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Predicting structural features of selected flavonoids responsible for neuroprotection in a *Drosophila* model of Parkinson's disease

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Abstract

Nature-derived bioactive compounds have emerged as promising candidates for the prevention and treatment of diverse chronic illnesses, including neurodegenerative diseases. However, the exact molecular mechanisms underlying their neuroprotective effects remain unclear. Most studies focus solely on the antioxidant activities of natural products which translate to poor outcome in clinical trials. Current therapies against neurodegeneration only provide symptomatic relief, thereby underscoring the need for novel strategies to combat disease onset and progression. We have employed an environmental toxin-induced *Drosophila* Parkinson's disease (PD) model as

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Author contributions

U.M. and L.C. conceived research idea. U.M. designed and performed experiments and analyzed data. J.C. and M.M.O assisted in survival and mobility assays. U.M. and L.C. wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

Contribution to the field statement

Parkinson's disease (PD) and other age-associated chronic disorders affect millions worldwide and have emerged as one of the greatest medical challenges of the 21st century. Despite years of extensive research, the available treatment options are limited. Recent studies have highlighted the role of plant-derived natural compounds in the prevention and treatment of neurodegenerative diseases. However, the exact molecular mechanisms underlying their neuroprotective effects remain unclear. In this study, we have identified a specific group of flavonoids known as flavones displaying protection against environmental toxin-induced neurodegenerative phenotypes in a *Drosophila* model. The flavones contain specific structural features, including the presence of α,β-unsaturated carbonyl group, and the lack of a functional group substitution at the C3 position in the flavonoid skeleton. Moreover, flavones-mediated induction of antioxidant properties through nuclear factor erythroid 2–related factor 2 (Nrf2) is not solely responsible for neuroprotection but also requires regulation of innate immune IMD pathways involving NFκB. In this study, we have examined specific structural features of selected flavonoids that provide neuroprotection in an *in vivo* model against environmental toxin-induced PD pathogenesis that can be further explored for novel therapeutic interventions.

Declaration of interests

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

an inexpensive *in vivo* screening platform to explore the neuroprotective potential of selected dietary flavonoids. We have identified a specific group of flavonoids known as flavones displaying protection against paraquat (PQ)-induced neurodegenerative phenotypes involving reduced survival, mobility defects, and enhanced oxidative stress. Interestingly, the other groups of investigated flavonoids, namely, the flavonones and flavonols failed to provide protection indicating a requirement of specific structural features that confer protection against PQ-mediated neurotoxicity in *Drosophila*. Based on our screen, the neuroprotective flavones lack a functional group substitution at the C3 and contain α,β-unsaturated carbonyl group. Furthermore, flavones-mediated neuroprotection is not solely dependent on antioxidant properties through nuclear factor erythroid 2–related factor 2 (Nrf2) but also requires regulation of the immune deficiency (IMD) pathway involving NFκB and the negative regulator poor Imd response upon knock-in (Pirk). Our data have identified specific structural features of selected flavonoids that provide neuroprotection against environmental toxin-induced PD pathogenesis that can be explored for novel therapeutic interventions.

Keywords

flavonoid; Parkinson's disease; *Drosophila* ; neuroinflammation; neuroprotection

Introduction

The incidence of chronic metabolic non-communicable ailments such as neurodegenerative diseases (ND) is rising and has become one of the most challenging global threats and economic burdens of the 21st century (1). Currently, there are no treatment options available to cure NDs, and the approved therapies only manage disease symptoms (2). Parkinson's disease (PD) is the second most prevalent ND after Alzheimer's disease (AD). The interaction of genetic and environmental factors has been linked to the pathogenesis of PD. However, much of the previous research has almost solely focused on targeting the pathophysiology of protein aggregation using PD models, which do not permit the study of the pre-symptomatic therapeutic interventions that delay or prevent neurodegenerations (3). It also remains unclear if compounds effective in counteracting toxic effects of α-synuclein accumulation in dopaminergic neurons can also prevent neurodegeneration in sporadic PD. Identification of therapeutic agents conferring protection from neuronal degeneration and death would lead to the development of preventive interventions for idiopathic and familial PD. Numerous factors have been linked with the increased risk of developing idiopathic ND (4). For example, exposure to environmental toxins, such as pesticides or certain microbial metabolites, induces neurodegeneration in multiple model organisms (5, 6). Paraquat (PQ), a previously widely used herbicide, has been linked to pathophysiological changes associated with Parkinson's and neurodegeneration (7–15).

A score of epidemiological studies strongly points to the fact that lifestyle modifications such as regular physical activity and/or following certain types of diet, such as the Mediterranean diet, have been linked to lowering the risk of AD or PD (16–24). Numerous recent studies have also linked a diet rich in flavonoids to the decreased risk of developing AD or PD (25–27). Flavonoids are commonly present in plants and have received

massive attention in the scientific literature, including the investigation of neuroprotective properties (1, 2). However, the exact molecular mechanism of action of flavonoids providing neuroprotection remains elusive (1, 28). For the past fifty years, the predominant hypothesis in the field promulgated the antioxidant activities of polyphenols as majorly responsible for their beneficial effects (1). However, numerous *in vivo* studies, including clinical trials disproved the direct antioxidant activity of polyphenols (1). Additionally, the upregulation of the antioxidant response element signaling pathway involving the Nuclear factor erythroid 2-related factor 2 (Nrf2) has also been shown as insufficient to provide neuroprotection in certain animal models of NDs (29).

Despite many years of research on flavonoids, none of the investigated compounds has been successfully registered as a drug (2, 28). This has led to the classification of these compounds as invalid/improbable metabolic panaceas (IMPs) lacking drug-like characteristics, such as an exquisitely specific interaction with a definable receptor (2, 28, 30). Additionally, flavonoids have often been classified as pan-assay interference compounds (PAINs) due to their ability to interact with numerous targets involving *in vitro* assays (31). NDs are considered multifactorial diseases caused by complex interactions between genetic and environmental factors (32, 33). Single compounds displaying pleiotropic effects, such as flavonoids, have been suggested to be more effective in the prevention or treatment of multifactorial diseases (2). Seigler et al. proposed that flavonoids and coumarins may play an essential role in human nutrition, following the concept of vitamin P by Szent-Györgyi (28).

Previous structure-activity relationship studies focused majorly on the antioxidant effects of flavonoids in *in vitro* assays (34, 35). There is only a very limited number of published studies focusing on understanding how the structure of flavonoids impacts their biological effects in the context of a living organism. Many biologically active flavonoids contain electrophilic α,β-unsaturated carbonyl group similarly to the oxidation products of essential omega-3 fatty acids (2). The presence of a highly reactive electrophilic group in flavonoids used at supraphysiological concentrations, may be responsible for the false positive results reported in the literature. Therefore, it is of crucial importance to investigate the biological activity of flavonoids, including interactions with multiple targets, in the context of a living organism at physiologically relevant concentrations.

We have employed a well-established paraquat-induced *Drosophila* model to screen selected flavonoids against PD symptoms in the context of a living organism (36). This fly model mimics the clinical hallmarks of PD in humans, including motor deficits, increased neuroinflammation, and oxidative stress and serves as an inexpensive platform to screen plant-derived flavonoids through diet (2, 37). The flavonoids were selected based on their structural similarity to gardenin A, which we previously identified as neuroprotective in this *Drosophila* model of PD (29). We investigated the neuroprotective potential of a group of selected flavonoids in our attempt to identify the structural elements linked to neuroprotection and decipher the possible molecular mechanism of action of these compounds.

Materials and methods

Drosophila culture and stocks

The wild-type strain *Canton S* was purchased from the Bloomington Drosophila Stock Center at Indiana University and used in all experiments. The fly stocks were raised on standard Nutri-fly BF medium (Genesee Scientific) containing cornmeal, corn syrup, yeast, and agar at 25 °C under a 12 h of light and 12 h of darkness cycle. Age-matched male flies three to five days old post-eclosion were used for all assays.

Chemicals

The flavonoids used in this study and the following chemicals were purchased from Sigma-Aldrich: Tangeretin Catalog# T8951 (>95% purity), Nobiletin N1538 (>97% purity), Apigenin 42251 (>95%), Sinensetin SML1787 (>98%), Luteolin 72511, Fisetin PHL82542, Eupatilin SML1689 (>98%), Naringenin 52186 (>95%), Hesperetin PHL89222, Kaempferol 96353, Quercetin Q4951 (>95%), Myricetin 70050 (>96%), Naringin 71162 (95%), Hesperidin H5254, Sucrose, and paraquat (methyl viologen dichloride hydrate). TRIzol reagent and the primers were obtained from Thermo Fisher Scientific. The Direct-zol RNA microprep kits for total RNA isolation were purchased from Zymo Research, and the iQ SYBR Green Supermix was from Biorad.

Drosophila feeding regimen

Adult male flies aged 3–5 days post-eclosion were maintained in vials (10/vial) and fed daily on filter paper saturated with specified concentrations of paraquat with or without flavonoids in 2.5% sucrose or with 2.5% sucrose only. Mortality was monitored daily upon PQ exposure (Day 0) in both pre- and co-feeding modes until all flies were dead. Survival assays were performed with at least ten independent biological replicates of 10 males each for different feeding conditions. The following concentrations of flavonoids were used for the feeding assays: 10 μM nobiletin, 5 μM sinensetin, 10 μM apigenin, 5 μM eupatilin, 100 μM luteolin, 20 μM tangeretin, 25 μM fisetin, 20 μM kaempferol, 25 μM myricetin, 25 μM quercetin, 20 μM hesperitin, 20 μM hesperidin, 20 μM naringenin, 25 μM naringin.

CantonS flies were fed either 2.5% sucrose or specified concentrations of different flavonoids containing the blue food dye (1% FD&C Blue#1) to measure food intake quantitatively. The flies were washed and homogenized in 1X PBS containing 1% Triton X-100 followed by centrifugation and the supernatants were measured at OD 630 nm. Data were analyzed using the two-tailed *Student's* t-test and error bars indicate the standard deviation.

Mobility assay

Negative geotaxis assays were performed to detect the mobility defects in adult male flies from different feeding groups. Ten flies per feeding condition were placed in an empty plastic vial and gently tapped to the bottom. The percentage of flies that crossed a line 5 cm from the bottom of the vial in 20 sec was recorded to compare different feeding conditions. Climbing assessment was performed at 48 h time point post PQ exposure in both pre- and co-feeding experiments. Five independent biological replicates were assayed three

times at 5 mins intervals and the average percentages were calculated and plotted. Statistical significance between different feeding conditions was calculated using one-way analysis of variance (ANOVA) for $P < 0.05$.

