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RESEARCH ARTICLE

Epigallocatechin Gallate and Punicalagin Combination Reduces Aβ Aggregation and Promotes Neurogenesis in Adult Zebrafish Brain

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline, memory impairment, and behavioral alterations. The pathogenesis of AD involves the accumulation of amyloid-beta (Aβ) plaques and the hyperphosphorylated tau proteins, which disrupt neuronal function and trigger neuroinflammation. This study explores the therapeutic potential of epigallocatechin gallate (EGCG) and punicalagin (PU) in mitigating Aβ-induced toxicity using an adult zebrafish model of AD. Our results demonstrate that the EGCG + PU combination significantly reduces Aβ accumulation, protects against cellular damage, suppresses acetylcholinesterase (AChE) activity, and normalizes the expression of amyloidogenic and AD-related genes. Additionally, EGCG + PU treatment alleviates neuroinflammation by suppressing glial activation, including reductions in L-plastin and proinflammatory cytokine expression, while promoting neuronal recovery through mechanisms of neurogenesis and neuroprotection. Notably, the combination treatment restored neuronal density and improved behavioral outcomes by alleviating anxiety- and aggression-like behaviors associated with Aβ toxicity. These results underscore the synergistic neuroprotective effects of EGCG + PU, highlighting their potential as a novel therapeutic approach for mitigating the pathological, behavioral, and inflammatory aspects of AD.

1 | Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory impairment, cognitive decline, and behavioral changes (Breijyeh and Karaman 2020; Nazli et al. 2025). The pathogenesis of AD is driven by complex molecular mechanisms, with two key pathological hallmarks: the accumulation of amyloid-beta (Aβ) plaques and the hyperphosphorylation of tau proteins, leading to the formation of neurofibrillary tangles (Abubakar et al. 2022; Raulin et al. 2022). Aβ plaques result from the abnormal cleavage of the amyloid precursor protein (APP), a transmembrane protein, by β-secretase (BACE1) and γ-secretase, at incorrect sites (Hampel et al. 2021). This process generates β-amyloid peptides that aggregate into toxic plaques, a hallmark of AD. Presenilin, the catalytic core of the γ-secretase complex, plays a critical role in the APP cleavage and Aβ generation. These peptides accumulate between neurons, disrupting synaptic communication and triggering cellular toxicity (Scheltens et al. 2016; Lane et al. 2018; Soria Lopez et al. 2019; Karabicici et al. 2021). Simultaneously, hyperphosphorylated tau proteins destabilize the neuronal cytoskeleton, promoting the formation of intracellular neurofibrillary tangles that impair neuronal function (Zhang et al. 2021). Together, these pathological changes initiate a cascade of detrimental

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Significance Statement

Alzheimer's disease (AD) is a growing global health challenge with limited treatment options that can halt or reverse disease progression. This study demonstrates that a combination of two naturally occurring compounds, epigallocatechin gallate (EGCG) and punicalagin (PU), markedly reduces amyloid-beta–induced brain toxicity in an adult zebrafish model of AD. The treatment not only decreased amyloid accumulation and neuroinflammation but also protected neurons and improved anxiety- and aggression-like behaviors. By targeting multiple disease mechanisms simultaneously, EGCG+PU shows promise as a multi-target, potentially safe therapeutic strategy. These findings highlight the potential of combining bioactive dietary compounds as an accessible and innovative approach for addressing key pathological and behavioral features of AD.

events, including oxidative stress, mitochondrial dysfunction, and widespread neuronal loss (Sengupta and Kayed 2022). The hippocampus and cerebral cortex, regions critical for cognitive function, are particularly vulnerable to these pathological alterations. Their vulnerability underlies the clinical symptoms of AD, including the progressive deterioration of memory and cognition (Ossenkoppele et al. 2022).

AD is the most prevalent form of dementia worldwide. According to the World Health Organization (WHO), dementia affects an estimated 55 million people globally, with AD accounting for 60%–70% of these cases (WHO 2023). The progressive nature of AD not only causes significant cognitive decline but also increases the need for long-term care, imposing substantial economic and social burdens on individuals, families, and healthcare systems (Wong 2020; Kosaner Kließ et al. 2021). Given its widespread prevalence and profound impact, research focused on understanding the pathophysiology, management, and treatment of AD is an urgent global priority (Breijyeh and Karaman 2020; Jucker and Walker 2023; Rafe et al. 2024).

Although there is no definitive cure for AD, pharmacological treatments are available to manage symptoms and temporarily mitigate cognitive decline. These treatments primarily fall into two categories: AChE inhibitors (e.g., donepezil, rivastigmine, galantamine) and NMDA receptor antagonists (e.g., memantine). AChE inhibitors enhance cognitive function by increasing acetylcholine levels in synaptic regions, while memantine regulates excessive glutamate release, protecting neurons from excitotoxicity (Guo et al. 2020; Marucci et al. 2021; Zuin et al. 2022). However, these therapies do not halt disease progression and provide only symptomatic relief. Recent research has shifted toward biological therapies targeting Aβ and tau proteins, with monoclonal antibodies and immunotherapies emerging as promising approaches to slow disease progression. Despite their potential, clinical efficacy remains under active investigation (Valiukas et al. 2022; Cummings et al. 2024; Dantas et al. 2024).

Beyond central nervous system (CNS)–intrinsic mechanisms, growing evidence implicates peripheral contributions to AD pathophysiology, particularly via the gut–brain axis, a bidirectional network linking the gastrointestinal tract and brain through neural, immune, endocrine, and metabolic pathways (Toledo et al. 2022; Bicknell et al. 2023). Alterations in gut microbiota composition have been associated with increased Aβ accumulation, neuroinflammation, and cognitive impairment, while both experimental and clinical studies demonstrate that microbiota modulation can reduce cerebral Aβ burden and improve cognitive performance (Qu et al. 2024; Zhang et al. 2025). Mechanistically, gut dysbiosis disrupts intestinal barrier integrity, promoting systemic inflammation and peripheral immune activation that drive microglial activation and neuroinflammatory signaling in the brain (Fung et al. 2017; Morais et al. 2021; Qu et al. 2023). In contrast, restoration of microbial diversity reinforces barrier function, attenuates inflammatory signaling, and supports synaptic and cognitive integrity. Together, these findings highlight the gut–brain axis as a key contributor to AD and a promising therapeutic target. In this context, plant-derived polyphenols have attracted attention for their antioxidant and anti-inflammatory properties and their ability to modulate gut microbiota and strengthen intestinal barrier function, suggesting that polyphenol-based interventions may confer neuroprotection through both direct neuronal and microbiota-mediated mechanisms (Filosa et al. 2018; Reddy et al. 2020).

Given the limitations of current treatments, natural compounds such as polyphenols have gained interest for their anti-aging, antioxidant, anti-inflammatory, and neuroprotective properties. Studies have shown that polyphenols, including flavonoids, curcuminoids, stilbenes, and catechins, exhibit beneficial effects on AD pathology (Calderaro et al. 2022; El Gaamouch et al. 2022; Kabir et al. 2023). For example, epigallocatechin gallate (EGCG), the primary catechin in tea leaves, has been shown to exert neuroprotective effects by modifying toxic Aβ structures, targeting catalytic enzymes such as α-, β-, and γ-secretases, inhibiting Aβ fibril elongation, and reducing AChE activity (Rezai-Zadeh et al. 2005; Cox et al. 2015; Unno et al. 2017; Xicota et al. 2017). Similarly, punicalagin (PU), the most abundant bioactive compound in pomegranate peel, has been reported to reduce lipopolysaccharide-induced Aβ1–42 production by downregulating APP and BACE1 expression (Kim et al. 2017; Siddiqui et al. 2024). Pomegranate peel extracts also inhibit Aβ plaque density and AChE activity (Calderaro et al. 2022; Cordiano et al. 2024). Our previous findings demonstrated that EGCG and PU, individually and in combination, can cross the blood–brain barrier (BBB) and disrupt Aβ plaque structures in an in vitro BBB model (Serdar et al. 2021). Building on this evidence, in this study, we explore the neuroprotective effects of these polyphenols in an in vivo model. Zebrafish (*Danio rerio*) has emerged as a valuable model organism for AD research due to its significant genetic and biological similarities to humans. With 70%–80% genetic homology to human genes, zebrafish exhibit neuroanatomical and neurochemical characteristics comparable to the human brain (Saleem and Kannan 2018; Wang et al. 2021; Demirci et al. 2022). Genetically engineered zebrafish carrying AD-associated mutations, such as APP, PSEN1, and PSEN2, have elucidated the roles of these genes in AD progression (Kiper and Freeman 2022). Additionally, nongenetically modified zebrafish have proven effective for modeling AD pathology and evaluating potential therapeutic compounds (Bhattarai et al. 2017; Nadiga et al. 2024).

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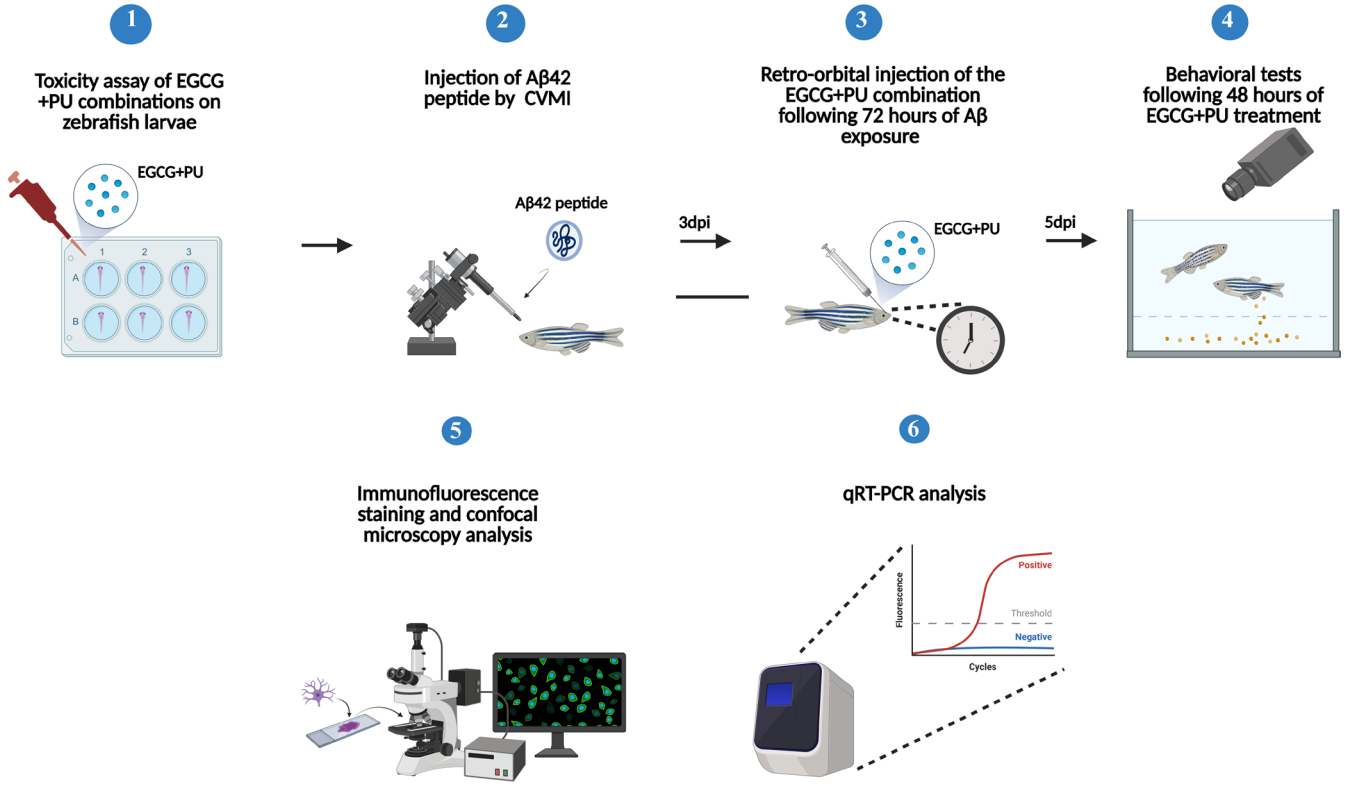


FIGURE 1 | Experimental workflow for evaluating the neuroprotective effects of EGCG and PU in a zebrafish model of Alzheimer's disease. (1) Toxicity assays of EGCG + PU combinations were performed on zebrafish larvae to determine safe concentrations. (2) Adult zebrafish were injected with Aβ42 peptide using cerebroventricular microinjection (CVMI) to induce Alzheimer's-like pathology. (3) Following Aβ42 administration, a retro-orbital injection of the EGCG + PU combination was performed at 3 days postinjection (dpi). (4) Behavioral tests were conducted at 5 dpi to evaluate anxiety- and aggression-like behaviors. (5) Immunofluorescence staining and confocal microscopy analysis were utilized to assess Aβ aggregation, neuroinflammation, and neuronal density. (6) Gene expression analysis of amyloidogenic pathways and inflammatory markers was performed using quantitative real-time PCR (qRT-PCR).

