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Neuroprotective and antioxidant activities of Colombian plants against paraquat and C2-ceramide exposure in SH-SY5Y cells

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Abnormal production of reactive oxygen species (ROS) has been implicated in the physiopathology of neuronal cell death. Increased ROS levels are associated with exacerbated peptide aggregation, inflammation, and mitochondrial dysfunction, which facilitate the triggering of specific cell death pathways. Antioxidant molecules are potentially useful in the amelioration of neurodegeneration. In this regard, natural products are an invaluable source of antioxidants. Therefore, we investigate the antioxidant and neuroprotective activities of four Colombian angiosperm extracts. Antioxidant activity was evaluated by phytochemical assays using TLC techniques with Dragendorff reagent, ninhydrin, and chloranil in dioxane, NH_3, Fast Blue, and $FeCl_3$, together with bioautography using DPPH and β-carotene. *In vitro* neuroprotective activity, cell death, and ROS accumulation were evaluated by MTT and flow cytometry in the SH-SY5Y cell line exposed to paraquat and C2-ceramide. We found that *Zanthoxylum rhoifolium* Lam, *Zanthoxylum martinicense*, *Nectandra membranacea*, and *Nectandra reticulata* extracts have antioxidant activity higher than quercetin under a β-carotene bleaching assay and protect SH-SY5Y cells against paraquat and C2-ceramide associated with a reduction in ROS. In conclusion, these extracts have a strong neuroprotective potential, and the precise mechanism requires more evaluation.

KEYWORDS

Zanthoxylum, *Nectandra*, reactive oxygen species, antioxidative effect, neuroprotection, plant extract

1 Introduction

The study of reactive oxygen species (ROS) is important because they are involved in many cellular processes, including cell cycle regulation, phagocytosis, and enzymatic activation, and have been implicated in neurodegenerative diseases (Di Meo et al., 2016). An imbalance between the production and scavenging of ROS can lead to the generation of oxidative stress that can damage DNA, lipids, and proteins (Verbon et al., 2012), leading to cell death. Antioxidant enzymatic enzymes, such as superoxide dismutase

TABLE 1 Extracts isolated.

Code	Family	Genus	Species	Part	Isolation
1	Rutaceae	*Zanthoxylum*	*rhoifolium* Lam	Bark	Chloroformic*
2	Rutaceae	*Zanthoxylum*	*martinicense*	Root	Chloroformic*
3	Lauraceae	*Nectandra*	*membranacea*	Leaves	Ethanolic
4	Lauraceae	*Nectandra*	*reticulata*	Bark	Chloroformic

*Alkaloidal extraction.

TABLE 2 MTT result.

Code	Maximum viable concentration [µg/mL]	% viability	SEM[a]
1	25	*99.12*	*3.51*
2	25	*99.41*	*3.91*
3	25	*94.33*	*1.81*
4	25	*98.41*	*0.28*

[a]SEM, standard error of the mean.

(SOD), glutathione peroxidases (GPX), glutaredoxins, thioredoxins, and catalases, act as free radical scavengers (Manoharan et al., 2016)

In Alzheimer's disease (AD) models, it has been shown that the increase in ROS levels causes different pathogenic events and leads to alterations in the mitochondrial electron transport chain (Zhao and Zhao, 2013), leading to a vicious cycle of oxidative damage that generates more ROS (Zhang et al., 2023). Recently, ROS can also stimulate the production and accumulation of amyloid-beta (Aβ) peptides and phosphorylation of the tau protein, which are the major hallmarks of AD pathology (Holubiec et al., 2022). Consequently, the accumulation of these peptides induces a positive feedback loop where Aβ increases oxidative stress-inducing lipid peroxidation, protein oxidation, and DNA oxidation (Gandhi and Abramov, 2012), and reduces antioxidant potential (Zhao and Zhao, 2013; Kim et al., 2015).

Similarly, in PD models, oxidative stress is associated with mitochondrial dysfunction, impaired protein degradation systems, and dopaminergic neuronal death (Duan et al., 2023). An important physiopathological characteristic of PD is the inhibition of the complex I of the electron transport chain, which increases ROS levels and further reduces the activity of these complexes. In addition, the imbalance in ROS can produce mutations in the mitochondrial DNA. These changes are finally associated with the induction of apoptosis of dopaminergic neurons (Swerdlow et al., 1996; Lin and Beal, 2006; Hauser and Hastings, 2013; Blesa et al., 2015; Pallikonda et al., 2019). In accordance with this, targeting oxidative stress and enhancing antioxidant defenses have emerged as potential therapeutic approaches for mitigating neurodegenerative pathology by modulating ROS levels, restoring redox balance, and preserving neuronal function.

Cellular and animal models treated with molecules that induce ROS accumulation at toxic levels have been used to study ROS pathways. In these regards, most cellular and animal models of ROS are based on the toxic effect of inhibition of mitochondrial complex I, including the 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) and paraquat (PQ) exposure models, which increase ROS, induce mitochondrial dysfunction, and subsequently lead to dopaminergic cell death (William Langston, 1985; Nisticò et al., 2011). On the other hand, ceramides, a group of signaling lipids, are implicated in the generation of ROS by the induction of mitochondrial dysfunction that promotes the production of free radicals that induce cell death related to the progression of neurodegeneration in different contexts (Pan et al., 2023). Ceramides can also cause inhibition of the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) cell survival pathway and, therefore, have been used as an *in vitro* model of neuronal death (Arboleda et al., 2009; Chaurasia and Summers, 2015; Jazvinšćak Jembrek et al., 2015). As is well known, the SH-SY5Y neuroblastoma cells have been used in neurobiology because they have phenotype-like catecholaminergic neurons, are grown easily, and can be differentiated with retinoic acid (Kovalevich and Langford, 2013). SH-SY5Y cells give us an approximation of the neuronal response to an abnormal ROS concentration.

Historically, natural products have been the main sources of molecules with potential therapeutic applications due to the abundance of diverse molecules that can have multiple cellular targets (Atanasov et al., 2021). It is possible to isolate small molecules with structural and functional features that are difficult to obtain by biochemical synthesis (Zhao and Zhao, 2013), including molecules like curcumin (isolated from *Curcuma longa* L), quercetin (isolated from *Dimorphandra mollis*), berberine (isolated from *Berberis aquifolium* Purch), rivastigmine (isolated from *Physostigma venenosum* Balf. F), and galantamine (isolated from *Galanthus woronowii* Losinsk). All these molecules have important antioxidant activity and other bioactive functions, including anti-inflammation Aβ aggregation inhibition, and acetylcholinesterase (AChE) inhibition (Marco-Contelles

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TABLE 3 Antioxidant capacity of the extracts.

| Code | Antioxidant activity | | Developers | | | | | | | | | | | | |
| | Bioautography | | Dragendorff | | Ninhydrin | | Chloranil | | FeCl₃ | | Fast Blue | | NH₃ | | |
	DPPH	β-carotene	Rf	Result	Rf	Result	Rf	Result	Rf	Result	Rf	Result	Rf	Result	S. metabolites*
1	+	+	0.9 / 0.1	+	0.5 / 0.3	+	0.9 / 0.3	+	−	−	0.8	+	−	−	−
2	+	+	0.8 / 0.3	+	−	−	0.8 / 0.3 / 0.2	+	−	−	−	−	−	−	−
3	+	+	−	−	−	−	−	−	0.05	+	1 / 0.5	+	0.4 / 0.1	Blue / Blue	Isoflavone / Isoflavone
4	+	+	−	−	0.1	+	−	−	0.05	+	0.05	+	0.8 / 0.4	Light blue / Green	Isoflavone, aurone, and flavanone flavonoid
Control	Quercetin		Berberine		L-tryptophan				Quercetin						
	+	+	+	+	+	+	+	+	0.05	+	0.05	+	0.05	Green	Flavonoid
vh	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+, presence of colored spots; −, absence of colored spots; *, possible secondary metabolite taken from Mabry et al. (1970).
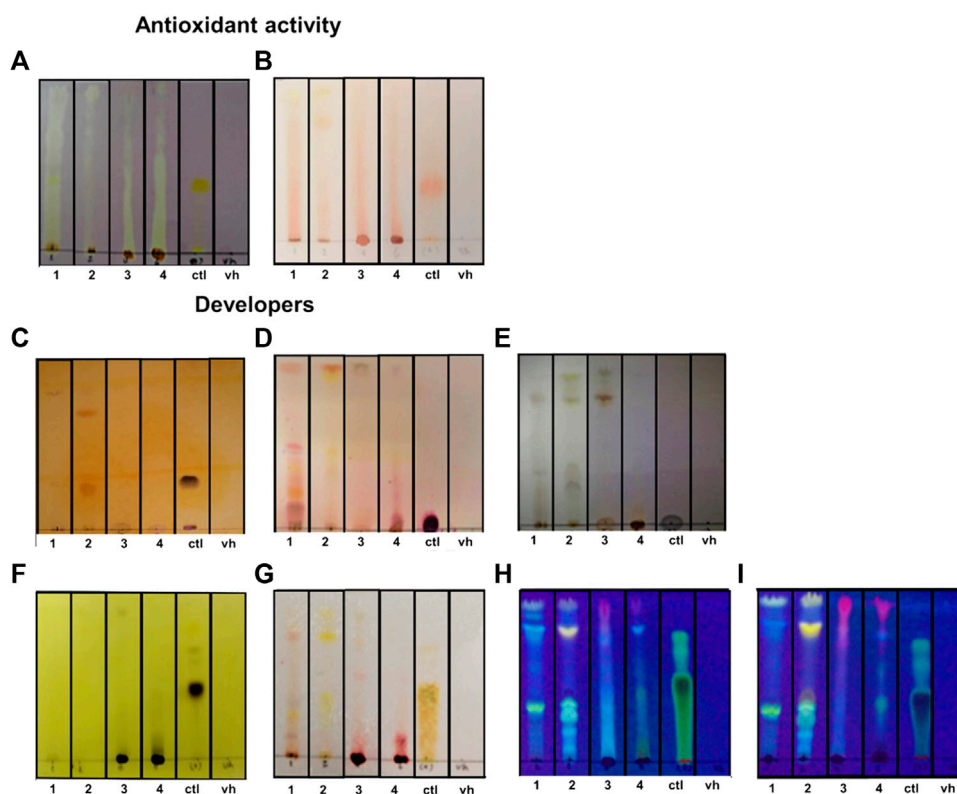
FIGURE 1
Antioxidant capacity of extracts and **fractions. (A, B)** DPPH bioautography **(A)** and β-carotene bioautography **(B)** to evaluate the antioxidant activity.
(C) Chromatoplate developed with Dragendorff reagent to determine alkaloids. **(D)** Chromatoplate developed with ninhydrin to determine amino acids.
(E) Chromatoplate developed with chloranil in dioxane to determine primary and secondary amines. **(F)** Chromatoplate developed with FeCl$_3$ to
determine the presence of phenolic molecules. **(G)** Chromatoplate developed with Fast Blue to determine the presence of a flavonoid. **(H, I)**
Chromatoplates developed without NH$_3$ vapor **(H)** and with NH$_3$ vapor **(I)** at 365 nm to see the possible presence of flavonoids. UV development codes, 1:
Zanthoxylum rhoifolium Lam., 2: *Zanthoxylum martinicense*, 3: *Nectandra membranacea*, 4: *Nectandra reticulata*. ctl: control (quercetin for A, B, F, G, H,
and I; berberine for C; L-tryptophan for D and E); and vh: blank or solvent vehicle.