Quantitative real time RT-PCR

Total RNA was extracted from the heads of 25–30 adult male flies using TRIzol Reagent and Direct-zol RNA microprep kit (Zymo Research) followed by cDNA preparation from 0.5–1 μg total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The resulting cDNA samples were diluted, and specific transcripts were detected using the iQ SYBR Green Supermix (Biorad) in a StepOnePlus Real time PCR System according to the manufacturer's protocols. The relative levels of specified transcripts were calculated using the ΔΔCt method, and the results were normalized based on the expression of *ribosomal protein L32* (*RpL32/RP49*) as the endogenous control within the same experimental setting. At least three independent biological replicates were performed in triplicates for the specified feeding conditions and compared to the control sucrose-fed flies (assigned a value of 1) for data analysis. The primer sequences are: *rp49*(F): 5' AGA AGC GCA AGG AGA TTG TC 3'; *rp49*(R): 5' ATG GTG CTG CTA TCC CAA TC 3'; *relish*(F): 5' GGC ATC ATA CAC ACC GCC AAG AAG 3; *relish*(R): 5' GTA GCT GTT TGT GGG ACA ACT CGC 3; *cncC*(F): 5' GAG GTG GAA ATC GGA GAT GA 3'; *cncC*(R): 5' CTG CTT GTA GAG CAC CTC AGC 3'; *gstD1*(F): 5' GGC CGC CTT CGA GTT CCT GA 3'; *gstD1*(R): 5' CGG TTG CCA CCA GGG CAA TG 3'; *pirk*(F): 5' CGA TGA CGA GTG CTC CAC 3'; *pirk*(R): 5' TGC TGC CCA GGT AGA TCC 3'.

Statistical analysis

Data were analyzed using the GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA). Statistical significances of gene expression between different groups were determined using a non-parametric Mann Whitney U-test. For survival and mobility assays, log-rank test and one-way analysis of variance (ANOVA) were used respectively, to compare the differences between specified feeding groups. Results are expressed as mean ± SEM, and $p < 0.05$ was considered statistically significant. Details of the specific analysis are mentioned in the figure legends.

Results

A specific group of flavonoids known as flavones exerts protection against paraquat-induced toxicity in a *Drosophila* PD model.

We employed an environmental toxin-induced *Drosophila* PD model as a screening platform to identify flavonoids with neuroprotective potential. Exposure to the herbicide paraquat (PQ) has been previously shown to result in reduced survival, impaired climbing abilities, and dopaminergic neuron loss in the wild-type *Drosophila* strain, *Canton S* (36, 38–40). Adult male flies were used in this study due to their higher sensitivity to PQ toxicity leading to PD phenotypes earlier than female flies, thereby mimicking the higher prevalence of PD in human male patients (38). Adult male *Canton S* wild type flies were fed 5 mM PQ dissolved in 2.5% sucrose solution on filter paper to induce parkinsonian symptoms, including reduced survival and enhanced mobility defects. This *Drosophila* PD model was

used to screen three groups of flavonoids, namely flavones, flavonones and flavonols as listed in Table 1. Flavonoids consist of different groups of polyphenolic compounds that are ubiquitously present in plants and are one of the most common groups of phytochemicals found in the human diet (41). Flavonoids are divided into different subgroups depending on the presence of methoxy or hydroxy groups, the degree of unsaturation and the oxidation status of the C-ring (41, 42). Flavones have a double bond between positions 2 and 3 and a ketone in the position 4 of the C-ring. Some flavones tested in this model are polymethoxylated. Flavonols have a hydroxyl group in the position 3 of the C-ring and they are diverse in methylation and hydroxylation patterns. On the other hand, flavonones have the C-ring saturated between positions 2 and 3 as compared to flavones (42, 43). The selected flavonoids are structurally related to each other but differ in the functional groups attached to the parent flavonoid skeleton and unsaturation of the C-ring. Different groups of flavonoids were investigated to unravel the structural features that are associated with protection against PQ-induced toxicity using survival assays.

We used two modes of feeding regime for all the flavonoids; Group I: pre-treatment with flavonoids for 4 days followed by continuous exposure to PQ and Group II: co-treatment with flavonoids along with PQ together as shown in Fig. 1A. For Group I, adult male flies were pre-fed for 4 days with either 2.5% sucrose solution as control or specified flavones alone diluted in 2.5% sucrose solution, and then exposed to 5 mM PQ continuously and survival was scored every 24 h after PQ exposure, and for Group II, the flies were co-fed with flavonoids diluted in 2.5% sucrose solution along with 5 mM and survival was scored every 24 h. Both pre- and co-feeding modes were used for all the investigated compounds. The duration of 4 days of pre-treatment and concentration range were chosen based on our earlier findings that showed neuroprotection by another polymethoxyflavonoid, Gardenin A against PQ toxicity using the *Drosophila* PD model (29). We selected the specified concentrations of the flavonoids ranging from 5–100 μM after stringent preliminary screening that gave optimal reproducible data in survival assays and were considered physiologically relevant in the micromolar concentration ranges.

Consistent with earlier findings, exposure to PQ leads to a gradual increase in mortality (Fig. 1B) (36). However, the group of flavones, nobiletin, sinensetin and apigenin conferred protection when pre-fed for 4 days prior to PQ exposure. Interestingly, nobiletin and sinensetin were protective when the flies were pre-exposed to flavones intermittently for 4 days (flavones on Days 1 and 3; sucrose on Days 2 and 4 for the pre-treatment duration prior to PQ exposure) while apigenin improved survival against PQ-toxicity when pre-fed continuously for 4 days prior to PQ exposure as depicted in Fig. 1B. We performed intermittent pre-feeding for 4 days with the flavonoids that did not show protection with continuous pre-feeding. In contrast, the other group of flavones, eupatilin, luteolin and tangeretin were protective against PQ-induced toxicity only in co-feeding mode using survival assays as shown in Fig. 1C. In both pre- and co-treatment groups, the flavones significantly improved the median survival to PQ and extended the survival rate by 1–3 days as compared to only PQ-treated groups (Figs. 1B&C).

The same pre- and co-feeding regimens were followed for the group of flavonones (hesperitin, hesperidin, naringenin, naringin) and flavonols (fisetin, kaempferol, myricetin,

quercetin) to identify compounds that were protective against PQ-induced toxicity. Flavonoids can be found in plants in both glycoside-bound and free aglycon forms. Both flavonone glycosides (hesperidin and naringin) and aglycones (hesperetin and naringenin) were included in the screen to evaluate the effects on PQ-mediated morbidity in flies. As shown in Figs. 1D and E, the data suggest that none of the selected compounds in the groups of flavonones and flavonols were able to rescue against PQ-induced toxicity in survival assays using both pre- and co-feeding modes of treatment. Additionally, we observed that only aglycones exert protective effects in our model.

Feeding assays rely on the amount of food consumed by the flies and the taste and odor of different flavonoids mixed with the control food might influence the food intake ability. Therefore, we performed feeding assays using the dye (1% FD&C Blue#1) mixed with food to ensure that exposure to different flavonoids does not lead to significant differences in the food intake abilities as compared to the sucrose-fed *CantonS* wild-type controls. Our data confirm comparable levels of food consumption in flies exposed to different groups of flavonoids as shown in Fig. 2A and B. Overall, the results suggest that the specific group of flavonoids known as flavones confer protection against PQ toxicity in *Drosophila*.

Identification of specific structural features of flavonoids required to rescue PQ-induced mobility defects.

Our next goal was to determine the effect of different groups of flavonoids on PQ-mediated locomotion defects using a negative geotaxis assay. Severe locomotion impairment is a common symptom of PD caused by the deterioration of dopaminergic neurons in the midbrain (36, 38). The same feeding regimen was followed as specified in Fig. 1A and climbing assessment was same feeding regimen was followed as specified in Fig. 1A and climbing assessment was performed at 48 h time point post PQ exposure in both pre- and co-feeding experiments. The flies were either pre-fed for 4 days with sucrose or different concentrations of flavonoids as specified, followed by continuous exposure to PQ or co-fed with PQ and climbing assessment was performed at 48 h time points post-exposure. As shown in Fig. 3A, PQ exposure significantly reduced climbing abilities to 52% as compared to the 92% of the sucrose-fed control group. Interestingly, pre-treatment with specific group of flavones, including nobiletin, sinensetin and apigenin, significantly restored mobility impairment after PQ exposure. Interestingly, nobiletin and seninsetin among all the tested flavones appear to confer protection against PQ-induced toxicity only with intermittent pre-feeding in both survival and mobility assays. Consistent with the survival data, co-feeding PQ with the flavones, eupatilin, luteolin and tangeretin also significantly improved PQ-induced mobility defects as shown in Fig. 3B. However, the structurally related flavonoids, flavonones (hesperitin, hesperidin, naringenin, naringin) and flavonols (fisetin, kaempferol, myricetin, quercetin) failed to improve mobility defects in flies exposed to PQ (Fig. 3C and D). Our data suggest that certain structural features of flavones, including the presence of a double bond at the C2-C3 position (α,β-unsaturated carbonyl) of the flavonoid skeleton and the lack of a functional group substitution at the C3 position, are essential to confer protection against PQ-mediated toxicity in *Drosophila*. The compounds belonging to the flavonones and flavonols subgroups do not comply with these structural features and thus fail to provide protection against PQ exposure. Our findings confirm that only specific group

of flavonoids known as flavones are protective against PQ-induced toxicity and mobility defects, which could potentially be attributed to the structural features like the degree of unsaturation and functional groups attached to the C-ring of the parent flavonoid structure.

The antioxidant properties of flavones are not sufficient to confer protection against PQ-induced oxidative stress.

Increased oxidative stress has been linked to the onset and progression of neurodegenerative diseases (ND), including AD and PD (29, 44). Exposure to the herbicide PQ is known to induce oxidative stress leading to clinical symptoms of PD in both mammalian and invertebrate models (29, 45). Flavonoids have been shown to reduce oxidative stress by inducing the antioxidant response through the nuclear factor erythroid 2–related factor 2 (Nrf2), which regulates the expression of several downstream antioxidant genes and detoxifying proteins such as thioredoxins, glutathione synthetase, and glutathione S-transferases to counterbalance the accumulation of free radicals within the cells (43, 46). Nrf2 homologs belong to the *cap'n'collar (cnc)* subfamily of leucine zippers and known as the *cnc* gene in *Drosophila* (47). Therefore, we evaluated the effect of flavones that were protective in either pre- or co-feeding modes on the genes involved in the antioxidant response pathways. The flies were pre-fed for 4 days with either sucrose or flavones (nobiletin, sinensetin and apigenin) as specified earlier in the pre-treatment regime followed by RNA extraction and qRTPCR with primers specific to *cncC* and its downstream target, *gstD1*. We have previously shown induction of both *cncC* and *gstD1* transcripts in response to PQ exposure and used that as the positive control (29). As shown in Fig. 4A, the group of flavones (nobiletin, sinensetin and apigenin) that conferred protection with the pre-feeding mode of treatments failed to upregulate the expression of either *cncC* or *gstD1* in the absence of a stressor like PQ. The pre-fed flavones also failed to further induce the expression of *cncC* or *gstD1* upon PQ exposure compared to PQ alone since they were only used for the first four days before PQ-exposure (Fig. 4B). Next, we determined the effects of flavones (eupatilin, luteolin and tangeretin) in the co-feeding regimen along with PQ on the antioxidant genes, *cncC* and *gstD1*. Interestingly, co-treatment with flavones in the presence of PQ resulted in further induction of the *cncC* and *gstD1* transcript levels compared to PQ alone, thereby supporting their role in activating the antioxidant response to counteract against PQ-mediated oxidative stress (Fig. 4C). The data suggest that the presence of both flavones and a stressor like PQ that induces oxidative stress are required together to activate the antioxidant response pathway, since the pre-treatment feeding regime failed to upregulate the expression of both genes (*cncC* and *gstD1*) involved in the antioxidant response pathway. Based on the differential regulation of antioxidant genes by flavones, our findings suggest that the protective effects of flavones against PQ toxicity are not solely dependent on the antioxidant activities of flavonoids.

