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Lumbee traditional medicine: Neuroprotective activities of medicinal plants used to treat Parkinson's disease-related symptoms

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Abstract

Ethnopharmacological relevance—Parkinson's disease (PD) is a neurodegenerative disorder characterized by a loss of dopaminergic neurons in the *substantia nigra pars compacta* and the presence in surviving neurons of Lewy body inclusions enriched with aggregated forms of the presynaptic protein α -synuclein (α Syn). Although current therapies provide temporary symptomatic relief, they do not slow the underlying neurodegeneration in the midbrain. In this study, we analyzed contemporary herbal medicinal practices used by members of the Lumbee tribe to treat PD-related symptoms, in an effort to identify safe and effective herbal medicines to treat PD.

Aim of the study—The aims of this study were to (i) document medicinal plants used by Lumbee Indians to treat PD and PD-related symptoms, and (ii) characterize a subset of plant candidates in terms of their ability to alleviate neurotoxicity elicited by PD-related insults and their potential mechanisms of neuroprotection.

Materials and Methods—Interviews of Lumbee healers and local people were carried out in Pembroke, North Carolina, and in surrounding towns. Plant samples were collected and prepared as water extracts for subsequent analysis. Extracts were characterized in terms of their ability to induce activation of the nuclear factor E2-related factor 2 (Nrf2) antioxidant response in cortical

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astrocytes. An extract prepared from *Sambucus caerulea* flowers (elderflower extract) was further examined for the ability to induce Nrf2-mediated transcription in induced pluripotent stem cell (iPSC)-derived astrocytes and primary midbrain cultures, to ameliorate mitochondrial dysfunction, and to alleviate rotenone- or aSyn-mediated neurotoxicity.

Results—The ethnopharmacological interviews resulted in the documentation of 32 medicinal plants used to treat PD-related symptoms and 40 plants used to treat other disorders. A polyphenol-rich extract prepared from elderflower activated the Nrf2-mediated antioxidant response in cortical astrocytes, iPSC-derived astrocytes, and primary midbrain cultures, apparently via the inhibition of Nrf2 degradation mediated by the ubiquitin proteasome system. Furthermore, the elderflower extract rescued mitochondrial functional deficits in a neuronal cell line and alleviated neurotoxicity elicited by rotenone and aSyn in primary midbrain cultures.

Conclusions—These results highlight potential therapeutic benefits of botanical extracts used in traditional Lumbee medicine, and they provide insight into mechanisms by which an elderflower extract could suppress neurotoxicity elicited by environmental and genetic PD-related insults.

Graphical Abstract

1. Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disease affecting 1–2% of individuals over the age of 60 and 5% of the population over the age of 85 (Shulman et al., 2011). PD symptoms include motor disturbances such as slow movement, resting tremor, and loss of balance, as well as non-motor symptoms including depression and anxiety (Fahn, 2003; Massano and Bhatia, 2012). Histopathological hallmarks include a loss of dopaminergic neurons in the *substantia nigra pars compacta* and the presence in surviving neurons of Lewy bodies enriched with aggregated forms of the pre-synaptic protein α -synuclein (aSyn) (Rochet et al., 2012; Shulman et al., 2011; Spillantini et al., 1997). The post-mortem brains of PD patients are also characterized by evidence of mitochondrial dysfunction (in particular, a decrease in complex I activity) and oxidative damage (Betarbet et al., 2000; Sanders and Greenamyre, 2013). Mutations in the SNCA gene encoding aSyn, including gene duplications and substitutions (A30P, E46K, H50Q, G51D, A53E, and A53T) (Petrucci et al., 2016), have been linked to familial forms of PD and are thought to promote aSyn aggregation (Conway et al., 2000; Khalaf et al., 2014; Rochet et al., 2012;

Ysselstein et al., 2015). A number of other genes have been found to be mutated in familial, monogenic forms of PD, including the genes encoding parkin, PINK1, DJ-1, ATP13A2, LRRK2, and VPS35, and genes with polymorphisms that increase the risk of PD have been identified via GWAS analysis (Hernandez et al., 2016; Trinh and Farrer, 2013). Epidemiological evidence suggests that exposure to environmental toxins, including the pesticide rotenone and the herbicide paraquat (PQ), results in an increased risk of PD (Tanner et al., 2011). Both rotenone (an inhibitor of mitochondrial complex I) and PQ (a redox-cycling agent) trigger an accumulation in neurons of reactive oxygen species (ROS) that in turn stimulate the conversion of aSyn to oxidatively modified species with an enhanced ability to form potentially neurotoxic oligomers (Conway et al., 2001; Mirzaei and Regnier, 2006; Rochet et al., 2012). Current therapeutic options for PD patients consist of dopamine replacement strategies that only result in a temporary relief of symptoms, with no effect on the underlying loss of dopaminergic neurons (Fahn, 2003). Hence, there is an urgent need for new therapies that could slow or stop the neurodegenerative process.

Epidemiological data suggest that the consumption of berries rich in anthocyanins and proanthocyanidins may lead to a reduced risk of developing PD (Gao et al., 2012). In support of these observations, we reported that anthocyanin- and proanthocyanidin-rich extracts and a number of individual anthocyanins alleviated neurodegeneration triggered by rotenone in a primary midbrain culture model relevant to PD (Strathearn et al., 2014). Moreover, other polyphenols have been reported to mitigate neurotoxicity in cellular and animal models of PD (Song et al., 2012), including stilbenes (Blanchet et al., 2008; Jin et al., 2008; Khan et al., 2010; Strathearn et al., 2014), isoflavones (Chen et al., 2008; Kyuhou, 2008; Li et al., 2013a; Zhu et al., 2014), and curcumin (Rajeswari and Sabesan, 2008; Spinelli et al., 2015; Tripanichkul and Jaroensuppaperch, 2012). Because polyphenols generally accumulate in the brain at low levels compared to endogenous antioxidants, it is hypothesized that they carry out their neuroprotective effects via mechanisms unrelated to ROS scavenging (Del Rio et al., 2013; Milbury and Kalt, 2010; Williams et al., 2004), including stimulation of cellular antioxidant responses mediated by the transcription factor nuclear factor E2-related factor 2 (Nrf2) (Colin-Gonzalez et al., 2012; Kraft et al., 2004; McWalter et al., 2004; Na and Surh, 2008; Satoh et al., 2013; Yan et al., 2015), amelioration of mitochondrial dysfunction (Morin et al., 2003; Zini et al., 2002), and suppression of inflammatory responses associated with glial activation (Guo et al., 2007; Kao et al., 2009; Lau et al., 2007).

The Lumbee Indian tribe of North Carolina is the eighth largest Native American tribe in the United States, consisting of more than 65,000 members as reported by the 2011–2015 US Census Bureau (US Census Bureau). The Lumbee tribe, recognized by the State of North Carolina since 1885, derives its name from the Lumbee River, which is of critical importance to the cultural identity and spiritual life of the tribe and a symbol for its people (Knick, 2003). Lumbee Indians primarily reside in southeastern North Carolina in the four counties of Robeson, Hoke, Cumberland, and Scotland (Bryant and LaFromboise, 2005). Over hundreds of years of migration and interactions with different cultures, the Lumbee Indians have modified and adapted their way of life, and as a result their cultural heritage has been imprinted with European customs including certain Christian practices (Bryant and LaFromboise, 2005; Dial and Eliades, 1996; Lowery, 2010). Similarly, contemporary

Lumbee traditional medicine is a combination of herbal and Christian faith healing (Boughman and Oxendine, 2004; Steedly, 1979). Traditionally, Lumbee healers harvested medicinal plants in natural areas close to where they lived, including fields and ditches (Boughman and Oxendine, 2004; Croom, 1982). Robeson and surrounding counties are located in the Southeastern Atlantic coastal plain of North Carolina and benefit from a unique ecological environment. Characteristic landscapes include swamp forests and sandhill, pine, and oak forests with a high floristic diversity (Boyle, 2009). However, agriculture and industrialization have continuously modified these wetlands, leading to the decline of certain medicinal plants (Boughman and Oxendine, 2004; Croom, 1982).

This article is aimed at documenting contemporary herbal medicinal practices used by the Lumbee Indians to treat symptoms related to PD and other CNS disorders. We report the use of 32 medicinal plants to treat PD-related symptoms, and we describe a system of traditional medicine based on a combination of herbal knowledge and faith healing. In addition, we describe the characterization of a subset of plants in terms of their ability to activate the cellular antioxidant response, protect against neuronal death induced by PD-related insults, and preserve mitochondrial function. Our findings suggest that medicinal plants used in Lumbee traditional medicine could form the basis of PD therapies, and they yield insights into mechanisms by which the plants could interfere with neurodegeneration in the brains of patients.

2. Materials and methods

2.1. Materials

Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Minimal Essential Media (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA). Nuserum was purchased from Thermo Fisher Scientific (Waltham, MA). The SH-SY5Y cells were purchased from ATCC (Manassas, VA). Human induced pluripotent stem cell (iPSC)-derived astrocytes (iCell astrocytes) were obtained from Cellular Dynamics International (Madison, WI). The vector pSX2_d44_luc (Alam et al., 1999) was provided by Dr. Ning Li (UCLA) with the permission of Dr. Jawed Alam (LSU Health Sciences Center), and the GFPu reporter adenovirus was provided by Dr. Xuejun Wang (University of South Dakota). Plant specimens were collected and deposited as described below.

2.2. Antibodies

The following antibodies were used in this study: chicken anti-MAP2 (catalog number CPCA-MAP2, EnCor Biotechnology, Gainesville, FL); rabbit anti-TH (catalog number 2025-THRAB, PhosphoSolutions, Aurora, CO); mouse anti-GFAP (catalog MAB360, EMD Millipore, Billerica, MA); rabbit anti-Iba-1 (catalog number 019-19741, Wako Chemicals USA, Richmond, VA); and anti-mouse IgG-Alexa Fluor 488, anti-rabbit IgG-Alexa Fluor 488, and anti-chicken IgG-Alexa Fluor 594 (Invitrogen, Carlsbad, CA).

