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DIETARY POLYPHENOLS AND THEIR GUT
MICROBIOTA METABOLITES AS POTENTIAL
TREATMENTS FOR ALZHEIMER'S DISEASE

BY

KENNETH N. ROSE

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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IN
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DOCTOR OF PHILOSOPHY IN INTERDISCIPLINARY NEUROSCIENCE
OF
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2019

ABSTRACT

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that has no cure, no therapies to stop the disease progression, and limited palliative care options. As the 6th leading cause of death in the United States, it is necessary to discover therapeutics which can alter disease progression to alleviate the burden on patients, caregivers, and healthcare systems. In the search of therapeutics for AD, dietary polyphenols have become the topic of research as epidemiological evidence suggests that exposure to dietary polyphenols, such as a Mediterranean diet, over one's life, reduces the risk of dying from AD. Additionally, evidence from rodent models of AD suggests that dietary polyphenols can reduce the severity of cognitive decline and attenuate neuroinflammation, which is thought to be a contributing factor to disease progression. Because of these findings, several dietary polyphenols have entered the clinic in an attempt to elucidate their potential beneficial effects. For example, curcumin, from the curry spice turmeric (*Curcuma longa*), and resveratrol, a phytoalexin stilbene found in a variety of plants, have been investigated in AD disease populations in phase I and II clinical studies. However, these agents have only had moderate to little success which increases the demand for novel dietary polyphenols leads that may have potentially beneficial effects in the treatment of AD. Due to this demand, this dissertation investigates novel polyphenols and gut metabolites of dietary polyphenols from a variety of dietary sources for potential efficacy in a *Caenorhabditis elegans* (*C. elegans*) model of AD. This dissertation also employs molecular biology and mass spectrometry techniques to elucidate the potential mechanisms of action of select compounds found to be efficacious in a *C. elegans* model of AD. Finally, a polyphenol-enriched extract

was evaluated in a murine model of AD for the potential to reduce neuroinflammation when administered orally.

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“All there is to thinking is seeing something noticeable which makes you see something you weren’t even noticing which makes you see something that isn’t even visible” -Norman Maclean, *A River Runs Through It and Other Stories*

I would like to thank my parents, Jonathan Rose and Donna Rose, for their support and encouragement. Surely none of my accomplishment would have been possible without their help. I would like to thank my grandparents, Robert Mikson and Carolyn Mikson, for teaching me the meaning of hard work and indulging my interests over the years. They have had a transformative impression on my life that I will cherish forever.

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PREFACE

This dissertation has been written in manuscript style format.

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SATEMENT OF THE PROBLEMS

Alzheimer's disease poses a substantial risk to human health, a risk that is growing steadily as the population ages. Nevertheless, few reliable treatments exist to slow the progression of, mitigate symptoms, or cure this disease. In recent years, dietary polyphenols have been evaluated for their therapeutic potential. These compounds have been shown to reduce inflammation, disaggregate amyloid deposits, and reduce hyperphosphorylated tau. In animal models of Alzheimer's disease, polyphenols have shown to reverse the cognitive deficits associated with the disease. An emerging field in the area of polyphenol research is the potential therapeutic benefit of gut microbiota metabolites of polyphenols. Given the chemical nature of polyphenols, their penetration across the blood-brain barrier is limited. However, gut microbiota metabolites undergo several methylation, dehydroxylation and various other catabolic reactions which increases the likelihood of blood-brain barrier penetration due to reduced polarity and molecular weight. Herein, this dissertation describes the identification of potential therapeutics from dietary polyphenols and gut microbiota metabolism of these polyphenols, the mechanisms of action responsible for these natural products' potentially beneficial effects, and the efficacy of one of these potential therapeutics in an animal model of Alzheimer's disease.

CHAPTER 1

EFFICACY OF DIETARY POLYPHENOLS AND GUT METABOLITES IN A *CAENHORBADITIS ELEGANS* MODEL OF ALZHEIMER'S DISEASE AND INFLAMMATION

Manuscript 1 appears as published:

Isolation, identification, and biological evaluation of phenolic compounds from a traditional North American confectionary, maple sugar. Liu, Yougqiang. Rose, Kenneth N.; DaSilva, Nicholas A.; Johnson, Shelby L.; Seeram, Navindra P. (2017) Journal of Agricultural and food chemistry. 65, 4289-4295.

Manuscript 2 appears as published:

Development of a neuroprotective potential algorithm for medicinal plants. Liu, Weixi; Ma, Hang; DaSilva, Nicholas, A.; Rose, Kenneth N.; Johnson, Shelby L.; Zhang, Lu; Wan, Chunpeng; Dain, Joel A.; Seeram, Navindra, Seeram P. (2016). Neurochemistry International. 100, 164-177.

Manuscript 3 appears as published:

Phenolic compounds isolated and identified from Amla (*Phyllanthus emblica*) juice powder and their antioxidant and neuroprotective activities. Rose, Kenneth N.; Wan, Chunpeng; Thomas, A; Seeram, Navindra P.; Ma, Hang. (2018). Natural Product Communications. 13, 1309-1311.

ABSTRACT

Dietary polyphenols and gut microbial metabolites of dietary polyphenols offer a diverse and large library of compounds that are potentially beneficial for the treatment of neurodegenerative diseases, which potentially increases the likelihood of an effective compound to be discovered and exploited clinically. This also offers a significant scientific challenge. With such a vast library to search, there must be efficient methods of determining which compounds should be further investigated and which should be discarded as not relevant to the study. Herein, this chapter describes the use of wild-type and a transgenic *C. elegans* model, which expressed the human β -amyloid₃₋₄₂ gene, to evaluate polyphenols and gut microbial metabolites derived from a variety of plant sources.

The sources of polyphenols and their gut microbial metabolites used in this chapter are from three plants that have been identified as being potentially beneficial for the treatment of neurodegenerative diseases. The first is a maple syrup as an standardized, phenolic-extract of maple syrup has recently been demonstrated to reduce neuroinflammation markers in human SH-SY5Y neuroblastomas and reduced the LPS-induced inflammation in murine BV-2 microglia.

The second is an extract from the fruit of *Punica granatum* (Pomegranate) as previous work has demonstrated that the extract can attenuate hydrogen peroxide-induced toxicity in murine microglia BV-2. Furthermore, pomegranate ellagitannins are known to be the parent compound of the gut microbial metabolites urolithins. Previous research has identified that urolithin treatment increases the lifespan of CL4176 *C. elegans* and improves cellular respiration and induce mitophagy leading to overall improved mitochondrial health.

The third is an extract from the fruit of *Phyllanthus emblica* (Amla or Indian Gooseberry) as it is commonly used in the Ayurvedic system of traditional medicine. The pure chemical constituents of the Indian Gooseberry plant are well known and have been described as antioxidants. As with the pomegranate extract, the amla extract contains ellagic acid and several ellagic acid derivatives which are known to form urolithins upon ingestion. The extract of Indian Gooseberry has been identified as being potentially neuroprotective based on its antioxidant properties and its ability to reduced β -amyloid fibrillization.

This chapter describes the investigation of these sources of natural products for their potentially efficacious effects involved treating *C. elegans* with the various compounds and extracts in their growth media and examining differences in survival rates over time.

MANUSCRIPT 1

Manuscripts 1 appears as published in the Journal of Agriculture and Food Chemistry.

Isolation, identification, and biological evaluation of phenolic compounds from a traditional North American confectionary, maple sugar. Liu, Yougqiang. Rose, Kenneth N.; DaSilva, Nicholas A.; Johnson, Shelby L.; Seeram, Navindra P. (2017) Journal of Agricultural and Food Chemistry. 65, 4289-4295.

