for 24 h with PMA (10  $\mu$ g/ml) for differentiation to  
47  
48 microglial-like cells. After the differentiation, cells were incubated with the fluorescent probe  
49  
50 H<sub>2</sub>DCF-DA (10  $\mu$ g/mL) for 30 min at room temperature. At the end of incubation, cells were  
51  
52 treated with various concentrations of chrysin (1.25 – 5  $\mu$ M) and *t*-BuOOH (100  $\mu$ M) for 30  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 min. The ROS formation was measured (excitation at 485 nm and emission at 535 nm) using  
2 a VICTOR™ X3 multilabel plate reader. Data are expressed as AUF.  
3

4 The antioxidant activity of chrysin against the oxidative stress induced by  
5 lipopolysaccharides (LPS) was evaluated in microglial THP-1 cells. Briefly, cells were seeded  
6 in cultures dishes at  $2.5 \times 10^6$  cells/dish, incubated for 24 h with PMA (10  $\mu\text{g}/\text{mL}$ ) and  
7 subsequently, treated for 24 h with chrysin (5  $\mu\text{M}$ ) and LPS (1 $\mu\text{g}/\text{mL}$ ). At the end of incubation,  
8 cells were loaded with the fluorescent probe H<sub>2</sub>DCF-DA (10  $\mu\text{g}/\text{mL}$ ) for 30 min at room  
9 temperature. The ROS formation was measured (excitation at 485 nm and emission at 535 nm)  
10 using a VICTOR™ X3 multilabel plate reader. Data are expressed as AUF.  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23

#### 24 **2.2.5 Anti-inflammatory activity in microglial THP-1 cells**

25 The anti-inflammatory activity of chrysin in microglial THP-1 activated by LPS was  
26 performed as previously described by Di Martino et al., 2020. Briefly, THP-1 cells were seeded  
27 in 60 mm dishes at  $2.5 \times 10^6$  cells/dish, incubated for 24 h with PMA (10  $\mu\text{g}/\text{mL}$ ) and  
28 subsequently, treated for 24 h with chrysin (5  $\mu\text{M}$ ) and LPS (1 $\mu\text{g}/\text{mL}$ ). Afterward, the cell  
29 suspension was pelleted, and RNA was extracted by the PureLink RNA Mini Kit (Life  
30 Technologies, Carlsbad, CA, USA) according to the manufacturer's guidelines. A total of 1  $\mu\text{g}$   
31 of RNA were used to synthesize cDNA using the SuperScript VILO MasterMix (Invitrogen,  
32 Carlsbad, CA, USA). Quantitative RT-PCR was carried out using SYBR Select Master Mix  
33 (Invitrogen), and relative normalized expression was calculated by comparing the cycle  
34 threshold (Ct) of the target gene (Inducible nitric oxide synthase, iNOS; Interleukin 1 beta; IL-  
35 1 $\beta$ ; Tumour Necrosis Factor-alpha; TNF- $\alpha$ ) to that of the reference genes  $\beta$ -Actin and  
36 glyceraldehyde-3-phosphate dehydrogenase protein (GAPDH, Life Technologies). All  
37 reactions had three technical replicates, and each condition had three biological replicates.  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Relative quantification was calculated according to the  $\Delta\Delta C_t$  method ( $2^{-\Delta\Delta C_t}$ ) with untreated cells as control. Primer sequences used in this study are listed in Table 1.

### **2.2.6 Neuroprotective activity in neuronal SH-SY5Y cells**

The neuroprotective activity of chrysin was evaluated in neuronal SH-SY5Y cells in terms of cytostatic effect and necrosis using trypan blue and propidium iodide (PI), respectively, as previously described by Pruccoli et al., 2020. Cells were seeded in 60 mm dishes at  $1 \times 10^6$  cells/dish, incubated for 24 h, and subsequently treated for 24 h with chrysin (5  $\mu$ M) and AlCl<sub>3</sub> (50  $\mu$ M). At the end of incubation, cells were collected and the cytostatic effect, in terms of the number of total cells, was detected by using trypan blue (1:10). In parallel, cells were loaded with PI (25  $\mu$ g/mL) for 5 min at room temperature. The necrosis was detected by using an inverted fluorescence microscope (Eclipse Ti-E, Nikon Instruments Spa, Florence, Italy). The total cells were counted in a bright field, then only the red necrotic cells were counted using TRITC filters (EX 535/50, BS 575, EM 590LP). Data are expressed as a percentage of necrotic cells versus total cells.

## **2.3 In vivo tests**

### **2.3.1 Animals**

Experiments were conducted using male Swiss mice (25 – 30 g) about two months old. Animals were maintained at a stable temperature of  $22 \pm 2$  °C and a controlled 12 hours light/dark cycle, with free access to food and water. All manipulations were carried out between 08:00 and 16:00. All the protocols and experimentations were approved by the Brazilian College of Animal Experimentation (COBEA) and were approved by the local Ethics in Research Committee (protocol number: 056/2016) of the Federal University of Goiás (UFG).

### 2.3.2 Experimental design

The animals were randomized into five groups (n = 14 – 16): control; aluminum chloride 100 mg/kg (AlCl<sub>3</sub>) dissolved in water; chrysin 10 mg/kg (Chrysin 10); chrysin 30 mg/kg (Chrysin 30) and chrysin 100 mg/kg (Chrysin 100). The control group received water, while all other groups received AlCl<sub>3</sub> 100 mg/kg/day for 90 days (Treatment 1). On the 46<sup>th</sup> day, the control and AlCl<sub>3</sub> groups received normal saline (adjusted pH 7.4) while all the other groups received chrysin until the 90<sup>th</sup> day (Treatment 2). All treatments were administered daily by gavage at a volume of 10 µL/1 g.

The chrysin doses were chosen based on a previous work of He et al., 2012 and Sarkaki et al., 2019. The Al dose and treatment periods were chosen based on previous work of our group (Oliveira et al., 2018; Thomaz et al., 2018).

At the end of the behavioral tests, the number of animals per group was subdivided for the biochemical assays (n = 8) and the histopathological and morphometric analysis (n = 5).

## 2.4 Behavioral tests

### 2.4.1 Open field test (OFT)

Exploratory activity was measured in the OFT (Montgomery, 1956) at protocol day 90, previously to the memory task. The floor of the open field was divided into nine squares. Each animal was placed individually in the center of the arena, and the number of segments crossed (four-paw criterion) was recorded in a 5 min session.

### 2.4.2 Chimney test (CT)

Locomotor activity was measured in the CT (Boissier, 1960) also at protocol day 90. This test consisted of evaluating the ability of animals to climb back up through an acrylic tube. The animals were introduced in the tube horizontally, and when they reach the opposite

1 extremity, the tube was put in the vertical position, and the activity of the animals to climb for  
2 30 sec was observed.  
3  
4  
5  
6

### 7 **2.4.3 Step-down avoidance test (SDPAT)**

8  
9 The SDPAT has been used to study nonspatial long-term memory (Kameyama et al.,  
10 11 1986). The apparatus consisted of a single box where the floor was made of a metal grid  
12 13 connected to a shock scrambler. A safe platform was also placed in the box. The training  
14 15 session (acquisition) day 91, consisted in putting the mice gently on the platform and upon  
16 17 stepping with four paws onto the grid floor, an electric shock of 0.5 mA/s was delivered. The  
18 19 latency of the step-down motion into the grid was recorded in the training session. Some  
20 21 seconds later (~5), the mouse was removed from the SDPAT apparatus and returned to its home  
22 23 cage. The retention trial was performed 24 h after training on day 92. For this, mice were placed  
24 25 on the platform, but no shock was given when they went down. The criterion for learning was  
26 27 taken as an increase in the transfer latency time on the retention trial as compared to the  
28 29 acquisition trial. Therefore, short transfer latencies indicate poor retention. The box was  
30 31 illuminated throughout the experimental period.  
32  
33  
34  
35  
36  
37  
38  
39  
40

## 41 **2.5 Ex vivo tests**

### 42 **2.5.1 Tissue collection and preparation**

43  
44 Twenty-four hours after the last behavioral test, the animals were anesthetized  
45 46 intraperitoneally with ketamine and xylazine hydrochloride. They were subsequently sacrificed  
47 48 by decapitation and the brain cortex and hippocampus were removed and dissected. Cerebral  
49 50 tissue samples were homogenized in 0.1 mol/L potassium phosphate buffer (KPB), pH = 7.4  
51 52 in a 1:10 (w/v) ratio. The homogenate was then centrifuged at 8000 x g for 10 min to yield the  
53 54 low-speed supernatant (S1) fractions that were used in the assays.  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## **2.6 Biochemical assays**

### **2.6.1 Cholinesterase (ChEs) activity**

Enzyme activities were carried out according to the method of Ellman et al., 1961. The S1 fractions were incubated with 0.3 mmol/L DTNB, and the enzymatic reaction was initiated by the addition of 0.45 mmol/L of ATCh and BTCh as the substrate for AChE and BChE, respectively. Enzyme activities were spectrophotometrically measured at 412 nm for 3 min. Results are expressed in  $\mu\text{mol ACTh}/\text{min}/\text{mg}$  protein and  $\mu\text{mol BCTh}/\text{min}/\text{mg}$  protein for AChE and BChE, respectively.

### **2.6.2 Lipid peroxidation (LPO) levels**

For LPO measurement the method of thiobarbituric acid reactive substances (TBARS) was used. The TBARS estimation was spectrophotometrically performed following the method described by Ohkawa et al., 1979, with some modifications. The S1 fractions were incubated with thiobarbituric acid, acetic acid (pH 3.4), and SDS at 95 °C for 60 min. The reaction product was determined at 532 nm. For the interpretation of the results, a MDA curve was performed and the data are expressed as equivalents of MDA in nmol/mg protein.

### **2.6.3 Carbonylated protein (CP) levels**

Protein carbonyl derivatives were measured following the method described by Levine et al., 1990, with some modifications. The S1 fractions were incubated with DNPH prepared in 2 mol/L HCl. The mixture was kept in the dark for 1 h and vortexed each 15 min. Denaturation buffer, ethanol, and hexane were then added to each tube and the final mixture was vortexed for 40 sec and centrifuged at 3000 x g for 10 min. The supernatant obtained was discarded. The pellet was washed with ethanol-ethyl acetate (1:1 v/v) and re-suspended in a

1 denaturation buffer. The sample was vortexed for 5 min and was used to measure absorbance  
2 at 370 nm. Results are expressed as nmol of carbonyl content/mg protein.  
3  
4  
5  
6

#### 7 **2.6.4 Superoxide dismutase (SOD) activity**

8  
9 The brain cortex and hippocampus SOD activities were spectrophotometrically  
10 determined according to the method of Misra and Fridovich, 1972. The principle of this method  
11 is the ability of the superoxide dismutase enzyme to inhibit the autoxidation of epinephrine.  
12 The S1 fractions were incubated with 60 mmol/L of epinephrine bitartrate (pH 10) and the  
13 sample color intensity was measured at 480 nm. The enzymatic activity was expressed in units  
14 (U) of SOD/mg of protein.  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25

#### 26 **2.6.5 Catalase (CAT) activity**

27  
28 The brain cortex and hippocampus CAT activities were spectrophotometrically  
29 determined by the H<sub>2</sub>O<sub>2</sub> decomposition at 240 nm according to the method described by Aebi,  
30 1984, with some modifications. The S1 fractions were incubated with 86 mmol/L H<sub>2</sub>O<sub>2</sub> and  
31 sodium phosphate buffer (pH 7.0). The enzymatic activity was expressed in U of CAT/mg of  
32 protein. One U of enzyme thus decomposed one μmol of H<sub>2</sub>O<sub>2</sub>/min at pH 7.0 at 25 °C.  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43

#### 44 **2.6.6 Protein content determination**

45  
46 Total protein concentration was measured by the method described by Bradford, 1976  
47 using bovine albumin serum as the standard.  
48  
49  
50  
51  
52

### 53 **2.7 Histopathological and morphometric analysis**

54  
55 The brain cortex and hippocampus were fixed by immersion in 4 % paraformaldehyde  
56 (buffered in 0.1 M phosphate, pH 7.2) for 24 h. The tissues were then dehydrated through a  
57  
58  
59  
60  
61  
62  
63  
64  
65



1 crescent ethanol series, clarified in xylol, embedded in paraplast (Histosec, Merck, Darmstadt,  
2 Germany), and sectioned at 5  $\mu\text{m}$  on a Leica microtome (Leica RM2155, Nussloch, Germany).  
3  
4 Sections were stained by thionine acetate and analyzed in a Zeiss Axioscope A1 light  
5 microscope (Zeiss, Germany). Morphometric analysis was performed to quantify the  
6 neurodegeneration in terms of the percentage (%) of eosinophilic neurons (necrosis-like cells)  
7 in the brain cortex and hippocampus (CA1 area). For this, 30 photomicrographic fields were  
8 obtained (6 fields/animal,  $n = 5$  animals/group). The percentage (%) of necrotic neurons was  
9 obtained to the total number of neurons per photomicrographic field. All analyses were  
10 conducted using Image Pro-Plus program version 6.1 (Media Cybernetics Inc., Silver Spring,  
11 MD, USA).  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25

## 26 **2.8 Data presentation and statistical analysis**

27  
28 All experiments results are given as the mean (s)  $\pm$  standard error mean (SEM).  
29 Statistical analyses were performed using Student t-test or one-way ANOVA followed by  
30 Tukey's multiple comparisons test, Dunnett's test, Bonferroni's test, or Student's t-test as  
31 appropriate. Values of  $p < 0.05$  were considered statistically significant. Calculations were  
32 performed using the software GraphPad Prism 8.0. San Diego, CA USA.  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## 66 **3. Results**

