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Neuroprotective Potential of the Flavonoids Quercetin and Epicatechin in a *C. elegans* Tauopathy Model

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ABSTRACT

The prevalence of cognitive disorders such as Alzheimer's disease (AD) is increasing due to the global rise in longevity. The accumulation of amyloid β (Aβ) deposits and hyperphosphorylated Tau protein (p-Tau) are considered the main hallmarks of AD. A growing body of evidence suggests that the regular intake of flavonoid-rich foods could reduce the risk of developing AD or mitigate its progression. This study explores the potential of quercetin (Q) and epicatechin (EC) as effective molecules against AD-like pathology, using the *Caenorhabditis elegans* BR5270 strain, which expresses the pro-aggregant F3DK280 fragment of the human Tau protein. The results showed that after exposure to 150 μM of EC or Q, worms exhibited increased lifespan, improved chemotaxis, and delayed age-related decline in locomotion. To explore the molecular mechanisms involved, the expression of genes associated with the inhibition of p-Tau proteotoxicity were measured by RT-qPCR. It was found that Q and EC significantly increased the expression levels of autophagy-related genes and of a key gene for de novo synthesis of α-tubulin. EC and Q delay neurodegeneration in the *C. elegans* tauopathy model, suggesting their potential to reduce the risk of AD progression.

1 | Introduction

The rise in life expectancy has led to illnesses associated with advanced age, including Alzheimer's disease (AD), a prevalent cause of dementia in individuals aged over 65 [1]. Actually, age is the primary factor influencing the progression of AD, and its incidence is expected to increase [2]. AD is characterized by two main hallmarks: amyloid plaques formed from amyloid-β peptide accumulation and neurofibrillary tangles (NFTs) composed of hyperphosphorylated Tau protein (p-Tau) [3]. Specifically, Tau protein is a microtubule-associated protein (MAP) that regulates tubulin assembly and stabilizes microtubules, ensuring proper neuronal function and axonal transport. Any abnormality or change in the Tau protein such as hyperphosphorylation, results in microtubule depolymerization. Hyperphosphorylation of Tau modifies its conformation, enabling self-aggregation, which leads to paired helical filaments (PHF) and, finally, the formation of NFTs [4, 5].

Currently, there are only pharmacological medications that provide modest symptomatic relief for the pathology of AD. However, the risk of developing dementia depends on several

factors that can potentially be modified [6, 7]. Changing certain dietary behaviors and adopting healthier lifestyles could lead to a reduction in the incidence of dementia and AD [8, 9]. Several studies have pointed to plant polyphenols as compounds that may have protective roles in neurodegenerative disorders, owing to their antioxidant and anti-inflammatory capacities [10–12].

Polyphenols are a group of plant secondary metabolites, among which flavonoids, widely distributed in fruits and vegetables, appear to be particularly promising in reducing cognitive impairment [13]. Flavonoid-rich diets may help protect against cognitive decline being associated with a reduced lower risk of developing dementia [14, 15]. These bioactive molecules have been suggested to have a positive impact on AD pathogenesis by diminishing amyloid beta (Aβ) toxicity, tauopathy, and microgliosis [16–18]. Quercetin (Q) and epicatechin (EC), two of the major flavonoids present in the diet, have shown antioxidant, neuroprotective, and anti-inflammatory activities in many in vitro and in vivo studies [19–21]. For instance, quercetin intraperitoneal injection (25 mg/kg every 48 h, 3 months) in 21–24 month-old-triple transgenic AD model (3xTg-AD) mice significantly decreased PHF, βA1-40, and βA1-42 levels and improved the spatial learning and memory task performance of the mice [22]. Quercetin was also seen to attenuate endoplasmic reticulum (ER) stress that is involved in p-Tau neuronal injury, as well as to be able to inhibit phosphorylation of tau protein in SH-SY5Y cells exposed to okadaic acid, a compound known to lead to Alzheimer-like hyperphosphorylation and accumulation of p-tau [23]. In the same way, the administration of EC orally (~18 mg/day, 21 days) inhibited Tau phosphorylation and decreased the levels of oligomeric Tau in 4 and 6.5-month-old rTg4510 mice [24]. It was also demonstrated that epicatechin reduced the levels of phosphorylated relative protein in the hippocampus of aged C57BL6 male mice [25].

According to the literature, the expression of autophagy-related genes, mitochondrial function, cellular apoptotic processes, unfolded protein response (UPR) pathways, or tubulin synthesis can impact AD [26–28]. The modulation of these targets through flavonoid treatment could be a therapeutic strategy for tauopathies [29, 30]. However, it is still unclear whether and how Q and EC may impact Tau pathology at the molecular level, so further research is needed.

The nematode *Caenorhabditis elegans* is commonly used as a model organism to study a range of biological processes, including aging, and neurodegenerative pathologies. Its capacity for genetic manipulation enables the simulation of human disorders, making this nematode a useful tool for providing information about molecular mechanisms and potential treatments for AD [31, 32].

In the present study, the strain BR5270 of *C. elegans* is used as a tauopathy model to assess the capacity of EC and Q to reduce AD progression in vivo, as well as to explore the possible molecular mechanisms involved in the neuroprotection. This mutant strain integrates pan-neuronally the amyloidogenic F3DK280 fragment of human Tau, expressing pro-aggregant Tau species in the nervous system of *C. elegans* [33].

