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ORIGINAL PAPER

Neuroprotective Effects of Methanolic Extract of Avocado *Persea americana* (var. Colinred) Peel on Paraquat-Induced Locomotor Impairment, Lipid Peroxidation and Shortage of Life Span in Transgenic *knockdown* Parkin *Drosophila melanogaster*

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder associated with oxidative stress. Therefore, finding new antioxidant sources might be beneficial for its treatment. Avocado *Persea americana* is a fruit widely cultivated in tropical and subtropical climates worldwide. Although avocado by-products in the form of peel, seed coat and seeds are currently of no commercial use, they constitute a natural source of bioactive compounds. Methanolic (80%) extract obtained from lyophilized ground peels, seed coats, and seeds of the avocado Hass, Fuerte, Reed and Colinred varieties were analyzed for their total phenolic content (TPC) and their correlations with antioxidant capacity (AC) were assessed by ABTS, FRAP, and ORAC assays. For all varieties, the var. Colinred peel shows the highest TPC and AC. Further analysis showed that the var. Colinred peel presented major phenolic compounds B-type procyanidins and epicatechin according to HPLC–MS. The antioxidant effect of peel extract was evaluated upon in vivo oxidative stress (OS) model. We show for the first time that the peel extract can protect and/or prevent transgenic *parkin Drosophila melanogaster* fly against paraquat-induced OS, movement impairment and lipid peroxidation, as model of PD. Our findings offer an exceptional opportunity to test natural disease-modifying substances from avocado's by-products.

Keywords Antioxidant capacity · Avocado · Methanolic extracts · *Persea americana* · Total phenolic content · HPLC–MS

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder associated with a selective loss of the dopaminergic (DAergic) neurons in the *substantia nigra* pars compacta, which results in the characteristic motor features such as hypokinesia or akinesia, postural instability, and tremor [1]. Although the cause(s) of the disorder is (are) not yet fully established, it has been shown that oxidative stress (OS) plays a critical role in neuronal dismiss [2]. Accordingly, pharmacological antioxidant therapy has been explored as a potential strategy to prevent neural death and disease progression (e.g., Ref. [3]). Similarly, the traditional herbal medicines that are compounds extracted from natural plant products have proved an invaluable source of therapeutic agents for drug development for PD [4]. Unfortunately, modification of the disease course by antioxidant and/or neuroprotective therapy is an important unmet clinical need [5]. Therefore, finding new natural antioxidant sources, which can be less toxic, may have reduced side effects and inexpensive that the synthetic molecules/drugs, might be critical for its treatment [6].

Mounting evidence has shown an association between the increase in the risk for developing PD and the use of pesticides and fungicides (e.g., paraquat, PQ^{2+} [7, 8]). PQ^{2+} is a redox cycling neurotoxic compound widely used for in vivo model of PD [9–11]. Indeed, PQ^{2+} causes selective degeneration of dopaminergic neurons in the fly *Drosophila*

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melanogaster through OS [12], reproducing an important pathological feature of PD [13]. Specifically, PQ^{2+}-induced locomotor impairment, lipid peroxidation (LPO), and decreased life span in the transgenic knockdown (KD) parkin *Drosophila melanogaster* through OS [14, 15]. Therefore, *Drosophila* can be used as a screening platform to assess potential phytochemicals [16] that can reduce or halt the OS burden at which DAergic neurons are exposed [15, 17].

Avocado (*Persea americana*, Lauraceae) is one of the earliest edible fruits in Mesoamerica [18]. The fruit is widely cultivated in tropical and subtropical climates worldwide, and consists of numerous hybrids or varieties [19]. Colombia is the third avocado producer in the world and the common varieties of use in commerce include Hass, Fuerte, Reed, and Colinred, among other varieties. At present, the avocado pulp is exploited only for human consumption, generating a huge quantity of peel, seed coat and seed as by-products. Since the avocado seed and peel have high amounts of extractable bioactive compounds such as polyphenols [20], this has attracted pharmaceutical and medical research. In fact, seed and peel provide several secondary metabolites including alkanos, terpenoid glycosides, furan ring-containing derivatives, and flavonoids [21]. Specifically, flavonoids are phenolic compounds characterized by fifteen carbon atoms distributed in two benzene rings connected by a chain of three carbon atoms, that can form a third ring [22]. Interestingly, polyphenols have demonstrated antioxidant properties, anti-inflammatory, and regulation of autophagy in important human neurodegenerative disorders including PD [23]. Therefore, avocado constitutes an alternative natural source of bioactive polyphenolic compounds [24] that might be cost effective for the treatment of PD [25]. Although the antioxidant activity and phenolic profile of avocado have been previously described in peels and seeds of Hass and Fuerte varieties [26–28], detailed information of avocado's sub-products is still scarce in Colombian's avocado cultivars. Therefore, the first aim of the present study was to determine the morphometric features, the total phenolic content (TPC), and antioxidant capacity (AC) of methanolic extracts from the peels, seed coats and seeds from four avocado varieties, namely Hass, Fuerte, Reed and Colinred. Subsequently, the variety and avocado's part with the highest TPC and AC (i.e., var. Colinred, peel) was selected for further phenolic compound characterization and content profile.

Since avocado by-products might be a natural source of antioxidants for PD treatment, the second aim was to establish whether the methanolic extract avocado var. Colinred peel was capable to protect and/or prevent transgenic parkin fly against PQ^{2+}-induced OS, movement impairment and LPO. Our findings offer an exceptional opportunity to test natural disease-modifying substances from avocado's by-products for the treatment of PD in the well-recognized *Drosophila* model of PD [29, 30].

Materials and Methods

Chemicals and Materials

Polyphenols gallic acid (GA), epicatechin (EC), the solvent used for the sample extraction methanol, and other reagents, unless specified otherwise, were purchased from Sigma Aldrich (St. Louis, MO, USA) and all reagents were commercial products of the highest purity grade available.

Plant Material and Extracts Preparation

Avocado fruits (*Persea americana* Mill.) varieties Hass, Fuerte, Reed and Colinred were obtained from the farm "Las Guacamayas" north of Antioquia, Colombia at 2420 meters above sea level (m.a.s.l.), with relative humidity of 80%, ambient temperature Min: 6.9 °C and Max: 25.7 °C with an average of 16.3 °C. Soil temperature was min: 15.6 °C and max: 22.1 °C. The fruits were kept at room temperature until they reached ready-to-eat ripeness. The avocado fruits were morphometrically recorded and the parts were dissected in peels, seed coats and seeds. The extraction of phenolic compounds was carried out according to Ref. [27].

Total Phenolic Content

The total phenol content (TPC) of methanolic extract of *Persea americana* was determined spectrophotometrically by the Folin–Ciocalteu method [31].

Analysis of In Vitro Antioxidant Capacity

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was carried out according to Ref. [32] with minor modifications. The AAPH, Trolox and Fluorescein were used as a peroxyl radical generator, standard reagent and fluorescent probe, respectively. The relative ORAC values were calculated using the differences of areas under the decay curves (AUC) and the results are expressed as mmol Trolox equivalents (0–0.2 mM, final concentration) per 100 g sample (mmol TE/100 g). Data are reported as the mean values ± standard deviation (SD) of three measurements.

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS).

The antioxidant capacity assay was determined as described in Ref. [33].

Ferric Reducing Antioxidant Power (FRAP).