2.3. Ethnopharmacological interviews

The interview phase of this study was carried out in Robeson County, NC and in the city of Pembroke, NC, the political center of the tribe. Ethnopharmacological interviews were carried out with the approval of the Purdue Institutional Review Board. The participants were informed about the anonymity of the survey and the fact that they could terminate the interview process at any time. The interview objectives and process were defined as described previously (de Rus Jacquet et al., 2014) and in the accompanying article (de Rus Jacquet et al., 2017). Briefly, open-ended interviews were performed to question the participants about medicinal plants used to treat symptoms related to PD. Our approach was to ask the participants about medicinal plants used to treat both motor symptoms (tremor, weakness, and paralysis) and non-motor symptoms (inflammation, aches, anxiety, depression, and mental disorders). Our rationale for this approach was that medicinal plants used to treat these symptoms could potentially show protective activity in preclinical models relevant to PD pathogenesis. The choice of inflammation as a non-motor symptom for this study is based on evidence that (i) peripheral inflammation can play a role in perturbations of the innate immune response in the brain, including microglial activation; and (ii) peripheral anti-inflammatory compounds can mitigate these perturbations (Couch et al., 2011; Sanchez-Guajardo et al., 2013). Mental health conditions including anxiety and depression were considered relevant because therapies for these disorders likely cross the blood brain barrier (BBB) to target various regions of the brain, including the midbrain (Chaudhury et al., 2013). Information about herbal medicines used to treat cancers was also considered relevant based on evidence that pathogenic mechanisms such as inflammation, oxidative stress, and the dysregulation of certain proteins are common to both PD and some cancers (de Rus Jacquet et al., 2014; Devine et al., 2011; West et al., 2005). To obtain a broader perspective on relationships between Lumbee Indians and their natural environment (Croom, 1982), we asked participants to describe memories relating to the use of natural areas and plants for work, leisure, or social activities such as berry picking or medicinal plant harvesting.

2.4. Identification of plant species

When available, voucher specimens were collected, assigned a collection number, and deposited at the Purdue Kriebel Herbarium for future reference. In the case of plants for which voucher specimens were not available, informants were asked to identify the plants using photographs. We examined two photographic records. The first of these records contained photographs illustrating traditionally used plants and was provided by the participants. The second record contained photographs of plants and herbarium specimens (USDA Plant database, University of Florida Herbarium Collection Catalog) and was used to formally identify the plant species illustrated in the first photographic record. All plant names were verified using the weblink www.theplantlist.org (date accessed: 04-21-2016).

2.5. Preparation of botanical extracts

Botanical extracts were prepared as described in the accompanying article (de Rus Jacquet et al., 2017). Plant material was harvested or obtained in dried form from Friends of the Trees Society (Hot Springs, MT). Freshly harvested plant material was dried at 37 °C with a food

processor. To remain faithful to traditional practices, we avoided the use of potentially destructive techniques at each step of the extraction process. The use of water decoctions remains faithful to most traditional practices and avoids exposure of the plant materials to organic solvents or nontraditional extraction methods, both of which can change the chemical composition of the extract and lead to variations in pharmacological activity (Lapornik, 2005; Sithisarn et al., 2006). For each sample, 10 g of plant material was extracted in 50 to 100 mL of deionized water by incubating for 45 min at 50 to 60 °C with constant stirring. To remove plant debris, samples were centrifuged at $19,800 \times g$ for 30 to 60 min. The supernatant was then filtered through a 0.2 micron membrane, freeze-dried, and stored at -80 °C. The samples were protected from light during all steps of the extraction procedure. Prior to each experiment, freeze-dried extracts were dissolved in sterile deionized water.

2.6. Folin-Ciocalteu assay

The total polyphenolic content of the extracts was determined using the Folin-Ciocalteu assay (Strathearn et al., 2014; Waterhouse, 2002). A mixture of 2 μ L of botanical extract or gallic acid standard, 10 μ L of Folin-Ciocalteu reagent, and 158 μ L of H_2O was prepared in a 96-well format and incubated for 5 min at 22 °C. Subsequently, 30 μ L of Na_2CO_3 (200 g/L) was added with careful mixing, and the solution was incubated for 1 h at 22 °C. Absorbance measurements were carried out at 765 nm with a Synergy 4 microplate reader (BioTek Instruments, Inc.). The total polyphenol content (in %) was estimated as the gallic acid equivalent.

2.7. HPLC-TOF-MS profiling of an elderflower extract

HPLC-TOF-MS analyses were conducted on an Agilent 6220a TOF-MS, equipped with a 1200 series HPLC (Agilent Technologies, Santa Clara, CA) with a Waters X-Bridge column, 4.6×100 mm, 3.5 μ m (Waters, Milford, MA) using a gradient method with a mobile phase consisting of 0.1% (v/v) formic acid in H_2O (A) and 0.1% (v/v) formic acid in CH_3CN (B): 0–5 min, 2% B; 14 min, 40% B; 17–18 min, 100% B; 20–24 min, 2% B. TOF parameters included a drying gas of 10 L/min, nebulizer pressure of 45 psi, capillary voltage of 3.5 kV, fragmentor voltage of 80 V, and a mass range of $m/z = 100$ –3000 in both positive and negative ion modes. The extract was analyzed at 10 mg/mL with an injection volume of 10 μ L.

2.8. Adenoviral ARE-EGFP reporter construct

An adenovirus encoding enhanced green fluorescent protein (EGFP) downstream of the minimal promoter of the mouse heme oxygenase-1 (HO-1) gene (Alam et al., 1999) was prepared as described in the accompanying article (de Rus Jacquet et al., 2017). The construct, which has two antioxidant response elements (AREs) in the SX2 enhancer and is referred to as pAd-ARE-EGFP-TKpolyA, was packaged into virus via lipid-mediated transient transfection of the HEK293A packaging cell line.

2.9. Preparation of cortical astrocytes

Cultures enriched with cortical astrocytes were prepared via dissection of E17 embryos obtained from pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) using methods approved by the Purdue Animal Care and Use Committee. The cortical regions were isolated stereoscopically, and the cells were dissociated with trypsin (final concentration, 13 µg/mL in 0.9% (w/v) NaCl). The cells were plated in a 175 cm² flask pre-treated with rat collagen (25 µg/mL) at a density of $\sim 14.5 \times 10^6$ cells per dish in media consisting of DMEM, 10% (v/v) FBS, 10% (v/v) horse serum (HS), penicillin (10 U/mL), and streptomycin (10 µg/mL). After 48 h, new media consisting of DMEM, 10% (v/v) Nuserum, 10% (v/v) HS, penicillin (20 U/mL), and streptomycin (20 µg/mL) was added to selectively propagate the astrocyte population (which at this stage consisted of clusters of cells attached to the dish) and remove unattached neurons. The media was replaced with fresh media every 2 days until most of the astrocytes had spread out on the plate, generally by 7 days in vitro (DIV). The astrocyte-rich culture was passaged at least once (and no more than twice) before being used for the described experiments.

2.10. Preparation of primary midbrain cultures

Primary midbrain cultures were prepared via dissection of E17 embryos obtained from pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) using methods approved by the Purdue Animal Care and Use Committee (Strathearn et al., 2014; Ysselstein et al., 2015). Briefly, 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). For experiments that involved monitoring activation of the Nrf2 pathway (see section 2.11), the dissociated cells were plated in the wells of a 96-well black clear-bottom plate (pretreated with poly-L-lysine, 10 µg/mL) at a density of 92,650 cells per well in midbrain culture media, consisting of DMEM, 10% (v/v) FBS, 10% (v/v) HS, penicillin (10 U/mL), and streptomycin (10 µg/mL). After 5 DIV, the cultures were treated with AraC (20 µM, 72 h) to slow the proliferation of glial cells. For experiments that involved analysis of neuroprotective activity (see section 2.13), the dissociated cells were plated in the wells of a 48- or 96-well plate (pretreated with poly-L-lysine, 5 µg/mL) at a density of 163,500 or 81,750 cells per well (respectively) in midbrain culture media. After 5 DIV, the cultures were treated with AraC (20 µM, 48 h) before initiating experimental treatments.

The relative percentages of neurons, astrocytes, and microglia in the cultures were estimated at DIV = 11 via immunocytochemical analysis. The cultures were fixed, permeabilized, and blocked as described (Strathearn et al., 2014; Ysselstein et al., 2015). After washing with PBS (136 mM NaCl, 0.268 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4), the cells were treated for 24 h at 4 °C with primary antibodies specific for (i) microtubule-associated protein 2 (MAP2) (chicken, 1:2,000) and glial fibrillary acidic protein (GFAP) (mouse, 1:500); or (ii) MAP2 (chicken, 1:2,000) and ionized calcium-binding adapter molecule 1 (Iba-1) (rabbit, 1:500). The cells were then washed with PBS and treated for 1 h at 22 °C with (i) a goat anti-chicken antibody conjugated to Alexa Fluor 594 (1:1,000) and a goat anti-mouse antibody conjugated to Alexa Fluor 488 (1:1,000); or (ii) a goat anti-chicken antibody conjugated to Alexa Fluor 594 (1:1,000) and a goat anti-rabbit antibody

conjugated to Alexa Fluor 488 (1:1,000). After washing with PBS, the cells that had been treated with antibodies specific for MAP2 and Iba-1 were stained with DAPI (300 nM in PBS) for 5 min prior to a final round of washing with PBS.

2.11. Treatment of astrocytes or mixed midbrain cultures with ARE-EGFP reporter adenovirus

Activation of the Nrf2 pathway was monitored in cortical astrocytic cultures and primary midbrain cultures using a reporter adenovirus derived from the construct pAd-ARE-EGFP-TKpolyA. Primary cortical astrocytes were plated on a 96-well black clear-bottom plate (pretreated with rat collagen, 25 µg/mL) at a density of 5,000 cells/well. After 24 h, the cells were transduced with Nrf2 reporter virus at a multiplicity of infection (MOI) of 25. Additional experiments were carried out with human iCell astrocytes generated at Cellular Dynamics International (Madison, WI) via differentiation of an iPSC line that was reprogrammed from a fibroblast source obtained from an apparently healthy female individual, without mutations in known PD-related genes. iCell astrocytes were plated on a 96-well black clear-bottom plate (pretreated with rat collagen, 25 µg/mL) at a density of 10,000 cells/well. After 24 h, the cells were transduced with Nrf2 reporter virus at an MOI of 25. In other experiments, primary midbrain cultures (8 DIV) prepared as described above (see section 2.10) were transduced with Nrf2 reporter virus at an MOI of 10.