### 67 **3.1 In vitro studies**

#### 68 **3.1.1 Neurotoxicity**

69 The cytotoxicity of chrysin was evaluated in neuronal SH-SY5Y cells to define the  
70 range of concentrations not associated with toxicity. After 24 h of treatment with various  
71 concentrations of chrysin (1.25 – 40  $\mu\text{M}$ ), the neuronal viability was measured using MTT

1 assay. As reported in Fig. 2A, the treatment with concentrations up to 5  $\mu\text{M}$  did not affect  
2 neuronal viability. Therefore, we selected the range of 1.25 – 5  $\mu\text{M}$  for the following  
3 experiments.  
4  
5  
6

### 7 8 9 **3.1.2 Antioxidant activity in neuronal SH-SY5Y and microglial THP-1 cells**

10  
11  
12 The antioxidant activity of chrysin was first evaluated in neuronal SH-SY5Y cells  
13 against the ROS formation induced by  $\text{H}_2\text{O}_2$ . After 30 min of treatment with chrysin (1.25 – 5  
14  $\mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ), the ROS formation was detected using the fluorescent probe  $\text{H}_2\text{DCF}$ -  
15 DA. As reported in Fig. 2B, chrysin significantly reduced the ROS formation in neuronal SH-  
16 SY5Y cells with all the concentrations used ( $p = 0.0001$ ). The antioxidant activity was dose-  
17 correlated with  $\text{EC}_{50}$  (concentration in which the 50% of ROS formation is reduced) 4  $\mu\text{M}$  and  
18 maximum activity at 5  $\mu\text{M}$  (51% of ROS formation inhibition). The antioxidant activity of  
19 chrysin was then evaluated in both neuronal SH-SY5Y and microglial THP-1 cells against the  
20 ROS formation induced by *t*-BuOOH, which is known to generate ROS from lipid peroxidation  
21 (Christine et al., 2018). After 30 min of treatment with chrysin (1.25 – 5  $\mu\text{M}$ ) and *t*-BuOOH  
22 (100  $\mu\text{M}$ ), the ROS formation was detected using the fluorescent probe  $\text{H}_2\text{DCF}$ -DA. As  
23 reported in Fig. 3A, chrysin significantly decreased the ROS formation in neuronal SH-SY5Y  
24 cells with all the concentrations used ( $p = 0.0011$ ). The antioxidant activity was dose-correlated  
25 with  $\text{EC}_{50}$  2  $\mu\text{M}$  and maximum activity at 5  $\mu\text{M}$  (73% of ROS formation inhibition). To better  
26 evaluate the ability of chrysin to exert its antioxidant activity at the neuronal membrane level,  
27 we measured the TAA of cytosolic and membrane-enriched fractions of neuronal SH-SY5Y  
28 cells treated with 5  $\mu\text{M}$  of chrysin for 2 h. The membrane fractions, but not the cytosolic  
29 fractions, obtained from SH-SY5Y cells treated with chrysin showed a significant increase in  
30 TAA in comparison to untreated cells (untreated cells =  $44.57 \pm 0.3526 \mu\text{molTE}/\text{mg prot}$  vs  
31 cells treated with chrysin =  $68.90 \pm 1.238 \mu\text{molTE}/\text{mg prot}$ ;  $p = 0.0001$ ).  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 The antioxidant activity of chrysin was also evaluated in microglial THP-1 cells in the  
2 same experimental conditions. As reported in Fig. 3B, Only the concentration of 5  $\mu$ M  
3 significantly decreased the ROS formation induced by *t*-BuOOH in microglial THP-1 cells  
4 (36% of ROS inhibition) ( $p = 0.0011$ ). Driven by these results, the concentration of 5  $\mu$ M was  
5 selected for the following experiments.  
6  
7  
8  
9  
10

### 11 **3.1.3 Antioxidant and inflammatory activity in microglial THP-1 cells**

12  
13 After 24 h of treatment with chrysin (5  $\mu$ M) and LPS (1  $\mu$ g/ml), the ROS formation was  
14 detected using the fluorescent probe H<sub>2</sub>DCF-DA. As reported in Fig. 4A, chrysin significantly  
15 reduced the ROS formation evoked by 24 h of treatment with LPS ( $p = 0.0020$ ). In parallel, the  
16 expressions of pro-inflammatory cytokines iNOS, IL-1 $\beta$ , and TNF $\alpha$  were evaluated in  
17 microglial THP-1 cells after 24 h of treatment with chrysin and LPS by RT-PCR. As reported  
18 in Fig. 4B-D, chrysin significantly decreased iNOS, IL-1 $\beta$ , and TNF $\alpha$  expression at the  
19 transcriptional level, suggesting its strong anti-inflammatory activity ( $p = 0.0001$ ).  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32

### 33 **3.1.4 Antioxidant activity against Fenton reaction/AlCl<sub>3</sub> in neuronal SH-SY5Y cells**

34 The antioxidant activity of chrysin was also evaluated against the pro-oxidant activity  
35 of AlCl<sub>3</sub> on Fenton reaction in neuronal SH-SY5Y cells using the fluorescent probe H<sub>2</sub>DCF-  
36 DA. As shown in Fig. 5, the adding of AlCl<sub>3</sub> (50  $\mu$ M) to FeSO<sub>4</sub>/H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M/200  $\mu$ M) reaction  
37 for 15 min further increased the ROS formation in SH-SY5Y cells. In these experimental  
38 conditions, chrysin (5  $\mu$ M) significantly abolished the pro-oxidant activity evoked by AlCl<sub>3</sub> ( $p$   
39 = 0.0001). In parallel, chrysin and AlCl<sub>3</sub> alone did not modify the basal level of ROS supporting  
40 the ability of chrysin to counteract specific ROS generated by catalytic effects of AlCl<sub>3</sub> on  
41 Fenton reaction.  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56

### 57 **3.1.5 Neuroprotective activity in neuronal SH-SY5Y cells**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

To determine the neuroprotective effects of chrysin in neuronal SH-SY5Y cells, the cytotoxicity induced by AlCl<sub>3</sub> in terms of cytostatic effect and necrosis was measured using trypan blue and PI, respectively. As reported in Fig. 6A, chrysin (5 μM) significantly counteracted the cytostatic effect induced by 24 h of treatment with AlCl<sub>3</sub> in terms of the number of total cells (p = 0.0041). In the same experimental conditions, chrysin significantly reduced the necrosis induced by AlCl<sub>3</sub> (Fig. 6B-C) (p = 0.0007).

### 3.2 *In vivo* studies

#### 3.2.1 Behavioral test

##### 3.2.1.1 OFT and CT

Mice performances in the OFT and CT are presented in Table 2. No effects on spontaneous locomotor (p = 0.9662) or exploratory (p = 0.9518) activities were observed due to any of the treatments.

##### 3.2.1.2 SDPAT

Mice performance in the SDPAT is presented in Fig. 7. No difference among groups was observed in the transfer latency time in the acquisition phase. Statistical analysis yielded a significant difference among the groups (p = 0.001) in the retention phase. Post-hoc comparisons revealed that the AlCl<sub>3</sub> group presented a lower transfer latency time (~87%) than the control group. Chrysin treatment at all tested doses (10, 30 and 100 mg/kg) prevented this impairment (p = 0.031, p = 0.037 and p = 0.032, respectively). All doses tested were not different when comparing the three chrysin doses (10, 30, and 100 mg/kg) between them (p > 0.05).

#### 3.2.2 Biochemical assays

##### 3.2.2.1 ChEs activity

1 The brain cortex and hippocampus AChE activities are presented in Fig. 8A. Statistical  
2 analysis of the brain cortex AChE activity showed no significant difference among the groups  
3 (p = 0.8028). Statistical analysis of the hippocampus AChE activity showed a significant  
4 difference among the groups (p = 0.001). Post-hoc comparisons revealed that AlCl<sub>3</sub> increased  
5 (~38%) the brain cortex AChE activity when compared to the Control group. All doses of  
6 chrysin showed a similar significant ability to normalize the AChE activity.  
7  
8  
9  
10  
11  
12  
13

14 The brain cortex and hippocampus BChE activities are presented in Fig. 8B. Statistical  
15 analysis of the brain cortex BChE activity showed no significant difference among the groups  
16 (p = 0.1166). Statistical analysis of the hippocampus BChE activity showed a significant  
17 difference among the groups (p = 0.001). Post-hoc comparisons revealed that AlCl<sub>3</sub> increased  
18 (~31%) the brain cortex AChE activity when compared to the control group. All doses of  
19 chrysin showed a similar significant ability to normalize the BChE activity.  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30

### 31 **3.2.2.2 LPO levels**

32 The brain cortex and hippocampus LPO levels are presented in Fig. 9A. Statistical  
33 analysis of the brain cortex LPO levels showed a significant difference among the groups (p =  
34 0.002). Post-hoc comparisons revealed that AlCl<sub>3</sub> increased (~97%) the brain cortex LPO  
35 levels when compared to the control group. Chrysin treatment at all tested doses prevented this  
36 increase. Statistical analysis of the hippocampus LPO levels showed a significant difference  
37 among the groups (p = 0.0001). Post-hoc comparisons revealed that AlCl<sub>3</sub> increased (~48%)  
38 the hippocampus LPO levels when compared to the control group. All doses of chrysin showed  
39 a similar significant antioxidant activity.  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55

### 56 **3.2.2.3 CP levels**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

The brain cortex and hippocampus CP levels are presented in Fig. 9B. Statistical analysis of the brain cortex CP levels showed a significant difference among the groups ( $p = 0.0167$ ). Post-hoc comparisons revealed that  $AlCl_3$  increased (~31%) the brain cortex CP levels when compared to the control group. Chrysin treatment could not prevent this increase in the brain cortex. Statistical analysis of the hippocampus CP levels showed a significant difference among the groups ( $p = 0.004$ ). Post-hoc comparisons revealed that  $AlCl_3$  increased (~36%) the hippocampus CP levels when compared to the control group. All doses of chrysin showed a similar significant antioxidant activity.

#### 3.2.2.4 SOD activity

The brain cortex and hippocampus SOD activities are presented in Fig. 9C. Statistical analysis of the brain cortex SOD activity showed a significant difference among the groups ( $p = 0.0001$ ). Post-hoc comparisons revealed that  $AlCl_3$  increased (~70%) the brain cortex SOD activity when compared to the control group. Chrysin treatment in all tested doses prevented this increase. Statistical analysis of the hippocampus SOD activity showed a significant difference among the groups ( $p = 0.0001$ ). Post-hoc comparisons revealed that  $AlCl_3$  inhibited (~28%) the hippocampus SOD activity when compared to the control group. All doses of chrysin showed a similar significant ability to normalize the SOD activity.

#### 3.2.2.5 CAT activity

The brain cortex and hippocampus CAT activities are presented in Fig. 9D. Statistical analysis of the brain cortex CAT activity showed no significant difference among the groups ( $p = 0.2743$ ). Statistical analysis of the hippocampus CAT activity showed a significant difference among the groups ( $p = 0.0001$ ). Post-hoc comparisons revealed that  $AlCl_3$  inhibited

1 (~30%) the hippocampus CAT activity when compared to the control group. All doses of  
2 chrysin showed a similar significant ability to normalize the CAT activity.  
3  
4  
5  
6

### 7 **3.2.3 Histopathology and morphometric analysis**

8  
9 Histopathological analysis showed that AlCl<sub>3</sub> exposure induced tissue damage to the  
10 cerebral cortex and the hippocampus, causing an increased incidence of degenerating neurons.  
11  
12 All groups exposed to AlCl<sub>3</sub> showed some neurons with nuclear disintegration and atrophic  
13 cytoplasm. These neurons stained intensely in pink using the hematoxylin method (data not  
14 shown), or in dark purple using the thionine acetate method, a pattern that is compatible with  
15 eosinophilic neuronal death. As reported in Fig. 10A, morphologically, all chrysin treatments  
16 decreased the occurrence of degenerative neurons with necrosis phenotype. The frequency (%)  
17 of the necrotic cell in the brain cortex and CA1 hippocampus are presented in Fig. 10B.  
18  
19 Quantitative analysis of the brain cortex indicated that treatment with chrysin caused a  
20 significant reduction in the frequency of necrotic neurons, especially in the chrysin 100 group.  
21  
22 Similar results were observed in the CA1 hippocampus, where all groups treated with chrysin  
23 had a lower frequency of necrotic neurons when compared to the AlCl<sub>3</sub> group, being the most  
24 significant reduction found in the chrysin 100 group.  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43

## 44 **4. Discussion**

45  
46 In this study, we showed the ability of chrysin to counteract the early oxidative stress  
47 evoked by different oxidant treatments, including H<sub>2</sub>O<sub>2</sub>, *t*-BuOOH, and Fenton reaction in  
48 presence of AlCl<sub>3</sub> as well as the late neurotoxicity triggered by AlCl<sub>3</sub> in neuronal SH-SY5Y  
49 cells. Regarding the antioxidant activity of chrysin, several studies show that the presence of  
50 two hydroxyl groups in the chrysin molecule is an important factor determining its antioxidant  
51 potency against the oxidation at a cellular level (Huang et al., 2012). Dynamic distribution of  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 chrysin at a cellular level can contribute to antioxidant effects recorded in neuronal SH-SY5Y  
2 cells. A recent *in vitro* study reported a quick uptake or metabolic degradation of chrysin by  
3 human hepatocytes after 2 h of incubation in a culture system (Huang et al., 2012).  
4 Interestingly, we demonstrated in similar experimental conditions the scavenger activity of  
5 chrysin against the ABTS<sup>•+</sup> radical at membrane levels in neuronal SH-SY5Y cells, suggesting  
6 the dynamic distribution of chrysin in the membrane. It was reported the membrane localization  
7 and distribution of various flavonoids, including chrysin, can improve the antioxidant activity  
8 in proximity to the double bonds of the cell membrane lipids (Günther et al., 2015). These  
9 pieces of evidence may underlie the ability of chrysin to reduce the ROS formation elicited by  
10 *t*-BuOOH in both neuronal SH-SY5Y and microglial THP-1 cells. Since *t*-BuOOH is an  
11 oxidant compound that mimics the LPO in neuronal tissues, these results also suggest the  
12 ability of chrysin to counteract the LPO at the neuronal and microglial membrane levels.  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28

29 The antioxidant activity of chrysin at the membrane level could also modulate the redox  
30 signaling in microglial cells. It is noted that NADPH oxidases present in the microglial  
31 membrane are a source of ROS, such as H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup>, involved in pro-inflammatory  
32 microglial activation (Haslund-Vinding et al., 2017). In this regard, we demonstrated that  
33 chrysin reduced both the ROS formation and activation of pro-inflammatory gene expression,  
34 such as iNOS, IL-1 $\beta$ , and TNF $\alpha$ , stimulated by LPS in microglial THP-1 cells, suggesting its  
35 potential ability to modulate the redox signaling at the membrane level. These findings  
36 corroborate with the ability of chrysin to counteract the oxidative stress-driven  
37 neuroinflammation in various experimental models of focal cerebral ischemia/reperfusion  
38 injury, Parkinson's disease and isoniazid-induced neurotoxicity (Yao et al., 2014; Goes et al.,  
39 2018; Çelik et al., 2020). However, we cannot exclude that chrysin exerts anti-inflammatory  
40 effects through other molecular mechanisms such as the inhibition of nuclear factor-kappa B,  
41 which is a signaling molecule involved in neuroinflammation (Ha et al., 2010; Li et al., 2019).  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

In this regard, further studies are needed to elucidate the anti-inflammatory mechanisms of chrysin.