et al., 2006; Cai et al., 2016; Dighe et al., 2019; Voulgaropoulou
et al., 2019).

The genus *Zanthoxylum* has been shown to have *in vitro*
antioxidant effects and the ability to inhibit acetylcholinesterase
in AD models (Plazas et al., 2018). According to ethnobotanical
records, this genus is known to be distributed worldwide and is
mainly used in Asia, Africa, and the Americas as an antidote to snake
venom, analgesics, bactericides, and fungicides, among others
(Okagu et al., 2021). The genus *Nectandra* can be found in the
neotropics, and it has been reported to be used for antifungal, anti-
inflammatory, antimalarial, analgesic, treating ulcers, and febrifuge
purposes, among others (Macías-Villamizar et al., 2015).
Furthermore, their potential as a biological source of bioactive
molecules stems from the rich composition of flavonoid,
polyphenolic, and alkaloid metabolites in both genera.

In the present work, we have demonstrated the antioxidant activity
of extracts from several Colombian plants. Using bioautography, we
found that plant extracts from *Zanthoxylum rhoifolium* Lam,
Zanthoxylum martinicense, *Nectandra membranacea*, and *Nectandra
reticulata* have strong antioxidant activity. We also showed that these
extracts 1) have a protective effect on the β-carotene peroxidation to a
higher level than quercetin and 2) increase the survival of cells exposed

to paraquat and ceramide. This suggests that the extracts analyzed have
a strong antioxidant activity that has neuroprotective capacity.

2 Materials and methods

2.1 Plant material and preparation of the extracts

Rutaceae and *Lauracea* species were collected from different
regions of Colombia (Table 1). To obtain the ethanolic extracts,
plant material was separated in parts, dried at room temperature,
macerated using 96% ethanol, and then concentrated under a
vacuum at 40°C. For alkaloid extraction, the classical acid–base
methodology was used (Grycová et al., 2007; Doncheva et al., 2015).
In brief, the ethanol-extracted plant was evaporated under reduced
pressure at 35–40°C. Then, 3 g of each dried residue was resuspended
with 10 mL of 2% HCl solution three times. The acidic phases were
filtered, and the pH was adjusted to 10 with 10% NaOH. The extraction
was performed with CHCl$_3$ to obtain the alkaloid extracts in CHCl$_3$.
Finally, the solvent was evaporated, and the residual aqueous phases
were neutralized and lyophilized (Table 1).
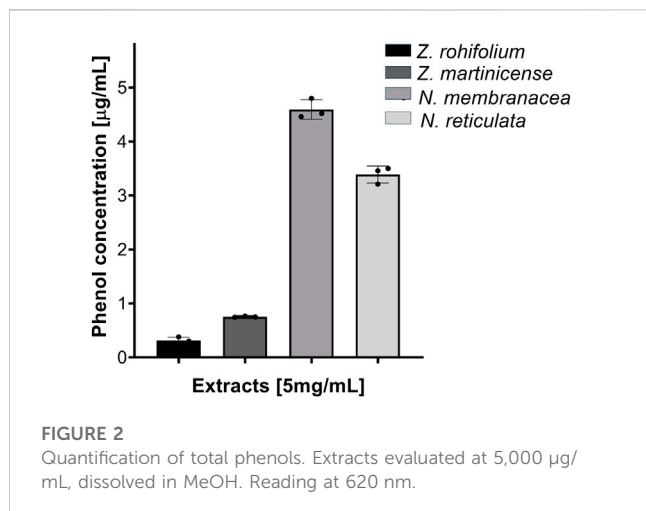
FIGURE 2
Quantification of total phenols. Extracts evaluated at 5,000 μg/mL, dissolved in MeOH. Reading at 620 nm.

2.2 Phytochemical qualitative analysis of extracts by TLC and TLC bioautography

The extracts were seeded on silica gel 60 F254 chromatofolds (ref. 1055540001, Merck, United States) eluted with $CHCl_3$-MeOH mixture (9:1) (solvents AR grade). Once the TLC development protocol was ready and dry, some chromoplates were stained with NH_3, $FeCl_3$, ninhydrin, Fast Blue, and Dragendorff reagents to identify what type of secondary metabolite was present. Other groups of chromoplates were used to evaluate the preliminary antioxidant activity. The developed chromoplates were dried and sprayed with 0.2% 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol or a 0.05% solution of β-carotene in chloroform (β-carotene bleaching assay), following the protocol adapted from Takao et al. (1994) and Miller (1971) and described in detail previously by Plazas et al. (2018), Miller (1971), Takao et al. (1994), and Plazas et al. (2018).

2.3 Quantification of total phenols using the Folin–Ciocalteu reagent

To quantify total phenols, 5 mg of extract was dissolved in 1 mL of 95% methanol and then filtered using a 0.22-μm membrane (solution 1). Then, 50 μL of solution A was mixed with 200 μL of 10% Folin–Ciocalteu reagent, shaken for 1 min, allowed to stand for 1 min more; then, 800 μL of 700 mM of sodium carbonate (solution B) was added. It was incubated for 1 h at room temperature. A 200 μL aliquot of solution B was transferred to a 96-well plate, and the absorbance at 620 nm was read.

2.4 Quantification of antioxidant capacity using β-carotene

The quantification of antioxidant capacity was performed by adapting Miller's method (Miller and Rice-Evans, 1996). This experiment measures the antioxidant capacity of extracts by quantifying the change in color of the carotene solution when this solution is oxidized by linoleic acid (yellow-uncolored). First, we prepared an emulsion with a 500 μL aliquot of 0.02% β-carotene

in $CHCl_3$ solution. The solvent was removed by rota-evaporation, and 10 mg of linoleic acid (0.9 mg/mL), 100 mg of tween-20, and 20 mL of distilled water were added. Vigorous shaking was necessary to homogenize the emulsion. Second, 230 μL of the emulsion and 20 mL of the extract (5 μg/mL) were added to a 96-well plate. The assay was read at 450–490 nm every 6 s to 180 min at 50°C. Physostigmine was used as a positive standard.

2.5 Cell culture

The human neuroblastoma SH-SY5Y cell line was obtained from the ATCC (CRL-2266, American Type Culture Collection, MD, United States). Cells were cultured in Dulbecco modified Eagle's Minimal Essential Medium (DMEM) and F12 with 10% serum fetal bovine (SFB) and 1% penicillin/streptomycin (100 units penicillin/mL; 100 μg streptomycin/ml; P/S) at 37°C in a humidified atmosphere of 5% CO_2. To evaluate cytotoxicity, 10,000 cells were seeded per well in a 96-well plate in DMEM F12 containing 10 mM retinoic acid, with 1% SFB and 1% P/S. The cells were allowed to differentiate for 3 days.

2.6 MTT assay

MTT is a colorimetric method to determine cell viability indirectly by a reduction reaction made by complex II of viable mitochondria of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (MTT), which is yellow, in the formazan salt (E, Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan, which is purplish-blue. The SH-SY5Y cells were incubated for 24 h with a 100 μL dilution of the plant extracts, which were added in quadruplicate at concentrations of 25, 75, 125, and 250 mg/mL in the cell maintenance medium (1% P/S and 2% SFB in DMEM F12). Then, 10 μL of the MTT solution (5 mg/mL in PBS) was added for 4 h, the medium was removed, and 100 μL of cell lysis buffer (40% v/v of dimethylformamide, 5% v/v glacial acetic acid, and 16% w/v SDS in H_2Odd) was added to each well. The plate was shaken in an orbital shaker at a speed of 65 rpm for 15 min, and absorbance was read at 540 nm on a plate reader (TekanSunrise™, Austria) (Table 2).