2 | Experimental Section

2.1 | Standards and Reagents

Quercetin dihydrate (Q) was acquired from Alfa Aesar (Karlsruhe, Germany). (-)-Epicatechin (EC), cholesterol, ampicillin sodium salt, nystatin, sodium azide, 5-Fluoro-2′-deoxyuridine (FUdR), agar, yeast extract, 2-mercaptoethanol, and RNaseZAP were purchased from Sigma-Aldrich (Madrid, Spain). Tryptone glucose yeast extract agar and peptone were from Fluka Analytical (Madrid, Spain), and tryptone medium from Merck (Darmstadt, Germany). Ethanol, dimethyl sulfoxide (DMSO), magnesium sulphate heptahydrate ($MgSO_4$), potassium dihydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (HK_2PO_4), and di-sodium hydrogen phosphate dodecahydrate (Na_2HPO_4) were from VWR Chemicals (Leuven, Belgium). Sodium chloride (NaCl) was from Labkem (Barcelona, Spain), and calcium chloride ($CaCl_2$) and isoamyl alcohol (IA) from Panreac (Barcelona, Spain). Type II water was obtained from Autwomatic Plus 1+2 purification system from Wasserlab (Navarra, Spain).

2.2 | Strains and Culture Conditions

Escherichia coli OP50, BR5270 (byIs161 [rab-3p::F3(delta)K280 + myo-2p::mCherry]) and BR5271 (byIs162 [rab-3p::F3(delta)K280 I277P I380P + myo-2p::mCherry]) strains were provided by the Caenorhabditis Genetics Center (CGC). Nematodes were grown at 20°C on standard nematode growth medium (NGM) plates with *E. coli* OP50 as a food source. To evaluate the neuroprotective role of flavonoids, synchronized worms were cultivated from eggs on OP50 seeded plates containing EC or Q 150 μM as treatment or 0.1% DMSO as control. The flavonoids were incorporated directly into the NGM while it was still in its liquid state, following established preparation protocols. In brief, after supplementation of the medium with the required nutrients, a volume of epicatechin or quercetin stock solution in DMSO was added at a ratio of 1:1000 relative to the total NGM volume to obtain a final flavonoid concentration of 150 μM. The same volume of DMSO was added to the control plates to maintain a consistent final DMSO concentration of 0.1% under all conditions. This way, the compounds were uniformly distributed throughout the solidified NGM, ensuring homogeneity across the entire plate rather than just on the surface. Synchronized populations were obtained by allowing gravid hermaphrodites to lay eggs on fresh plates for 6–8 h. M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1 M $MgSO_4$, H_2O to 1 L) was used for the collection and washing of nematodes from NGM-seeded plates.

2.3 | Lifespan Assay

Around 80 nematodes of the BR5270 strain, divided over four 3.5 cm plates, were used for each treatment. Animals were cultivated from eggs to death in the presence of 150 μM EC or Q, or vehicle (DMSO as control). Worms on the first day of adulthood were placed on plates with or without treatment (EC, Q, or DMSO) and FUdR 75 μM to prevent the development of progeny and the mixing of generations. The lifespan assay was carried out

TABLE 1 | Oligonucleotide primers used in RT-qPCR studies.

Gene	Forward	Reverse
pink-1	AGCATATCGAATCGCAAATGAGTTAG	TCGACCGTGGCGAGTTACAAG
pdr-1	AGCCACCGAGCGATTGATTGC	GTGGCATTTTGGGCATCTTCTTG
epg-8	GCGGTAAACGCTACACAAAGA	CCATCCGCTGAGATTCCTGG
hsp-70	CAAGACTTTGGAGCCGGTTG	GGAGCAGTTGAGGTCCTTCCC
xbp-1	TGGACACCTCGAACAAATCC	CTTGAACAAGACGCTGCATAAC
rpn-6.1	AATATTGGAAAAGCACCTGAAATGT	TTTGATGTGGAAGTGAAGTCATTGT
sut-1	CGAGCAGTGGAAGGAGAATAAT	CTCGCCTCCACTTCTTTCTC
sut-2	CAGGAGCATGCAGAAATCCA	GGCCGGCTTATGGTAGAAATAG
ced-13	AGCTCCCTGTTTATCACTTCTC	GTCAAACTCGTCGCACATAAC
ced-7	GAAGTGGCAGATTCCGAAAGA	TCAAGGGAGGACTGAGCTAATA
tbce-1	GGTGTCCATGCCTATGAGATT	CTGCAGACCTTCGTTCTACTT
act-1	CCAGGAATTGCTGATCGTATG	GGAGAGGGAAGCGAGGATAG

by transferring surviving worms onto fresh plates every 2–3 days. Worms were scored as dead if they did not respond to stimulation with a platinum wire pick. Animals that experienced internal hatching were not included in the analysis.

2.4 | Chemotaxis Assay

Chemotaxis to volatile compounds was measured as described previously by Kauffman et al. [34]. and Fang et al. [35]. Briefly, 1-day-old adults treated with or without each flavonoid from eggs were washed with M9 buffer 3 times to remove *E. coli* OP50. About 200 animals for each trial were scored during this assay. Washed worms were placed at the origin of a 9 cm NGM plate; spots of 1% isoamyl alcohol (IA), as attractant odorant (diluted in water) and ethanol (EtOH), as neutral compound, were deposited on two opposite points equidistant from the origin. After 1 h at room temperature, worms were counted on both IA and EtOH spots containing 1 μL sodium azide (NaN_3) 1 M to anesthetize the animals. The chemotaxis index (CI) was calculated using the formula [nIA − nEtOH] / [nIA + nEtOH + nOrigin].