The ability of the samples to reduce the TPTZ–Fe(III) complex was measured according to the method reported in Ref. [34].

Qualitative and Quantitative Analyses of Phenolics and Flavonoids Compounds by HPLC

HPLC analyses were carried out according to Ref. [35] using an Agilent 1200 Series Rapid Resolution Liquid Chromatography system (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, an autosampler, a quaternary pump and a Diode-Array Detector (DAD). Separation of the compounds was performed using an Agilent Zorbax SB Rapid Resolution High Throughput® (RRHT) C18 (50 mm × 4.6 mm, with a 1.8-µm particle size) column, with a flow rate of 1.2 mL/min at 47.5 °C. To identify the flavanol oligomers, an LC–MS method was developed using an Agilent 1200 LC-MSD-Q (Agilent Technologies, Palo Alto, CA, USA) with an electrospray ionization (ESI) interface, using DAD (280 nm) and MS detection simultaneously. The ionization of flavanol monomers and oligomers was performed using the ESI source on negative mode with the following settings: drying gas flow (N2) 5 L/min, nebulizer pressure 60 psi, drying gas temperature 350 °C, vaporizer temperature 400 °C, capillary voltage 3 kV, and fragmentor 150 V. Selective detection was performed by single ion monitoring (SIM) of the parent ions [M–H] for catechin and epicatechin (m/z 289), as well as procyanidin dimers, trimers, tetramers, pentamers (m/z 577, m/z 865, m/z 1153, and m/z 1441, respectively) and chlorogenic and neochlorogenic acids (m/z 353).

Fly Stock and Culture

Directed suppression of *parkin*-related transgenes was achieved using GAL4/UAS (upstream activating sequence) system, with lines described as follows: WT (Bloomington Stock Center #3605, genotype: w^{1118}); *TH-GAL4* (BSC #8848,: *w[*];; P{w[+mC]=ple-GAL4.F}3*) and *UAS-parkin-RNAi* (Vienna Drosophila RNAi stock center #47636,: W^{1118}*; P{GD5543}v47636*). Stock vials of *Drosophila melanogaster* were raised at 25 °C on 12 h light/dark cycle in bottles containing Nutri-Fly™ (Flystuff–Genesee scientific) fly food medium. Propionic acid was added to prevent fungal growth (Merck–Schuchardt OHG D-85662 Hohenbrunm Germany). Knockdown (KD) *parkin* female flies F1 (fF1) *TH>parkin-RNAi/+*(w[+]; UAS-parkin-RNAi/+;TH-GAL4/+) and *TH/+*(w[+];+;TH-GAL4/+) were obtained by crossing female (VDRC #47636; BSC#3605) with male (BSC #8848). KD *parkin* fF1 were collected under brief CO_2 anesthesia from 2 to 3 days after eclosion for further experiments.

Paraquat Toxicity, Protection, Prevention, Survival and Locomotion Assay

The paraquat toxicity, protection, prevention, survival and locomotion Assays were performed on virgin 2- to 3-day-old fF1 flies as exactly described in Ref. [9].

Western Blotting Analysis

Adult fly heads (n = 100) were homogenized at 4 °C in a lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/mL leupeptin with protease inhibitor PFMS 1 mM), samples were place at − 80 °C for 5 min and then centrifuged for 15 min × 13,000 rpm at 4 °C, supernatant was recovered and then stored at − 80 °C. The resulting protein supernatants were subjected by the method of Bicinchoninic acid assay (BCA) using bovine serum albumin as the standard to ensure equal protein loading. The protein supernatants (30 µg) were then resolved on 8–12% SDS/PAGE Bis–Tris gels and transferred to Hybond ECL 0.45 µm Nitrocellulose membranes (GE Healthcare Life Sciences). The membranes were blocked in TBS (pH 7.4, 10 mM TrisHCl/150 mM NaCl/0.1%) containing 5% nonfat milk. The membranes were incubated overnight at 4 °C with the primary antibody anti-tyrosine hydroxylase (anti-TH, ab112, Abcam Inc; 1:1000 dilution) and anti-Parkin (Sigma-Aldrich, SAB1300355; 1: 1000 dilution). β-Actin (anti-actin, ab50412, Abcam, Inc; 1: 5000 dilution) was used as a loading control. Proteins were detected by IR fluorescence with an Odyssey imager (LICOR Biosciences). The WB analysis was assessed three times in independent experiments.

Lipid Peroxidation (LPO) Assay

Quantification of lipid peroxidation involving TBARS (thiobarbituric acid reactive substance) was performed according to Ref. [17].

Statistical Analysis

Morphometric, TPC, AC data are presented as Means ± Standard Deviation (SD) for at least three replications for each sample. Data were analyzed by one-way ANOVA statistical model with Tukey's posttest using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA). Differences were considered statistically significant when $p < 0.05$.

Results

Morphometric Features of Avocado

Due to phenotypic variability of cultivars [36], the size of the fruits has to be determining in a case-by-case basis. Supplementary Table 1 shows the morphometric measurements of the whole fruit of var. Hass and Fuerte, which are a Mexican–Guatemalan hybrid; var. Reed, which possibly resulted from a cross between 'Anaheim' (Guatemalan hybrid originated in California) and 'Nabal' (Guatemalan hybrid); and var. Colinred (Guatemalan X West Indian races). The varieties show common morphologic characteristics such as oval form, pale green pulp, 1 large seed only, rough or smooth surface. The pear-shaped fruit show no statistically significant difference in length, width and weight among the studied varieties (Suppl. Table 1).

Total Phenolic Content of the Methanolic Extracts of *Persea americana* and Its Relation to the Antioxidant Activity In Vitro ORAC, ABTS and FRAP

Methanol has extensively been used as solvent to extract the most representative phenolic compounds from avocado (e.g., [27, 37]), and had the strongest antioxidant activity when compared to other solvents [26, 38]. We therefore used methanol (80%) to extract the secondary metabolites from avocado samples. The initial extraction procedure is aimed generally at maximizing the amount and concentration of the compounds of interest. Because extraction can be considered a very critical step, we followed a strict step-by-step procedure (Suppl. Figure 1a–h) to obtain lyophilized vegetal material (Suppl. Figure 1i) under standard laboratory conditions. The plant material (dry weight, g) was used for total phenolic content (TPC) analysis measured by Folin Ciocalteu's colorimetric assay. As described in Suppl. Table 2, peels from the four varieties displayed almost 3- to 8-fold increase of TPC (e.g., Colinred 315.50 ± 2.19 mg GAE/g TPC) when compared to seed coats (e.g., Reed 106.60 ± 0.98 mg GAE/g TPC) and seeds (e.g., Colinred 39.27 ± 1.47 mg GAE/g TPC).