2.7 Flow cytometry

To approach the mechanisms of neuroprotection, flow cytometry was performed to follow ROS accumulation and the cell death mechanism using the Muse Cell Analyzer platform (Muse-Guava, Luminex). In brief, 100,000 cells per well were seeded in 12-well plates in a cell culture medium and exposed to different extract concentrations for 1 h or 6 h. Later, paraquat (PQ) or ceramide (C2) was added to the cells for 23 h. Then, 150 μL of 0.5% trypsin-EDTA solution (Gibco—ref. 15,400–054) was added for 3 min, followed by inactivation with 350 μL of 10% FBS in DMEM F12. Two dilutions were made from this mixture: 1) the first was adjusted to 1×10^5–5×10^5 cells per mL for staining with Annexin-V, following the standardized steps of the Muse™ Annexin V Dead Cell kit (Millipore Ref. MCH100105); 2)
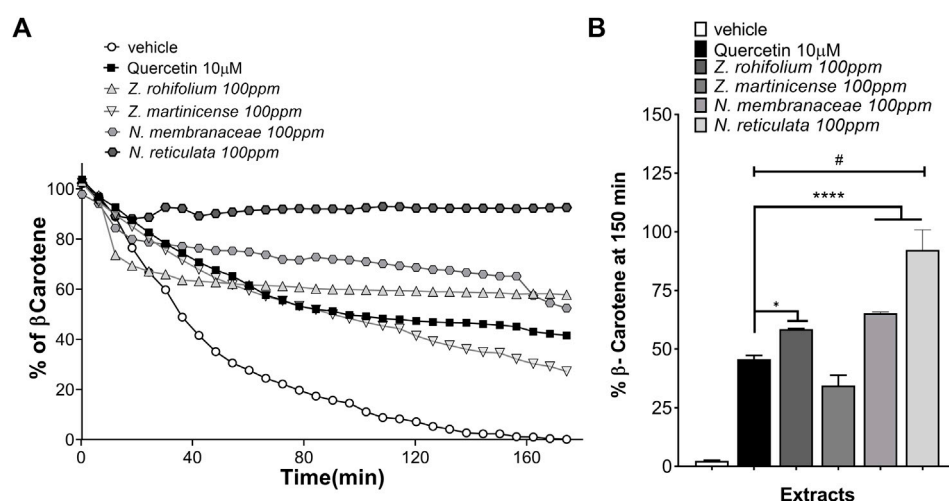
FIGURE 3
Antioxidant and protective activities of β-carotene. **(A)** Quantification of the percentage of β-carotene over 180 min, adding extracts of *Zanthoxylum rhoifolium* Lam, *Zanthoxylum martinicense*, *Nectandra membranacea*, and *Nectandra reticulata*, the solution used as vehicle and quercetin used as the positive control. **(B)** Quantification of the percentage of β-carotene using the extracts at 150 min; the graphic shows the mean and standard error of the mean (SEM). Statistical analysis performed by one-way ANOVA, followed by a multiple comparisons test of Tukey, where significant differences are shown: *($p < 0.05$); ****($p < 0.0001$), in relation to quercetin; #($p < 0.0001$), in relation to the vehicle.

the second was adjusted to $1 \times 10^6 – 5 \times 10^7$ cells per mL and stained following the product recommendation Muse® Oxidative Stress Kit (Millipore Ref. MCH100111) (Guava Muse Cell Analyzer, Luminex).

2.8 Statistical analysis

The statistical analysis was performed using Graph Pad Prism software version 6 (Graph Pad Software Inc.). All quantification values in this study are expressed as the mean ± standard error of the mean (SEM). *In vitro* experiments were evaluated using at least three independent assays. For statistical significance between multiple groups, the parametric data were analyzed using a multivariable 2-way or a 1-way ANOVA, followed by Tukey's *post hoc* test for comparisons between several independent groups. Levels of significance were *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3 Results

3.1 Determination of working concentration of plant extracts

Four Colombian plant extracts were selected to be evaluated by different approaches for their antioxidant capacity. The selected extracts were *Z. rhoifolium* Lam (code 1), *Z. martinicense* (code 2), *N. membranacea* (code 3), and *N. reticulata* (code 4). As a preliminary assay, we determined the optimal working concentration of each extract that did not affect the cell survival of SH-SY5Y cells by MTT. We found that 25 μg/mL was the working concentration for all extracts (Table 2).

3.2 Presence of free radical scavenging and antioxidant capacity of plant extracts

Free radical scavenging capacity was evaluated using the DPPH and β-carotene bleaching assays by bioautography. The DPPH assay is a stable and sensitive method to determine the presence of antioxidant activity in plant extracts. We found inhibition zones from Rf 0.1 to 1 (Table 3), meaning that all extracts have free radical scavenging capacity (Figure 1A; Table 3). The β-carotene bleaching assay is used to evaluate the potential inhibition of the lipid peroxidation of β-carotene exposed to UV light. We found that the bioautography assay showed dots in the TLC for all the extracts, so the extracts will act as lipid peroxidation protectors in a biological environment (Figure 1B).

Normally, the antioxidant activity is related to the presence of phenols and flavonoids, but some studies suggest that alkaloids also have antioxidant activity (Plazas et al., 2018). Because we used two methods of extraction (alkaloidal and ethanolic), we decided to stain the chromoplates with specific reagents to the alkaloids, amino acids, primary and secondary amines, phenols, and flavonoids; these analyses were a preliminary study of the composition of the substances responsible for the antioxidant capacity of the extracts (Figure 1). To confirm this, we first used Dragendorff staining in the chromoplates. We found that only extract codes 1 (Rf 0.9) and 2 (Rf 0.8, 0.3) have dark spots similar to the control, which means they have alkaloids in their composition (Figure 1C; Table 3). Because the structures of alkaloids have nitrogenated groups, we decided to evaluate the presence of primary and secondary amines (chloranil in dioxane) and amino acids (ninhydrin) in the extracts to corroborate the Dragendorff stain. We found that extract codes 1 (retention factor (Rf) 0.9) and 2 (Rf 0.8, 0.3) probably have primary and secondary amines that could be part of the alkaloid structure (Figure 1E; Table 3). Additionally, extract code 1 had a positive
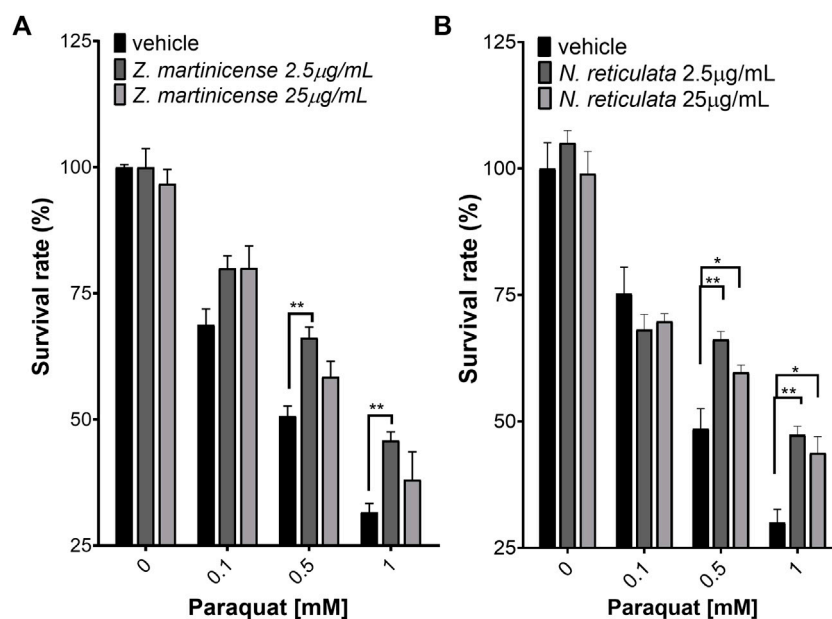
FIGURE 4
Neuroprotection assay evaluated by the variation of mitochondrial activity (MTT). **(A)** SHSY5Y cells pretreated for 1 h with the alkaloidal fraction (chloroform) of *Zanthoxylum martinicense* and exposed to PQ for 23 h. **(B)** SH-SY5Y cells pretreated for 1 h with *Nectandra reticulata* extract and exposed to PQ for 23 h. The graphic shows the mean and standard error of the mean (SEM). Statistical analysis performed by two-way ANOVA, followed by a multiple-comparison test of Tukey, where significant differences are shown as follows: *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$); ****($p < 0.0001$).

spot for amino acids (Figure 1D), but these spots were not in the Rfs shown in the Dragendorff staining (0.5, 0.1).

