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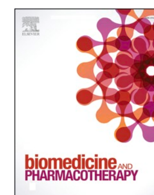

# Neuroprotective effects of polyphenol-rich extracts obtained from agricultural by-products in an induced cognitive decline model of zebrafish larvae and in human neurons

Ángela Castillo-Moral [a,b], Job Tchoumtchoua [c], Kevin Leonard [c], Josep M. Del Bas [a,d,e], Nàdia Ortega [a,f,g], Xavier Escoté [a,d,h,i,*], Joan Teichenné [a,**]

[a] Eurecat-Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus 43204, Spain
[b] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology, Tarragona 43007, Spain
[c] Biomass Valorisation Platform, Extraction Department, CELABOR Srl, Herve 4650, Belgium
[d] Institute of Health Pere Virgili (IISPV), Center of Environmental, Food and Toxicological Technology—TecnATox, Rovira i Virgili University, Reus 43201, Spain
[e] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology Nutrigenomics Research Group, Tarragona 43007, Spain
[f] Department of Food Technology, Engineering and Science, University of Lleida, Spain
[g] Agrotecnio CERCA Centre, Av. Alcalde Rovira Roure, 191, 25198, Spain
[h] Universitat Rovira i Virgili, Department of Biochemistry and Biotechnology Nutrition and Metabolic Health Research Group, Reus 43201, Spain
[i] CIBER in Physiopathology of Obesity and Nutrition (CIBEROBN), Carlos III Health Institute, Madrid 28029, Spain

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ABSTRACT

Neurodegenerative diseases are closely associated with chronic neuroinflammation and oxidative stress, which contribute to progressive neuronal dysfunction and cell death. Due to their antioxidant and anti-inflammatory properties, polyphenols have gained attention as potential neuroprotective agents. Agricultural by-products represent a promising and sustainable source of polyphenols, yet their neuroprotective value remains under-explored. In this study, we evaluated four polyphenol-rich extracts derived from red onion peels (ROPE), olive pruning (OPE), vineyard pruning (VPE) and chicory leaves (CLE), obtained by subcritical water extraction. Their effects were tested in two complementary models of neurodegeneration: *in vitro* human neurons (SH-SY5Y cells) exposed to D-galactose and a basic cognitive decline model of zebrafish larvae exposed to aluminium chloride ($AlCl_3$). All extracts exhibited anti-inflammatory effects *in vitro*, significantly reducing *IL-1β* and *IL-8* mRNA expression, at doses ranging 12.5–50 μg/mL in cell medium. In the zebrafish model, treatment with 100 μg/mL ROPE or VPE in medium restored the normal sensorimotor pattern in the Dark-Light-Dark test, while ROPE treatment additionally rescued basal startle responses and enhanced habituation indexes, even surpassing healthy control larvae. Overall, these results highlight the potential of polyphenol-rich agri-food extracts, particularly ROPE, as neuroprotective and cognitive-enhancing compounds and support their further investigation as natural and sustainable interventions to slow or prevent neurodegenerative processes.

## 1. Introduction

Neurodegenerative diseases are a group of disorders characterized by the progressive degeneration of the nervous system, leading to a decline in cognitive function and motor abilities. Disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) are prominent examples. These diseases pose significant challenges to both patients and healthcare systems due to their debilitating nature and the lack of effective treatments. The involvement of oxidative stress and inflammation in the development and progression of neurodegenerative disorders is well documented [1,2]. Oxidative stress refers to an imbalance between the production of reactive oxygen species (ROS) and antioxidant arsenal, which initiates lipid peroxidation of neuronal membranes, leading to the release of reactive aldehydes such as 4-HNE and MDA that form covalent adducts with proteins and disturb proteostasis [3]. These oxidative modifications precipitate protein misfolding

* Corresponding author at: Eurecat-Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus 43204, Spain.
** Corresponding author.
*E-mail addresses:* xavier.escote@urv.cat (X. Escoté), joan.teichenne@eurecat.org (J. Teichenné).

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and ER stress, activating the unfolded protein response and fostering aggregation of disease-linked proteins (e.g. tau, α-synuclein) [4]. Oxidative damage to mitochondrial lipids and respiratory chain proteins impairs the function of complexes I and III, leading to reduced ATP production and disruption of $Ca^{2+}$ homeostasis. This dysfunction enhances ROS generation, creating a vicious cycle of mitochondrial impairment [5]. Furthermore, protein aggregates can aggravate mitochondrial stress by impairing organelle dynamics and bioenergetics, thereby amplifying oxidative stress [6]. The accumulation of oxidized biomolecules and mitochondrial fragments triggers microglial activation via TLR/NLR pathways and the release of inflammatory cytokines such as *IL-1β*, *IL-6* and *TNFα*, which further exacerbates neurodegeneration by promoting synaptic dysfunction and neuronal loss [7]. This harmful interplay between oxidative damage and inflammatory signalling contributes to hallmark features of neurodegenerative diseases, reinforcing the potential of targeting redox and immune pathways in therapeutic interventions.