Antioxidant Capacity

Table 1 shows that all parts of the avocado varieties (peel, seed coat, and seed) presented antioxidant activity according to the ORAC, ABTS and FRAP assays. Peels from var. Colinred and seeds from var. Reed showed the highest ORAC, ABTS and FRAP antioxidant activity. However, Colinred

Table 1 Antioxidant capacity (ORAC, ABTS, FRAP) of peel, seed coat and seed extracts of avocado varieties hass, fuerte, reed and colinred

Part of fruit	Variety	ORAC		ABTS		FRAP	
		mmol TE/100 g[1]	± SD	mmol TE/100 g[1]	± SD	mmol Fe^{2+}/100 g[2]	± SD
Peel	Hass[A]	733.29[b,c,d]	0.449	743.00[b,c,d]	0.042	732.28[b,c,d]	0.043
	Fuerte[B]	759.77[a,c,d]	0.238	660.00[a,c,d]	0.084	707.10[a,c,d]	0.073
	Reed[C]	523.16[a,b,d]	0.634	587.00[a,b,d]	0.155	555.39[a,b,d]	0.132
	Colinred[D]	**788.63[a,b,c]**	**0.295**	**766.00[a,b,c]**	**0.141**	**774.40[a,b,c]**	**0.178**
Seed Coat	Hass[A]	130.83[b,c,d]	0.189	**378.50[b,c,d]**	**0.049**	**200.10[b,c,d]**	**0.061**
	Fuerte[B]	95.43[a,c,d]	0.063	95.50[a,c,d]	0.007	94.69[a,c,d]	0.011
	Reed[C]	100.43[a,b,d]	0.135	271.50[a,b,d]	0.035	195.30[a,b,d]	0.041
	Colinred[D]	**136.58[a,b,c]**	**0.072**	174.50[a,b,c]	0.035	141.91[a,b,c]	0.039
Seeds	Hass[A]	45.72[b,c,d]	0.025	37.40[b,c,d]	0.008	42.53[b,c,d]	0.014
	Fuerte[B]	28.49[a,c,d]	0.071	23.20[a,c,d]	0.004	26.42[a,c,d]	0.009
	Reed[C]	**57.49[a,b,d]**	**0.0104**	**46.45[a,b,d]**	**0.002**	**52.90[a,b,d]**	**0.007**
	Colinred[D]	40.30[a,b,c]	0.087	29.20[a,b,c]	0.005	34.70[a,b,c]	0.010

Numbers in bold represent the highest value of antioxidant activity

ABTS 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, *FRAP* ferric reducing antioxidant power, *GAE* gallic acid equivalents, *ORAC* oxygen radical absorbance capacity, *TE* trolox equivalents, *SD*standard deviation

Values are means of three replicates ± standard deviation

Capital letters (A, B, C, D) represent avocado varieties. Means followed by different minor letters (a, b, c, d) in the same column are significantly different from each other by the Tukey's test ($p < 0.05$)

[1]Expressed as mmol Trolox equivalents per g of avocado peel, seed coat, and seed (lyophilized)

[2]Expressed as mmol Fe^{2+} per g of avocado peel, seed coat, and seed (lyophilized)

peels showed higher AC than Reed seeds (i.e., Colinred had 13.72, 16.49 and 14.88-fold increase mmol TE/100 g in ORAC, ABTS and FRAP assays, respectively when related to var. Reed). In addition, we found a direct, almost perfect, correlation between the amount of TPC and AC in vitro ORAC, FRAP and ABTS extract of *Persea americana* var. Colinred peel (Pearson correlation index = TPC vs ORAC: 0.956 $P \leq 0.001$; TPC vs ABTS 0.971 $P \leq 0.001$; TPC vs FRAP 0.993 $P \leq 0.001$).

Identification and Quantification of Phenolic Compounds of Methanolic Extracts of *Persea americana* var. *Colinred* by HPLC–MS-DAD

Since the peel from Colinred variety showed the highest TPC and AC, it was selected for further analysis. The HPLC–MS-DAD fingerprinting of its methanolic extract revealed the presence of three unidentified compounds ($t_R = 0.634$ min, Peak 1; $t_R = 0.785$ min, peak 2 and $t_R = 2.576$ min, peak 4) and 16 peaks were identified as neochlorogenic acid ($t_R = 1.537$ min, peak 3), chlorogenic acid ($t_R = 2.900$ min, peak 5) (+)-catechin ($t_R = 3.358$ min, peak 6) (-)-epicatechin ($t_R = 5.722$ min, peak 9), procyanidin type B-dimer ($t_R = 4.428$ min, peak 7; $t_R = 5.036$ min, peak 8 and $t_R = 10.716$ min, peak 19), procyanidin type B-trimer ($t_R = 7.037$ min, peak 11; $t_R = 7.350$ min, peak 12 and $t_R = 8.551$ min, peak 15), and procyanidin type B tetramer ($t_R = 6.053$ min, peak 10; $t_R = 7.835$ min, peak 13; $t_R = 8.166$ min, peak 14; $t_R = 9.018$ min, peak 16, $t_R = 9.707$ min, peak 17 and $t_R = 10.113$ min, peak 18). Chromatographic peaks were identified (Fig. 1a and Suppl. Table 3) based on the elution order according to reference standards. According to quantitative HPLC (Table 2), the majority of components in the methanolic extract were B-type procyanidins (~55%, dimer, trimer and tetramer), flavanols monomers (e.g. (-)-epicatechin (~32%) and (+)-catechin (~7%), Fig. 1b), with a minor percentage (<5%) of chlorogenic acid and neochlorogenic acid (Fig. 1c).

Methanolic Extracts of *P. americana* var. Colinred Peel Increases Life Span and Locomotor Activity, and Decreases Lipid Peroxidation in Transgenic Knockdown Parkin *Drosophila melanogaster* Treated with PQ

We wanted to evaluate whether avocado Colinred peel administration alleviate transgenic KD fly against PQ toxicity. Initially, we assessed the *GAL4>UAS-RNAi* system to suppress *parkin* in the *Drosophila's* brain. We crossed the tyrosine hydroxylase driver *TH-GAL4* with the *UAS-parkin-RNAi* or WT (w^{1118}) to obtain the transgenic *TH>parkin-RNAi/+*and *TH/+*(control) flies, respectively (Suppl. Figure 2A, B). Western blot revealed a dramatic reduction of the levels of parkin protein in the transgenic flies compared with control (Fig. 2a, b). Further analysis of TH expression—a specific dopaminergic (DAergic) neuron marker showed that the *GAL4>UAS-RNAi* system altered neither the integrity of DAergic neurons in transgenic *TH>parkin-RNAi/+*nor in *TH/+*flies (Fig. 2c, d). It is concluded that the *GAL4>UAS-RNAi* system efficiently reduced the expression of *parkin* (>95%) in *Drosophila melanogaster* with no apparent damage of DAergic neurons.

Next, we treated *TH>parkin-RNAi/+*flies with PQ (1 mM)-a well-known neurotoxin to affect survival life span and locomotor activity in this fly line [14]. Consequently, PQ reduced survival and movement activity of knockdown flies (Fig. 3). Indeed, 50% KD flies exposed to PQ die by day 4th (Fig. 3a, *red line*). Likewise, PQ affected climbing activity of KD flies (Fig. 3b, 50% climbing diminished by day 3rd). We also confirmed that PQ did not affect the expression of TH protein according to Western blot analysis (Fig. 4, track 1 and 2). These observations comply with the notion that PQ provokes neurodegeneration rather cell loss in transgenic *parkin* flies [12].