Abstract

Maple sap, collected from the sugar maple (*Acer saccharum*) tree, is boiled to produce the popular plant-derived sweetener, maple syrup, which can then be further evaporated to yield a traditional North American confectionery, maple sugar.

Although maple sap and maple syrup have been previously studied, the phytochemical constituents of maple sugar are unknown. Herein, 30 phenolic compounds, 1–30, primarily lignans, were isolated and identified (by HRESIMS and NMR) from maple sugar. The isolates included the phenylpropanoid-based lignan tetramers (erythro,erythro)-4'',4'''-dihydroxy-3,3',3'',3'''-5,5'-hexamethoxy-7,9';7',9'-diepoxy-4,8'';4',8'''-bisoxo-8,8'-dineolignan-7'',7''',9'',9'''-tetraol, 29, and (threo,erythro)-4'',4'''-dihydroxy-3,3',3'',3'''-5,5'-hexamethoxy-7,9';7',9'-diepoxy-4,8'';4',8'''-bisoxo-8,8'-dineolignan-7'',7''',9'',9'''-tetraol, 30, neither of which have been identified from maple sap or maple syrup before. Twenty of the isolates (selected on the basis of sample quantity available) were evaluated for their potential biological effects against lipopolysaccharide-induced inflammation in BV-2 microglia in vitro and juglone-induced oxidative stress in *Caenorhabditis elegans* in vivo. The current study increases scientific knowledge of possible bioactive compounds present in maple-derived foods including maple sugar.

1.0 Introduction

Maple syrup and maple sugar are traditional North American sweeteners commercially produced only in eastern North America, predominantly in the province of Quebec in Canada. They are obtained by boiling the sap collected from the sugar maple (*Acer saccharum*) species which is endemic to this part of the world. Maple syrup is obtained by boiling sap (approx. 40 L sap yields 1 L of syrup) and on further slow evaporation, under low heat, yields the crystallized maple sugar product (1 L of syrup yields ca. 950 g of sugar). In the seventeenth and eighteenth centuries, maple sugar was an important economic and political weapon in the fight to abolish slavery since cane sugar was associated with slave labor.¹ Nowadays, it is highly regarded as a specialty natural sweetener and confectionery with the characteristic flavor and odor of pure maple syrup which is highly desired by many consumers. Moreover, similar to maple syrup, maple sugar is of significant cultural and economic importance to this region of the world.

Over recent years, a number of chemical compositional and biological studies have been conducted on maple-derived food products, namely, maple sap (functional beverage applications)², maple syrup (food/sweetener applications),³⁻¹⁰ and maple syrup-derived extracts (nutraceutical/functional food applications).¹¹⁻¹⁹ Notably, maple syrup and its derived extracts and pure compounds have been reported to show a wide range of biological effects in vitro and in vivo including antioxidant, anti-inflammatory, anticancer, antidiabetic, and neuroprotective properties.¹¹⁻²¹ Therefore, given these promising data as well as the worldwide popularity and consumption of maple-derived food products, continued research to increase scientific knowledge of these natural products is necessary.

Our laboratory has had an ongoing research program focused on the isolation, identification, and biological evaluation of compounds from maple-derived foods.^{2-5,9-14,18-19} During these works, we reported on the isolation and structure elucidation (by NMR) of sixty-three compounds, primarily phenolics, as well as inulin, from these foods.^{2-5,10,13} Given that maple sugar has not been previously investigated, herein, our objectives were: 1) isolate and identify the phytochemicals in maple sugar using a combination of high resolution mass spectrometry (HRESIMS; by comparison to authentic standards previously isolated by our laboratory) and nuclear magnetic resonance (NMR; for those compounds for which we lacked authentic standards), and 2) evaluate the isolates for their effects against inflammation in murine BV-2 microglia in vitro and oxidative stress in *Caenorhabditis elegans* in vivo. This is the first study to isolate, identify, and biologically evaluate the phytochemicals present in maple sugar.

2.0 Materials and Methods

2.1 General Experimental Procedures

All nuclear magnetic resonance (^1H and ^{13}C NMR) were acquired on a Bruker 300 MHz instrument using deuterated methanol (CD_3OD) as solvent. High resolution electrospray ionization mass spectral (HRESIMS) data were acquired by direct infusion of the pure compounds on a Triple TOF 4600 (Applied Biosystems/MDS Sciex; Framingham, MA, USA) mass spectrometer equipped with a DuoSpray Ion Source.

2.2 Chemicals

ACS grade ethyl acetate was obtained from Pharmco-AAPER through Wilkem Scientific (Pawcatuck, RI, USA). The standards used for HRESIMS characterization were previously isolated and identified (by NMR and HRESIMS) by our laboratory.²⁻⁵ MCI resin and LC-MS grades of acetonitrile and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The C_{18} resin was purchased from Varian (Palo Alto, CA, USA).

2.3 Maple sugar

Maple sugar (25 Kg) was produced by the Federation of Maple Syrup Producers of Quebec (Longueil, Quebec, Canada) as previously reported by our laboratory.⁹ Briefly, maple syrup (containing < 1 % inverted sugar to ensure crystallization) was slowly heated in baking pans (22-24°C) which were then cooled on a cold surface (ca. 1 min per L) followed by immersion in cold water (for 2-5 min) to promote formation of the sugar crystals. As soon as crystals started to form, the mixture was stirred with a mechanical mixer by continuously scraping the bottom and sides of the pan until the

temperature dropped to below 32°C. Once stirring was completed, the maple sugar crystals were sieved to desired particle size.

2.4 Isolation of compounds 1-30 from maple sugar

Maple sugar (1250 g) was dissolved in DI water (2.5 L) and extracted with ethyl acetate (3 x 2.5 L) at room temperature. The combined ethyl acetate extracts were concentrated under reduced pressure *in vacuo* to yield a dried maple sugar ethyl acetate extract (463 mg). The maple sugar ethyl acetate extract (463 mg) was initially purified by C₁₈ resin medium pressure liquid column chromatography (glass column: 350 mm in height and 50 mm in diameter) by eluting with a gradient solvent system of 5%, 15%, 30%, 40%, 50%, 60%, 80%, and 100% of MeOH:H₂O to yield nine fractions, A-I, respectively. Fractions A-F were further individually purified by MCI resin column chromatography (glass column: 305 mm in height and 25 mm in diameter) using gradient solvent systems of MeOH:H₂O to yield compounds **1-30** as follows. Fraction A afforded compound **1** (0.1 mg); fraction B afforded compounds **2** (0.5 mg), **3** (1.0 mg), **4** (0.1 mg), **5** (0.1 mg), **6** (0.1 mg), **7** (0.1 mg), **8** (0.7 mg), **9** (1.1 mg), **10** (0.2 mg) and **11** (0.5 mg); fraction C afforded compounds **12** (1.0 mg), **13** (1.1 mg), **14** (4.6 mg), **15** (1.2 mg), **16** (3.8 mg), **17** (0.9 mg), **18** (0.4 mg) and **19** (1.0 mg); fraction D afforded compounds **20** (1.1 mg), **21** (0.7 mg), **22** (0.9 mg) and **23** (0.9 mg); fraction E afforded compounds **24** (0.8 mg), **25** (1.5 mg), **26** (0.5 mg), **27** (1.2 mg) and **28** (1.2 mg); and fraction F afforded compounds **29** (0.2 mg) and **30** (0.2 mg).