Polyphenols are well-known substances eliciting strong antioxidant and anti-inflammatory properties, which have been linked to neuroprotective effects in preclinical and clinical studies [8]. In plants, polyphenols are synthesized as secondary metabolites that play key ecological and physiological roles, such as protection against ultraviolet radiation, pathogens, and oxidative stress, as well as signalling molecules in plant defence responses [9]. Structurally, they comprise a diverse family—including phenolic acids, flavonoids, stilbenes, lignans, and tannins—characterized by one or more aromatic rings bearing hydroxyl groups [9]. They are abundant in fruits, vegetables, tea, coffee, wine, cereals, and agricultural by-products, where they contribute to colour, flavour, and antioxidant potential. Beyond their physiological role in plants, dietary polyphenols exert beneficial effects in humans by modulating oxidative and inflammatory pathways, improving endothelial and mitochondrial function, and offering protection against chronic diseases such as cardiovascular, metabolic, and neurodegenerative disorders [10]. Some polyphenols, such as resveratrol, caffeic acid, homovanilic acid or quercetin, are able to cross the blood-brain barrier (BBB) and reach brain cells, with variable ability to accumulate in the central nervous system (CNS), depending on their properties related to their chemical structure [11–13]. Consequently, novel dietary interventions rich in polyphenols may offer a promising avenue for managing neurodegenerative diseases. Agricultural by-products are an underutilized reservoir of high-value bioactive compounds, particularly polyphenols. In recent years, growing interest has emerged in their recovery and valorisation, not only due to their environmental advantages, such as waste reduction and support for a circular economy, but also because of their promising applications across the food, nutraceutical, and pharmaceutical sectors [14,15]. Various sustainable extraction methods have been developed to efficiently recover these valuable compounds. Methods such as subcritical water extraction (SWE), microwave-assisted extraction, hot-pressurized liquid extraction, supercritical fluid extraction, ultrasound-assisted extraction, and enzyme-assisted extraction enable the effective isolation of polyphenols and other bioactive compounds. These approaches not only minimize the use of hazardous organic solvents and lower energy consumption but also enhance the economic value of agricultural residues [15–18]. Among these techniques, SWE offers several advantages, since it uses water under controlled temperature and pressure conditions, eliminating the need for toxic organic solvents and substantially reducing environmental impact. Furthermore, by tuning the temperature and pressure, water's polarity can be adjusted to optimize the solubility and selectivity for targeted bioactive compounds, resulting in high yields and improved extract purity while consuming less energy [18,19].

The human neuroblastoma cell line SH-SY5Y is commonly used to assess the anti-inflammatory and neuroprotective effects of polyphenols or plant-based compounds [20,21]. This cell line has been widely employed in neurodegenerative research, particularly in models of AD and PD [22,23]. Its ability to undergo differentiation to neurons and respond to oxidative and inflammatory stimuli makes it a suitable *in vitro* model to mimic key cellular processes involved in neuronal damage and aging. SH-SY5Y cells are frequently used in D-galactose (D-gal)-induced models of neurosenescence, which simulate aging-related oxidative stress, mitochondrial dysfunction, and apoptotic responses [24]. On the other hand, zebrafish (*Danio rerio*) has emerged as a powerful model organism in biomedical research in recent years, particularly in the study of neurodegenerative diseases. Its high genetic and physiological homology to humans, sharing more than 80 % of known disease-related genes, makes it a valuable vertebrate model for translational neuroscience. Zebrafish larvae possess a fully developed central nervous system and display complex, quantifiable behaviours such as learning, memory, and sensorimotor responses, allowing the assessment of cognitive and neurobehavioral alterations associated with neurodegeneration [25], and a variety of neurodegenerative models can be chemically induced in zebrafish adults and larvae. For example, aluminium exposure has been shown to induce AD-related symptoms in both adult and larval zebrafish [26,27]. Neurodegeneration in zebrafish larvae can be assessed through several tests, such as the Dark-Light-Dark (DLD) test, a behavioural assay commonly used to evaluate sensorimotor function of zebrafish and neurobehavioral responses to chemical toxins and drugs [28], and the startle response test, useful to understand whether the larvae have proper sensory and motor stimuli which are critical for survival, as well as to evaluate habituation when larvae are exposed to continuous stimuli [29].

In the present study, we aimed to discover the potential neuroprotective effects of four polyphenol-rich extracts, obtained by SWE and resin adsorption techniques from four agricultural by-products (red onion peels, vineyard pruning, olive pruning, and chicory leaves), in the human neuroblastoma cell line SH-SY5Y exposed to D-gal, as a model of neurosenescence, and in an $AlCl_3$-induced model of neurodegeneration in zebrafish larvae.

## 2. Materials and methods

### 2.1. Plant materials, extraction of polyphenols

Four polyphenol-rich extracts were obtained by SWE from chicory (*Cichorium intybus var. sativum)* leaves (CLE), red onion (*Allium cepa*) peels (ROPE), olive (*Olea europaea var. verdale aglandau*) leaves and branches pruning (OPE), and vineyard (*Vitis vinifera var. caberbnet franc)* branches pruning (VPE), as previously described [30]. Briefly, dried raw materials were milled (2–4 mm sieve) and dropped into a 6 L stainless steel insert. The insert was introduced in a reactor, and the system was closed. Water was pumped and heated (120–150 °C) through a heat exchanger until the system was filled and reached the targeted pressure of 15 bars. Automatic valves were closed, and the recirculation pump was powered on to recirculate the water in the extraction loop at a flow rate of 1000 g/min. Recirculation was maintained for 30 min; then the liquid extract was cooled prior to system depressurization. The total liquid extract was flushed in the collector using a nitrogen flow to drain the system. Final extracts were recovered from the extract collector. Two polyphenol liquid extracts (Chicory leaves and olive pruning) were directly subjected to a further enrichment step using hydrophobic adsorption resin to increase their purity in batch mode. Two complete cycles of extraction were performed and pooled together prior to drying step by spray-drying to obtain the polyphenol extracts. The extracts were characterized for their total phenolic content and their antioxidant activity, and the identification and quantification of polyphenols in the dried extracts were performed by Ultra-High Performance Liquid Chromatography (UHPLC) coupled to mass spectrometry [30]. The different extracts were then dissolved in DMSO in a concentration of 100 mg/mL, and serial dilutions were subsequently prepared in order to be used in the different mediums at different work concentrations.

### 2.2. In vitro cell culture

The human neuroblastoma cell line SH-SY5Y (ATCC, CRL-2266) was cultured and maintained in 5 % $CO_2$ at 37 ºC in DMEM/F12 medium (Sigma-Aldrich Corp., St. Louis, MO, USA), supplemented with 10 % FBS and antibiotics (Corning; 30–002-CI, Corning, NY, USA). Cells were subcultured every 3–4 days, when cell density achieved 80–90 % of the growth surface.