To characterize the effect of CRE, *TH>parkin-RNAi/+*flies were exposed to low (1 mg/mL) and high (5 mg/mL) CRE alone or in combination with PQ (Suppl. Figure 3A). Here, we show for the first time that the extract of Colinred peel protects *TH>parkin-RNAi/+*against PQ exposure in an inverse concentration fashion. Indeed, Fig. 3 shows that low (*green line*), but not high (*grey line*), CRE significantly increases both survival (Fig. 3a) and climbing activity (Fig. 3b) of KD flies when compared to untreated KD flies (Fig. 3a, b, *blue line*). Interestingly, in 50% of the transgenic flies treated with low CRE and PQ the percentage of survival slightly increased (by 2 days, Fig. 3a *black line*) and climbing performance significantly augmented (by 6 days, Fig. 3b) compared to flies treated with PQ. In contrast, KD flies treated with high CRE and PQ diminished life span and locomotor activity to comparable survival and climbing percentages of flies treated with PQ alone (Fig. 3a, b). Further analysis showed that CRE alone (Fig. 4, track 3) or with PQ (Fig. 4, track 5) did not alter the expression of TH marker according to Western blot analysis.

Previous work has shown that PQ induced LPO—as evidence of OS in KD *parkin* Drosophila [15]. Therefore, we assessed the effect of CRE on PQ-induced LPO (Suppl. Figure 3). As shown in Fig. 5a, KD flies exposed to PQ and CRE significantly reduced the LPO when compared to KD flies treated with PQ alone. This observation suggests that CRE might be an effective antioxidant agent to protect *TH>parkin-RNAi/+*against PQ. Because the EC was the second most abundant component of CRE (Table 2), we evaluated EC (0.1 and 0.5 mM, Suppl. Figure 3F) in the present PQ-induced toxicity paradigm. We found that both

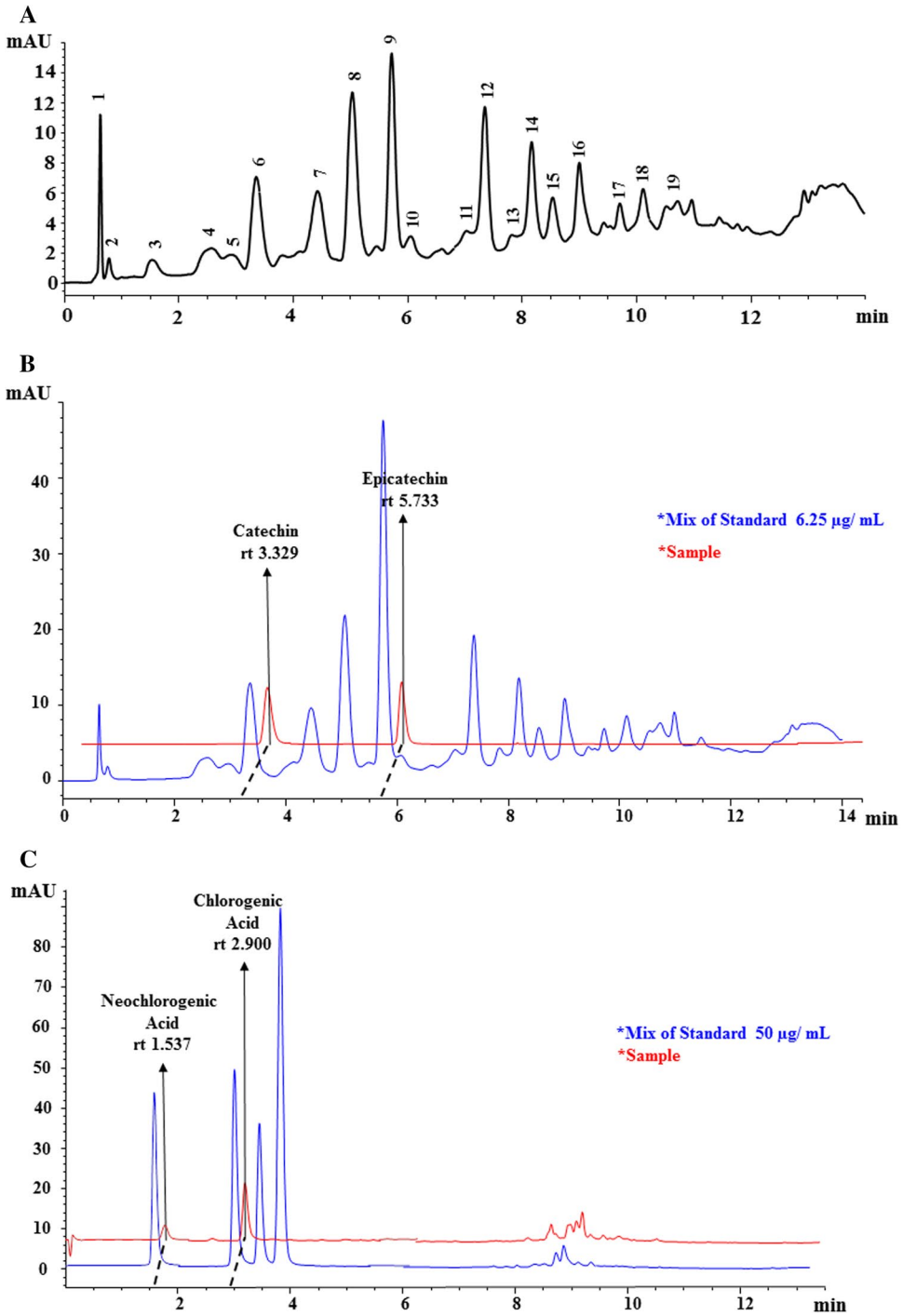

Fig. 1 **a** Chromatogram of polyphenols of avocado var. Colinred peel using UV detector with excitation at a wavelength of 280 nm. Numbers 1, 2 and 4 on the peak denote unidentified compounds, neochlorogenic acid (peak 3), chlorogenic acid (peak 5), catechin (peak 6), procyanidins type B-dimer (peak 7, 8 and 19), epicatechin (peak 9), procyanidins type B-tetramer (peak 10, 13, 14, 16, 17 and 18), procyanidins type B-trimer (peak 11, 12 and 15). **b** Chromatogram of polyphenols compounds in peel of Colinred avocado using HPLC–DAD. Representative chromatogram of catechin (retention time 3.329 min) and epicatechin (r.t. 5.733 min). **c** Chromatogram of polyphenols compounds in peel of Colinred avocado using HPLC–DAD. Representative chromatogram of chlorogenic (r.t. 4.400 min) and neochlorogenic acid (r.t. 3.349 min)

EC concentrations significantly increased life span (Fig. 3c and Table 3) and locomotor activity (Fig. 3d and Table 3) in transgenic flies treated with PQ when compared to KD flies treated with PQ only. Additionally, EC (0.5 mM) did not affect dopaminergic TH levels (Fig. 4, track 4 vs. 6), and significantly reduced LPO index compared to treated flies with PQ (Fig. 5a).

Methanolic Extracts of *P. americana* var. Colinred Peel Prevent Transgenic Knockdown Parkin Drosophila against PQ-Induced Locomotor Impairment, Shortened of Life Span and Increased of LPO

To further analyze the effect of CRE (peel) on parkin deficient *Drosophila,* we exposed KD flies to CRE 5 days before PQ treatment, which lasted for an additional 10 days (Suppl.