In this study, we investigated the therapeutic potential of the EGCG + PU combination in mitigating the pathological, cellular, and behavioral effects of Aβ42 toxicity using a zebrafish model of AD. Our findings revealed that EGCG + PU effectively reduced Aβ aggregation, normalized amyloidogenic gene expression, and alleviated neuroinflammation by suppressing glial activation. Furthermore, EGCG + PU restored neuronal density, reduced cellular damage markers, and promoted neuronal recovery. Behaviorally, the combination treatment alleviated Aβ42-induced anxiety- and aggression-like behaviors, restoring normal locomotor patterns and reducing excessive aggression. Collectively, these data demonstrate that EGCG + PU not only protects against Aβ42-induced neurotoxicity but also promotes recovery by targeting key pathological and behavioral aspects of Alzheimer's-like conditions.

2 | Materials and Methods

2.1 | Zebrafish Husbandry and Experimental Design

Zebrafish used in this study were obtained from the Izmir Biomedicine and Genome Center (IBG) Zebrafish Core Facility. They were maintained on a 14-h light/10-h dark cycle at 28.5°C, following the guidelines of the IBG Animal Care and Use Committee. All animal experiments adhered to the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the IBG Animal Experiments Local Ethics Committee (IBG-AELEC) with protocol number 2020-034. To determine the required sample size for each experimental group, a statistical power analysis was performed using G*Power 3.1.9.7. Based on an effect size of 0.8, an alpha level of 0.05, and a statistical power of 0.8, the analysis indicated that six biological replicates per group were required (Faul et al. 2007). This effect size estimate was derived from our preliminary pilot data. Ethical regulations also limit the number of zebrafish that can be sacrificed per experimental conditions, and this permissible limit aligns with the sample size determined by power analysis. Accordingly, six zebrafish were allocated to each group, with three used for confocal imaging and three used for molecular analyses (quantitative real-time PCR = qRT-PCR), providing complementary readouts from biologically independent samples. The experimental workflow included the toxicity assay for the epigallocatechin gallate (EGCG) and punicalagin (PU) combinations in zebrafish larvae, generation of an Aβ toxicity model via cerebroventricular microinjection, of EGCG + PU combination through retro-orbital injection, behavioral testing, tissue preparation for immunofluorescence analysis, and gene expression analysis by qRT-PCR (Figure 1).

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2.2 | Toxicity Assay and Dose Selection

A toxicity assay was conducted to determine the maximum nontoxic concentrations of EGCG (E4143; Sigma-Aldrich, MO, USA), PU (P0023; Sigma-Aldrich), and the clinically approved AChE inhibitor donepezil (D6821-10MG; Sigma-Aldrich), which was included as a positive control in subsequent experiments. Zebrafish larvae at 24 h postfertilization (hpf) were used for the assay, which was performed in six-well plates under standard rearing conditions. Following dechorionation, combinations of EGCG and PU, as well as donepezil at two different concentrations, were prepared in E3 embryo medium and administered to the larvae. A total of seven EGCG + PU combination doses and two donepezil doses (10 and 20 μM) were tested (Table S1) (Best et al. 2008; Whyte-Fagundes et al. 2024). Larvae were maintained at 28.5°C throughout the experiment, with the exposure media refreshed daily. Embryonic development, morphology, and survival were monitored at 24- and 48-h postdose application using a stereo microscope (SZX2 Series Stereo Microscope; Olympus, Tokyo, Japan). Images were captured during these observations as previously described (Hesaraki et al. 2022; Köktürk et al. 2022; Kokturk et al. 2023). The assay was terminated 48 h after compound application.

Drug concentrations were selected based on previously reported effective ranges of EGCG and PU in neuronal cell culture systems, in conjunction with our preliminary toxicity screening results (Serdar et al. 2021). Larval range-finding assays were used to identify the no-observed-effect concentrations, which informed—but did not solely dictate—the doses applied in adult zebrafish experiments. Adult exposure levels were further validated to ensure tolerability, with no mortality, overt behavioral abnormalities, or visible signs of toxicity observed throughout the treatment period. Due to solubility constraints, PU stock solutions were initially prepared in 50% methanol; however, following serial dilution in embryo medium, the final methanol concentration in exposure wells was approximately 0.0005%. This concentration is well below levels reported to induce physiological or behavioral effects in zebrafish larvae and was therefore considered biologically inert, obviating the need for a separate solvent control group.

2.3 | Peptide Preparation

The Aβ42 peptide (DAEFRHDSGYEVHHQKLVFFAEDV GSNKGAIIGLMVGGVVIA) purchased from PeptiTeam (Ankara, Türkiye) was initially dissolved at a concentration of 20 mg/mL in a 1:1 mixture of distilled deionized water and acetonitrile, as described previously (Bhattarai et al. 2017; Nadiga et al. 2024). For injection, the peptide was further diluted in phosphate-buffered saline (PBS) to achieve a final concentration of 40 μM.

2.4 | Cerebroventricular Microinjection of Aβ

Cerebroventricular microinjection (CVMI) was performed according to the established protocols (Kizil and Brand 2011; Kizil et al. 2015; Bhattarai et al. 2017). To create an Aβ toxicity model, adult zebrafish (6–10 months old, body length 25–32 mm) were anesthetized in system water containing tricaine (50 mg/mL, tricaine methane sulfonate, TMS, MS-222, MESAB). A 1-mm hole was created in the skull using a 30 G needle, and 2 μL of 40 μM Aβ42 peptide was injected through the hole using glass needles connected to a microinjection system. For the control group, 2 μL of PBS was injected. After the injections, zebrafish were housed in tanks containing system water, with daily water changes, for 3 days to establish the Aβ toxicity model.

2.5 | Retro-Orbital Injection of Polyphenol Combinations

Zebrafish with the Aβ42 toxicity model were divided into seven groups ($n = 6$ per group). The polyphenol doses specified in Table S1 were administered to each group via retro-orbital injection, a method shown to allow compounds to cross the BBB (Pugach et al. 2009). In parallel, a positive control group received donepezil at the concentration determined in the toxicity assay, administered via retro-orbital injection using the same procedure. Control zebrafish ($n = 6$), which had previously received PBS through CVMI, were also injected with PBS via retro-orbital injection.

2.6 | Measurement of Lactate Dehydrogenase Activity

LDH activity in zebrafish brains was measured using the lactate dehydrogenase (LDH) Activity Assay Kit (Elabscience Bionovation Inc., TX, USA) according to the manufacturer's instructions. Brain tissue was weighed and homogenized at a 1:9 (w/v) ratio as per the kit protocol. Pyruvate concentration in the samples was determined by measuring absorbance at 450 nm. LDH activity was calculated using the formula provided in the kit protocol.

2.7 | Measurement of Acetylcholinesterase Activity

Acetylcholinesterase (AChE) activity was quantified using Ellman's colorimetric method, adapted for zebrafish brain lysates. Briefly, adult zebrafish brains were dissected and homogenized in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with a protease inhibitor cocktail. Homogenates were incubated on ice for 20 min and subsequently centrifuged at 14,000 × g for 15 min at 4°C. The resulting supernatants were collected, and total protein concentrations were determined using the bicinchoninic acid (BCA) assay according to the manufacturer's instructions. For AChE activity measurements, 50 μL of each brain lysate was added to a reaction mixture containing 50 μL of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as the chromogenic reagent and 100 μL of 75 mM acetylthiocholine iodide (ATCI) as the substrate, prepared in phosphate buffer. The formation of the yellow 5-thio-2-nitrobenzoate (TNB) anion was monitored kinetically by measuring absorbance at 412 nm

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for 5 min at 25°C using a microplate reader. AChE activity was calculated from the linear rate of absorbance change over time using the molar extinction coefficient of TNB (13,600/M/cm) and expressed as units per milligram of protein (U/mg protein) after normalization to total protein count.

2.8 | Tissue Preparation and Cryosectioning

Zebrafish were euthanized in ice-cold water, and the severed heads of three zebrafish from each group were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C for 2 days, as described previously (Demirci et al. 2020; Azbazdar et al. 2023). Following fixation, the heads were incubated in a 20% sucrose-20% EDTA solution at 4°C for 2 days, then transferred to a 30% sucrose-20% EDTA solution for another 2 days. After the 6-day process, the heads were embedded in 20% sucrose-7.5% gelatin, frozen using dry ice, and stored at −80°C for sectioning. Transverse sections of 14 μm thickness were obtained from the telencephalon region using a cryostat (CM1850; Leica, Wetzlar, Germany). The sections were incubated at 65°C for 2 days and stored at −20°C until further analysis.

2.9 | Immunofluorescence Staining, Imaging, and Quantitative Image Analysis

Immunofluorescence staining was performed as described previously (Demirci et al. 2020; Azbazdar et al. 2023). Cryostat-prepared slides stored at −20°C were dried at room temperature (RT) for 20 min and sequentially washed in PBSTX (PBS/0.3% Triton X-100) for 2×10 min, followed by PBS washes for 2×5 min. Antigen retrieval was performed by incubating the sections in 10 mM sodium citrate at 85°C for 15 min, followed by further washing in PBS (2×5 min) and PBSTX (2×10 min). Sections were incubated overnight at +4°C with primary antibodies diluted in PBSTX. The following primary antibodies were used: Rabbit anti-β-Amyloid (1:500, D54D2; Cell Signaling Technology, MA, USA), mouse anti-HuC/HuD (1:500, A-21271; Thermo Fisher Scientific, MA, USA), mouse anti-GFAP (1:500, ab154474; Abcam, Cambridge, UK), rabbit anti-L-Plastin (1:200, 55208-1-AP; Proteintech, CA, USA). The following day, sections were washed in PBSTX for three 10-min intervals and incubated with secondary antibodies diluted in PBSTX at RT for 2 h. The following secondary antibodies were used: Rhodamine (TRITC)-AffiniPure donkey anti-rabbit IgG (1:500, 711–025-152; Jackson Immunoresearch Laboratories, PA, USA) and Cy5 AffiniPure donkey antimouse IgG (H + L) (1:500, 712–175-150; Jackson Immunoresearch Laboratories). Nuclear counterstaining was performed using 4′,6-diamidino-2-phenylindole (DAPI; 4083S; Cell Signaling Technology). Sections were subsequently washed in PBS (3×5 min) and mounted with 70% glycerol. To verify antibody specificity, negative control sections without the primary antibody were included in parallel. These controls exhibited no detectable fluorescence, indicating minimal nonspecific secondary antibody binding.

Imaging was performed using an LSM 900 laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany). Three biological replicates (brains) were analyzed per experimental group, and three anatomically comparable sections per brain without tissue loss were selected for quantification. To ensure consistency across samples, a predefined region of interest (ROI) of identical dimensions was systematically applied to all sections. For cell-based markers, immunopositive cells were identified based on spatial co-localization with DAPI-labeled nuclei and manually counted by an investigator blinded to experimental group assignment. Cell counts were normalized to the ROI surface area and expressed as cell density (cells per unit area). For fluorescence intensity-based measurements, confocal image stacks were acquired and processed in 16-bit format to preserve the full dynamic range. Background fluorescence was corrected using the sliding paraboloid subtraction algorithm in Fiji, with identical parameters applied across all samples. Integrated density values were then extracted from the predefined ROI as a measure of total fluorescence signal. Mean values were calculated per biological replicate and subsequently used for group-level statistical comparisons. Statistical significance was determined using an unpaired *t*-test (Table S3).

2.10 | RNA Isolation and cDNA Synthesis

Zebrafish were sacrificed 48 h after retro-orbital injection by immersion in ice-cold water. Heads from three zebrafish in each group were removed, and the telencephalon regions of their brains were dissected and pooled. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. cDNA synthesis was performed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Inc., CA, USA) as described previously (Azbazdar et al. 2019; Cark et al. 2025).

2.11 | Quantitative Real-Time PCR and Statistical Analysis

Quantitative real-time PCR (qRT-PCR) was conducted in triplicate using the GoTaq qPCR Master Mix (Promega, WI, USA) on an Applied Biosystems 7500 Fast Real-Time PCR System (Katkat et al. 2023). Gene expression levels were normalized to the zebrafish ribosomal protein L13a (*rpl13a*) gene, and relative expression was calculated. Data were analyzed using GraphPad Prism 8 software (GraphPad Software Inc., CA, USA) and presented as the mean ± SD (standard deviation) from three independent samples. Statistical significance was evaluated using an unpaired *t*-test. Primer sequences for the analyzed genes (*appa*, *appb*, *bace*, *psen1*, *psen2*, *il1β*, *il2rga*, *il6st*, *il8*, and *rpl13a*) are listed in Table S2.