Regulatory effects of flavones on the neuroinflammatory responses associated with PD pathogenesis.

Recent studies indicate that chronic neuroinflammatory responses play a critical role in PD pathogenesis (48). Our previous data demonstrate that PQ exposure activates the innate immune transcription factor, Relish, the *Drosophila* orthologue of mammalian NFκB (36). In addition, *relish* knockdown leads to improved survival, climbing ability and rescue of

dopaminergic neurons against PQ-mediated toxicity in *Drosophila* (36). Consistent with these findings, increased NFκB activation has been linked to PD pathogenesis in diverse mammalian models (49–52). Therefore, we next determined the effect of flavones on the transcript levels of *relish*. As observed in earlier studies, *relish* transcript levels were induced 2.64-fold in response to PQ treatment (Fig. 5A). Interestingly, pre-treatment with the following flavones (nobiletin, sinensetin and apigenin) significantly reduced the levels of relish transcripts. The same trend was observed in the co-feeding mode of treatment with the flavones, eupatilin, and tangeretin (Fig. 5B). However, luteolin failed to suppress the PQ-mediated induction of *relish* transcripts. It is interesting to note that luteolin was protective at a higher micromolar concentration (100 μM) compared to the rest of the flavones ranging from 1–20 μM. We speculate that it might confer protection by activating the antioxidant response pathways as observed in our study and probably through some other unknown mediators.

Negative regulation plays a critical role in preventing prolonged hyperactivation of the immune response that gradually leads to chronic inflammation, which is associated with diverse disease pathologies, including PD. Our earlier studies demonstrated PQ-mediated regulation of *pirk* (poor Imd response upon knock-in), which is a negative player of the IMD pathway. Therefore, we determined the effect of flavones on the transcript levels of *pirk* which is also known to be regulated by Relish in response to microbial infection through the IMD pathway. As shown in Fig 5C and D, the transcript levels of *pirk* were induced around 2.2-fold in response to PQ treatments in both the pre- and co-feeding modes. Interestingly, pre-treatment with the following flavones (nobiletin, sinensetin and apigenin) resulted in significant induction of *pirk* transcripts as compared to PQ treatment alone. The same trend was observed in the co-feeding mode of treatment with the flavones, eupatilin, luteolin, and tangeretin (Fig. 5D). Our data suggest that flavone-mediated protection against PQ toxicity is dependent on the downregulation of the transcription factor, Relish, through the negative regulator Pirk thereby supporting the role of flavones as anti-inflammatory mediators.

Discussion

In this study, we used toxin-induced *Drosophila* model of PD to investigate the neuroprotective activity of selected dietary flavonoids. Several compounds representing different classes of flavonoids were tested to understand how the structure of these molecules influences their neuroprotective activity *in vivo*. Numerous epidemiological studies have shown that a diet rich in flavonoids decreases the risk of developing neurodegenerative diseases, including PD (17, 21, 24–27, 33). Neuroprotective activity of several flavonoids has also been proven in numerous animal studies, however, the molecular mechanism of action of these compounds remains elusive (2, 28). It also remains unknown whether all classes of flavonoids are characterized by similar neuroprotective activity. Previously, we showed that the polymethoxyflavone gardenin A is a multitarget neuroprotective compound against PQ-induced PD symptoms in *Drosophila* (29). Here a selected group of flavonoids structurally related to gardenin A but differing in the number and positions of hydroxyl and methoxy groups, and the level of saturation of heterocyclic ring C were tested (Table 1). Our data show that pretreatment with apigenin, nobiletin, and sinsensetin, and co-treatment with eupatilin, luteolin and tangeretin significantly improves

survival and mobility defects against PQ-induced parkinsonian symptoms (Figs. 1&3). The mechanisms of action between the pre- and co-fed neuroprotective flavones against PQ-toxicity may be partly explained due to the differential regulation of downstream molecular pathways involving antioxidant and anti-inflammatory responses (Fig 6). The use of pre- and co-feeding modes highlight the common and unique molecular gene signature patterns regulated by different groups of flavonoids to confer neuroprotection against environment toxin. Future studies are underway to understand the different modes of action between the specific groups of flavones. Interestingly, neuroprotective activities of nobiletin and sinensetin were only observed upon intermittent pretreatment compared to continuous apigenin pretreatment for four days prior to PQ exposure. Previously, Kirkland and others have shown that treatment with senolytic drugs, capable of removing senescent cells, was most effective with intermittent administration of these compounds (53, 54). Several polyphenols, including flavonoids have been investigated as potential senolytics, for example: quercetin, fisetin, luteolin, or curcumin (53). The mechanistic nature of this phenomenon is not yet understood and requires further investigation.

All compounds which improved survival and mobility defects in the toxin-induced fly model of PD belonged to one class of flavonoids, namely flavones. Compounds representing other classes of tested flavonoids (flavanones and flavonols as well as all flavonoid glycosides) provided no protection against PQ-induced parkinsonian symptoms. Our data suggest that the presence of α,β-unsaturated carbonyl group, and the lack of substitution at the C3 position are required to confer protection against PQ-mediated toxicity in *Drosophila*.

To the best of our knowledge, this is for the very first time that neuroprotective structure-activity relationships of flavonoids are investigated *in vivo* in the context of a living organism. Previous studies investigating structure-activity relationships for flavonoids have primarily focused on understanding the direct antioxidant activity of these compounds assessed using *in vitro* assays, predominantly in the presence of free radicals (34, 35). Most *in vitro* antioxidant studies indicate that the number and position of hydroxyl groups is the most significant determinant of the antioxidant activity of flavonoids (34, 35). A 3′4′-catechol structure in the B-ring has been indicated as a feature significantly increasing the direct antioxidant activity of flavonoids *in vitro* (34, 35). Additional structural features associated with high free radical scavenging potential *in vitro* include, for example, the presence of a free 3-OH group or the lack of O-methylated groups (34, 35). Many *in vitro* studies concluded that 2–3 unsaturation and a 4-carbonyl group might be dispensable, although others indicate the presence of these structural features enhances *in vitro* antioxidant activity (34, 35).. Our data indicate that both electrophilic α,β-unsaturated carbonyl group and the lack of substitution at the C3 position are essential to confer protection in the PQ-induced model of PD in flies. This data opposes many *in vitro* findings suggesting flavonols, with a hydroxyl group at C3 position are the most biologically active antioxidants (38). Lipophilic flavones with methoxy groups provided better neuroprotection in our model compared to those with hydroxyl groups. This data contradicts the *in vitro* data suggesting that the introduction of methoxy groups decreases the biological activity of flavonoids based on *in vitro* antioxidant assays. However, methylation of flavones was previously shown to enhance their anti-inflammatory activities (55).

Numerous studies, including several clinical trials, disproved the direct antioxidant activity of flavonoids *in vivo* (1). Furthermore, it has been shown that antioxidant activity assessed using in *vitro* assays does not predict the antioxidant effects of these compounds *in vivo* (1). *In vitro* studies assume direct interaction between free radicals formed in the cellular environment and flavonoids. This scenario requires high concentrations of antioxidants present in cellular compartments in the close vicinity of free radical production sites. Additionally, many of these studies do not take into consideration the fact that cells contain high concentrations of other molecules involved in antioxidant defense, such as glutathione (1, 2).

The disproval of the direct antioxidant activity hypothesis of natural compounds led to the emergence of other hypotheses explaining the beneficial effects of plant specialized metabolites, including flavonoids. Mattson and his collaborators postulated that many specialized plant metabolites evolved as natural pesticides that protect plants from herbivores and omnivores (1, 56–59). Plants synthesize many compounds that dissuade potential predators from eating plant parts rich in these compounds. To exclude the possibility that any of the tested compounds affects the amount of food ingested by *Drosophila*, we measured the amount of food uptake upon exposure to different flavonoids compared to the control food. Data presented in Fig. 2 clearly indicate that none of the tested compounds significantly changed food uptake. Therefore, the observed protective effects cannot be attributed to other mechanisms like calorie restriction, which has been previously shown to be neuroprotective in multiple animal models (60).

Mattson suggested that most natural compounds are recognized by cells as detrimental and activate multiple evolutionary conserved stress response and other cellular signaling pathways (1). He proposed that natural neuroprotective compounds, including flavonoids act as neurohormetics, providing protection at lower concentrations while exerting detrimental effects at higher concentrations (1, 56–59). Among several molecular pathways, Nrf2 activation has been considered as one of the major mechanisms by which numerous plant specialized metabolites exert beneficial effects (1). We previously showed that antioxidant activity of gardenin A through Nrf2 activation is not sufficient to provide neuroprotection in PQ-induced *Drosophila* model of PD (29). Our data showed that pretreatment with nobiletin, sinensetin and apigenin alone does not result in the upregulation of *Drosophila* Nrf2 orthologue and one of its downstream targets, *gstD1*. This further confirms that antioxidant activity through Nrf2 activation alone is not sufficient to provide protective effects in a toxin-induced model of PD. However, co-treatment with eupatilin, luteolin and tangeretin further increased transcript levels of Nrf2 orthologue and *gstD1*, compared to PQ alone. Our findings suggest flavones increase antioxidant response in the presence of stressors such as PQ (Fig. 6). Additionally, our data disprove both the neurohormetic and xenohormetic hypotheses in the context of the toxin-induced fly model, which imply that low concentrations of phytochemicals induce adaptive cellular stress response pathways such as Nrf2 signaling pathway (1, 61).

Following the neurohormesis hypothesis, Mattson suggested that certain phytochemicals should also activate NFκB, similarly to hormetic doses of glutamate and provide protective effects (1). Most known flavonoids inhibit the transcriptional activities of NFκB in

cell culture and *in vivo* models and no phytochemicals activating NFκB have been identified to date (62–64). Here we showed that the specific flavones providing protective effects in toxin-induced model of PD, decreased transcript levels of NFκB orthologue in *Drosophila*. Dysregulation of NFκB has been associated with increased neuroinflammation and pathogenesis of several neurodegenerative diseases including PD (65). In addition, the flavones also increased the transcript levels of the negative regulator of the IMD pathway, *pirk*. In *Drosophila*, overexpression of *pirk* has been shown to reduce the IMD pathway response in the context of microbial infection. Earlier studies have demonstrated that loss of *pirk* leads to the hyperactivation of *relish* which subsequently increases PQ susceptibility. Moreover, hyperactive immune pathways are associated with neurodegeneration in fly models. Our data suggest that flavones confer neuroprotection against PQ-induced PD phenotypes through modulation of the innate immune IMD pathway. However, among all tested protective flavones, luteolin, failed to decrease transcript levels of NFκB orthologue. Luteolin provided protection at a concentration approximately 5 times higher compared to other flavones. Luteolin is the most polar compound from among all neuroprotective flavones (xlogP values: luteolin – 1.4; nobiletin – 3.0; sinensetin – 3.0; tangeretin – 3.0; eupatilin – 2.9; apigenin – 1.7) with the highest number of free hydroxyl groups. Interestingly, luteolin has been shown to be protective in a transgenic *Drosophila* model of Alzheimer's disease (66). It is possible that the neuroprotective effects observed for luteolin against PQ-toxicity may be due to the combination of Nrf2 antioxidant effects and its interaction with other unidentified targets. Further studies are warranted to dissect the mechanistic role of *pirk* in mediating the protective potential of flavones against PQ toxicity.

