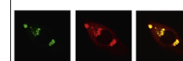


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Research Report

Neuroprotective effects of anthocyanin- and proanthocyanidin-rich extracts in cellular models of Parkinson's disease

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ABSTRACT

Neuropathological evidence indicates that dopaminergic cell death in Parkinson's disease (PD) involves impairment of mitochondrial complex I, oxidative stress, microglial activation, and the formation of Lewy bodies. Epidemiological findings suggest that the consumption of berries rich in anthocyanins and proanthocyanidins may reduce PD risk. In this study, we investigated whether extracts rich in anthocyanins, proanthocyanidins, or other polyphenols suppress the neurotoxic effects of rotenone in a primary cell culture model of PD. Dopaminergic cell death elicited by rotenone was suppressed by extracts prepared from blueberries, grape seed, hibiscus, blackcurrant, and Chinese mulberry. Extracts rich in anthocyanins and proanthocyanidins exhibited greater neuroprotective activity than extracts rich in other polyphenols, and a number of individual anthocyanins interfered with rotenone neurotoxicity. The blueberry and grape seed extracts rescued rotenone-induced defects in mitochondrial respiration in a dopaminergic cell line, and a purple basal extract attenuated

Abbreviations: A β , amyloid- β ; ANC, anthocyanin; BB, blueberry; BC, blackcurrant; BCA, bichinchonic acid; C3G, cyanidin-3-O-glucoside; C3Sa, cyanidin-3-O-sambubioside; C3So, cyanidin-3-O-sophoroside; D3G, delphinidin-3-O-glucoside; D3Sa, delphinidin-3-O-sambubioside; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's minimal essential media; DMSO, dimethyl sulfoxide; EU, endotoxin units; FBS, fetal bovine serum; GS, grape seed; LC-MS, liquid chromatography-mass spectrometry; LPS, lipopolysaccharide; M3G, malvidin-3-O-glucoside; MAP2, microtubule-associated protein 2; MPP⁺, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine; PA, phenolic acid; PAC, proanthocyanidin; PB, purple basil; PBS, phosphate buffered saline; PD, Parkinson's disease; PTFE, polytetrafluoroethylene; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; SPE, solid-phase extraction; TFA, trifluoroacetic acid; TH, tyrosine hydroxylase

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nitrite release from microglial cells stimulated by lipopolysaccharide. These findings suggest that anthocyanin- and proanthocyanidin-rich botanical extracts may alleviate neurodegeneration in PD via enhancement of mitochondrial function.

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that involves a loss of dopaminergic neurons in a region of the midbrain referred to as the *substantia nigra pars compacta* (SNpc). A neuropathological hallmark of PD is the presence in some surviving neurons of Lewy bodies, cytosolic inclusions rich in fibrillar forms of the presynaptic protein α -synuclein (Spillantini et al., 1997). The postmortem brains of PD patients are also characterized by reduced activity of complex I, an enzyme of the mitochondrial electron transport chain (Betarbet et al., 2000). This complex I defect causes a 'leakage' of electrons from the transport chain, leading to the accumulation of reactive oxygen species (ROS) that promote the formation of aSyn aggregates (Betarbet et al., 2000; Rochet et al., 2012). Dopaminergic neurons of the SNpc contain relatively high basal levels of ROS resulting from the metabolism and auto-oxidation of dopamine (Betarbet et al., 2000; Graham, 1978). Therefore, these neurons are thought to be particularly susceptible to pathogenic mechanisms that up-regulate ROS in PD. Moreover, the SNpc has a relatively high density of microglia compared to other brain regions, and microglial activation likely contributes to neurodegeneration in PD by triggering neuroinflammation (Block et al., 2007). Current PD therapies act by controlling the disease symptoms but do not slow the underlying neurodegeneration in the brains of PD patients.

Epidemiological evidence suggests that PD risk increases as a result of chronic exposure to environmental pollutants, including rotenone, a complex I inhibitor used as an insecticide and as a pesticide to control fish populations (Tanner et al., 2011). Rats or primates subjected to prolonged, low-dose rotenone exposure develop a PD-like phenotype characterized by motor dysfunction, a loss of dopaminergic neurons, the formation of Lewy-like inclusions, and microglial activation (Betarbet et al., 2000; Sherer et al., 2003a). In addition, rotenone triggers preferential dopaminergic cell death and aSyn aggregation in primary midbrain cultures (Liu et al., 2008a, 2008b). Rotenone is thought to elicit neurotoxicity by disrupting mitochondrial electron transport, thereby causing a buildup of ROS (Sherer et al., 2003b). In turn, this increase in ROS levels promotes the conversion of aSyn to oxidatively modified species with a high propensity to form potentially neurotoxic oligomers (Conway et al., 2001; Mirzaei et al., 2006; Rochet et al., 2012).

Multiple lines of evidence suggest that diets rich in polyphenols may have neuroprotective effects that result in a lower risk of neurodegenerative disorders including PD (Albarracin et al., 2012; Chao et al., 2012; Lau et al., 2007b). A number of phytochemicals have exhibited neuroprotective effects in cellular and animal models of PD (Chao et al., 2012;

Song et al., 2012), including curcumin (Zbarsky et al., 2005), green tea flavan-3-ols (Choi et al., 2002; Guo et al., 2007; Levites et al., 2001; Mercer et al., 2005), and stilbenes including resveratrol and oxyresveratrol (Blanchet et al., 2008; Chao et al., 2008; Khan et al., 2010). Although polyphenolic compounds are well known for their ROS scavenging ability, the fact that their peak concentrations in the brain are lower than endogenous glutathione levels has led to the suggestion that they may alleviate neurodegeneration via additional protective mechanisms (Del Rio et al., 2013; Milbury and Kalt, 2010; Williams et al., 2004). Consistent with this idea, polyphenols have been found to exhibit an array of neuroprotective activities independent of ROS scavenging (reviewed in Chao et al., 2012; Ramassamy, 2006; Song et al., 2012), including suppression of oxidative stress via effects on mitochondrial respiratory chain function (Morin et al., 2003; Zini et al., 2002) and alleviation of inflammatory responses associated with glial activation (Guo et al., 2007; Kao et al., 2009; Lau et al., 2007a).

Recent epidemiological findings suggest that the consumption of berries (e.g. blueberries, strawberries) rich in two classes of polyphenols, anthocyanins (ANC) and proanthocyanidins (PAC), may reduce the risk of PD (Gao et al., 2012). Although a number of polyphenolic extracts or individual polyphenols have been tested for neuroprotective activity in PD models as outlined above, much less is known about the effects of botanical extracts rich in ANC and/or PAC on PD-related neurodegeneration, or how the neuroprotective activities of these extracts compare to those of extracts rich in other classes of polyphenols. In this study we characterized ANC- and PAC-rich extracts and a number of individual ANC in terms of their ability to alleviate neurotoxicity in primary midbrain cultures exposed to rotenone, and we examined potential underlying mechanisms. Our findings suggest that extracts rich in ANC and PAC protect against rotenone neurotoxicity by alleviating mitochondrial dysfunction.

2. Results

2.1. Study design

The underlying hypothesis of this study was that botanical extracts rich in ANC and/or PAC have neuroprotective activity against PD stresses (Gao et al., 2012). To address this hypothesis we characterized a series of extracts with high levels of ANC and/or PAC, in addition to significant amounts of phenolic acids (PA) and stilbenes (Table 1) (Del Rio et al., 2013; Ramassamy, 2006), in terms of their ability to alleviate neuronal cell death elicited by the PD-related neurotoxin, rotenone. We also examined resveratrol and a Chinese

Table 1 – Botanical extracts characterized in this study.

Extract	Botanical source	Major polyphenols
Blackcurrant (BC)	<i>Ribes nigrum</i>	ANC, PA, PAC
Black plum	<i>Prunus domestica</i>	ANC, PA, PAC
Black plum, dried (prunes)	<i>Prunus domestica</i>	PA, PAC
Blueberry (BB)	<i>Vaccinium corymbosum</i>	ANC, PA, PAC
Chinese mulberry	<i>Morus australis</i>	S
Grape seed (GS)	<i>Vitis vinifera</i>	PAC
Grape skin	<i>Vitis vinifera</i>	ANC, PAC, S
Hibiscus	<i>Hibiscus sabdariffa</i>	ANC
Purple basil (PB)	<i>Ocimum basilicum</i>	ANC, PA

mulberry extract containing high levels of oxyresveratrol (Table 1) to obtain an estimate of the relative neuroprotective activity of ANC and/or PAC compared to that of stilbenes previously shown to alleviate neurotoxicity in PD models (Blanchet et al., 2008; Chao et al., 2008; Khan et al., 2010). After examining a series of extracts with relatively complex polyphenolic profiles, we then tested a simpler ANC-rich extract from a hibiscus plant and four individual ANC for their ability to alleviate rotenone neurotoxicity. Extracts were analyzed via HPLC coupled with photodiode array detection or mass spectrometry (LC-MS) to identify and quantify their polyphenolic constituents (polyphenol identities were determined by analyzing both UV and MS data). In addition, the total polyphenol content in each extract was determined spectrophotometrically using the Folin–Ciocalteu assay (Waterhouse, 2002) (Table 2).

2.2. Neuroprotective activities of ANC-rich extracts

The first set of experiments was carried out to assess the neuroprotective activities of three ANC-rich botanical extracts from blueberry (BB), blackcurrant (BC), and purple basil (PB) (Table 1). Analysis of the BB and BC extracts by reversed phase HPLC coupled with UV and MS detection confirmed that ANC were the predominant polyphenolic components in each extract (Tables 3 and 4; Fig. 1). Whereas the BB extract contained 17 ANC encompassing different cyanidin, delphinidin, malvidin, peonidin, and petunidin glycosides, the BC extract was characterized by a less complex ANC profile consisting of cyanidin and delphinidin glycosides. The PB extract was previously shown to consist of 14 ANC encompassing malonyl or p-coumarylglycoside derivatives of cyanidin and peonidin (Phippen and Simon, 1998). Versions of the extracts recovered after a polyphenol enrichment step on a C18 solid-phase extraction (SPE) column (referred to here as ‘post-C18 extracts’) contained much higher polyphenol levels compared to the initial crude extracts (Tables 3 and 4).