2.5 Identification of compounds 1-30 from maple sugar

Our laboratory has previously isolated and identified (by NMR and HRESIMS) sixty-three compounds from maple sap, maple syrup, and maple syrup-derived extracts.^{2-5,13} Therefore, using these authentic standards, compounds **1-28** were identified by

HRESIMS (see Figure 1). Compounds **29** and **30**, which were not previously isolated from these aforementioned maple-derived foods, were identified by NMR and HRESIMS (see below).

Compound **29**: (-) HRESIMS, m/z 809.3026 $[M-H]^-$, calcd. for molecular formula $C_{42}H_{50}O_{16}$; 1H -NMR (CD_3OD , 300 MHz) δ 3.24 (m, H-8, 8'), 3.36 (m, H-9''b, 9'''b), 3.61 (m, H-9''a, 9'''a), 3.84 (s, OCH_3 -3'', 3''', 5'', 5'''), 3.85 (s, OCH_3 -3, 5, 3', 5'), 3.87 (m, H-9b, 9'b), 3.91 (m, H-8''b, 8'''b), 4.30 (m, H-9a, 9'a), 4.78 (m, H-7, 7'), 4.90 (m, H-7'', 7'''), 6.70 (s, H-2, 6, 2', 6'), 6.74 (brs, H-6'', 6'''), 6.79 (brs, H-5'', 5'''), 6.99 (brs, H-2'', 2'''); ^{13}C -NMR (CD_3OD , 75 MHz) δ 55.7 (C-8, 8'), 56.3 (OCH_3 -3'', 3''', 5'', 5'''), 56.7 (OCH_3 -3, 3', 5, 5'), 61.7 (C-9'', 9'''), 73.1 (C-9, 9'), 74.1 (C-7'', 7'''), 87.2 (C-7, 7'), 104.3 (C-2, 6, 2', 6'), 111.4 (C-2'', 2'''), 115.6 (C-5'', 5'''), 120.7 (C-6'', 6'''), 133.8 (C-1'', 1'''), 136.1 (C-4, 4'), 138.9 (C-1, 1'), 146.9 (C-4'', 4'''), 148.6 (C-3'', 3'''), 154.5 (C-3, 5), 154.5 (C-3', 5').²²

Compound **30**: (-) HRESIMS, m/z 809.3053 $[M-H]^-$, calcd. for molecular formula $C_{42}H_{50}O_{16}$; 1H -NMR (CD_3OD , 400 MHz) δ 3.24 (m, H-8, 8'), 3.34 (m, H-9'''b), 3.45 (m, H-9''b), 3.68 (m, H-9'''a), 3.83 (s, OCH_3 -3''', 5'''), 3.84 (s, OCH_3 -3'', 5''), 3.85 (m, H-9''a), 3.88 (s, OCH_3 -3, 5), 3.89 (s, OCH_3 -3', 5'), 3.93 (m, H-9b, 9'b), 3.96 (m, H-8'''), 4.11 (m, H-8''), 4.29 (m, H-9a, 9'a), 4.78 (m, H-7, 7'), 4.99 (brs, H-7'', 7'''), 6.71 (brs, H-2, 6, 2', 6'), 6.74 (brs, H-5'', 5'''), 6.87 (brs, H-6'', 6'''), 6.99 (brs, H-2'', 2'''). ^{13}C -NMR (CD_3OD , 75 MHz) δ 55.7 (C-8, 8'), 56.3 (OCH_3 -3'', 3''', 5'', 5'''), 56.7 (OCH_3 -3, 3', 5, 5'), 61.7 (C-9''), 61.8 (C-9'''), 73.0 (C-9, 9'), 74.0 (C-7''), 74.3 (C-7'''), 87.2 (C-7, 7'), 87.3 (C-8''), 88.7 (8'''), 104.2 (C-2', 6'), 104.3 (C-2, 6), 111.4 (C-2''), 111.6 (C-2'''), 115.6 (C-5''), 115.8 (C-5'''), 120.1 (C-6''), 120.7 (C-6'''), 133.5 (C-1''), 133.8 (C-

1'''), 136.1 (C-4), 136.7 (C-4'), 138.9 (C-1), 139.1 (C-1'), 146.8 (C-4''), 147.1 (C-4'''), 148.7 (C-3''), 148.7 (C-3'''), 154.3 (C-3', 5'), 154.5 (C-3, 5).²²

2.5 Analytical High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD)

HPLC-DAD analyses were performed on a Hitachi Elite LaChrom system (Pleasanton, CA, USA) consisting of a L2130 pump, a L-2200 autosampler, and a L-2455 Diode Array Detector, operated by EZChrom Elite software. Chromatographic separation was performed on a Waters Sunfire C18 column (250 mm × 4.6 mm i.d., 5 µm, Waters, Massachusetts, U.S.A.) with a Waters Sunfire C18 VanGuard Pre-Column (20 mm × 4.6 mm i.d., 5 µm, Waters, Massachusetts, U.S.A.). The mobile phase consisted of 0.1% (v/v) formic acid/ water (A) and methanol (B), with a gradient elution at room temperature as follow: 0-5 min, 5% B; 5-165 min, from 5% to 53% B; 165-212 min, from 53% to 100% B; 212-224 min, 100% B. The flow rate was at 1.0 mL/min, the injection volumes were 20 µL, and the detection wavelength was 280 nm.

2.6 Ultra-Fast Liquid Chromatography Mass Spectrometry (UFLC-ESI-TOF-MS)

UFLC-ESI-TOF-MS analyses were performed on a SHIMADZU Prominence UFLC system (Marlborough, MA, USA) consisting of two LC-20AD pumps, a DGU-20A degassing unit, SIL-20AC auto sampler, CTO-20AC column oven and CBM-20A communication bus module. Chromatographic separation was performed on a Waters XBridge® BEH C₁₈ column (100 mm × 2.1 mm i.d., 2.5 µm, Milford, MA, USA). The mobile phase consisted of 0.1% (v/v) formic acid:acetonitrile (solvent A) and 0.1% (v/v) formic acid:H₂O (solvent B) with a gradient elution of 3-27% A from 0-96 min,

27-97% A from 96-97 min, and 97% A from 97-110 min. The column temperature was 40 °C, flow rate was 0.2 mL/min, and injection volume was 4 µL. Mass spectrometry was performed using a Triple TOF 4600 system from Applied Biosystems/MDS Sciex (Framingham, MA, USA) coupled with an electrospray ionization (ESI) interface. Nitrogen was used in all cases. In this study, the parameters were optimized as follows: ESI voltage, -4500 V; nebulizer gas, 40; auxiliary gas, 60; curtain gas, 30; turbo gas temperature, 600°C; declustering potential, -70V. Instrument calibration was carried out according to the manufacturer's instructions. The mass range was set from m/z 100 to 1000 and the mass range for product ion scan was m/z 100-1000. The data were acquired and processed using Analyst TF 1.7 Software.

2.7 Sample preparation for HPLC-DAD and UFLC-ESI-TOF-MS analyses

The maple sugar ethyl acetate extract (20 mg) was dissolved in 4 mL 60% aqueous methanol (LC-MS grade). The solution was stored at 4 °C and filtered (0.22 micron filter) before analyses.

2.8 Isolates for bioassays

Based on availability of the pure compounds isolated from maple sugar (obtained in quantities > 0.5 mg), we evaluated twenty-one of the thirty isolates as follows: **2, 3, 8, 9, 11-17, 19- 28.**

2.9 Cell culture conditions

Murine BV-2 microglia were a kind gift from Dr. Grace Sun (University of Missouri, Columbia, MO, USA). The cells were cultured in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (Gibco, Grand Island, NY, USA) and penicillin/ streptomycin antibiotics (1%) (Gibco, Grand Island, NY, USA). Cells were cultured and allowed to incubate within a humidified chamber at

approximately 37°C under 5% CO₂. Prior to seeding, cells were examined for their viability and counted using trypan blue staining and a hemocytometer.