### 2.3. MTT assay

Cell viability was quantified by the MTT assay, a colorimetric assay that measures the activity of enzymes that transform the MTT molecule to insoluble formazan, which has a purple colour [31]. Cells were seeded in a 96-well plate at a seeding density of $2.5 \times 10^4$ cells/well 24 h before treatments in DMEM/F12 medium supplemented with 10 % FBS and 1 % antibiotics. Then the dissolved extracts were applied for 48 h at different concentrations. The vehicle for treatments was DMEM/F12 supplemented with 1 % FBS and 1 % antibiotics, and all treatments included 0.2 % DMSO, including the controls. After treatments, cell medium was aspirated and 100 μL of MTT 1 mg/mL (Sigma; M2128), diluted in EMEM, were added to the wells. Cells were then incubated at 35 ºC for 3 h. The MTT solution was aspirated, formazan crystals were dissolved in 100 μL of DMSO, and absorbance was measured at 570 nm in a SPECTROstar Nano absorbance microplate reader (BMG Labtech). Mean values of controls were considered as 100 % cell viability.

### 2.4. mRNA expression analysis

SH-SY5Y cells ($2.5 \times 10^5$ cells/well) were cultured in 12 well-plates and the differentiation protocol was followed. Cells were seeded in maintenance medium and then exposed to three days of 5 μM of retinoic acid (RA) and 3 days of brain derived neurotrophic factor (50 ng/mL) and RA in 1 % FBS supplemented DMEM/F12. After this, cells were exposed to the different extracts (12.5 μg/mL for ROPE, 25 μg/mL for both OPE and CLE and 50 μg/mL for VPE) for 24 h, a group of samples were treated with just the vehicle (differentiation medium), other group with 300 mM D-gal and four groups with the right concentration of each extract combined with 300 mM of D-gal, using 0.1 % of DMSO as a vehicle.

Total RNA was extracted using the HigherPurity™ Total RNA Extraction Kit (Canvax Biotech, Córdoba, Spain) following the manufacturer's protocol instructions. RNA concentration and purity was then assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

For reverse transcription, 10 μL of RNA at a concentration of 100 ng/mL was used to synthesize cDNA with the High-Capacity cDNA RT kit (Fisher Scientific). The reaction was carried out in a thermal cycler under the conditions of 25ºC for 10 mins, 28ºC for 120 min, and 85ºC for 5 min. The resulting cDNA was stored at −20ºC until used in quantitative PCR (qPCR).

qPCR was performed using SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a LightCycler® 480 II (Roche Diagnostics) with specific primers (0.2 μM) (Table 1). Data were analyzed using the $2^{\Delta\Delta Ct}$ method [32] and normalized to *GAPDH* mRNA expression.

**Table 1**
List of oligonucleotides pairs sequences used for mRNA expression analyses.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *GAPDH* | CATGAGAAGTATGACAACAGCCT | AGTCCTTCCACGATACCAAAGT |
| *CCL2* | AGCAGCAAGTGTCCCAAAGA | GGTGGTCCATGGAATCCTGA |
| *IL−1β* | GGGACAGGATATGGAGCAACA | TTTCAACACGCAGGACAGGTA |
| *IL−8* | TGGCAGCCTTCCTGATTTCT | TTCTCAGCCCTCTTCAAAAACTTC |
| *TNFα* | CCTCTCTCTAATCAGCCCTCTG | GAGGACCTGGGAGTAGATGAG |
| *GAP43* | ATAACTCGCCGTCCTCCAAG | CAGGCATGTTCTTGGTCAGC |
| *BDNF* | AAAACCATAAGGACGCGGACTT | AAAGAGCAGAGGAGGCTCCAA |

### 2.5. Animals

Zebrafish (*Danio rerio*) embryos were obtained from an external supplier (Aprende con Danio, Spain) and received at 24 h post-fertilization (hpf). Embryos corresponded to a wild-type population, not belonging to a genetically defined laboratory strain. According to the provider, zebrafish are maintained under breeding conditions that avoid excessive inbreeding and therefore preserve the genetic variability and phenotypic robustness typical of wild-type zebrafish. This approach minimizes the colony-related abnormalities often associated with long-term inbred laboratory lines.

Upon arrival, embryos were maintained in E3 medium under standard laboratory conditions (28 °C; 14:10 h light–dark cycle) until 3 days post-fertilization (dpf), when they were randomly distributed for experimental treatments. All procedures were performed in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes.

### 2.6. Neurodegeneration zebrafish model and treatments

Zebrafish larvae were exposed to $AlCl_3$ 50 μM from 3 to 5 dpf with the presence or absence of 100 μg/mL of each extract in Petri dishes. The vehicle for treatments was E3 medium, and all treatments included 0.2 % DMSO, including the healthy and $AlCl_3$ controls. Treatments were renewed every day. Larvae were incubated at 28 ºC with a 14–10 h light–dark period (lights on: 08.00–22.00 h). At 5 dpf, treatments were removed and replaced by E3 medium. Then, 13–14 normally moving, noncurved, and healthy-looking larvae from each treatment were transferred from the Petri dishes into a 96-well plate (1 larva per plate in 100 μL of medium). The 96-well plate was introduced in the observation chamber of the tracking system (Daniovision, Noldus Information Technology B.V., Wageningen, The Netherlands) and maintained at 28 ºC for 30 min for acclimation with lights on before the behavioural paradigms started. After the behavioural analysis, larvae were euthanized by placing the plate on ice for 1 h.

### 2.7. DLD and Startle response paradigms

After the 30 min acclimation, the DLD test started. This test consisted of 10 additional minutes of acclimation (lights off) followed by 3 cycles consisting of 10 min with lights on and 10 min with lights off. Total distance moved was measured per block of 10 min. Dark/Light distance ratio was calculated by dividing the total distance moved during the 3 dark blocks by the total distance moved during the 3 light blocks.