2.12 | Behavioral Testing

Behavioral tests were conducted 48 h after retro-orbital injection of polyphenol combinations. The selection of behavioral paradigms and endpoints was guided by recent methodological consensus and best-practice frameworks for zebrafish-based Alzheimer's disease modeling, which emphasize anxiety-related behavior, locomotor activity, and social/aggressive responses as translationally relevant readouts of AD-associated neurobehavioral dysfunction (Wang et al. 2023). For each group, zebrafish ($n = 9$–10 per group) were individually placed

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into a trapezoidal test tank (27 cm × 10 cm × 17 cm) filled with 3.5 L of system water. Testing was conducted between 10:00 a.m. and 4:00 p.m., with the tank water replaced with fresh system water maintained at 27°C ± 1°C after each test. The tests were recorded simultaneously using two cameras, and the videos were analyzed with Panlab Smart v3.0 software (Harvard Apparatus, MA, USA). In addition to quantitative behavioral endpoints, individual swimming trajectories and spatial occupancy heatmaps were generated to visualize inter-individual variability and group-level behavioral patterns; these data are presented in Figure S1.

2.13 | Novel Tank Diving Test

The novel tank test is a widely used behavioral test to evaluate anxiety and locomotor activity in zebrafish. This assay measures fish behavior in a novel environment, often used to assess stress and anxiety responses or study disease models undergoing drug trials (Haghani et al. 2019; Fontana et al. 2022). The test was performed as described previously, using a tank filled with water to a height of 15 cm, divided into three horizontal zones: top, middle, and bottom, each 5 cm in height (Azbazdar et al. 2023). Recording began immediately after the fish were introduced into the tank. Observations were made at 1-min intervals during the following time periods: 0–5, 6–10, 11–15, 16–20, 21–25, and 26–30 min. Data analysis included the distance traveled in the bottom zone (cm), the distance traveled in the top zone (cm), and the number of entries into the top zone.

2.14 | Mirror-Biting Test

The mirror-biting test assesses aggression levels in zebrafish by observing their reaction to their reflection, perceived as another individual. This test is commonly used in neuropsychiatric and neurodevelopmental disorder studies (Audira et al. 2020; Ogi et al. 2020). During the acclimatization period, a mirror positioned at one end of the tank was covered with an opaque plastic sheet to prevent reactions to the reflection. After 5 min, the opaque sheet was removed. The tank was divided into two zones: the mirror-close zone and the mirror-far zone (Audira et al. 2020). The fish were recorded for 15 min, and the collected data were analyzed for the following parameters: Average speed (cm/s), time spent in the mirror-close zone (s), average speed in the mirror-close zone (cm/s), and distance traveled in the mirror-close zone (cm).

2.15 | Statistical Analysis of Behavioral Data

All statistical analyses were conducted in R version 4.5.1 (R Core Team 2025). Behavioral outcomes from the novel tank diving test, comprising six repeated 5-min time bins per fish over a 30-min period, were analyzed using linear mixed-effects models (LMMs) implemented with the *lme4* package (Bates et al. 2015). Experimental group, time, and interaction were included as fixed effects, and FishID was modeled as a random intercept to account for within-subject correlations. Type III analysis of variance (ANOVA) tables with Satterthwaite-adjusted degrees of freedom were generated using the *lmerTest* package (Kuznetsova et al. 2017). When significant omnibus effects were detected, post hoc pairwise contrasts were performed using *emmeans* (Lenth 2020), restricted to biologically relevant comparisons (Control vs. Aβ-induced toxicity model; Aβ-induced toxicity model vs. Aβ model + Dose 4), with p value adjustment for multiple testing using the Sidak method. Outcomes from the mirror-biting test were analyzed using one-way ANOVA when assumptions of normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test) were satisfied ($p > 0.05$). In cases of unequal variances, Welch's ANOVA was applied, while non-normally distributed datasets were evaluated using the Kruskal–Wallis test. Post hoc analyses were conducted using Tukey's honestly significant difference (HSD) test for one-way ANOVA, the Games–Howell test for Welch's ANOVA, and Dunn's test with Holm adjustment for Kruskal–Wallis comparisons (Ogle et al. 2025). Data visualization was performed using *ggplot2* (Wickham 2016). Statistical significance was defined as $p < 0.05$, and detailed assumption checks, model diagnostics, and summary statistics are provided in Table S4.

3 | Results

3.1 | High Doses of EGCG + PU Induce Mortality in Zebrafish Larvae

High concentrations of EGCG and PU have been shown to cause liver toxicity in rodent models and promote oxidative stress in vitro (Lambert et al. 2010; Čolić et al. 2022). To evaluate the potential toxicity of the EGCG and PU combination in zebrafish and determine optimal experimental doses, seven different concentrations were tested. Larval viability, hatching rate, and developmental progress were monitored at 24- and 48-h post-treatment (24/48 hpt). Larvae treated with Doses 1 through 5 exhibited a 100% survival rate throughout the 48-h observation period, with normal hatching dynamics and no visible morphological abnormalities (Figure 2A and Figure S2A). In contrast, all larvae exposed to Dose 7 perished within 24 h, while those treated with Dose 6 did not survive beyond 48 h (Figure S2A,B). Based on these findings, lower doses (Dose 3, Dose 4, and Dose 5) were selected for subsequent experiments (Figure 2B). These results demonstrate that the EGCG and PU combination exhibits dose-dependent toxicity in zebrafish larvae. In parallel, donepezil toxicity was assessed at two concentrations (10 and 20 μM) to serve as a pharmacological reference (Best et al. 2008; Whyte-Fagundes et al. 2024). No adverse effects were observed at 10 μM; however, larvae exposed to 20 μM displayed marked developmental abnormalities, including cardiac edema and spinal curvature, indicative of dose-dependent toxicity (Figure S2C).

3.2 | EGCG + PU Treatment Reduces Aβ Accumulation in Adult Zebrafish Brain

Injection of Aβ42 via CVMI has been shown to induce Aβ accumulation in the zebrafish brain (Bhattarai et al. 2017). To validate this toxicity model and assess the effects of EGCG + PU combination treatment on amyloid beta aggregation and clearance, we administered the combination via retro-orbital injection—an efficient method for systemic drug delivery with rapid

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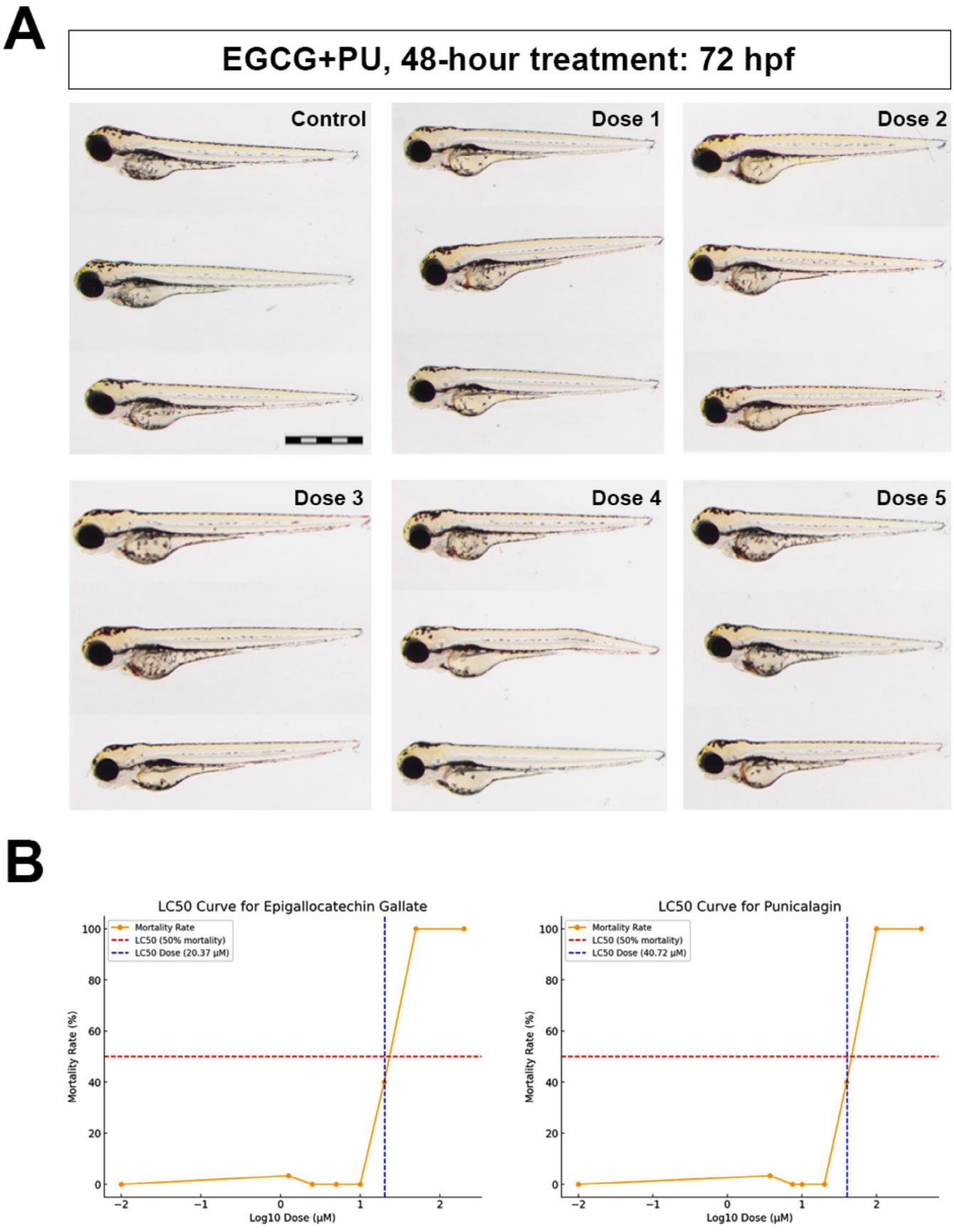


FIGURE 2 | Legend on next page.

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FIGURE 2 | High doses of EGCG + PU induce mortality in zebrafish larvae. (A) Representative images of zebrafish larvae treated with increasing doses of EGCG + PU combinations for 48 h, starting at 24 h postfertilization (hpf) and observed at 72 hpf. The doses are labeled as Dose 1 through Dose 5, with untreated larvae serving as the control. No significant morphological abnormalities were observed up to a certain concentration, indicating a safe therapeutic range. Scale bar: 1 mm. Images are representative of at least three independent experiments. (B) Lethal concentration 50% (LC_{50}) curves for epigallocatechin gallate (EGCG) and punicalagin (PU), derived from mortality rate data across varying concentrations. The LC_{50} values for EGCG and PU were determined to be 20.37 and 40.72 μM, respectively (indicated by the blue dashed lines). The red dashed line marks the 50% mortality threshold.

circulation and minimal tissue damage (Pugach et al. 2009)—into adult zebrafish previously injected with Aβ42 into the brain. Following treatment, we performed immunofluorescent staining on brain sections using an anti-β-amyloid antibody to detect total Aβ. No Aβ accumulation was detected in the PBS-injected group, whereas widespread Aβ deposition was evident in the brains of zebrafish injected with Aβ42, confirming the validity of the model (Figure 3A). Treatment with Dose 3, Dose 4, and Dose 5 resulted in a dose-dependent reduction in Aβ accumulation. Dose 4 exhibited the most potent Aβ-clearance effect, while treatment with Dose 5 led to postinjection recovery failure followed by mortality in adult zebrafish, indicating limited tolerability at this concentration (Figure 3A). In contrast, donepezil-treated zebrafish displayed persistent Aβ accumulation, with fluorescence intensity levels comparable to those observed in the Aβ42-induced toxicity group (Figure 3A). Quantitative analysis of β-amyloid fluorescence intensity confirmed these observations, demonstrating a significant reduction in amyloid burden following EGCG + PU treatment at Dose 4, whereas donepezil treatment did not result in a meaningful decrease relative to the Aβ42 group (Figure 3B). Based on the combined qualitative and quantitative assessments, we selected EGCG + PU Dose 4 for subsequent experiments due to its superior efficacy in reducing Aβ accumulation and its acceptable safety profile.

3.3 | EGCG + PU Treatment Reduces AChE Activity, LDH Activity, and Expression of Amyloidogenic Pathway Genes

To evaluate the effects of Aβ-induced toxicity and to assess the protective potential of EGCG + PU within the cholinergic axis, we first measured AChE activity as a key biochemical indicator of neuronal dysfunction. AChE activity was markedly elevated in the Aβ-induced toxicity model, indicating disruption of cholinergic homeostasis and reflecting a characteristic feature of AD pathology (Figure 4A). To assess therapeutic responsiveness, we next examined whether pharmacological interventions could reverse this elevation. Both donepezil and EGCG + PU significantly reduced AChE activity relative to the untreated toxicity group; however, EGCG + PU produced a greater degree of inhibition than donepezil, indicating an enhanced capacity to restore cholinergic balance (Figure 4A).

Next, we assessed LDH activity to evaluate the downstream effects of Aβ toxicity on cellular damage. LDH activity, a marker of cellular injury and membrane integrity loss, was higher in the Aβ-induced toxicity group, reflecting severe cellular damage (Figure 4B). EGCG + PU treatment reduced LDH activity, bringing it closer to levels observed in the control group and highlighting its protective effect against Aβ-induced damage.