2.5 | Locomotion Assay

This assay scored the number of body bends of the worms from the young to the old adult stage. Measurements in young, middle-aged, and old nematodes (1, 3, 5, 8, and 11 days of adulthood) were made by placing at least 25 worms for each condition on a non-seeded plate containing a drop of M9 buffer. Each worm was allowed to adapt for 30 s, after which the number of bends was hand-counted for 30 s [36].

2.6 | Gene Expression Assay

Synchronized BR5270 eggs were treated with or without EC or Q 150 μM. At the 3–4 day of adulthood, nematodes were collected and washed with M9 buffer, followed by isolation of total RNA using the RNAspin Mini Kit (product no. 25050071, Cytiva). The homogenization of nematodes was carried out in a FastPrep-24 5G at 5.5 m/s for 10 s 7 times, with stainless steel beads (2 mm).

For complementary DNA synthesis, SuperScript IV VILO Master Mix (Invitrogen) was used at 25°C for 10 min, 50°C for 10 min, and 85°C for 5 min. cDNA samples were then used for standard reverse transcription quantitative real-time PCR (RT-qPCR) to quantify mRNA levels of the genes *pink-1, pdr-1, epg-8, tbce-1, hsp-70, xbp-1, rpn-6.1, sut-1, sut-2, ced-13*, and *ced-7* using the primers described in Table 1. cDNA Specific Target Preamplification (STA reaction) was performed using the QIAGEN Multiplex PCR kit (Hilden, Alemania) by heating to 95°C for 15 min, cycling 20 times between 95°C for 15 s and 60°C for 4 min. A 1.25 μL aliquot of each cDNA was mixed with 0.5 μL of pooled DeltaGene primers (500 nM), 0.75 μL of nuclease-free water, and 2.5 μL of Multiplex Mix. Finally, exonuclease I was added to eliminate unincorporated primers at 37°C for 30 min and 85°C for 15 min. Preamplified cDNA was then used for RT-qPCR reactions completed in a BioMark HD System (Fluidigm, South San Francisco, CA, USA). A 2.25 μL aliquot of each amplified cDNA was mixed with 2.5 μL of 2X SsoFast EvaGreen Supermix with Low ROX (Bio-Rad) and with 0.25 μL of 20X DNA Binding Dye Sample Loading Reagent (Fluidigm). Individual RT-qPCR primer pairs (100 μM) were diluted 1:10 with Tris-EDTA (2.5 μL total volume) and mixed with 2.5 μL Assay Loading Reagent (Fluidigm). Each sample was individually pipetted onto the GE 96.96 Dynamic Array integrated fluidic circuit (IFC) (Fluidigm). Gene expression data were analyzed using the comparative $2^{-\Delta\Delta Ct}$ method with *act-1* housekeeping gene as internal control [37, 38]. Four to six independent experiments were performed.

2.7 | Statistical Analysis

GraphPad Prism 9.3.0 software (GraphPad Software, Boston, MA, USA) was used for statistical analyses. For the lifespan assay, *p* was determined by log-rank test (Kaplan-Meier) to evaluate the differences in survival curves between treated and control groups. For maximum lifespan, chemotaxis assay, and RT-qPCR assays, *p* was analyzed by one-way analysis of variance (ANOVA) Fisher's

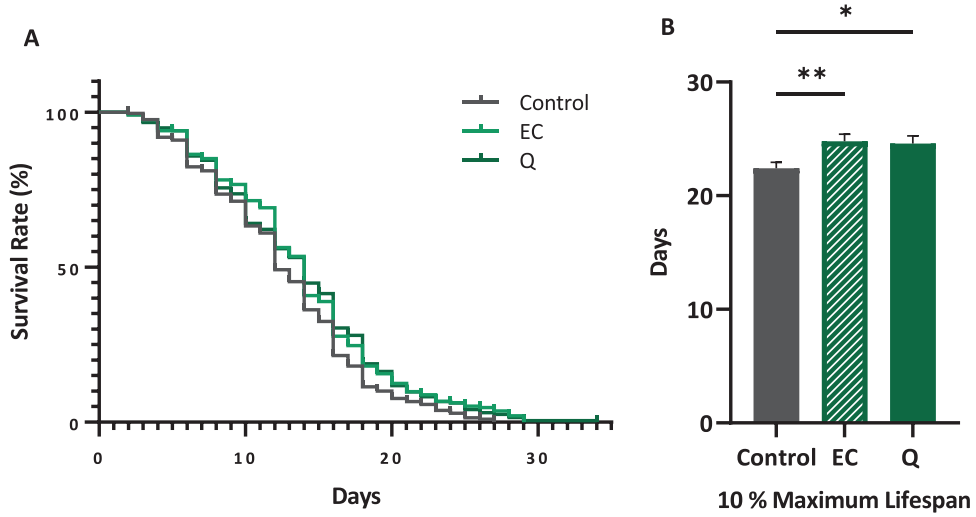

FIGURE 1 | **(A)** Kaplan-Meier representation of the survival rate of BR5270 nematodes grown in the presence of EC or Q 150 μM. **(B)** Average maximum life (10% longest survivors of each population) of BR5270 worms is represented as bars ± SEM. Data were analyzed by log-rank test and one-way ANOVA Fisher's LSD multiple-comparisons test from three independent assays ($n = 80$ per assay and treatment; $^{*}p < 0.05$, $^{**}p < 0.01$).

TABLE 2 | Influence of EC and Q on the lifespan of BR5270 *C. elegans*.