Lastly, we demonstrated for the first time the antioxidant activity of chrysin against the pro-oxidant ability of Al in neuronal SH-SY5Y cells. The oxidative damage of Al observed in various *in vitro* and *in vivo* studies is a feature not expected for a metal without a redox capability (Singla and Dhawan, 2013). Several studies suggest that the increase of  $O_2^{\cdot-}$  - lifetime mediated by Al -  $O_2^{\cdot-}$  - complex coupled to Fenton reaction gives oxidative stress in biological environments (Singla and Dhawan, 2013). In this context, a recent study demonstrated that chrysin has a good scavenging ability for  $O_2^{\cdot-}$ . This is due to the nucleophilic reaction taking place between the unsaturated carbonyl group of chrysin and  $O_2^{\cdot-}$  - as well as the single-electron transfer between the phenolic hydroxyl group and  $O_2^{\cdot-}$  (Zheng et al., 2005). We can therefore assume that chrysin breaks the pro-oxidant activity of Al through its ability to neutralize the  $O_2^{\cdot-}$  - downstream of the oxidative process triggered by Al.

The cell membrane is an important biological target of  $Al^{3+}$ , and the damage caused to membrane components may impair important functions. In particular,  $Al^{3+}$  ions altered the lipid profile and fluidity of the neuronal membrane, leading to a loss of membrane integrity (Singla and Dhawan, 2013). According to these evidence, we recorded the membrane integrity loss in terms of necrosis in neuronal SH-SY5Y cells treated with  $AlCl_3$  for 24 h. Remarkably, chrysin also counteracted the necrosis induced by  $AlCl_3$  in neuronal SH-SY5Y cells suggesting its ability to preserve the neuronal membrane integrity.

Taken together, *in vitro* results suggest that antioxidant and neuroprotective effects of chrysin against the toxicity of  $AlCl_3$  share the same neuronal membrane target. The chrysin may act as a membrane shield against early oxidative events that contribute to progressive neurodegeneration and neuronal death triggered by  $AlCl_3$  exposure.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Considering the limitations of the *in vitro* models to predict the *in vivo* response, the ability of chrysin to counteract the oxidative stress and neuronal death induced by Al was further evaluated in a mouse model of neurotoxicity induced by chronic exposure of ninety days to AlCl<sub>3</sub>. Taken together, the *in vivo* results indicate that daily oral chrysin administration during forty-five consecutive days prevented non-spatial learning and memory impairment elicited by AlCl<sub>3</sub>. In addition, the chrysin supplementation also normalized the increased activities of AChE and BChE, decreased the oxidative damage, in terms of LPO and protein carbonylation, as well as cell necrosis in the brain cortex and hippocampus.

It is well-known that in neurodegenerative disorders the CNS sensory and motor regions damage can be present (Albers et al., 2015). This kind of impairment can induce false results when behavioral memory tests are assessed. We, therefore, evaluated the motor activity of animals. The OFT and CT performance showed that AlCl<sub>3</sub> treatment did not affect their motor activity and interference in behavioral tests as well as the SDPAT acquisition phase. However, AlCl<sub>3</sub> affected the non-spatial long-term memory as recorded by the latency time decrease in the step down during the retention phase. This cognitive impairment in long-term memory was then recovered by chrysin treatment. In this context, AlCl<sub>3</sub> increased the AChE activity in the hippocampus, but not in the brain cortex, interfering with the processes of memory consolidation as observed in SDPAT. These findings suggest that the impairment of AChE activity in hippocampus structure elicited by AlCl<sub>3</sub> is enough to establish a cognitive deficit. A similar ChE activity pattern in the brain cortex and hippocampus after the different treatments were also recorded for BChE.

Several pre-clinical studies recorded flavonoid-inhibitor effects on ChEs activity indicating their potential use for the treatment of motor neuron and dementia disorders, such as myasthenia gravis, Lewy body dementia, and Alzheimer's disease (Khan et al., 2018). Among the plant-derived flavonoids, chrysin showed the ability to inhibit electric eels' AChE

1 activity as well as human AChE and BChE in *in vitro* systems (Balkis et al., 2015; Taslimi et  
2 al., 2017).  
3

4 It is established that Al promotes ROS and LPO in different tissues, including the brain  
5 (Samarghandian et al., 2019). As expected, the AlCl<sub>3</sub> chronic administration to mice induced a  
6 significant LPO, in terms of TBARS levels, in the brain cortex and hippocampus. Remarkably,  
7 the chrysin treatment decreased the LPO elicited by AlCl<sub>3</sub> in both brain regions confirming its  
8 ability to protect the tissue membrane from oxidative damage. These *in vivo* results corroborate  
9 with the *in vitro* antioxidant profile of chrysin recorded in neuronal SH-SY5Y cells.  
10  
11  
12  
13  
14  
15  
16  
17  
18

19 Along with the LPO elevation, the ROS formation also causes protein oxidation with  
20 the formation of various carbonyl species. In this regard, AlCl<sub>3</sub> chronic exposure increased the  
21 CP levels in the brain cortex and hippocampus, indicating that the pro-oxidant action of Al  
22 targets both the polyunsaturated fatty acids and proteins. Remarkably, the chrysin treatment  
23 decreased the CP levels suggesting the ability of this flavonoid to prevent the triggering of the  
24 protein carbonylation process. It is noted that the protein carbonylation is non-enzymatic  
25 leading to irreversible modifications in proteins (Ferdorova et al., 2014).  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35

36 The role and effectiveness of the first-line defense antioxidants that include SOD, CAT,  
37 and glutathione peroxidase (GPX) are important and indispensable in the entire defense  
38 strategy of antioxidants, including flavonoids (Ighodaro and Akinloye, 2017). Under oxidative  
39 stress conditions, SOD acts as a first-line defense against superoxide as it converts the O<sub>2</sub><sup>·-</sup>  
40 radical to H<sub>2</sub>O<sub>2</sub> and molecular oxygen. Then CAT and the GPX, using reduced glutathione,  
41 converts the H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O (Maya et al., 2016). Considering that, Al can strengthen the  
42 oxidative capacity of O<sub>2</sub><sup>·-</sup>, directly or indirectly through catalysis of the Fenton reaction, we,  
43 therefore, assessed the activity of SOD and CAT, two enzymes involved in the first extremely  
44 efficient reactions of O<sub>2</sub><sup>·-</sup> detoxification.  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 The conflicting effects promoted by Al on CAT and SOD activities in different rodent  
2 brain regions has been described in the literature (Atienzar et al., 1998; Yuan et al., 2012;  
3 Jelenković et al., 2014; Benyettou et al., 2017; Sadauskiene et al., 2020), highlighting critical  
4 experimental conditions, such as Al concentration, treatment time, species and age of animals.  
5 Our *in vivo* studies showed that the AlCl<sub>3</sub> treatment for 90 days inhibited CAT and SOD  
6 activities in the hippocampus and increased the SOD activity in the cortex. The contradictory  
7 increase of SOD can be interpreted as an adaptive neuronal response of the frontal cortex  
8 involving a strong oxidative stress increase elicited by Al (Navarro et al., 2009). The different  
9 composition of lipids between brain cortex and hippocampus and cortex could explain a greater  
10 O<sub>2</sub><sup>·-</sup> formation induced by Al and antioxidant response in terms of SOD levels in the  
11 hippocampus. However, the chrysin treatment was able to reverse these enzymes activities  
12 alterations, corroborating with the ability of chrysin to counteract early oxidant events at a brain  
13 level.  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30

31 Chronic oxidative damage induced by Al also leads to neuronal death, predominant in  
32 necrosis (Feng et al., 2014; Yamawaki et al., 2019). The neuronal death in the brain cortex and  
33 hippocampus can affect the learning and memory processes (Matthews, 2015; Yamawaki et  
34 al., 2019). Our histological results in mice recorded that the treatment with AlCl<sub>3</sub> induces  
35 significant neurodegeneration in the brain cortex and hippocampus. In particular, the  
36 histological analysis showed focal and neurodegenerative changes along with the increased  
37 number of degenerative neurons with necrosis phenotype. In this regard, the necrotic neuron  
38 frequency in the brain cortex was higher than hippocampus suggesting that the cortex is the  
39 most sensitive brain area to neuronal damage elicited by AlCl<sub>3</sub>. Interestingly, a recent study  
40 recorded that the Al content of different human brain tissues, including cortex, in AD, autism  
41 spectrum disorder, and multiple sclerosis was significantly elevated suggesting a critical role  
42 of this metal in the etiology of these neurodegenerative diseases (Exley and Mold, 2019). The  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 neurodegeneration in terms of necrosis was attenuated by chrysin in both the brain cortex and  
2 hippocampus.  
3

4  
5 Taken together, *in vivo* results show that oral administration of chrysin can prevent and  
6  
7 counteract oxidative damage and neurodegeneration in the brain cortex and hippocampus  
8  
9 prompted by chronic exposure to AlCl<sub>3</sub>. In particular, all the doses (10, 30, and 100 mg/kg) of  
10  
11 chrysin used showed significant antioxidant and neuroprotective effects in both brain regions.  
12  
13 However, the dose of 10 mg/kg chrysin already exhibited the maximum antioxidant and  
14  
15 neuroprotective activity at a brain level. This evidence suggests that the BBB crossing  
16  
17 processes could become saturated limiting the increase of the chrysin levels in the brain. After  
18  
19 oral administration, the chrysin is highly biotransformed in the liver, during which conjugated  
20  
21 metabolites chrysin-7-sulfate and chrysin-7-glucuronide are formed in mice (Mohos et al.,  
22  
23 2020). Despite these conjugates appear at higher concentrations in the circulation than chrysin,  
24  
25 they probably do not contribute to the cerebral effects because they cannot cross the BBB as  
26  
27 predicted by *in silico* studies (Daiana and Zoete, 2016; Daiana et al., 2016). These evidence,  
28  
29 therefore, support that chrysin is predominant in brain action. It is probably that rising the dose  
30  
31 of chrysin also increases its metabolites that limit the availability of chrysin in the circulation  
32  
33 to cross the BBB, explaining the same neuroprotective effects recorded between 10 and 100  
34  
35 mg/kg.  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45

## 46 **5. Conclusion**

47  
48 This study demonstrates the ability of chrysin by oral administration to protect critical  
49  
50 brain regions for cognitive function from oxidative damage and neurodegeneration induced by  
51  
52 chronic AlCl<sub>3</sub> exposure. Regarding the mechanisms of neuroprotective action, chrysin showed  
53  
54 the ability to act as a membrane shield against early oxidative events mediated by O<sub>2</sub><sup>·-</sup> and  
55  
56 other ROS that contribute to neuronal death triggered by AlCl<sub>3</sub> exposure.  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

These findings support the potential use of chrysin as a food supplement for the prevention of neurological and neurodegenerative diseases or the maintenance of a healthy brain.

### **Conflict of interest**

The authors disclose no actual or potential conflicts of interest, including financial, personal, or relationships with other people or organizations. All authors have contributed to the work and agreed with the presented findings.

### **Acknowledgments**

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG).