To monitor the effects of the extracts on neurotoxicity, primary midbrain cultures were pre-treated with each extract or vehicle and then exposed to the complex I inhibitor rotenone. The cultures were stained for tyrosine hydroxylase (TH), a marker of dopaminergic neurons, and microtubule-associated protein 2 (MAP2), a general neuronal marker, and relative dopaminergic cell survival was assessed by determining the percentage of MAP2⁺ neurons that were also

Table 2 – Total phenolic concentration of crude and polyphenol-enriched (post-C18) botanical extracts^a.

Extract	× 10 ² mg/g	
	Crude	Post-C18
Blueberry	0.12	2.8
Blackcurrant	3.8	4.4
Purple basil	0.90	4.3
Grape seed	8.3	8.2
Plums	0.10	5.7
Prunes	0.08	4.4
Chinese mulberry	1.5	4.3
Grape skin	0.25	2.2
Hibiscus (SN26)	0.30	N/A
Hibiscus (dark red)	N/A	4.6

^a Determined using the Folin–Ciocalteu assay.

TH⁺. Cultures treated with rotenone plus extracts derived from BB or BC (but not PB) had a higher relative number of dopaminergic neurons than cultures treated with rotenone plus vehicle (Figs. 2A and 3). The BB extract was neuroprotective at a concentration of 0.01 µg/mL but not 0.1 µg/mL, apparently because the extract’s neuroprotective activity was offset by toxicity at the higher dose. Additional data revealed that the BB extract (0.01 µg/mL) induced an increase in dopaminergic cell viability when removed from the cultures 6 h prior to the rotenone treatment (Fig. 2B), suggesting that the extract did not merely carry out a neuroprotective effect by disrupting toxicant entry into the cells. The BB extract also alleviated the rotenone-induced loss of neurites from TH⁺/MAP2⁺ neurons (Fig. 2C and D), implying that BB-mediated increases in TH⁺ cell counts in Fig. 2A and B reflect an enhancement of dopaminergic cell viability rather than just an increase in TH expression. Collectively, these data suggest that BB polyphenols interfere with neurotoxicity in rotenone-treated midbrain cultures.

2.3. Neuroprotective activities of extracts rich in PAC and PA

The next set of experiments was designed to test the neuroprotective activities of three extracts with high levels of PAC and/or PA: (i) a grape seed (GS) extract rich in PAC (Wu et al., 2005); (ii) a plum extract with high levels of ANC,

Table 3 – Polyphenolic composition of blueberry extracts.

Polyphenol	Crude (mg/g)	Post-C18 (mg/g)
ANC ^a		
1. Delphinidin-3-O-galactoside	0.57	18.8
2. Delphinidin-3-O-glucoside	0.28	9.37
3. Cyanidin-3-O-galactoside	0.18	6.23
4. Delphinidin-3-O-arabinoside	0.33	11.3
5. Cyanidin-3-O-glucoside	0.09	2.96
6. Cyanidin-3-O-arabinoside	0.38	15.9
7. Petunidin-3-O-glucoside	0.37	9.67
8. Peonidin-3-O-galactoside	–	2.49
9. Petunidin-3-O-arabinoside	0.19	6.58
10. Malvidin-3-O-galactoside	1.05	36.3
11. Malvidin-3-O-glucoside	0.73	26.5
12. Malvidin-3-O-arabinoside	0.54	21.3
13. Delphinidin-3-O-(6"-acetyl-glucoside)	0.14	6.05
14. Cyanidin-3-O-(6"-acetyl-glucoside)	–	2.13
15. Malvidin-3-O-(6"-acetyl-galactoside)	0.32	9.91
16. Petunidin-3-O-(6"-acetyl-glucoside)	0.11	5.26
17. Malvidin-3-O-(6"-acetyl-glucoside)	0.41	15.2
Total ANC	5.7	206
PAC ^b		
Catechin	n.d.	9.6
Epicatechin	n.d.	0.8
Total PAC	n.d.	10.4
PA ^b		
Chlorogenic acid	2.5	220

n.d., not detected.

^a ANC concentrations were estimated by HPLC as the cyanidin-3-O-glucoside equivalent.^b Quantified by HPLC using reference standards.**Table 4 – Polyphenolic composition of extracts prepared from blackcurrant, plums, and prunes.**

Polyphenol	Blackcurrant		Black plums		Dried plums	
	Crude (mg/g)	Post-C18 (mg/g)	Crude (mg/g)	Post-C18 (mg/g)	Crude (mg/g)	Post-C18 (mg/g)
ANC ^a						
1. Delphinidin-3-O-glucoside	41.2	50.4	n.d.	n.d.	n.d.	n.d.
2. Delphinidin-3-O-rutinoside	126	153	n.d.	n.d.	n.d.	n.d.
3. Cyanidin-3-O-glucoside	20.9	25.8	0.18	14.3	n.d.	0.04
4. Cyanidin-3-O-rutinoside	127	153	0.14	10.8	n.d.	0.05
Total ANC	315	382	0.32	25.1		0.09
PAC ^b						
Catechin	n.d.	4.32	4.70	27.6	0.30	11.1
Epicatechin	n.d.	1.90	1.60	9.20	0.10	3.70
Polymeric PAC	n.d.	n.d.	11.2	239	3.90	161
Total PAC		6.20	17.5	275	4.30	176
PA ^b						
Caffeic acid	n.d.	16.5	0.83	5.40	n.d.	2.60
Chlorogenic acid	n.d.	n.d.	3.40	9.90	n.d.	35.5
Neochlorogenic acid	n.d.	n.d.	3.60	24.6	2.20	85.2
Total PA		16.5	7.83	39.9	2.20	123

n.d., not detected.

^a ANC concentrations were estimated by HPLC as the cyanidin-3-O-glucoside equivalent.^b Quantified by HPLC using reference standards.

PAC, and PA; and (iii) a dried plum (prune) extract rich in PA but with lower relative amounts of PAC and undetectable ANC (Table 4) (ANC are heat sensitive and have been reported

to degrade during drying of plums (Piga et al., 2003)). Our rationale for testing the effects of a GS extract was that this approach would enable us to assess the neuroprotective

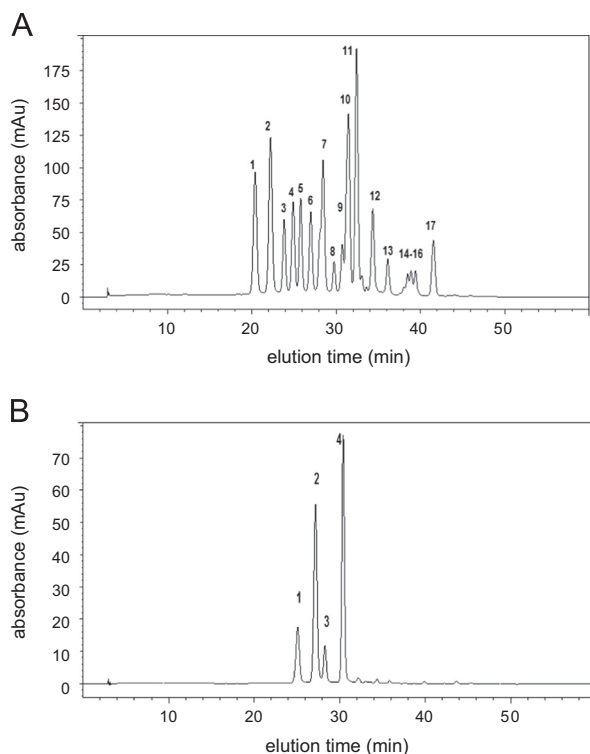


Fig. 1 – RP-HPLC chromatograms showing the ANC composition of blueberry and blackcurrant extracts. Identities of ANC peaks revealed by analysis of the blueberry extract (A) and the blackcurrant extract (B) are shown in Tables 3 and 4, respectively. Absorbance was monitored at 520 nm in (A) and (B). ANC were identified by UV, MS, MS/MS, and comparison with available standards.

activity of PAC with little contribution from other polyphenols. Moreover, a comparison of the effects of plum and prune extracts might yield insight into the neuroprotective activity of PA (Shukitt-Hale et al., 2009). RP-HPLC analysis confirmed the presence of the expected polyphenolic classes in the plum and prune extracts and yielded quantitative data relating to individual constituents (Table 4). The GS extract was previously shown to consist of 22 PAC with different degrees of polymerization and gallate substitution (Wu et al., 2005). Cultures treated with rotenone plus GS extract (Fig. 4A) but not plum or prune extract (Fig. 4C and D) exhibited greater dopaminergic cell survival than cultures treated with rotenone plus vehicle. In addition, the GS extract alleviated rotenone-induced neurite loss from TH⁺/MAP2⁺ neurons (Fig. 4B). The total polyphenol concentration of the GS extract was approximately 80-fold greater than that of the plum extract (Table 2), and this difference may explain why the GS extract but not the plum extract interfered with rotenone neurotoxicity in this set of experiments, despite the fact that both extracts are rich in PAC. Alternatively, the GS extract may consist of different PAC with greater neuroprotective activity compared to those of the plum extract. The total PA level of the plum and prune extracts was similar to that of the BB extract (Tables 3 and 4), implying that BB PA (primarily chlorogenic acid) do not play a major role in suppressing

rotenone-mediated neurodegeneration. These results suggest that PAC alleviate dopaminergic neuron death and neurite loss in rotenone-treated cultures, whereas PA are apparently less neuroprotective.

2.4. Neuroprotective activities of extracts rich in stilbenes

Previous studies revealed that the stilbenes resveratrol and oxyresveratrol protect against neurotoxicity in cellular and animal models of PD (Blanchet et al., 2008; Chao et al., 2008; Khan et al., 2010). Here, we characterized a Chinese mulberry bark extract rich in oxyresveratrol (Table S1 and Fig. S1A), a grape skin extract rich in resveratrol (Table S1 and Fig. S1B) (Careri et al., 2003), and pure resveratrol in terms of their ability to alleviate dopaminergic cell death in primary mid-brain cultures exposed to rotenone. Cultures treated with rotenone plus the mulberry extract or resveratrol (but not the grape skin extract) exhibited greater dopaminergic cell viability than cultures treated with rotenone plus vehicle (Fig. 5). Together, these data suggest that some (but not all) stilbene-rich extracts can protect against rotenone neurotoxicity.