Treatment	Mean (days)	*p* vs. Control (log Rank)	Maximum 10% (days)	*p* vs. Control (ANOVA)
Control	12.7 ± 0.37		22.6 ± 0.51	
EC	13.9 ± 0.41	0.014	24.8 ± 0.62	0.008
Q	13.8 ± 0.42	0.018	24.6 ± 0.69	0.02

Note: Results are expressed as mean ± SEM. The differences were considered significant at $p < 0.05$.

LSD multiple-comparisons test. For the locomotion assay, two-way ANOVA followed by Fisher's LSD multiple-comparisons test was used for data analysis.

All experiments were performed at least three times independently. p value < 0.05 was considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

3 | Results

3.1 | Longevity Assays

Figure 1 and Table 2 show the results obtained in the lifespan assays conducted on BR5270 nematodes expressing the pro-aggregant F3ΔK280 fragment of human Tau protein. As shown, treatment of the worms with 150 μM of EC or Q increased mean lifespan by 9.47% and 8.60%, respectively, compared with the untreated group. Furthermore, EC and Q also induced an extension of the maximum lifespan (considered as the 10% longest survivors of each population) from 22.57 days to 24.79 and 24.58 days, respectively.

3.2 | Chemotaxis Assays

The effect of EC and Q on the cognitive ability of BR5270 nematodes was examined through their chemotaxis behavior toward isoamyl alcohol used as an odorant compared to ethanol (neutral compound). The anti-aggregant p-Tau BR5271 strain was used as a negative control. The chemotaxis index of the pro-aggregant BR5270 nematodes was reduced by 52% compared to the anti-aggregant strain (Figure 2). However, when treated (EC or Q 150 μM) and untreated BR5270 worms (positive control) were compared, the chemotactic index significantly improved by 19.4% and 22.9%, respectively (Figure 2). This indicates that treated BR5270 nematodes can better link the olfactory signal to the odorant, suggesting that these flavonoids might reduce the pathology associated with the aggregation of hyperphosphorylated Tau.

3.3 | Locomotion Assays

The locomotor capacity of BR5270 strain was assessed by counting the number of body bends per 30 s for each of the worms present in a drop of M9 buffer deposited on a non-seeded NGM plate. Treatment with EC or Q resulted in a significant improvement in the locomotion of the young (3-day old), middle-aged (5-day old), and old (8- and 11-day old) worms (Figure 3). This result reveals a delay in the age-related decline in mobility in the groups of nematodes treated with the flavonoids, suggesting that the lifespan extension mediated by flavonoids could also be associated with an enhanced healthspan.

3.4 | Gene Expression Assays

To better understand the molecular mechanisms underlying the improved healthspan induced by EC and Q, the expression levels

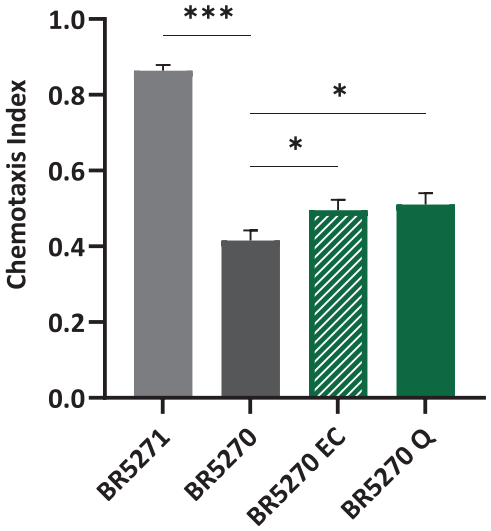

FIGURE 2 | Effect of 150 μM EC or Q treatment on chemotaxis behavior of Tau-expressing BR5270 worms. Anti-aggregant BR5271 and pro-aggregant BR5270 controls were cultured in the presence of DMSO (vehicle). The bars represent the chemotaxis index toward isoamyl alcohol. Data are represented as mean± SEM and analyzed by one-way ANOVA Fisher's LSD multiple-comparisons test. Five assays were performed independently ($n = 200$ nematodes per group and trial). *$p <$ 0.05, ***$p < 0.001$.

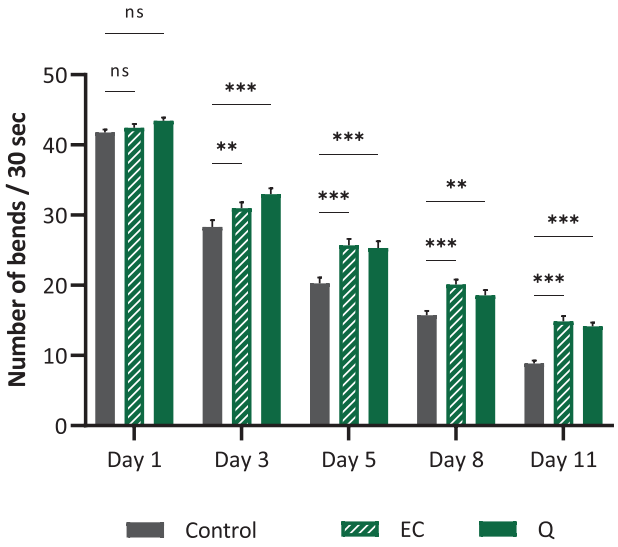

FIGURE 3 | Representation of the average of body bends at different ages of BR5270 worms treated with EC or Q 150 μM. Assays were performed in triplicate ($n = 25$ per condition and independent trial) and analyzed by two-way ANOVA Fisher's LSD multiple-comparisons test. Data are represented as mean ± SEM. * $p < 0.05$, **$p < 0.01$, and *** $p < 0.001$.

of genes that could be potential targets associated with the inhibition of p-Tau proteotoxicity were measured. In particular, genes that could act as molecular modulators of mitophagy, autophagy, apoptosis, UPR processes, Tau suppression, or tubulin synthesis were explored, since these mechanisms are associated with AD pathology [39–41].