### **6. References**

- Aebi, H., 1984. Catalase. *Enzyme Act Oxidoreductases*. 51, 674–684.  
<https://doi.org/10.1086/330448>.
- Albers, M.W., Gilmore, G.C., Kaye, J., Murphy, C., Wingfield, A., Bennett, D.A., Boxer, A.L., Buchman, A.S., Cruickshanks, K.J., et al. 2015. At the interface of sensory and motor dysfunctions and Alzheimer’s disease. *Alzheimers Dement*. 11, 70–98.  
<https://doi.org/10.1016/j.jalz.2014.04.514>.
- Atienzar, F., Desor, D., Burnel, D., Keller, J. M., Lehr, P., Vasseur, P., 1998. Effect of aluminum on superoxide dismutase activity in the adult rat brain. *Biological Trace Element Research*. 65, 19–30. <https://doi.org/10.1007/BF02784111>.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- Balkis, A., Tran, K., Lee, Y.Z., Ng, K., 2015. Screening flavonoids for inhibition of acetylcholinesterase identified baicalein as the most potent inhibitor. *The Journal of Agricultural Science*. 7, 26–35. <https://doi.org/10.5539/jas.v7n9p26>.
- Benyettou, I., Kharoubi, O., Hallal, N., Benyettou H.A., Tair, K., Belmokhtar, M., Aoues, A., Ozaslan, M., 2017. Aluminium-induced behavioral changes and oxidative stress in developing rat brain and the possible ameliorating role of omega-6/omega-3 ratio. *Journal of Biological Sciences*. 17, 106-117. <https://doi.org/10.3923/jbs.2017.106.117>.
- Boissier, J., Tardy, J., Diverres, J.C., 1960. Une nouvelle méthode simple pour explorer l'action «tranquillisante»: le test de la cheminée. *Journal of Experimental Medicine*. 3, 81–84. <https://doi.org/10.1159/000134913>.
- Borowska S, Brzóska MM., 2015. Metals in cosmetics: implications for human health. *Journal of Applied Toxicology*. 35(6), 551-72. <https://doi.org/10.1002/jat.3129>.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 72, 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
- Çelik, H., Kucukler, S., Çomaklı, S., Caglayan, C., Özdemir, S., Yardım, A., Karaman, M., Kandemir, F.M., 2020. Neuroprotective effect of chrysin on isoniazid-induced neurotoxicity via suppression of oxidative stress, inflammation and apoptosis in rats. *Neurotoxicology*. 81, 197-208. <https://doi.org/10.1016/j.neuro.2020.10.009>.
- Christine, W., Dagmar, F., Berenike, L., Christian, T., Teodora, N., John, B., Peter, G., Peter, W., Anna, S.S., Stefan, K., Cornelia, D., 2018. T-BuOOH induces ferroptosis in human and murine cell lines. *Archives of Toxicology*. 92, 759–775. <https://doi.org/10.1007/s00204-017-2066-y>.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- Daiana, A., and Zoete, V., 2016. A boiled- egg to predict gastrointestinal absorption and brain penetration of small molecules. *Chemistry Europe*. 11, 1117–1121. <https://doi.org/10.1002/cmdc.201600182>. Available: <http://www.swissadme.ch/>.
- Daiana, A., Michielin, O., Zoete, V., 2016. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific reports*. 7:42717, 1–12. <https://doi.org/10.1038/srep42717>. Available: <http://www.swissadme.ch/>.
- Di Martino, R.M.C., Pruccoli, L., Bisi, A., Gobbi, S., Rampa, A., Martinez, A., Pérez C., Martinez-Gonzalez, L., Paglione, M., Di Schiavi, E., Seghetti, F., Tarozzi, A., Belluti, F., 2020. Novel curcumin-diethyl fumarate hybrid as a dualistic gsk-3 $\beta$  inhibitor/nrf2 inducer for the treatment of Parkinson's disease. *ACS Chemical Neuroscience*. 2020, 1-30. <https://doi.org/10.1021/acchemneuro.0c00363>.
- Drobyshev, E. J., Solovyev, N. D., Gorokhovskiy, B.M., Kashuro, V. A., 2018. Accumulation patterns of sub-chronic aluminum toxicity model after gastrointestinal administration in rats. *Biological Trace Element Research*. 185, 384–394. <https://doi.org/10.1007/s12011-018-1247-8>.
- El-Askarya, H., Haggagb, M., Husseina, D., Husseinb, S., Sleemd, A., 2017. Bioactivity-guided study of *Passiflora caerulea L.* leaf extracts. *Iranian Journal of Pharmaceutical Research*. 16, 46–57. PMID:29844775.
- Ellman, G.L., Courtney, K.D., Andres, V., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*. 7, 88–95. [https://doi.org/10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9).
- Exley, C., 2013. Human exposure to aluminium. *Environmental Science: Processes & Impacts*. 15, 1785–1970. <https://doi.org/10.1039/c3em00374d>.



- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- Farhat, S.M., Mahboob, A., Ahmed, P., 2019. Oral exposure to aluminum leads to reduced nicotinic acetylcholine receptor gene expression, severe neurodegeneration and impaired hippocampus dependent learning in mice. *Drug and Chemical Toxicology*. 54, 1–9. [https://doi.org/ 10.1080/01480545.2019.1587452](https://doi.org/10.1080/01480545.2019.1587452).
- Farkhondeh, T., Abedi, F., Samarghandian, S., 2019. Chrysin attenuates inflammatory and metabolic disorder indices in aged male rat. *Biomedicine & Pharmacotherapy*. 109, 1120–1125. <https://doi.org/10.1016/j.biopha.2018.10.059>.
- Fedorova, M., Bollineni, R.C., Hffmann, R., 2014. Protein carbonylation as a major hallmark of oxidative damage: update of analytical strategies. *Mass Spectrometry Reviews*. 33, 79–97. [https://doi.org/ 10.1002/mas.21381](https://doi.org/10.1002/mas.21381).
- Feng, X., Qin, H., Shi, Q., Zhang, Y., Zhou, F., Wu, H., Ding, S., Niu, Z., Shen, P., 2014. Chrysin attenuates inflammation by regulating M1/M2 status via activating PPAR $\gamma$ . *Biochemical Pharmacology*. 89, 503–514. <https://doi.org/10.1016/j.bcp.2014.03.016>.
- Fernandes, R.M., Corrêa, M.G., Aragão, W.A., Nascimento, P.C., Cartágenes, S.C., Rodrigues, C.A., et al., 2020. Preclinical evidences of aluminum-induced neurotoxicity in hippocampus and pre-frontal cortex of rats exposed to low doses. *Ecotoxicology and Environmental Safety*. 15:206, 111 - 139. <https://doi.org/10.1016/j.ecoenv.2020.111139>.
- Garza-Lombó, C., Posadas, Y., Quintanar, L., Gonsebatt, M.E., Franco, R., 2018. Neurotoxicity linked to dysfunctional metal ion homeostasis and xenobiotic metal exposure: redox signaling and oxidative stress. *Antioxidants & Redox Signaling*. 28(18), 1669-1703. <https://doi.org/10.1089/ars.2017.7272>.
- Goes, A.T., Jesse, C.R., Antunes, M.S., Lobo, F.V, Lobo, A.B., Luchese, C., Paroul, N., Boeira, S.P., 2018. Protective role of chrysin on 6-hydroxydopamine-induced neurodegeneration a mouse model of Parkinson's disease: Involvement of neuroinflammation and

neurotrophins. *Chemical-Biological Interactions.* 5:279, 111-120.

<https://doi.org/10.1016/j.cbi.2017.10.019>.

Günther, G., Berríos, E., Pizarro, N., Valdés, K., Montero, G., Arriagada, F., Morales, J., 2015.

Flavonoids in microheterogeneous media, relationship between their relative location and their reactivity towards singlet oxygen. *Plus One.* 10, 729-749. <https://doi.org/10.1371/journal.pone.0129749>.

Ha, S.K., Moon, E., Kim, S.Y., 2010. Chrysin suppresses LPS-stimulated proinflammatory responses by blocking NF- $\kappa$ B and JNK activations in microglia cells. *Neuroscience Letters.* 26:485, 143-147. <https://doi.org/10.1016/j.neulet.2010.08.064>.

Hadjmohammadi, Saman, S., M., Nazari, J., 2010. Separation optimization of quercetin, hesperetin and chrysin in honey by micellar liquid chromatography and experimental design. *Journal of Separation Science.* 33, 3144–3151. <https://doi.org/10.1002/jssc.201000326>.

Haslund-Vinding, J., McBean, G., Jaquet, V., Vilhardt, F., 2017. NADPH oxidases in oxidant production by microglia: activating receptors, pharmacology and association with disease. *British Journal of Pharmacology.* 174(12), 1733-1749. <https://doi.org/10.1111/bph.13425>.

He, X., Wang, Y., Bi, M., Du, G., 2012. Chrysin improves cognitive deficits and brain damage induced by chronic cerebral hypoperfusion in rats. *British Journal of Pharmacology.* 680, 41–48. <https://doi.org/10.1016/j.ejphar.2012.01.025>.

Huang, C.S., Lii, C.K., Lin, A.H., Yeh, Y.W., Yao, H.T., Li, C.C., Wang, T.S., Chen, H.W., 2012. Protection by chrysin, apigenin, and luteolin against oxidative stress is mediated by the Nrf2-dependent up-regulation of heme oxygenase 1 and glutamate cysteine ligase in rat primary hepatocytes. *Archives of Toxicology.* 87(1), 167-178. <https://doi.org/10.1007/s00204-012-0913-4>.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- Ighodaro, O.M., Akinloye, O.A., 2017. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*. 2017, 1-7. <https://doi.org/10.1016/j.ajme.2017.09.001> 209.
- Jelenković, A, Jovanović MD, Stevanović I, Petronijević N, Bokonjić D, Zivković J, Igić R., 2014. Influence of the green tea leaf extract on neurotoxicity of aluminium chloride in rats. *Phytotherapy Research*. 28(1):82-87. <https://doi.org/10.1002/ptr.4962>.
- Kalogeropoulos, N., Yanni, A., Koutrotsios, G., Aloup, M., 2013. Bioactive microconstituents and antioxidant properties of wild edible mushrooms from the island of Lesbos, Greece. 55, 378–385. <https://doi.org/10.1016/j.fct.2013.01.010>.
- Kameyama, T., Nabeshima, T., Kozawa, T., 1986. Step-down-type passive avoidance- and escape-learning method - Suitability for experimental amnesia models. *Journal of Pharmacological and Toxicological Methods*. 16, 39–52. [https://doi.org/10.1016/0160-5402\(86\)90027-6](https://doi.org/10.1016/0160-5402(86)90027-6).
- Khan, H., Marya, S., Amin, M.A., Kamal, S., Patel, S., 2018. Flavonoids as acetylcholinesterase inhibitors: Current therapeutic standing and future prospects. *Biomedicine & Pharmacotherapy*. 101, 860–870. <https://doi.org/10.1016/j.biopha.2018.03.007>.
- Klotz K, Weistenhöfer W, Neff F, Hartwig A, van Thriel C, Drexler H., 2017. The Health Effects of Aluminum Exposure. *Deutsches Ärzteblatt Internationa* 114(39), 653-659. <https://doi.org/10.3238/arztebl.2017.0653>.
- Krewski, D., Yokel, R., Nieboer, E., Borchelt, D., Cohen, J., Harry, J., Kacew, S., Lindsay, J., Mahfouz, A., Rondeau, V., 2007. Human health risk assessment for aluminium, aluminium oxide, and aluminium hydroxide. *Journal of Toxicology and Environmental Health*. 10, 1–269. <https://doi.org/10.1080/10937400701597766>.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, S., Shaltiel, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology*. 186, 464–478. [https://doi.org/10.1016/0076-6879\(90\)86141-H](https://doi.org/10.1016/0076-6879(90)86141-H).
- Li, R., Zang, A., Zhang, L., Zhang, H., Zhao, L., Qi, Z., Wang, H., 2014. Chrysin ameliorates diabetes-associated cognitive deficits in Wistar rats. *Neurological Sciences*. 35, 1527–1532. <https://doi.org/10.1007/s10072-014-1784-7>.
- Li, Z., Chu, S., He, W., Zhang, Z., Liu, J., Cui, L., Yan, X., Li, D., Chen, N., 2019. A20 as a novel target for the anti-neuroinflammatory effect of chrysin via inhibition of NF-κB signaling pathway. *Brain, Behavior, and Immunity*. 79, 228-235. <https://doi.org/10.1016/j.bbi.2019.02.005>.
- Lukiw, W.J., Kruck, T.P.A., Percy, M.E., Pogue A.I., Alexandrov, P.N. Walsh, W.J, Sharfman N.M., Jaber, V.R., Zhao, Y., Li, W., Bergeron, C., Culicchia, F., Fang, Z., McLachlan, D.R.C., 2018. Aluminum in neurological disease - a 36-year multicenter study. *Journal of Alzheimers Disease and Parkinsonism*. 8, 457–469. <https://doi.org/10.4172/2161-0460.1000457>.
- Mani, R., and Natesan, V., 2018. Chrysin: Sources, beneficial pharmacological activities, and molecular mechanism of action. *Phytochemistry*. 145, 187–196. <https://doi.org/10.1016/j.phytochem.2017.09.016>.
- Manzoli, E.S., Serpeloni, J.M., Grotto, D., Bastos, J.K., Antunes, L.M., Barbosa, J.F., Barcelos, G.R., 2015. Protective effects of the flavonoid chrysin against methylmercury-induced genotoxicity and alterations of antioxidant status, in vivo. *Oxidative Medicine and Cellular Longevity*. 2015, 1-8. <https://doi.org/10.1155/2015/602360>.
- Matthews, B.R., 2015. Memory dysfunction. *Behavioral Neurology and Neuropsychology*. 21, 613–626. <https://doi.org/10.1212/01.CON.0000466656.59413.29>.

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- Maya S., Prakash, T., Madhu, K.D., Goli D., 2016. Multifaceted effects of aluminium in neurodegenerative diseases: A review. *Biomedicine & Pharmacotherapy*. 83, 746-754. <https://doi.org/10.1016/j.biopha.2016.07.035>.
- Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry*. 247(10):3170-5. PMID: 4623845
- Mohos, V., Fliszár-Nyúl, E., Schilli, G., Hetényi, C., Lemli, B., Kunsági-Máté, S., Bognár B., Poór, M., 2018. Interaction of Chrysin and Its Main Conjugated Metabolites Chrysin-7-Sulfate and Chrysin-7-Glucuronide with Serum Albumin. *International Journal of Molecular Sciences*. 19, 1-15. <https://doi.org/10.3390/ijms19124073>.
- Mohos, V., Fliszár-Nyúl, E., Ungvári, O., Bakos, É., Kuffa, K., Bencsik, T., Zsidó, B.Z., Hetényi, C., Telbisz, Á., Özvegy-Laczka, C., Poór, M., 2020. Effects of chrysin and its major conjugated metabolites chrysin-7-sulfate and chrysin-7-glucuronide on cytochrome p450 enzymes and on OATP, P-gp, BCRP, and MRP2 transporters. *Drug Metabolism & Disposition*. 48(10), 1064-1073. <https://doi.org/10.1124/dmd.120.000085>.
- Montgomery, K.C., 1955. The relation between fear induced by novel stimulation and exploratory behavior. *Journal of Comparative Psychology*. 254–260. <https://doi.org/10.1037/0033-2909.126.1.78>.
- Nabavi, F.S., Braidy, N., Habtemariam, S., Erdogan, I., Daglia, M., Manayi, A., Gortzi, O., Nabavi, S.M., 2015. Neuroprotective effects of chrysin: From chemistry to medicine. *Neurochemistry International*. 90, 224–231. <https://doi.org/10.1016/j.neuint.2015.09.006>.
- Navarro A, Boveris A, Báñez M., Sánchez-Pino M., Gómez C, Muntané G, Ferrer I., 2009. Human brain cortex: mitochondrial oxidative damage and adaptive response in Parkinson

disease and in dementia with Lewy bodies. Free Radical Biology & Medicine. 46(12):1574-1580. <https://doi.org/10.1016/j.freeradbiomed.2009.03.007>.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 95, 351–358. [https://doi.org/10.1016/0003-2697\(79\)90738-3](https://doi.org/10.1016/0003-2697(79)90738-3).