Aβ42 is known to regulate the expression of enzymes involved in its production, creating a positive feedback loop that amplifies amyloidogenic signaling (Guglielmotto et al. 2011). To further characterize molecular responses to treatment, we examined the expression of key amyloidogenic pathway genes. The Aβ-induced toxicity model exhibited significantly elevated transcript levels of *appa*, *appb*, *bace*, *psen1*, and *psen2*, consistent with enhanced amyloidogenic drive (Figure 4C and Figure S3). Both EGCG + PU and donepezil attenuated these transcriptional changes, indicating therapeutic responsiveness. Notably, EGCG + PU treatment resulted in a stronger downregulation of *appa*, *appb*, and *bace*, suggesting more effective interference with amyloid precursor processing. In contrast, donepezil produced a more pronounced reduction in *psen1* and *psen2* expression, implying a differential influence on γ-secretase-associated pathways. Collectively, these findings demonstrate that EGCG + PU not only suppresses AChE activity and limits Aβ-induced cellular damage but also reshapes amyloidogenic gene expression profiles through a molecular action pattern that is distinct from that of donepezil.

3.4 | EGCG + PU Mitigates Aβ42-Induced Neuroinflammation and Promotes Neuronal Recovery

Neuroinflammation, a hallmark of AD, is triggered by Aβ plaque accumulation and is characterized by an increase in reactive glial cells (RGCs) in the zebrafish brain (Park et al. 2023; Firdous et al. 2024). Glial fibrillary acidic protein (GFAP) is commonly used as a marker for glial activation in RGCs. To investigate the neuroinflammatory response induced by Aβ42, we performed GFAP immunofluorescence staining on adult zebrafish brain sections. Aβ42 injection resulted in a pronounced increase in GFAP immunoreactivity, particularly in the central region of the dorsal telencephalon, compared with control brains, indicating robust glial activation and neuroinflammation (Figure 5A). Following 48 h of EGCG + PU treatment (Dose 4), GFAP signal intensity in both dorsal and ventral telencephalic regions was markedly reduced and restored to levels comparable to controls. In contrast, an equivalent 48-h treatment with donepezil did not produce a detectable reduction in GFAP immunoreactivity, suggesting limited efficacy in suppressing glial activation (Figure 5A).

Given the established link between neuroinflammation and neuronal loss in AD, we next analyzed neuronal integrity in the telencephalon using immunofluorescence staining for the neuronal marker HuC/D (Balistreri and Monastero 2023; Zhang, Xiao, et al. 2023). Aβ42-injected zebrafish exhibited a significant reduction in HuC/D-positive neuronal density across the

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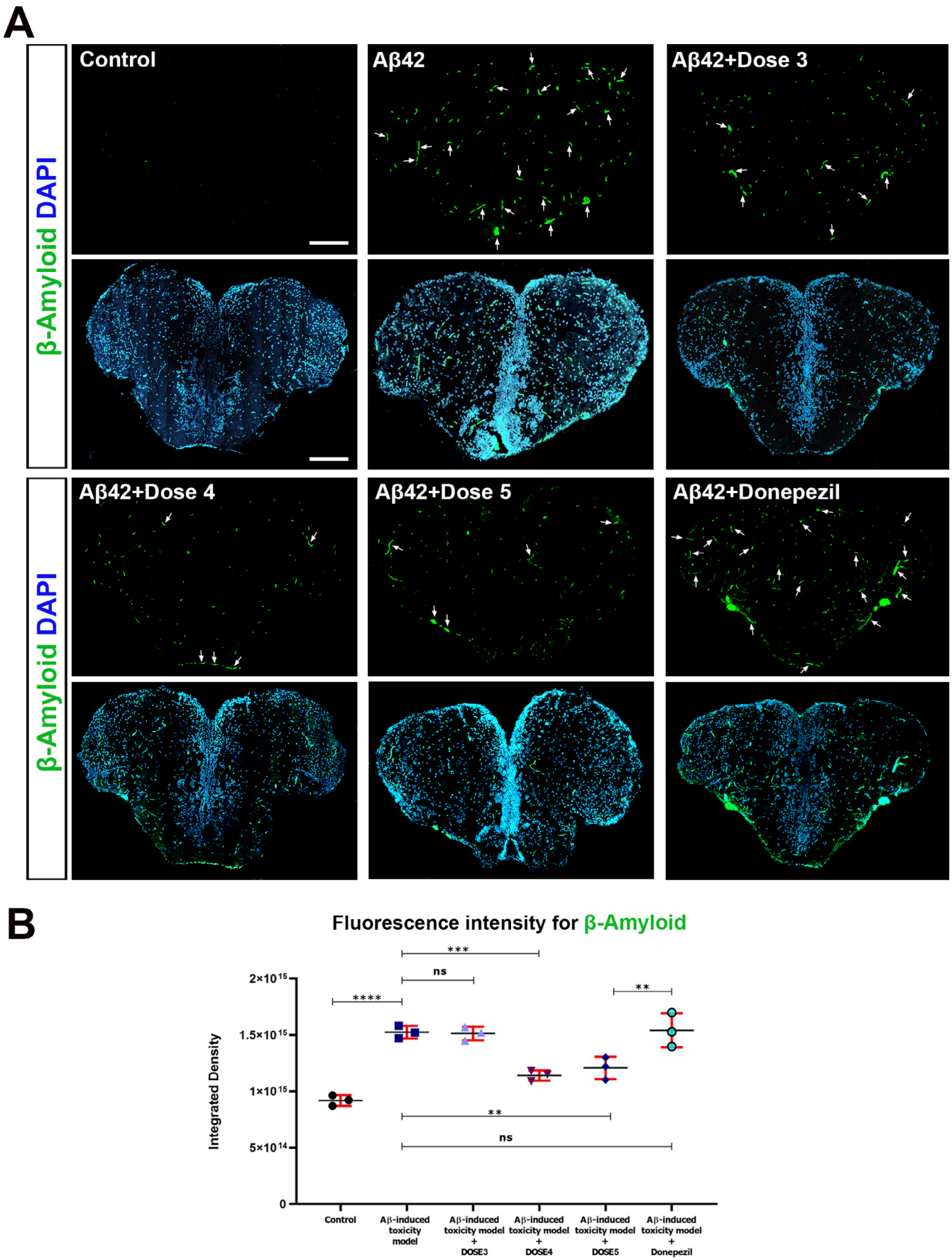


FIGURE 3 | Legend on next page.

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FIGURE 3 | EGCG + PU treatment reduces Aβ accumulation in adult zebrafish brain. (A) Representative immunofluorescence images of zebrafish telencephalon sections showing β-amyloid (Aβ) plaques (green) stained with an antibody against Aβ, and nuclei counterstained with DAPI (blue). Sections are from control, Aβ42-treated, and Aβ42-treated groups co-administered with increasing doses of EGCG + PU combination (Dose 3, Dose 4, and Dose 5) and donepezil. Aβ42 treatment resulted in an increase in β-amyloid plaque deposition (arrows), which was attenuated in a dose-dependent manner by treatment with the test compound. Scale bars: 200 μm. Images are representative of at least three independent experiments. (B) Quantification of fluorescence intensity for β-amyloid staining, presented as integrated density values. Aβ42-treated samples show significantly increased fluorescence compared to the control group. Treatment with EGCG + PU combination reduced β-amyloid intensity in a dose-dependent manner. Data are presented as mean ± standard deviation (SD, $n = 6$). Statistical significance was determined using an unpaired *t*-test. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

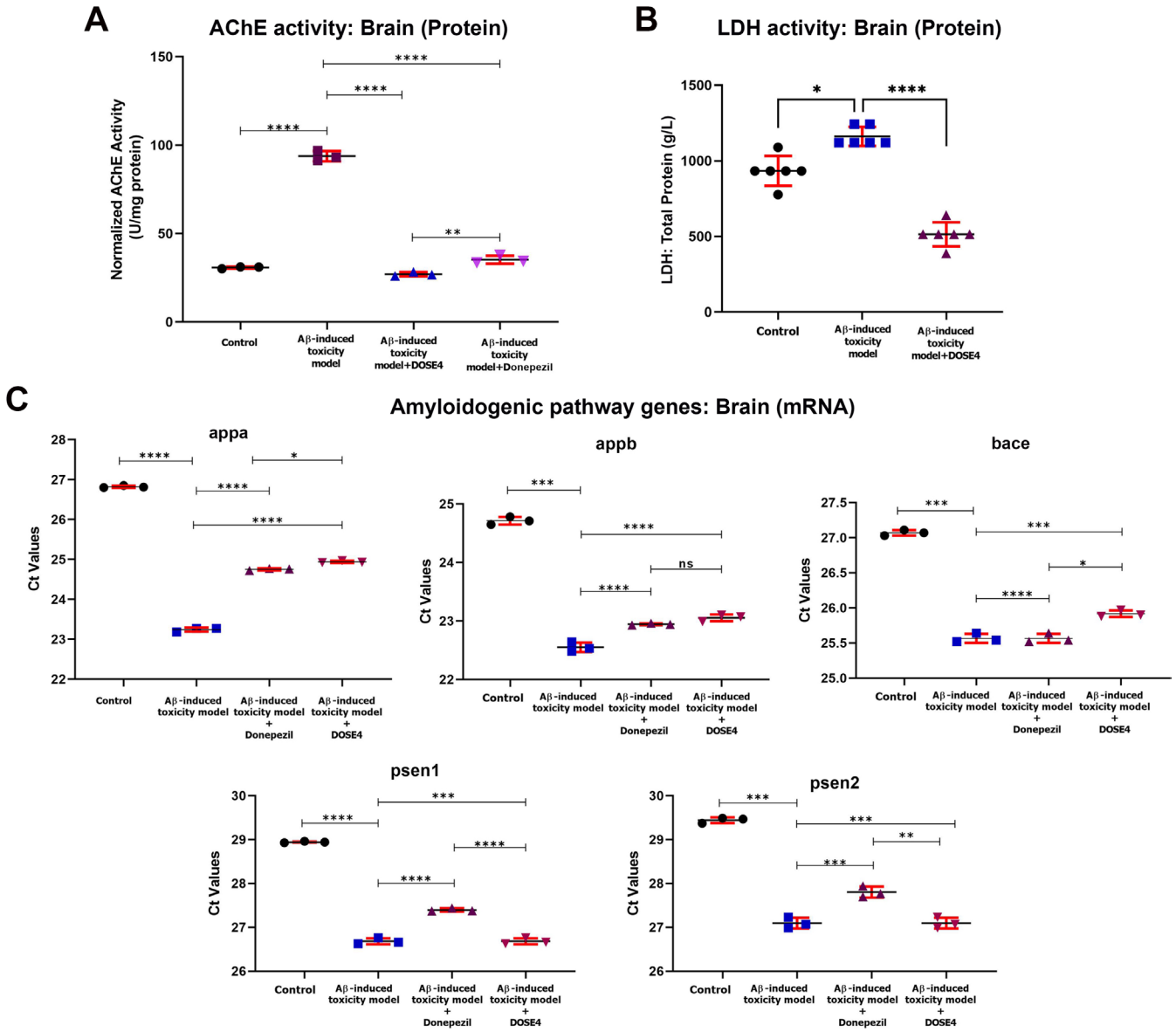


FIGURE 4 | EGCG + PU treatment reduces Aβ aggregation, LDH activity, and expression of amyloidogenic pathway genes. (A) Quantification of AChE activity (U/mg protein) in zebrafish brain tissues using Ellman's colorimetric assay. Aβ-induced toxicity markedly increased AChE activity compared with the control group. Treatment with EGCG + PU (Dose 4) significantly suppressed AChE activity, whereas Donepezil also reduced AChE activity but to a lesser extent under identical experimental conditions. (B) Measurement of LDH activity, a marker of cellular damage, in zebrafish brain tissues using a colorimetric method. Aβ-induced toxicity resulted in a significant increase in LDH activity relative to controls, indicating increased cellular injury. Treatment with EGCG + PU (Dose 4) significantly reduced LDH activity toward control levels. (C) Relative mRNA expression (Ct values) of key amyloidogenic pathway genes (*bace*, *appa*, *appb*, *psen1*, and *psen2*) in zebrafish brain tissues. Aβ-induced toxicity significantly upregulated all analyzed genes compared with the control group. Treatment with EGCG + PU (Dose 4) significantly downregulated these genes, whereas donepezil induced more limited gene-specific modulation. Data are presented as mean ± standard deviation (SD, $n = 3$). Statistical significance was determined using an unpaired *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