It was found that both Q and EC significantly increased the expression levels of different mitophagy (*pdr-1* and *pink-1*) and autophagy (*epg-8*) related genes (Figure 4A), while the expression of genes related to unfolded protein response (*hsp-70*, *xbp-1*, and *rpn-6.1*) were decreased (Figure 4B). However, no effect was observed on apoptosis (*ced-7* and *ced-13*) and Tau suppression (*sut-1* and *sut-2*) related genes (Figure 4C,D). These results indicated that EC and Q induce autophagy and mitophagy, improving the health status in the *C. elegans* tauopathy model. The decreased expression of mRNA levels of UPR-related genes might be due to the existence of a lower amount of misfolded Tau protein, possibly as a result of the protection produced, although further assays are needed to verify it. Furthermore, the upregulated expression of *tbce-1* by EC, a key gene for de novo synthesis of α-tubulin, might prevent the abnormal phenotype of *C. elegans* tauopathy model.

4 | Discussion

AD is the most common type of dementia worldwide, and its prevalence is expected to continue increasing [42]. One characteristic of AD is the presence of neurofibrillary tangles, which are formed by misfolded and hyperphosphorylated Tau protein. This is a common feature in the pathogenesis of AD and other tauopathies [43]. In this study, the protective effect of the flavonoids epicatechin and quercetin against p-Tau pathogenesis was evaluated using the transgenic BR5270 strain of *C. elegans* that expresses the hyperphosphorylated human Tau protein.

In previous studies on oxidative stress and aging models carried out by our group, the most effective dose of EC and Q in the culture media was determined to be 200 μM, at which significant lifespan extension and enhanced resistance to thermal and oxidative stress in the wild-type N2 strain of *C. elegans* were observed [44–47]. However, when we shifted our focus to neurodegeneration models, specifically the CL4176 strain, which expresses Aβ-peptide in muscle cells, among the different doses tested of EC and Q (50, 100, 150, and 200 μM), it was found that only 150 μM significantly delayed paralysis [48]. Furthermore, as in most studies on flavonoids conducted by our and other research groups, a hormetic response was pointed out, with hardly any or no effects at low doses, while negative effects become evident after a certain threshold. This biphasic response has been widely documented in the literature [49]. Based on these findings, 150 μM was selected as the working concentration for this study. The obtained results further support this choice, as in the assayed *C. elegans* tauopathy model, that is, the BR5270 strain, both EC- and Q-treated animals showed significantly enhanced mean lifespan and maximum survival (Figure 1), as well as an improvement in the chemotaxis index (Figure 2) and the prevention of the locomotion decline (Figure 3). These outcomes suggest that EC and Q could mitigate the toxicity of p-Tau aggregates, leading to an improvement in the healthspan in *C. elegans* tauopathy model. Studies carried out by other authors using a different tauopathy worm model (strain BR5706) also reported improved swimming speed when treated with plant extracts rich in polyphenols, specifically Mexican marigold extract (*Tagetes erecta*) and olive leaf extract enriched in oleuropein [50, 51].

Given the significant delay in the age-related decline in mobility observed in the locomotion assay from the third day of adulthood