2.5. Neuroprotective activities of extracts purified by SPE

A potential complication in interpreting differences in the biological effects of the botanical extracts outlined above is that different extracts may have different amounts of non-active components including sugars, and these could interfere with neuroprotective activity. To address this issue, we fractionated each extract on a C18 SPE column. In addition to removing sugars, elution of the extracts through a C18 column concentrated the polyphenolic constituents relative to levels in the initial crude extracts (Tables 3 and 4). Analysis of the post-C18 GS extract by LC-MS revealed the presence of a series of proanthocyanidins with different degrees of polymerization and gallate substitution (data not shown) (Wu et al., 2005). Next, we examined whether botanical extracts that failed to suppress dopaminergic cell death prior to the C18 enrichment (grape skin extract and extracts derived from prunes, plums, and purple basil) became neuroprotective after solid-phase extraction. The post-C18 extracts were tested for their ability to alleviate rotenone neurotoxicity at the same polyphenol concentrations as those used to test the neuroprotective effects of the crude extracts (based on the polyphenol concentrations determined using the Folin-Ciocalteu assay; Table 2). Our data revealed that the post-C18 extracts, like the initial crude extracts, were unable to alleviate rotenone-induced dopaminergic cell death (Fig. 6A). These findings suggest that the lack of neuroprotection by the crude extracts did not result from the suppression of polyphenol bioactivity by sugars or impurities – rather it reflected the limited ability of the polyphenolic constituents in these extracts to mitigate rotenone neurotoxicity.

2.6. Neuroprotective activities of post-C18 extracts normalized to polyphenol content

Our next objective was to determine which of the extracts that were found to alleviate neurotoxicity in crude form (BB, BC, GS, and mulberry; Figs. 2–5) had the greatest neuroprotective activity

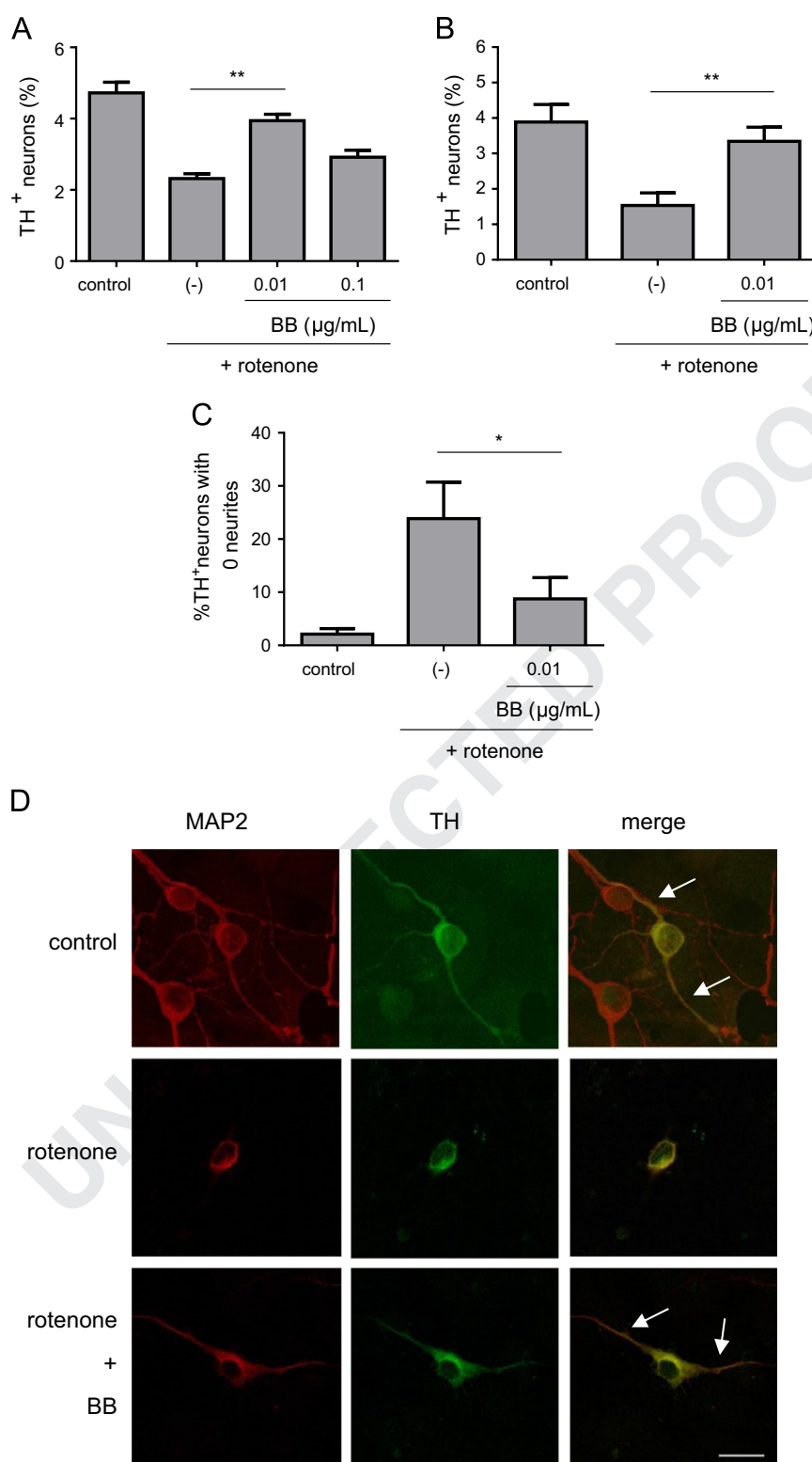


Fig. 2 – A blueberry (BB) extract alleviates rotenone-induced neurotoxicity and neurite loss. Primary midbrain cultures incubated in the absence or presence of a BB extract for 72 h were exposed to rotenone (100 nM) in the absence or presence of extract for 24 h (A). Alternatively, primary cultures incubated in the absence or presence of the BB extract for 66 h were incubated in fresh media (minus extract) for 6 h and then exposed to rotenone (100 nM) in the absence of extract for 24 h (B)–(D). Control cells were incubated in the absence of rotenone or extract. The cells were stained with antibodies specific for MAP2 and TH and scored for relative dopaminergic cell viability (A, B) or neurite loss (C). Representative fluorescence micrographs show the protective effect of the BB extract against rotenone-induced neurite loss (D) (scale bar, 20 μm). The data in panels (A)–(C) are presented as the mean ± SEM; *n* = 3 (A), *n* = 2 (B), or *n* = 4 (C); **p* < 0.05 and ***p* < 0.01.

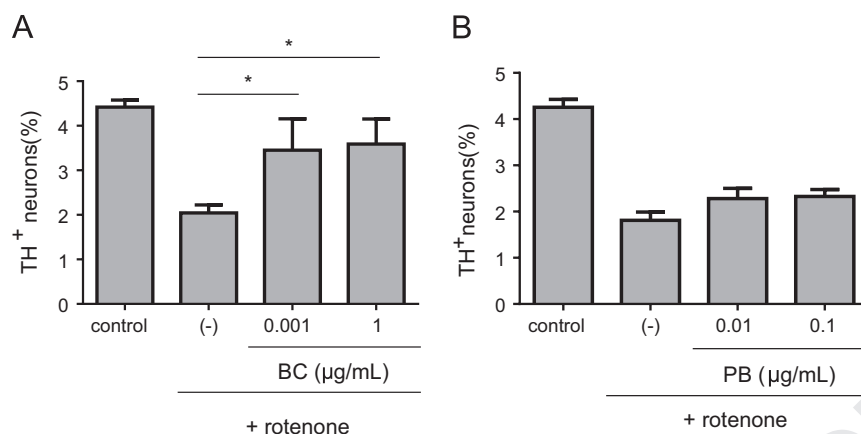


Fig. 3 – Different ANC-rich extracts have different abilities to alleviate rotenone neurotoxicity. Primary midbrain cultures were incubated in the absence or presence of a blackcurrant (BC) extract (A) or a purple basil (PB) extract (B) for 72 h. The cells were then exposed to rotenone (100 nM) in the absence or presence of extract for 24 h. Control cells were incubated in the absence of rotenone or extract. The cells were stained with antibodies specific for MAP2 and TH and scored for relative dopaminergic cell viability. The data are presented as the mean \pm SEM; $n=2-3$ (A) or $n=2-4$ (B); * $p < 0.05$.