After 48 h, the virus-containing media was removed from the astrocytes or midbrain cultures, and the cells were incubated in fresh media supplemented with botanical extract for 24 h. Control cells were transduced with the ARE-EGFP virus for 48 h and then incubated in fresh media for another 24 h, in the absence of extract (negative control) or in the presence of curcumin (5 µM) (positive control). The cells were incubated in the presence of Hoechst nuclear stain (2 µg/mL in HBSS) for 15 min at 37 °C, washed in HBSS, and imaged in HBSS at 37 °C using a Cytation 3 Cell Imaging Reader (BioTek Instruments, Winooski, VT) equipped with a 4X objective. Quantification of EGFP and Hoechst fluorescence was carried out using the Gen5 2.05 data analysis software (BioTek Instruments, Winooski, VT). To quantify EGFP fluorescence, regions of interest (ROIs) were generated by the software based on the cellular size range (40 to 400 µm for astrocytes, 20 to 400 µm for mixed midbrain cultures) and a designated fluorescence intensity threshold. For each experiment, the threshold was adjusted so that the overall fluorescence intensity in the curcumin-treated culture was 1.5- to 2.5-fold greater than in the negative-control culture. To quantify Hoechst fluorescence, ROIs were generated by the software based on a cellular size range of 10 to 40 µm. The fluorescence intensity threshold was set so that most of the nuclei stained with Hoechst were included among the detected ROIs. Finally, the number of ROIs for EGFP was divided by the total cell number (ROIs obtained from Hoechst fluorescence) for each condition and normalized to the control value.

2.12. UPS reporter assay

The function of the ubiquitin-proteasome system (UPS) was monitored using an adenoviral reporter construct encoding the surrogate UPS substrate GFPu, a variant of GFP with a C-terminal fusion of the degron CL1 (Tian et al., 2014). Under basal conditions, GFPu is rapidly degraded by the UPS, whereas in cells with decreased UPS function, GFPu

accumulates, producing an increase in cellular fluorescence (Bence et al., 2001). Cortical astrocytes were plated on a 96-well black clear-bottom plate at a density of 5,000 cells/well in media consisting of DMEM, 10% (v/v) Nuserum, 10% (v/v) HS, penicillin (20 U/mL), and streptomycin (20 µg/mL). After 24 h, the cells were transduced with the GFPu reporter adenovirus at an MOI of 2. After 48 h, the cells were incubated in fresh media containing elderflower extract for an additional 24 h. Control cells were transduced with the reporter virus for 48 h and then incubated in fresh media for another 24 h, in the absence of extract (negative control) or in the presence of MG132 (2 µM) (positive control). The cells were incubated in the presence of Hoechst nuclear stain (2 µg/mL in HBSS) for 15 min at 37 °C, washed in HBSS, and imaged in HBSS at 37 °C using a Cytation 3 Cell Imaging Reader equipped with a 4X objective. To quantify GFP fluorescence, ROIs were generated by the Gen5 2.05 software based on the cellular size range (20 to 400 µm) and a designated fluorescence intensity threshold. For each experiment, the threshold was adjusted so that a 6- to 7-fold increase in the number of ROIs above the threshold was observed for the MG132-treated culture compared to the negative-control culture. Finally, the number of ROIs for GFP was divided by the total cell number (ROIs obtained from Hoechst fluorescence) for each condition and normalized to the control value.

2.13. Analysis of neuroprotective activity of elderflower extract in primary midbrain cultures

In one set of experiments, primary midbrain cultures (7 DIV) were incubated in the absence or presence of botanical extract for 72 h. Next, the cultures were incubated in fresh media containing rotenone (50 nM) in the absence or presence of extract for another 24 h. In a second set of experiments, midbrain cultures were transduced with an adenovirus encoding A53T (A53T Ad) (Ysselstein et al., 2015) at an MOI of 15 for 72 h in the absence or presence of extract, and the cells were then incubated in fresh media with or without extract for another 24 h. Control cultures were incubated in media without rotenone, A53T Ad, or extract. The cultures were then fixed, permeabilized, and blocked (Strathearn et al., 2014; Ysselstein et al., 2015). After washing with PBS, the cells were treated for 48 h at 4 °C with primary antibodies specific for MAP2 (chicken, 1:2,000) and tyrosine hydroxylase (TH) (rabbit, 1:1,000). The cells were then washed with PBS and treated with a goat anti-chicken antibody conjugated to Alexa Fluor 594 (1:1,000) and a goat anti-rabbit antibody conjugated to Alexa Fluor 488 (1:1,000) for 1 h at 22 °C. After a final round of washing with PBS, prolong gold antifade reagent with DAPI was applied to each culture well before sealing with a coverslip.

Relative dopaminergic cell viability was assessed by counting MAP2- and TH-immunoreactive neurons in a blinded manner using images taken with an automated Cytation 3 Cell Imaging Reader equipped with a 4X objective. A minimum of 12 images were taken, and approximately 500–1000 MAP2⁺ neurons were counted per experiment for each treatment. Each experiment was conducted at least 3 times using embryonic cultures prepared from different pregnant rats. The data were expressed as the percentage of MAP2⁺ neurons that were also TH⁺ (thus ensuring that the data were normalized for variations in cell plating density).

Neurite length measurements were carried out on images taken with an automated Cytation 3 Cell Imaging Reader equipped with a 4X objective (in general these images were the same as those used to determine dopaminergic cell viability as outlined above). Lengths of MAP2⁺ processes extending from TH⁺/MAP2⁺ neurons with an intact cell body (~90 neurons per sample) were assessed in a blinded manner using the manual length measurement tool of the NIS Elements software (Nikon Instruments, Melville, NY).

2.14. O₂ consumption assay

O₂ consumption was monitored as described previously with minor modifications (Olofinsae et al., 2014). Human SH-SY5Y neuroblastoma cells were grown in DMEM without glucose, supplemented with 15% (v/v) Nuserum, 10 mM HEPES, 10 mM galactose, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were plated in a 10 cm dish at a density of 1.5×10^6 cells/plate. After 4 days, the cells were incubated in the absence or presence of elderflower extract (1 µg/mL) for 19 h and then treated with rotenone (30 nM) for 5 h. Control cells were incubated in the absence of rotenone or extract for 24 h. The cells were harvested via centrifugation ($350 \times g$, 5 min, 22 °C) and suspended in O₂ consumption buffer (10 mM MgCl₂, 20 mM HEPES pH 7.2, 8.6% (w/v) sucrose, 0.026% (w/v) KH₂PO₄) at a density of 9×10^6 cells/mL (determined using a hemocytometer). Cellular respiration was measured using a Clark-type oxygen electrode attached to a voltmeter (Digital Model 10 Controller, Rank Brothers, Ltd, Cambridge, UK). The electrode was allowed to stabilize in O₂ consumption buffer for 30 min at 37 °C to ensure air saturation. To normalize the background current, the voltmeter was set to zero using a polarizing voltage of 0.60 V (with the electrode disconnected). An aliquot of 4.5×10^6 cells was loaded into the respiration chamber, and the sensitivity control was set to 1 V. This setting corresponded to 100% of the O₂ concentration (200×10^3 pmol/mL) in the air-saturated reaction medium before the start of respiration. The sample was constantly stirred at 840 rpm using a magnetic stir bar located inside the chamber. Using the Pico Technology software program (PicoTechnology, Ltd., Cambridgeshire, UK), the O₂ level remaining in the chamber at any time during respiration was automatically logged (with 10 sec intervals) as a voltage, V_{O2}, which corresponded to the voltage generated by the reaction of O₂ with the electrode and steadily decreased as O₂ was consumed. The rate of O₂ consumption was calculated as previously described using the following formula (Herst and Berridge, 2007):

$$\text{Rate of O}_2\text{consumption}(\text{pmol/s per } 10^6 \text{ cells}) = \frac{(-\text{slope}[V/s]) \times 200 \times 10^3 (\text{pmol/ml})}{\text{number of cells/ml} (\times 10^6)}$$

where 200×10^3 pmol/mL is the O₂ concentration in the saturated solution and the number of cells/mL is 9.0×10^6 . Mean O₂ consumption rates determined from 3 independent experiments were normalized to control values. Experiments showing a rotenone-induced reduction in O₂ consumption of less than 30% of the control value were excluded.

2.15. Analysis of mitochondrial membrane potential

Mitochondrial membrane potential was measured using tetramethylrhodamine methyl ester (TMRM) as described previously (Chazotte, 2011), with minor modifications. SH-SY5Y cells grown in galactose-supplemented media as described in section 2.14 were plated on a 96-well black clear-bottom plate at a density of 10,000 cells/well. After 48 h, the media was replaced with fresh media with or without elderflower extract (1 µg/mL). The cells were incubated for 19 h and then exposed to rotenone (30 nM) for 5 h. Control cells were incubated in the absence of rotenone or extract for 24 h. The cells were then treated with TMRM (60 nM) for 20 min at 37 °C in HBSS. The media was replaced with fresh media containing TMRM (30 nM) and Hoechst nuclear stain (2 µg/mL in HBSS), and the cells were imaged at 37 °C using a Cytation 3 Cell Imaging Reader equipped with a 4X objective. Quantification of TMRM and Hoechst fluorescence was carried out using the Gen5 2.05 software. For quantification of TMRM fluorescence, ROIs were generated by the software based on the cellular size range (5 to 40 µm) and a designated fluorescence intensity threshold. For each experiment, the threshold was adjusted so that the rotenone-treated cells exhibited a reduction in the number of ROIs of about 40% compared to control cells treated with vehicle. For quantification of Hoechst fluorescence, ROIs were generated by the software based on a cellular size range of 10 to 40 µm. The fluorescence intensity threshold was set so that most of the nuclei stained with Hoechst were included among the detected ROIs. Finally, the number of ROIs for TMRM was divided by the total cell number (ROIs obtained from Hoechst fluorescence) for each condition and normalized to the control value.

2.16. Statistical analysis

Data obtained from measurements of primary neuron viability, mitochondrial O₂ consumption rates, or mitochondrial membrane potential were analyzed via one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test using GraphPad Prism 6.0 (La Jolla, CA). Prior to carrying out these ANOVA analyses, the data were subjected to a square root transformation (in the case of percentage neuron viability data) or a log transformation (in the case of O₂ consumption rate data or membrane potential data) to conform to ANOVA assumptions. Neurite length data were subjected to a log transformation to account for skewness in the data. The log-transformed data were analyzed using an approach that accounts for (i) the possibility of multiple neurites arising from a single cell, and (ii) comparison across experiments conducted on different days. Log-transformed neurite lengths for multiple treatment groups were compared using a general linear model implemented in the GLM procedure of SAS Version 9.3 followed by Tukey's multiple comparisons *post hoc* test (Cary, NC). For measurements of ARE-EGFP or GFPu fluorescence, fold-change values were log-transformed, and the transformed data were analyzed using GraphPad Prism 6.0 via a one-sample *t*-test to determine whether the mean of the log(fold-change) was different from the hypothetical value of 0 (corresponding to a ratio of 1) (Motulsky, 2014). Unless otherwise specified, 'n' values listed in the figure legends correspond to the number of biological replicates (i.e. independent experiments involving cultures prepared at different times).