We also evaluate the presence of phenols and flavonoids in the extracts. We applied the ferric chloride test to corroborate the presence of a phenol. We found black spots like the positive control in extract codes 3 and 4 (Figure 1F; Table 3). To determine the presence of flavonoids, we used the Fast Blue test. We found reddish spots in extract codes 3 and 4 (Figure 1G; Table 3). In addition, when comparing chromoplates before (Figure 1H) and after NH_3 vapor (Figure 1I), we corroborated the presence of a phenol in the extracts. We found an increase in the intensity color or changes in color after NH_3 exposure when the chromoplates were measured at 365 nm (Table 3). Together, these results indicate the presence of flavonoids in the extracts (Mabry et al., 1970; Tenorio, 2016).

3.3 Determination of total phenols

To confirm the presence of phenols and corroborate the presence of flavonoids as principal antioxidant secondary metabolites in the extracts, we evaluated the total phenol concentration in samples using the Folin–Ciocalteu assay. We found that the extracts of *N. membranacea* (code 3) and *N. reticulata* (code 4) had greater concentrations of phenols (4.593 µg/mL and 3.390 µg/mL, respectively) (Figure 2). These suggest that the antioxidant activity could be explained by the presence of the phenol and confirm the results observed in the chromoplates developed with $FeCl_3$ (Figure 1F).

3.4 Antioxidant and protective activity of β-carotene

To confirm the antioxidant potential and to evaluate the protective effect of extracts over time, we analyzed the kinetics of the β-carotene bleaching assay. We determine that the β-carotene present in the vehicle was fully oxidized after 160 min; however, when β-carotene was mixed with an extract or quercetin, the percentage of β-carotene was never less than 35% (Figure 3A), reflecting antioxidant activity. At 150 min, we found that all extracts protected β-carotene against oxidation (F (5,12) = 163.6; $p < 0.001$) (Figure 3B). Interestingly, the *post hoc* analysis showed that the extracts of *Z. rhoifolium* Lam ($p = 0.0233$), *N. membranacea* ($p < 0.001$), and *N. reticulata* ($p < 0.001$) exceed the effect of quercetin, a flavonoid that has been established as a powerful antioxidant widely used in the phytopharmaceutical industry (Figure 3B). These results again corroborate that the extracts could be considered potent antioxidant agents, especially *N. reticulata*, which presents the highest antioxidant effect, almost blocking the oxidizing effects (Figure 3A).

3.5 Extracts decrease the toxic effect of PQ on SH-SY5Y cells

We wanted to know if the antioxidant activity seen in the previous results could have a protective effect over SH-SY5Y cells exposed to toxic concentrations of PQ, which induces oxidative
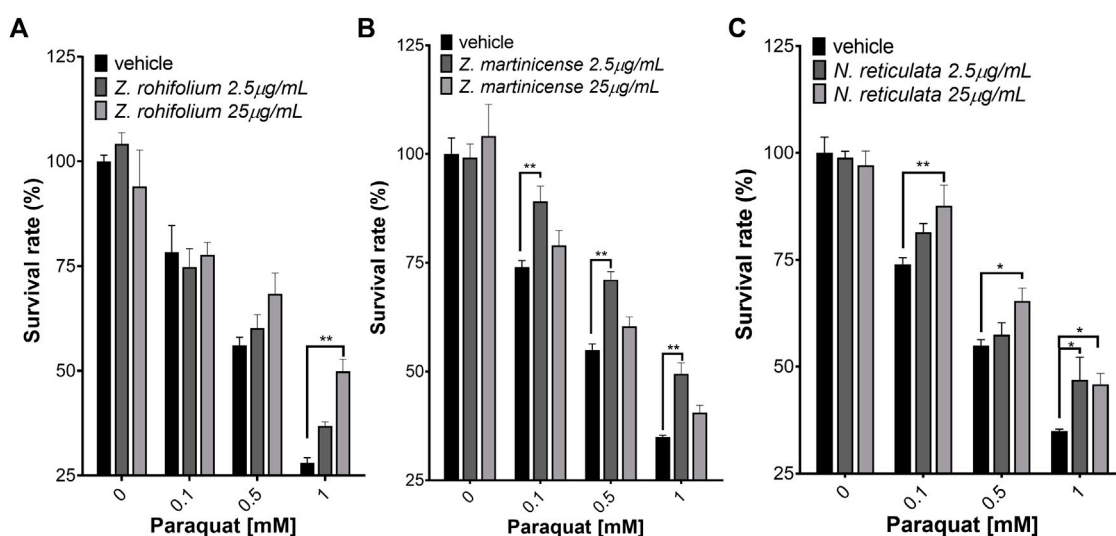
FIGURE 5
Neuroprotection assay assessed by MTT. **(A)** SH-SY5Y cells were pretreated for 6 h with *Zanthoxylum rhoifolium* extract and exposed to PQ for 23 h.
(B) SH-SY5Y cells pretreated for 6 h with *Zanthoxylum martinicense* extract and exposed to PQ for 23 h. **(C)** SH-SY5Y cells pretreated for 6 h with
Nectandra reticulata extract and exposed to PQ for 23 h. The figure shows the mean and standard error of the mean (SEM). Statistical analysis performed
by two-way ANOVA, followed by a multiple comparison test of Tukey, where significant differences are shown as follows: *($p < 0.05$), **($p < 0.01$),
($p < 0.001$), and *($p < 0.0001$).

stress (Figure 4). As expected, we found that PQ triggers cellular death in a dose-dependent manner, with cells having a less-than-30% survival rate with 1 mM PQ (Figure 4). To investigate the protective effect of extracts against the induction of new free radicals, we used pretreatments of extracts of 1 h or 6 h before exposure to PQ.

When the cells were pretreated with extracts for 1 h, only the extract of *Z. martinicense* (Figure 4A) and *N. reticulata* (Figure 4B) had a protective effect against different PQ concentrations. The extract of *Z. martinicense* reduced the toxic effect of PQ, preventing the decrease in cell viability compared to cells that only had the vehicle and were exposed to the toxin (F (2, 36) = 11.30; $p = 0.0002$) (Figure 4A). The pretreatment with a concentration of 25 μg/mL of the extract of *Z. martinicense* exposed to 0.1 mM PQ significant increase the viability of cells (t (36) = 2.622; $p = 0.025$). Interestingly, a smaller concentration of this extract (2.5 μg/mL) significantly increased the viability of cells exposed to 0.1 mM (t (36) = 2.597; $p < 0.05$), 0.5 mM (t (36) = 3.605; $p < 0.001$), and 1 mM (t (36) = 3.295; $p < 0.001$) concentrations of PQ (Figure 4A).

Likewise, the extract of *N. reticulata* also decreased the toxic effect of PQ on cell viability (F (2, 36) = 6.328; $p = 0.0044$) (Figure 4B). We found that both 2.5 and 25 μg/mL concentrations of extract reduced the 0.5 mM and 1 mM PQ effect. The *post hoc* test indicated that this extract at 2.5 μg/mL presented a protective effect against 0.5 and 1 mM PQ (t (36) = 3.801; $p = 0.0011$; t (36) = 3.728; $p = 0.0013$). Additionally, pretreatment of *N. reticulata* at 25 μg/mL also decreased the effect of 0.5 mM and 1 mM PQ on cell viability (t (36) = 2.407; $p = 0.0422$; t (36) = 2.957; $p = 0.0109$) (Figure 4B).

When the extracts were used as pretreatment for 6 h, *Z. martinicense* and *N. reticulata* extracts again showed a protective effect, and additionally, the extract of *Z. rhoifolium* had protective

effects on SH-SY5Y cell survival (Figure 5). The treatment with the extract of *Z. rhoifolium* (25 μg/mL) only reduced the cytotoxic effect of PQ (1 mM) in cell culture (F (2, 9) = 32.2; $p < 0.0001$) (Figure 5A). The treatment with 2.5 μg/mL *Z. martinicense* significantly reduced the cytotoxic effect of PQ (F (2, 36) = 12.60; $p < 0.0001$) at 0.1, 0.5, and 1 mM PQ ($p = 0.0036$, $p = 0.0019$, and $p = 0.053$) (Figure 5B). Finally, the extract of *N. reticulata* at 25 μg/mL reduced the cytotoxic effect of PQ (F (2, 36) = 7.215; $p = 0.0023$) at 0.01, 0.5, and 1 mM PQ ($p = 0.0061$, $p = 0.0387$, and $p < 0.0305$). Moreover, 2.5 μg/mL of *N. reticulata* extract had a protective effect on cell viability against 1 mM PQ ($p = 0.0169$) (Figure 5C).

To verify the cellular protective effects observed by MTT, we performed flow cytometry to directly evaluate the cellular viability by labeling with Annexin V and 7AAD. Additionally, the dihydroethidium marker was used to stain intracellular ROS by flow cytometry and evaluate the relative change in the free radical with or without extracts. The concentrations of PQ, C2, and extracts used for this experiment were the concentrations that yielded the best results in the neuroprotection assay (Figure 6). The untreated SH-SY5Y cells show 99.38% living cells (Figure 6A) with a low basal level of ROS (17.91%) (Figure 7A). When the cells were treated with PQ, the percentage of living cells decreased in a concentration-dependent manner: 86.76% living cells at 0.1 mM PQ, 54.19% living cells at 0.5 mM PQ, and 27.70% living cells at 1 mM PQ (Figures 6B–D). Evaluation of ROS levels showed that cells treated with PQ increased the ROS levels in a concentration-dependent manner: 0.1 mM PQ: 32.61% ROS; 0.5 mM PQ: 50.58% ROS; and 1 mM PQ: 82.98% ROS (Figures 7B–D).