Lipophilic neuroprotective flavones share similar structural features with anti-inflammatory lipid-derived oxidation products of essential fatty acids (2). Figure 7 compares the structures of anti-inflammatory nobiletin, identified in our study, and the structure of anti-inflammatory 13-EFOX-L_2 (13-oxoHODE). 13-EFOX-L_2 is linoleic acid derivative that showed anti-inflammatory effects in a *Drosophila* metainflammation blood tumor model (67). Both molecules represent the class of anti-inflammatory and antioxidant lipophilic soft electrophiles with α,β-unsaturated carbonyl group. Oxidative stress and chronic inflammation have been shown to be underlying hallmarks of numerous non-communicable diseases, including neurodegenerative ailments (33). We postulate that lipophilic flavones along with oxidation products of polyunsaturated fatty acids may constitute a group of pleiotropic soft electrophiles involved in the process of resolution of inflammation through the activation of Nrf2 and NFκB inhibition.

Recent studies have shown that other small molecule soft electrophiles may constitute a viable source of new drug hits and leads for the treatment of numerous diseases (68). For example, dimethyl fumarate, a small-molecule electrophile (Fig. 7C) was recently approved for the treatment of relapsing multiple sclerosis (69). Although molecular mechanism of action of dimethyl fumarate is yet not fully understood, it has been proposed that beneficial effects of this molecule stem from its ability to activate Nrf2 and inhibition of NFκB.

The electrophilic pro-resolving molecules may constitute a class of compounds that should be investigated in the prevention and treatment of multifactorial diseases such as PD. Additionally, we suggest that rather than pursuing electrophilic phytochemicals as drugs, we

should give them proper consideration as important or even essential nutrients, for example vitamin P, as suggested by Seigler *et al*. that would potentially help to stop or delay the onset and progression of NDs (28).

Conclusions

To the best of our knowledge, this is the first study that explored neuroprotective structure-activity relationships of flavonoids *in vivo* using the *Drosophila* environmental toxin-induced PD model. Since many *in vitro* studies performed with phytochemicals fail to predict *in vivo* effects, we stress the importance of testing these molecules in the context of a living organism. Our data suggest that the presence of α,β-unsaturated carbonyl group, and the lack of substitution at the C3 position are required to confer protection against PQ-mediated toxicity in *Drosophila*.

Future studies should further explore the concept of essential soft electrophiles and their ability to interact with nucleophiles. Although this study only considered the potential antioxidant and anti-inflammatory properties of flavonoids, other possible targets relevant to PD pathogenesis should be investigated in detail. Similar studies should be extended to additional groups of flavonoids and other classes of phytochemicals in the context of both fly and mammalian models.

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References

1. Lee J, Jo DG, Park D, Chung HY, Mattson MP. Adaptive cellular stress pathways as therapeutic targets of dietary phytochemicals: focus on the nervous system. Pharmacol Rev. 2014;66(3):815–68. [PubMed: 24958636]
2. Maitra U, Stephen C, Ciesla LM. Drug discovery from natural products - Old problems and novel solutions for the treatment of neurodegenerative diseases. J Pharm Biomed Anal. 2022;210:114553.
3. Eisele YS, Monteiro C, Fearns C, Encalada SE, Wiseman RL, Powers ET, et al. Targeting protein aggregation for the treatment of degenerative diseases. Nat Rev Drug Discov. 2015;14(11):759–80. [PubMed: 26338154]
4. Chin-Chan M, Navarro-Yepes J, Quintanilla-Vega B. Environmental pollutants as risk factors for neurodegenerative disorders: Alzheimer and Parkinson diseases. Front Cell Neurosci. 2015;9:124. [PubMed: 25914621]
5. Di Monte DA, Lavasani M, Manning-Bog AB. Environmental factors in Parkinson's disease. Neurotoxicology. 2002;23(4–5):487–502. [PubMed: 12428721]
6. Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, et al. Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. Neurology. 1997;48(6):1583–8. [PubMed: 9191770]
7. Nistico R, Mehdawy B, Piccirilli S, Mercuri N. Paraquat- and rotenone-induced models of Parkinson's disease. Int J Immunopathol Pharmacol. 2011;24(2):313–22. [PubMed: 21658306]
8. Zeng XS, Geng WS, Jia JJ. Neurotoxin-Induced Animal Models of Parkinson Disease: Pathogenic Mechanism and Assessment. ASN Neuro. 2018;10:1759091418777438.

9. Bove J, Prou D, Perier C, Przedborski S. Toxin-induced models of Parkinson's disease. NeuroRx. 2005;2(3):484–94. [PubMed: 16389312]
10. Chen L, Yoo SE, Na R, Liu Y, Ran Q. Cognitive impairment and increased Abeta levels induced by paraquat exposure are attenuated by enhanced removal of mitochondrial H(2)O(2). Neurobiol Aging. 2012;33(2):432 e15–26.
11. Yan D, Zhang Y, Liu L, Yan H. Pesticide exposure and risk of Alzheimer's disease: a systematic review and meta-analysis. Sci Rep. 2016;6:32222.
12. Cassar M, Issa AR, Riemensperger T, Petitgas C, Rival T, Coulom H, et al. A dopamine receptor contributes to paraquat-induced neurotoxicity in Drosophila. Hum Mol Genet. 2015;24(1):197–212. [PubMed: 25158689]
13. Li B, He X, Sun Y, Li B. Developmental exposure to paraquat and maneb can impair cognition, learning and memory in Sprague-Dawley rats. Mol Biosyst. 2016;12(10):3088–97. [PubMed: 27460631]
14. Munoz-Soriano V, Paricio N. Drosophila models of Parkinson's disease: discovering relevant pathways and novel therapeutic strategies. Parkinsons Dis. 2011;2011:520640.
15. Soares JJ, Rodrigues DT, Goncalves MB, Lemos MC, Gallarreta MS, Bianchini MC, et al. Paraquat exposure-induced Parkinson's disease-like symptoms and oxidative stress in Drosophila melanogaster: Neuroprotective effect of Bougainvillea glabra Choisy. Biomed Pharmacother. 2017;95:245–51. [PubMed: 28843913]
16. Kimura Y, Yoshida D, Ohara T, Hata J, Honda T, Hirakawa Y, et al. Long-term association of vegetable and fruit intake with risk of dementia in Japanese older adults: the Hisayama study. BMC Geriatr. 2022;22(1):257. [PubMed: 35351024]
17. Yuan C, Fondell E, Bhushan A, Ascherio A, Okereke OI, Grodstein F, et al. Long-term intake of vegetables and fruits and subjective cognitive function in US men. Neurology. 2019;92(1):e63–e75. [PubMed: 30464030]
18. Martucci M, Ostan R, Biondi F, Bellavista E, Fabbri C, Bertarelli C, et al. Mediterranean diet and inflammaging within the hormesis paradigm. Nutr Rev. 2017;75(6):442–55. [PubMed: 28595318]
19. Szarc vel Szic K, Declerck K, Vidakovic M, Vanden Berghe W. From inflammaging to healthy aging by dietary lifestyle choices: is epigenetics the key to personalized nutrition? Clin Epigenetics. 2015;7:33. [PubMed: 25861393]
20. Ruiz-Nunez B, Pruimboom L, Dijck-Brouwer DA, Muskiet FA. Lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low-grade inflammation in an evolutionary context. J Nutr Biochem. 2013;24(7):1183–201. [PubMed: 23657158]
21. Morris MC, Tangney CC, Wang Y, Sacks FM, Bennett DA, Aggarwal NT. MIND diet associated with reduced incidence of Alzheimer's disease. Alzheimers Dement. 2015;11(9):1007–14. [PubMed: 25681666]
22. Agarwal P, Wang Y, Buchman AS, Holland TM, Bennett DA, Morris MC. MIND Diet Associated with Reduced Incidence and Delayed Progression of ParkinsonismA in Old Age. J Nutr Health Aging. 2018;22(10):1211–5. [PubMed: 30498828]
23. Baranowski BJ, Marko DM, Fenech RK, Yang AJT, MacPherson REK. Healthy brain, healthy life: a review of diet and exercise interventions to promote brain health and reduce Alzheimer's disease risk. Appl Physiol Nutr Metab. 2020;45(10):1055–65. [PubMed: 32717151]
24. Maraki MI, Yannakoulia M, Stamelou M, Stefanis L, Xiromerisiou G, Kosmidis MH, et al. Mediterranean diet adherence is related to reduced probability of prodromal Parkinson's disease. Mov Disord. 2019;34(1):48–57. [PubMed: 30306634]
25. Gao X, Cassidy A, Schwarzschild MA, Rimm EB, Ascherio A. Habitual intake of dietary flavonoids and risk of Parkinson disease. Neurology. 2012;78(15):1138–45. [PubMed: 22491871]
26. Yeh TS, Yuan C, Ascherio A, Rosner BA, Willett WC, Blacker D. Long-term Dietary Flavonoid Intake and Subjective Cognitive Decline in US Men and Women. Neurology. 2021;97(10):e1041-e56.
27. Zhang X, Molsberry SA, Yeh TS, Cassidy A, Schwarzschild MA, Ascherio A, et al. Intake of Flavonoids and Flavonoid-Rich Foods and Mortality Risk Among Individuals With Parkinson Disease: A Prospective Cohort Study. Neurology. 2022;98(10):e1064-e76.