3. Results and Discussion

3.1. Cultural identity

Similar to other Native American tribes, Lumbee Indians share an ancestral heritage of rituals and ceremonies. The Cultural Center, located in Red Banks, NC, is a site for cultural and religious gatherings where a number of traditional rituals continue to be performed by elders and holy men. During our interviews, respondents provided information relating to the central role of the natural environment in providing sacred plants to perform various ritualistic activities (Supplementary Table 1). In addition, Lumbee participants documented traditional uses of herbal products for the preparation of various artifacts and tools as summarized in Supplementary Table 2. Finally, our results highlight the importance of wild or domesticated foods harvested in Lumbee traditions (Supplementary Table 3), and participants mentioned the harvesting of plants as a way to socialize and strengthen familial and neighborhood relationships.

3.2. Lumbee traditional medicine

This documentation of Lumbee traditional medicine involved interviews of three categories of participants, as observed in other studies of Lumbee Indians (Croom, 1982). Participants of the first category are traditional healers who learned herbal medicine from their ancestors or received the “gift” of healing and practice a combination of herbal and Christian faith healing. Information provided by one healer highlighted the importance of praying rituals in Lumbee traditional medicine, consistent with the results of a previous study (Boughman and Oxendine, 2004) and with practices reported by other Native American groups including the Pikuni-Blackfeet Indians (de Rus Jacquet et al., 2017). Participants of the second category undertake a combination of medicinal practices inherited from their family or learned from herbal books and health stores. Participants of the third category do not necessarily practice herbal medicine themselves but have learned some customs from their parents and grandparents.

3.2.1. Medicinal plants used to treat PD-related symptoms—Healers and local members of the Lumbee tribe provided information that enabled us to document the medicinal uses of plants, with a focus on plants used to treat PD-related symptoms. The uses of 72 medicinal plants, including 32 plants used to treat PD-related symptoms (Table 1, Supplementary Table 4, Supplementary Fig. 1) and 40 plants used to treat other disorders (Supplementary Table 5) were documented. The plants are described along with the ailments treated, plant parts used, and modes of administration. The preparation of herbal teas is a common form of administration, although the use of capsules from herbal stores is occasionally proposed as an alternative when the required plant material is not available. Most of the documented plants are available locally and harvested from neighboring forests and ditches, although participants mentioned a reduction in the number and variety of medicinal plants found locally, potentially resulting from urban and economic developments (Alig, 2010). In addition to herbal preparations, Lumbee Indians reported the use of natural products such as vinegar, turpentine, healing water, and honey to increase their therapeutic options (Supplementary Table 6).

The most common PD-related treatments documented include medicines with anti-inflammatory effects (7 plants), analgesic properties (8 plants), and sedative activities (9 plants). Antiinflammatory plants include cabbage, collards, horsemint, mullein, pineapple, sage, and white oak. Cabbage and collards are both from the Brassicaceae family, and chemical profiling of several plant species in this family has revealed the presence of health-promoting natural products such as organosulfur compounds (e.g. sulforaphane and other isothiocyanates), quercetin, kaempferol, and several anthocyanins (mostly cyanidin derivatives) (Chun et al., 2004; Liang, 2006; Lin et al., 2008a). Interestingly, these compounds were found to exhibit anti-inflammatory effects via modulation of pro-inflammatory factors. Sulforaphane was shown to alleviate LPS-induced inflammation in macrophages, an effect that was likely mediated through modulation of NF κ B and Nrf2 (Heiss et al., 2001; Lin et al., 2008b). Chang liver cells and murine macrophages incubated with quercetin or kaempferol exhibited a down-regulation of pro-inflammatory factors such as NF κ B, inducible nitric oxide synthase (iNOS), and/or cyclooxygenase-2 (COX-2) (Garcia-Mediavilla et al., 2007; Hamalainen et al., 2007). In addition, cyanidin and cyanidin derivatives interfered with LPS-induced inflammation in RAW 264.7 cells and carrageenan-induced inflammation in air pouches in BALB/c mice (Min et al., 2010). An extract prepared from mullein (*Verbascum* sp.) and several fractions and pure compounds derived from the extract were shown to mitigate inflammation in a mouse model of paw edema and in LPS-stimulated macrophages (Dimitrova et al., 2012). In another study, harpagoside, a biologically active iridoid glycoside isolated from *Verbascum* sp., and an extract prepared from *Verbascum phoeniceum* were found to alleviate inflammation and the production of COX-1 and COX-2 in mouse models of acute and chronic inflammation (Dimitrova, 2013). Ilwensisaponin C, a compound found in *Verbascum* sp., was found to have a significant anti-inflammatory effect in mouse models of inflammation (Akkol et al., 2007). The anti-inflammatory effects of the pineapple plant and its protease cocktail bromelain have been studied and validated in animal models of inflammatory diseases and in clinical studies (Darshan and Doreswamy, 2004; Hale et al., 2005; Maurer, 2001). Finally, the plant extract of sage as well as its constituents carnosol and ursolic acid were reported to have anti-inflammatory activities by inhibiting the Croton oil-induced ear edema in mice (Baricevic et al., 2001) and reducing the production of pro-inflammatory factors in LPS-stimulated macrophages (Lo et al., 2002; Mueller, 2010).

Plants used for their analgesic effects include arnica, boneset, comfrey, mullein, potatoes, and white oak. The analgesic effects of arnica have been described (Ahmad, 2013), and randomized double-blind trials revealed that preparations of this plant exhibited a powerful analgesic effect in patients suffering from osteoarthritis but did not mitigate after-surgery pain (Knuesel et al., 2002; Stevinson et al., 2003; Widrig et al., 2007). Mullein (*Verbascum* sp.) is composed of several bioactive compounds including the phenylethanoid glycoside verbascoside, and a *Verbascum* extract and verbascoside were found to have analgesic properties in a carrageenan-induced hind paw edema mouse model (Akdemir et al., 2011).

Plants used for their sedative effects include lemon balm, maypop, peach, peppermint, rabbit tobacco, and sage. The sedative and calming effects of lemon balm have been validated by extensive research in humans. Clinical studies have revealed that the administration of lemon balm can result in self-rated “calmness”, ameliorate the negative mood effects of stressors in

adults, and alleviate the symptoms of children suffering from restlessness and nervous dyskoimesis (Kennedy et al., 2004; Kennedy et al., 2002; Muller and Klement, 2006). Another medicinal plant described as having sedative properties by Lumbee Indians is maypop, also known as passionflower (*Passiflora incarnata*). The use of *Passiflora* sp. for its sedative effect has been reported in multiple historical and contemporary ethnopharmacological studies (Dhawan et al., 2004). *Passiflora incarnata* is composed of a variety of bioactive chemicals such as flavonoids (e.g. quercetin, kaempferol, apigenin, and luteolin) and indole alkaloids (Dhawan et al., 2004). Data from animal studies support the idea that *Passiflora* extracts have anxiolytic and sedative effects, potentially via a mechanism involving stimulation of the GABA_A receptor (de Castro et al., 2007; Deng et al., 2010; Elsas et al., 2010; Lolli et al., 2007). Clinical studies of *Passiflora* extracts have revealed a significant improvement in sleep quality (Ngan and Conduit, 2011) and a reduction of preoperative anxiety in patients (Movafegh et al., 2008). The sedative properties of leaves and bark of *Prunus persica* (peach) reported by the Lumbee Indians were previously described in studies of traditional medicine practices in India (Nadkarni, 1976) and Pakistan (Wazir, 2004). Finally, extracts prepared from sage (*Salvia* sp.) were reported to have significant sedative and anxiolytic activities in rodents (Imanshahidi and Hosseinzadeh, 2006; Rabbani et al., 2005) and to ameliorate anxiety and enhance calmness in healthy participants of a double-blind human study (Kennedy et al., 2006).

Plants used for both analgesic and sedative effects include catnip and elderberry flowers and berries. Analyses of an essential oil prepared from *Nepeta cataria* (catnip) or *Nepeta pogonosperma* in rodent models revealed both anti-inflammatory and antinociceptive effects, possibly due to the presence of the iridoid nepetalactone (Ali et al., 2012; Ricci, 2010). Extracts prepared from elderflower contain high levels of quercetin, kaempferol, and their derivatives (Christensen, 2008; Mikulic-Petkovsek et al., 2015), and these polyphenols were found to have antinociceptive properties in several animal models of pain (De Melo et al., 2009; Filho et al., 2008; Kaur et al., 2005). Moreover, extracts prepared from elderberries are highly enriched in anthocyanins (Lee and Finn, 2007), and anthocyanin-rich extracts were reported to exhibit antinociceptive activities (Tall et al., 2004; Torri et al., 2007).

Interestingly, some of the botanicals documented in this article were also described in our study of Pikuni-Blackfeet traditional medicine (de Rus Jacquet et al., 2017). Notably, *Arnica* sp., *Hypericum perforatum*, *Mentha × piperita*, *Sambucus* sp., and *Valerian* sp. were reported by both the Pikuni-Blackfeet and Lumbee tribes as being used to treat PD-related symptoms. In addition, *Allium* sp. and *Filipendula ulmaria* were documented in our study of Lumbee traditional medicines as being used to treat symptoms unrelated to PD, whereas these botanicals were reported by the Pikuni-Blackfeet Indians and other tribes as being used to treat PD-related symptoms (de Rus Jacquet et al., 2017).

3.3. Neuroprotective activities and mechanisms of action of medicinal plants used to treat PD-related symptoms

3.3.1. Study design—We selected a total of 8 medicinal plants for extraction and further analysis based on their ethnopharmacological use and their availability at the time of the study. The selected botanicals were: catnip (sedative, analgesic), elderflower (analgesic,

sedative, anti-cancer), mullein (analgesic, inflammation), passionflower (mild sedative), peach bark and leaves (sedative), rabbit tobacco (sedative), rosemary (good for nerves, stimulant), and sassafras (anxiety, depression, tonic) (Table 1). As outlined in section 3.2.1, these medicinal plants and their chemical constituents were described in this study and others as being used to treat symptoms related to PD as defined in the Materials and Methods. In particular, elderflower was described as ‘good for everything’ by the Pikuni-Blackfeet Indians (de Rus Jacquet et al., 2017), and it was described as an analgesic, sedative, and anti-cancer remedy by the Lumbee Indians. Thus, it was selected as a plant candidate because of its documentation in our studies of both tribes and because of its high polyphenol content. At the time of the study, *Sambucus caerulea*, but not *Sambucus nigra*, was available and was therefore selected for further analysis.