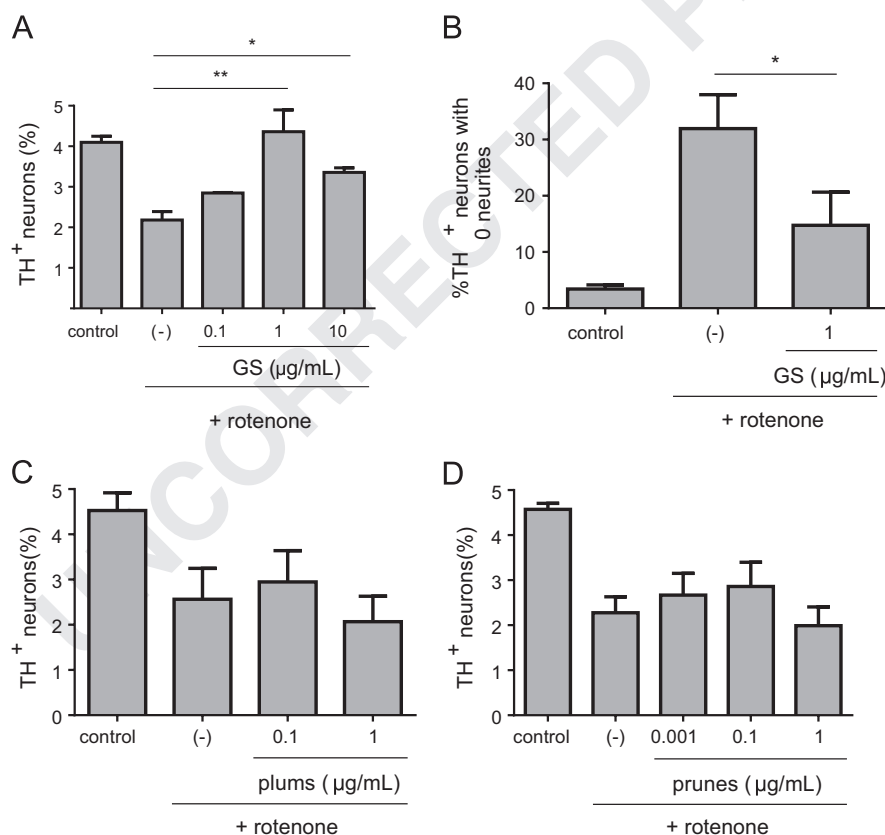
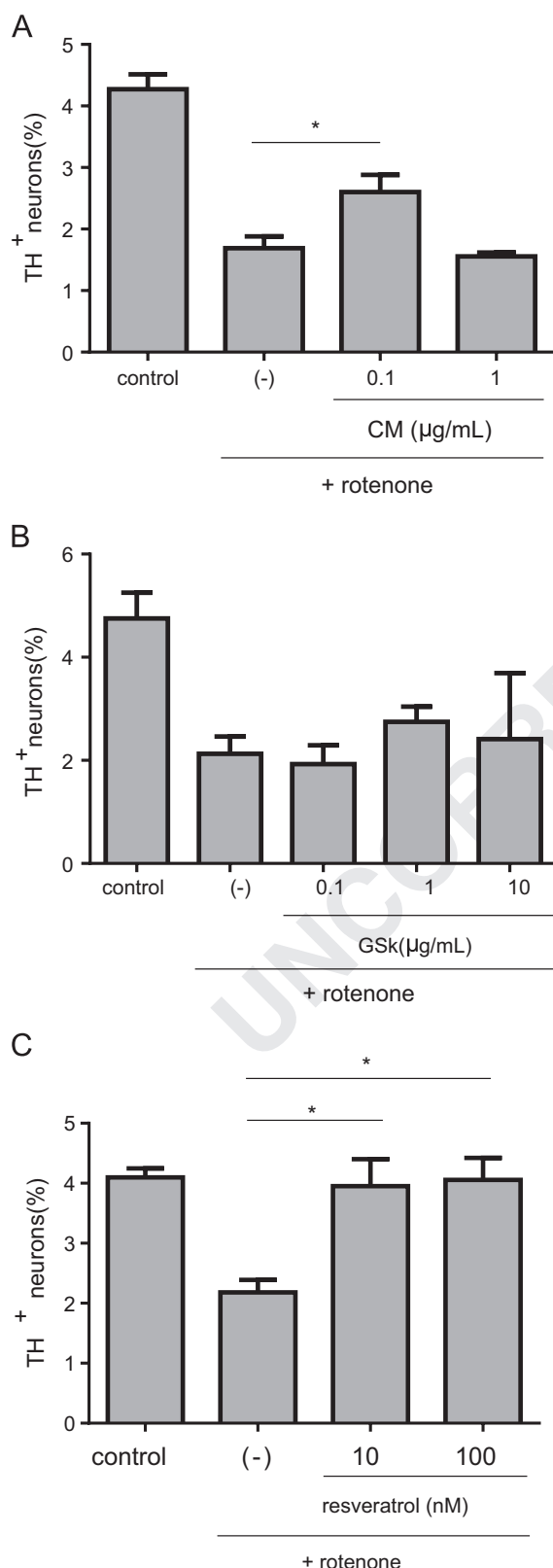


Fig. 4 – Different extracts rich in PAC and PA have different abilities to alleviate rotenone neurotoxicity. Primary midbrain cultures were incubated in the absence or presence of a grape seed (GS) extract (A, B), a plum extract (C), or a prune extract (D) for 72 h. The cells were then exposed to rotenone (100 nM) in the absence or presence of extract for 24 h, stained with antibodies specific for MAP2 and TH, and scored for relative dopaminergic cell viability (A, C, D) or neurite loss (B). The data are presented as the mean \pm SEM; $n=3$ (A), $n=4$ (B), $n=2-3$ (C), or $n=2-6$ (D); * $p < 0.05$ and ** $p < 0.01$.

as post-C18 extracts normalized to polyphenol content. Primary midbrain cultures were exposed to rotenone in the absence or presence of each post-C18 extract at a total polyphenol concentration of 30 ng/mL. Cultures treated with rotenone plus extract prepared from BB, BC, or GS (but not mulberry) exhibited greater

dopaminergic cell survival compared to cultures treated with rotenone plus vehicle (Fig. 6B). These results suggest that extracts with high levels of ANC and/or PAC inhibit rotenone-induced neurodegeneration with greater efficacy than extracts rich in other types of polyphenols, including PA and stilbenes.

Although the data in Table 2 and Figs. 2–5 suggested that the post-C18 extracts should be neuroprotective at polyphenol concentrations as low as 0.1 ng/mL (BB extract) or 0.4 ng/mL (BC extract), we found that the extracts only alleviated dopaminergic cell death at total polyphenol concentrations ≥ 30 ng/mL.



This observation suggests that the crude (pre-C18) extracts have greater neuroprotective activity per unit mass of total polyphenol than the post-C18 fractions.

2.7. Neuroprotective effects of individual ANC

Having shown that the ANC-rich BB and BC extracts have neuroprotective activity, we next examined the effects of individual ANC on rotenone neurotoxicity. In one set of experiments, we examined the effects of two commercially available ANC, malvidin-3-O-glucoside (M3G) and cyanidin-3-O-sophoroside (C3So), and two ANC isolated from a BC extract by counter-current chromatography, delphinidin-3-O-glucoside (D3G) and cyanidin-3-O-glucoside (C3G). Primary midbrain cultures exposed to rotenone plus M3G, C3So, and D3G (but not C3G) exhibited greater dopaminergic cell viability than cultures exposed to rotenone plus vehicle (Fig. 7). In a second set of analyses, we tested the effects of two versions of a hibiscus extract (a crude extract referred to as 'SN26' and a post-C18, dark red extract), each consisting primarily of two ANC, cyanidin-3-O-sambubioside (C3Sa) and delphinidin-3-O-sambubioside (D3Sa) (Table S2) (Juliani et al., 2009). We found that both extracts alleviated dopaminergic cell death in primary midbrain cultures exposed to rotenone (Fig. 8). Together, these results suggest that individual ANC (or mixtures of a small number of ANC) can protect against rotenone neurotoxicity.

2.8. Effects of ANC- and PAC-rich extracts on mitochondrial respiration

Multiple lines of evidence suggest that ANC and PAC (or extracts rich in these polyphenols) alleviate mitochondrial dysfunction via modulatory effects on electron transport in non-neuronal systems (Hokayem et al., 2013; Pajuelo et al., 2012; Xie et al., 2012). Accordingly, we hypothesized that ANC- and PAC-rich extracts mitigate toxicity associated with rotenone-mediated complex I inhibition by rescuing defects in mitochondrial respiration. To address this hypothesis, we tested the extracts listed in Table 1 for their effects on O₂ consumption in MES23.5 neuronal cells exposed to rotenone. The O₂ consumption assay involves plating the cells on a 96-well plate that contains an immobilized O₂-sensitive fluorophore. In the presence of functional mitochondria, O₂ is consumed, leading to a higher (i.e. less quenched) fluorescence signal than that associated with cells with impaired mitochondria. MES23.5 cells exposed to rotenone (100 nM, 24 h) exhibited a ~35% decrease in fluorescence compared to

Fig. 5 – Different stilbene-rich extracts have different abilities to alleviate rotenone neurotoxicity. Primary midbrain cultures were incubated in the absence or presence of extracts derived from Chinese mulberry (CM) (A) or grape skin (Gsk) (B) or the pure compound resveratrol (C) for 72 h. The cells were then exposed to rotenone (100 nM) in the absence or presence of extract or compound for 24 h. Control cells were incubated in the absence of rotenone, extract, or compound. The cells were stained with antibodies specific for MAP2 and TH and scored for relative dopaminergic cell viability. The data are presented as the mean \pm SEM; $n=2-3$ (A), $n=2-4$ (B), or $n=3$ (C); * $p<0.05$.

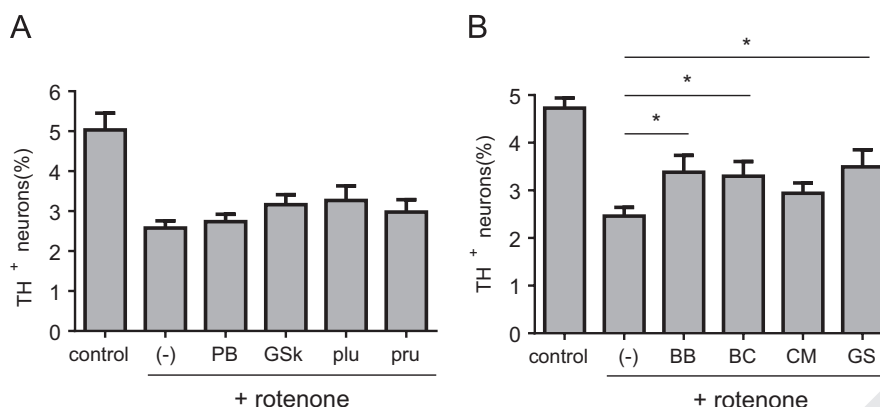


Fig. 6 – Extracts rich in ANC and/or PAC have greater neuroprotective activity than extracts containing other types of polyphenols. Primary midbrain cultures were incubated in the absence or presence of post-C18 extracts derived from purple basil (PB), grape skin (GSK), plums (plu), or prunes (pru) (A) or blueberry (BB), blackcurrant (BC), Chinese mulberry (CM), or grape seed (GS) (B). Each extract was diluted to a final, total polyphenol concentration of 30 ng/mL. The cells were then exposed to rotenone (100 nM) in the absence or presence of each extract for 24 h. Control cells were incubated in the absence of rotenone or extract. The cells were stained with antibodies specific for MAP2 and TH and scored for relative dopaminergic cell viability. The data are presented as the mean \pm SEM; $n=3$ (A) or $n=6$ (B); * $p<0.05$.

control cells (Fig. 9). Rotenone elicited minimal cell loss under these conditions (Liu, F. and Rochet, J.-C., unpublished data), indicating that the decrease in fluorescence mostly reflected a reduced rate of O_2 consumption rather than a decrease in cell viability. MES23.5 cells treated with rotenone plus crude BB or GS extract exhibited greater fluorescence than cells treated with rotenone plus vehicle (Fig. 9). The concentration at which the BB extract produced a fluorescence increase (10 μ g/mL) was substantially greater than the minimal concentration at which it alleviated rotenone neurotoxicity in primary midbrain cultures (0.01 μ g/mL) (Fig. 2). In contrast to the BB and GS extracts, extracts derived from BC, PB, plums, prunes, mulberry, grape skin, or hibiscus showed no effects in this assay (data not shown). These results suggest that the BB and GS extracts rescue rotenone-induced defects in mitochondrial O_2 consumption in MES23.5 neuronal cells.