28. Seigler DS, Friesen JB, Bisson J, Graham JG, Bedran-Russo A, McAlpine JB, et al. Do Certain Flavonoid IMPS Have a Vital Function? Front Nutr. 2021;8:762753.
29. Maitra U, Harding T, Liang Q, Ciesla L. GardeninA confers neuroprotection against environmental toxin in a Drosophila model of Parkinson's disease. Commun Biol. 2021;4(1):162. [PubMed: 33547411]
30. Bisson J, McAlpine JB, Friesen JB, Chen SN, Graham J, Pauli GF. Can Invalid Bioactives Undermine Natural Product-Based Drug Discovery? J Med Chem. 2016;59(5):1671–90. [PubMed: 26505758]
31. Baell J, Walters MA. Chemistry: Chemical con artists foil drug discovery. Nature. 2014;513(7519):481–3. [PubMed: 25254460]
32. Calabrese V, Santoro A, Monti D, Crupi R, Di Paola R, Latteri S, et al. Aging and Parkinson's Disease: Inflammaging, neuroinflammation and biological remodeling as key factors in pathogenesis. Free Radic Biol Med. 2018;115:80–91. [PubMed: 29080843]
33. Sivandzade F, Prasad S, Bhalerao A, Cucullo L. NRF2 and NF-B interplay in cerebrovascular and neurodegenerative disorders: Molecular mechanisms and possible therapeutic approaches. Redox Biol. 2019;21:101059.
34. Wolfe KL, Liu RH. Structure-activity relationships of flavonoids in the cellular antioxidant activity assay. J Agric Food Chem. 2008;56(18):8404–11. [PubMed: 18702468]
35. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free Radic Biol Med. 1997;22(5):749–60. [PubMed: 9119242]
36. Maitra U, Scaglione MN, Chtarbanova S, O'Donnell JM. Innate immune responses to paraquat exposure in a Drosophila model of Parkinson's disease. Sci Rep. 2019;9(1):12714.
37. Maitra U, Ciesla L. Using Drosophila as a platform for drug discovery from natural products in Parkinson's disease. Medchemcomm. 2019;10(6):867–79. [PubMed: 31303984]
38. Chaudhuri A, Bowling K, Funderburk C, Lawal H, Inamdar A, Wang Z, et al. Interaction of genetic and environmental factors in a Drosophila parkinsonism model. J Neurosci. 2007;27(10):2457–67. [PubMed: 17344383]
39. Soares JJ, Rodrigues DT, Gonçalves MB, Lemos MC, Gallarreta MS, Bianchini MC, et al. Paraquat exposure-induced Parkinson's disease-like symptoms and oxidative stress in Drosophila melanogaster: Neuroprotective effect of Bougainvillea glabra Choisy. Biomed Pharmacother. 2017;95:245–51. [PubMed: 28843913]
40. Arsac JN, Sedru M, Dartiguelongue M, Vulin J, Davoust N, Baron T, et al. Chronic Exposure to Paraquat Induces Alpha-Synuclein Pathogenic Modifications in Drosophila. Int J Mol Sci. 2021;22(21).
41. Dias MC, Pinto D, Silva AMS. Plant Flavonoids: Chemical Characteristics and Biological Activity. Molecules. 2021;26(17).
42. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. J Nutr Sci. 2016;5:e47. [PubMed: 28620474]
43. Ayaz M, Sadiq A, Junaid M, Ullah F, Ovais M, Ullah I, et al. Flavonoids as Prospective Neuroprotectants and Their Therapeutic Propensity in Aging Associated Neurological Disorders. Front Aging Neurosci. 2019;11:155. [PubMed: 31293414]
44. Trist BG, Hare DJ, Double KL. Oxidative stress in the aging substantia nigra and the etiology of Parkinson's disease. Aging Cell. 2019;18(6):e13031.
45. Wang Q, Ren N, Cai Z, Lin Q, Wang Z, Zhang Q, et al. Paraquat and MPTP induce neurodegeneration and alteration in the expression profile of microRNAs: the role of transcription factor Nrf2. NPJ Parkinsons Dis. 2017;3:31. [PubMed: 29071302]
46. Khan H, Tundis R, Ullah H, Aschner M, Belwal T, Mirzaei H, et al. Flavonoids targeting NRF2 in neurodegenerative disorders. Food Chem Toxicol. 2020;146:111817.
47. Pitoniak A, Bohmann D. Mechanisms and functions of Nrf2 signaling in Drosophila. Free Radic Biol Med. 2015;88(Pt B):302–13. [PubMed: 26117322]
48. Flood PM, Qian L, Peterson LJ, Zhang F, Shi JS, Gao HM, et al. Transcriptional Factor NF-κB as a Target for Therapy in Parkinson's Disease. Parkinsons Dis. 2011;2011:216298.

49. Bellucci A, Bubacco L, Longhena F, Parrella E, Faustini G, Porrini V, et al. Nuclear Factor-κB Dysregulation and α-Synuclein Pathology: Critical Interplay in the Pathogenesis of Parkinson's Disease. Front Aging Neurosci. 2020;12:68. [PubMed: 32265684]
50. Hunot S, Brugg B, Ricard D, Michel PP, Muriel MP, Ruberg M, et al. Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease. Proc Natl Acad Sci U S A. 1997;94(14):7531–6. [PubMed: 9207126]
51. Dolatshahi M, Ranjbar Hameghavandi MH, Sabahi M, Rostamkhani S. Nuclear factor-kappa B (NF-κB) in pathophysiology of Parkinson disease: Diverse patterns and mechanisms contributing to neurodegeneration. Eur J Neurosci. 2021.
52. Dutta D, Jana M, Majumder M, Mondal S, Roy A, Pahan K. Selective targeting of the TLR2/MyD88/NF-κB pathway reduces α-synuclein spreading in vitro and in vivo. Nat Commun. 2021;12(1):5382. [PubMed: 34508096]
53. Kirkland JL, Tchkonia T. Senolytic drugs: from discovery to translation. J Intern Med. 2020;288(5):518–36. [PubMed: 32686219]
54. Xu M, Pirtskhalava T, Farr JN, Weigand BM, Palmer AK, Weivoda MM, et al. Senolytics improve physical function and increase lifespan in old age. Nat Med. 2018;24(8):1246–56. [PubMed: 29988130]
55. During A, Larondelle Y. The O-methylation of chrysin markedly improves its intestinal anti-inflammatory properties: Structure-activity relationships of flavones. Biochem Pharmacol. 2013;86(12):1739–46. [PubMed: 24134915]
56. Mattson MP. Hormesis defined. Ageing Res Rev. 2008;7(1):1–7. [PubMed: 18162444]
57. Murugaiyah V, Mattson MP. Neurohormetic phytochemicals: An evolutionary-bioenergetic perspective. Neurochem Int. 2015;89:271–80. [PubMed: 25861940]
58. Mattson MP, Cheng A. Neurohormetic phytochemicals: Low-dose toxins that induce adaptive neuronal stress responses. Trends Neurosci. 2006;29(11):632–9. [PubMed: 17000014]
59. Mattson MP, Son TG, Camandola S. Viewpoint: mechanisms of action and therapeutic potential of neurohormetic phytochemicals. Dose Response. 2007;5(3):174–86. [PubMed: 18648607]
60. Kapahi P, Kaeberlein M, Hansen M. Dietary restriction and lifespan: Lessons from invertebrate models. Ageing Res Rev. 2017;39:3–14. [PubMed: 28007498]
61. Baur JA, Sinclair DA. What is Xenohormesis? Am J Pharmacol Toxicol. 2008;3(1):152–9. [PubMed: 26949380]
62. Shin SY, Woo Y, Hyun J, Yong Y, Koh D, Lee YH, et al. Relationship between the structures of flavonoids and their NF-kappaB-dependent transcriptional activities. Bioorg Med Chem Lett. 2011;21(20):6036–41. [PubMed: 21907578]
63. Eun SH, Woo JT, Kim DH. Tangeretin Inhibits IL-12 Expression and NF-kappaB Activation in Dendritic Cells and Attenuates Colitis in Mice. Planta Med. 2017;83(6):527–33. [PubMed: 27806407]
64. Shukla S, Shankar E, Fu P, MacLennan GT, Gupta S. Suppression of NF-kappaB and NF-kappaB-Regulated Gene Expression by Apigenin through IkappaBalpha and IKK Pathway in TRAMP Mice. PLoS One. 2015;10(9):e0138710.
65. Singh SS, Rai SN, Birla H, Zahra W, Rathore AS, Singh SP. NF-kappaB-Mediated Neuroinflammation in Parkinson's Disease and Potential Therapeutic Effect of Polyphenols. Neurotox Res. 2020;37(3):491–507. [PubMed: 31823227]
66. Ali F, Rahul, Jyoti S, Naz F, Ashafaq M, Shahid M, et al. Therapeutic potential of luteolin in transgenic Drosophila model of Alzheimer's disease. Neurosci Lett. 2019;692:90–9. [PubMed: 30420334]
67. Panettieri S, Paddibhatla I, Chou J, Rajwani R, Moore RS, Goncharuk T, et al. Discovery of aspirin-triggered eicosanoid-like mediators in a Drosophila metainflammation blood tumor model. J Cell Sci. 2019;133(5).
68. Jackson PA, Widen JC, Harki DA, Brummond KM. Covalent Modifiers: A Chemical Perspective on the Reactivity of alpha,beta-Unsaturated Carbonyls with Thiols via Hetero-Michael Addition Reactions. J Med Chem. 2017;60(3):839–85. [PubMed: 27996267]
69. Bomprezzi R. Dimethyl fumarate in the treatment of relapsing-remitting multiple sclerosis: an overview. Ther Adv Neurol Disord. 2015;8(1):20–30. [PubMed: 25584071]

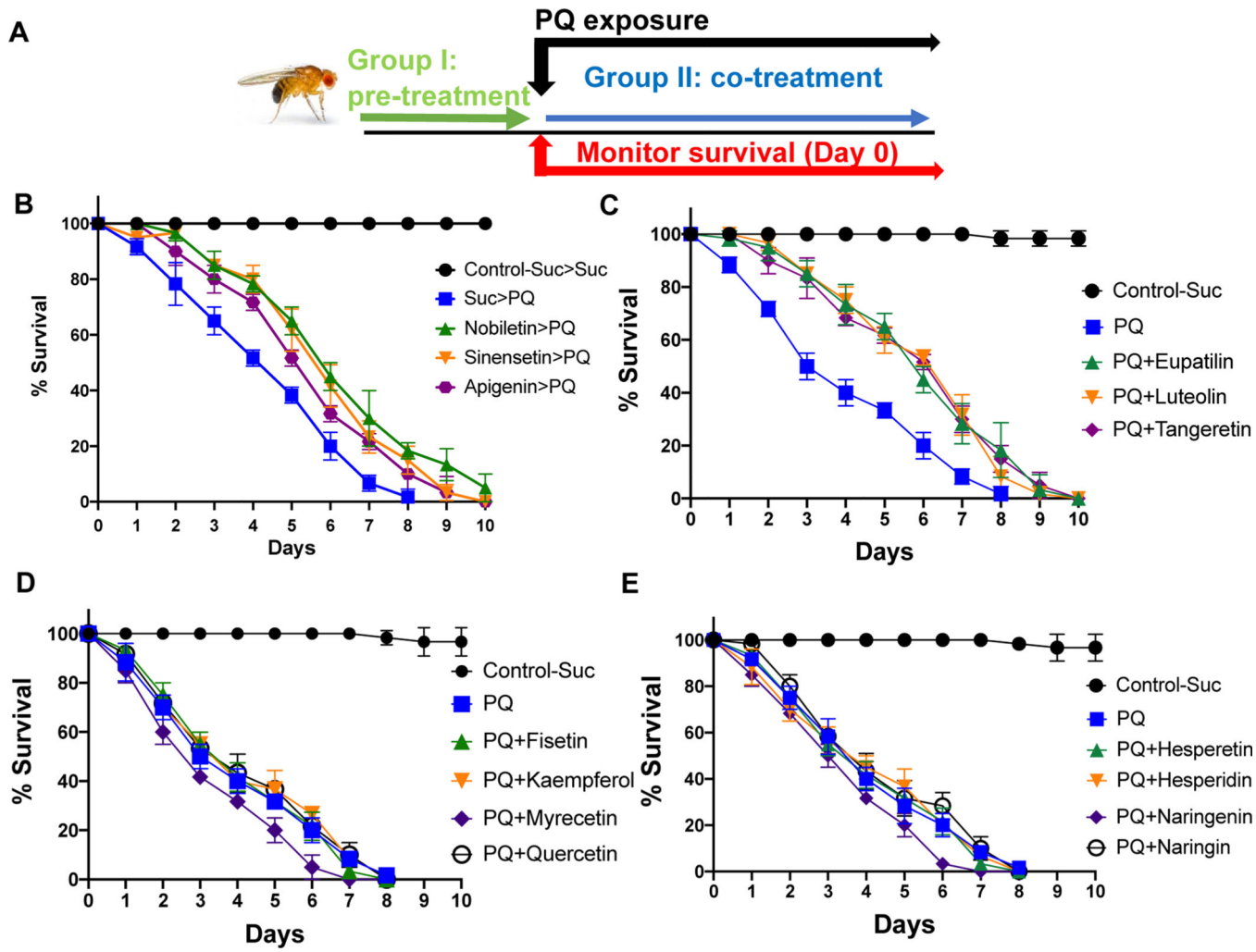


Figure 1. Effects of different groups of flavonoids against paraquat-induced toxicity in a *Drosophila* PD model.