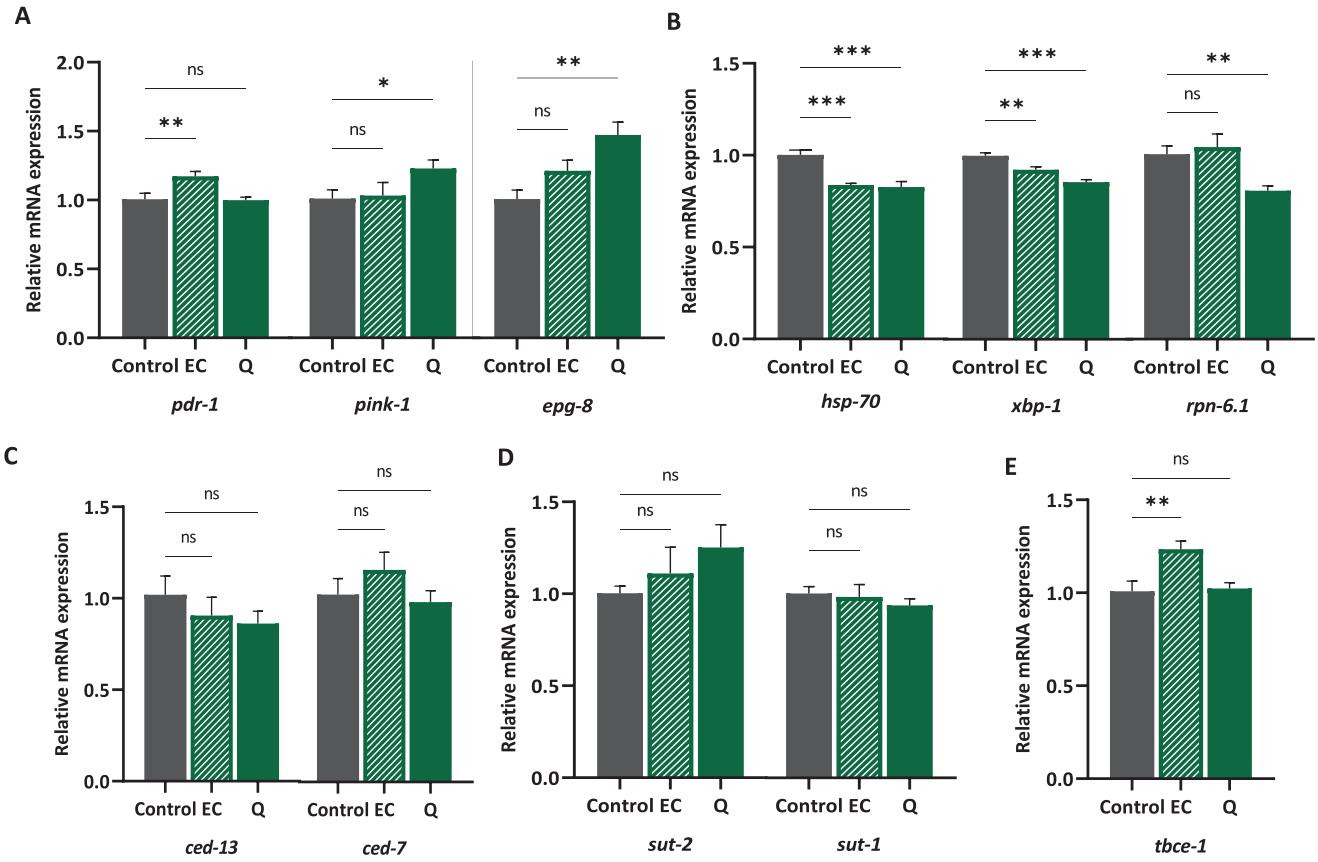

FIGURE 4 | Effects of EC and Q on the relative mRNA levels of *pink-1*, *pdr-1*, *epg-8, tbce-1*, *hsp-70*, *xbp-1*, *rpn-6.1*, *sut-1*, *sut-2*, *ced-13*, and *ced-7* genes in *C. elegans* BR5270; *act-1* was used as internal control. **(A)** Mitophagy and autophagy related genes. **(B)** Unfolded protein response related genes. **(C)** Apoptosis related genes. **(D)** Tau suppression related genes. **(E)** Tubulin synthesis related gene. Four to six independent assays were performed in each case. Data were analyzed by one-way ANOVA Fisher's LSD multiple-comparisons test. The results are represented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

(Figure 3), gene expression analyses were performed at this age (3–4-day-old nematodes) in order to see if there were changes in the mRNA levels of worms treated with the flavonoids. It was found that both EC and Q were able to induce mitophagy and autophagy pathways. In particular, overexpression of *pink-1* was found in nematodes treated with Q, while expression of *pdr-1* was enhanced in the group treated with EC (Figure 4A). Both *pink-1* (ortholog of human PINK1) and *pdr-1* (ortholog of human Parkin RBR E3 ubiquitin protein ligase) are mitophagy regulators that promote the clearance of damaged mitochondria [52]. Ubiquitination-dependent PINK1/Parkin-mediated mitophagy is essential for mitochondrial homeostasis, and its impairment is linked to synaptic dysfunction in AD. Under stress conditions, PINK1 becomes autophosphorylated and stabilized on the mitochondrial membrane, where it phosphorylates Parkin. This activation of Parkin's E3 ubiquitin ligase activity leads to the formation of phosphorylated poly-ubiquitin chains, which are recognized by autophagy receptors via binding to LC3, thereby facilitating the sequestration of damaged mitochondria into autophagosomes for subsequent lysosomal degradation [53]. Xie et al. [54], using nematode and rodent models of tauopathy, also identified mitophagy as the underlying molecular mechanism of neuroprotection in the anti-AD potential observed for kaempferol, a flavonol present in tea, broccoli, and kale. This reinforces the notion that mitophagy inducers might improve defective mitophagy pathways that drive Tau pathologies and AD progression, advancing the idea that research on bioactive compounds as inducers of mitophagy could have positive implications for brain health.

It was also found that *epg-8*, the homologue of the human ATG14 autophagy-related gene, was upregulated in Q-treated nematodes (Figure 4A). This gene plays an essential role in the autophagy pathway in *C. elegans*, a process that participates in the quality control system to eliminate aggregate-prone proteins [55]. ATG14 is crucial for the biogenesis of autophagosomes, double-membraned vesicles that engulf cytoplasmic components such as damaged organelles or misfolded proteins. During the autophagosome formation, ULK1/2 complex promotes the activation of the class III phosphatidylinositol 3-kinase complex I (PI3K complex). ATG14 is a specific subunit of the PI3K complex that generates the lipid phosphatidylinositol 3-phosphate (PI(3)P) on phagophore membranes, thereby promoting phagophore expansion. It has been reported that ATG14 is essential for PINK1/Parkin-mediated mitophagy and that loss of ATG14 blocks activation of the PI3K complex due to the failure in recruiting PI(3)P to mitochondria [56–57]. Zubčić et al. [58] examined the effects of quercetin on the activation of the PI3K signaling pathway on P19 neurons exposed to $CuSO_4$-induced oxidative damage and reported that quercetin promoted neuronal survival

by reducing ROS production and activating the PI3K cascade. The signal transduction through the PI3K complex may be considered a potential therapeutic target in neurodegeneration. In accordance with the literature and as observed in the present study, the upregulation of both *pink-1* and *epg-8* in Q-treated nematodes suggests the potential of quercetin to modulate autophagy pathways. The observed stimulation of *pink-1*, *pdr-1*, and *epg-8* expression by Q and EC may help maintain proper mitophagy and autophagy, which could contribute to the protective effects observed in the performed assays. By contrast, in a previous study of our group, the relative mRNA expression of *epg-8* was significantly decreased in the CL4176 strain (Aβ model) treated with EC [48]. A similar observation was made by Lin et al. [59], which found a delay in the Aβ-induced paralysis in the CL4176 strain using RNAi to decrease the expression of *epg-8*. The opposite results obtained among assays might be associated with the existence of different molecular mechanisms underlying AD pathology in the Aβ and Tau models.