Treatment of SH-SY5Y cells with 2.5 μg/mL of plant extracts did not affect their viability. We found values of living cells close to 99%, and in all cases, the percentage of basal ROS levels was close to untreated cells (Figures 6E,G; Figures 7E,G).
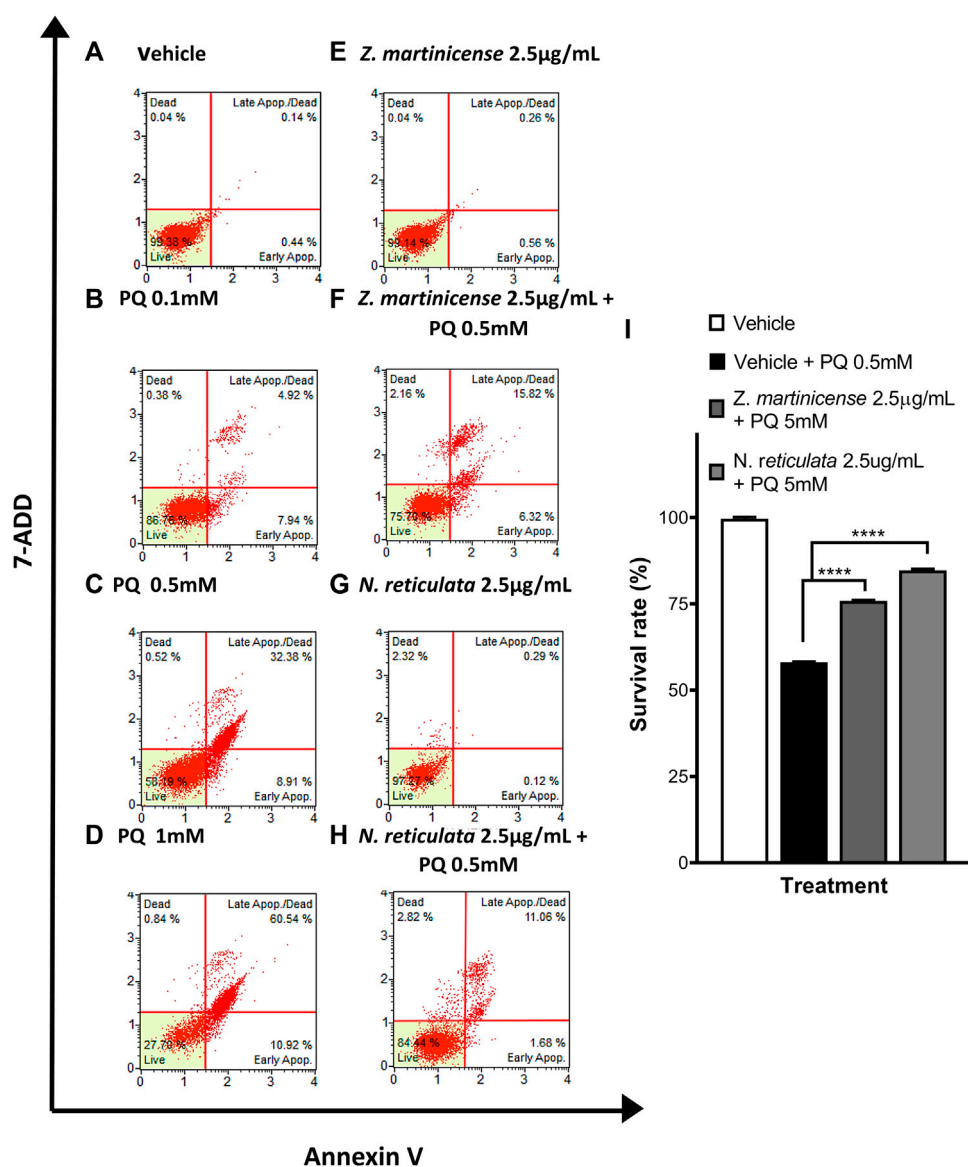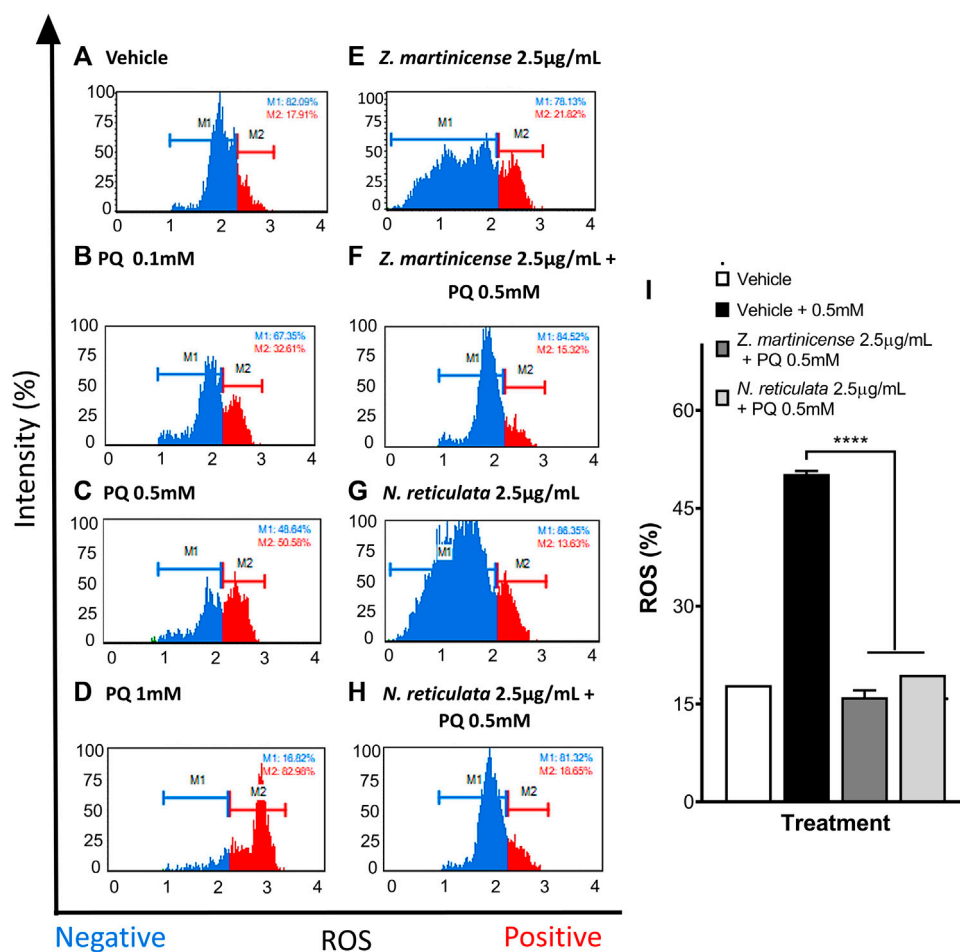
FIGURE 6
Evaluation of the neuroprotective effect of *Zanthoxylum martinicense* and *Nectandra reticulata* by flow cytometry. **(A–H)** Histograms of live, apoptotic cells (stained with annexin V) and necrotic cells (stained with 7-AAD). **(A)** Control cells cultured with the vehicle. **(B)** Cells exposed to PQ 0.1 mM for 23 h. **(C)** Cells exposed to PQ 0.5 mM for 23 h. **(D)** Cells exposed to PQ 1 mM for 23 h. **(E)** Cells exposed to the extract of *Zanthoxylum martinicense* 2.5 μg/mL for 24 h. **(F)** Cells pretreated with the extract of *Zanthoxylum martinicense* 2.5 μg/mL for 1 h and PQ 0.5 mM for 23 h. **(G)** Cells exposed to *Nectandra reticulata* 2.5 μg/mL extract for 24 h. **(H)** Cells pretreated with the extract of *Nectandra reticulata* 2.5 μg/mL extract for 1 h and PQ 0.5 mM for 23 h. **(I)** Bar chart of the percentage of live SH-SY5Y cells determined using annexin V and 7-AAD. The graphic shows the mean and standard error of the mean (SEM). Statistical analysis performed by one-way ANOVA, followed by a multiple comparison test of Tukey, where significant differences are shown: *(p < 0.05), **(p < 0.01), ***(p < 0.001), and ****(p < 0.0001).

Interestingly, the pretreatment with plant extracts induced a neuroprotective effect in the SH-SY5Y cells (F (2, 3) = 454; $p < 0.0001$) and decreased ROS levels (F (3, 4) = 783.3; $p < 0.0001$) upon PQ exposure (Figure 6; Figure 7). Pretreatment with 2.5 μg/mL of *Zanthoxylum martinicense* extract significantly reduced the cytotoxic effect of PQ (58.05% vs. 75.85%; q (3) = 62.45; $p < 0.0001$) (Figures 6F,I) and decreased the ROS level of cells exposed to 0.5 mM PQ (50.24% vs. 16.06%; q (3) = 36; $p < 0.0001$) (Figures 7F,I). Pretreatment with 2.5 μg/mL of *N. reticulata* extracts significantly increased cell viability (58.05%

vs. 84.72%; q (3) = 93.56; $p < 0.0001$) (Figures 6H,I) and decreased ROS levels (50.24% vs. 19.48%; q (3) = 32.4; $p < 0.0001$) upon 0.5 mM PQ exposure (Figures 7H,I).