(A) Schematic representation of the pre- and co-feeding regimen. In the pre-treatment mode, adult male flies were either pre-fed for 4 days with 2.5% sucrose or specified flavonoids followed by continuous exposure to 5 mM PQ and were scored daily for survival. In the co-treatment mode, adult male flies were exposed to both flavonoids and 5 mM PQ at the same time and were scored daily for survival. Survival was scored upon PQ exposure in both pre- and co-treatment regimes and Day 0 in the figure is when PQ exposure starts (red arrow). Survival data for the pre- and co-feeding experiments are representative of ten independent biological replicates with 10 male flies per feeding conditions. (B) Survival assays were performed using adult male flies pre-fed with sucrose or specified flavones followed by continuous exposure to 5 mM PQ as outlined above and the number of live flies was recorded every 24 h until all of the flies were dead and the average survival percentages were plotted. Statistical significances between the PQ-fed and the flavone pre-fed groups followed by PQ exposure were determined using the log-rank test (Suc>PQ vs Nobiletin>PQ, Suc>PQ vs Sinensetin>PQ ***$p<0.001$; Suc>PQ vs Apigenin>PQ **$p<0.01$). (C) Survival assays were performed using the co-feeding mode in which adult male flies were exposed

to both 5 mM PQ or specified flavones and the number of live flies was recorded every 24 h and the average survival percentages were plotted. Statistical significances between the PQ-fed and the co-fed PQ+flavone groups were determined using the log-rank test ***p<0.001. (D) Survival assays were performed using the co-feeding mode in which adult male flies were exposed to both 5 mM PQ or specified flavonols and the number of live flies was recorded every 24 h and the average survival percentages were plotted. No statistical significances between the PQ-fed and the flavonol-fed groups were observed using the log-rank test. (E) Survival assays were performed using the co-feeding mode in which adult male flies were exposed to both 5 mM PQ or specified flavonones and the average survival percentages were plotted. No statistical significances between the PQ-fed and the flavonone-fed groups were observed using the log-rank test.

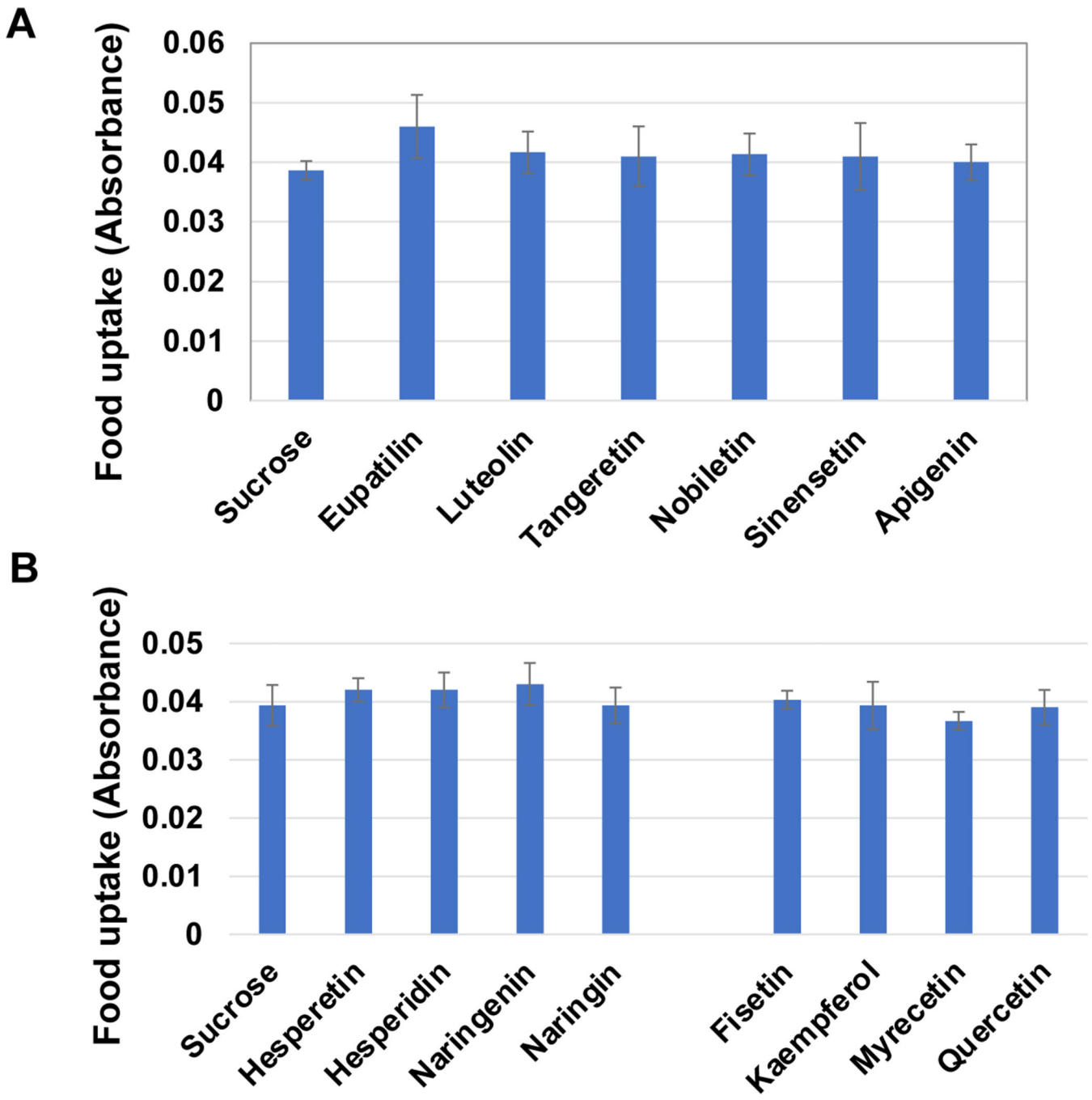


Figure 2. Feeding assays to quantitate food uptake upon exposure to different flavonoids.

Adult male flies were fed either sucrose or specified flavonoids (A) flavones (B) flavonones or flavonols mixed with the blue food dye (1% FD&C Blue#1) and the dye content was measured spectrophotometrically at 630 nm. Data are representative of three independent biological replicates using10 male flies per group. Error bars indicate the standard deviation. No statistical significances between the control sucrose-fed group and the specified flavonoid-fed groups were observed using the *Student's* t-test. $p>0.05$.

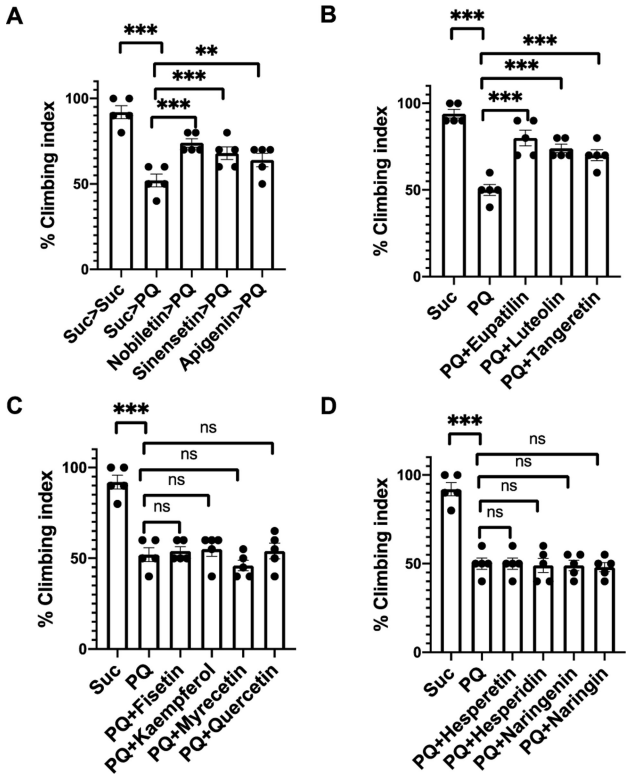


Figure 3. Effects of different groups of flavonoids against paraquat-induced mobility defects.

Negative geotaxis assays were used to determine the effect of flavonoids on the climbing abilities of flies exposed to PQ. The number of flies able to cross 5 cm within 20 s were recorded and plotted at 48 h post PQ exposure. Data are representative of at least five independent experiments involving three to five technical repeats using10 male flies per group. (A) Adult male flies were either pre-fed for 4 days with 2.5% sucrose or flavones (nobiletin, sinensetin and apigenin) followed by continuous exposure to 5 mM PQ and the % climbing index was plotted at 48 h. (B) Mobility assays were performed using the co-feeding mode in which adult male flies were exposed to both 5 mM PQ or specified flavones (eupatilin, luteolin, and tangeretin) and data were analyzed at 48 h. The protective effects of specified flavonoids on the climbing abilities of flies exposed to PQ were determined by mobility assays. (C) flavonols (fisetin, kaempferol, myricetin, quercetin) and D. flavonones (hesperitin, hesperidin, naringenin, naringin). $^{**}p < 0.01$; $^{***}p < 0.001$ based on one-way ANOVA between indicated feeding conditions.