Table 2 Quantitation of polyphenol compounds in the methanolic extract of *Persea americana* var. Colinred by HPLC–MS-DAD

Compound	*Persea americana*		
	mg/kg	± SD	RSD (%)
(+)- Catechin	4.77	0.07	1.40
(−)- Epicatechin	20.85	0.90	4.44
Chlorogenic acid	2.79	0.03	1.05
Neochlorogenic acid	1.12	0.02	1.50
Total procyanidins	35.61	1.10	3.10

Results are expressed as mean ± standard deviations (SD) and relative standard deviation (RSD) of three determinations

Figure 3B). PQ reduced the (50%) survival (Fig. 6a) and locomotor activity (Fig. 6b) of *TH>parkin-RNAi/+*flies by day 8th and 8th respectively (Table 3, treatment # 11). However, when KD flies were exposed to PQ and CRE (Table 3, treatment # 12(control) vs. treatment #13 and #14), the survival of *parkin* deficient flies dramatically increased by day 13th and day 14th, respectively (Fig. 6a, b). Noticeably, CRE treatment significantly augmented the locomotor activity in more than 65% flies by day 15th (Fig. 6b and Table 3, treatment # 13, 14). A significant reduction on LPO was observed in KD flies treated with PQ and CRE (Fig. 5b). Interestingly, a significant increased on survival (Fig. 6c), locomotor (Fig. 6d) or decreased LPO index (Fig. 5b) were also observed when KD flies were exposed to EC 7 days before PQ exposure (Table 3, treatment # 16, 17) compared to KD flies treated with EC alone (Table 3, treatment # 15).

Discussion

Here, we report for the first time that CRE peel can protect *parkin* deficient flies against PQ-induced neurodegeneration. However, care should be paid in the dosage of CRE provided as prophylactic because the extract at high concentrations (e.g., 5 mg/mL) might operate as pro-oxidant [39] whereas at low concentrations (e.g., 1 mg/mL) might exert antioxidant activity [40]. Our results also suggested that EC might be involved in the protective effect of avocado peel in *Drosophila* against PQ insult. Since B-type procyanidins are polymeric chains of catechin and epicatechin (containing 2–7 monomeric units linked through C4–C6 or C4–C8 bonding [22], it is conceivable that metabolism of procyanidins provides the monomeric flavanols [41]. However, we do not discard the possibility that procyanidins, catechin [42], chlorogenic acid (an ester of caffeic acid and (−)-quinic acid, a minor compound of CRE) and neochlorogenic acid (minor compound of CRE) may contribute to or exert antioxidant activity under the present experimental conditions. At present, prevention with antioxidants is one of the most

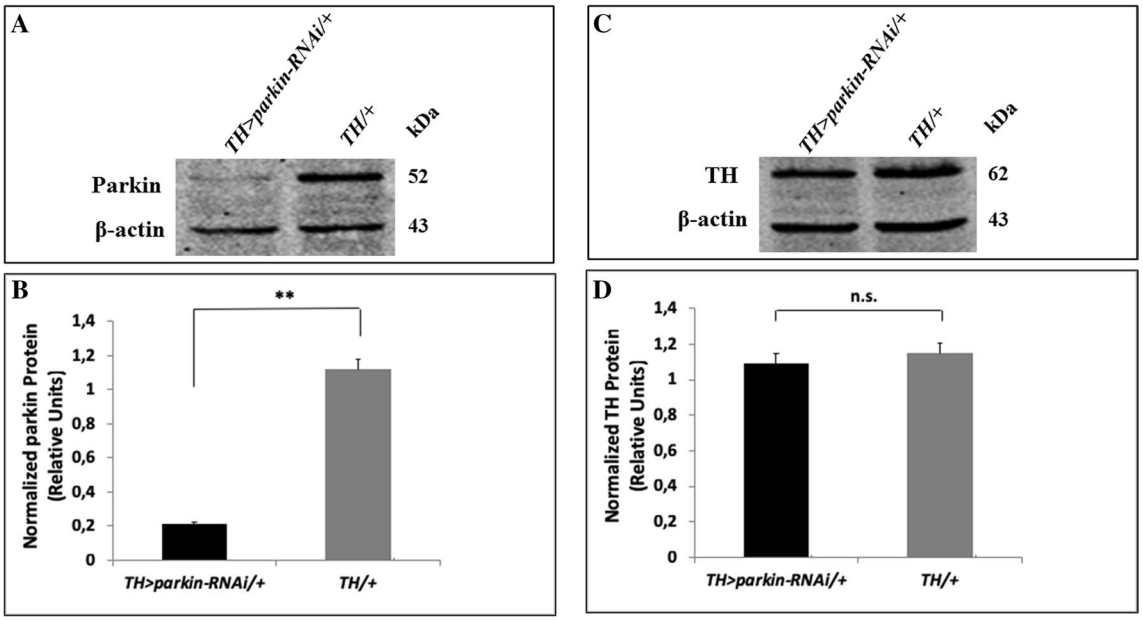

Fig. 2 The binary GAL4>UAS-RNAi system effectively reduced the levels of Parkin protein in the DAergic neurons in the transgenic Parkin Drosophila. The driver TH-GAL4 was crossed with reporter UAS-parkin-RNAi or WT w^{1118} to obtain female (f) F1 *TH>parkin-RNAi/+*and *TH/+*flies, respectively. Proteins in extracts from transgenic *Drosophila's* brain were blotted with primary antibody anti-parkin and anti-tyrosine hydroxylase (TH) as described in "Methods" section. Representative Western blot image for parkin (**a**) and TH (**c**) protein and β-actin. The intensities of the bands in Western blotting were measured (**b** and **d**) by an infrared imaging system (Odyssey, LI-COR), and the intensity was normalized to that of β-actin. Data are represented as mean relative expression ratios ± SD. Data are expressed as mean ± SD *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ was considered significant