Because the *Z. rhoifolium* plant extract showed neuroprotection after 6 h of pretreatment, we decided to evaluate cell death and ROS levels in SH-SY5Y cells by flow cytometry. We found that 25 μg/mL of extract did not affect the cell viability (98.72% of a living cell) (Figure 8C). When cells were exposed to 1 mM PQ, the percentage of living cells decreased to 27.79% (Figure 8B), and 6 h of pretreatment with the extract increased the cell viability (28.95%
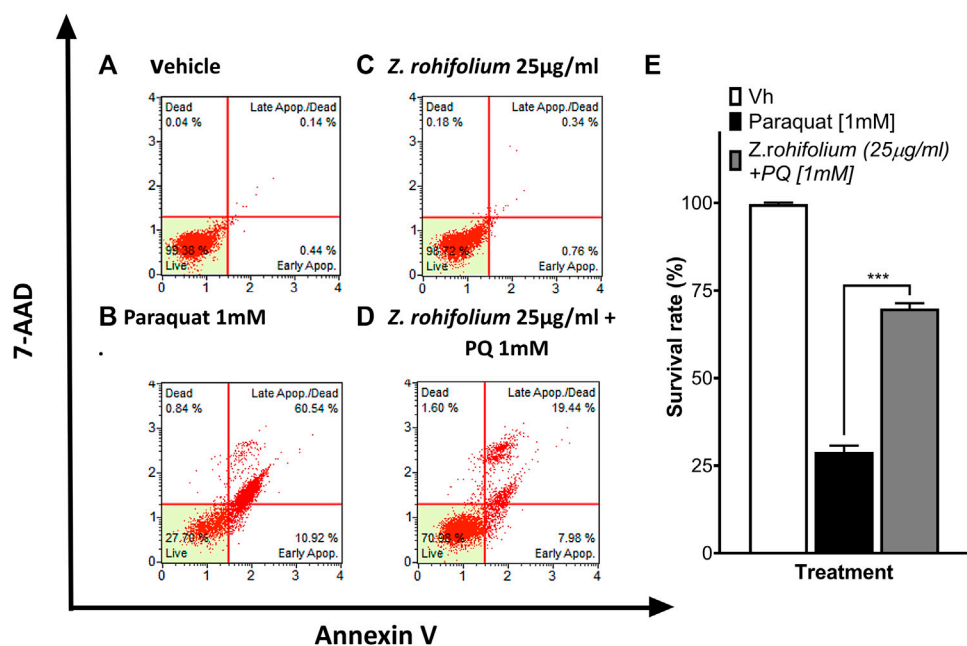
FIGURE 7
Evaluation of the antioxidant effect of *Zanthoxylum martinicense* and *Nectandra reticulata* by flow cytometry. **(A–H)** Histograms of SH-SY5Y cells labeled with dihydroethidium to evaluate the presence (red) or absence (blue) of ROS in cells. **(A)** Control cells cultured with the vehicle. **(B)** Cells exposed to PQ 0.1 mM for 23 h. **(C)** Cells exposed to PQ 0.5 mM for 23 h. **(D)** Cells exposed to PQ 1 mM for 23 h. **(E)** Cells exposed to the extract of *Zanthoxylum martinicense* 2.5 μg/mL for 24 h. **(F)** Cells pretreated for 1 h with the extract of *Zanthoxylum martinicense* 2.5 μg/mL and PQ 0.5 mM for 23 h. **(G)** Cells exposed to the extract of *Nectandra reticulata* 2.5 μg/mL for 24 h. **(H)** Cells pretreated for 1 h with the extract of *Nectandra reticulata* 2.5 μg/mL, followed by PQ 0.5 mM for 23 h. **(I)** Bar diagram of the percentage of SH-SY5Y cells with positive ROS levels identified by labeling ROS with ethidium bromide. The graphic shows the mean and standard error of the mean (SEM). Statistical analysis performed by one-way ANOVA, followed by a multiple comparison test of Tukey where significant differences are shown: *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$).

vs. 69.88%; $F_{(2, 3)} = 133$; $p < 0.0001$; $q_{(3)} = 29.76$; $p = 0.002$) upon 1 mM PQ exposure (Figures 8B,D,E). We found that cells exposed to PQ have a high level of ROS (83.09%) (Figure 9B), and 6 h of pretreatment with the extract induced a significant decrease in ROS levels (22.41%; $F_{(2, 3)} = 803.7$; $p < 0.0001$). Remarkably, treatment with 25 μg/mL of *Z. rhoifolium* extract by itself increased ROS levels (30.99%) (Figure 9C), without affecting cell viability (98.72%) (Figure 8C).

3.6 Extracts decrease the toxic effect of C2-ceramide on SH-SY5Y cells

C2-ceramide (C2) induced the generation of ROS mediated by alteration of membrane phospholipids, disruption of mitochondria, and inactivation of intracellular signaling

pathways, including PI3K/AKT and MAPK. To evaluate the effect of the plant extracts against C2, we used MMT assays and flow cytometry to measure cell death. We found that C2 decreases cell survival in a dose-dependent manner (Figure 10). However, pretreatment for 6 h with *Z. rhoifolium* ($F_{(3, 32)} = 24.76$ $p < 0.0001$) and *N. reticulata* ($F_{(3, 32)} = 13.15$; $p < 0.0001$) extracts induced a neuroprotective effect (Figure 10). We found that pretreatment with 0.25 μg/mL ($t_{(32)} = 6.442$; $p < 0.0001$) and 2.5 μg/mL of *Z. rhoifolium* extract ($t_{(32)} = 5.573$; $p < 0.0001$) protected cells against the toxic effect of 10 μM of C2 (44.82% living cells in C2; 77.39% living cells at 0.25 μg/mL + C2; and 72.99% living cells at 2.5 μg/mL + C2) (Figure 10A). Additionally, similar results were found with *N. reticulata* extract. Pretreatment with 0.25 μg/mL ($t_{(32)} = 3.584$; $p = 0.0033$) and 2.5 μg/mL of *N. reticulata* ($t_{(32)} = 3.040$; $p = 0.0140$) extract protected cells against the toxic effect of 10 μM
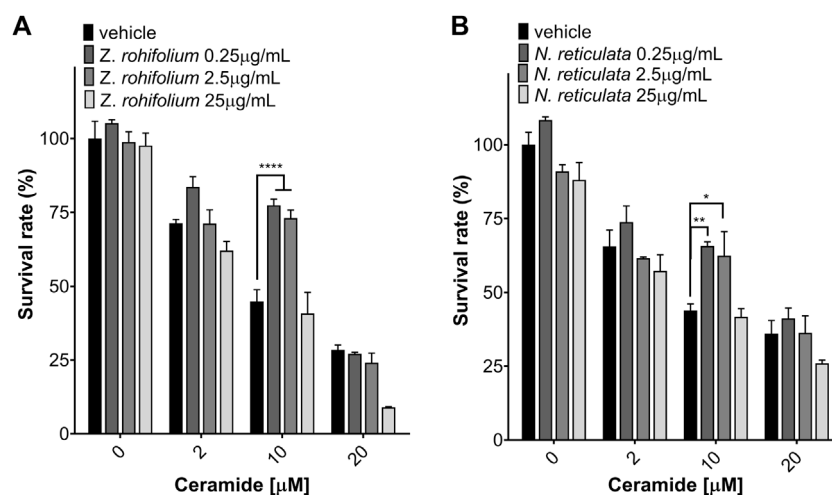
FIGURE 8
Evaluation of the neuroprotective effect of Z. rhoifolium by flow cytometry. **(A–D)** Histograms of SH-SY5Y live cells, apoptotic cells (stained with annexin V), and necrotic cells (stained with 7-AAD). **(A)** Control cells cultured with the vehicle. **(B)** Cells exposed to PQ 1 mM for 23 h. **(C)** Cells exposed to the extract of *Zanthoxylum rhoifolium* 25 µg/mL for 29 h. **(D)** Cells pretreated with the extract of *Zanthoxylum rhoifolium* 25 µg/mL for 1 h and exposed to PQ 1 mM for 23 h. **(E)**. Bar chart of the percentage of live SH-SY5Y cells determined by analysis using annexin V and 7-AAD. The graphic shows the mean and standard error of the mean (SEM). Statistical analysis performed by one-way ANOVA, followed by a multiple comparison test of Tukey, where significant differences are shown: *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$).
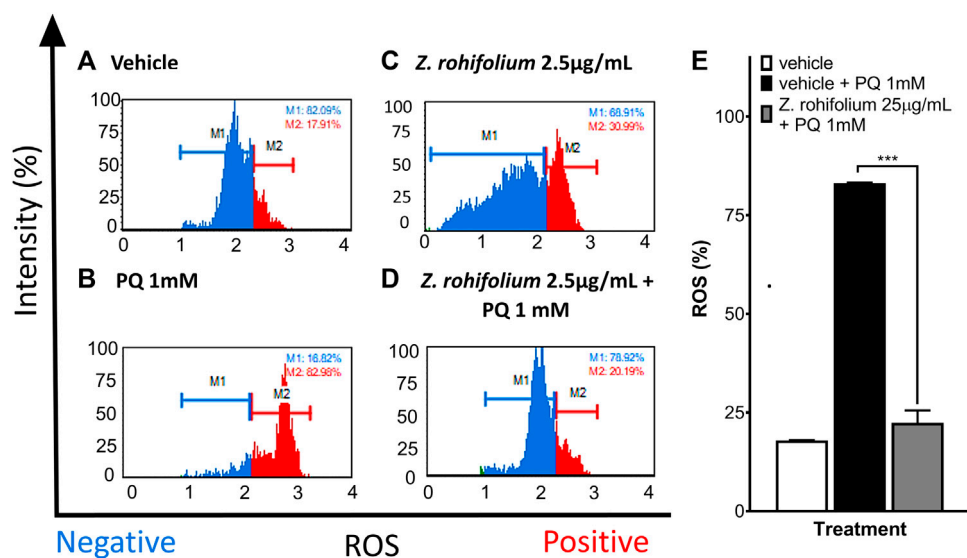
FIGURE 9
Evaluation of the antioxidant effect of *Zanthoxylum rhoifolium* by flow cytometry. **(A–D)** Histograms of SH-SY5Y cells labeled with dihydroethidium to evaluate the presence or absence of ROS in the cells. **(A)** Control cells cultured with the vehicle. **(B)** Cells exposed to PQ 1 mM for 23 h. **(C)** Cells exposed to the extract of *Zanthoxylum rhoifolium* 25 µg/mL for 29 h. **(D)** Cells pretreated with the extract of *Zanthoxylum rhoifolium* 25 µg/mL for 1 h and exposed to PQ 1 mM for 23 h. **(E)** Bar diagram of the percentage of SH-SY5Y cells with positive ROS levels identified by labeling ROS with ethidium bromide. The graphic shows the mean and standard error of the mean (SEM). Statistical analysis performed by one-way ANOVA, followed by a multiple comparison test of Tukey, where significant differences are shown: *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$).