The 8 medicinal plants outlined above were screened for the ability to activate the cellular antioxidant response mediated by Nrf2 in cortical astrocytes. Nrf2 is a transcription factor that regulates the expression of more than 200 genes encoding cytoprotective proteins involved in the cellular antioxidant response and the production of phase II detoxification enzymes (Kumar et al., 2014). In unstressed cells, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) and targeted for degradation by the UPS (Bryan et al., 2013). However, under conditions of oxidative stress, Keap1 becomes modified, resulting in Nrf2 stabilization and translocation to the nucleus where it binds the antioxidant response element (ARE) in the 5' flanking region of its target genes (Erlank et al., 2011; Kumar et al., 2014; Satoh et al., 2013). In the brain, Nrf2 is most active in astrocytes, and the Nrf2-mediated expression of astrocytic enzymes involved in glutathione synthesis promotes neuronal antioxidant responses via the production of glutathione metabolites that are taken up and reassembled by neighboring neurons (Shih et al., 2003). Multiple lines of evidence suggest that activation of the Nrf2/ARE pathway leads to increased neuronal survival in toxin-related and genetic models of PD (Chen et al., 2009; Gan et al., 2012; Kumar et al., 2012). Accordingly, the extract that showed the most robust activation of the Nrf2-mediated response was further examined for its ability to alleviate neurotoxicity in primary midbrain cultures exposed to two PD-related insults, rotenone and aSyn-encoding adenovirus.

3.3.2. Identification of botanical extracts that activate Nrf2/ARE signaling—The panel of 8 medicinal plants described above was examined for the ability to activate Nrf2 signaling using a fluorescence-based reporter assay. Primary cortical astrocytes were transduced with a reporter adenovirus encoding EGFP downstream of an enhancer element encompassing two AREs and the HO-1 minimal promoter. Astrocytes treated with water extracts of catnip leaves, elderflower, mullein leaves, and rosemary leaves exhibited an increase in EGFP fluorescence compared to untreated cells, suggesting that these extracts induced activation of Nrf2 signaling (Fig. 1). An increase in Nrf2-mediated transcription was also observed in human astrocytes derived from iPSCs (Fig. 2A), and a trend towards such an increase was observed in astrocytes in primary neuron-glia cultures from rat midbrain (Fig. 2B), after incubation of the cells in the presence versus the absence of the elderflower extract. In contrast, primary cortical astrocytes treated with passionflower

leaves, peach bark, peach leaves, rabbit tobacco whole plant, or sassafras roots did not exhibit a significant increase in EGFP fluorescence (Fig. 3).

To our knowledge this is the first report of Nrf2 activation by a rosemary extract in primary astrocytes, or by a catnip, elderflower, or mullein extract in any cellular context. A number of botanicals such as green tea and extracts prepared from garlic, broccoli or rosemary have been shown to modulate the Nrf2/ARE pathway, an activity largely attributed to the polyphenolic constituents of these extracts (Colin-Gonzalez et al., 2012; de Rus Jacquet et al., 2017; Kraft et al., 2004; McWalter et al., 2004; Na and Surh, 2008; Satoh et al., 2013; Yan et al., 2015). The total polyphenolic content (determined with a Folin-Ciocalteu assay) was found to be highest in the extracts prepared from elderflower, peach bark, and rosemary (Table 2). Two of these extracts (rosemary and elderflower) exhibited the highest degree of activation of the Nrf2-mediated response in our primary screen, whereas the third extract (prepared from peach bark) showed no activity in this assay. Accordingly, we infer that the total polyphenolic content and chemical profile are both important factors that determine the ability of an extract to activate Nrf2 signaling.

A rosemary crude extract was previously shown to activate the Nrf2-mediated antioxidant response in a cell culture model of colon cancer (Yan et al., 2015). Moreover, a number of phenolic compounds present in rosemary extract, including the terpenes carnosic acid and carnosol and the phenolic acid rosmarinic acid, are potent activators of the Nrf2/ARE pathway (Jin et al., 2013; Martin et al., 2004; Satoh et al., 2013). Chemical profiling of aerial parts of mullein (*Verbascum salviifolium*) revealed the presence of several polyphenolic compounds including luteolin glucosides, apigenin glucosides, and chrysoeriol glucosides (Tatli et al., 2008), some of which have been shown to activate the Nrf2 pathway (Huang et al., 2013; Song and Park, 2014). However, the presence of these polyphenols seems to vary among *Verbascum* species (Dalar et al., 2014).

An HPLC-TOF-MS analysis of the elderflower extract revealed the presence of 15 major components that were identified or tentatively identified by their masses, UV maxima, and information in the literature about compounds reported to be elderflower constituents (Table 3). Most of these compounds (8 out of 15) were flavonoid glycosides, including six derivatives of isorhamnetin. Many of these polyphenolic constituents were reported to promote the Nrf2/ARE antioxidant response (as outlined in detail below) and could individually or synergistically activate Nrf2 signaling in our cellular models. Chlorogenic acid and extracts with high amounts of this phytochemical have been shown to activate the nuclear translocation of Nrf2 and transcription of target genes such as NQO1 in cell culture systems (Feng et al., 2005; Kim et al., 2012). Epicatechin, a ubiquitous flavanol found in a variety of foods and herbal remedies such as cocoa and tea (Khokhar and Magnusdottir, 2002; Sanchez-Rabaneda et al., 2003), was reported to activate cytoprotective and antioxidant responses via modulation of the Nrf2/ARE pathway in a brain ischemia model (Shah et al., 2010) and in cortical astrocytes (Bahia et al., 2008). Quercetin and its metabolite isorhamnetin were shown to increase Nrf2-dependent expression of HO-1, NQO1, and GCL in cell culture models (Tanigawa et al., 2007; Yang et al., 2014). Our study was focused on the neuroprotective activity of a crude elderflower extract, based on the fact that traditional herbal medicines are taken as crude preparations. It is hypothesized that the

synergistic activity of compounds in a crude extract can potentially enhance individual activities (Wagner, 2011; Williamson, 2001). In support of this idea, quercetin and kaempferol were reported to act synergistically to activate the cellular antioxidant response in HepG2-C8 cells (Saw et al., 2014).

Although the extract prepared from peach bark contained high levels of total polyphenols, it did not activate Nrf2 signaling in our assay. The major polyphenolic constituents of the peach bark extract likely consisted of high-molecular-weight polymerized polyphenolic species such as proanthocyanidins (PAC) (condensed tannins) (Jerez et al., 2006; Saha et al., 2012; Tanaka et al., 2008), and our findings suggest that these phytochemicals lack the ability to induce Nrf2-mediated transcription in cortical astrocytes (although a previous study revealed that some PAC can activate Nrf2-signaling (Chen et al., 2015)). Collectively, these observations highlight the fact that the specific polyphenolic composition of an extract, and not necessarily its total polyphenolic content, is critical for the extract's ability to activate signaling through the Nrf2/ARE pathway.

In summary, we identified four botanical extracts used by the Lumbee Indians to treat PD-related symptoms with the ability to induce up-regulation of Nrf2 signaling in cortical astrocytes. One of these extracts, prepared from elderberry flowers, also activated (or showed a trend towards activating) Nrf2-mediated transcription in human astrocytes derived from iPSCs and in astrocytes in mixed neuron-glia cultures from rat midbrain. The up-regulation of Nrf2 signaling by dietary and/or pharmacological means is recognized as a potential neuroprotective strategy (de Vries et al., 2008; Kumar et al., 2012; Satoh et al., 2013). Accordingly, we infer that polyphenols in the four bioactive extracts identified in our reporter-based screen could potentially alleviate neurodegeneration in PD via up-regulation of the Nrf2-mediated cellular antioxidant response. In contrast, the five extracts that are used by the Lumbee Indians to treat PD-related symptoms but failed to activate Nrf2 signaling could potentially achieve neuroprotective effects in PD brain via other mechanisms.

3.3.3. Effect of an elderflower extract on UPS function in rat primary cortical astrocytes—Polyphenols such as those described in the previous section are thought to activate Nrf2 signaling via a range of post-translational mechanisms. As one possibility, they may cause a modification of Keap1, resulting in a disruption of the Nrf2-Keap1 interaction, Nrf2 accumulation, and Nrf2 translocation into the nucleus. Some polyphenols have been reported to engage in redox cycling reactions, resulting in a build-up of ROS that react with key regulatory cysteine residues of Keap1, whereas others are thought to activate Nrf2 transcriptional activity by reacting directly with Keap1 (Erlank et al., 2011; Kumar et al., 2014; Satoh et al., 2013). As a second possible mechanism, some polyphenols have been shown to inhibit the UPS (Chang, 2009; Murakami, 2013; Pettinari et al., 2006). Nrf2 is degraded by the UPS, and inhibition of the UPS has been linked to up-regulation of Nrf2 activity and an increase in glutathione levels (Chen and Regan, 2005; Yamamoto et al., 2007).

In the next phase of our study, we characterized the elderflower extract, which induced the most robust up-regulation of Nrf2 signaling in cortical astrocytes compared to the other extracts (Fig. 1), in terms of its ability to elicit UPS inhibition as a potential mechanism for

Nrf2 activation. To address this problem, we developed an assay involving the use of a reporter adenovirus encoding GFPu, a form of GFP that is linked to the CL1 degron sequence and is thus rapidly degraded by a normally functioning UPS (Bence et al., 2001; Tian et al., 2014). In cortical astrocytes expressing GFPu, inhibition of the UPS results in accumulation of GFP and hence increased cellular fluorescence. Primary cortical astrocytes transduced with the GFPu adenovirus exhibited greater fluorescence following treatment in the presence versus the absence of the elderflower extract (Fig. 4), suggesting that polyphenols in this extract inhibit UPS activity.