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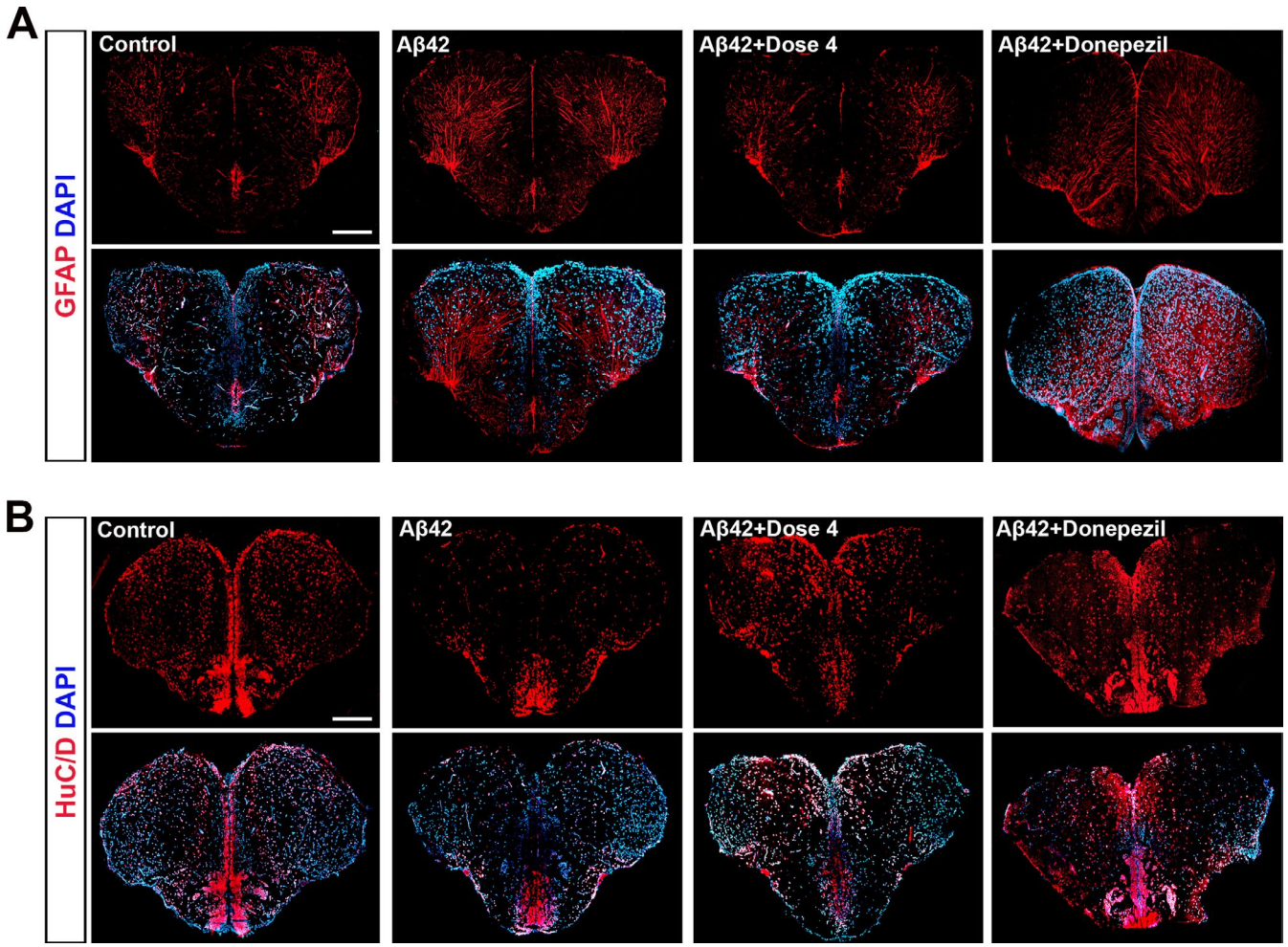


FIGURE 5 | EGCG + PU diminishes Aβ42-induced glial activation and promotes neuronal recovery. (A) Representative immunofluorescence images of zebrafish telencephalon sections stained for GFAP (glial fibrillary acidic protein; red) and DAPI (nuclei; blue). Control brains exhibit basal GFAP expression. Aβ42 injection induced pronounced glial activation, reflected by increased GFAP staining. Treatment with EGCG + PU (Dose 4) markedly reduced GFAP expression, indicating attenuation of glial activation. (B) Representative immunofluorescence images of zebrafish telencephalon sections stained for the neuronal marker HuC/D (red) and DAPI (nuclei; blue). Control brains show widespread neuronal staining. Aβ42 treatment resulted in a substantial reduction in HuC/D-positive cells, suggesting neuronal loss or damage. Treatment with EGCG + PU (Dose 4) and donepezil restored neuronal staining, indicating neuronal recovery. Scale bars: 200 μm. Images are representative of at least three independent experiments.

telencephalon compared with controls, indicating widespread neuronal degeneration (Figure 5B). Treatment with EGCG + PU for 48 h resulted in a clear recovery of HuC/D-positive neurons, consistent with neuroprotective and regenerative effects. Notably, donepezil treatment also led to a partial restoration of neuronal density, despite the absence of a measurable reduction in GFAP expression, suggesting that donepezil confers neuronal protection without directly attenuating glial activation (Figure 5B).

To further characterize the inflammatory response, we evaluated macrophage activation by analyzing L-plastin expression. Accumulation of Aβ is known to promote macrophage activation and the release of pro-inflammatory mediators in the brain (Wyss-Coray 2006; Wang et al. 2015; Bhattarai et al. 2016; LaRocca et al. 2021). Confocal imaging revealed a significant increase in L-plastin-positive cells in the Aβ42-injected group compared with controls (Figure 6A). Both EGCG + PU and donepezil treatments reduced L-plastin immunoreactivity, indicating attenuation of immune cell accumulation. Quantitative analysis confirmed a significant reduction in the number of L-plastin–positive cells following EGCG + PU (Dose 4) and donepezil treatment (Figure 6B).

To further assess the molecular inflammatory profile, we analyzed the expression of immune- and inflammation-related genes in brain tissue using qRT-PCR. Aβ42-injection significantly upregulated the pro-inflammatory cytokine *interleukin-1β* (*il1b*), *interleukin-6 signal transducer* (*il6st/glycoprotein 130*), *interleukin-6* (*il8*), and the cytokine receptor *interleukin 2 receptor gamma* (*il2rga*), consistent with an activated inflammatory state (Figure 6C and Figure S4). Treatment with EGCG + PU markedly suppressed the expression of all analyzed inflammatory markers, indicating broad anti-inflammatory activity at the transcriptional level. In contrast, while donepezil reduced immune cell accumulation as reflected by L-plastin staining,

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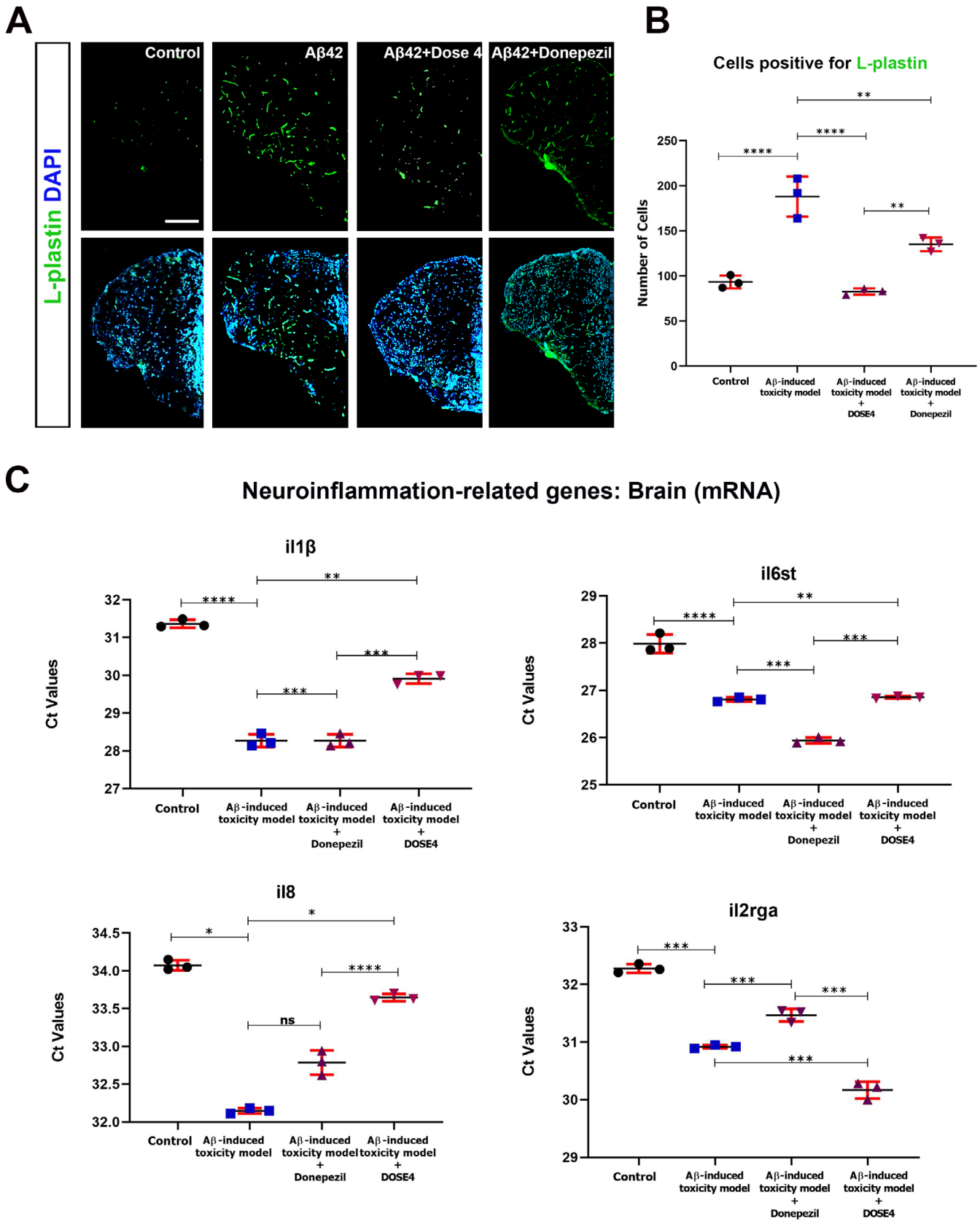


FIGURE 6 | Legend on next page.

its effects on cytokine gene expression were comparatively limited. Collectively, these findings demonstrate that EGCG + PU exerts a multifaceted anti-inflammatory effect by reducing glial and macrophage activation and suppressing proinflammatory gene expression, while also promoting neuronal recovery. Donepezil, although providing neuronal protection, displays a

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FIGURE 6 | EGCG + PU combination mitigates Aβ42-induced neuroinflammation. (A) Representative immunofluorescence images of zebrafish telencephalon sections stained for L-plastin (green) and DAPI (nuclei; blue). Control brains exhibit minimal basal L-plastin immunoreactivity. Aβ42 injection induces robust neuroinflammatory activation, evidenced by a marked increase in L-plastin-positive cells. Treatment with EGCG + PU (Dose 4) reduces L-plastin staining, indicating attenuation of glial/immune activation, whereas donepezil treatment produces a partial but less pronounced reduction. Scale bar: 200 μm. Images and analyses are representative of at least three independent experiments. (B) Quantification of L-plastin-positive cells in the zebrafish telencephalon. Aβ42 treatment significantly increased the number activated immune cells compared with controls. EGCG + PU (Dose 4) significantly reduced L-plastin-positive cell counts, while donepezil resulted in an intermediate reduction. Data are presented as mean ± standard deviation (SD, $n = 4$). Statistical significance was determined using an unpaired *t*-test. ****$p < 0.0001$. (C) Relative mRNA expression (Ct values) of neuroinflammation-related genes (*il1b*, *il6st*, *il8*, and *il2rga*) in zebrafish brain tissue. Aβ-induced toxicity markedly upregulated all analyzed inflammatory markers. EGCG + PU (Dose 4) significantly suppressed their expression compared with the Aβ42 group, whereas donepezil produces a partial normalization. Data are presented as mean ± SD ($n = 3$). Statistical significance was determined using an unpaired *t*-test. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

more restricted anti-inflammatory profile, highlighting distinct and complementary mechanisms of action between the two interventions.

3.5 | EGCG + PU Ameliorates Anxiety- and Aggression-Like Behaviors in Aβ Toxicity Model of Zebrafish

In humans, neurodegeneration in the brain contributes to emotional and behavioral symptoms in AD. Among these symptoms, anxiety and depression manifest in approximately 75% cases, while aggression and agitation occur in about 20% (Okamura et al. 2018; El Haj et al. 2020; Johansson et al. 2020). To investigate these behavioral effects in the Aβ toxicity model of adult zebrafish, we performed behavioral tests to evaluate anxiety- and aggression-like behaviors. As no prior studies have examined these behaviors in the zebrafish AD model, we developed a behavioral test to address this gap.