The ER is an organelle specialized in the regulation of protein homeostasis, including the protein folding and assembly. However, when ER homeostasis is disrupted (ER stress), unfolded or misfolded proteins accumulate within the ER lumen, thereby activating the UPR. ER stress can be induced by factors such as deficient autophagy, impaired proteasomal degradation, or increased ROS levels, among others. The UPR helps the cell to adapt to ER stress through mechanisms that include the nuclear translocation of XBP1, which enhances protein folding and promotes degradation pathways [60]. The expression of UPR-related genes *hsp-70*, *xbp-1*, and *rpn-6.1* was downregulated in the worms treated with EC and Q (Figure 4B). Similarly, it was observed that the treatment with EC also reduced the expression of *hsp-70* in the transgenic strain CL4176 model of Aβ [48]. Both UPR and autophagy play important roles in preventing and mitigating AD [61]. UPR is expected to enhance the autophagy process, which efficiently eliminates large protein aggregates and damaged organelles through the lysosomal pathway [62]. Thus, the observed downregulation of UPR-related genes might be related to the stimulation of mitophagy and autophagy with the subsequent reduction in Tau aggregates, making removal unnecessary. The HSP70 class of chaperones typically identifies client proteins during the initial stages of folding. If the client protein cannot fold correctly, HSP70 associates with additional co-chaperones to facilitate the degradation of the misfolded protein [63]. The fact that the relative mRNA level of *hsp-70* was significantly lower in treated nematodes compared with the control group could suggest that EC and Q protect against the formation of p-Tau aggregates. Similar reasoning can be applied in the cases of genes *xbp-1* and *rpn-6.1*. The key proteasomal factor, *rpn-6.1*, and one of the main UPR transcription factors, *xbp-1*, were identified as target genes in *C. elegans* to correct deficiencies in protein homeostasis characteristic of neurodegenerative diseases such as AD [64, 65]. Additional analysis showed that the BR5270 tauopathy model overexpressed these UPR-related genes compared to the wild-type N2 strain, suggesting that the treatment with EC or Q may restore expression levels closer to the undamaged situation (Figure S1). Thus, the observation of lower relative mRNA levels of *hsp-70*, *xbp-1*, and *rpn-6.1* could once more indicate the capability of EC and Q to diminish the toxicity associated with Tau protein hyperphosphorylation and p-Tau accumulation. It seems that the accumulation of misfolded proteins in neurons directly contributes to ER stress in neurodegenerative diseases such as AD, and although the important role of UPR pathways in preventing AD has been demonstrated, the precise mechanisms that trigger UPR signaling during the disease pathogenesis remain unclear [60]. Therefore, investigating the potential of EC or Q to alleviate ER stress could represent a promising strategy for reducing neurodegeneration.

No effects were observed in the expression of apoptosis related genes *ced-7* and *ced-13* (Figure 4C). Schumacher et al. [66] described *ced-13* as an apoptosis promoter in *C. elegans*. That gene is promoted after the *cep-1* activation cascade (the homolog of the p53 gene in mammals), which mediates apoptosis and meiotic chromosome separation under DNA damage condition [67, 68]. In turn, *ced-7* is an ATP-binding cassette (ABC) transporter that facilitates the recognition of apoptotic cells by the *ced-1* phagocyte receptor by promoting the generation of extracellular phosphatidylserine (PS) vesicles on the membrane [69]. However, it seems that the apoptotic process is not the major mechanism of cell death in hyperphosphorylated Tau protein pathologies [70, 71]. This could be the reason why no significant differences have been found in the relative mRNA levels of the apoptosis related genes between treated and untreated nematodes. Nonetheless, the role of flavonoids as apoptosis modulators has been reported in both in vitro and in vivo assays, not only in neurodegenerative diseases but also in other pathologies like cancer, taking part in the initiation of the cell death process [72, 73]. In fact, previous studies by our group showed a significant decrease in the expression of *ced-7* in the Aβ-induced CL4176 strain of *C. elegans* treated with EC, suggesting that this flavonoid could act as a regulator of the apoptosis pathway [48]. Nevertheless, Sierksma et al. [74] showed that the transcriptional response to cell death pathways in Aβ and Tau pathology models can be differentially expressed, which could explain the results obtained herein.

Sut-1 and *sut-2* have been defined as tau suppressor-associated genes in *C. elegans* models of tauopathies. Both *sut-1* and *sut-2* have been identified as RNA-binding proteins that reside in nuclear speckles and are required for tau pathology-driven neurodegeneration in *C. elegans*. The loss of function of *sut-1* and *sut-2* prevents tau-mediated neurodegeneration and protects against progressive motor incoordination [75–77]. On the other hand, *sut-2* overexpression aggravates Tau accumulation, making *C. elegans* neurons more vulnerable to Tau toxicity [78]. The mammalian ortholog of *sut-2*, *MSUT2*, has also been demonstrated to regulate p-Tau toxicity in mouse models of tauopathy [79]. In the present study, no effects were found on the mRNA expression levels of *sut-1* and *sut-2* in treated nematodes compared with the control group (Figure 4D). However, as far as our understanding goes, this work is the first study that investigates the influence of flavonoids on genes related to Tau suppression. Therefore, it would be interesting to expand the knowledge about the Tau suppression cascade upstream or downstream of SUT in further research.