After the DLD test, larvae underwent the startle response test. After a 10 min acclimation period, 10 tapping stimuli were administered with a 20-s interstimulus interval (ISI). Following another 10 min rest period, larvae were exposed to 30 stimuli with a 1-s ISI. The test was conducted under illuminated conditions. The maximum velocity (mm/s) within 1 s following each stimulus was recorded. Additionally, the habituation index was calculated for each stimulus by dividing the maximum velocity of the stimulus by that of the first stimulus during the 20-second ISI. Lower habituation index values indicate greater habituation to stimuli. Larvae that exhibited no response to the initial stimulus were excluded from the analysis.

### 2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). For the cell viability and relative mRNA expression in SH-SY5Y cells, outliers were discarded before the following analysis (as calculated by Grubb's analysis). In order to compare each group, a Kruskal-Wallis or one-way ANOVA (1wANOVA) was performed depending on a previous normality test. If statically significant differences ($p < 0.05$) were found, an uncorrected Dunn's test or LSD post-hoc analysis was performed.

For the DLD test, individuals showing outliers values (as calculated

by Grubb's analysis) for the Dark/Light distance ratio were discarded before subsequent analyses. When comparing distance moved in the DLD test in the total dark time and total light time, a two-way ANOVA (2wANOVA) analysis was performed, with Treatments (T) and Light (L) as factors. If significant interaction between T and L was found (TxL), a DMS post-hoc analysis was performed to detect significant differences versus healthy controls within each light condition.

For the startle response test, individuals showing outlier values in the area under the curve (AUC) of the habituation index's (as calculated by Grubb's analysis) were discarded before subsequent analyses. When comparing habituation index over time in the 20-s ISI and 1-ISI, a non-parametrical Friedman test was performed. If statically significant differences ($p < 0.05$) were found, an uncorrected post-hoc analysis was performed.

Statistical analyses and graphical representations were performed using GraphPad Prism software (version 10.2.1).

## 3. Results

### *3.1. Anti-inflammatory effects of polyphenol extracts in senescence neurons*

SH-SY5Y cells were treated for 48 h with serial dilutions (from 100 mg/mL) of the different phenolic extracts, and the cell viability was assessed. All extracts showed significant toxicity at 100 µg/mL (Fig. 1**A-D**). At 50 µg/mL, CLE and ROPE also showed a statistically significant decrease in cell viability, while OPE showed a statistically trend ($p = 0.063$) towards significant toxicity. Although the decrease in cell viability was not significant for ROPE at 25 µg/mL, a working concentration of 12.5 µg/mL was selected for gene expression to avoid possible negative effects. The other selected working concentrations were 25 µg/mL for both OPE and CLE and 50 µg/mL for VPE.

D-gal exposition induced a pronounced inflammatory response in SH-SY5Y cells, as evidenced by significantly elevated expression levels of key pro-inflammatory markers, including *IL-1β* (41-fold increase), *IL-8* (108-fold increase), *CCL2* (5.4-fold increase), and *TNFα* (17-fold increase) (Fig. 2**A–D**). Co-treatment with all polyphenolic extracts significantly reduced *IL-1β* expression compared to the control D-gal group, with CLE showing the most pronounced effect (70 % reduction, $p < 0.001$), followed by OPE (48 % reduction, $p = 0.013$), ROPE (48 % reduction, $p = 0.0219$), and VPE (43 % reduction, $p = 0.030$) (Fig. 2**A**).

All extracts also showed a significant mitigating effect on *IL-8* expression. Each treatment group exhibited a partial restoration of *IL-8* levels relative to the D-gal group, with significant reductions observed: CLE (59 % reduction, $p < 0.001$), OPE (52 %, $p < 0.001$), ROPE (62 %, $p < 0.001$), and VPE (77 %, $p < 0.001$) (Fig. 2**C**).

As for *CCL2*, although no significant differences were found in cells treated with extracts when compared to the D-gal control, a trend toward reduced expression was observed in cells treated with CLE (27 % reduction) (Fig. 2**B**).

In the case of *TNFα*, none of the experimental groups showed statistically significant differences when compared to the D-gal group, although numerically lower expression levels of this gene were found with both CLE and ROPE *vs.* D-gal+Vehicle (CLE $p = 0.195$; ROPE $p = 0.212$) (Fig. 2**D**).

Exposure to D-gal also induced dysregulation in the expression of neural-related genes in SH-SY5Y cells, including a 44 % reduction in *GAP43* and a 5.13-fold increase in BDNF levels. Although no statistically significant differences were observed between extract-treated groups and the D-gal control group, a trend toward increased *GAP43* expression was noted in cells treated with CLE (21 % increase, $p = 0.171$) and OPE (26 % increase, $p = 0.075$) (Supplementary Fig. 1).