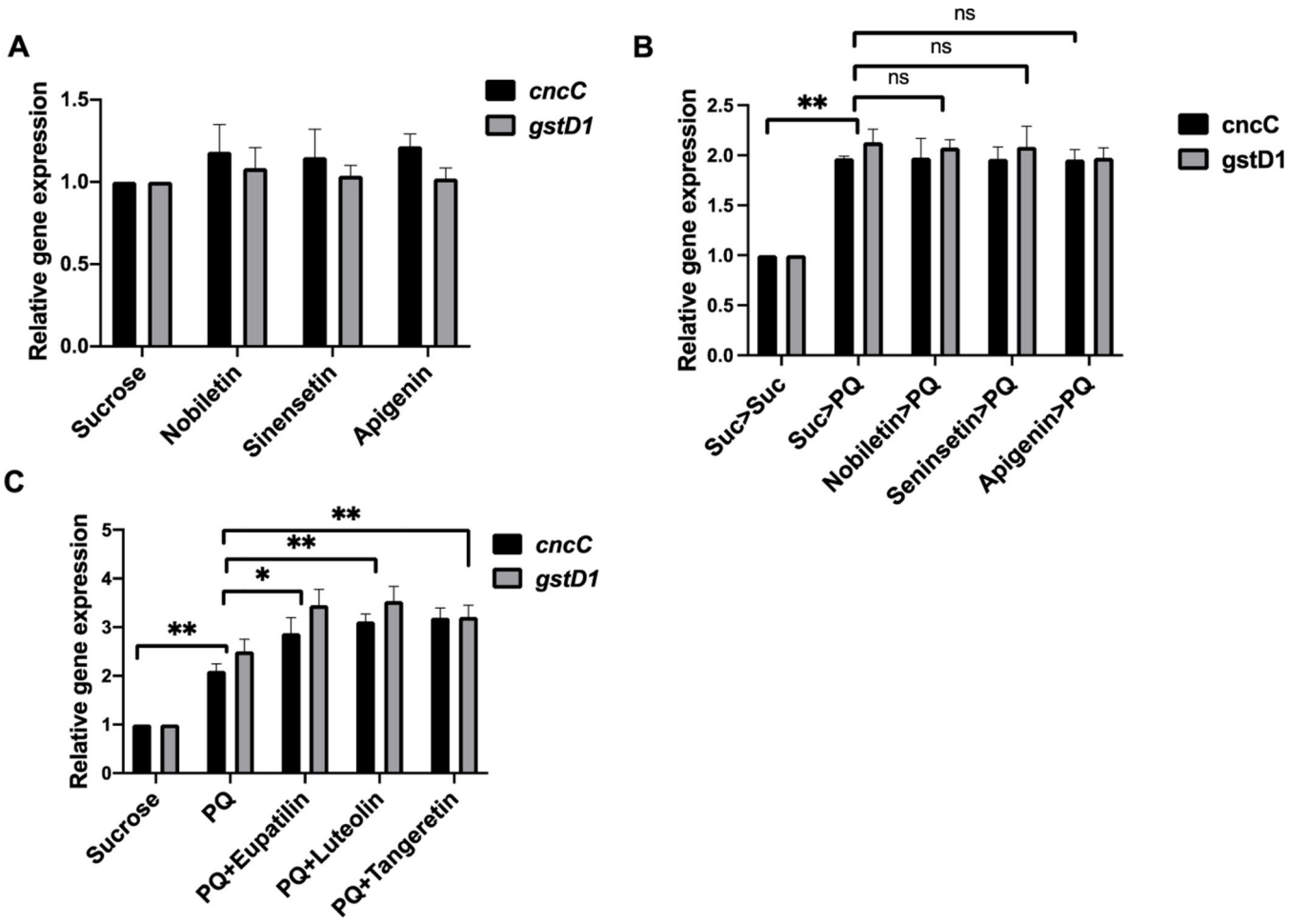


Figure 4. The protective effect of flavones against PQ toxicity is not solely dependent on antioxidant activities.

Effects of flavones on the transcript levels of *cncC*, the human Nrf2 orthologue, and its downstream target *gstD1* in *Drosophila* using qRT-PCR. (A) Adult male flies were either pre-fed for 4 days with 2.5% sucrose or flavones (nobiletin, sinensetin and apigenin) followed by RNA isolation from the heads of adult flies and processed for qRT-PCR. The transcript levels of *cncC* and *gstD1* were analyzed and plotted after normalization with *rp49* levels as the internal control. Each data point represents mean ± SEM. The mRNA fold changes are normalized to the sucrose-fed (Suc) flies (assigned a value of 1). (B) Adult male flies were either pre-fed for 4 days with 2.5% sucrose or flavones (nobiletin, sinensetin and apigenin) followed by PQ exposure for 24 h. Total RNA was isolated from the heads of adult flies and processed for qRT-PCR. The transcript levels of *cncC* and *gstD1* were analyzed and plotted after normalization with *rp49* levels as the internal control. Each data point represents mean ± SEM. The mRNA fold changes are normalized to the sucrose-fed (Suc>Suc) flies (assigned a value of 1). $^{**}p < 0.01$; ns: not significant between different feeding conditions based on Mann-Whitney U test. (C) Relative gene expression using qRT-PCR were performed using the co-feeding mode in which adult male flies were exposed to both 5 mM PQ or specified flavones (eupatilin, luteolin and tangeretin) for 24 h. The transcript levels of *cncC* and *gstD1* were analyzed and plotted after normalization

with *rp49* levels as the internal control. Each data point represents mean ± SEM. The mRNA fold changes are normalized to the sucrose-fed (Suc) flies (assigned a value of 1). Data represents three independent biological replicates including three technical repeats per specified feeding condition and compared to the control sucrose-fed flies (assigned a value of 1) for data analysis. *$p<0.05$; **$p < 0.01$ between different feeding conditions based on the Mann-Whitney U test.

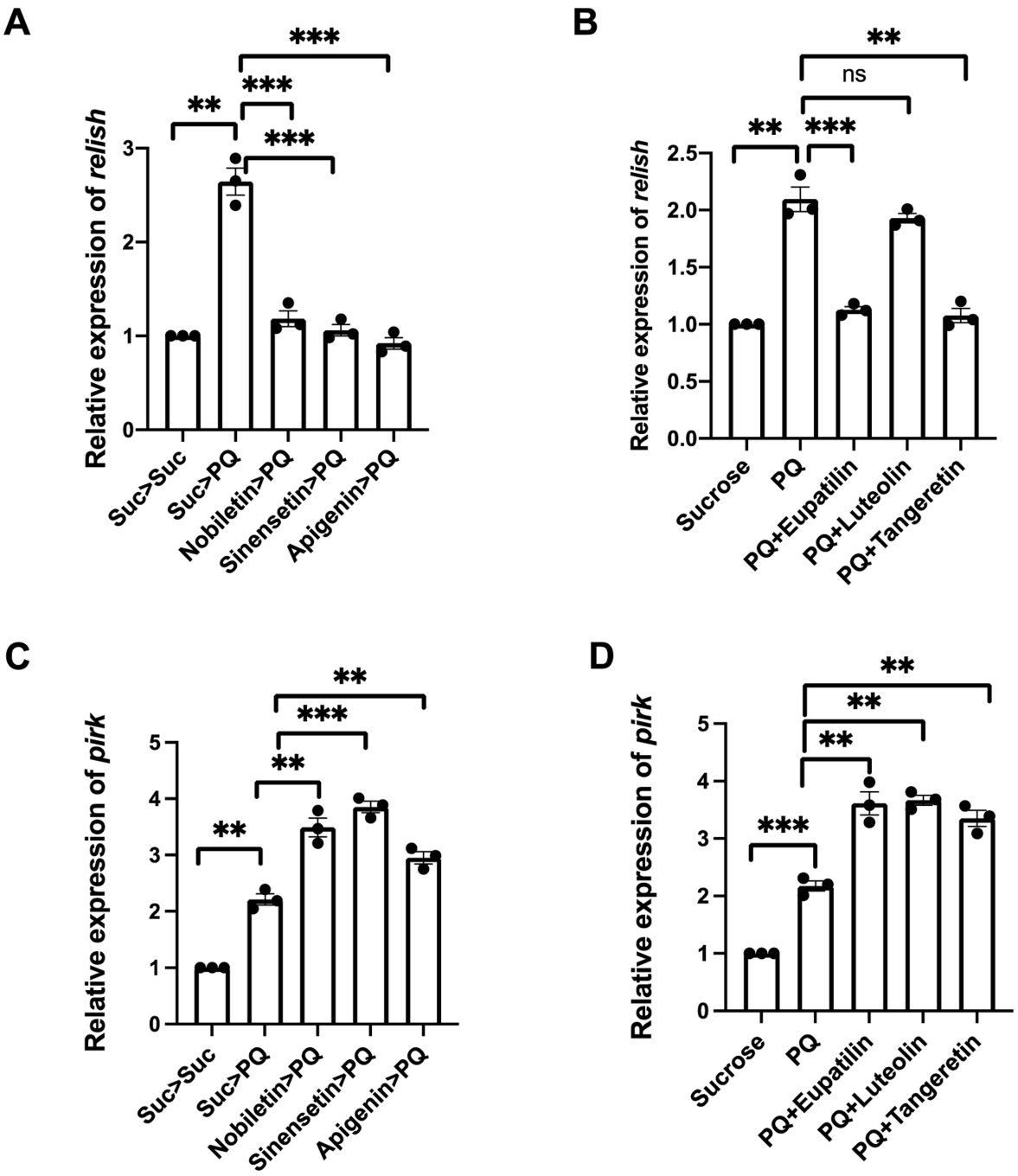


Figure 5. A specific group of flavones confer protection against PQ-induced neurotoxicity through modulation of the neuroinflammatory responses in *Drosophila*.

(A) Effects of different flavones on the transcript levels of *relish*, the human NFκB orthologue in *Drosophila* using qRT-PCR. A. Adult male flies were either pre-fed for 4 days with 2.5% sucrose or flavones (nobiletin, sinensetin and apigenin) followed by PQ exposure for 24 h. Total RNA was isolated from the heads of adult flies and processed for qRT-PCR. The transcript levels of *relish* were analyzed and plotted after normalization with *rp49* levels as the internal control. Each data point represents mean ± SEM. The mRNA fold changes are normalized to the sucrose-fed (Suc>Suc) flies (assigned a value of 1). $^{**}p < 0.01$; $^{***}p$

< 0.001 between different feeding conditions based on Mann-Whitney U test. (B) Relative gene expression using qRT-PCR were performed using the co-feeding mode in which adult male flies were exposed to both 5 mM PQ or specified flavones (eupatilin, luteolin and tangeretin). The transcript levels of *relish* were analyzed and plotted after normalization with *rp49* levels as the internal control. Each data point represents mean ± SEM. The mRNA fold changes are normalized to the sucrose-fed (Suc) flies (assigned a value of 1). Data represents three independent biological replicates including three technical repeats per specified feeding condition and compared to the control sucrose-fed flies (assigned a value of 1). Statistical differences between the specified feeding groups were analyzed using the nonparametric Mann-Whitney U test $^{**}p < 0.01$; $^{***}p < 0.001$. (C) Effects of different flavones on the transcript levels of *pirk*, the negative regulator of the IMD pathway in *Drosophila* using qRT-PCR. A. Adult male flies were either pre-fed for 4 days with 2.5% sucrose or flavones (nobiletin, sinensetin and apigenin) followed by RNA isolation from the heads of adult flies and processed for qRT-PCR. The transcript levels of *pirk* were plotted after normalization with *rp49* levels as the internal control. Each data point represents mean ± SEM. The mRNA fold changes are normalized to the sucrose-fed (Suc>Suc) flies (assigned a value of 1). $^{**}p < 0.01$; $^{***}p < 0.001$ between different feeding conditions based on Mann-Whitney U test. (D) Relative gene expression using qRT-PCR were performed using the co-feeding mode in which adult male flies were exposed to both 5 mM PQ or specified flavones (eupatilin, luteolin and tangeretin). The transcript levels of *pirk* were analyzed and plotted after normalization with *rp49* levels as the internal control. Each data point represents mean ± SEM. The mRNA fold changes are normalized to the sucrose-fed (Suc) flies (assigned a value of 1). Data represents three independent biological replicates including three technical repeats per specified feeding condition and compared to the control sucrose-fed flies (assigned a value of 1). Statistical differences between the specified feeding groups were analyzed using the nonparametric Mann-Whitney U test $^{**}p < 0.01$; $^{***}p < 0.001$.