This is the first report of UPS inhibition by an elderflower extract. A number of polyphenols, including elderflower polyphenols listed in Table 3, have been shown to interfere with UPS activity (Chang, 2009; Shen et al., 2012). The flavonol quercetin has been shown to inhibit the chymotrypsin-like activity of purified 20S and 26S proteasome in Jurkat T cells (Chen et al., 2005). Furthermore, ester-bond containing catechin polyphenols such as epigallocatechin-3-gallate (EGCG) have been reported to inhibit proteasome activity (Nam et al., 2001). UPS impairment by elderflower polyphenols could potentially contribute to Nrf2 stabilization in cortical astrocytes via a mechanism involving direct interference with UPS-mediated Nrf2 degradation. Alternatively, inhibition of the UPS could lead to a compensatory induction of the macroautophagy pathway (Korolchuk et al., 2010; Pandey et al., 2007). During autophagy, the adaptor protein p62 (also known as SQSTM1/A170) forms complexes with ubiquitylated cargoes and subsequently undergoes mTORC1-dependent phosphorylation (Ichimura et al., 2013; Katsuragi et al., 2015). In turn, phosphorylated p62 sequesters Keap1 to the p62/cargo complexes, which are ultimately eliminated (along with Keap1) by autophagy, resulting in Nrf2 stabilization. Accordingly, inhibition of the UPS by elderflower polyphenols could induce Nrf2 activation via a mechanism involving the up-regulation of autophagy, leading to p62-mediated Keap1 degradation.

In summary, our observation that an elderflower extract interfered with GFPu degradation implies that polyphenols in this extract could induce activation of the Nrf2/ARE pathway by eliciting UPS inhibition. In addition, it is possible that some elderflower polyphenols activate Nrf2-mediated transcription via a mechanism involving Keap1 modification, either by ROS or by the polyphenols themselves (Erlank et al., 2011; Kumar et al., 2014; Satoh et al., 2013) (this possibility will be addressed in future studies).

3.3.4. Effects of an elderflower extract on neurotoxicity elicited by PD-related insults in primary midbrain cultures—Our next objective was to measure the neuroprotective activity of the elderflower extract in a rat primary midbrain culture model. An advantage of using midbrain cultures to monitor neuroprotection is that they contain post-mitotic neurons, astrocytes, and microglia (Supplementary Fig. 2), and thus they provide a native-like environment similar to the mesencephalic region affected in PD patients. The distribution of different cell types in our cultures at DIV = 11 was as follows: neurons positive for MAP2 staining, ~15%; astrocytes positive for GFAP staining, ~70%; and microglia positive for Iba-1 staining, ~15%. The neuronal population consisted of dopaminergic neurons positive for TH staining (~4–10%) and GABAergic neurons that were stained with an antibody specific for GABA (~90–95%) (Liu et al., 2008).

Because the elderflower extract showed a strong trend towards activating astrocytic Nrf2 signaling in primary midbrain cultures (Fig. 2B), we hypothesized that it should alleviate neurotoxicity elicited by pro-oxidant insults in the same model (Lee et al., 2003; Shih et al., 2003). To address this hypothesis, we characterized the elderflower extract in terms of its ability to alleviate preferential toxicity to dopaminergic neurons in midbrain cultures exposed to two PD-related insults: (i) rotenone, an environmental toxicant epidemiologically linked to increased PD risk (Tanner et al., 2011); and (ii) adenovirus encoding A53T aSyn, a mutant form of aSyn that is expressed in the brains of patients with early-onset, familial PD (Polymeropoulos et al., 1997) and exhibits accelerated fibrillization (Conway et al., 1998) and enhanced neurotoxicity (Ysselstein et al., 2015) compared to WT aSyn.

In one set of experiments, primary midbrain cultures were exposed to rotenone in the absence or presence of elderflower extract. The cultures were co-stained for MAP2, a general marker of neurons, and TH, a specific marker of dopaminergic neurons, and scored for the percentage of neurons co-expressing TH and MAP2, providing a measure of relative dopaminergic neuron viability (Strathearn et al., 2014; Ysselstein et al., 2015). The relative number of TH⁺ neurons was greater in cultures treated with rotenone plus extract compared to cultures treated with rotenone alone (Fig. 5A). Neurite length analyses revealed a decrease in the lengths of processes extending from MAP2⁺/TH⁺ neurons in cultures exposed to rotenone compared to control cultures, and this decrease was attenuated in cultures treated with rotenone in the presence of elderflower extract (Fig. 5B). Together, these results suggest that polyphenols in the elderflower extract alleviated dopaminergic cell death and neurite retraction in rotenone-treated midbrain cultures.

In a second set of experiments, primary midbrain cultures were transduced with A53T adenovirus in the absence or presence of elderflower extract. Previous data from our lab indicated that (i) the adenoviral transduction efficiency was >90% for both TH⁺ and MAP2⁺ neuronal populations; and (ii) transduction of primary midbrain cultures with the A53T virus results in selective toxicity to MAP2⁺/TH⁺ neurons, whereas MAP2⁺/TH⁻ neurons are unaffected (Ysselstein et al., 2015). The cultures were co-stained for MAP2 and TH and scored for relative dopaminergic cell viability as outlined above. The relative number of TH⁺ neurons was greater in cultures treated with virus plus extract compared to cultures treated with virus alone (Fig. 5C), suggesting that elderflower polyphenols interfered with aSyn-mediated dopaminergic cell death.

These data reveal for the first time that an elderflower extract alleviates neurotoxicity elicited by rotenone or mutant aSyn in a primary midbrain culture model. Chemical profiling of the elderflower extract revealed the presence of high levels of polyphenols, a class of compounds widely studied for their health-promoting effects, including the flavonoids quercetin (and its derivatives), isorhamnetin (and its derivatives), and chlorogenic acid (Table 3, Fig. 6). Evidence suggests that the consumption of polyphenols results in a reduced risk of diseases such as PD and other neurological disorders (Bhullar and Rupasinghe, 2013; Gao et al., 2012), presumably because many polyphenols activate the cellular antioxidant response (Erlank et al., 2011; Kumar et al., 2014; Satoh et al., 2013). Based on our finding that the elderflower extract showed a strong trend towards activating astrocytic Nrf2 signaling (Fig. 2B) and alleviated neurotoxicity elicited by rotenone (Fig. 5A,B) and A53T

(Fig. 5C) in primary midbrain cultures, we infer that up-regulation of the Nrf2/ARE antioxidant response could play a key role in the extract's neuroprotective activity. In support of this idea, Nrf2-mediated transcription has been shown to attenuate neurodegeneration triggered by rotenone or aSyn in cellular and animal models (Gan et al., 2012; Lee et al., 2003).

Our observation that an elderflower extract alleviated rotenone- or aSyn-mediated dopaminergic cell loss is consistent with the results of other studies showing protective effects of polyphenol-rich extracts and individual polyphenols against cytotoxicity elicited by rotenone (Li et al., 2013b; Song et al., 2012; Strathearn et al., 2014) or aSyn (Bieschke et al., 2010; Macedo et al., 2015; Takahashi et al., 2015; Teraoka et al., 2012). Notably, catechin and quercetin have been shown to alleviate rotenone-induced neurodegeneration in cellular and animal models (Karuppagounder et al., 2013; Mercer et al., 2005), and evidence suggests that chlorogenic acid, EGCG, kaempferol, and quercetin inhibit aSyn fibrillization and cytotoxicity (Bieschke et al., 2010; Caruana et al., 2011; Ono and Yamada, 2006; Teraoka et al., 2012). Finally, our observation that the elderflower extract caused a modest inhibition of UPS function (Fig. 4), together with evidence that UPS impairment can lead to a compensatory up-regulation of macroautophagy (Korolchuk et al., 2010; Pandey et al., 2007), suggests that elderflower polyphenols may have interfered with A53T-mediated neurodegeneration by stimulating the autophagic clearance of toxic aSyn species.

In summary, our findings indicate that an elderflower extract alleviates dopaminergic cell loss elicited by environmental and genetic, PD-related insults. A number of the extract's polyphenolic constituents, including quercetin, kaempferol, and isorhamnetin (as either intact forms or metabolites), have been shown to penetrate the BBB (Ferri et al., 2015; Ishisaka et al., 2011; Rangel-Ordóñez et al., 2010). Accordingly, we infer that the elderflower extract could potentially be of clinical benefit in reducing PD risk or slowing neurodegeneration in the brains of patients.

3.3.5. Effect of an elderflower extract on mitochondrial function—Evidence suggests that the neurotoxic effects of rotenone and aSyn are related at least in part to a disruption of mitochondrial respiration that can result in an increase in oxidative stress (Betarbet et al., 2000; Di Maio et al., 2016; Dryanovski et al., 2013; Nakamura et al., 2011). Accordingly, we hypothesized that the elderflower extract could protect against neurotoxicity elicited by rotenone or A53T aSyn by alleviating mitochondrial functional deficits triggered by these insults, in addition to activating the Nrf2-mediated antioxidant response. To address this hypothesis, we characterized the extract in terms of its effects on mitochondrial O₂ consumption in human SH-SY5Y neuroblastoma cells challenged with rotenone. The cells were conditioned to grow in media containing galactose instead of glucose to ensure that they relied on mitochondria for energy production via oxidative phosphorylation and were thus sensitized to the toxic effects of rotenone (Marroquin et al., 2007; Swiss and Will, 2011). Galactose-conditioned SH-SY5Y cells treated with rotenone showed a mean decrease in the rate of cellular O₂ consumption of 36% compared to control cells (Fig. 7A). Cells cultured in the presence of rotenone plus elderflower extract exhibited an increase in the O₂ consumption rate compared to cells exposed to rotenone alone, suggesting that the extract mitigated the rotenone-induced interference with mitochondrial

respiration. In additional experiments, we examined the effects of the extract on the mitochondrial membrane potential (monitored using the fluorescent cationic dye TMRM) in galactose-conditioned SH-SY5Y cells exposed to rotenone. Cells treated with rotenone showed a 40% decrease in TMRM fluorescence compared to control cells, and this decrease was partially reversed in the case of cells treated with both rotenone and elderflower extract (Fig. 7B). These results suggest that the extract alleviated the rotenone-induced loss of mitochondrial membrane potential.

Our data reveal for the first time that an elderflower extract can ameliorate mitochondrial functional deficits triggered by rotenone in a neuronal cell line. These findings are consistent with the results of other studies (including data from our group) suggesting that plant extracts and polyphenols can mitigate mitochondrial functional impairments (Karuppagounder et al., 2013; Lagoa et al., 2011; Strathearn et al., 2014). In one study, quercetin was found to induce an increase in mitochondrial complex I activity in rotenone-treated rats (Karuppagounder et al., 2013). Moreover, quercetin, kaempferol, and epicatechin were reported to inhibit rotenone-induced increases in mitochondrial H₂O₂ production and compete for ubiquinone binding sites in complex I of the electron transport chain (Lagoa et al., 2011). Data from another study revealed that expression levels of PGC1- α , a transcriptional co-activator involved in mitochondrial biogenesis, were increased in mouse cortical neuron cultures co-treated with quercetin and epicatechin (Nichols et al., 2015). Collectively, these observations suggest that elderflower polyphenols could potentially rescue mitochondrial dysfunction by disrupting the inhibitory effects of rotenone on complex I and/or by activating mitochondrial biogenesis pathways.