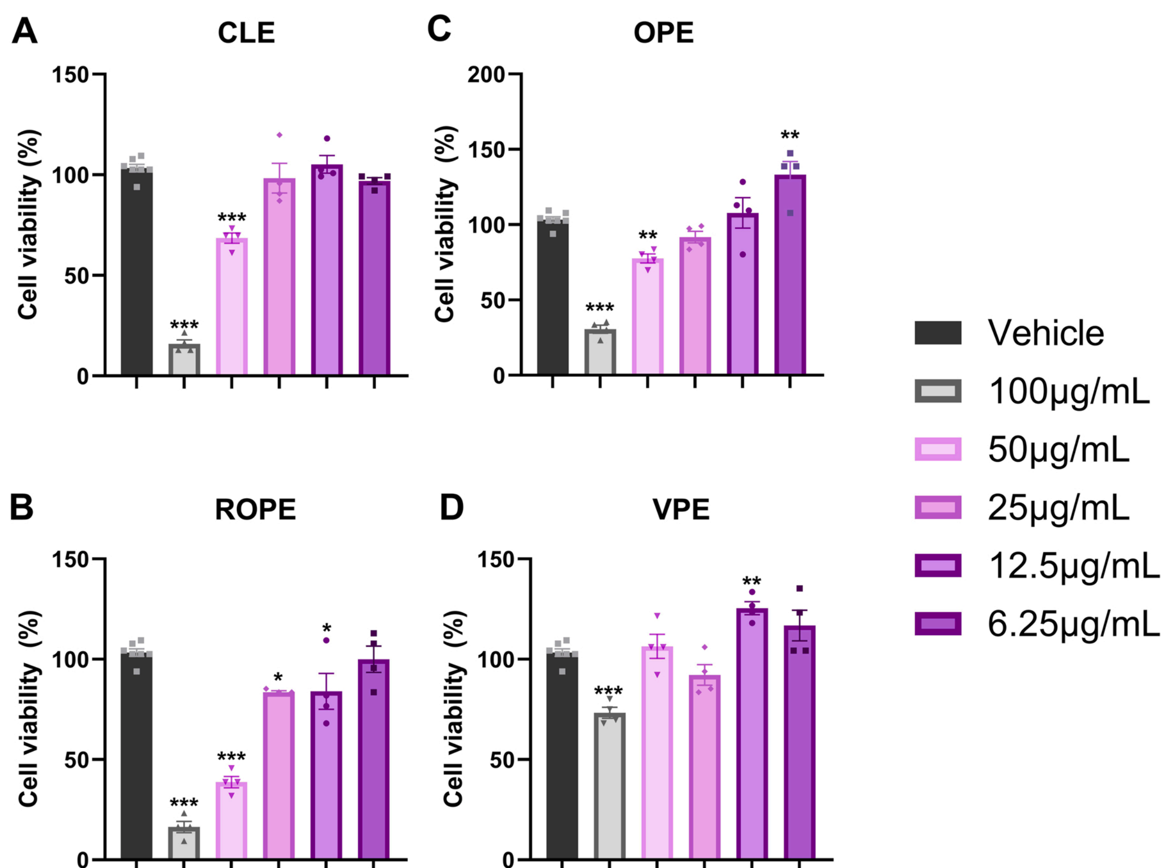


**Fig. 1.** Cell viability at different concentrations of chicory leaves extract (CLE) (A), olive pruning extract (OPE) (B), red onion peel extract (ROPE) (C) and vineyard pruning extract (VPE) (D). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *vs* vehicle. n = 4/group.

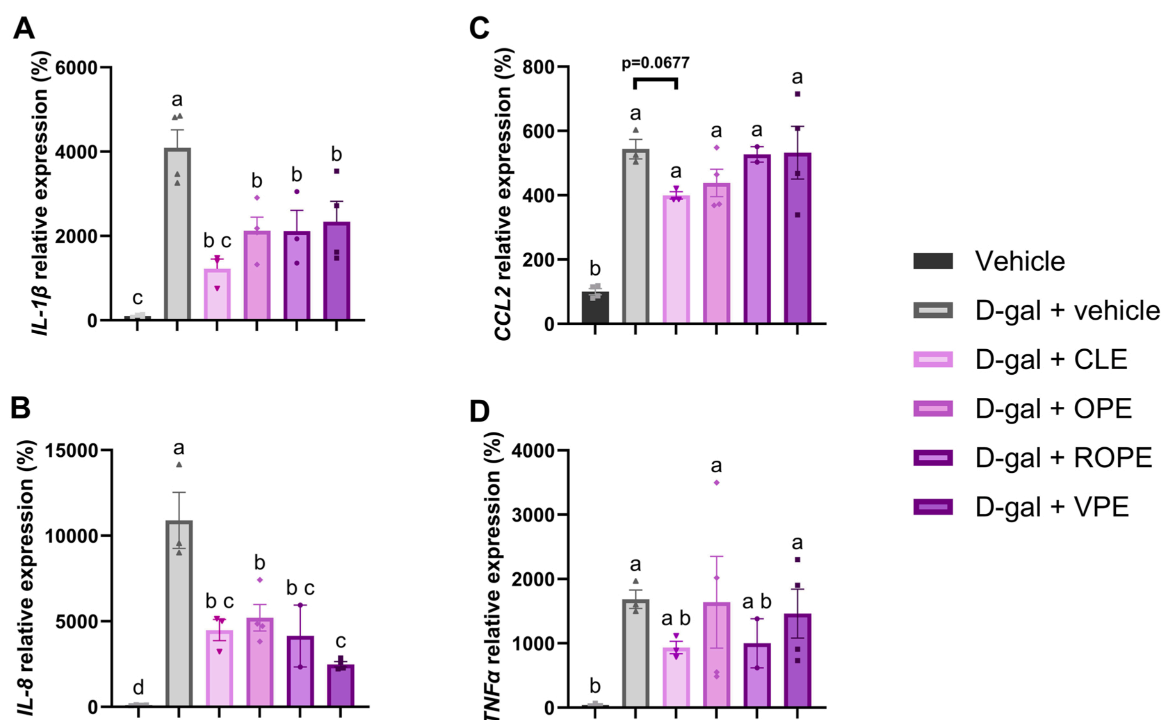


**Fig. 2.** Relative expression levels of the inflammation-related genes *IL-1β* (A), *CCL2* (B), *IL-8* (C) and *TNFα* (D). Data are expressed as means ± SEM (n = 2–4/group). [abc], experimental groups sharing a letter do not present significant differences ($p < 0.05$ in post-hoc analysis). Red onion peels (ROPE), olive pruning (OPE), vineyard pruning (VPE) and chicory leaves (CLE).