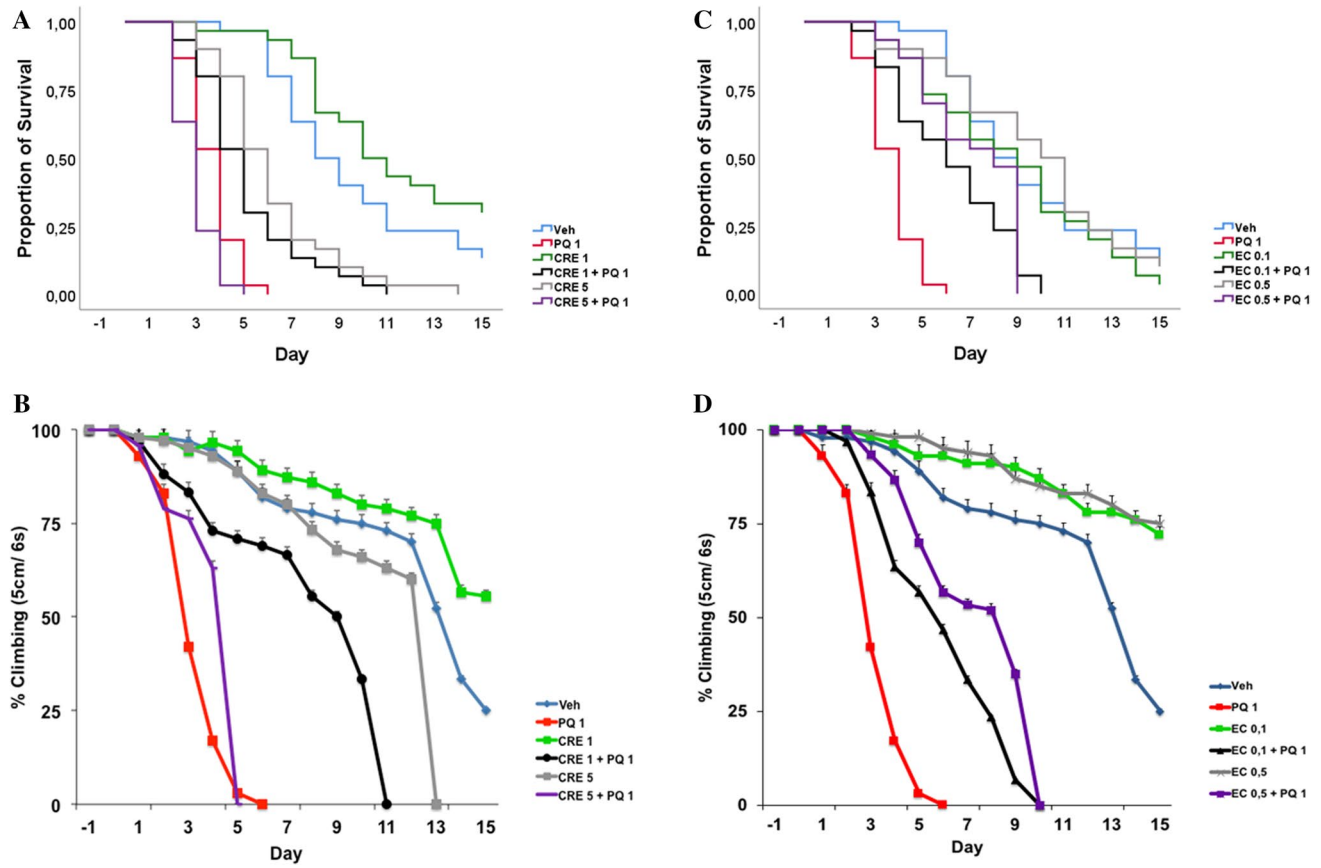

Fig. 3 Methanolic extracts of *Persea americana* var. colinred peel (CRE) and epicatechin (EC) protects *Drosophila melanogaster* TH>parkin-RNAi/+exposed to paraquat, increase life span and locomotor activity. Female flies (n = 60 per treatment) were treated as described in "Materials and Methods". The graphs show that the proportion of survival (**a**, **c**) and climbing performance (**b**, **d**) were significantly increased in *TH-parkin-RNAi/+*flies treated with methanolic extract of *P. americana* var. collinred (CRE) at 1 mg/mL (**a**, **b**), polyphenol EC (**c**, **d**), whereas 5 mg/mL CRE (**a**, **b**) is more toxic to fly. Statistical comparisons between untreated (vehicle, Veh) and treated flies showed a $P \leq 0.05$ by the log-rank test (Color figure online)

innovative therapeutic strategies for PD progression [43]. We report for the first time that avocado *P. americana* var. Colinred peel can prevent transgenic KD *parkinDrosophila* against PQ-induced locomotor impairment, shortened of life span and increased of LPO. Taken together, these observations suggest that polyphenols in CRE might serve as prophylactics in preventive antioxidant therapy in PD.

Horticulturally, it is accepted that the size of avocados depends on its origin. Indeed, while Mexican race produces fruits of small size (fruit is 4–12 cm long, weight 90–240 g), the Guatemalan race produces fruits of medium to large size (fruit is 10–18 cm long, weight 240–1000 g). Cultivation of the West Indian produces fruits of large size (10–25 cm long, weight>1000 g) [19]. In contrast to Rios-Castaño and Tafur Reyes [44], who reported the size order Fuerte (12.30 cm long/9.40 width)>Reed (10.60 l/8.70 w)>Colinred (10.34 l/8.82 w)>Hass (9.16 l/6.79 w), and Colinred as the heaviest one (438.15 g) followed by Reed (430 g), Fuerte (350 g) and Hass (285 g), the varieties studied in the present study showed common morphologic characteristics and no statistically significant difference in length, width and weight. One possible explanation for this discrepancy is that differences in site cultivation locations (e.g., municipality La Candelaria Valle-Colombia (at 950 m.a.s.l.) vs. farm "Las Guacamayas" Antioquia-Colombia (at 2420 m.a.s.l.). Further studies however are required to establish whether phenotypic variability obeys genetic diversity [45] or post-harvest selection. Despite the fact that the avocado has a long history of cultivation in Central and South America, likely beginning as early as 500 BC [19], these observations suggest that the varieties cultivated under similar conditions conserve the typical phenotypic features of each variety, and that the differences among them most probably are due to genetic imprinting and/or epigenetic phenomena.

Interestingly, we found higher TPC values than previously published in peels from Hass [26, 27] and Fuerte [27]. However, we found comparable TPC values in seeds from "Hass" variety (32.33 ± 1.09 mg GAE/g (this work)

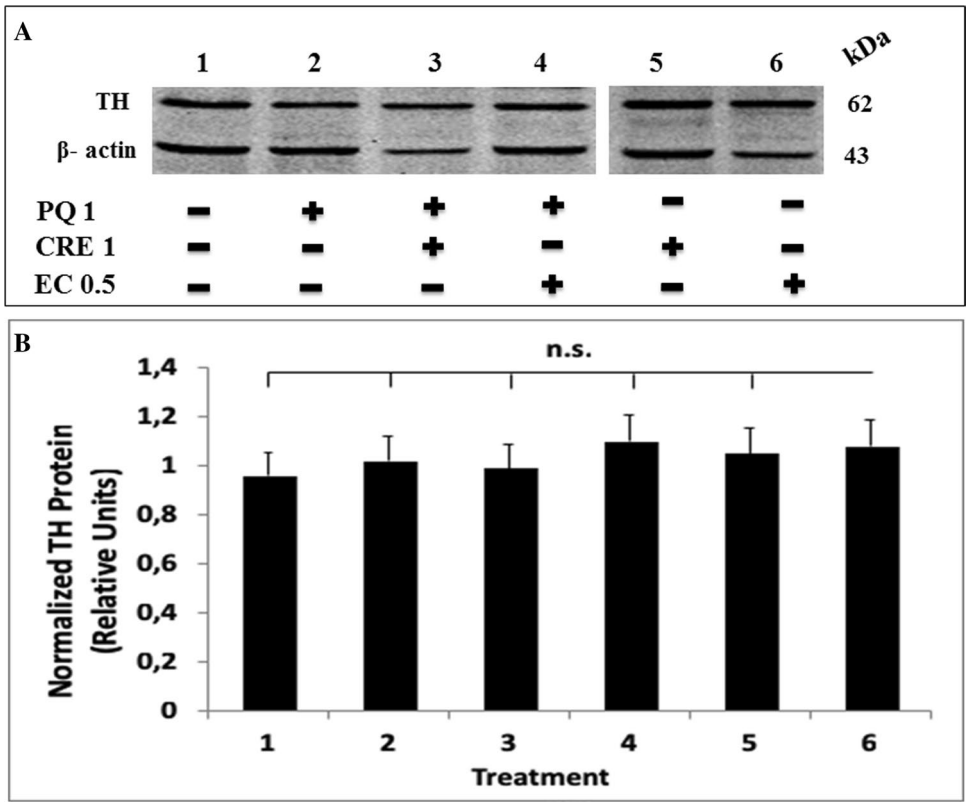

Fig. 4 Effect of paraquat, methanolic extract of *Persea americana* var. Colinred peel and epicatechin (EC) treatments on the expression of the TH protein in *TH>parkin-RNAi/+Drosophila melanogaster* fly heads. Proteins in extracts from transgenic *Drosophila's* brain were blotted with primary antibody anti-tyrosine hydroxylase (TH) and anti-actin as described in "Methods" section. **a** Representative Western blots images for TH protein and β-actin. The protein bands were visualized by an infrared imaging system (Odyssey, LI-COR). Densitometric units of the TH protein bands were normalized to the densitometric units of the corresponding β-actin protein bands on each blot for quantification. (**b**) Data are represented as mean relative expression ratios ± SD. Female adult fly heads from F1 *Drosophila melanogasterTH>parkin-RNAi/+*were used. Lane 1 to 6 represent different treatments e.g., PQ 1 = PQ 1 mM; CRE 1 = CRE 1 mg/mL; EC 0.5 = EC 0.5 mM; (−) = absence; (+) = presence of reagent