Zebrafish naturally exhibit bottom-dwelling behavior when introduced to a new environment, followed by exploratory activity after a brief adaptation period (Egan et al. 2009; Parker et al. 2014). To assess whether Aβ toxicity influences anxiety-like behaviors, we utilized the novel tank test to measure locomotor activity. In the Aβ toxicity model, zebrafish displayed significantly reduced top-dwelling behavior (Figure 7A) and increased bottom-dwelling behavior (Figure 7B). The distance traveled at the bottom was significantly greater than in the control group, reflecting heightened anxiety. Zebrafish treated with the EGCG + PU combination showed markedly reduced bottom-dwelling behavior from the first minute, accompanied by a significant increase in top-dwelling behavior, approaching levels observed in the control group. To further evaluate anxiety-like behaviors, we analyzed the number of entries into the top zone, which reflects exploratory behavior and reduced anxiety. In the Aβ toxicity model, zebrafish exhibited a significantly lower number of entries to the top zone compared with the control group. Conversely, zebrafish treated with the EGCG + PU combination showed a higher number of top-zone entries, even surpassing the control group (Figure 7C and Table S4).

To assess aggression-like behaviors, we employed the mirror-biting test, which measures social aggression in zebrafish. Zebrafish in the Aβ toxicity model displayed heightened aggression, evidenced by increased time spent, distance traveled, and average speed in the mirror-close zone (Figure 7D–F). The average speed across all regions was also significantly elevated compared to the control group (Figure 7G). Treatment with the EGCG + PU combination significantly reduced locomotor activity in the mirror-close zone and decreased the average speed across all regions, indicating a reduction in aggression-like behaviors. These findings demonstrate that the EGCG + PU combination effectively alleviates Aβ42-induced anxiety- and aggression-like behaviors in zebrafish by restoring normal locomotor patterns and reducing excessive aggression, suggesting its potential to mitigate emotional and behavioral symptoms associated with AD (Table S4).

4 | Discussion

4.1 | Therapeutic Potential of EGCG + PU as a Multi-Target Modulator in Aβ-Induced Pathology

AD is a progressive neurodegenerative disorder that profoundly affects individuals' quality of life while placing significant economic and social burdens on healthcare systems. The escalating prevalence of AD, combined with its high care costs and the lack of effective disease-modifying treatments, underscores the urgent need for innovative therapeutic strategies. In this regard, naturally occurring flavonoids have gained considerable attention due to their cost-effectiveness, neuroprotective properties, and favorable safety profiles. In this study, we explored the therapeutic potential of the combined use of EGCG and PU in an Aβ42-induced zebrafish model of AD. Our findings demonstrate that the EGCG + PU combination (i) exhibited a strong safety profile without inducing toxicity in zebrafish larvae across various tested concentrations; (ii) significantly decreased Aβ accumulation, oxidative stress markers, LDH levels, and AChE activity, alleviating key contributors to Aβ toxicity; (iii) normalized the expression of amyloidogenic pathway genes, reversing molecular disruptions induced by Aβ toxicity; (iv) supported neuronal preservation and recovery by mitigating neuroinflammation-associated neuronal loss; (v) improved behavioral outcomes, including anxiety- and aggression-like behaviors, and restored locomotor activity (Figure 8). Collectively, these findings support the therapeutic potential of EGCG + PU as a multi-target modulator of Aβ-induced pathology.

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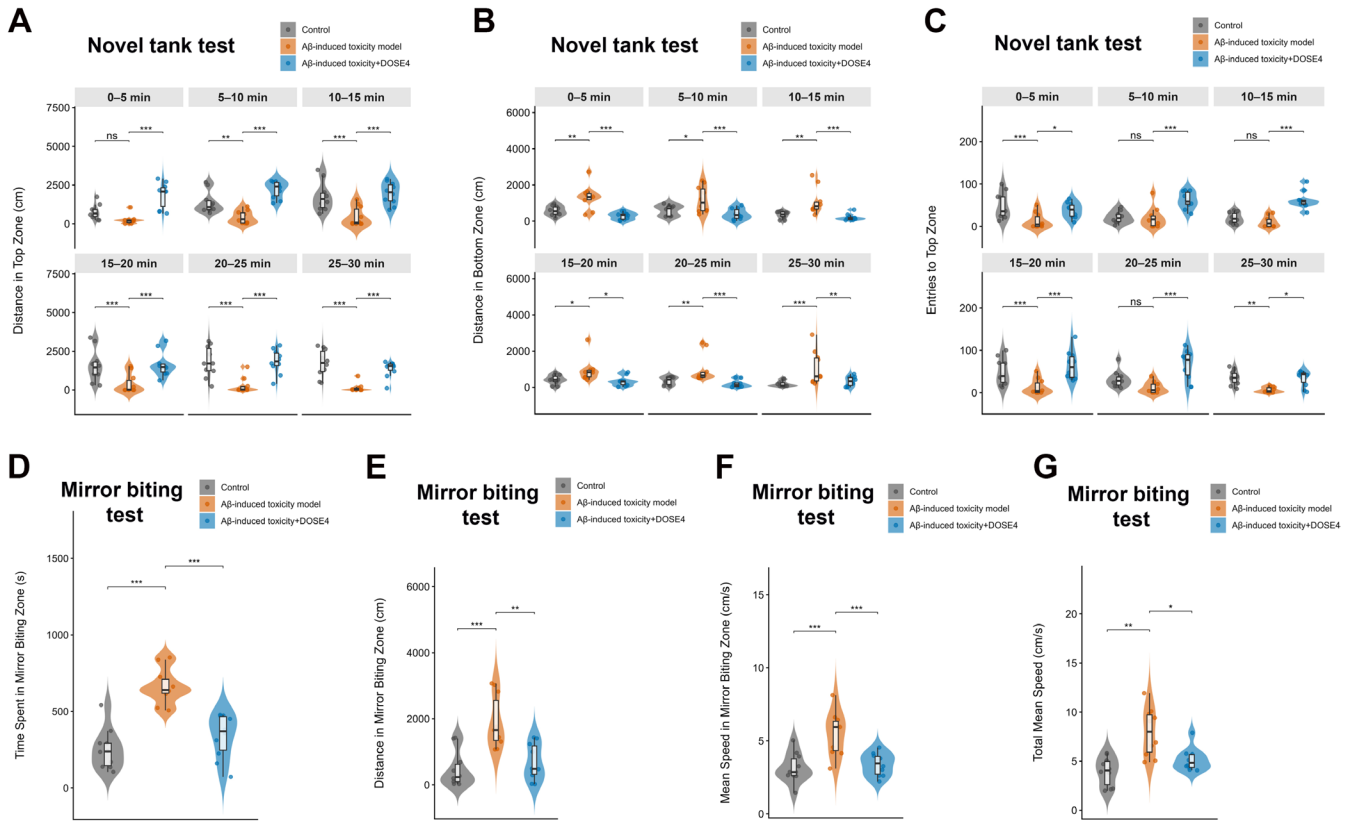


FIGURE 7 | EGCG + PU ameliorates anxiety- and aggression-like behaviors in the Aβ toxicity model of zebrafish. (A–C) Novel tank test: Behavioral responses were assessed in three metrics. (A) Distance traveled in the top zone, (B) distance traveled in the bottom zone, and (C) number of entries to the top zone, over 30 min. Control zebrafish displayed typical exploratory behaviors, with greater time spent in the top zone. $A\beta_{42}$ exposure induced significant anxiety-like behavior, marked by reduced activity in the top zone and increased bottom-dwelling. Treatment with EGCG + PU (Dose 4) significantly restored exploratory behaviors, increasing activity in the top zone. (D–G) Mirror-biting test: Aggression-like behaviors were quantified using, (D) time spent in the mirror-biting zone, (E) distance traveled in the mirror-biting zone, (F) mean speed in the mirror-biting zone, and (G) total mean speed. Aβ42 exposure significantly increased aggressive behaviors and hyperactivity compared to the control, as shown by heightened metrics in the mirror-biting zone. Treatment with EGCG + PU (Dose 4) significantly reduced these metrics, indicating attenuation of aggression-like and hyperactive behaviors. Statistical significance was assessed using linear mixed-effects models for repeated-measures novel tank outcomes (A–C), and one-way ANOVA (D, F), Kruskal–Wallis test (E), or Welch's ANOVA (G), followed by appropriate post hoc pairwise comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and ns, nonsignificant. Data are presented as mean ± standard deviation (SD; $n = 10$).

4.2 | Modulation of Amyloidogenic Pathways by EGCG + PU

The pathological accumulation of Aβ exacerbates oxidative stress, activating amyloidogenic pathway genes through a positive feedback loop involving inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (Combs et al. 2001; Bazan and Lukiw 2002; Yamamoto et al. 2007; Schmidt et al. 2008; Lahiri and Maloney 2010; Tabaton et al. 2010; Guglielmotto et al. 2011; Meraz-Ríos et al. 2013; Sato et al. 2021). This feedback loop promotes Aβ production, neuroinflammation, and neuronal dysfunction, driving AD progression. Polyphenols such as EGCG have been shown to modulate Aβ production by enhancing α-secretase activity and inhibiting BACE1 (Shimmyo et al. 2008; Lee et al. 2009). Notably, in vitro experiments with neuronal cell lines expressing human APP demonstrate that EGCG significantly reduces Aβ production (Rezai-Zadeh et al. 2005). However, EGCG alone is insufficient to completely block BACE activity in vivo (Cheng et al. 2012). Interestingly, when combined with other modulators like ferulic acid, EGCG effectively inhibits BACE activity and reduces amyloidosis in APP/PS1 mouse models (Mori et al. 2013, 2019). Consistent with these reports, our study demonstrates that the EGCG + PU combination treatment significantly reduces intracellular Aβ accumulation and aggregated Aβ levels in the adult zebrafish Aβ-induced toxicity model. Additionally, the treatment led to a notable reduction in the expression levels of *bace*, *appa*, *appb*, *psen1*, and *psen2* genes, emphasizing the ability of this combination to modulate the amyloidogenic pathway.

4.3 | Restoration of Cholinergic Homeostasis Through AChE Suppression

AChE is the principal catalytic enzyme responsible for the rapid hydrolysis of acetylcholine within the synaptic cleft, thereby ensuring precise temporal control of cholinergic neurotransmission. In AD, aberrant upregulation of AChE activity exacerbates cholinergic hypofunction, disrupts synaptic efficacy, and contributes to the progressive deterioration of

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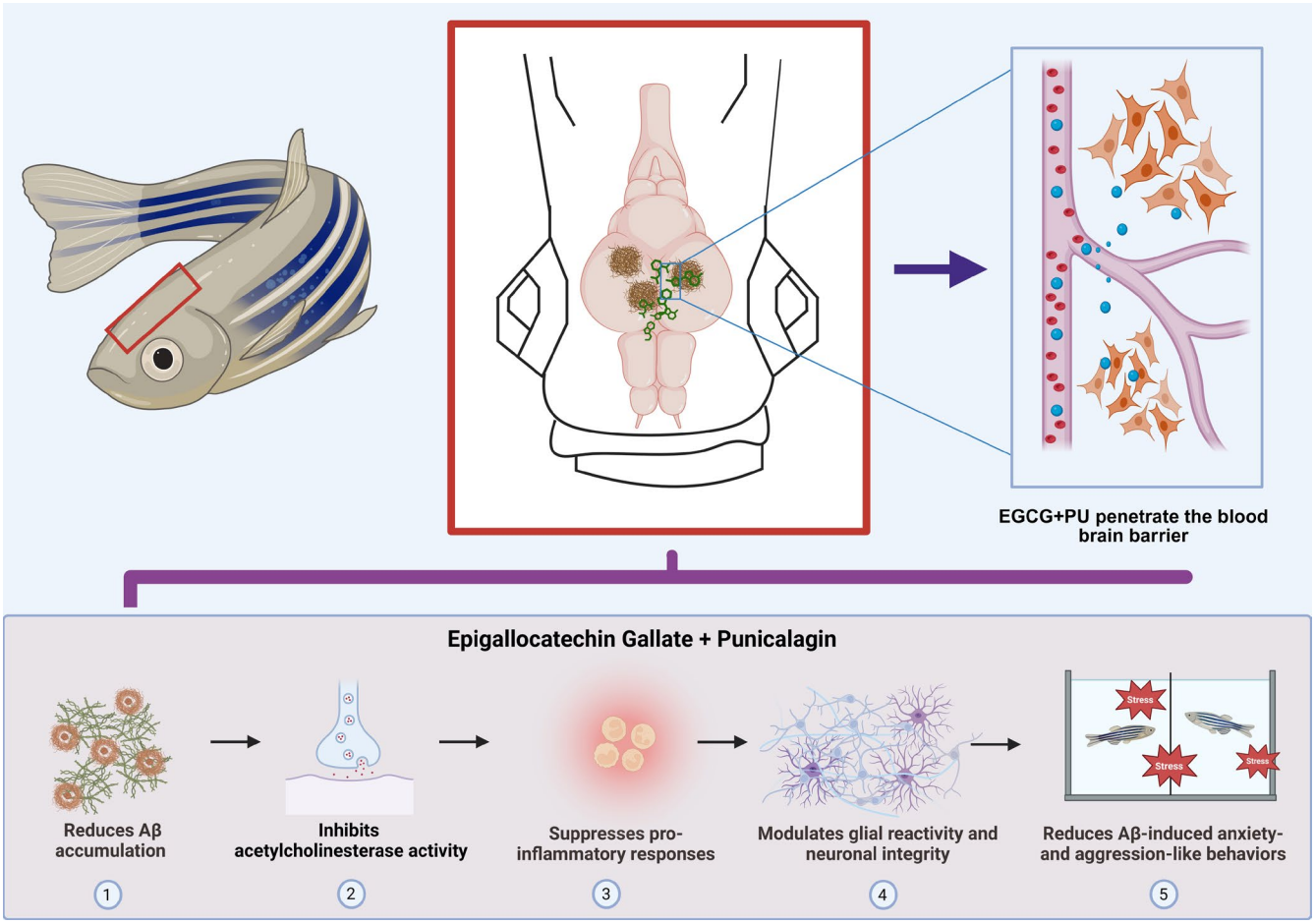