4. Conclusion

This ethnopharmacological study was aimed at (i) documenting plants used in Lumbee traditional medicine to treat PD-related symptoms, and (ii) obtaining insights into the relationships between the Lumbee Indians and their local natural environment. Information provided by the respondents suggested that although the Lumbee Indians have adapted to modern ways of life, they continue to use local plants and herbs for artistic, utilitarian, or medicinal purposes according to their traditions. Sassafras, mullein and sage were the most frequently cited medicinal plants and can easily be found locally. An important priority for future research will be to carry out epidemiological studies aimed at determining whether exposure to particular plant species results in a reduced risk of developing PD.

Based on our ethnopharmacological survey, a total of 8 plant species were selected for further characterization in terms of their ability to modulate Nrf2/ARE signaling in a primary screen. The botanical preparation that exhibited the most robust Nrf2 response was an extract prepared from elderberry flowers, reported by the Lumbee Indians to be used for analgesic, sedative, and anti-cancer effects (medicinal properties that were classified as being relevant to a potential neuroprotective PD treatment in our study). The elderflower extract was found to protect dopaminergic neurons against toxicity elicited by rotenone or A53T α Syn, and this neuroprotective activity could involve the activation of Nrf2-mediated transcription (potentially via UPS inhibition, resulting in Nrf2 stabilization) and/or a rescue of mitochondrial dysfunction by the extract's polyphenolic constituents. Future studies

should be focused on elucidating other mechanisms by which elderflower polyphenols could mitigate neurotoxicity elicited by PD-related insults, including inhibition of aSyn aggregation (Caruana et al., 2011; Ono and Yamada, 2006) and up-regulation of macroautophagy as a consequence of UPS impairment (Korolchuk et al., 2010; Pandey et al., 2007). Importantly, a number of the extract's polyphenolic constituents or their derivatives have been shown to penetrate the BBB (Ferri et al., 2015; Ishisaka et al., 2011; Rangel-Ordóñez et al., 2010), suggesting that they could alleviate neurodegeneration in the brains of PD patients. Our findings provide a solid biological rationale for the use of a crude elderflower preparation by the Lumbee Indians as a traditional medicine to treat PD-related symptoms, and they lay the foundation for further characterization of the extract in terms of its potential clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A subset of botanical extracts activate the Nrf2/ARE antioxidant response in rat cortical astrocytes

Rat primary cortical astrocytes were transduced with an ARE-EGFP reporter adenovirus and incubated in the presence of botanical extract. Control cells were transduced with the reporter virus and incubated in the absence of extract. Graphs show an increase in EGFP fluorescence (fold change relative to control) in astrocytes treated with extract prepared from catnip leaves (A), elderflower (B), mullein leaves (C), or rosemary leaves (D).

Representative images show an increase in fluorescence in astrocytes treated with elderflower extract (E). The data in (A–D) are presented as the mean $\pm$ SEM; $n = 2$ –5; $*p < 0.05$ versus a predicted ratio of 1; log transformation followed by one-sample t -test. Scale bar in E, 300 μm .

Figure 2. An elderflower extract activates the Nrf2/ARE antioxidant response in human astrocytes and shows a trend towards activating this response in rat mesencephalic cultures Human iPSC-derived astrocytes (A) and rat primary midbrain cultures (B) were transduced with an ARE-EGFP reporter adenovirus and incubated in the presence of elderflower extract. Control cells were transduced with the reporter virus and incubated in the absence of extract. The data are presented as the mean $\pm$ SEM; $n = 3-7$; * $p < 0.05$ versus a predicted ratio of 1; log transformation followed by one-sample t -test.

Figure 3. A subset of botanical extracts failed to activate the Nrf2/ARE antioxidant response Primary cortical astrocytes were transduced with an ARE-EGFP reporter adenovirus and incubated in the presence of botanical extract. Control cells were transduced with the reporter virus and incubated in the absence of extract. Graphs show no increase in EGFP fluorescence (fold change relative to control) in astrocytes treated with extract prepared from passionflower leaves (A), peach bark (B), peach leaves (C), rabbit tobacco whole plant (D), or sassafras roots (E). The data are presented as the mean $\pm$ SEM; $n = 3-6$; *** $p < 0.001$ versus a predicted ratio of 1; log transformation followed by one-sample t -test.

Figure 4. An elderflower extract inhibits the UPS

Primary cortical astrocytes were transduced with a GFPu reporter adenovirus and incubated in the presence of elderflower extract. Control cells were transduced with the reporter virus and incubated in the absence of extract. The data are presented as the mean $\pm$ SEM; $n = 3-4$; * $p < 0.05$ versus a predicted ratio of 1; log transformation followed by one-sample t -test.

Figure 5. An elderflower extract alleviates neurotoxicity elicited by PD-related insults

Primary midbrain cultures were exposed to rotenone (50 nM) (A, B) or A53T adenovirus (MOI 15) (C) in the absence or presence of elderflower extract. Control cells were incubated in the absence of rotenone, A53T, or extract. The cells were stained with antibodies specific for MAP2 and TH and scored for relative dopaminergic cell viability (A, C) or for the lengths of neurites extending from dopaminergic neurons (B). The data are presented as the mean $\pm$ SEM; $n = 5$ (A) or $n = 3$ (B, C); (A, C) $*p < 0.05$, $***p < 0.001$, square root transformation, one-way ANOVA with Tukey's multiple comparisons *post hoc* test; (B) $**p < 0.01$, $****p < 0.0001$, Tukey's multiple comparisons *post hoc* test after log transformation and general linear model implementation.

Figure 6. Chemical structures of major polyphenolic compounds identified in an elderflower extract

See also Table 3.

Figure 7. An elderflower extract rescues rotenone-induced mitochondrial dysfunction

Galactose-conditioned SH-SY5Y cells pre-incubated in the absence or presence of an elderflower extract (1 $\mu\text{g/mL}$) were exposed to rotenone (30 nM) in the absence or presence of extract. Control cells were incubated in the absence of rotenone or extract. (A) O₂ consumption was monitored in a suspension of cells with a Clark-type oxygen electrode attached to a voltmeter. (B) Mitochondrial membrane potential was monitored via fluorescence microscopy imaging of cells incubated with TMRM. The data are presented as the mean $\pm$ SEM; $n = 4$ (A) or $n = 6$ total replicates from 3 independent experiments (B); * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ versus rotenone alone (-); log transformation, one-way ANOVA with Tukey's multiple comparisons *post hoc* test.

Table 1

Medicinal uses of plants relevant to PD-related symptoms

Scientific name (Family) Local, name	Medicinal use	Part used	Mode of use
<i>Acorus</i> sp. (Acoraceae), Calamus	Anti-cancer *	Rhizomes	Make a tea with hot, non boiling water. The rhizome can also be eaten fresh
<i>Ananas comosus</i> (L.) Merr. (Bromeliaceae), Pineapple	Anti-inflammatory *	Fruits	The fruit is used to treat inflammation
<i>Arnica</i> sp. (Asteraceae), Arnica	Analgesic *	Roots	Make a tea of roots and use as a wash for backache. It can be dangerous is taken internally. Use it as a rub or salve for sore muscle, sprains etc...
<i>Brassica oleracea</i> L. (Brassicaceae), Cabbage	Cleanse, mild laxative, anti-inflammatory *, treatment for glaucoma, pneumonia and breast feeding	Leaves	Make chow chow. The recipe can vary but the main ingredients are cabbage, pepper and tomatoes made in vinegar. It is food and medicine. For glaucoma, place clean and fresh leaves (no pesticides) over the eye. For pneumonia, place clean and fresh plant on the chest. Leave overnight, the leaves will turn yellow. Cabbage can also be used if lactation problems (due to inflammation)
<i>Brassica oleracea</i> L. (Brassicaceae), Collards	Congestion, antipyretic, anti-inflammatory *, treatment for glaucoma and pneumonia	Leaves	Place the leaves on the body. To treat a fever, take fresh collards leaves, add vinegar and place/tie around the areas of the body to heal. It will draw the fever out. For glaucoma, place a clean and fresh plant (no pesticides) over the eye. For pneumonia, place a clean and fresh plant on the chest. Leave overnight, the leaves will turn yellow
<i>Brassica rapa</i> L. (Brassicaceae), Turnip	Anti-cancer *, cold medicine	Roots	Make a turnip soup (food): roots of the turnip (some people use the greens), add cornmeal and pepper. Make a soup and drink 1 cup/day. The broth is the most important. It can be canned for conservation
<i>Capsicum annuum</i> L. (Solanaceae), Red pepper, Cayenne	Astringent, treatment for breast cancer *, women medicine, stimulant *	Fruits and seeds	Use capsules or power. Cayenne pepper is said to reduce the menstrual flow without stopping it
<i>Diospyros virginiana</i> L. (Ebenaceae), Persimmons	Anti-cancer *, cough and asthma medicines	Bark	To treat coughs and asthma, make a syrup. Clean persimmon bark, red oak bark and wild cherry bark and boil in water. Strain and cook the juice with brown sugar to make the syrup
<i>Eupatorium perfoliatum</i> L. (Asteraceae), Boneset	Analgesic *, cold medicine, antipyretic, stomachache	-	Make a tea of boneset to treat various ailments
<i>Hypericum perforatum</i> L. (Hypericaceae), St John's Wort	Antidepressant *		St John's Wort is used for its antidepressant properties
<i>Lavandula</i> sp. (Lamiaceae), Lavender	Soporific *	Flowers	Make a tea of lavender flowers for a soporific effect
<i>Melissa officinalis</i> L. (Lamiaceae), Lemon balm	Sedative *	Leaves	The leaves of lemon balm are used as a calming tea. The leaves are not boiled but infused in hot water
<i>Mentha x piperita</i> L. (Lamiaceae), peppermint	Cough and cold medicine, soporific *, digestive disorders, energizer *, colon	Candy, Essential oil	To treat a cough or induce sleep, take a 1 peppermint candy stick, lemon juice and let it sit in liquor. Take one teaspoon/day for 2–3 days. To treat a cold, take a large