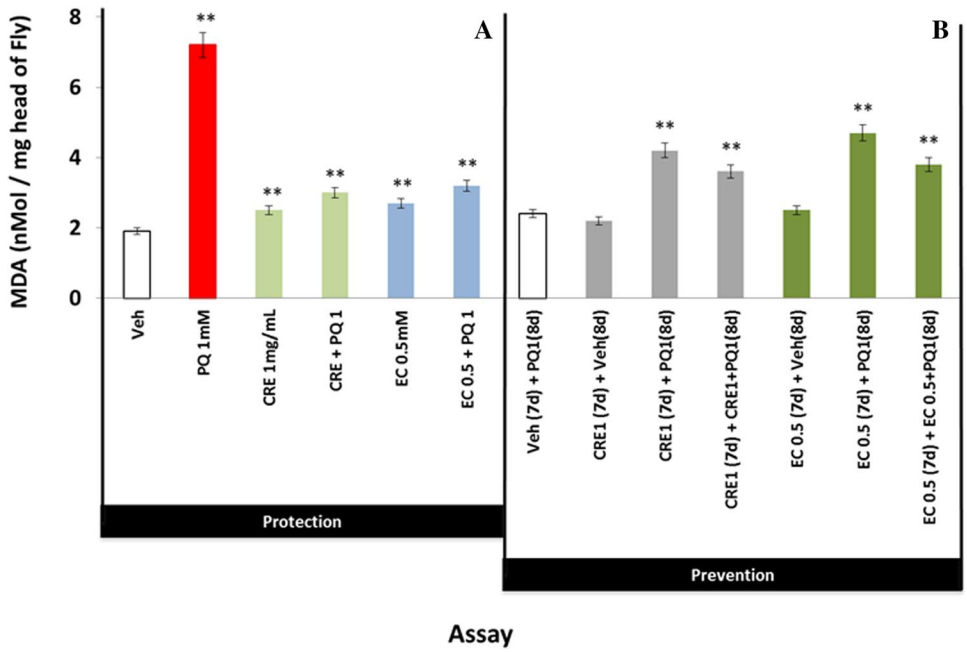

Fig. 5 Methanolic extracts of *Persea americana* var. Colinred peel and polyphenol epicatechin (EC) reduce the lipid peroxidation (LPO) in transgenic *TH-parkin-RNAi/+Drosophila melanogaster* treated with paraquat (PQ). Quantification of lipid peroxidation involving TBARS (Thiobarbituric acid reactive substance) was performed according to Ref. [32] for protection (**a**) and prevention (**b**) feeding schedule. The data are presented as the mean amount of malondialdehyde measured as MDA (nmol/mg fly heads) ± SEM per treatment group. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001

Table 3 Methanolic extracts of *Persea americana* var. *Colinred* peel (CRE) and epicatechin (EC) increases survival and improves locomotor activity of *TH>parkin-RNAi/+*flies treated with paraquat (PQ)

Assays	Treatment	#	*Concentration* mg/mL mM	Effect on			
				Survival (50%)[a]	K–M *p*	Climbing (50%)[b]	χ^2 *p*
Protection	PQ	1	0	9	1 vs. 2*	13	1 vs. 2*
		2	1	4	n.a	3	n.a
	CRE	3	1	11	2 vs. 3*	>	2 vs. 4*
	CRE+PQ	4	1+1	5	3 vs. 4*	9	3 vs. 4*
	CRE	5	5	6	2 vs. 5*	12	2 vs. 6*
	CRE+PQ	6	5+1	3	5 vs. 6*	4	5 vs. 6*
	EC	**7**	**0.1**	**9**	**2 vs. 7***	**>**	**2 vs. 7***
	EC+PQ	**8**	**0.1+1**	**6**	**7 vs. 8***	**6**	**7 vs. 8***
	EC	9	0.5	11	2 vs. 9*	>	2 vs. 9*
	EC+PQ	10	0.5+1	8	9 vs. 10*	8	9 vs. 10*
Prevention	Veh (7d)+PQ (8d)	11	1%+1	8	n.a	8	n.a
	CRE (7d)+Veh (8d)	12	1+1%	15	11 vs. 12*	>	11 vs. 12*
	CRE (7d)+PQ (8d)	13	1+1	13	11 vs. 13*	>	11 vs. 13*
					12 vs. 13*		12 vs. 13*
	CRE (7d)+CRE+PQ (8d)	14	1+1+1	14	11 vs. 14*	>	11 vs. 14*
					12 vs. 14*		12 vs. 14*
					13 vs. 14*		13 vs. 14*
	EC (7d)+Veh (8d)	15	0.5+1%	13	11 vs. 15*	>	11 vs. 15*
	EC (7d)+PQ (8d)	16	0.5+1	13	11 vs. 16*	>	11 vs. 16*
					15 vs. 16*		15 vs. 16*
	EC (7d)+EC+PQ (8d)	17	0.5+0.5+1	14	11 vs. 17*	>	11 vs. 17*
					15 vs. 17*		15 vs. 17*
					16 vs. 17*		16 vs. 17*

Letters and numbers in bold represent data not shown in Fig. 6

PQ Paraquat, *CREColinred* extract, *d* day, *Veh* vehicle solution, *K–M* Kaplan–Meier test, *n.a.* not apply

[a]Represents number of days in which 50% of total flies have been killed

[b]Represents number of days in which 50% of climbing ability is impaired

*$P \leq 0.005$

vs. 35.11 ± 9.98 mg GAE/g [26]. Therefore, the variety that presented the highest TPC in the seeds was the Reed or Colinred varieties (39.73 and 39.27 mg GAE/g, respectively), for the seed coat was the Reed (106.60 mg GAE/g) and for the peel was the Colinred variety (315 ± 2.19 mg GAE/g) (Suppl. Table 2). To the best of our knowledge, this work is the first to report TPC values in peels, seed coats and seeds from Reed and Colinred varieties. Likewise, we report the highest antioxidant capacity from studied avocado varieties when compared to previous published data (e.g., ABTS [26] and ORAC [27]) assay). Taken together, these results comply with the notion that the avocado by-products, mainly the peels, might be rich sources of phenolic compounds. Effectively, in agreement with Wang et al. [20], who reported phenolic compounds of avocado peels from several avocado varieties including Slimcado, Simmonds, Loretta, Choquette, Booth 7, Booth 8, and Tonnage, we found that the majority of compounds identified in Colinred pertain to B-type procyanidins. In contrast to Kosinska et al. [27], who report peels of Shepard variety devoid of (+)-catechin and procyanidin dimers, we detected not only (+)-catechin but also procyanidins in different degrees of polymerization. The amount of (−)-epicatechin was second in abundance (20.85 mg/kg) being procyanidins the major components (35.61 mg/kg) in peel of *P. americana* var. Colinred. Interestingly, studies based on the phytochemical composition of avocado peel have reported similar compositions of approximately 16 compounds as reported here [20, 24, 26–28]. We therefore anticipated that avocado peels from Hass, Fuerte and Reed varieties cultivated in Colombia might produce similar identifiable and quantifiable compounds as observed in Colinred (this work). Therefore, avocado peel Colinred might be considered as potential source of phenolic nutraceuticals.