2.9. Effects of ANC- and PAC-rich extracts on microglial activation

Based on evidence in the literature (Guo et al., 2007; Kao et al., 2009; Lau et al., 2007a), we hypothesized that botanical extracts with high levels of ANC and/or PAC mitigate neurotoxicity by suppressing neuroinflammation associated with microglial activation. To address this hypothesis, we tested the effects of botanical extracts on the production of NO, a neuroinflammatory agent, by the BV2 mouse microglial cell line. BV2 cells were treated with lipopolysaccharide (LPS), a classic inducer of microglial activation (Lau et al., 2007a), in the absence or presence of the extracts listed in Table 1 (post-C18 extracts at a normalized polyphenol concentration of 10 μ g/mL). The culture media was then assayed for levels of the stable NO metabolite, nitrite (Lau et al., 2007a). Nitrite levels were markedly lower in the media from cells exposed to LPS plus the PB extract compared to cells exposed to LPS alone (Fig. 10). The dark red hibiscus extract also appeared to reduce LPS-induced nitrite release, although this effect did

not reach statistical significance (Fig. S2). The other extracts listed in Table 1 had no significant effect on LPS-induced nitrite release in this assay, which showed considerable variability (data not shown). Similar results were obtained when examining the crude (pre-C18) forms of the extracts at a polyphenol concentration of 1–100 μ g/mL (data not shown). None of the extracts had a significant impact on superoxide production in LPS-treated BV2 cells (data not shown). From these data, we infer that ANC and/or PA in the PB extract interfere with microglial activation to a greater extent than polyphenols in the other extracts.

3. Discussion

In this study, we characterized a number of botanical extracts rich in ANC and/or PAC in terms of their neuroprotective activities in primary midbrain cultures exposed to rotenone. An advantage of using this primary cell culture model is that it consists of a mixed population of glial cells and post-mitotic, dopaminergic and non-dopaminergic neurons, similar to the midbrain region affected in PD (Liu et al., 2008b). Accordingly, primary midbrain cultures provide a powerful platform to identify candidate neuroprotective extracts or compounds than can be validated in subsequent in vivo experiments. Moreover, given the reported link between rotenone exposure and PD risk (Tanner et al., 2011), our approach of testing extracts for the ability to alleviate rotenone neurotoxicity has the potential to reveal chemical entities with neuroprotective activity in human PD.

3.1. ANC- and PAC-rich extracts protect primary dopaminergic neurons against rotenone neurotoxicity

A key result from our study is the observation that ANC-rich extracts prepared from BB and BC (but not PB) protected dopaminergic neurons against rotenone toxicity. This finding

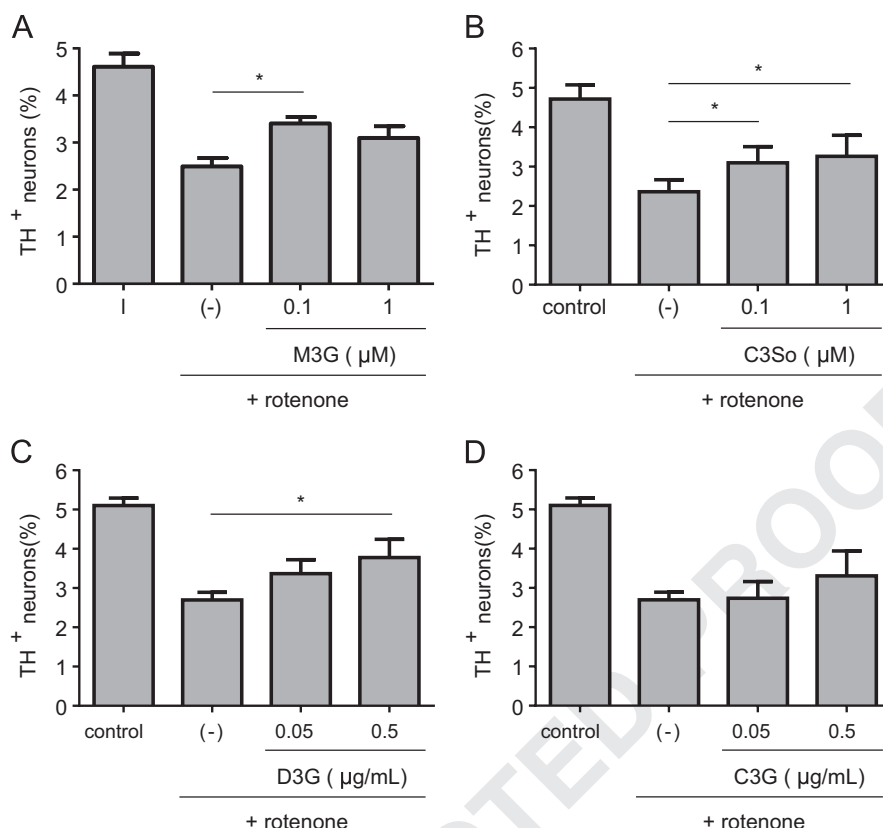


Fig. 7 – Different ANC have different abilities to alleviate rotenone neurotoxicity. Primary midbrain cultures were incubated in the absence or presence of malvidin-3-O-glucoside (M3G) (A), cyanidin-3-O-sophoroside (C3So) (B), delphinidin-3-O-glucoside (D3G) (C), or cyanidin-3-O-glucoside (C3G) (D) for 72 h. (M3G and C3So were purchased compounds, whereas D3G and C3G were isolated from a blackcurrant extract. The concentrations of D3G and C3G are expressed in μg/mL because these ANC were not 100% pure. Given that the purity of these compounds was >95%, their doses of 0.05 and 0.5 μg/mL were approximately equal to 0.1 and 1 μM). The cells were then exposed to rotenone (100 nM) in the absence or presence of each ANC for 24 h. Control cells were incubated in the absence of rotenone or ANC. The cells were stained with antibodies specific for MAP2 and TH and scored for relative dopaminergic cell viability. The data are presented as the mean ± SEM; *n*=6 (A, B) or *n*=5 (C, D); **p*<0.05.

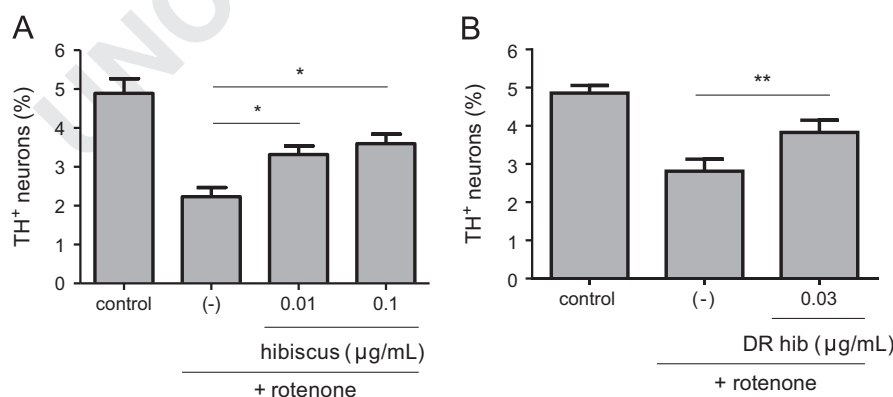


Fig. 8 – Hibiscus extracts containing two major ANC alleviate rotenone neurotoxicity. Primary midbrain cultures were incubated in the absence or presence of a crude hibiscus extract (A) or a post-C18, dark red hibiscus extract (DR hib) (B) for 72 h. The cells were then exposed to rotenone (100 nM) in the absence or presence of extract for 24 h. Control cells were incubated in the absence of rotenone or extract. The cells were stained with antibodies specific for MAP2 and TH and scored for relative dopaminergic cell viability. The data are presented as the mean ± SEM; *n*=2–4 (A) or *n*=6 (B); **p*<0.05 and ***p*<0.01.

is consistent with previous data showing that a mulberry extract from *Morus alba* L. (Moraceae) interfered with dopaminergic cell death in mice exposed to 1-methyl-4-phenyl-

1,2,3,6-tetrahydropyridine (MPTP) and in primary mesencephalic cultures treated with 6-hydroxydopamine (6-OHDA) or the MPTP metabolite, MPP⁺ (Kim et al., 2010). The mulberry

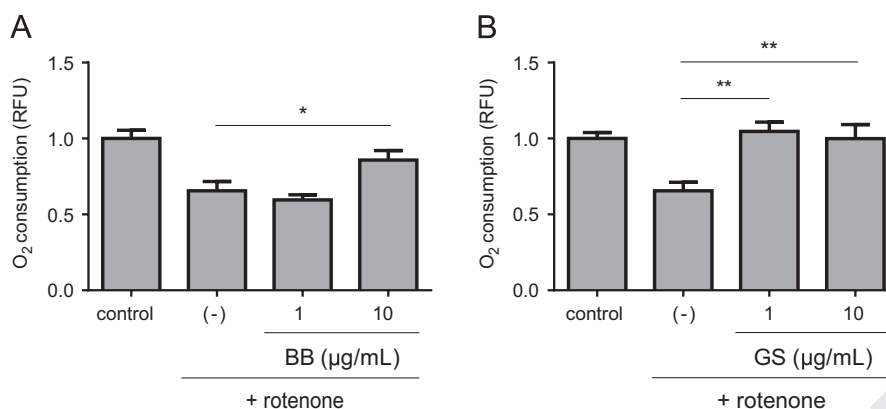


Fig. 9 – Blueberry (BB) and grape seed (GS) extracts alleviate rotenone-induced defects in mitochondrial respiration. MES23.5 cells were plated in a 96-well Oxygen Biosensor plate and treated with rotenone (100 nM) in the absence or presence of a crude BB extract (A) or a crude GS extract (B) for 24 h. Control cells were incubated in the absence of rotenone or extract. Mean fluorescence data were obtained from duplicate or triplicate wells in three independent experiments and normalized to control values. The data are presented as the mean \pm SEM; $n=6-8$ (A) or $n=7-9$ (B); * $p<0.05$ and ** $p<0.01$.