FIGURE 8 | Schematic overview of the proposed multitarget neuroprotective actions of EGCG + PU in the adult zebrafish Aβ-induced toxicity model. Systemic administration of epigallocatechin gallate (EGCG) and punicalagin (PU) enables their penetration across the blood–brain barrier and distribution to the zebrafish brain. Within the central nervous system, the EGCG + PU combination exerts coordinated protective effects by (1) reducing Aβ accumulation, (2) suppressing proinflammatory responses, (3) modulating glial reactivity and preserving neuronal integrity, (4) limiting degeneration-driven apoptosis while supporting regenerative processes, and (5) alleviating Aβ-induced anxiety- and aggression-like behaviors. Together, these interconnected actions illustrate the multilevel, disease-modifying potential of EGCG + PU against molecular, cellular, and behavioral features of AD–like pathology.

cognitive processes (Mufson et al. 2008; Hampel et al. 2021; Chen et al. 2022). Beyond its canonical role in neurotransmitter turnover, elevated AChE activity has also been implicated in promoting amyloidogenic processes and amplifying neurotoxic signaling cascades, underscoring its multifaceted contribution to AD pathophysiology (Inestrosa et al. 1996; Carvajal and Inestrosa 2011; Grabowska et al. 2025). Consistent with these mechanisms, our findings demonstrate that EGCG + PU markedly suppresses AChE activity in the Aβ42-induced zebrafish model, supporting restoration of cholinergic homeostasis. Previous studies have similarly reported that EGCG reduces elevated AChE activity and improves cholinergic function in diverse experimental models of cognitive impairment, including streptozotocin-induced dementia and anesthesia- or AD-related rodent models (Biasibetti et al. 2013; Okello and Mather 2020; Kim et al. 2021; Nan et al. 2021). In parallel, PU and pomegranate peel–derived polyphenols have been shown to exert anti-cholinesterase effects, inhibiting AChE activity and lowering brain AChE levels in vivo, further supporting a direct contribution of PU-rich preparations to cholinergic modulation (Morzelle et al. 2016; Amri et al. 2017; Chen, Chen, et al. 2023). Collectively, these observations indicate that EGCG + PU provides robust cholinergic support, complementing its broader protective actions against Aβ-induced neurotoxicity and reinforcing its multitarget therapeutic profile in AD-related pathology.

4.4 | Blood–Brain Barrier Penetration and Pharmacokinetic Considerations

The BBB is a selectively permeable membrane that regulates the exchange of substances between the bloodstream and the brain, maintaining neural homeostasis while restricting potentially harmful agents. Effective BBB penetration, therefore, is a critical factor in the development of therapies for neurodegenerative diseases, as many candidate compounds fail to reach therapeutically relevant concentrations within the CNS. In the present study, the dose-dependent reduction in Aβ accumulation following retro-orbital injection of the EGCG + PU combination provides indirect but compelling evidence of BBB penetration, allowing these compounds to exert central

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effects. Consistent with this interpretation, we previously demonstrated that EGCG and PU, both individually and in combination, can traverse an in vitro BBB model and directly disrupt Aβ plaque structures (Serdar et al. 2021). Moreover, EGCG has been shown to penetrate the BBB and reach functionally relevant brain areas (Singh et al. 2008; Cascella et al. 2017; Pervin et al. 2017), a property essential for its neuroprotective effects, including modulation of Aβ aggregation. Additional support for the selected dosing strategy comes from pharmacokinetic studies reporting measurable brain concentrations of EGCG and neuroactive PU-derived metabolites following systemic administration, indicating that circulating polyphenols or their metabolites can achieve central exposure sufficient to engage pathological targets (Van Amelsvoort et al. 2001; Chow et al. 2003; Andreu-Fernández et al. 2020; Shabir et al. 2024; Chang et al. 2025). Together, these findings support the BBB permeability of the EGCG + PU combination and highlight its potential as a promising therapeutic strategy for Aβ-related disorders.

4.5 | Neuroprotective Actions of PU and Synergistic Effects With EGCG

PU has emerged as an independent neuroactive polyphenol rather than a passive adjunct, with accumulating evidence demonstrating its capacity to modulate multiple pathological processes relevant to AD. PU and PU-rich pomegranate preparations have been shown to exert potent antioxidant and anti-inflammatory effects in neural and glial systems, including suppression of oxidative stress, inhibition of proinflammatory cytokine production, and attenuation of microglial activation through modulation of NF-κB, MAPK, and NLRP3 inflammasome signaling pathways (Hartman et al. 2006; Olajide et al. 2014; Kim et al. 2017; Lo et al. 2022; Chen, Guo, and Zhou 2023). These reported actions closely align with our observations that EGCG + PU treatment significantly reduced oxidative stress markers, decreased LDH activity, and markedly attenuated neuroinflammation in the Aβ42-induced zebrafish model. In vivo studies further demonstrate that PU improves learning and memory performance, restores redox homeostasis, and suppresses neuroinflammatory signaling in aging and inflammation-driven models (Hartman et al. 2006; Chen, Chen, et al. 2023; Cordiano et al. 2024), consistent with the behavioral improvements and neuronal recovery observed in our study following EGCG + PU administration. Importantly, PU has also been reported to modulate amyloidogenic signaling by downregulating APP and BACE1 expression and to inhibit AChE activity, thereby reducing Aβ generation and supporting cholinergic function (Kim et al. 2017; Calderaro et al. 2022; Alami et al. 2024), mechanisms that mirror our findings of reduced Aβ accumulation, normalization of amyloidogenic gene expression, and suppression of AChE activity. Following systemic administration, PU undergoes metabolic conversion into smaller, bioactive ellagitannin-derived metabolites that are proposed to mediate central neuroprotective effects through reinforcement of antioxidant defenses and interference with protein aggregation pathways (Hartman et al. 2006; Čolić et al. 2022; Chen, Guo, and Zhou 2023). Within this mechanistic framework, the combined administration of PU with EGCG—an established BBB-permeable flavonoid—provides additive or complementary benefits by engaging overlapping yet distinct molecular pathways governing amyloid burden, neuroinflammation, synaptic integrity, and behavioral dysfunction.

4.6 | Mitigation of Metabolic Dysfunction and Cellular Injury via LDH Modulation

LDH is critical for neuronal energy metabolism and cellular homeostasis. In the brain, astrocytes produce lactate from glucose, which neurons utilize as a key energy source (Beard et al. 2021). The LDH-B isoform in neurons converts lactate into pyruvate for mitochondrial oxidative phosphorylation, essential for ATP production (Wu et al. 2023). Under hypoxic or metabolically stressed conditions, neurons shift to lactic acid fermentation, increasing LDH-A activity (Benarroch 2024). In AD, disrupted energy metabolism and oxidative stress lead to elevated LDH activity, exacerbating neuronal dysfunction and vulnerability (Zhang et al. 2018; Perluigi et al. 2024). EGCG has been reported to regulate cellular metabolism, including reducing lactate production and downregulation of LDH expression in cancer models, indicating its capacity to modulate glycolytic flux and cellular energy balance (Lu et al. 2015; Wei et al. 2018). Similarly, PU exhibits pronounced cytoprotective effects in models of oxidative or toxic injury, where it reduces LDH release and activity in both in vivo and in vitro systems, reflecting its ability to preserve membrane integrity and limit cellular stress responses (Chen et al. 2012; Ding et al. 2017; Ye et al. 2019; Yanpar et al. 2025). Consistent with these reports, we observed that elevated LDH levels in the Aβ-induced toxicity model were significantly reduced in zebrafish treated with the EGCG + PU combination. This reduction suggests that the combined intervention effectively mitigates the metabolic dysregulation associated with Aβ-induced toxicity. To our knowledge, this is the first study to demonstrate the effects of EGCG on LDH levels in an AD model. Collectively, these findings highlight the potential of EGCG + PU as a therapeutic strategy to address disrupted energy metabolism in AD.

4.7 | Suppression of Neuroinflammation and Glial Activation

Neuroinflammation, driven by activated microglia and astrocytes in response to Aβ plaques, plays a pivotal role in AD pathogenesis (De Marchi et al. 2024; Pelkmans and Gispert 2024; Zhang, Yang, et al. 2024). In the zebrafish brain, neuroinflammation is characterized by the release of pro-inflammatory cytokines such as il-1β, il6st and il8, along with chemokines that recruit and activate immune cells (Grimaldi et al. 2019; Brenet et al. 2024; Lu and Hyde 2024). These cytokines activate RGCs and microglia, which in turn secrete additional cytokines, creating a positive feedback loop that amplifies inflammation, induces neurotoxicity and promotes neurodegeneration (Heneka et al. 2015; Heppner et al. 2015). Importantly, increased oxidative stress is known to exacerbate inflammatory signaling and contribute to neurodegenerative progression, and emerging evidence highlights a tight mechanistic coupling between oxidative damage and neuroinflammatory cascades in aging and AD models (He et al. 2024). Studies show that treatments with EGCG and PU effectively suppress microglial activation and

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shift microglia toward an anti-inflammatory phenotype in AD models (Lee et al. 2013; Olajide et al. 2014; Lo et al. 2022; Chen, Chen, et al. 2023; Li et al. 2024). In vitro experiments have revealed that both EGCG and PU inhibit the expression of proinflammatory mediators such as TNFα, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS) while simultaneously restoring intracellular antioxidant levels, including Nrf2 and HO-1 (Cheng-Chung Wei et al. 2016; El-Missiry et al. 2018; Huang et al. 2021). Additionally, EGCG has been shown to reduce microglial neurotoxicity by suppressing the release of pro-inflammatory factors associated with Aβ-induced proinflammatory microglial phenotypes (Cascella et al. 2017). Consistent with these findings, our study observed a significant reduction in proinflammatory cytokines and glial activation following EGCG + PU treatment in the Aβ-induced AD toxicity model.

4.8 | Promotion of Neuronal Survival, Recovery, and Neurogenesis

EGCG and PU are widely recognized for their neuroprotective and neurorescue effects. EGCG has been shown to enhance cell viability, reduce reactive oxygen species levels, and decrease apoptotic markers (Schroeder et al. 2009; Han et al. 2014; Yao et al. 2014; Zhang et al. 2014). Notably, it also promotes neurite outgrowth in serum-deprived PC12 neuronal cells, highlighting its potential role in neuronal repair and regeneration (Reznichenko et al. 2005). In mammalian studies, EGCG has been demonstrated to stimulate neural progenitor cell proliferation in the adult hippocampus, promote neuronal differentiation and proliferation in the subventricular zone after ischemic stroke through the PI3K/AKT signaling pathway, and enhance neural stem cell survival, proliferation, and differentiation by modulating the TLR4-NF-κB pathway to alleviate LPS-induced neuroinflammation and reduce proinflammatory cytokine production (Wang et al. 2012; Ortiz-López et al. 2016; Seong et al. 2016; Zhang et al. 2017). PU complements these effects by enhancing the Krebs cycle via activation of AMP-activated protein kinase and preventing diabetes-related neuronal death by increasing 5-hydroxymethylcytosine levels in diabetic mice (He et al. 2022). Additionally, PU provides significant neuroprotection against cerebral ischemia/reperfusion injury in a middle cerebral artery occlusion rat model (Yaidikar et al. 2014). These findings align with our results, which demonstrate that the EGCG + PU combination enhances neurogenesis in the Aβ-induced toxicity model. By counteracting the increased neuronal degeneration associated with Aβ toxicity, the EGCG + PU treatment mitigates its detrimental effects, underscoring the therapeutic potential of this combination in combating neurodegenerative diseases such as AD.

4.9 | Behavioral Recovery and Neuropsychiatric Symptom Modulation

Neurodegenerative conditions, including AD, are often accompanied by neuropsychiatric symptoms such as anxiety and aggression, driven by alterations in neurotransmitter systems and neuroinflammatory responses (Scheltens et al. 2016; Sakakibara et al. 2018; Latif-Hernandez et al. 2019; Zhang, Liu, et al. 2023). In our study, the adult zebrafish Aβ-induced toxicity model exhibited anxiety-like and aggressive behaviors. These findings are consistent with previous studies in rodent models, which demonstrated Aβ pathology and neuroinflammation significantly contribute to behavioral changes (Alexander et al. 2011; Bories et al. 2012; Jawhar et al. 2012; Braun and Feinstein 2019; Kosel et al. 2021). Moreover, behavioral analyses in rats with AD-like symptoms induced by aluminum chloride have similarly revealed reduced exploratory and locomotor activities (Singh et al. 2018). Notably, treatment with EGCG-loaded nanoparticles alleviated these neurobehavioral impairments, underscoring the therapeutic potential of EGCG. Consistent with these reports, our findings demonstrate that EGCG + PU treatment significantly improved locomotor performance and reduced anxiety-like behaviors in the Aβ-induced toxicity model, supporting its neuroprotective and neurorescue potential.