Oliveira, T.S., Thomaz, D.V., da Silva, H.F., Cerqueira, L.B., Garcia, L.F., Gil, H.P.V., Pontarolo, R., Campos, F.R., Costa, E.A., Santos, F.C.S., Gil, E.S., Ghedini, P.C., 2018. Neuroprotective effect of *Caryocar brasiliense* camb. Leaves is associated with anticholinesterase and antioxidant properties. Oxidative Medicine and Cellular Longevity. 1–12. <https://doi.org/10.1155/2018/9842908>.

Ortiz, C.J.C., Damasio, C.M., Pruccoli, L., Nadur, N.F., de Azevedo, L.L., Guedes, I.A., Dardenne, L.E., Kümmerle, A.E., Tarozzi, A., Viegas, C. J., 2020. Cinnamoyl-n-acylhydrazone-donepezil hybrids: synthesis and evaluation of novel multifunctional ligands against neurodegenerative diseases. Neurochemical Research. 45, 3003–3020. <https://doi.org/10.1007/s11064-020-03148-2>.

Pruccoli, L., Morroni, F., Sita, G., Hrelia, P., Tarozzi, A., 2020. Esculetin as a bifunctional antioxidant prevents and counteracts the oxidative stress and neuronal death induced by amyloid protein in SH-SY5Y cells. Antioxidants. 9(6), 551-566. <https://doi.org/10.3390/antiox9060551>.

Reis, J.S., Oliveira, G.B., Monteiro, M.C., Machado, C.S., Torres, Y.R., Prediger, R.D., Maia, C.S., 2014. Antidepressant- and anxiolytic-like activities of an oil extract of propolis in rats. Phytomedicine. 21, 1466–1472. <https://doi.org/10.1016/j.phymed.2014.06.001>.

Ruipérez, F., Mujika, J.I., Ugalde, J.M., Exley, C., Lopez, X., 2012. Pro-oxidant activity of aluminum: promoting the Fenton reaction by reducing Fe(III) to Fe(II). Journal of Inorganic Biochemistry. 117, 118-123. <https://doi.org/10.1016/j.jinorgbio.2012.09.008>.

- 1 Sadauskiene, I., Liekis, A., Staneviciene, I., Naginiene, R., Ivanov, L., 2020. Effects of long-  
2 term supplementation with aluminum or selenium on the activities of antioxidant  
3 enzymes in mouse brain and liver. *Catalysts*. 10(5), 585.  
4  
5 <https://doi.org/10.3390/catal10050585>.  
6  
7  
8  
9 Sairazi, N.S.M., Sirajudeen, K.N.S., Asari, M.A., Mummedy, S., Muzaimi, M., Sulaiman, S.A.  
10 2017. Effect of tualang honey against KA-induced oxidative stress and  
11 neurodegeneration in the cortex of rats. *BMC Complementary Medicine and Therapies*.  
12 17–31. <https://doi.org/10.1186/s12906-016-1534-x>.  
13  
14  
15  
16  
17  
18 Samarghandian, S., Farkhondeh, T., Azimi-nezhad, M., Mohammad, A., Shahri. P., 2019.  
19 Antidotal or protective effects of honey and chrysin, its major polyphenols, against  
20 natural and chemical toxicities. *Acta Biomedica*. 90, 2–18.  
21  
22 <https://doi.org/10.23750/abm.v90i4.7534>.  
23  
24  
25  
26  
27  
28 Sarkaki, A., Farbood, Y., Mansouri, S.M.T., Badavi, M., Khorsandi, L., Dehcheshmeh, M.G.,  
29 Shooshtari, M.K., 2019. Chrysin prevents cognitive and hippocampal long-term  
30 potentiation deficits and inflammation in rat with cerebral hypoperfusion and reperfusion  
31 injury. *Life Science*. 226, 2002–209. <https://doi.org/10.1016/j.lfs.2019.04.027>.  
32  
33  
34  
35  
36  
37  
38 Singla, N., Dhawan, D.K., 2013. Zinc protection against aluminium induced altered lipid  
39 profile and membrane integrity. *Food and Chemical Toxicology*. 55, 18-28.  
40  
41 <https://doi.org/10.1016/j.fct.2012.12.047>. Epub 2013 Jan 9. PMID: 23313339.  
42  
43  
44  
45 Tal, Y., Anavi, S., Reisman, M., Samach, A., Tirosh, O., Troen, A.M., 2016. The  
46 neuroprotective properties of a novel variety of passion fruit. *Journal of Functional*  
47 *Foods*. 23, 359–369. <https://doi.org/10.1016/j.jff.2016.02.039>.  
48  
49  
50  
51  
52  
53 Tarozzi, A., Bartolini, M., Piazzzi, L., Valgimigli, L., Amorati, R., Bolondi, C., Djemil, A.,  
54 Mancini, F., Andrisano, V., Rampa, A., 2014. From the dual function lead AP2238 to  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1 AP2469, a multi-target-directed ligand for the treatment of Alzheimer's disease.  
2 Pharmacology Research & Perspectives. 2(2), 1-14. <https://doi.org/10.1002/prp2.23>.  
3  
4 Tarozzi, A., Morroni, F., Bolondi, C., Sita, G., Hrelia P., Djemil, A., Cantelli-Forti, G., 2012.  
5 Neuroprotective effects of erucin against 6-hydroxydopamine-induced oxidative damage  
6 in a dopaminergic-like neuroblastoma cell line. International Journal of Molecular  
7 Sciences. 13(9), 10899–1091. <https://doi.org/10.3390/ijms130910899>  
8  
9 Taslimi, P., Caglayan, C., Gulcin, I., 2017. The impact of some natural phenolic compounds  
10 on carbonic anhydrase, acetylcholinesterase, butyrylcholinesterase, and  $\alpha$ -glycosidase  
11 enzymes: An antidiabetic, anticholinergic, and antiepileptic study. Journal of  
12 Biochemical and Molecular Toxicology. 31, 1–7. <https://doi.org/10.1002/jbt.21995>.  
13  
14 Thomaz, D.V., Peixoto, L.F., Oliveira, T.S., Fajemiroye, J.O., Neri, H.F.S., Xavier, C.H.,  
15 Costa, E.A., Santos, F.C.A., Gil, E.S., Ghedini, P.C., 2018. Antioxidant and  
16 neuroprotective properties of *Eugenia dysenterica* leaves. Oxidative Medicine and  
17 Cellular Longevity. 2018, 1–10. <https://doi.org/10.1155/2018/3250908>.  
18  
19 Willhite, C.C., Karyakina, N.A., Yokel, R.A., Yenugadhati, N., Wisniewski, T.M., Arnold,  
20 I.M., Momoli, F., Krewski, D., 2014. Systematic review of potential health risks posed  
21 by pharmaceutical, occupational and consumer exposures to metallic and nanoscale  
22 aluminum, aluminum oxides, aluminum hydroxide and its soluble salts. Critical Reviews  
23 in Toxicology. 44, 1–80. <https://doi.org/10.3109/10408444.2014.934439>  
24  
25 Yamawaki, N., Corcoran, K.A., Guedea, A.L., Shepherd, G.M., Radulovic, G., 2019.  
26 Differential contributions of glutamatergic hippocampal - retrosplenial cortical  
27 projections to the formation and persistence of context memories. Cerebral Cortex. 29,  
28 2728–2736. <https://doi.org/10.1093/cercor/bhy142>.  
29  
30 Yao, Y., Chen, L., Xiao, J., Wang, C., Jiang, W., Zhang, R., Hao, J., 2014. Chrysin protects  
31 against focal cerebral ischemia/reperfusion injury in mice through attenuation of  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



oxidative stress and inflammation. *International Journal of Molecular Sciences*. 13:15,  
20913 - 20926. <https://doi.org/10.3390/ijms151120913>.

Yuan CY, Lee YJ, Hsu G.S., 2012. Aluminum overload increases oxidative stress in four  
functional brain areas of neonatal rats. *Journal of Biomedical Science*. 19(1), 30-51.  
<https://doi.org/10.1186/1423-0127-19-51>.

Zheng, J.B., Zhang, H.F., Gao, H., 2005. Investigation on electrochemical behavior and  
scavenging superoxide anion ability of chrysin at mercury electrode. *Chinese Journal of  
Chemistry*. 23(8), 1042-1046. <https://doi.org/10.1002/cjoc.200591042>.

## Figure captions

**Figure 1.** Chemical structure of chrysin (IUPAC: 5,7-Dihydroxy-2-phenyl-4H-chromen-4-one, CAS registry number: 480-40-0).

**Figure 2.** Cytotoxicity and antioxidant activity of chrysin against H<sub>2</sub>O<sub>2</sub>-induced ROS formation in neuronal SH-SY5Y cells. **(A)** Cells were incubated for 24 h with various concentrations of chrysin (1.25 – 40 μM). At the end of incubation, the neuronal viability was measured using MTT assay. **(B)** Cells were incubated for 30 min with the fluorescent probe H<sub>2</sub>DCF-DA. At the end of incubation, cells were treated with various concentrations of chrysin (1,25 – 5 μM) and H<sub>2</sub>O<sub>2</sub> (100 μM). Data are represented as mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. <sup>a</sup>p = 0.0031, <sup>b</sup>p = 0.0001, and <sup>c</sup>p = 0.0001 when compared to cells treated with H<sub>2</sub>O<sub>2</sub>.

**Figure 3.** Antioxidant activity of chrysin against *t*-BuOOH-induced ROS formation in neuronal SH-SY5Y **(A)** and microglial THP-1 cells **(B)**. Cells were incubated for 30 min with the fluorescent probe H<sub>2</sub>DCF-DA. At the end of incubation, cells were treated for 30 min with various concentrations of chrysin (1,25 – 5 μM) and *t*-BuOOH (100 μM). Data are represented as mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's test. **(A)** <sup>a</sup>p = 0.0335, <sup>b</sup>p = 0.0449, and <sup>c</sup>p = 0.0118 when compared to cells treated with *t*-BuOOH; **(B)** <sup>a</sup>p = 0.0035 when compared to cells treated with *t*-BuOOH.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

**Figure 4.** Effects of chrysin against the oxidative stress and inflammation induced by LPS in microglial THP-1 cells. **(A)** Cells were incubated for 24 h with chrysin (5  $\mu$ M) and LPS (1  $\mu$ g/mL). At the end of incubation, ROS formation was measured using the fluorescent probe H<sub>2</sub>DCF-DA. Data are represented as mean  $\pm$  SEM of three independent experiments. Statistical analysis was performed using Student's t-test. <sup>a</sup>p = 0.0020 when compared to cells treated with LPS. **(B-D)** Cells were incubated for 24 h with chrysin (5  $\mu$ M) and LPS (1  $\mu$ g/mL). At the end of incubation, iNOS **(B)**, IL-1 $\beta$  **(C)** and TNF $\alpha$  **(D)** expression was measured by quantitative RT-PCR. Data are represented as mean  $\pm$  SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's test. **(B)** <sup>a</sup>p = 0.0001 when compared to untreated cells and <sup>b</sup>p = 0.0154 when compared to cells treated with LPS; **(C)** <sup>a</sup>p = 0.0001 when compared to untreated cells and <sup>b</sup>p = 0.0122 when compared to cells treated with LPS; **(D)** <sup>a</sup>p = 0.0001 when compared to untreated cells and <sup>b</sup>p = 0.0038 when compared to cells treated with LPS.

35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

**Figure 5.** Antioxidant activity of chrysin against the pro-oxidant effect of AlCl<sub>3</sub> on Fenton reaction in neuronal SH-SY5Y cells. Cells were incubated for 30 min with the fluorescent probe H<sub>2</sub>DCF-DA. At the end of incubation, cells were first treated for 15 min with FeSO<sub>4</sub>/H<sub>2</sub>O<sub>2</sub>/AlCl<sub>3</sub> (50  $\mu$ M, 200  $\mu$ M, and 50  $\mu$ M, respectively) and then treated for 15 min with chrysin (5  $\mu$ M). Data are represented as mean  $\pm$  SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's test. <sup>a</sup>p = 0.0031 when compared to untreated cells, <sup>b</sup>p = 0.0022 when compared to cells treated with FeSO<sub>4</sub>/H<sub>2</sub>O<sub>2</sub> and <sup>c</sup>p = 0.0012 when compared to cells treated with FeSO<sub>4</sub>/H<sub>2</sub>O<sub>2</sub>/AlCl<sub>3</sub>.