2.10 Anti-inflammatory assay (by measuring nitric oxide release with the Griess reagent)

The murine BV-2 microglia cells were cultured as previously described by our laboratory.¹⁸ In brief, microglia were seeded into 24-well plates at a density of approximately 100,000 cells/mL. Cells were serum starved after following a 24 h incubation period to ensure adherence. Cells were then exposed to either pure compounds (at 10 µM), the positive control, resveratrol (RESV; at 10 µM), or vehicle control for 1 hr. Lipopolysaccharide (LPS; at a concentration of 1 µg/mL) was then added directly to the cells and allowed to incubate for 23 hours. Following this incubation period, the total nitric oxide released into cell culture media was quantified using the Greiss Reagent System (Promega, Madison, WI, USA).

2.11 *Caenorhabditis elegans* maintenance

Wild-type (N2) *Caenorhabditis elegans* were obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota (Minneapolis, MN, USA). Worm cultures were maintained on Nematode Growth Medium (NGM) (1.7% Agar, 0.3% NaCl, 0.25% peptone, 1 mM CaCl₂, 1 mM MgSO₄, 5 mg/L cholesterol in ethanol, and 2.5 mM KPO₄) in petri dishes at 20°C with UV-killed OP50 *Escherichia coli* as a food source. Age synchronous cultures were obtained by the standard hypochlorite method as previously reported by our laboratory.¹⁸ For sample treatment, *C. elegans* were washed from their plates with liquid nematode growth medium (0.3% NaCl, 0.25% peptone, 1 mM CaCl₂, 1 mM MgSO₄, 5 mg/L cholesterol in ethanol, and

2.5 mM KPO₄) and transferred to culture flasks containing UV-killed OP50 *E. coli* as a food source.

2.12 *Caenorhabditis elegans* oxidative stress resistance assay

To determine if oxidative stress resistance is improved in *C. elegans*, a method to induce oxidative stress was adapted as previously reported with minor modifications.²³ Briefly, age synchronous culture of N2 (L2) worms in liquid NGM were transferred into the wells of a 96-well plate. To each individual well, compounds were added to achieve a final concentration of 10 µM. A solvent control well was made by adding DMSO/water solution to achieve a final DMSO concentration of 0.01%. The cultures were incubated in the dark for 24 h. After incubation with the compounds, the cultures were exposed to 400 µM of 5-hydroxy-1,4-naphthoquinine (also known as juglone). Immediately after the addition of juglone, a baseline count was taken with a dissecting microscope after which the worms were counted every hour for 6 hours.

3.0 Results and Discussion

3.1 Isolation and identification of compounds in maple sugar

Our laboratory has conducted extensive studies on maple sap and maple syrup which has led to the isolation and identification (by NMR) of sixty-three compounds, predominantly phenolics, belonging to the lignan sub-class.^{2-5,13} Given our group's access to these authentic maple standards (many of which are not commercially available), we initially compared the HPLC-DAD profiles of the ethyl acetate extracts of maple syrup and maple sugar. As expected, there were marked similarities in the chromatograms of maple syrup and maple sugar suggesting that the phenolic compounds present in maple syrup were preserved in the slow heating evaporation process required for its transformation to maple sugar. This was in support of our previous findings where we reported that the natural phenolic compounds present in maple sap are also preserved in the intensive heating/boiling process required in its transformation to maple syrup.² Thus, while flavor/odor compounds are created during the cooking/boiling process of transforming maple sap to maple syrup, it was apparent that the majority of the phenolic compounds originally present in the xylem sap of the maple tree persist in maple syrup and maple sugar.

In our previous research projects on maple sap and maple syrup,^{2-5,13} we obtained the majority of the isolates in limited quantities which have since been exhausted thereby hampering their evaluation in bioassays. Therefore, although we did not seek to 'comprehensively re-isolate' all of the compounds in maple sugar, we were able to isolate (using chromatography) and identify (using HRESIMS and NMR) thirty (**1-30**) of its constituents from a maple sugar ethyl acetate extract. **Figure 1** shows the total ion chromatography (TIC) of the maple sugar ethyl acetate extract with the accompanying

identities of compounds **1-30** shown in Table **1** and their structures shown in **Figure 2**. It should be noted that compounds **1-28** were identified by HRESIMS (by comparison with authentic standards previously isolated by our laboratory^{2-5,13}) while compounds **29** and **30** (for which we lacked authentic standards) were identified by NMR (further described below).

Thus, based on retention times (tR) and accurate *m/z* of pseudo molecular ions (by comparison to authentic standards), compounds **1-28** were identified as 2,3-dihydroxy-1-(3,4-dihydroxyphenyl)-1-propanone (**1**), *C*-veratroylglycol (**2**), 2,3-dihydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone (**3**), (*erythro,erythro*)-1-[4-(2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-1-(hydroxymethyl)ethoxy)-3-methoxyphenyl]-1,2,3-propanetriol (**4**), 1,2-diguaiacyl-1,3-propanediol (**5**), (*erythro,erythro*)-1-[4-[2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-1-(hydroxymethyl)ethoxy]-3,5-dimethoxyphenyl]-1,2,3-propanetriol (**6**), epicatechin (**7**), (*threo,erythro*)-1-[4-[2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-1-(hydroxymethyl)ethoxy]-3,5-dimethoxyphenyl]-1,2,3-propanetriol (**8**), 3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)propan-1-one (**9**), coumaric acid (**10**), leptolepisol D (**11**), guaiacylglycerol- β -O-4'-coniferyl alcohol (**12**), lyoniresinol (**13**), *threo*-guaiacylglycerol- β -O-4'-dihydroconiferyl alcohol (**14**), isolariciresinol (**15**), *erythro*-guaiacylglycerol- β -O-4'-dihydroconiferyl alcohol (**16**), [3-[4-[(6-deoxy- α -L-mannopyranosyl)oxy]-3-methoxyphenyl]methyl]-5-(3,4-dimethoxyphenyl)-dihydro-3-hydroxy-4-(hydroxymethyl)-2(3H)-furanone (**17**), *erythro*-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2,6-dimethoxyphenoxy]-1,3-propanediol (**18**), 5-(3'',4''-dimethoxyphenyl)-3-hydroxy-3-(4'-hydroxy-3'-methoxybenzyl)-4-(hydroxymethyl)dihydrofuran-2-one (**19**), secoisolariciresinol (**20**), icaraside E4 (**21**),