FIGURE 10
Neuroprotection assay of *Zanthoxylum rhoifolium* and *Nectandra reticulata* against C2-ceramide evaluated using MTT. **(A)** SH-SY5Y cells were pretreated for 6 h with *Zanthoxylum rhoifolium* extract and exposed to C2-ceramide for 23 h. **(B)** SH-SY5Y cells were pretreated for 6 h with the *Nectandra reticulata* extract and exposed to C2-ceramide for 23 h. The graphic shows the mean and standard error of the mean (SEM). Statistical analysis performed by two-way ANOVA, followed by a multiple comparisons test of Tukey where significant differences are shown as follows: *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$); ****($p < 0.0001$).

of C2 (43.94% living cells in C2; 65.72% living cells at 0.25 μg/mL + C2; and 62.41% living cells at 2.5 μg/mL + C2) (Figure 10B).

Cell death and ROS levels in SH-SY5Y cells were evaluated by flow cytometry upon C2 exposure. C2 induced a dose-dependent cell death effect (2 μM C2: 74.35% living cells; 10 μM C2: 29.91% living cells; 20 μM C2: 3.15% living cells) (Figures 11B–D). Treating SH-SY5Y cells with 0.25 μg/mL of *Z. rhoifolium* or *N. reticulata* extract did not affect the viability of the cells (98.72% and 99.38% living cells) (Figures 11E,G). Pretreatment for 6 h with the plant extracts induced a neuroprotective effect upon C2 exposure (F (2, 3) = 639.2; $p = 0.0001$). Pretreatment with 0.5 ug/mL of *Z. rhoifolium* and *N. reticulata* extracts protected the SH-SY5Y cells against 10 μM of C2 (30.02% vs. 47.02%; q (3) = 23.72; $p = 0.0003$; 30.02% vs. 55.12%; q (3) = 35.03; $p = 0.0001$, respectively) (Figures 11F,H,I). Together, the results showed that the extracts were a potent cellular protector against C2 exposure.

4 Discussion

Extracts derived from diverse Colombian plants evaluated in the present paper have an important antioxidant and neuroprotective activity against PQ and C2, two toxins that are widely described to be involved in the physiopathology of various neurodegenerative diseases (Fukushima et al., 2002; Arboleda et al., 2009; Chaurasia and Summers, 2015; Jazvinšćak Jembrek et al., 2015; Yan et al., 2016; Cruciani-Guglielmacci et al., 2017; Islam et al., 2019). We proposed that the beneficial activities of these extracts could be attributed to three potential types of biomolecules: alkaloids, phenols, and flavonoids (Mabry et al., 1970). We found that the extracts with the best results were *N. membranacea* and *N. reticulata*.

The Fast Blue assay showed the presence of reddish spots in the chromoplate for the extracts *N. membranacea* and *N. reticulata*.

These spots developed as the result of the formation of a red complex for the diazonium salt because of the presence of phenols in the extract. Additionally, we used NH$_3$ vapors to confirm the presence of phenols. Because the ammonia interacting with phenols increases their electron density, we can see an increase in the intensity of a color change read at 365 nm (Mabry et al., 1970; Lock Sing de Ugaz, 1997; Tenorio, 2016). We again found that the extracts were associated with phenols and flavonoids. In addition, a relationship was found between the results of the FeCl$_3$ (Figure 1F) and the quantification of total phenols by a Folin–Ciocalteu assay (Figure 2). On both assays, the extracts of *N. membranacea* and *N. reticulata* had a higher concentration of phenols that could be directly associated with the antioxidant effect observed in the bioautographies. These results confirm our hypothesis that secondary metabolites of the flavonoid type give the antioxidant characteristic to these extracts. Previous research had demonstrated that the genus *Nectandra* is rich in metabolites, such as polyphenols, flavonoids, and alkaloids, that confer analgesic, anti-inflammatory, and antioxidant activities (Le Quesne et al., 1980; Filho et al., 2004; Oliveira De Melo et al., 2006; Moreno et al., 2011; Ponci et al., 2015; Espitia Corredor et al., 2016). Additionally, some investigations suggested that some polyphenols have biological activities, such as transcription factors agonists (LXR agonist), BACE inhibition, and anti Aβ aggregation activity, that give the extracts from *Nectandra* potential action against AD (Park et al., 2004; Ondeyka et al., 2005; Wang and Du, 2009; Paula et al., 2019).