57  
58  
59  
60  
61  
62  
63  
64  
65

**Figure 6.** Neuroprotective effects of chrysin against the cytotoxicity induced by AlCl<sub>3</sub> in neuronal SH-SY5Y cells. Cells were incubated for 24 h with chrysin (5  $\mu$ M) and AlCl<sub>3</sub> (50

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

$\mu\text{M}$ ). At the end of incubation, the cytotoxicity of  $\text{AlCl}_3$  in terms of cytostatic effect (**A**) and necrosis (**B**) was assessed using trypan blue and propidium iodide, respectively. (**C**) Representative bright field (BF) and PI fluorescence images. Scale bars: 100  $\mu\text{M}$ . Data are represented as mean  $\pm$  SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's test. (**A**) <sup>a</sup>  $p = 0.0033$  when compared to untreated cells and <sup>b</sup>  $p = 0.0432$  when compared to cells treated with  $\text{AlCl}_3$ ; (**B**) <sup>a</sup>  $p = 0.0010$  when compared to untreated cells and <sup>b</sup>  $p = 0.0071$  when compared to cells treated with  $\text{AlCl}_3$ .

**Figure 7.** Effect of chrysin on  $\text{AlCl}_3$  induced memory impairment in the step-down passive avoidance task. Data are represented as mean  $\pm$  SEM. for  $n = 14$  to 16 animals per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. <sup>a</sup>  $p < 0.05$  when compared to the control group; <sup>b</sup>  $p = 0.0310$  when compared to the  $\text{AlCl}_3$  group. <sup>c</sup>  $p = 0.0370$  when compared to the  $\text{AlCl}_3$  group; <sup>d</sup>  $p = 0.0320$  when compared to the  $\text{AlCl}_3$  group.

**Figure 8.** Effect of chrysin on brain cortex and hippocampus AChE (**A**) and BChE (**B**) activities of mice treated with  $\text{AlCl}_3$ . Data are represented as mean  $\pm$  SEM. for  $n = 8$  animals per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. (**A**) <sup>e</sup>  $p = 0.0013$  when compared to the control group; <sup>f</sup>  $p = 0.0118$ , <sup>g</sup>  $p = 0.0187$ , and <sup>h</sup>  $p = 0.0215$  when compared to the  $\text{AlCl}_3$  group for hippocampus AChE activity; (**B**) <sup>e</sup>  $p < 0.0002$  when compared to the control group; <sup>f</sup>  $p = 0.111$ , <sup>g</sup>  $p = 0.0115$ , and <sup>h</sup>  $p = 0.0194$  when compared to the  $\text{AlCl}_3$  group for hippocampus BChE activity.

**Figure 9.** Effect of chrysin on brain cortex and hippocampus LPO levels (**A**), CP levels (**B**), SOD activities (**C**) and CAT activities (**D**) of mice treated with  $\text{AlCl}_3$ . Data are represented as

1 mean  $\pm$  SEM. for n = 8 animals per group. Statistical analysis was performed using one-way  
2 ANOVA followed by Tukey's test. (A) <sup>a</sup> p = 0.0001 when compared to the control group, <sup>b</sup> p =  
3 0.001, <sup>c</sup> p = 0.032, and <sup>d</sup> p = 0.010 when compared to the AlCl<sub>3</sub> group, for brain cortex MDA  
4 levels; <sup>e</sup> p = 0.0005 when compared to the control group; <sup>f</sup> p = 0.0002, <sup>g</sup> p = 0.0001, and <sup>h</sup> p =  
5 0.0001 when compared to the AlCl<sub>3</sub> group, for hippocampus MDA levels. (B) <sup>a</sup> p = 0.0001  
6 when compared to the control group for brain cortex CP levels; <sup>e</sup> p = 0.0153 when compared  
7 to the control group; <sup>f</sup> p = 0.001, <sup>g</sup> p = 0.027, and <sup>h</sup> p = 0.021 when compared to the AlCl<sub>3</sub>  
8 group, for hippocampus CP levels. (C) <sup>a</sup> p = 0.0001 when compared to the control group; <sup>b</sup> p =  
9 0.0014, <sup>c</sup> p = 0.0385, and <sup>d</sup> p = 0.0395 when compared to the AlCl<sub>3</sub> group, for brain cortex  
10 SOD activity; <sup>e</sup> p = 0.0015 when compared to the control group; <sup>f</sup> p = 0.0084, <sup>g</sup> p = 0.0001, and  
11 <sup>h</sup> p = 0.0001 when compared to the AlCl<sub>3</sub> group, for hippocampus SOD activity. (D) <sup>e</sup> p =  
12 0.0012 when compared to the control group; <sup>f</sup> p = 0.0064, <sup>g</sup> p = 0.0001, and <sup>h</sup> p = 0.0002 when  
13 compared to the AlCl<sub>3</sub> group, for hippocampus CAT activity.  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30

31  
32  
33  
34 **Figure 10.** Histological sections of the frontal cerebral cortex (left column) and CA1 region of  
35 the hippocampus (right column) (A). Thionine acetate staining method. Arrows indicate  
36 degenerating neurons with necrosis appearance. These neurons stain intensely in dark purple  
37 and show nuclear degeneration and atrophic cytoplasm. All chrysin treatments promoted a  
38 marked reduction in degenerative neurons in both cerebral cortex and hippocampus. Frequency  
39 of degenerative neurons in the brain cortex and hippocampus (B). Data are represented as mean  
40  $\pm$  SEM. for n = 5 animals per group. Statistical analysis was performed using one-way ANOVA  
41 followed by Tukey's test. <sup>a</sup> p = 0.0001 when compared to the control group; <sup>b</sup> p = 0.0001, <sup>c</sup> p =  
42 0.0001, and <sup>d</sup> p = 0.0001 when compared to the AlCl<sub>3</sub> group, for brain cortex necrotic cells  
43 frequency. <sup>e</sup> p = 0.0041 when compared to the control group; <sup>f</sup> p = 0.0334, <sup>g</sup> p = 0.0387, and <sup>h</sup>  
44 p = 0.0003 when compared to the AlCl<sub>3</sub> group, for hippocampus necrotic cells frequency.  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

## Tables

### Tables 1

Primer sequences for quantitative RT-PCR.

Primer	5' to 3' Sequence	
	Forward	Reverse
iNOS	TGAACTACGTCCTGTCCCCT	CTCTTCTCTTGGGTCTCCGC
IL-1 $\beta$	TGATGGCTTATTACAGTGGCAATG	GTAGTGGTGGTTCGGAGATTCG
TNF- $\alpha$	ATCTTCTCGAACCCCGAGTG	GGGTTTGCTACAACATGGGC
B-actin	GCGAGAAGATGACCCAGATC	GGATAGCACAGCCTGGATAG
GAPDH	GGTCGGAGTCAACGGATTTG	GGAAGATGGTGGTGGGATTTC

### Table 2

Spontaneous locomotor and exploratory activity in the open field and chimney test of mice

	Open Field (num)	Chimney (s)
	Crossings	Climbing
Control	102.0 $\pm$ 5.30	13.92 $\pm$ 2.08
AlCl <sub>3</sub>	110.8 $\pm$ 11.51	15.70 $\pm$ 3.05
Chrysin 10	109.6 $\pm$ 19.21	16.16 $\pm$ 1.69
Chrysin 30	115.4 $\pm$ 23.35	16.44 $\pm$ 2.08
Chrysin 100	107.8 $\pm$ 12.97	15.14 $\pm$ 2.25

Data are represented as mean  $\pm$  SEM for n = 14 -16 animals per group.

Figures

Figure 1

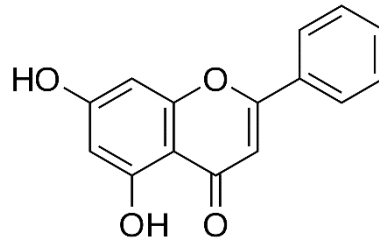
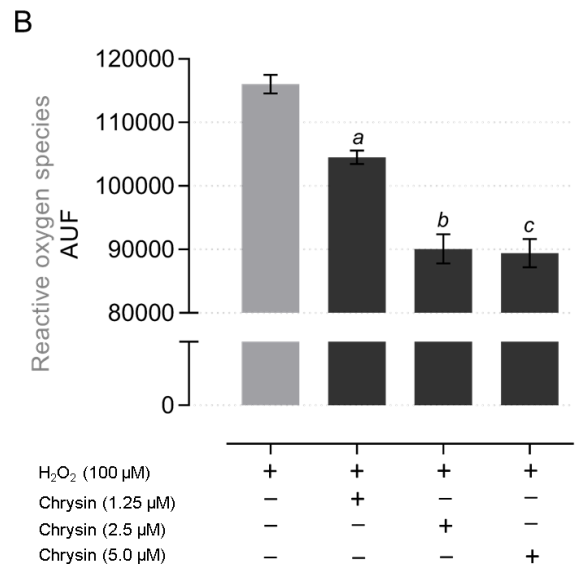
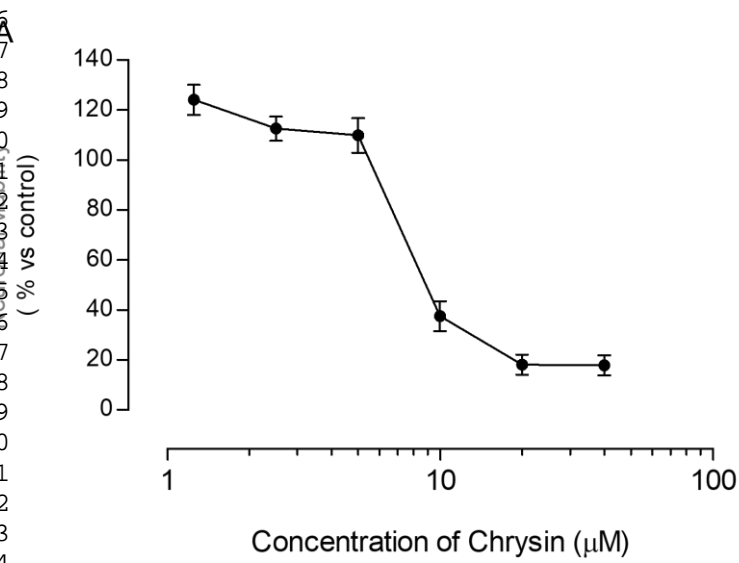
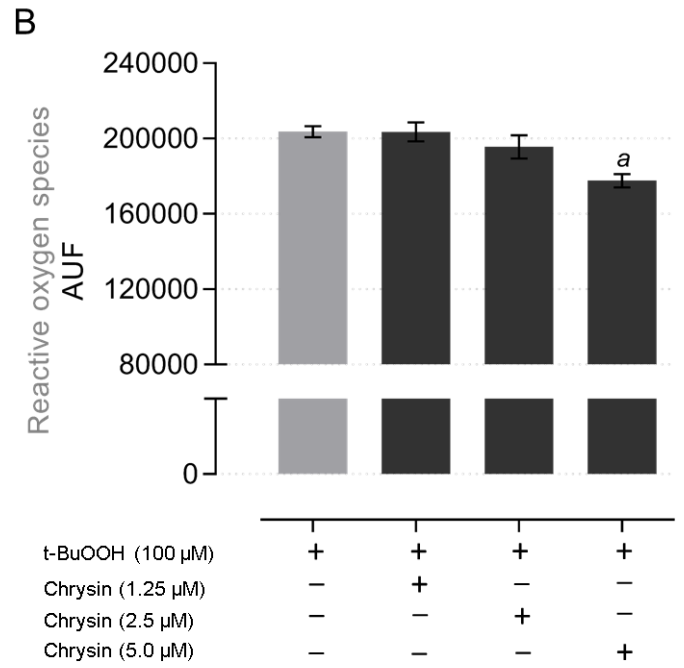
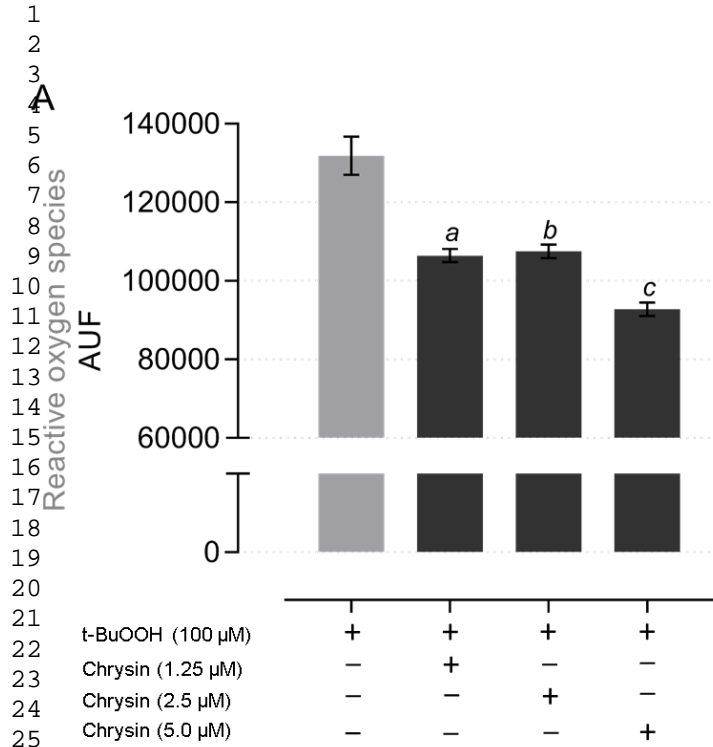