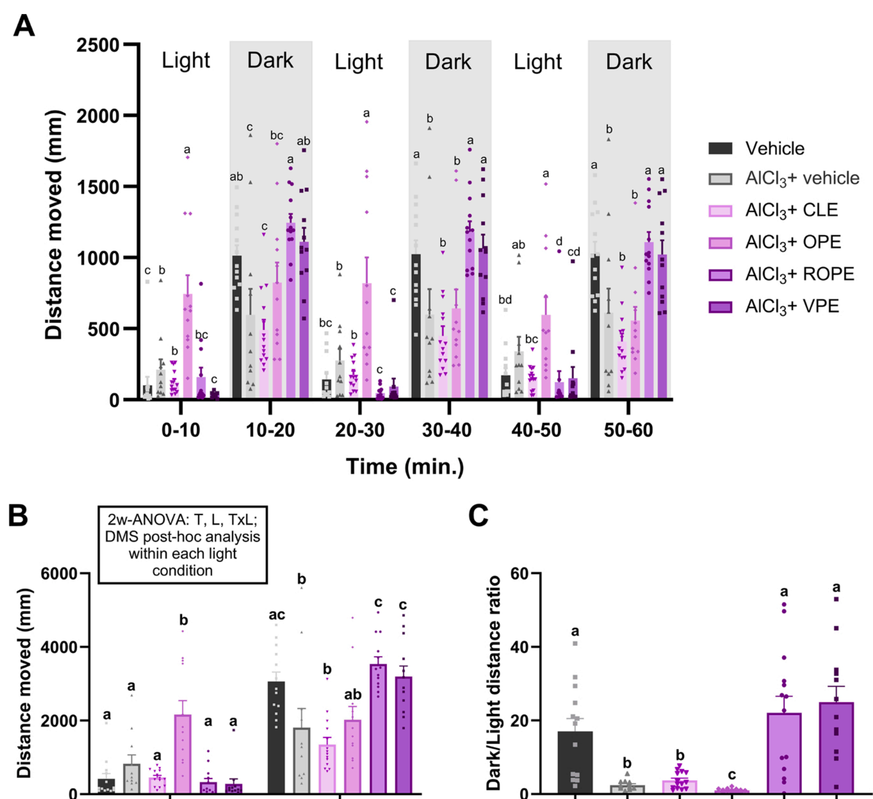


**Fig. 3.** Dark-Light-Dark paradigm results. A) Distance moved for each 10-min interval. Grey highlighting means darkness. Kruskal-Wallis ANOVA test between groups on each interval. B) Total distance moved during the 3 light and the 3 dark periods. 2w-ANOVA: T, L, TxL; C) Dark/Light distance ratio, calculated by dividing the total distance moved during darkness by the total distance moved during brightness. Data are expressed as means ± SEM (n = 11–14/group). [abc], experimental groups sharing a letter do not present significant differences ($p < 0.05$ in the corresponding post-hoc analysis). For 2w-ANOVA analysis: T, significant ($p < 0.05$) effect for treatment factor; L, significant effect for light factor; TxL, significant interaction between treatment and light factors. Red onion peels (ROPE), olive pruning (OPE), vineyard pruning (VPE) and chicory leaves (CLE).

### 3.2. Red onion peel extract and Vineyard pruning extract restore a normal activity pattern in response to light changes in neurodegenerative zebrafish larvae

To test the potential neuroprotective effect of the polyphenolic extracts in a basic cognitive decline model, zebrafish larvae were exposed to $AlCl_3$ and cotreated with the different extracts from 3 to 5 dpf. Larvae were then subjected to two distinct behavioural tests. The results of the DLD test in zebrafish larvae are shown in Fig. 3. This test revealed a typical activity pattern in healthy control larvae, with consistent low activity levels during the light intervals and clearly increased activity levels during the dark intervals, as expected (Fig. 3**A-B**). Larvae exposed to $AlCl_3$ showed an abnormal activity pattern when compared to healthy larvae, showing significantly decreased activity levels in the dark periods and a significantly lower dark/light distance ratio (Fig. 3**C**), which suggests a perturbation in the cognitive function of the larvae and the recognition of light and darkness.

Regarding the effect of the treatments with the polyphenolic extracts obtained, CLE and OPE did not appear to restore the normal activity pattern in zebrafish larvae. While CLE-treated larvae exhibited a pattern similar to that of $AlCl_3$ control larvae, indicating no positive nor negative effect of the treatment, OPE-treated larvae showed a significantly higher activity index in the dark compared to all the other treatments, with no differences in activity between dark and light periods (Fig. 3**B-C**), thus indicating a possible negative effect of this extract on the activity pattern and the recognition of light and dark environments.

Notably, zebrafish larvae treated with ROPE or VPE following $AlCl_3$ exposure demonstrated a clear recovery of the visual motor response (VMR), showing significantly increased activity levels during the dark periods and an increased dark/light distance ratio compared to $AlCl_3$ control larvae (Fig. 3**B-C**). Importantly, activity levels of animals treated with these extracts were at the same level as that of healthy control larvae, suggesting that these extracts could have a neuroprotective effect against $AlCl_3$-induced neurodegeneration.

### 3.3. ROPE restores the basal startle response and improves habituation index in neurodegenerative zebrafish larvae

In the startle response paradigm, we first analysed the larvae's reaction to the first stimulus, aiming to evaluate the fundamental operation of the Mauthner-cell startle circuit (Fig. 4**A**). This analysis revealed a decreased reaction to the stimulus in the larvae exposed to the neurodegenerative element $AlCl_3$, suggesting differences in the basal functioning of the Mauthner-cells-based startle circuitry. Interestingly, the ROPE seemed to revert this abnormal functioning, showing a similar first response to that of the healthy controls and a trend to a significantly increased response when compared to $AlCl_3$ controls ($p = 0.088$). By contrast, VPE seemed to exert an inhibiting effect in the basal startle response, since only 46 % of the larvae (6/13) reacted to the first stimulus, compared to 93 % of responding larvae in the control group or 85 % in the $AlCl_3$ control group, and larvae that reacted showed the lowest response of all groups to the first stimulus.