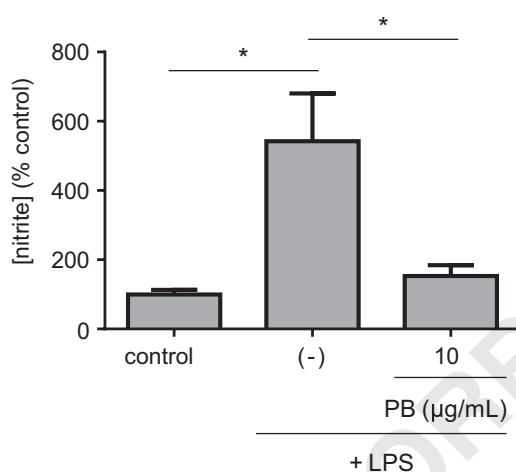


Fig. 10 – A purple basil (PB) extract alleviates LPS-induced microglial activation. Mouse BV2 microglial cells were treated with LPS (120 EU/mL) for 24 h in the absence or presence of a post-C18 PB extract (total polyphenol concentration, 10 µg/mL). Control cells were incubated in the absence of LPS or extract. Conditioned media was recovered from each well and assayed spectrophotometrically for nitrite levels, and values were normalized to the protein concentration of the BV2 cell lysate from the same well (control nitrite levels were ~ 0.7 µM in 1 mL of media, equivalent to ~ 7.5 µmol/g protein). The data are presented as the mean \pm SEM; $n=3$; * $p<0.05$.

extract described in this earlier report was found to contain C3G and presumably also included other ANC (Du et al., 2008), although in contrast to the results presented here a complete ANC profile was not determined. ANC-rich botanical extracts have also been shown to alleviate neurodegeneration in models of excitotoxicity related to cognitive impairment (Duffy et al., 2008) and amyloid- β (A β) neurotoxicity associated with Alzheimer's disease (Brewer et al., 2010). The major ANC present in the BB and BC extracts were malvidin, delphinidin, and cyanidin glycosides (Tables 3 and 4). In

contrast, the predominant ANC in the PB extract were malonyl or p-coumarylglycoside derivatives of cyanidin or peonidin (Phippen and Simon, 1998), and these compounds were absent from the BB and BC extracts. From these data, we infer that the BB, BC, and PB extracts had different neuroprotective activities as a result of differences in their total ANC content, their ANC profiles, and/or their distributions of less abundant polyphenols (e.g. PAC, PA, stilbenes) that were below the limit of detection of our RP-HPLC analysis.

Our study also revealed that a PAC-rich extract from GS alleviated dopaminergic cell death in rotenone-treated mid-brain cultures. Consistent with this finding, a PAC-rich cocoa extract was found to alleviate nigral dopaminergic cell death and striatal dopamine depletion in rats exposed to 6-OHDA, whereas catechin-rich extracts from the seeds of red or white grapes showed little evidence of neuroprotective activity in the same model (Datla et al., 2007). Another study revealed that a PAC-rich fraction from the bark of *Croton celtidifolius* Baill attenuated motor deficits and nigral dopaminergic cell death in rats receiving an intranasal infusion of MPTP (Moreira et al., 2010). A number of groups have shown that monomeric PAC such as catechin and (-)-epigallocatechin-3-gallate and extracts rich in these polyphenols but lacking polymeric PAC, including tea extracts, are neuroprotective in cellular and rodent models of PD (Choi et al., 2002; Guo et al., 2007; Levites et al., 2001; Mercer et al., 2005). Monomeric but not polymeric PAC have been shown to ameliorate cognitive deficits when administered orally in a transgenic mouse model of Alzheimer's disease, and only monomeric PAC metabolites (glucuronide derivatives) accumulate in rodent brain (Abd El Mohsen et al., 2002; Wang et al., 2012). Collectively, these observations and the data presented here imply that (i) PAC-rich extracts can alleviate neurodegeneration in PD models, although the degree of neuroprotection likely depends on the polyphenol content of the extract and/or the nature of the model; and (ii) monomeric PAC or their metabolites from extracts with neuroprotective activity (including the GS extract examined here) may slow neurodegeneration in rodent PD models and in human brain.

Additional experiments revealed that an oxyresveratrol-rich mulberry extract (in addition to the pure compound resveratrol) alleviated rotenone neurotoxicity in primary midbrain cultures. These results are consistent with previous data showing that oxyresveratrol or resveratrol attenuated neuronal cell death triggered by PD-related insults in cellular or animal models (Blanchet et al., 2008; Chao et al., 2008; Khan et al., 2010). Together with recently reported evidence that resveratrol glucuronide can be detected in the brains of mice fed a grape polyphenol preparation (Wang et al., 2013), our findings suggest that stilbenes or their metabolites may have neuroprotective activity in PD brain. In contrast, we found that extracts prepared from grape skin, plums, or dried plums (prunes) failed to protect against rotenone neurotoxicity. The grape skin extract may have failed to suppress rotenone-induced dopaminergic cell death because the concentration of resveratrol in this extract was below a minimal concentration required for neuroprotection. The data in Fig. 5C imply that this minimal concentration was ≤ 10 nM, whereas the results in Table S1 and Fig. 5B indicate that the final resveratrol concentration ranged from 0.05 to 5 nM in the grape skin extract samples examined here. The inability of the plum and prune extracts to carry out a neuroprotective effect suggests that PA abundant in these extracts may have a weak ability to alleviate neurotoxicity in the rotenone midbrain culture model. Consistent with this idea, a plum juice extract containing PA, ANC, and PAC, but not a dried plum powder consisting primarily of PA, interfered with age-related cognitive decline in rats (Shukitt-Hale et al., 2009).

In this study, we show for the first time that extracts rich in ANC and/or PAC are more neuroprotective than extracts containing other types of polyphenols (based on normalized total polyphenol levels) in a PD cell culture model. This finding suggests that ANC and PAC activate pro-survival pathways which are essential for the viability of neurons exposed to rotenone (e.g. pathways involved in mitochondrial function – see below). In contrast, other polyphenol classes (including PA and stilbenes) may not activate identical pathways. Instead, they may trigger different responses involved in neuroprotection against other PD-related insults. Our results are consistent with previous data showing that ANC- and PAC-rich fractions of a BB extract, but not pure chlorogenic acid, rescued deficits in Ca^{2+} buffering in hippocampal cultures exposed to dopamine, $\text{A}\beta_{42}$, or LPS (Joseph et al., 2010). Moreover, we found that our crude (pre-C18) extracts exhibited higher neuroprotective activity per unit mass of total polyphenol than the corresponding post-C18 extracts. This observation, also consistent with previous findings (Joseph et al., 2010), suggests that chemical entities removed during the C18 purification step may be neuroprotective and/or may enhance the protective activity of ANC and/or PAC in unfractionated extracts via synergistic effects.

3.2. A subset of individual ANC protect against rotenone neurotoxicity

Another key outcome of our study was the demonstration that the individual ANC M3G, C3So, and D3G (but not C3G) attenuated dopaminergic cell death in rotenone-treated midbrain cultures. In addition, we found that a hibiscus extract

mitigated rotenone neurotoxicity, suggesting that C3Sa and/or D3Sa were also likely neuroprotective. In a previous report, the ANC pelargonidin was shown to alleviate motor deficits and nigral dopaminergic cell death in 6-OHDA-treated rats (Roghani et al., 2010). To our knowledge the study presented here is the first to compare a range of ANC in terms of neuroprotective activity in a PD model. Our observation that an ANC-rich PB extract failed to improve dopaminergic neuron survival in rotenone-treated midbrain cultures (in contrast to the BB and BC extracts) suggests that some ANC may not be neuroprotective in the rotenone model. Notably, ANC that are abundant in the PB extract but absent from the BB and BC extracts, including malonyl or p-coumarylglycoside derivatives of cyanidin and peonidin, are predicted to have a weak ability to suppress rotenone neurotoxicity. The only PB ANC that was also detected in the BB and BC extracts, C3G, failed to produce a significant neuroprotective effect in rotenone-treated cultures. Importantly, these findings demonstrate that the identity of the sugar moiety attached to the anthocyanidin core plays a key role in determining the degree to which a given ANC alleviates neurotoxicity (e.g. C3So is neuroprotective, whereas C3G is not). We note, however, that the profile of protective versus inactive ANC may vary in different PD models or in patients with different forms of the disease (e.g. idiopathic versus familial). Moreover, although our data demonstrate that individual ANC can interfere with neurotoxicity, synergistic interactions involving multiple ANC may be required for maximal neuroprotection (Carey et al., 2013). Thus, an ANC that is inactive when tested individually may in fact contribute to the overall neuroprotective activity of an ANC-rich extract. Finally, our results showing evidence of ANC-mediated neuroprotection are likely relevant to in vivo protective effects, given that a number of ANC have been detected as intact glycosides in the brains of rats (Andres-Lacueva et al., 2005; Ho et al., 2013; Janle et al., 2010; Talavera et al., 2005; Wang et al., 2013) and pigs (Milbury and Kalt, 2010) fed diets supplemented with ANC-rich extracts.

3.3. Mechanisms by which ANC and PAC alleviate neurodegeneration

In this study we addressed two potential mechanisms by which extracts rich in ANC and/or PAC could suppress rotenone neurotoxicity: amelioration of mitochondrial dysfunction, and interference with microglial activation. Our rationale for focusing on these two mechanisms was that: (i) rotenone is known to cause defects in mitochondrial respiration by inhibiting complex I of the electron transport chain (Betarbet et al., 2000); and (ii) microglial activation plays a role in the death of dopaminergic neurons elicited by rotenone in cell culture (Gao et al., 2002) and in rat brain (Sherer et al., 2003a).