Figure 2



**Figure 3**





**Figure 4**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

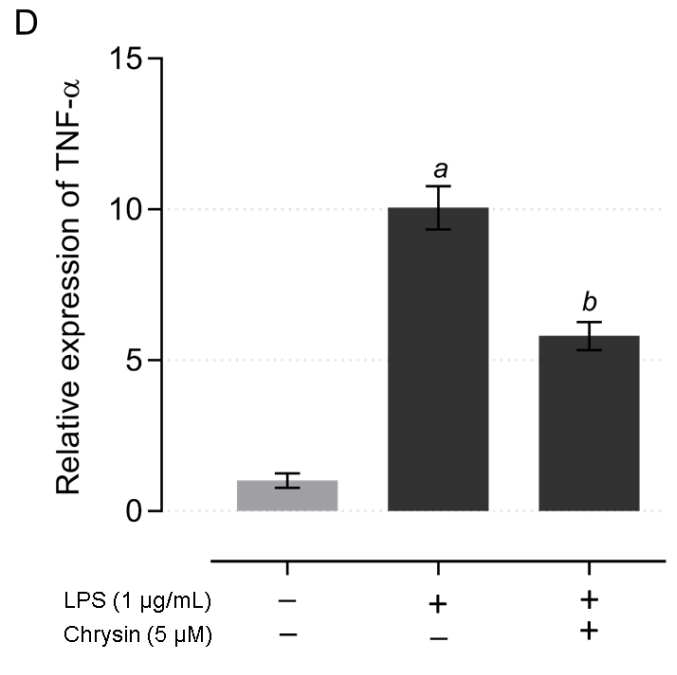
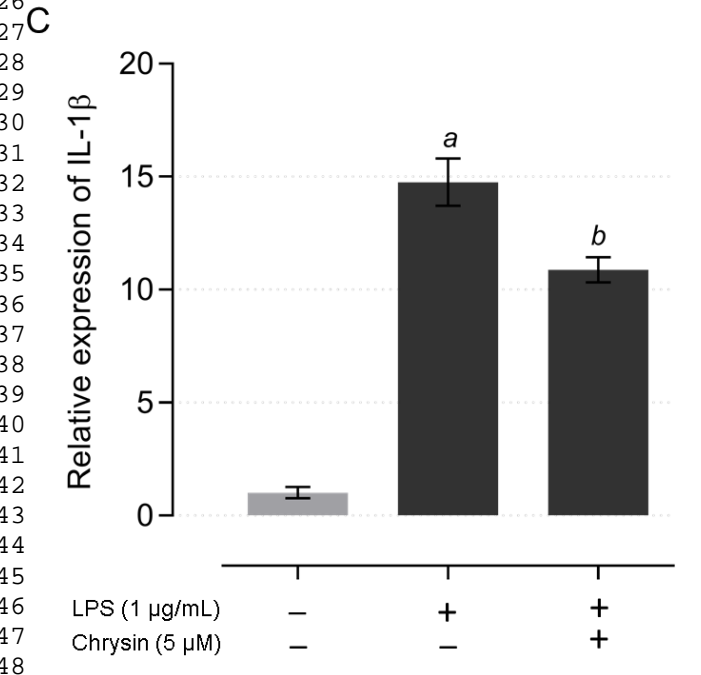
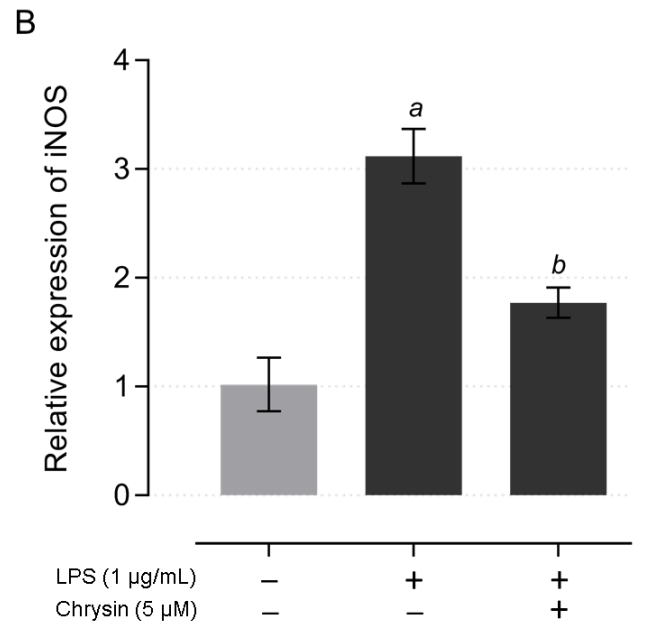
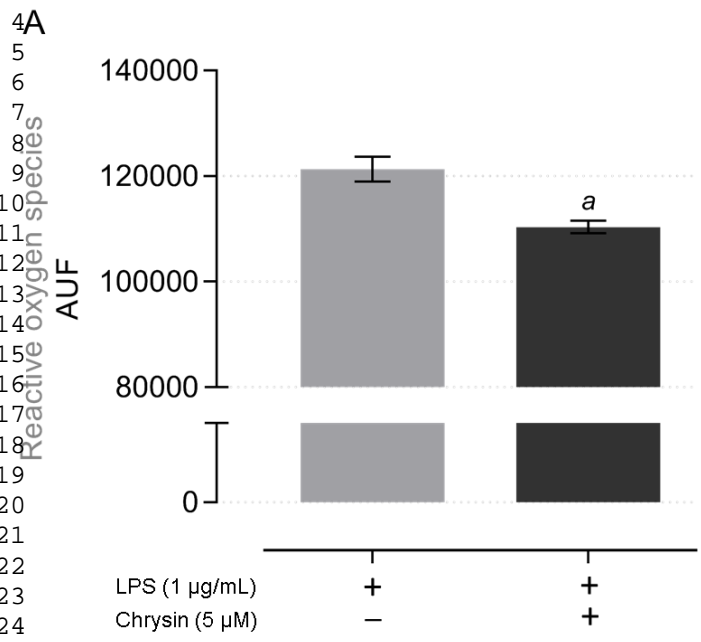
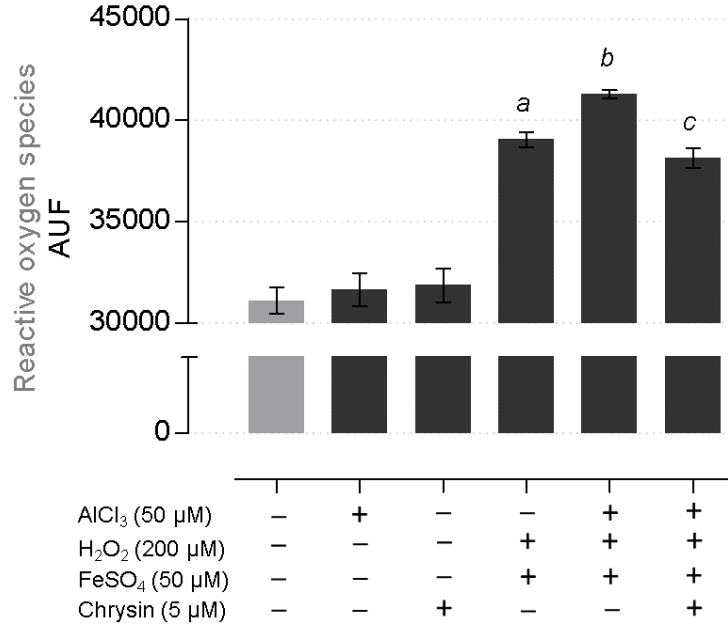
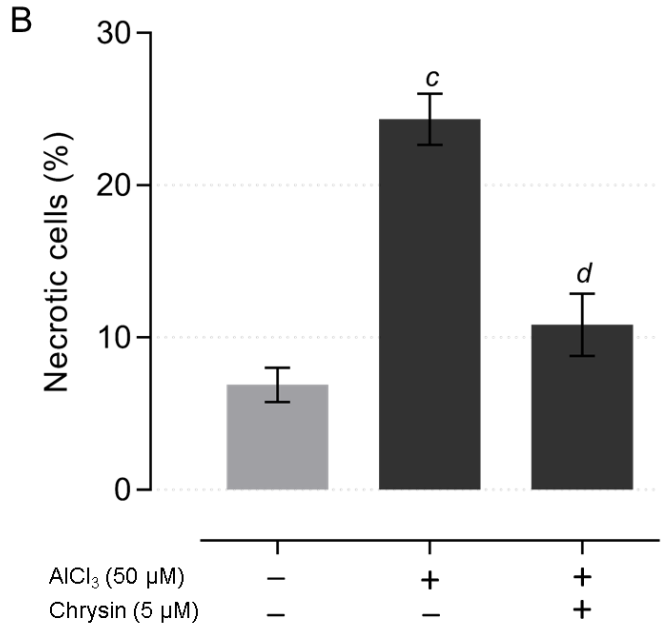
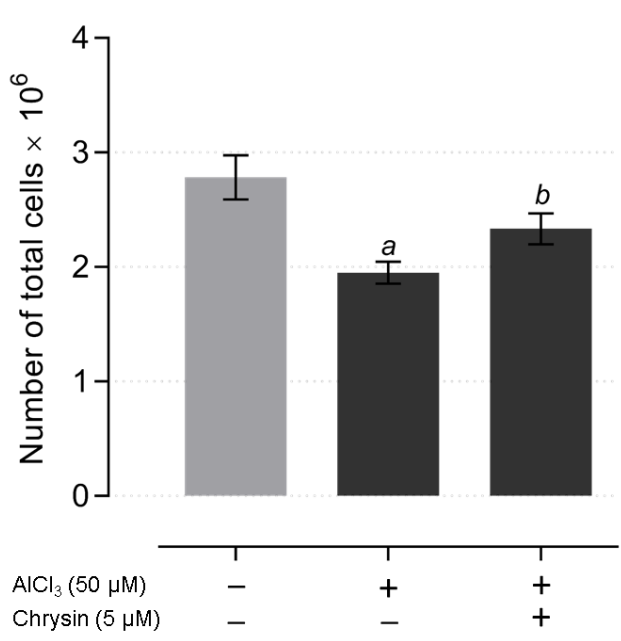


Figure 5

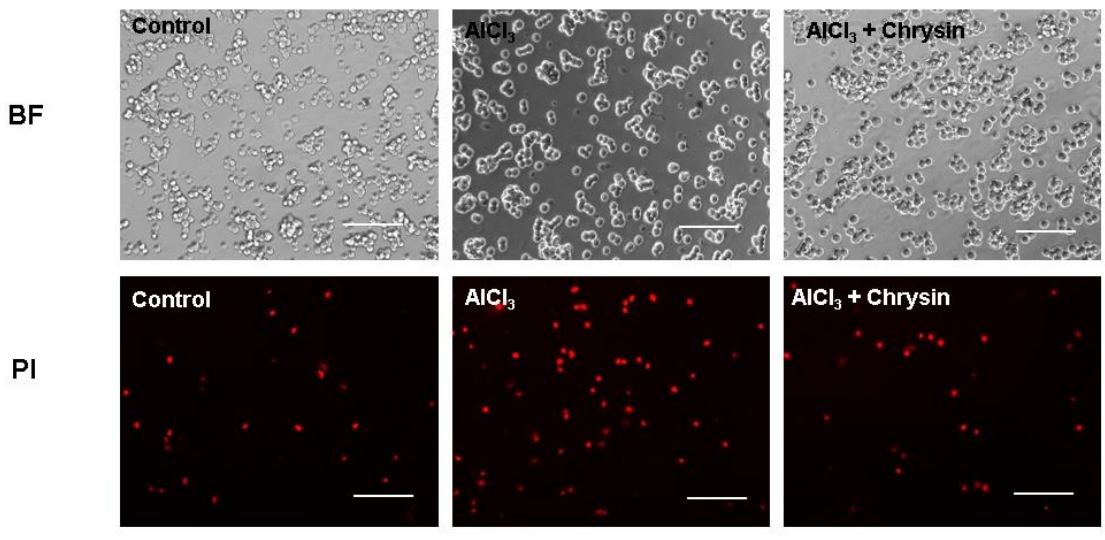


**Figure 6**

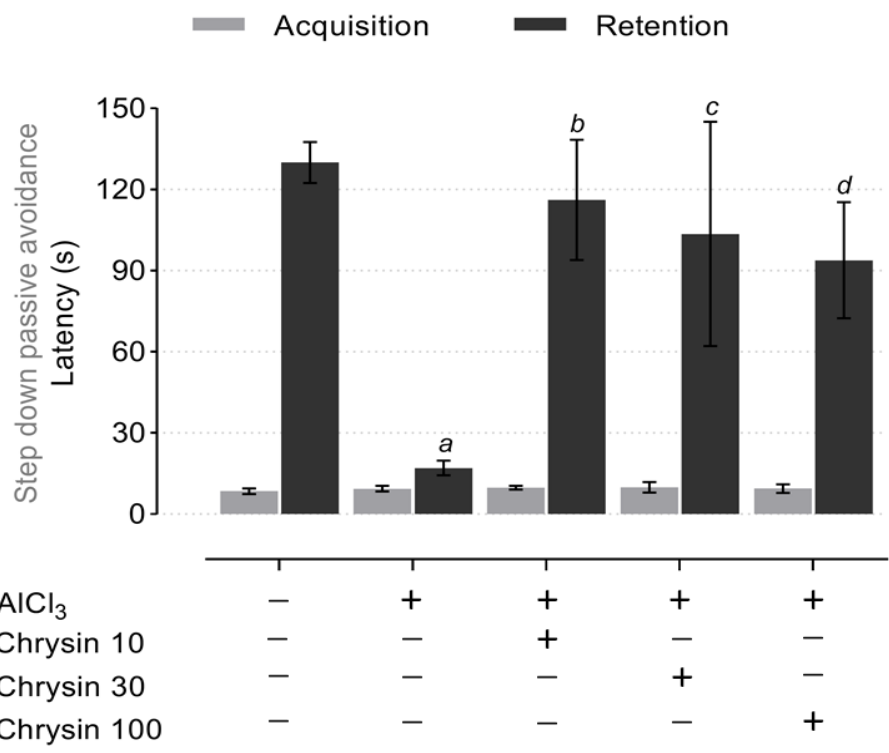
1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