Then, we evaluated the startle habituation by analysing the larvae's response after continuous stimuli in the 20-s ISI paradigm and the 1-s ISI paradigm. Regarding the analysis of the startle response habituation to the 20-s ISI stimuli (Fig. 4**B**), it revealed a significantly higher habituation index of $AlCl_3$ control larvae over time compared to healthy control larvae ($p = 0.009$) (Supplementary Fig. 2), indicating lower habituation/memory of these larvae. While vineyard and olive extracts did not show differences in the habituation index compared to the $AlCl_3$ control, chicory and red onion extracts showed significantly lower habituation indexes over time ($p = 0.031$ and $p = <0.001$, respectively) (Supplementary Fig. 2). Noteworthily, ROPE-treated larvae, which displayed a normal first stimulus reaction, showed the lowest habituation indexes over time, even compared to healthy control larvae ($p = 0.094$), suggesting an improvement in the startle response habituation due to this polyphenolic extract. The analysis of the area under the curve (AUC) of the 20-s ISI habituation index showed similar results, further indicating enhanced habituation in ROPE-treated larvae. (Fig. 4**B**).

With respect to the results of the second phase of the startle response paradigm (30 stimuli with 1-s ISI after a 10-min rest period) (Fig. 4**C**), the effects of the neurodegenerative model were more discrete, since 1-s ISI leads to stronger habituation. Nonetheless, $AlCl_3$ control larvae also showed a trend to higher habituation index (indicating reduced habituation) over time compared to healthy control larvae ($p = 0.062$) (Supplementary Fig. 3). Consistent with observations from the 20-s ISI stimuli phase, larvae treated with the red onion extract showed a lower habituation index (indicating enhanced habituation) over time compared to $AlCl_3$ controls ($p < 0.001$) and even compared to healthy controls ($p = 0.045$) (Supplementary Fig. 3). By contrast, in this phase of the startle response, treatment with the chicory extract did not show amelioration of the habituation when compared to $AlCl_3$ controls.

## 4. Discussion

This study demonstrates that polyphenolic extracts derived from agricultural by-products can exert protective effects in different models of neurodegeneration, with particular relevance in both inflammatory response modulation and behavioural recovery. D-gal exposure induced a strong inflammatory response in SH-SY5Y cells, as evidenced by increased expression of *IL-1β, IL-8, CCL2* and *TNFα.* Co-treatment with polyphenol-rich extracts significantly reduced the expression of some of these markers, particularly *IL-1β* and *IL-8*. CLE showed the most consistent anti-inflammatory activity, which may be attributed to the presence of compounds such as chicoric acid, luteolin and apigenin, previously identified in chicory and known for their ability to inhibit pro-inflammatory cytokine production and MAPK/NF-kB activation [33]. These effects are consistent with previous findings demonstrating the ability of such compounds to reduce oxidative stress and neuroinflammation in cellular models of neurodegeneration [34,35].

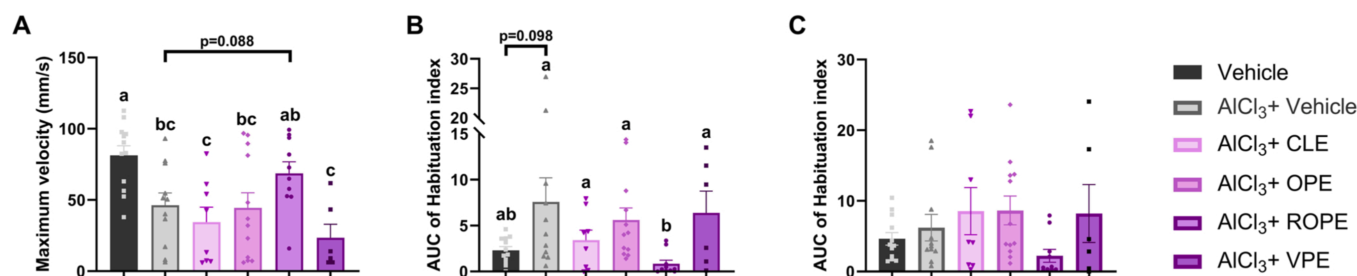


**Fig. 4.** Startle response paradigm results. A) Maximum velocity within the 1st second after the stimulus. B) Habituation index for the 10 stimuli of the 20-s ISI block. The area under the curve was calculated for the habituation index of the stimuli 2–10. C) Habituation index for the 30 stimuli of the 1-s ISI block. The area under the curve was calculated for the habituation index of the 30 stimuli (Kruskal-Wallis test, $p = 0.091$). Data are expressed as means ± SEM (n = 6–12/group). [abc], experimental groups sharing a letter do not present significant differences. ROPE, red onion peels; OPE, olive pruning; VPE, vineyard pruning; CLE, chicory leaves.

ROPE also significantly attenuated inflammation in treated SH-SY5Y cells. This extract is known to be rich in quercetin and its glycosides, as well as anthocyanins, which have been shown to modulate oxidative and inflammatory signalling in neural cells and may penetrate the blood-brain barrier [35]. While *CCL2* and *TNFα* levels were not significantly altered, downward trends observed with CLE and ROPE may indicate a potential role in chronic inflammation control. Such effects align with reports on the modulatory capacity of polyphenols on cytokine expression in glial and neuronal cells [36].

In the zebrafish model of neurodegeneration, ROPE and VPE were able to restore the altered visual motor response during the dark periods of the DLD test. These findings suggest a protective role of these extracts in visual-motor integration and possibly cognitive functions involving environmental changes [37,38]. In the case of ROPE, its high anthocyanins and quercetin content may underlie this effect, as both compound classes have shown the capacity to improve spatial learning and recognition memory in rodent models [39,40]. Anthocyanins have also been associated with increased synaptic plasticity and BDNF expression, which may explain the behavioural recovery observed [34].