The antioxidant activity of *Z. rhoifolium* and *Z. martinicense* extracts could be explained by the presence of some alkaloids because they were obtained from the alkaloidal isolation before starting with the analysis. However, we found dark spots in the chromoplates of these extracts when they were developed with the Dragendorff reagent. Additionally, the chromoplates of these extracts showed spots when they were developed with ninhydrin.
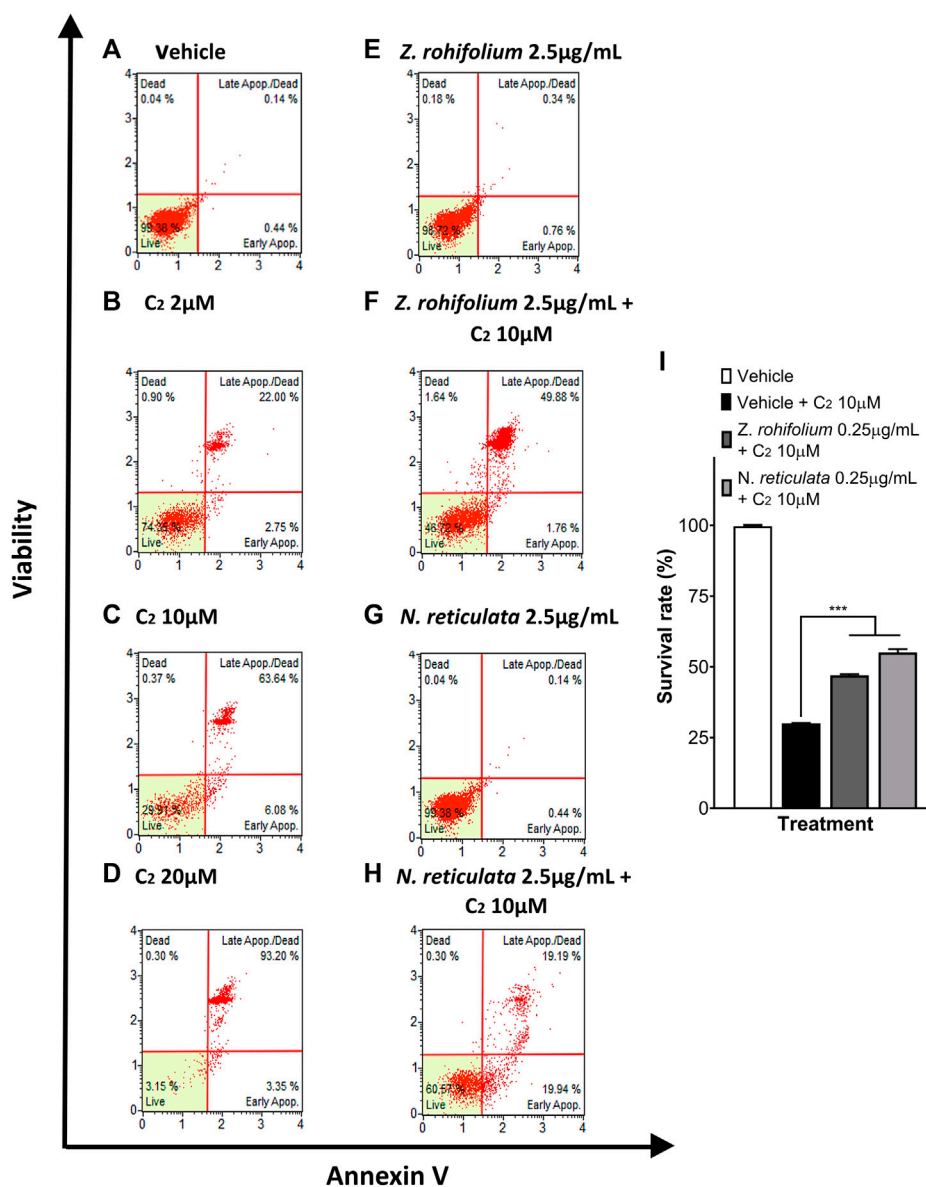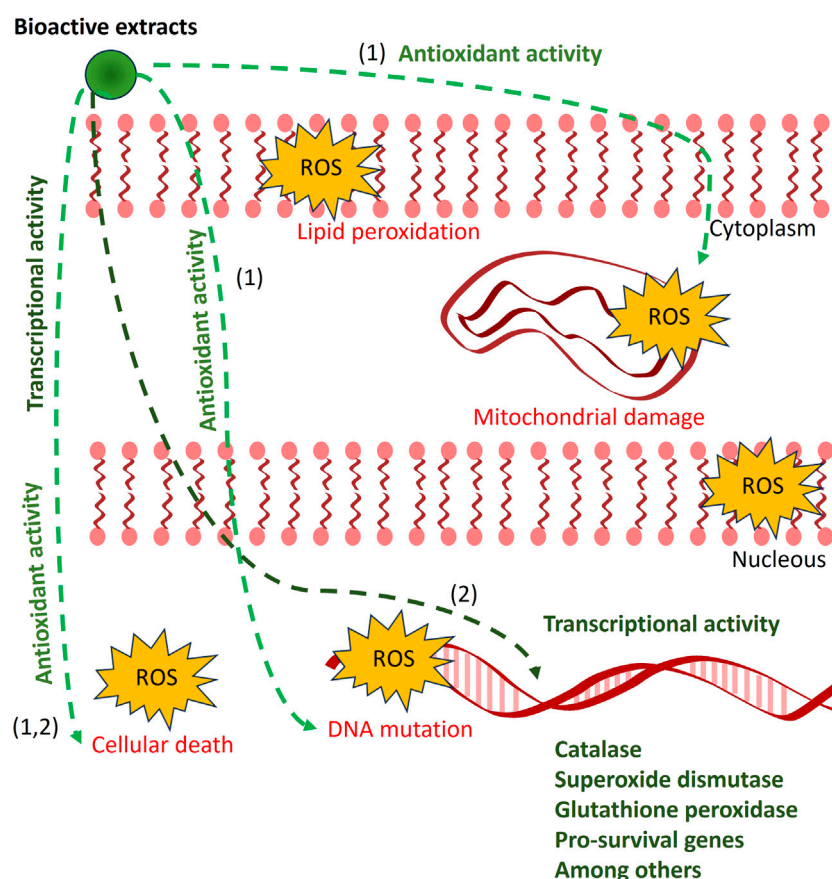
FIGURE 11
Evaluation of the neuroprotective capacity of *Zanthoxylum rhoifolium* and *Nectandra reticulata* extracts against C2-ceramide. **(A–H)** Histograms of live, apoptotic SH-SY5Y cells stained with annexin V and necrotic cells stained with 7-AAD. **(A)** Control cells cultured with the vehicle. **(B)** Cells exposed to C2 2 μM for 23 h. **(C)** Cells exposed to C2 10 μM for 23 h. **(D)** Cells exposed to C2 20 μM for 23 h. **(E)** SH-SY5Y cells treated for 29 h with the extract of *Zanthoxylum rhoifolium* 2.5 μg/mL. **(F)** SH-SY5Y cells pretreated for 1 h with the extract of *Zanthoxylum rhoifolium* 2.5 μg/mL and exposed to C2 10 μM for 23 h. **(G)** SH-SY5Y cells pretreated for 29 h with an extract of *Nectandra reticulata* 2.5 μg/mL. **(H)** SH-SY5Y cells pretreated for 1 h with an extract of *Nectandra reticulata* 2.5 μg/mL and exposed to C2 10 μM for 23 h. Bar chart of the percentage of live SH-SY5Y cells determined by analysis using annexin V and 7-AAD. Statistical analysis performed by two-way ANOVA, followed by a multiple comparison test of Tukey where significant differences are shown as follows: *(p < 0.05), **(p < 0.01), ***(p < 0.001), and ****(p < 0.0001).

Interestingly, most of the ninhydrin spots in the chromoplates match the spots developed by the Dragendorff reagent (see Rfs in Table 3). Moreover, recent research has shown that Colombian *Zanthoxylum* alkaloidal extracts have antioxidant capacity provided by alkaloid molecules (Plazas et al., 2018). Furthermore, these extracts showed low concentrations of phenols (<0.8 μg/mL). This is because the presence of flavonoids in the alkaloid fractions is very unlikely, but some

alkaloids that could also have an antioxidant effect in this type of extract have been reported (Gülçin et al., 2010; Rehman and Khan, 2017). Therefore, the genus *Zanthoxylum* is rich in alkaloids as fundamental metabolites with a variety of antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, cardiovascular protectant, and anti-cancer activities (Ethur et al., 1997; Nhiem et al., 2020). Some alkaloids, including dihydronitidine, 6-oxynitidine,

FIGURE 12
Proposed model of the antioxidant effect from bioactive extracts. Abnormal increases in ROS (red arrow) cause cellular death associated with lipid peroxidation, mitochondrial damage, and DNA mutation, among others. The bioactive extracts from *Zanthoxylum rhoifolium*, *Zanthoxylum martinicense*, *Nectandra membranacea*, and *Nectandra reticulata* reduced ROS levels (black and green arrows) and increased cell viability. We proposed two alternative models for the neuroprotective function of plant extracts depending on pretreatment duration: (1) in short treatments (1 h), the bioactive molecules could act directly as direct antioxidant agents against increased free ROS present in different cellular components such as membranes, mitochondria, and nuclei; and (2) in longer pretreatments (6 h), the bioactive molecules could directly or indirectly regulate gene transcriptional activity, increasing the expression of genes associated with antioxidant or pro-survival effects.

magnoflorine, bocconoline, nitidine, chelerythrine, and skimmianine, were isolated from *Z. rhoifolium.* (Ethur et al., 1997; Blanco-Ayala et al., 2014; Tavares et al., 2014; Plazas et al., 2018; Pallikonda et al., 2019; Nhiem et al., 2020). 6-Oxynitidine has been associated with anti-inflammatory activity, and skimmianine has strong AChE inhibitor activity (Tavares et al., 2014; Pallikonda et al., 2019). This neuroprotective effect of alkaloids could explain that the present results are related to their antioxidant capacity and additional cell survival properties.

Exposure of SH-SY5Y cells, a neuroblastoma cell line, to PQ decreased their viability and increased ROS levels. However, a short pretreatment (1 h) of the cell with the extracts of *N. membranacea* or *N. reticulata* demonstrated a neuroprotective effect upon toxin exposure, which was related to a decrease in cellular ROS levels. Although the mechanisms for neuroprotection were not studied in the present paper, we suggest that it could be due to a direct antioxidant activity over harmful ROS that can induce lipid peroxidation, mitochondrial damage, and DNA mutation. Additionally, the

bioactive extracts could induce post-transcriptional changes in proteins or molecules associated with the cellular response to increase free radical levels and therefore reduce their harmful effects on mitochondrial function and key cellular components. A longer pretreatment (6 h) with the extracts of *Z. rhoifolium* and *N. reticulata* also demonstrated a neuroprotective effect against PQ. In this case, we propose that these protections may be conferred, in part, by genomic regulation, increasing the transcription of genes related to free radical scavenging enzymes (catalase, superoxide dismutase, and glutathione peroxidase), pro-survival, and anti-apoptotic proteins that would confer resistance to the cells upon toxin exposure (Figure 12).

To test the additional cellular protective effect of the extracts, we exposed the cell to C2, which is a potent cellular toxin that activates different signaling pathways conducive to cell death and increases free radical generation. We found that only a longer pretreatment (6 h) with *Z. rhoifolium* and *N. reticulata* extracts showed a neuroprotective effect against toxic concentrations of C2. In this case, the extracts most probably regulate gene

transcription toward a cell phenotype that confers resistance to diverse cellular insults and favors cell survival. Finally, recent findings of our laboratory suggest that longer treatment with these plant extracts induced diverse changes in gene expression toward a neuroprotective phenotype than non-treated conditions (unpublished data). However, additional studies are required to support this hypothesis.

5 Conclusion

Colombian plant extracts from *Z. rhoifolium, Z. martinicense, N. membranacea*, and *N. reticulata* have important antioxidant and neuroprotectant activities against known ROS-producing toxins and cell death triggers such as PQ and C2. The main metabolites associated with this activity are probably flavonoids and alkaloids, molecules with extensive research confirming their antioxidant potential, and could be used to reduce the deleterious effects of ROS in neurodegeneration.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

AB-R: investigation, formal analysis, data curation, validation, and writing—original draft. JM-C: formal analysis, data curation, and writing—original draft. LC: writing—review and editing, and resources. GA: resources, visualization, and writing—review and editing. MA: visualization, methodology, resources, and writing—review and editing. AS-H: conceptualization, methodology, funding acquisition, project administration, visualization, and writing—review and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The author GA declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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