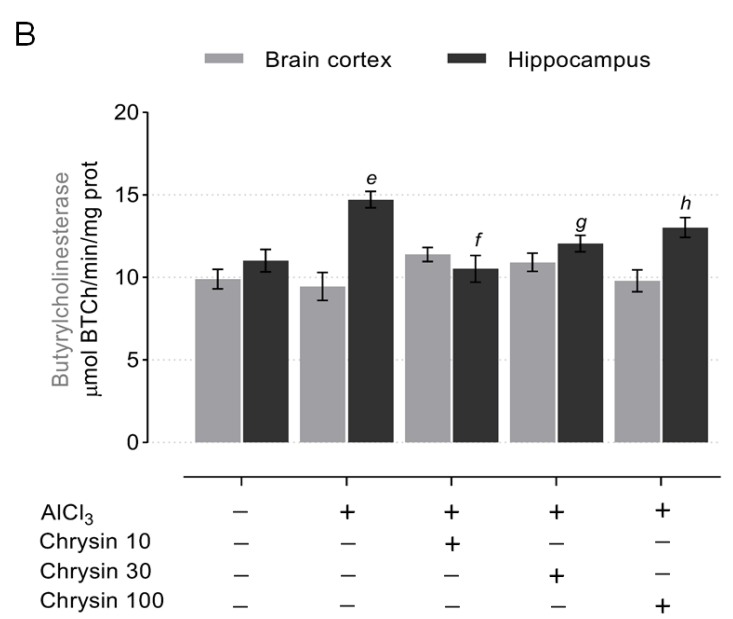
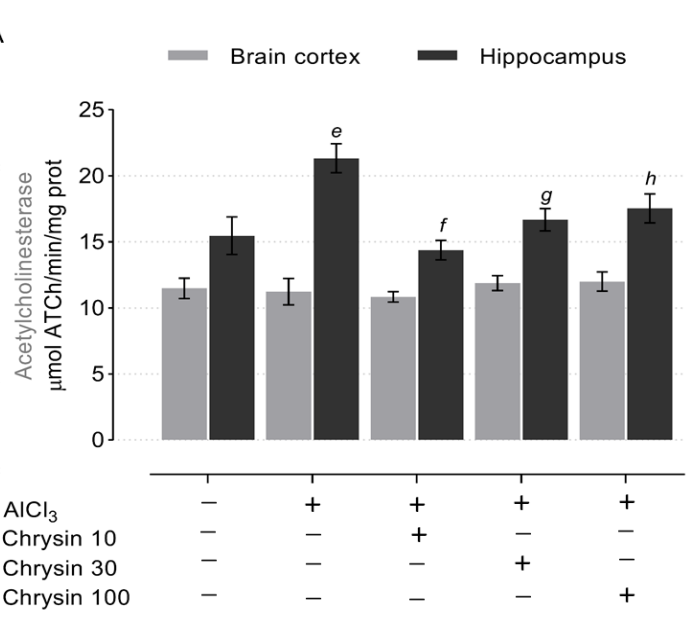
**C**



**Figure 7**



**Figure 8**



**Figure 9**

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

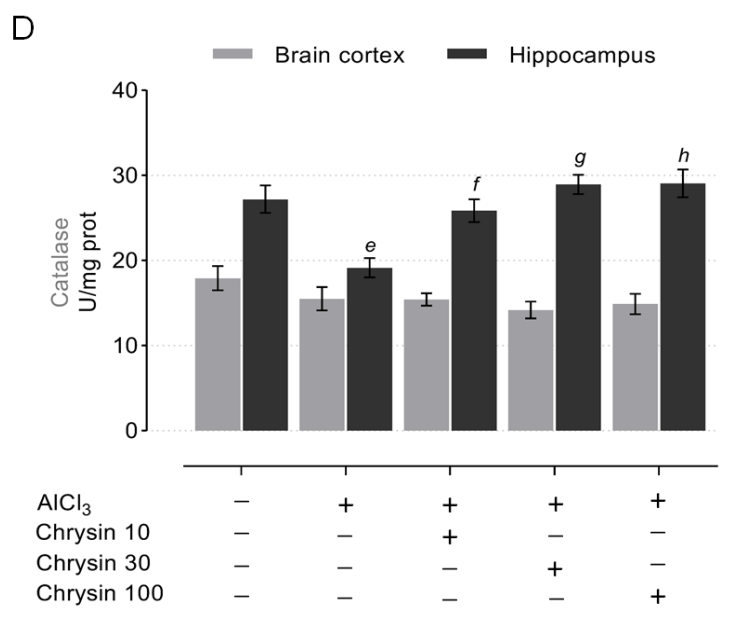
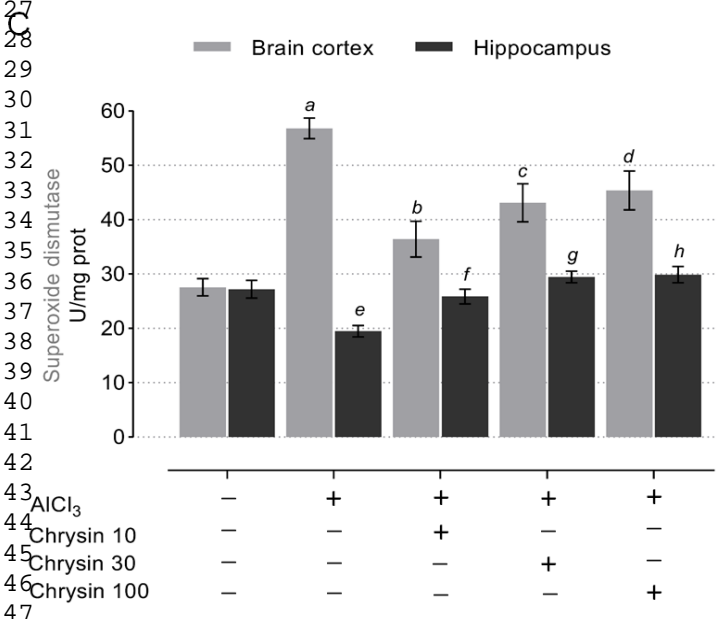
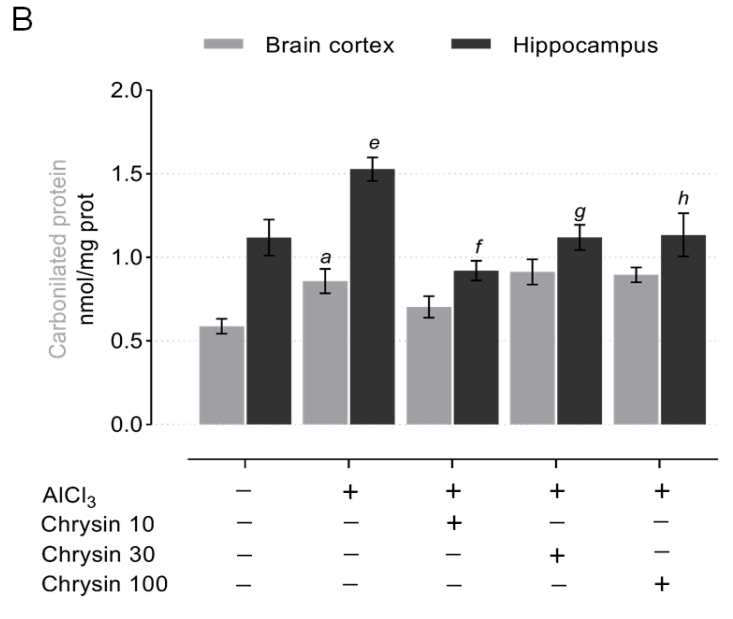
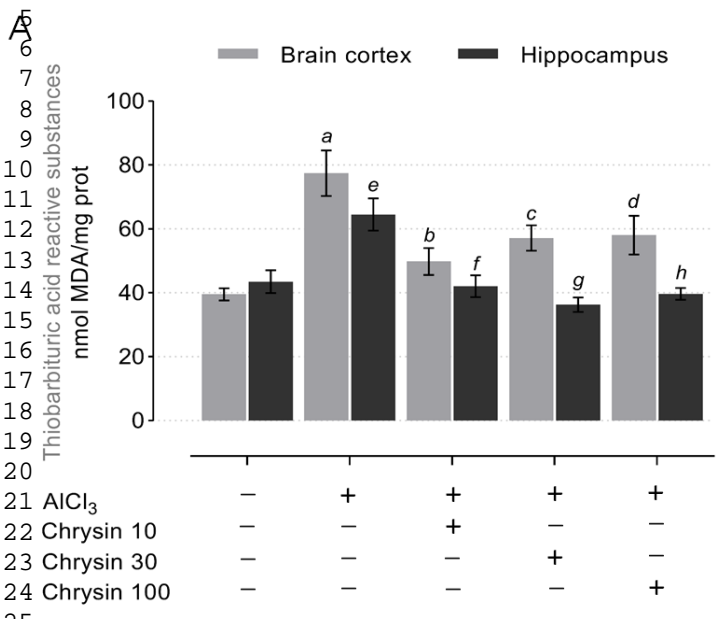
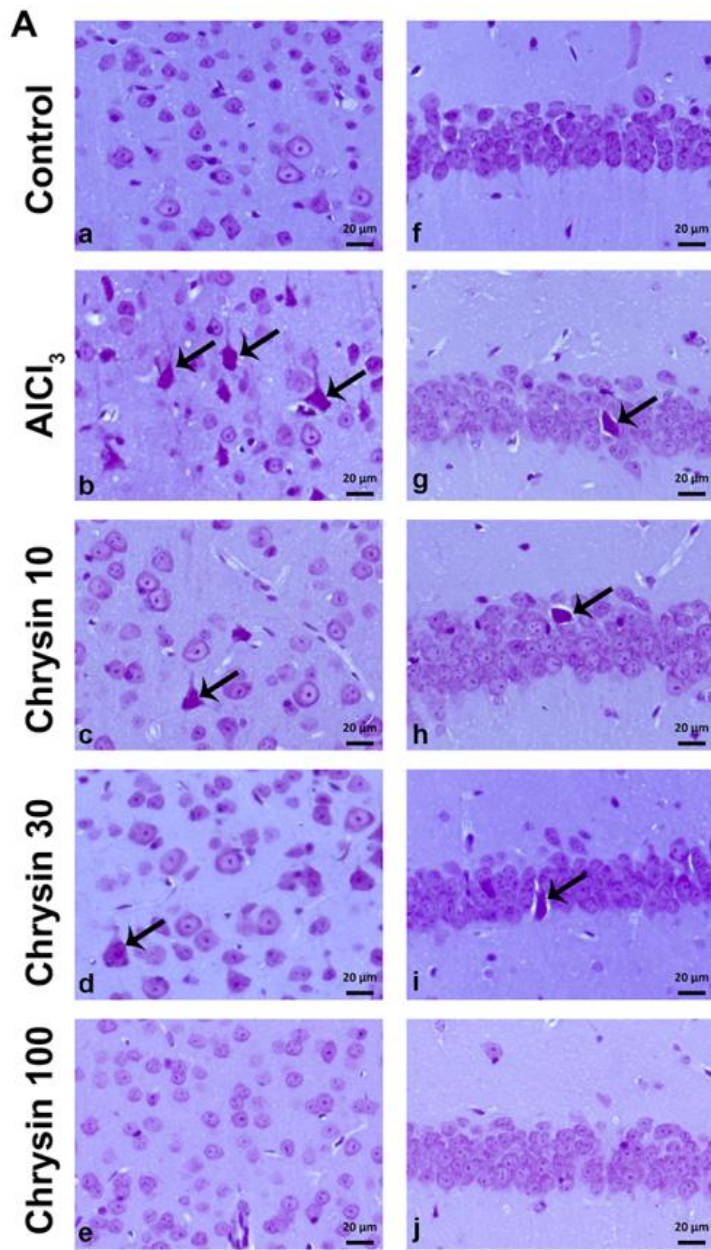
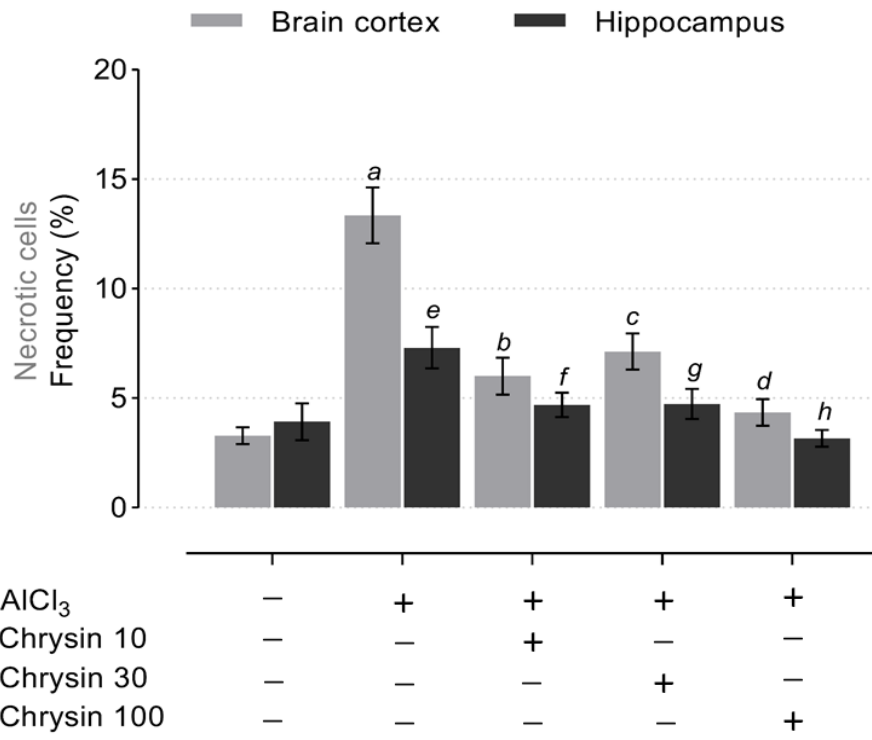


Figure 10



B



AlCl <sub>3</sub>	-	+	+	+	+
Chrysin 10	-	-	+	-	-
Chrysin 30	-	-	-	+	-
Chrysin 100	-	-	-	-	+