sakuraresinol (**22**), dehydroconiferyl alcohol (**23**), syringaresinol (**24**), acernikol (**25**), 2-[4-[2,3-dihydro-3-(hydroxymethyl)-5-(3-hydroxypropyl)-7-methoxy-2-benzofuranyl]-2,6-dimethoxyphenoxy]-1-(4-hydroxy-3-methoxyphenyl)-1,3-propanediol (**26**), (1*S*,2*R*)-2-[2,6-dimethoxy-4-[(1*S*,3*aR*,4*S*,6*aR*)-tetrahydro-4-(4-hydroxy-3,5-dimethoxyphenyl)-1*H*,3*H*-furo[3,4-*c*]furan-1-yl]phenoxy]-1-(4-hydroxy-3-methoxyphenyl)-1,3-propanediol (**27**) and buddlenol E (**28**). Since we lacked authentic standards for compounds **29** and **30**, they were identified as two di-lignans by NMR data (provided in the Methods section) as (7*R*,7'*R*,7''*S*,7'''*S*,8*S*,8'*S*,8''*S*,8'''*S*)-4'',4'''-dihydroxy-3,3',3'',3'''-5,5'-hexamethoxy-7,9';7', 9-diepoxy-4,8'';4',8'''-bisoxy-8,8'-dineolignan-7'',7''',9'',9'''-tetraol (**29**) and (7*R*,7'*R*,7''*R*,7'''*S*,8*S*,8'*S*,8''*S*,8'''*S*)-4'',4'''-dihydroxy-3,3',3'',3'''-5,5'-hexamethoxy-7,9';7', 9-diepoxy-4,8'';4',8'''-bisoxy-8,8'-dineolignan-7'',7''',9'',9'''-tetraol (**30**). The NMR data of these di-lignans were in agreement with published values.²² Whilst this is the first report of these di-lignans in a maple-derived food product, given that these are naturally occurring plant secondary metabolites, it is highly likely that they are also present in maple sap and maple syrup but were not isolated/obtained in our previous studies.^{2-5,13} As shown in the HPLC-DAD chromatograms of maple syrup and maple sugar, there are many minor peaks present in these foods and obtaining all of these compounds is not possible due to a variety of reasons including complicated matrix with multiple overlapping peaks, low yields of isolates, etc. Nevertheless, overall, similar to our previous observations for maple sap and maple syrup,^{2-5,13} the majority of the phenolic compounds found in maple sugar are also lignans.

3.2 Evaluation of anti-inflammatory effects of maple sugar compounds in murine BV-2 microglia

Our group has previously utilized a murine BV-2 microglia cell model to evaluate the anti-inflammatory effects of a phenolic-enriched maple syrup extract.¹⁸ However, as previously mentioned, because of limited sample quantities available, the pure maple compounds were not assayed in that study. Therefore, herein, the BV-2 murine microglia were pre-treated with twenty-one of the maple sugar isolates (obtained > 0.5 mg), namely, compounds **2**, **3**, **8**, **9**, **11-17**, and **19-28** (at 10 μ M) for one hour (**Figure 3A**). Resveratrol (RESV; at 10 and 20 μ M) was used as a positive control since this natural polyphenol is well-known for its anti-inflammatory properties.¹⁸ Immediately following pre-treatment with the compounds, lipopolysaccharide (LPS) was added to stimulate the production of nitric oxide (NO). Total NO concentrations found in cell culture media of LPS-treated cells were approximately 33.85 μ M $\pm$ 0.23 as compared to vehicle treated cells which released approximately 0.69 μ M $\pm$ 0.04 of NO. RESV produced significantly lower NO media levels by approximately 16.36% (28.31 μ M $\pm$ 0.33) and 33.76% (22.42 μ M $\pm$ 0.05) at 10 and 20 μ M respectively (**Figure 3A**). Among the isolates, compounds **3**, **8**, **9**, **17**, and **24** significantly decreased NO levels as follows 9.89% (30.5 μ M $\pm$ 0.14), 18.52% (27.58 μ M $\pm$ 0.72), 4.99% (32.16 μ M $\pm$ 0.23), 4.96% (32.17 μ M $\pm$ 0.41) and 9.57% (30.61 μ M $\pm$ 0.84), respectively (**Figure 3B**). Based on this data, it is not possible to make any structure activity related (SAR) inferences given the structural diversity of the compounds.

3.3 Evaluation of effects of maple sugar isolates on oxidative stress in *Caenorhabditis elegans* in vivo

Natural products, including resveratrol, have been shown to improve oxidative stress resistance and life span in *Caenorhabditis elegans* after exposure to the pro-oxidant, juglone.^{23,24} Therefore, using this in vivo model, Kaplan-Meier survival curves were

generated for the twenty-one maple sugar isolates and their Mantel-Haenszel Ratio (MHR) are listed in **Table 3**. Nine of the twenty-one compounds extended lifespan of *C. elegans* in a high-oxidative stress environment by a statistically significant margin ($P < 0.01$), namely, **16**, **17**, **20-24**, **26**, and **28** indicating that these compounds were mitigating the toxicity of juglone. Similar to the data accumulated from the in vitro BV-2 cell assay, it is not possible to make any SAR inferences given the structural diversity of the compounds.

In summary, this is the first study to isolate, identify, and biologically evaluate the phytochemicals present in the traditional North American confectionery, maple sugar. Similar to other maple-derived food products, maple sugar contains a variety of phenolic compounds among which the lignan sub-class predominate. The protective effects of the maple sugar isolates against inflammation (in BV-2 microglia in vitro) and oxidative stress (in *C. elegans* in vivo) is in agreement with a vast body of literature supporting similar effects for phenolic compounds found in plant foods. Whether these biological effects are translatable in vivo would require animal studies which is included in our group's future planned studies.

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Figure 1. Total ion chromatogram (TIC) of maple sugar ethyl acetate extract.

Figure 2. Compounds identified in maple sugar ethyl acetate extract.

Figure 3. Anti-inflammatory effects of maple sugar isolates assayed by measuring nitric oxide release by murine BV-2 microglia after exposure to lipopolysaccharide (LPS). **Fig. 3A** BV-2 microglia were pretreated with twenty-one isolates (10 μ M), positive control, resveratrol (RESV; 10 and 20 μ M) or vehicle control (DMSO) for one hour prior to lipopolysaccharide (LPS) stimulation at 1 μ g/mL for 23 hours. The total NO concentrations were assayed using the Greiss Assay. **Fig. 3B.** At 10 μ M, compounds **3**, **8**, **9**, **17**, and **24** and RESV significantly decreased NO release as compared to the cells treated with LPS-alone. Statistical significance as determined by student's t-test was determined as follows p < 0.05 * ; p < 0.01 ** ; p < 0.001 *** ; p < 0.0001 ****.

Table 1. UFLC-ESI-TOF-MS data for compounds identified in maple sugar ethyl acetate extract.

No .	Compounds	tR	HR-ESI(-)-MS	Error	Molecular
		(min)	[M-H] ⁻ m/z exptl (theor)	(ppm)	formula
1	2,3-dihydroxy-1-(3,4-dihydroxyphenyl)-1-propanone	4.71	197.0462 (197.0455)	3.3132	C ₉ H ₁₀ O ₅
2	C-veratroylglycol	8.84	211.0621 (211.0611)	4.2773	C ₁₀ H ₁₂ O ₅
3	2,3-dihydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone	11.6	241.0727 (241.0717)	3.8912	C ₁₁ H ₁₄ O ₆
4	(<i>erythro,erythro</i>)-1-[4-(2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-1-(hydroxymethyl)ethoxy)-3-methoxyphenyl]-1,2,3-propanetriol	13.2	409.1499 (409.1504)	-	C ₂₀ H ₂₆ O ₉
5	1,2-diguaiacyl-1,3-propanediol	15.8	319.1190 (319.1187)	0.9019	C ₁₇ H ₂₀ O ₆
6	(<i>erythro,erythro</i>)-1-[4-[2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-1-(hydroxymethyl)ethoxy]-3,5-dimethoxyphenyl]-1,2,3-propanetriol	17.9	439.1617 (439.1609)	1.6598	C ₂₁ H ₂₈ O ₁₀
7	epicatechin	22.7	289.0717 (289.0717)	-	C ₁₅ H ₁₄ O ₆
8	(<i>threo,erythro</i>)-1-[4-[2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-1-(hydroxymethyl)ethoxy]-3,5-dimethoxyphenyl]-1,2,3-propanetriol	24.2	439.1624 (439.1609)	3.2537	C ₂₁ H ₂₈ O ₁₀
9	3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)propan-1-one	25.4	225.0773 (225.0768)	2.0113	C ₁₁ H ₁₄ O ₅
10	coumaric acid	26.5	163.0408 (163.0400)	4.4910	C ₉ H ₈ O ₃
11	leptolepisol D	30.5	515.1941 (515.1922)	3.5496	C ₂₇ H ₃₂ O ₁₀
12	guaiacylglycerol- β -O-4'-coniferyl alcohol	39.9	375.1457 (375.1449)	2.0605	C ₂₀ H ₂₄ O ₇
13	lyoniresinol	40.4	419.1727 (419.1711)	3.7173	C ₂₂ H ₂₈ O ₈
14	<i>threo</i> -guaiacylglycerol- β -O-4'-dihydroconiferyl alcohol	42.3	377.1606 (377.1605)	0.0608	C ₂₀ H ₂₆ O ₇
15	isolaricresinol	43.9	359.1504 (359.1500)	1.0793	C ₂₀ H ₂₄ O ₆
16	<i>erythro</i> -guaiacylglycerol- β -O-4'-dihydroconiferyl alcohol	44.7	377.1609 (377.1605)	0.8562	C ₂₀ H ₂₆ O ₇