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	cleansing, sedative *, oxygenates the bloodstream		peppermint stick, moonshine and lemon juice. Cut the peppermint and add moonshine to fill half a jar. Add 1/4 of honey and 1/4 of lemon. For digestive disorders such as stomachaches, rub essential oil on the stomach but be careful not to touch your eyes. Peppermint is also indicated to increase production of digestive sucs
<i>Mentha</i> sp. (Lamiaceae), Mint	Sedative *, stomach disorders	Leaves	Mint leaves are used to relieve nervousness and stomach disorders
<i>Monarda fistulosa</i> L. (Lamiaceae), Horsemint	Backache, chills, antipyretic, anti-inflammatory *	Leaves	Collect and crush fresh horsemint leaves to prepare a maceration in cold water
<i>Morus</i> sp. (Moraceae), Mulberry	Antihelminthic, tonic *	Roots, Fruits	To eliminate kingworms and tapeworms or in case of weakness, drink root tea. To lower a fever, eat the fruits
<i>Nepeta cataria</i> L. (Lamiaceae), Catnip	Colic, thrush, flu and cold medicine, soporific *, treatment for measles and mumps, sedative *, analgesic *, stomach disorders, antiemetic	Leaves, Stems	For a colic or thrush, boil the leaves and stems until it turns a color. One can also make a tea of leaves. This preparation can be used for children. Capsules from the health store can also be taken, but it was not specified if capsules are suitable for children
<i>Passiflora incarnata</i> L. (Passifloraceae), Maypop	Heart medicine, nervine hypotensive, mild sedative *	Flowers, Fruits	Make a tea of flower or fruit
<i>Prunus persica</i> (L.) Stokes (Rosaceae), Peach	Inflamed bladder, laxative, sedative *, cleanse	Bark, Skin of the fruit or leaves	Peach bark has a soothing effect on the nerve. For a cleansing effect, make a tea of the dried peach skin and drink one cup per day. This recipe works for most fruit trees e.g. strawberries. Cleanse four times a year
<i>Pseudognaphalium obtusifolium</i> (L.) (Hilliard & B.L.Burt (Asteraceae), Rabbit tobacco	Treatment for cold and cramps, antipyretic, mild sedative *, treatment for pneumonia and sore throat, upset stomach	Leaves, Flowers	For a cold, boil and drink one or two cups until cured. Add sweet if necessary for the taste. For colds, fever and flu, collect rabbit tobacco stems and pine needles from the woods. Wash thoroughly and boil in a pint of water for about 10 minutes. Drink one cup a day until healthy. A participant indicated to add honey to taste and some whiskey to the preparation. Another participant suggested the use of pine tops alone
<i>Quercus virginiana</i> Mill. (Fagaceae), White oak	Diarrhea, anti-inflammatory *, analgesic *	Bark	To treat bleeding hemorrhoids and diarrhea, boil the bark and drink the preparation
<i>Rosmarinus officinalis</i> L. (Lamiaceae), Rosemary	Good for nerves *, stimulant *, treatment for upset stomach, hair care medicine	Leaves	Drink a tea of the stem and leaves
<i>Salvia officinalis</i> L. (Lamiaceae), Sage, Common sage	Anti-diabetes, anti-inflammatory *, cleanse, skin infection, cough and cold medicine, memory strengthener *, sedative *, women medicine, treatment for ulcer	Leaves	Boil the leaves to make a tea for diabetes. For inflammation and skin infection, drink 4 cups a day of tea leaves until healed. For a cleansing effect, boil the leaves and inhale the steam (leaves can also be burned). To treat coughs, colds and congestions, peel and shred fresh ginger (2 tablespoons). Add one leaf of fresh sage from the garden. Boil and add honey for taste. Sage leaves tea is a memory strengthener and sedative. It is also a women medicine used to treat menopause symptoms such as hot flashes. To treat

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<i>Sambucus nigra</i> L. (Adoxaceae), Elder, Elderberry	Cold medicine, analgesic *, sedative *, used to remove freckles and whiten skin, respiratory problem, anti-cancer *	Flowers or berries	ulcers, make a tea of leaves by pouring hot water and drink half a cup 3 times a day until relieved Make a tea of flowers or berries. For skin cancer, soak the flowers in witch hazel for 7 days and apply as a lotion or make a cream To treat anxiety, use the root of sassafras. For
<i>Sassafras albidum</i> (Nutt.) Nees (Lauraceae), Sassafras	Anxiety treatment *, blood purification, cleanse, cold medicine, anti-diabetes, treatment to reduce blood sugar, antidepressant *, tonic *, treatment for measles, kidney problems and upset stomach, anti-rheumatic, sores, antiseptic, strengthen the immune system	Roots	blood purification or cleansing, make a tea of the root or the inner part of the root. The tea can be taken for breakfast. To treat a cold, make a tea of roots or inner part of the roots. It can be taken for breakfast, and honey can be added to taste. Alternatively, one can make a syrup by adding onions and honey. Two tablespoons of syrup are taken at night. For diabetes and to control the blood sugar, drink 8 ounces of tea morning and evening as long as needed. Sassafras roots are also used to treat depression. To give energy and treat measles, make a tea of roots and drink anytime. For kidney problems, drink 8 ounces of a tea of roots morning and evening as long as needed. For upset stomach, dry the roots in the sun. Cut the dried roots and boil in a pot. Filter with a cheesecloth and add sugar, stir and drink 1 cup 2/3 times a day for 1 week if bad stomach. The dosage varies from participants, it has also been suggested to consume one cup a day for two days. To treat rheumatisms, sores and to use as antiseptic, use the inner part of the root
<i>Silybum marianum</i> (L.) Gaertn. (Asteraceae), Milk thistle	Good for nerves *, protects kidney, brain and other tissues from chemical toxins, stimulant *	Leaves	Drink a tea of the leaves
<i>Solanum tuberosum</i> (Solanaceae), Potato	Analgesic *	Tuber	To relieve a sore on legs, take white potatoes and cut in half. Put the cut half on the skin until it dries
<i>Symphytum officinale</i> L. (Boraginaceae), Comfrey	Assist torn flesh to grow back together, analgesic *, treatment for wounds	Leaves	For analgesic properties, make a paste or soak the comfrey leaves and wrap around the painful area
<i>Taraxacum officinale</i> (L.) Weber ex F.H. Wigg. (Asteraceae), Dandelion	Digestive disorders, tonic *	Roots, Leaves	To treat heartburns, drink a tea of roots. A tonic is prepared by making a tea of leaves
<i>Vaccinium</i> sp. (Ericaceae), Blueberries	Anti-cancer *, anti-diabetes	Berries, Leaves	For cancer and diabetes, eat the berries in small amount, or about one cup a day for diabetes. To treat diabetes, a tea of leaves can also be made and patients drink one cup a day, twice a month
<i>Valeriana officinalis</i> L. (Caprifoliaceae), Valerian	Soporific *	Roots	The roots of Valerian are used to induce sleep
<i>Verbascum thapsus</i> L. (Scrophulariaceae), Mullein	Asthma, treatment for respiratory disorders, arthritis and gout, analgesic *, anti-inflammatory *, blood purification, treatment for headaches	Leaves, Roots	To treat asthma and respiratory complains, make a poultice with the whole leaves. Also can make powder of dried leaves, fill up capsules from health store and take twice a day. Drink enough water with the pills to avoid acid reflux. Alternatively, smoke the dried leaves or prepare a medicine using one cup of common juniper berries, mullein, parsley leaves and

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Scientific name (Family) Local, name	Medicinal use	Part used	Mode of use
			pennyroyal. For coughs, boil the dried roots and make sweetened syrup and give to children. To treat arthritis, chop the leaves and stuff a jar. Fill with green alcohol, massage the joints when painful. To prepare a gout treatment, boil the roots and make a tea. You can also boil the leaves and filter with cheesecloth: you then apply the boiled leaves on the area. To relieve pain or inflammation, make a paste of leaves, soak or boil the leaves and wrap around the affected area. The leaves are also prepared as a tea for blood purification and headaches

* PD-related uses

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Table 2

Total polyphenol content of botanical extracts

Extract	Plant part	Total polyphenol content (%) ^I
Catnip	Leaves	7.7
Elderflower	Flowers	16.4
Mullein	Leaves	8.9
Passionflower	Leaves	3.6
Peach	Bark	32.6
Peach	Leaves	3.5
Rabbit tobacco	Whole plant	2.8
Rosemary	Leaves	12.0
Sassafras	Roots	10.2

^I % total polyphenols in extract (mass/mass) estimated with the Folin-Ciocalteu assay.

Table 3

HPLC-TOF-MS analysis of an elderflower extract

Retention time (min)	<i>m/z</i> negative mode	<i>m/z</i> positive mode	Formula	Identification
7.70	164.0731	166.0909	C ₉ H ₁₁ NO ₂	Phenylalanine
9.17	353.0937	355.1053	C ₁₆ H ₁₈ O ₉	Neochlorogenic acid
10.16	353.0922	355.1093	C ₁₆ H ₁₈ O ₉	Chlorogenic acid
11.17	289.0766	291.0925	C ₁₅ H ₁₄ O ₆	Catechin
11.86	595.1352	597.1494	C ₂₆ H ₂₇ O ₁₆	Quercetin 3-O-diglycoside
12.09	639.1594	641.1771	C ₂₈ H ₃₂ O ₁₇	Isorhamnetin3-O-diglycoside
12.70	463.0920	465.1096	C ₂₁ H ₂₀ O ₁₂	Isoquercetin
13.12	549.0881	551.1112	C ₂₄ H ₂₂ O ₁₅	Quercetin-3-O-malonyl-glycoside
13.39	477.1080	479.1278	C ₂₂ H ₂₂ O ₁₂	Isorhamnetin3-O-galactoside
13.52	477.1077	479.1274	C ₂₂ H ₂₂ O ₁₂	Isorhamnetin3-O-glucoside
13.98	563.1030	565.1276	C ₂₅ H ₂₄ O ₁₅	Isorhamnetin-3-O-malonyl-galactoside
14.18	563.1020	565.1273	C ₂₅ H ₂₄ O ₁₅	Isorhamnetin-3-O-malonyl-glucoside
14.74	519.1175	521.1387	C ₁₇ H ₂₈ O ₁₈	Isorhamnetin-3-O-acetyl-glycoside
16.18	301.0392	303.0555	C ₁₅ H ₁₀ O ₇	Quercetin
17.80	315.0552	317.0729	C ₁₆ H ₁₂ O ₇	Isorhamnetin