The O_2 consumption experiments revealed that the BB and GS extracts, both of which were neuroprotective in the primary cell culture model, rescued deficits in mitochondrial respiration triggered by rotenone in MES23.5 neuronal cells. The fact that the minimal effective concentration of the BB extract was greater in the O_2 consumption assay than in the primary neuron viability assay suggests (as one possibility) that BB polyphenols may rescue mitochondrial dysfunction

in dopaminergic neurons with greater potency than in MES23.5 cells. In contrast, three neuroprotective extracts (BC, mulberry, and hibiscus) had no effects on rates of O₂ consumption, suggesting that they antagonize rotenone neurotoxicity in primary midbrain cultures via alternative mechanisms. Our findings imply that the enhancement of mitochondrial function plays a role in the protective effects of some extracts against rotenone neurotoxicity, but this mechanism is not necessary for neuroprotection by all extracts. In contrast, stimulatory effects on mitochondrial respiration may be sufficient for neuroprotection, given that all of the non-protective extracts (PB, plums, prunes, and grape skin) failed to reverse rotenone-mediated deficits in O₂ consumption. The evidence presented here that the BB and GS extracts can enhance mitochondrial function is consistent with previous data showing that D3G mitigates the inactivation of enzymes of the electron transport chain in endothelial cells (Xie et al., 2012), and that grape polyphenols alleviate deficits in mitochondrial respiration in human and rat tissues (Hokayem et al., 2013; Pajuelo et al., 2012). BB and GS polyphenols may ameliorate rotenone-induced mitochondrial dysfunction by various mechanisms, two examples of which include (i) displacement of rotenone from its binding site on complex I (Lagoa et al., 2011), thereby abrogating the formation of ROS that cause oxidative damage to respiratory chain enzymes (Keeney et al., 2006); and (ii) activation or up-regulation of the transcriptional co-activator PGC1 α , a master regulator of mitochondrial biogenesis and oxidative metabolism (Lagouge et al., 2006; Valenti et al., 2013).

Analyses of nitrite release revealed that (i) the PB extract, which failed to protect against rotenone neurotoxicity in primary midbrain cultures, suppressed LPS-induced activation of BV2 microglial cells; and (ii) the hibiscus extract, which was protective against rotenone neurotoxicity, exhibited a trend towards alleviating BV2 cell activation, consistent with previous data (Kao et al., 2009). In contrast, none of the other extracts (either neuroprotective or non-protective) had a significant effect on nitrite release from LPS-treated BV2 cells. Our data differ from those in a recent report showing that a BB extract alleviated nitrite release from LPS-stimulated BV2 cells (Carey et al., 2013), presumably because the levels of BB extract examined here were lower. From these results, we infer that the ability of an extract to abrogate nitrite release from LPS-treated BV2 cells does not necessarily correlate with the extract's neuroprotective activity in rotenone-treated midbrain cultures. A previous study revealed that a BB extract suppressed microglial activation in the brains of 6-OHDA-treated rats (Stromberg et al., 2005), suggesting that ANC-rich extracts can alleviate neuroinflammation under some conditions. A goal for future research will be to test this hypothesis by monitoring a range of neuroinflammatory markers in mixed neuron/glia cultures or in the brains of rodents exposed to various PD-related insults in the absence or presence of extract.

3.4. Conclusions

In summary, our data show that extracts rich in ANC and/or PAC alleviate rotenone neurotoxicity to a greater extent than extracts rich in other types of polyphenols, including PA and stilbenes. We also identified a number of individual ANC that

enhance dopaminergic cell survival in rotenone-treated mid-brain cultures. These observations are consistent with recent epidemiological findings suggesting that the consumption of ANC- or PAC-rich berries reduces PD risk (Gao et al., 2012). Two extracts (prepared from BB and GS) were found to rescue rotenone-mediated decreases in mitochondrial respiration, whereas a PB extract interfered with LPS-induced microglial activation. In particular, the stimulatory effects of the BB and GS extracts on mitochondrial respiration may play a key role in the ability of these extracts to suppress rotenone-mediated neuronal loss and neurite retraction, given that (i) rotenone elicits neurotoxicity by inhibiting mitochondrial complex I, and (ii) mitochondrial function is critical for both neuron viability and neurite integrity (Celardo et al., 2013; Sherer et al., 2003b). Nevertheless, the enhancement of mitochondrial function does not fully account for the neuroprotective activities exhibited by the multiple ANC- and PAC-rich extracts studied here. Rather, multiple protective pathways may play a role in mitigating neurotoxicity. Thus, the primary cell culture model used in this study appears highly suitable for screening potentially neuroprotective extracts or compounds because it recapitulates a range of pro-survival mechanisms spanning multiple cell types. Importantly, neuroprotective ANC identified from such screens could alleviate nigral dopaminergic cell death in vivo given that they can be delivered as intact glycosides to mammalian brain (Andres-Lacueva et al., 2005; Ho et al., 2013; Janle et al., 2010; Milbury and Kalt, 2010; Talavera et al., 2005; Wang et al., 2013), and the bioavailability and neuroprotective activity of these compounds could be enhanced further through the development of semi-synthetic derivatives (Lee, 2010). ANC- and PAC-rich extracts or their individual polyphenolic constituents may prove beneficial as agents to lower the risk of PD and/or slow disease progression.

4. Experimental procedures

4.1. Materials

Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The MES23.5 mouse-rat hybrid dopaminergic cell line was provided by Dr. Dennis Selkoe (Brigham and Women's Hospital) with the permission of Dr. Stanley Appel (Baylor School of Medicine). The BV2 microglial cell line was developed in the laboratory of Dr. Elisabetta Blasi at the University of Perugia (Blasi et al., 1990) and provided by Dr. Linda Van Eldik (Northwestern University). Dulbecco's minimal essential media (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA, were obtained from Invitrogen (Carlsbad, CA). Pure M3G and C3So for analysis in primary midbrain cultures were purchased from Carbomer Inc. (San Diego, CA). Cyanidin, delphinidin, petunidin, and malvidin glycosides used as standards in HPLC analyses were obtained from Polyphenols Laboratories (Sandnes, Norway). Catechin and epicatechin standards were obtained from Chromadex (Laguna Hills, CA). Polytetrafluoroethylene (PTFE) filters were obtained from Fisher Scientific (Pittsburgh, PA). The bicinchoninic (BCA) protein assay kit was obtained from Pierce Biotechnology

(Rockford, IL). Oxygen Biosensor 96-well plates were purchased from BD Biosciences (San Jose, CA). The Griess nitrite assay kit was purchased from Promega (Madison, WI).

4.2. Antibodies

The following antibodies were used in these studies: chicken anti-MAP2 (EnCor Biotechnology; Gainesville, FL); rabbit anti-TH (Millipore, Billerica, MA); anti-rabbit IgG-Alexa Fluor 488 and anti-chicken IgG-Alexa Fluor 594 (Invitrogen, Carlsbad, CA).

4.3. Preparation of botanical extracts

Plant specimens were first authenticated relative to species identity either by botanical or chemical taxonomic identification. A BB extract was prepared from *Vaccinium corymbosum* berries (highbush variety from a local farm in Lowell, IN) as described (Joseph et al., 1999). Briefly, frozen blueberries were combined with H₂O in a 1:1 (w/v) ratio, and the mixture was homogenized in a blender. The homogenized suspension was spun down at 13,000 rpm for 20 min at 4 °C to remove plant debris. The supernatant was frozen and lyophilized, and the freeze-dried extract was stored at -80 °C. Plums and pitted prunes (Kirkland Sunsweet variety) were extracted with 80% (v/v) methanol, 0.3% (v/v) trifluoroacetic acid (TFA), the extract was filtered, and organic solvent was removed by evaporation. The remaining aqueous concentrate was freeze-dried to afford the crude extract. A BC extract was prepared by pressing fresh fruits obtained from Just the Berries Ltd. (Los Angeles, CA) in a grape press to separate skin from juice. Pressed skins were rinsed with water to remove sugars and extracted with 80% (v/v) ethanol. Solvent was removed and the BC concentrate was freeze dried. To manually prepare a grape skin extract, the skin of fresh purple table grapes (*Vitis vinifera*) was carefully peeled off the fruit, stored at -20 °C, and extracted using a kitchen model blender with methanol at a 1:1 ratio (w/v) (Wu et al., 2005). The extract was then lyophilized. A grape seed extract (*V. vinifera*) was obtained from iBioCeuticals LLC (Eastham, MA). Chinese mulberry (*Morus australis*) was first identified and then manually collected in Edison, New Jersey. An extract was prepared from freshly ground bark material by extraction with 80% (v/v) ethanol. Extracts were prepared from hibiscus (*Hibiscus sabdariffa*) using two sources of the variety Vimto (SN26 and dark red) as described (Juliani et al., 2009). Purple basil (PB) was prepared from field-grown basil variety Red Rubin (Johnny's Selected Seeds, Albion, ME) at the Rutgers Agricultural Experiment Station (Pittstown, NJ) as described (Juliani et al., 2009; Phippen and Simon, 1998; Wu et al., 2005).

For cell culture experiments, the hibiscus extract, resveratrol, M3G, and C3So were dissolved in dimethyl sulfoxide (DMSO). The PB and mulberry extracts were dissolved in 50% DMSO/ethanol (v/v). The plum and prune extracts were dissolved in 30% (v/v) ethanol. All other extracts were dissolved in water.

4.4. Folin-Ciocalteu assay to determine total phenolic concentrations

The total phenolic concentration in each extract was determined using the Folin-Ciocalteu assay (Waterhouse, 2002).

Briefly, 20 µL of botanical sample (0.1–10 mg/mL) or gallic acid (0.05–2 mg/mL) was mixed with 1.58 mL of H₂O and 100 µL of the Folin-Ciocalteu reagent in a 4.0 mL UV-vis cuvette. After incubating for 1–8 min, 300 µL of Na₂CO₃ (200 mg/mL) was added with careful mixing, and the solution was incubated for an additional 2 h. Absorbance was measured at 765 nm using a Cary 3 UV-vis spectrophotometer (Varian Inc., Cary, NC). The total phenolic concentration in each extract was calculated as the gallic acid equivalent.