4.10 | Integrated Mechanistic Model Underlying EGCG + PU Efficacy

A growing body of biochemical and structural evidence supports a unifying mechanistic framework that can link the multilevel improvements observed in the present study, although these relationships were not directly tested experimentally here (Ehrnhoefer et al. 2008; Bieschke et al. 2010; Palhano et al. 2013; Wang et al. 2017; Acharya et al. 2020; Alami et al. 2024). Beyond reducing Aβ production, EGCG has been shown to directly interact with misfolded amyloidogenic peptides and redirect them away from β-sheet–rich, synaptotoxic oligomeric assemblies toward unstructured, off-pathway species with markedly lower neurotoxicity (Schuwirth et al. 2006). Such structural redirection is predicted to reduce the pool of pathogenic Aβ species capable of initiating microglial activation, oxidative stress, and downstream inflammatory cascades. In this context, the observed reductions in proinflammatory cytokine expression, glial reactivity, and neuronal loss and cellular damage markers in our model can be interpreted as downstream consequences of diminished Aβ-driven immune activation. PU, through its ability to stabilize redox homeostasis, attenuate inflammatory signaling, and preserve BBB integrity, is likely to further constrain Aβ-mediated toxicity by creating a cellular environment less permissive to oxidative and inflammatory amplification. Together, these complementary actions support a mechanistic model in which EGCG + PU first attenuates toxic Aβ assemblies, thereby dampening neuroinflammatory responses, promoting neuronal preservation and recovery, and normalizing behavioral outputs such as anxiety- and aggression-like responses. While this integrated pathway remains to be experimentally validated through causal and temporal analyses, it provides a coherent framework linking amyloid burden, neuroinflammation, neuronal integrity, behavior, and underscores the therapeutic promise of EGCG + PU as a multi-target intervention in AD-related pathology.

In contrast to the broader mechanistic profile observed with EGCG + PU, donepezil exhibited more limited upstream effects in the present zebrafish model. Although donepezil reduced the expression of *psen1* and *psen2* and attenuated several inflammatory markers, these molecular changes did not translate into marked reductions in overall Aβ burden or substantial attenuation of GFAP-associated glial activation. Donepezil also reduced

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AChE activity, consistent with its established pharmacological action; yet the magnitude of this inhibition remained lower than that achieved by the EGCG + PU combination under identical experimental conditions, which may account for its comparatively modest effects on neuronal preservation and behavioral recovery. Together, these observations underscore a mechanistic divergence within this model system: whereas Donepezil primarily exerts symptomatic cholinergic modulation, EGCG + PU appears to engage multiple upstream pathways related to amyloid processing, neuroinflammation, and metabolic dysfunction. While these findings do not diminish the established clinical utility of Donepezil, they highlight the potential of EGCG + PU as a multitarget intervention with broader disease-modifying capacity in preclinical AD models.

5 | Limitations and Future Directions

While the present findings delineate a coherent mechanistic framework linking molecular, histological, and behavioral outcomes, definitive validation of causal relationships will require targeted follow-up studies. Approaches incorporating pathway-specific inhibition, cell-type-resolved analyses, and genetic manipulation will be necessary to establish mechanistic causality and to identify the cellular compartments that mediate the observed protective effects. Such studies will also be essential for refining mechanistic interpretation and strengthening the translational relevance of the findings.

5.1 | Model Scope and Translational Positioning of Zebrafish AD Paradigms

While zebrafish do not recapitulate the full structural and functional complexity of the mammalian brain, they retain multiple features that are highly relevant for modeling core aspects of AD biology, including conserved neuroanatomical regions, neurotransmitter systems, and amyloid-responsive cellular pathways. Accumulating evidence demonstrates that zebrafish neuroanatomy, cholinergic signaling, and cellular responses to amyloid pathology exhibit substantial conservation with human AD-related mechanisms (Cosacak et al. 2022; Dey et al. 2024). Importantly, zebrafish possess homologous amyloidogenic pathways encompassing APP processing, secretase activity, and Aβ-induced glial activation, and they reproduce hallmark AD-associated phenotypes such as neuroinflammation, neuronal loss, and cholinergic dysfunction (Saleem and Kannan 2018; Dey et al. 2024). In addition, zebrafish express a vertebrate-typical repertoire of cytochrome P450 enzymes and phase I/II metabolic pathways, supporting drug absorption, distribution, and biotransformation patterns that are broadly comparable to those of higher vertebrates (Cassar et al. 2020; Nawaji et al. 2020; Zhang, Xu, et al. 2024). From a translational perspective, these attributes position zebrafish as a valuable early-stage intermediary model within the preclinical pipeline, enabling rapid in vivo assessment of efficacy, toxicity, and behavioral outcomes while providing initial insight into pharmacodynamic responsiveness. At the same time, species-specific differences in drug metabolism, BBB properties, and higher-order cortical organization limit direct extrapolation of zebrafish findings to human disease. Consequently, outcomes obtained in zebrafish should be interpreted as supporting hypothesis generation, mechanistic validation, and dose refinement, rather than as definitive predictors of clinical efficacy.

5.2 | Sex as a Biological Variable

Sex-balanced cohorts were used throughout the study, and male and female zebrafish were intentionally pooled within each treatment group to minimize sex bias at the group level. However, because pooled analyses do not resolve sex-specific variance, the present study was not powered to detect sexually dimorphic behavioral or biochemical responses. This consideration is particularly relevant given accumulating evidence that sex can influence neuroinflammatory signaling, cholinergic function, and behavioral phenotypes in AD models. Although the observed group-level effects of EGCG + PU on amyloid burden, neuroinflammation, and behavior were robust, it remains possible that males and females differ in treatment sensitivity, response magnitude, or pathway engagement. Future studies incorporating sex-stratified analyses or sex-specific experimental designs will be necessary to determine whether EGCG + PU exhibits differential efficacy or mechanistic profiles across sexes.

5.3 | Resolution of Amyloid-β Conformational States

The immunofluorescence approach employed in this study quantifies total Aβ immunoreactivity and does not differentiate between monomeric, soluble oligomeric, or fibrillar conformational states. This distinction is biologically significant, as accumulating evidence indicates that soluble Aβ oligomers exhibit disproportionately greater synaptotoxic, neuroinflammatory, and behavioral effects compared with plaque-associated fibrillar species (Verma et al. 2015; Sengupta et al. 2016; Tolar et al. 2021). Consequently, while the observed reduction in total Aβ signal following EGCG + PU treatment reflects an overall decrease in amyloid burden, it does not allow definitive conclusions regarding selective modulation of specific Aβ conformers. Future studies incorporating conformation-specific antibodies, oligomer-sensitive fluorescent probes, or biochemical fractionation approaches will be required to determine whether EGCG + PU selectively targets the most neurotoxic oligomeric Aβ species. Such analyses would enable a more refined mechanistic interpretation by distinguishing whether the observed molecular, cellular, and behavioral improvements arise from global amyloid reduction or from selective suppression of highly pathogenic Aβ assemblies.

5.4 | Interpretation of Supranormal Behavioral Responses

While EGCG + PU treatment restored most behavioral parameters toward levels observed in untreated controls, certain endpoints—such as top-zone entries in the novel tank test—modestly exceeded control values. This pattern may reflect increased exploratory drive or reduced anxiety, consistent with reports that polyphenols can modulate stress responsivity, locomotor motivation, and affective regulation in neurodegenerative and stress-related contexts. Importantly, the bidirectional

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sensitivity of the novel tank paradigm represents a methodological advantage, as it enables detection of both pathological behavioral suppression and treatment-associated enhancement. Nevertheless, values exceeding the normal range may also arise from nonspecific arousal or heightened behavioral activation rather than true normalization and thus require cautious interpretation. Future studies incorporating additional behavioral dimensions such as swimming velocity, habituation dynamics across time bins, physiological stress markers, or complementary assays assessing impulsivity and arousal will be necessary to determine whether the observed enhancements reflect adaptive behavioral optimization or nonspecific excitation effects.

6 | Conclusion

Our findings demonstrate that the EGCG + PU combination exhibits promising neuroprotective effects in an Aβ-induced toxicity model of the adult zebrafish brain. This protective response is achieved through the reduction of Aβ plaques, the alleviation of neuroinflammation, and the normalization of proinflammatory cytokines and disease-associated gene expression. Moreover, EGCG + PU effectively mitigates both the biochemical and behavioral impairments associated with AD, underscoring its potential as a safe and effective therapeutic strategy.

Given the limited regenerative and reparative capacity of the mammalian brain, our results suggest that EGCG + PU or its derivatives may hold significant promise not only for counteracting AD pathology but also in promoting overall brain health in neurodegenerative diseases. Further investigation into the molecular mechanisms underlying its benefits could pave the way for novel therapeutic approaches. Besides, future research on EGCG + PU should focus on improving the drug form and evaluating long-term efficacy and safety. By targeting multiple pathological aspects of AD, these findings provide a solid foundation for advancing preclinical and clinical research into innovative treatment strategies for neurodegenerative disorders.

Author Contributions

D.N.: investigation, formal analysis, validation, data curation, visualization, writing – original draft. D.I.: investigation, formal analysis. Y.K.P.: investigation, formal analysis. K.C.: investigation, formal analysis. I.O.: investigation. E.T.S.: investigation. B.S.S.: formal analysis. S.K.: conceptualization, methodology, resources, supervision, writing – review and editing, funding acquisition. G.O.: conceptualization, methodology, resources, writing – review and editing, visualization, supervision, project administration, funding acquisition. All authors read and approved the final manuscript.

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Ethics Statement

The animal study protocol was approved by the Animal Experiments Local Ethics Committee of Izmir Biomedicine and Genome Center (IBG-AELEC) (2020-034).

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding authors S.K. and G.O.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Representative trajectory plots from the novel tank and mirror biting assays. Representative swimming trajectories of adult zebrafish recorded during the novel tank assay and mirror biting assay are shown for the control group, Aβ42-induced toxicity model, and the Aβ42-induced toxicity model treated with Dose 4. Each panel illustrates the movement patterns of individual fish during the test period, providing a qualitative visualization of exploratory behavior, anxiety-like responses, and aggression-related activity. In the novel tank assay, zebrafish in the Aβ42-induced toxicity model predominantly swam within the bottom zone, whereas treatment with Dose 4 displayed increased exploration of upper regions. In the mirror biting assay, the Aβ42-induced toxicity model exhibited swimming behavior largely confined to the mirror biting zone, while treatment with Dose 4 displayed a swimming pattern characterized by increased exploration of nonmirror regions. **Figure S2:** EGCG + PU exhibits dose-dependent developmental toxicity in zebrafish larvae. (A) Hatching and mortality rates of zebrafish embryos exposed to increasing doses of EGCG + PU at 24, 48, and 72 hpf. Higher EGCG + PU doses were associated with increased mortality. (B) Representative images of embryos treated with Dose 6 and Dose 7 showing developmental toxicity at 24 hpf. Exposure to Dose 6 and Dose 7 induced early lethality during development at 24 and 48 hpf. (C) Representative images of zebrafish larvae treated with increasing doses of Donepezil combinations for 48 h, starting at 24 h postfertilization (hpf) and observed at 72 hpf. Donepezil treatment at 20 μM induced mild developmental abnormalities. **Figure S3:** Relative mRNA expression levels of key amyloidogenic pathway genes (*bace*, *appa*, *appb*, *psen1*, and *psen2*) in zebrafish brain tissues. Aβ-induced toxicity significantly upregulates all analyzed genes compared to the control group. Treatment with Dose 4 of EGCG + PU significantly downregulates these genes compared to the Aβ-induced toxicity group. Data are presented as mean ± standard deviation of the mean (SD, $n = 3$). Statistical significance was determined using an unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. **Figure S4:** Relative mRNA expression levels of neuroinflammation-related genes (*il1b*, *il6st*, *il8*, and *il2rga*) in the zebrafish brain. Aβ-induced toxicity markedly upregulates the expression of these genes, while Dose 4 of EGCG + PU significantly reduces their expression. Data are presented as mean ± SD ($n = 3$). Statistical significance was determined using an unpaired *t*-test. $^{***}p < 0.001$ and $^{****}p < 0.0001$. **Table S1:** Combination doses of EGCG and PU administered to zebrafish larvae. **Table S2:** Forward and reverse primers used in qRT-PCR. **Table S3:** Comprehensive statistical analysis of all experimental data. **Table S4:** Statistical analyses for behavioral assays. **Appendix S1:** Transparent Science Questionnaire for authors.

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