17	[3-[4-[(6-deoxy- α -L-mannopyranosyl)oxy]-3-methoxyphenyl)methyl]-5-(3,4-dimethoxyphenyl)-dihydro-3-hydroxy-4-(hydroxymethyl)-2(3H)-furanone	50.9 0	[M-H+HCOOH] ⁻ 595.2057 (595.2032)	4.1499	C ₂₇ H ₃₄ O 12
18	<i>erythro</i> -1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2,6-dimethoxyphenoxy]-1,3-propanediol	52.2 2	407.1720 (407.1711)	2.1077	C ₂₁ H ₂₈ O 8
19	5-(3'',4''-dimethoxyphenyl)-3-hydroxy-3-(4'-hydroxy-3'-methoxybenzyl)-4-(hydroxymethyl)dihydrofuran-2-one	52.8 1	403.1409 (403.1398)	2.6253	C ₂₁ H ₂₄ O 8
20	secoisolaricresinol	54.7 5	361.1655 (361.1656)	- 0.4497	C ₂₀ H ₂₆ O 6
21	icariside E ₄	56.8 9	[M-H+HCOOH] ⁻ 551.2157 (551.2134)	4.1714	C ₂₆ H ₃₄ O 10
22	sakuraresinol	60.1 0	[M-H+HCOOH] ⁻ 509.2049 (509.2028)	4.0535	C ₂₄ H ₃₂ O 9
23	dehydroconiferyl alcohol	60.5 8	359.1512 (359.1500)	3.3068	C ₂₀ H ₂₄ O 6
24	syringaresinol	69.5 0	417.1566 (417.1554)	2.6567	C ₂₂ H ₂₆ O 8
25	acernikol	73.6 0	585.2364 (585.2341)	3.8683	C ₃₁ H ₃₈ O 11
26	2-[4-[2,3-dihydro-3-(hydroxymethyl)-5-(3-hydroxypropyl)-7-methoxy-2-benzofuranyl]-2,6-dimethoxyphenoxy]-1-(4-hydroxy-3-methoxyphenyl)-1,3-propanediol	78.2 7	585.2360 (585.2341)	3.1848	C ₃₁ H ₃₈ O 11
27	(1 <i>S</i> ,2 <i>R</i>)-2-[2,6-dimethoxy-4-[(1 <i>S</i> ,3 <i>aR</i> ,4 <i>S</i> ,6 <i>aR</i>)-tetrahydro-4-(4-hydroxy-3,5-dimethoxyphenyl)-1 <i>H</i> ,3 <i>H</i> -furo[3,4- <i>c</i>]furan-1-yl]phenoxy]-1-(4-hydroxy-3-methoxyphenyl)-1,3-propanediol	82.4 3	613.2307 (613.2290)	2.6894	C ₃₂ H ₃₈ O 12
28	buddlenol E	83.0 8	583.2200 (583.2184)	2.5959	C ₃₁ H ₃₆ O 11
29	(7 <i>R</i> ,7' <i>R</i> ,7'' <i>S</i> ,7''' <i>S</i> ,8 <i>S</i> ,8' <i>S</i> ,8'' <i>S</i> ,8''' <i>S</i>)-4'',4'''-dihydroxy-3,3',3'',3'''-5,5'-hexamethoxy-7,9';7',9'-diepoxy-4,8'';4',8'''-bisoxo-8,8'-dineolignan-7'',7''',9'',9'''-tetraol	91.6 5	809.3048 (809.3026)	2.7062	C ₄₂ H ₅₀ O 16
30	(7 <i>R</i> ,7' <i>R</i> ,7'' <i>R</i> ,7''' <i>S</i> ,8 <i>S</i> ,8' <i>S</i> ,8'' <i>S</i> ,8''' <i>S</i>)-4'',4'''-dihydroxy-3,3',3'',3'''-5,5'-hexamethoxy-7,9';7',9'-diepoxy-4,8'';4',8'''-bisoxo-8,8'-dineolignan-7'',7''',9'',9'''-tetraol	96.4 0	809.3053 (809.3026)	3.3241	C ₄₂ H ₅₀ O 16

Table 2. Effects of maple sugar isolates on oxidative stress induced by exposure to juglone in *Caenorhabditis elegans* Age synchronous *C. elegans* were treated with the maple sugar isolates (10 μ M) for 24 h after which the pro-oxidant, juglone was added (400 μ M). Living worms were counted every hour for six hours. Kaplan-Meier survival curves were constructed using GraphPad Prism 7. The Mantel-Haenszel ratios calculated from the curves were used as measures of statistical significance.

compound	Mantel-Haenszel ratio	95% CI	$p > 0.01$	compound	Mantel-Haenszel Ratio	95% CI	$p > 0.01$
2	0.8436	0.5025–1.416		17	2.547	1.465–4.426	***
3	1.258	0.7728–2.046		19	2.405	1.356–4.267	
8	1.996	1.208–3.297		20	3.332	1.959–5.670	***
9	2.131	1.382–3.286		21	4.415	2.596–7.506	***
11	1.971	1.048–3.286		22	4.773	2.676–8.515	***
12	2.552	1.453–4.379		23	2.842	1.616–5.000	***
13	1.949	1.126–3.372		24	2.577	1.505–4.412	***
14	2.601	1.465–4.617		25	2.054	1.207–3.494	
15	4.580	2.717–7.721	***	27	1.816	0.9762–3.380	
16	2.862	1.604–5.105	***	28	3.766	2.261–6.273	***
resveratrol	4.567	2.598–8.028	***				