4.5. HPLC analysis and purification of ANC

HPLC analysis of ANC in botanical extracts was carried out using an Agilent 1200 HPLC (Agilent Technologies Inc., Santa Clara, CA) with a diode array detector, an autosampler, and a controlled-temperature column compartment. Chemstation software was used to control the system and process the data. ANC were separated using a reversed phase Supelcosil-LC18 column, 250 mm × 4.6 mm × 5 µm (Supelco Inc., Bellefonte, PA). The mobile phase consisted of 5% (v/v) formic acid in H₂O (A) and 100% methanol (B). The flow rate was constant at 1 mL/min with a step gradient of 10%, 15%, 20%, 25%, 30%, 60%, 10% and 10% of solvent B at 0, 5, 15, 20, 25, 45, 47 and 60 min, respectively, with 30 °C constant temperature. Samples were prepared by dissolving 5 mg in 1 mL methanol and filtering through a 0.2 µm PTFE filter before injecting 10 µL onto the HPLC system. Five solutions of C3G at 0.5, 0.25, 0.125, 0.063, and 0.031 mg/mL were prepared in methanol and injected (5 µL) onto the HPLC system (prior to the ANC samples) as external standards. ANC were quantified using a linear regression of standard concentration and peak area monitored at 520 nm (Grace et al., 2009). C3G and D3G were purified (>95%) from a BC extract by high performance counter-current chromatography (Armen instrument, St-Ave, France) (Mbeunkui et al., 2012). The identities of ANC in fruit extracts and of purified C3G and D3G were confirmed by LC-MS as outlined in Section 4.6.

4.6. LC-MS analysis of ANC, PAC, and PA

LC-MS analysis of ANC, PAC, and PA was carried out using an Agilent accurate mass time-of-flight LC/MS instrument (Agilent Technologies, Wilmington, DE). The separation was performed using an Onyx monolithic C18 column (4.6 mm × 100 mm) (Phenomenex, Torrance, CA) with a binary solvent system composed of 0.1% (v/v) formic acid in H₂O (mobile phase A) and 0.1% (v/v) formic acid in methanol (mobile phase B). Samples (5 µL of each extract) were injected onto the HPLC system and eluted into the ion source at a flow rate of 0.8 mL/min with the following gradient parameters: 5–20% B over 5 min, 20–50% B over 10 min, 50–95% B over 2 min, isocratic at 95% B for 1 min, and return to 5% B over 2 min. The column was re-equilibrated under initial conditions (5% B) for 10 min prior to the next injection. The instrument was calibrated to <1 ppm error in mass accuracy with an external standard of ESI tuning mix (Agilent Technologies, Wilmington, DE). Agilent MassHunter Workstation software was used for data analysis.

Standard solutions of PAC and PA (10–100 µg/mL) were prepared in 50% (v/v) methanol/H₂O, and aliquots of each stock solution were diluted to different concentrations in the

calibration range of 40–200 ng/mL. A calibration curve was generated by plotting the mean peak area for each external standard (determined from three runs) against the concentration. The concentration of each compound in an extract identified on the basis of retention time and mass was calculated from its peak area using the standard calibration curve.

4.7. HPLC analysis of stilbenes

HPLC analysis of stilbenes was conducted using the Agilent 1200 HPLC system described in Section 4.5. The separation was performed using a reversed-phase Supelcosil-LC-18 column, 250 mm × 4.6 mm × 5 μm (Supelco, Bellefonte, PA). The mobile phase consisted of 5% (v/v) acetonitrile in H₂O (0.1% v/v acetic acid) (A) and 95% (v/v) acetonitrile in H₂O (0.1% v/v acetic acid) (B). The flow rate was 1 mL/min with the following step gradient of B in A: 5% (0–5 min), 30% (5–40 min), 60% (40–45 min), 90% (45–50 min), isocratic for 5 min, 0% (55–60 min) at a constant temperature (40 °C). Compounds were identified by comparison with reference standards and quantified as the resveratrol equivalent.

4.8. Polyphenolic enrichment by SPE

Polyphenolic enrichment was carried out using a C18 SPE cartridge type 1000 mg/6 mL (Grace Inc., Deerfield, IL). The cartridge was conditioned with 3–5 mL methanol and rinsed with 5 mL H₂O. The cartridge was loaded with freeze-dried botanical samples (~100 mg dissolved in ~2 mL H₂O) and washed with 20 mL deionized H₂O to remove sugars (ANC and other polyphenols were retained). Acidified methanol (0.3% TFA, v/v) was used to elute polyphenols from the column. The polyphenol-rich, sugar-free extract was dried down under reduced pressure. An aliquot (5 mg) dissolved in 100% methanol and filtered through a 0.2 μm PTFE membrane was analyzed via HPLC or LC–MS as outlined above.

4.9. Preparation of primary mesencephalic cultures

Primary midbrain cultures were prepared via dissection of E17 embryos obtained from pregnant Sprague–Dawley rats (Harlan, Indianapolis, IN) as described previously (Liu et al., 2008a, 2008b). All of the procedures involving animal handling were approved by the Purdue Animal Care and Use Committee. The mesencephalic region containing the substantia nigra and ventral tegmental area was isolated stereoscopically, and the cells were dissociated with trypsin (final concentration, 26 μg/mL in 0.9% [w/v] NaCl). The cells were plated in the wells of a 48-well plate (pretreated with poly-L-lysine, 5 μg/mL) at a density of 163,500 cells per well in media consisting of DMEM, 10% (v/v) fetal bovine serum, 10% (v/v) horse serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Five days after plating, the cells were treated with cytosine arabinofuranoside (20 μM, 48 h) to inhibit the growth of glial cells. At this stage (7 days in vitro), ~50% of the total cell population consisted of glial cells, and the neurons appeared differentiated with extended processes.

4.10. Treatment of primary midbrain cultures

Primary cultures (7 days in vitro) were incubated in the presence of botanical extract or compound (or the corresponding vehicle) for 72 h. Next, the cultures were incubated in fresh media containing rotenone (100 nM) plus extract, compound, or vehicle for an additional 24 h. Alternatively, primary cultures incubated in the absence or presence of extract for 66 h were incubated in fresh media (minus extract) for 6 h and then exposed to rotenone (100 nM) in the absence of extract for 24 h. Control cultures were incubated in media without rotenone, extract, or compound.

The cultures were fixed, permeabilized, and blocked as described (Liu et al., 2008a, 2008b). After washing with phosphate buffered saline (PBS) (136 mM NaCl, 0.268 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4), the cells were treated overnight at 4 °C with primary antibodies specific for MAP2 (1:1000) and TH (1:500). The cells were then washed with PBS and treated with a goat anti-chicken antibody conjugated to Alexa Fluor 594 and a goat anti-rabbit antibody conjugated to Alexa Fluor 488 (each at 1:1000) for 1 h at 22 °C. Prolong gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) was then applied to each culture well before adding a coverslip.

4.11. Measurement of primary neuron viability and neurite number

Relative dopaminergic cell viability was determined by counting MAP2- and TH-immunoreactive neurons using a Nikon TE2000-U inverted fluorescence microscope (Nikon Instruments, Melville, NY) with a 20× objective. The data were expressed as the percentage of MAP2⁺ neurons that were also TH⁺ (this ratiometric approach was used to correct for variations in cell plating density). Each experiment was carried out at least 2 times using embryonic cultures prepared from different pregnant rats. Approximately 300–500 MAP2⁺ neurons were counted per experiment for each condition.

Neurite numbers were assessed by determining the percentage of TH⁺ neurons with 0 neurites using a Nikon TE2000-U inverted fluorescence microscope with a 20× objective. Neurites of MAP2⁺/TH⁺ neurons were counted by examining the MAP2 stain based on the fact that MAP2 is a classic marker of the dendritic cytoskeleton (Bernhardt and Matus, 1984). To generate the fluorescence microscopy images in Fig. 2D, primary midbrain cultures were plated on coverslips (preincubated with poly-L-lysine, 5 μg/mL) in the wells of a 48-well plate and treated as described above. Representative images of immunostained neurons under different treatment conditions were taken using a Nikon A1R/A1 confocal microscope with a 60× objective.

4.12. O₂ consumption assay

MES23.5 neuronal cells derived from rat midbrain (Liu et al., 2008a) were seeded at 40,000 cells per well in a 96-well Oxygen Biosensor plate (embedded with an O₂-sensitive fluorophore) for 24 h. The cells were treated with rotenone (100 nM) in the absence or presence of botanical extract.

Control cells were incubated in the absence of rotenone or extract. After 24 h, the plate was analyzed with a Tecan Spectrafluor Plus microplate reader (excitation and emission wavelengths, 485 nm and 620 nm). Mean fluorescence data from duplicate or triplicate wells in three independent experiments were normalized to control values.

4.13. Nitrite release assay

Activation of the BV2 microglial cell line was monitored by assaying nitrite levels in the culture media (an indirect measurement of NO release) as described (Lau et al., 2007a). BV2 cells were seeded in duplicate wells of a 12-well plate at a cell density of 100,000 cells/well in media containing DMEM, 10% (v/v) FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL), pH 7.4. After 24 h, the cells were washed twice with PBS and then pre-incubated with botanical extract or vehicle for 2 h in phenol red-free, serum-free media. LPS (prepared as a 1 mg/mL stock solution in H₂O) was then added to a final concentration of 120 endotoxin units (EU)/mL, and the cells were incubated for 24 h. Controls cells were incubated in the absence of LPS or extract. Cell-conditioned media was removed and assayed spectrophotometrically for nitrite levels using a Griess kit (absorbance wavelength, 548 nm). The remaining cells in each well were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, protease inhibitor cocktail (Sigma), 1 mM phenylmethylsulfonylfluoride), and the protein concentration of the lysates was determined using a BCA kit. Nitrite concentrations were normalized to the protein concentration (µmol nitrite/g of protein) and expressed as a percentage of the control value.

4.14. Statistical analyses

Statistical analyses were carried out by one-way ANOVA with the Newman-Keuls post-test using GraphPad Prism, Version 5.0 (<http://www.graphpad.com/prism/Prism.htm>).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at: <http://dx.doi.org/10.1016/j.brainres.2014.01.047>.

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