Some reports have demonstrated that fruits (e.g., Blueberries (*Vaccinium corymbosum* L.), bilberries (*Vaccinium*

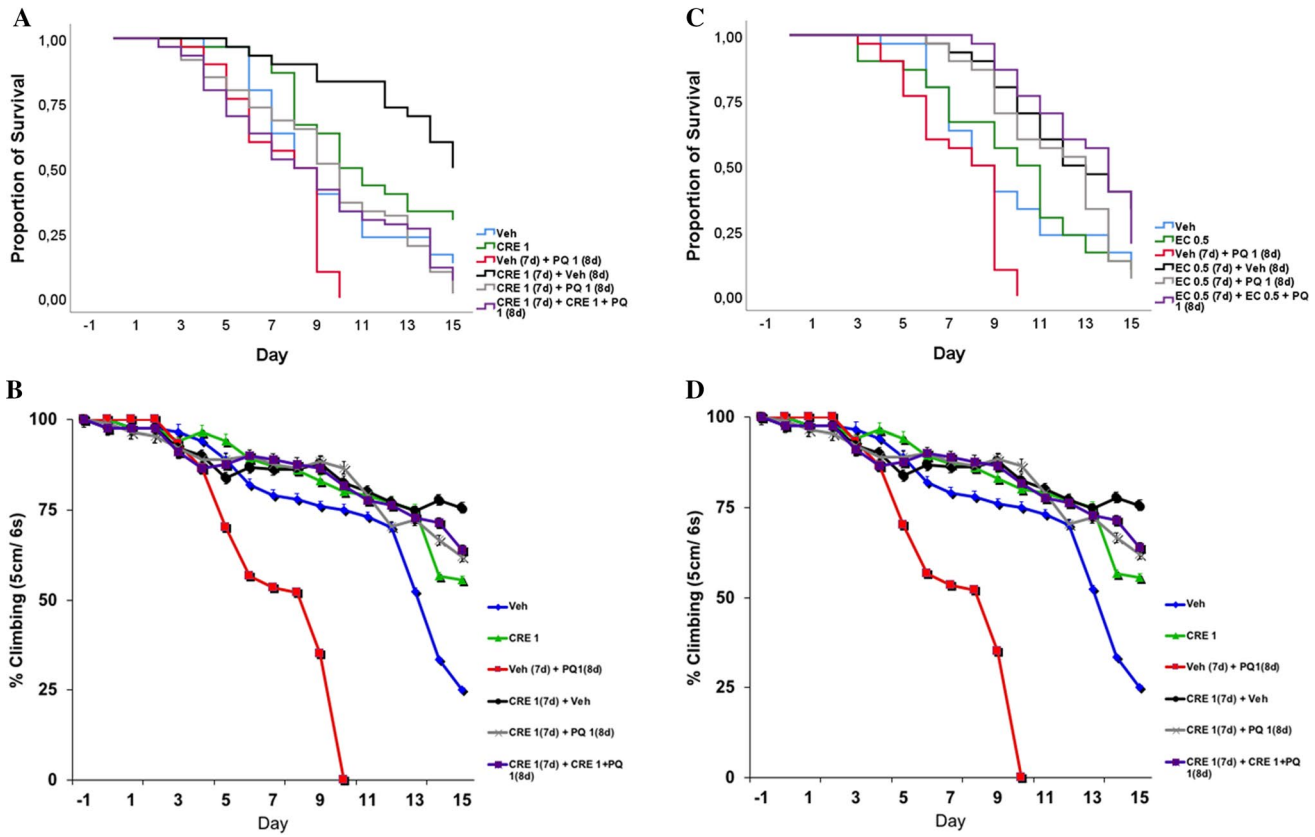

Fig. 6 Methanolic extracts of *Persea americana* var. Colinred peel and polyphenol epicatechin (EC) prevents transgenic *TH-parkinRNAi/+Drosophila melanogaster* against PQ-induced negative effect on life survival and movement alterations. Female flies (n = 60 per treatment) were treated as described in "Materials and Methods". The graphs show that the proportion of survival (**a**, **c**) and climbing performance (**b**, **d**) were significantly increased in *TH-parkinRNAi/+*flies treated with methanolic extract of *Persea americana* var. Colinred (CRE 1 mg/mL) (**a, b**) and treated with EC (0.5 mM) (**c**, **d**) until day 7th. Statistical comparisons between untreated (vehicle, Veh) and treated flies showed a $P \leq 0.05$ by the log-rank test

myrtillus L.), Mulberry (*Morus alba* L.) fruit; Pomegranate (*Punica granatum* L.), among others) have high antioxidant content and capacity, which are related to the high concentration of polyphenols present in them (e.g., [46–49]). Interestingly, Gu et al. [50] have found that mulberry fruit improves PD-related pathology by reducing α-synuclein (α-SN) and ubiquitin levels in a MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) probenecid model. Moreover, Mohammad-Beigi et al. [51] have recently found polyphenolic compounds from olive fruit extracts with high antioxidant activity, strong inhibition of αSN fibril nucleation and elongation with strong disaggregation activity on preformed fibrils and prevention of the formation of toxic αSN oligomers from in vitro assay. However, no data is available to establish whether bioactive molecules from fruits would have similar effects on in vivo assay as reported here. Taken together, these data suggest that fruit polyphenols, in addition to having antioxidant activity, may have therapeutic potential by acting on other pathologic factors of PD.

The present results had some limitations. While our findings show the effect of peel extract on OS-model of PD, the results did not evaluate the effect of the extract on the misfolding, aggregation and accumulation of proteins (e.g., α-SN), dysfunction of the ubiquitin–proteasome pathway, mitochondrial dysfunction, and neuroinflammation, which are part of the neuropathological hallmarks of PD [52]. Therefore, further preclinical (in vitro e.g., primary nerve cell cultures or in vivo e.g., mice) and clinical studies are necessary to confirm the antioxidant effect of *P. americana* avocado peel Colinred extract. Moreover, further investigation is required to reveal whether the extract can reduce other dysfunctional factors involved in the neurodegeneration process of PD.

In conclusion, our data suggest that extracts from avocado, especially the peel from the variety *Colinred* might be of potential use as antioxidant natural source to protect or prevent PD against OS. Our findings offer an exceptional opportunity to test natural disease-modifying substances from avocado's by-products in *Drosophila melanogaster*.

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Author Contributions MJ-Del-R and CV-P conceived and designed the experiments; HFO-A performed the experiments; HFO-A, CV-P, MJ-Del-R analyzed the data; MJ-Del-R contributed reagents/materials/analysis tools; CV-P, MJ-Del-R and HFO-A wrote and approved the paper.

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Compliance with Ethical Standards

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

Ethical Approval This study was carried out in accordance with National Legislation for Live Animal Experimentation (Colombia Republic, Resolution 08430, 1993). Experiments with Flies received the approval of the Ethics Committee for Animal Experimentation of the SIU-UdeA (act #83-2013).

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