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Abstract

Medicinal plants are promising candidates for Alzheimer's disease (AD) research but there is lack of systematic algorithms and procedures to guide their selection and evaluation. Herein, we developed an AD Neuroprotective Potential Algorithm (NPA) by evaluating twenty-three standardized and chemically characterized Ayurvedic medicinal plant extracts in a panel of bioassays targeting oxidative stress, carbonyl stress, protein glycation, amyloid beta (A β) fibrillation, acetylcholinesterase (AChE) inhibition, and neuroinflammation. The twenty-three herbal extracts were initially evaluated for: 1) total polyphenol content (Folin-Ciocalteu assay), 2) free radical scavenging capacity (DPPH assay), 3) ferric reducing antioxidant power (FRAP assay), 4) reactive carbonyl species scavenging capacity (methylglyoxal trapping assay), 5) anti-glycative effects (BSA-fructose, and BSA-methylglyoxal assays) and, 6) anti-A β fibrillation effects (thioflavin-T assay). Based on assigned index scores from the initial screening, twelve extracts with a cumulative NPA score ≥ 40 were selected for further evaluation for their: 1) inhibitory effects on AChE activity, 2) in vitro anti-inflammatory effects on murine BV-2 microglial cells (Griess assay measuring levels of lipopolysaccharide-induced nitric oxide species), and 3) in vivo neuroprotective effects on *Caenorhabditis elegans* post induction of A β ₁₋₄₂ induced neurotoxicity and paralysis. Among these, four extracts had a cumulative NPA score ≥ 60 including *Phyllanthus emblica* (amla; Indian gooseberry), *Mucuna pruriens* (velvet bean), *Punica granatum* (pomegranate) and *Curcuma longa* (turmeric; curcumin). These extracts also showed protective effects on H₂O₂ induced cytotoxicity in differentiated cholinergic human neuronal SH-SY5Y and murine BV-2 microglial cells and reduced tau protein levels in the SH-SY5Y neuronal cells. While published animal data support the neuroprotective

effects of several of these Ayurvedic medicinal plant extracts, some remain unexplored for their anti-AD potential. Therefore, the NPA may be utilized, in part, as a strategy to help guide the selection of promising medicinal plant candidates for future AD-based research using animal models.

1.0 Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorder characterized by the progression of cognitive decline leading to severe dementia (Buckner et al., 2005). The accumulation of senile plaques and neurofibrillary tangles in cerebral cortex and hippocampus are two major pathological hallmarks of AD (Ittner and Götz, 2011). However, due to the complexity of the disease, the precise factors which trigger the development of AD remains unknown (Alzheimer's Association, 2015). Moreover, given the unclear etiology of AD, current therapeutic approaches focus mainly on symptom management but no treatment is available to alter or reverse the course of the disease (Alzheimer's Association, 2015; Citron, 2010). Although the pathogenesis of AD is still under investigation, increasing evidence suggest that AD is a multifactorial disease which develops as a result of several risk contributors instead of a single cause alone (Norton et al., 2014; Reitz and Mayeux, 2014).

Oxidative stress and the production of reactive oxygen species (ROS) have been implicated in the pathogenesis of AD and are believed to be leading causative factors for neuronal cell dysfunction and cell death (Lin and Beal, 2006; Smith et al., 2000). It has been demonstrated that the products of protein oxidation and lipid peroxidation are elevated in AD patients (Christen, 2000). In addition, in the AD brain, the activities of antioxidant enzymes are altered, accompanied with a decline in the expression of these antioxidant enzymes (Christen, 2000; Smith et al., 2000). Given the established links between oxidative stress and AD, antioxidants, including those from natural products, are extensively studied for their neuroprotective abilities and constitute dietary intervention strategies for AD prevention and treatment (Alzheimer's Association, 2015; Choi et al., 2012; Praticò, 2008).

Apart from oxidative stress, carbonyl stress and the formation of advanced glycation end-products (AGEs) resulting from protein glycation are also believed to be vital contributors to AD (Srikanth et al., 2011; Vicente Miranda and Outeiro, 2010). Glycation is one type of post-translational modification of proteins, resulting in the formation of AGEs both intracellularly and extracellularly. Glycation and AGEs formation are associated with AD due to several reasons. First, AGEs bind to the transmembrane receptor, RAGE (receptor for AGEs), upregulate RAGE expression, and activate RAGE-mediated neuronal dysfunction and neuron damages (Srikanth et al., 2011). Second, RAGE mediates the transportation of beta amyloid (A β) across the blood brain barrier (BBB) (Donahue et al., 2006). Therefore, the activation of RAGE by AGE can cause A β accumulation in the brain. Third, during the course of glycation and AGE formation, ROS and reactive carbonyl species (RCS) are generated as by-products which, in turn, promote AGE formation and cause neurotoxicity (Ahmed et al., 2005; Münch et al., 2012; Picklo et al., 2002). Consequently, all of the factors involved in this positive feedback loop including AGEs, RCS, and ROS are considered to be promising targets for AD prevention and treatment.

Another common target for AD therapy is the A β peptide which consists of 40 to 42 amino acids and is generated from the cleavage of the A β precursor protein. A β is the major component of senile plaques and neurofibrillary tangles, two pathological hallmarks of AD (Buckner et al., 2005; Palop and Mucke, 2010). In AD patients, elevated A β levels were observed in both cerebrospinal fluid and blood (Mawuenyega et al., 2010). In addition, certain forms of A β , including fibrillated A β and glycated A β (A β -AGEs), have been shown to be neurotoxic (Butterfield, 2002; Koo et al., 1999; Li et al., 2013). Fibrillated A β can induce neurotoxicity by enhancing neuronal oxidative

stress and neuroinflammation (Butterfield, 2002; Koo et al., 1999). A β -AGEs can induce intracellular oxidative stress and inflammation by activating RAGE and upregulating RAGE expression in neuronal cells (Li et al., 2013). Therefore, considerable research efforts have been directed to finding inhibitors which may prevent or reverse the formation of A β fibrils and A β -AGEs. For example, aminoguanidine (AG), a synthetic glycation inhibitor, can reduce glycated A β formation, attenuate RAGE upregulation, and restore the cognitive deficit in AD animal models (Li et al., 2013). However, AG failed in human clinical trials due to severe side effects (Thornalley, 2003) leading to the search for non-toxic alternatives including medicinal plants and their derived natural products and botanical extracts (Solanki et al., 2016; Venigalla et al., 2016).

In addition to oxidative stress, glycation, and A β formation, neuroinflammation is another pivotal factor implicated in the development of neurodegenerative diseases with increased inflammation observed in AD (Eikelenboom et al., 2002). In addition, inflammatory stress leads to the activation of microglia cells, the immune cells in the central nervous system, which release nitric oxide species (NOS) including nitrates and nitrites. These NOS are neurotoxic and cause massive neuronal death further exacerbating neurodegenerative diseases (Eikelenboom et al., 2002; Eikelenboom et al., 2006).

For centuries, traditional systems of medicines such as Ayurveda [from India, a country which has one of the lowest incidences of AD worldwide (Chandra et al., 2001; Vas et al., 2001)] and traditional Chinese medicine (TCM) (Steele et al., 2013), have used medicinal plants to treat several ailments including neurodegenerative diseases. While neurochemical/biological studies have been conducted on some these traditional

medicinal plants, there is a lack of systematic procedures and algorithms to help guide the selection and evaluation of the most promising candidates for further AD-based research using animal models. This is urgently needed given the large variety of medicinal plant species (and combinations thereof) used worldwide in the traditional systems of medicines of various cultures. Furthermore, although medicinal plants are consumed as foods, herbs, spices, beverages, and botanical extracts, their underlying mechanisms of neuroprotective effects remain unclear. Therefore, given all of the aforementioned factors, herein, we utilized a panel of bioassays including total polyphenol contents, antioxidant capacities, anti-glycation effects, carbonyl scavenging abilities, anti-A β fibrillation, acetylcholinesterase (AChE) inhibition, and anti-neuroinflammatory activities to develop a Neuroprotective Potential Algorithm (NPA) to aid in the evaluation and selection of promising medicinal plant candidates for future AD based research using pre-clinical animal models (see **Figure 1**). We selected twenty-three commercially available and chemically characterized medicinal plant extracts (see **Table 1**), commonly consumed as foods (in India, and elsewhere), and used in Ayurveda, to develop the NPA.