Finally, an increase in the mRNA expression levels of *tcbe-1*, homologous to the human tubulin-folding cofactor E required for the synthesis of α-tubulin [80], was observed in the group of worms treated with EC (Figure 4E). This observation aligns with the results by Esser et al. [81], who, in a double-blind placebo-controlled clinical trial, demonstrated that epicatechin

supplementation in humans upregulated gene sets implicated in transcription and tubulin folding. In addition, a correlation between α-tubulin and mitophagy has been reported, with α-tubulin playing a crucial role in neuronal physiology through the regulation of microtubule assembly and mitochondrial distribution [82]. This observation is also consistent with our findings, as the *pdr-1* gene, the Parkin-induced mitophagy homologue, was also upregulated in the EC-treated group. Miyasaka et al. [40] demonstrated that knockdown of *tbce-1* by feeding RNAi directly intensified tau-induced neuronal dysfunction and reduced the expression of α-tubulin in a *C. elegans* tauopathy model. Microtubules are composed of α- and β-tubulin heterodimers, and AD patients with hyperphosphorylated tau proteins have low tubulin-binding activity [83]. Therefore, the stimulation of the tubulin synthesis by flavonoids can be a potential strategy to reduce the neuronal toxicity induced by p-Tau aggregation.

The differential effects observed in mRNA expression profiles between EC and Q can be attributed to differences in their chemical structure, making it likely that each flavonoid interacts with specific molecular targets, although the final reasons for these differential mechanisms remain unknown. A similar observation was made by Xie et al. [54], who found that kaempferol and the stilbenoid rhapontigenin (present in plants such as broccoli and grapes) increased the mRNA levels of different mitophagy/autophagy-related genes in the P301L strain of *C. elegans* (another p-Tau model). In this context, the present study could serve as a basis for further research to improve the understanding of flavonoid active sites and their potential impact on health by using virtual tools to predict the most stable compound-receptor interactions.

It is, however, necessary to take into account that *C. elegans* presents certain limitations that should be considered when trying to extrapolate results to humans. Thus, contrary to mammals, *C. elegans* lacks anatomical features such as a circulatory system, a blood-brain barrier, liver metabolism, kidney-based blood filtration, or DNA methylation. These elements may be crucial for tissue-specific signaling pathways or epigenetic effects [84]. In addition, while the present study employs the strain BR5270 of *C. elegans*, which expresses the hyperphosphorylated tau protein, one of the main hallmarks of AD, it is known that in human AD both oligomeric forms of Aβ and p-Tau act synergistically to induce synaptic dysfunction. Therefore, future studies could benefit from using a *C. elegans* double mutant (co-expressing Aβ and p-Tau) to further explore the effects and molecular mechanisms of flavonoids to protect against the neurotoxicity. A further limitation is that, although the dosage administered to the NGM is controlled with a certain precision, determining the exact concentration of the compound that is incorporated by the nematodes remains challenging. Previous studies carried out in our group confirmed the incorporation and biotransformation of flavonoids in wild-type nematodes cultured with quercetin-3-O-glucoside or quercetin, demonstrating a positive correlation between the concentrations in the medium and the levels of total metabolites in the animals determined via HPLC-DAD-ESI/MS analysis, indicating concentration-dependent uptake and effects [85].

Nevertheless, while more complex organisms may be required to model a specific process, *C. elegans* provides a link between in vitro studies and mammalian experiments that is essential for the preliminary screening and target characterization of the neuroprotective effects of bioactive compounds. Furthermore, *C. elegans* is employed as a valuable model organism capable of replicating certain aspects of human diseases. This facilitates understanding of underlying molecular mechanisms and the development of novel therapeutic strategies in the context of neurodegenerative disorders. For instance, the present study has observed the stimulation of mitophagy and autophagy processes as one of the possible molecular targets following treatment with EC or Q (Figure 4A). Additionally, *C. elegans* offers a low cost and faster investigation before investing in more expensive models [86].

5 | Conclusions

The BR5270 strain of *C. elegans*, expressing the F3DK280 fragment of human Tau, was used to evaluate the in vivo effects of two abundant dietary flavonoids (EC and Q). Both molecules were demonstrated to induce significant extension of lifespan, enhancement of chemotaxis responsiveness to odorant compounds, and attenuation of age-related decline in locomotor function in this transgenic strain. It was also found that Q or EC significantly increased the expression levels of genes associated with mitophagy, autophagy, and de novo synthesis of α-tubulin, while decreasing that of genes involved in the UPR. These findings pointed to Q and EC as potential neuroprotective agents against tauopathy in the BR5270 *C. elegans* model. However, differences in gene expression profiles between Q and EC-treated groups also suggest that different molecular mechanisms could be involved in countering p-Tau protein toxicity depending on the flavonoid. Taken together, the results obtained suggest that EC and Q could contribute to suppressing the toxicity of hyperphosphorylated Tau protein, one of the key hallmarks of AD. However, further investigation is still needed to confirm the promising potential of EC and Q as a dietary intervention strategy for mitigating Tau-associated diseases.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.