Vineyard pruning extracts, likely containing resveratrol, catechins, and gallic acid, have been shown to activate neuroprotective pathways such as SIRT1 and PGC-1α and to reduce oxidative damage in the CNS [41,42]. These mechanisms may also contribute to the improvement in dark-light response observed in this study. Interestingly, CLE, which was effective *in vitro*, did not significantly alter zebrafish behaviour, highlighting the possible divergence between cellular and organism outcomes due to factors such as bioavailability or metabolism. The extract from OPE, however, seemed to impair dark-light discrimination, possibly due to overactivation of sensory pathways, warranting further testing under non-degenerative conditions to evaluate the baseline effects.

The zebrafish model provided complementary *in vivo* data, particularly regarding the ability of the extracts to restore normal locomotor activity and sensorimotor responses. Sudden changes in illumination from light to dark during daytime hours evoke a scape response in zebrafish larvae with abrupt increases in swimming behaviour and increased activity, as larvae search the well for a way to return to the light [43,44]. Although the DLD test is often used to assess the anxiety-like behaviour (increased activity in the dark periods is considered proportional to the anxiety level of the larval zebrafish) and to determine the potential of anxiolytic candidates, neurodegeneration models such as exposure to aluminium may also lead to a reduction in the VMR, which could be indicative of a sensorimotor deficiency. In the present study, $AlCl_3$ exposure caused a reduction in the VMR, as previously described [27,45].

Additionally, to the best of our knowledge, the present study is the first to assess the effects of $AlCl_3$ exposure on the startle response paradigm in zebrafish larvae, revealing impairments in both initial sensory reactivity and habituation capacity. Larvae exposed to $AlCl_3$ showed reduced responsiveness to the first stimulus and impaired habituation, indicating both sensory and memory deficits, as described in adult zebrafish [46]. ROPE stood out by not only restoring the initial responsiveness to the first stimulus but also significantly improving habituation across both stimulus intervals (20 s and 1 s ISI). Remarkably, ROPE-treated larvae performed better than even healthy controls in some respects, suggesting enhanced neural plasticity or reduced baseline anxiety-like behaviour [37]. The underlying mechanism may involve the modulation of ion channels, such as voltage-gated potassium channels, and the enhancement of synaptic plasticity, as previously described for quercetin and related polyphenols [47,48]. While VPE also showed some benefits in the DLD test, it did not significantly improve startle response or habituation. Conversely, CLE displayed ambiguous effects – possibly due to the influence of the interindividual variability in larvae responsiveness – highlighting the need for further replicates or refined dosing strategies.

Altogether, ROPE emerged as the most consistently effective extract across all endpoints tested, displaying anti-inflammatory activity, behavioural normalization in visual motor response, and enhanced performance in habituation learning tasks. These broad-spectrum effects suggest that red onion peel-derived polyphenols, particularly quercetin derivates and anthocyanins, could be promising candidates for further development as neuroprotective agents. Future studies should focus on identifying the precise molecular targets of these compounds, assessing their pharmacokinetics and BBB permeability, and evaluating their long-term efficacy in more complex models of neurodegeneration.

## 5. Conclusions

This study highlights the therapeutic potential of polyphenol-rich extracts derived from agricultural waste byproducts in mitigating inflammation and neurodegeneration. All four tested extracts – CLE, OPE, ROPE and VPE- demonstrated the capacity to reduce pro-inflammatory gene expression *in vitro*, supporting their anti-inflammatory properties.

In the zebrafish neurodegeneration model, ROPE and VPE showed the most promising results in restoring behavioural responses disrupted by $AlCl_3$ exposure, particularly in the DLD transition test. ROPE also significantly improved sensorimotor responses and habituation patterns in the startle response test, suggesting a strong neuroprotective effect with potential cognitive benefits.

Among all extracts, ROPE exhibited the strongest and most consistent activity across both in vitro and in vivo models, supporting its further investigation as a lead candidate for sustainable cognitive health interventions.

Overall, these findings support the further exploration of polyphenolic extracts as valuable candidates for developing sustainable and effective interventions against neurodegenerative conditions. Future studies should focus on elucidating their molecular mechanisms and evaluating their efficacy in long-term and disease-specific models.

While the present study provides consistent evidence of neuroprotective and anti-inflammatory effects in both cellular and larval zebrafish models, the use of zebrafish larvae exclusively represents a limitation. Although larvae constitute an advantageous high-throughput and ethically efficient model, allowing high-throughput behavioural analysis, they may not completely reflect the full complexity of mature neural circuits and systemic physiology. Future work should therefore aim to validate these findings in adult zebrafish and complementary mammalian models, such as rodents, to confirm the translational relevance and durability of the observed effects across different stages of neurodevelopment and species.

## Author Contributions

Conceptualization, J.M Del Bas, N. Ortega, X. Escoté and J. Teichenné; Formal analysis, A. Castillo-Moral, J. Tchoumtchoua, K. Leonard and J. Teichenné; Funding acquisition, J.M Del Bas and N. Ortega; Investigation, A. Castillo-Moral, J. Tchoumtchoua, K. Leonard and J. Teichenné; Methodology, A. Castillo-Moral, J. Tchoumtchoua, K. Leonard and J. Teichenné; Project administration, J.M Del Bas, N. Ortega and X. Escoté; Writing – original draft, A. Castillo-Moral, X. Escoté and J. Teichenné; Writing – review and editing, A. Castillo-Moral, J. Tchoumtchoua, K. Leonard, J.M Del Bas, N. Ortega, X. Escoté and J. Teichenné.

## CRediT authorship contribution statement

**Ángela Castillo-Moral:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Job Tchoumtchoua:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Kevin Leonard:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Del Bas Josep Maria:** Writing – review & editing, Project administration, Funding acquisition,

Conceptualization. **Nàdia Ortega:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Xavier Escoté:** Writing – review & editing, Writing – original draft, Project administration, Conceptualization. **Joan Teichenné:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

## Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve language and readability. After using this tool, all the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118776.

## Data availability

Data will be made available on request.

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