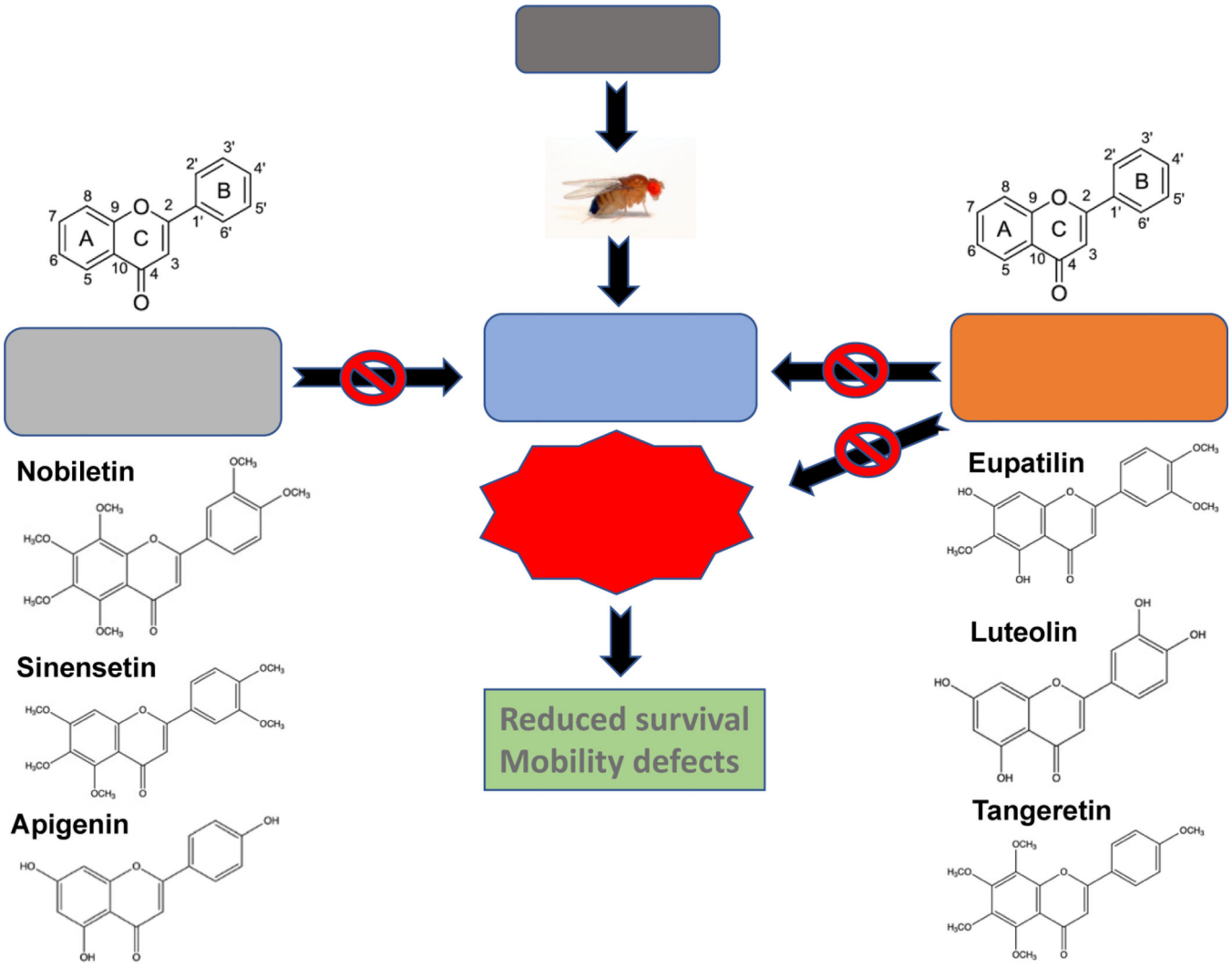


Figure 6. Schematic model of flavone-mediated protection against PQ in a *Drosophila* PD model.

Exposure to PQ induces PD symptoms involving increased mortality, mobility defects and progressive demise of dopaminergic neurons by activating oxidative stress and neuroinflammatory responses, including NFκB in adult male flies. A specific group of flavonoids known as flavones confer protection against PQ-induced neurotoxicity in pre- and co-feeding modes. Our data suggest that certain structural features of flavones including the presence of α,β-unsaturated carbonyl at the C2-C3 position of the flavonoid skeleton and the absence of a functional group substitution at the C3 position are essential to confer protection against PQ-mediated toxicity in *Drosophila*.

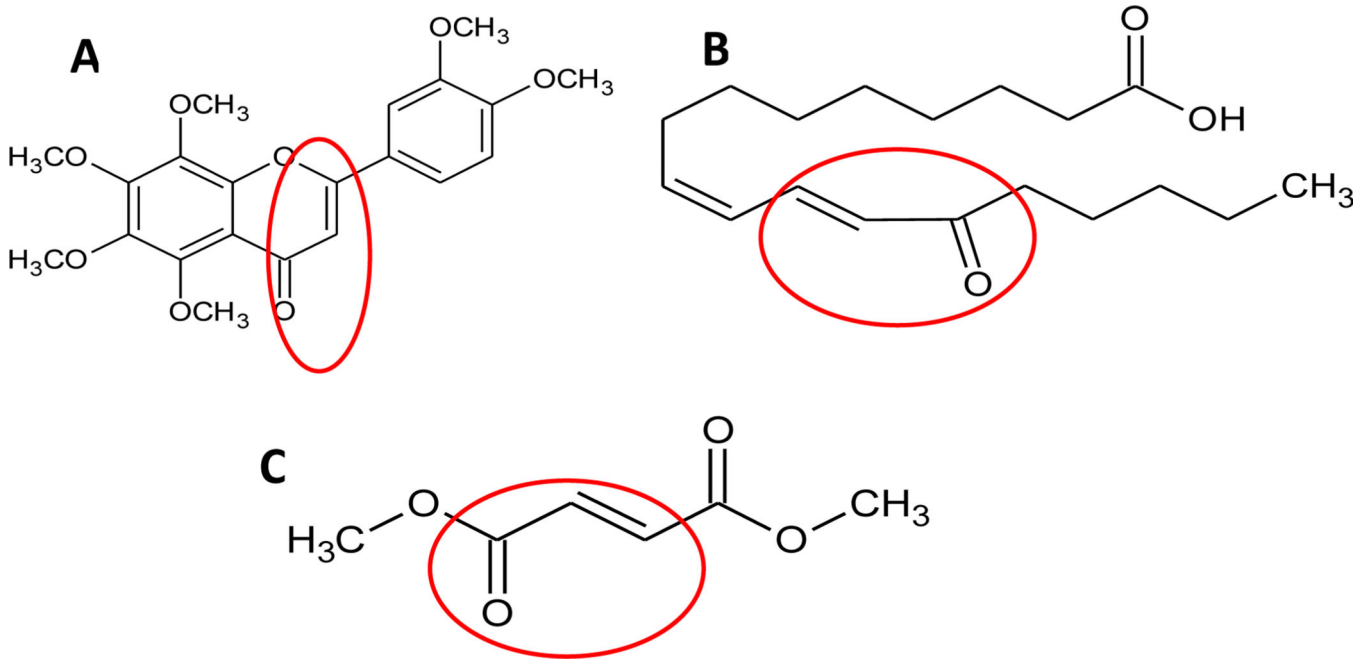
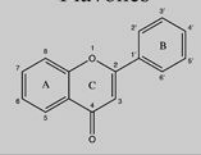
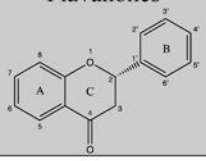
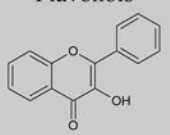
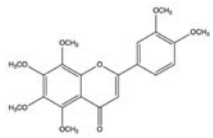
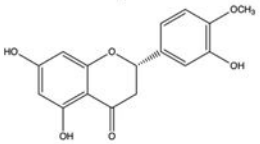
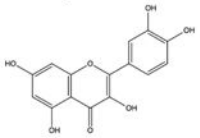
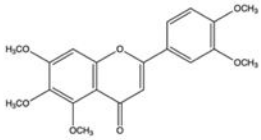
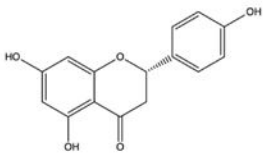
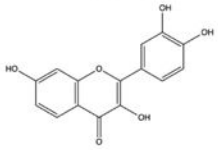
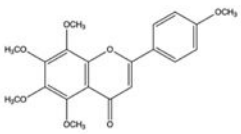
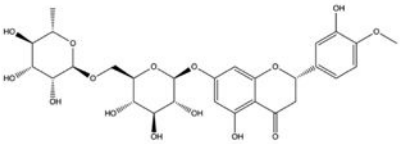
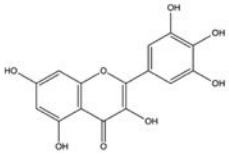
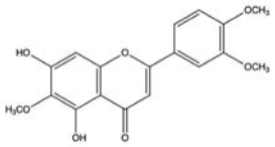
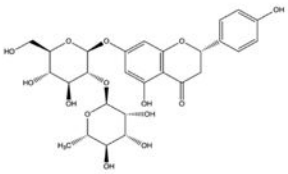
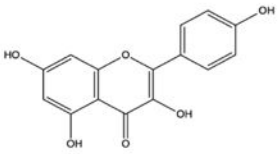


Figure 7. The comparison of structures of soft electrophiles with antioxidant and anti-inflammatory effects.

(A) Nobiletin (flavone). (B) 13-keto-9Z,11E-octadecadienoic acid (13-oxoHODE; 13-EFOX-L2; fatty acid derivative). (C) Dimethyl fumarate. Unsaturated α,β-carbonyl group is marked with red.

Table 1.

Structures of the investigated flavonoids.

Flavones	Flavanones	Flavonols
		
Nobiletin	Hesperitin	Quercetin
		
Sinensetin	Naringenin	Fisetin
		
Tangeretin	Hesperidin	Myrecetin
		
Eupatilin	Naringin	Kaempferol
		
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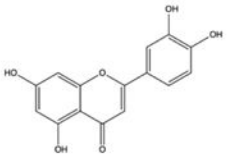		
Apigenin