2.0 Materials and Methods

2.1. Chemicals

The herbal extracts are botanically authenticated and chemically standardized GRAS (generally regarded as safe) extracts which are commercially available for human consumption and sourced from a single reputable natural products supplier, namely, Verdure Sciences (Noblesville, IN, USA), to ensure access to validated and consistent samples. The Latin binomial and common names, as well as the traditional uses, of the twenty-three medicinal plant species are provided in **Table 1**. Each extract was dissolved in DMSO (20.0 mg/mL) and diluted to test concentrations (10-2000 µg/mL) with 0.1 M phosphate buffer (pH=7.2). The following chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA): butylated hydroxytoluene (BHT), bovine serum albumin (BSA), methylglyoxal (MGO), aminoguanidine hydrochloride (AG), gallic acid, resveratrol (RESV), galanthamine, 1,2-phenylenediamine (PD), 2,3-dimethylquinoxaline (DQ), 2,4,6-tripyridyl-s-triazine, iron (III) chloride hexahydrate, L-ascorbic acid, HPLC-grade methanol and trifluoroacetic acid (TFA), thioflavin T agent (ThT), Tris-HCl buffer (pH 8.0), acetylcholinesterase (AChE; from electric eel), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and acetylthiocholine iodide (ATCI). Human beta amyloid 1-42 (A β ₁₋₄₂) peptide (Catalog #: AS-2027) was purchased from Anaspec (San Jose, CA, USA).

2.2. Antioxidant assays

2.2.1. Free radical scavenging activity (DPPH assay)

The extracts were evaluated for their free radical scavenging capacity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as previously reported by our laboratory (Ma et al., 2016). Briefly, 100 µL serial dilutions (10-2000 µg/mL) of each test sample were

mixed with 100 μ L of a 0.2 mM DPPH solution in a 96-well plate. After incubation at room temperature for 30 min, sample absorbance was read (at 517 nm) using a microplate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA).

2.2.2. Ferric reducing antioxidant power (FRAP assay)

The ferric reducing antioxidant power (FRAP) of the extracts were measured following published methods (Maksimović et al., 2005; Tsai et al., 2002). The FRAP reagent contained 100 mL acetate buffer (3 mM, pH 3.6), 10 mL 2,4,6-tripyridyl-s-triazine (10 mM) in 40 mM hydrochloric acid, and 10 mL $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mM). Briefly, 1.5 mL of freshly prepared FRAP reagent was mixed with 50 μ L of each extract (final concentration of 100 μ g/mL) and the absorbance was measured at 593 nm after 5 minutes using an UltroSpec 2100 instrument (Biochrom Ltd, Cambridge, UK). L-ascorbic acid (0.1 - 3 mM) was used as the positive control and to generate a calibration curve. The FRAP capacity of each extract was expressed as ascorbic acid equivalents (AAE)/mg where AAE is defined as follows: the reducing power of 1 mg dry plant material is equivalent to the reducing power of 1 nM of L-ascorbic acid (Maksimović et al., 2005). All data were the average of three individual experiments.

2.3. Total polyphenol content

The extracts were quantified for total phenolic content using the Folin–Ciocalteu method and expressed as gallic acid equivalents (GAEs) as previously reported by our laboratory (Jean-Gilles et al., 2012). Briefly, each sample (5 mg) was dissolved in 500 μ L of 50% aqueous methanol, and 100 μ L of each sample was incubated with 50 μ L of the Folin–Ciocalteu reagent for 5 min at room temperature. Then 150 μ L of a 20% sodium carbonate (Na_2CO_3) solution and 250 μ L of distilled water were added to each sample and the resulting solution was vortexed. The reaction mixtures were further

incubated for 30 min at 40 °C and then immediately cooled to room temperature. The standard (i.e. gallic acid) was prepared in parallel with the samples. Each sample was centrifuged and 200 µL of supernatant was added to a 96-well plate and absorbance read at 756 nm on a Spectramax plate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA).

2.4. BSA-fructose assay

The extracts were evaluated for their anti-glycation effects using the BSA-fructose assay based on previously reported methods by our laboratory (Liu et al., 2014; Ma et al., 2015). Briefly, 100 µg/mL of each extract was added to the glycation reaction mixture containing 10 mg/mL BSA and 100 mM D-fructose and incubated at 37 °C for 21 days. The intrinsic fluorescence of each sample was measured at excitation and emission wavelengths of 360 nm and 435 nm, respectively. The synthetic anti-glycating agent, aminoguanidine (AG), at an equivalent concentration of 100 µg/mL, served as the positive control.

2.5. BSA-MGO assay

The extracts were evaluated for their inhibitory effects on carbonyl species induced AGEs formation using a reaction model containing BSA (10 mg/mL), MGO (5 mM), and sample (100 µg/mL) as previously reported by our laboratory (Sun et al., 2016). Each sample was measured for intrinsic fluorescence after 7 days incubation at 37 °C with excitation and emission wavelengths of 360 nm and 435 nm, respectively. The aforementioned wavelengths were found optimal for the detection of MGO derived AGEs. The positive control, AG, was evaluated at an equivalent concentration of 100 µg/mL.

2.6. MGO trapping assay

The extracts were measured for their trapping capacity of RCS as previously reported by our laboratory (Liu et al., 2014). Briefly, each reaction solution contained MGO (5 mM), and test sample (100 µg/mL), or the positive control, AG (1000 µg/mL), and the mixtures were incubated at 37 °C for 4 hours. Afterwards, the derivatization reagent, PD (20 mM), and internal standard, DQ (5 mM), in 0.1 M phosphate buffer, pH 7.2 were added to the reaction mixture, and the remaining MGO was quantified by HPLC-DAD. The percentage decrease of MGO was calculated using the following equation:
$$\text{MGO decrease \%} = [1 - (\text{MGO amounts in solution with tested sample} / \text{MGO amounts in control solution})] \times 100\%.$$

2.7. Thioflavin T assay

The thioflavin T (ThT) assay was used to measure the inhibitory effects of the extracts on A β ₁₋₄₂ fibrillation as previously reported by our laboratory (Yuan et al., 2015). In each sample, the final concentrations of A β ₁₋₄₂ and test sample were adjusted to 50 µM and 100 µg/mL, respectively. Resveratrol (RESV; at 100 µg/mL) served as the positive control. To induce A β ₁₋₄₂ fibrillation, two individual protocols were followed. In the thermo induced fibrillation assay, A β ₁₋₄₂ solutions at a concentration of 50 µM were incubated at 37 °C for 72 hours with or without the herbal extracts. In the MGO-induced fibrillation assay, 1 mM MGO was added to the reaction mixture and all samples were incubated at 37 °C for 72 hours. Before and after incubation, 100 µL of each sample was added to an equal volume of ThT solution (50 µM) and fluorescence was measured on a plate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA) at excitation and emission wavelengths of 450 nm and 490 nm, respectively.

2.8. A β ₁₋₄₂-AGEs assay

In this assay, each extract was evaluated for its inhibitory effect against A β ₁₋₄₂-AGE formation induced by methylglyoxal (MGO), a reactive carbonyl species. Briefly, 50 μ M A β ₁₋₄₂ solution was mixed with 1 mM MGO to produce A β ₁₋₄₂-AGEs following published methods (Li et al., 2013). Treatments included either 100 μ g/mL of each extract or 100 μ g/mL of the positive control, AG. After 72-hour incubation at 37 °C, the A β ₁₋₄₂-AGEs level was measured by intrinsic fluorescence using a plate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA) at excitation and emission wavelengths of 360 and 435 nm, respectively.

2.9. Acetylcholinesterase (AChE) inhibition assay

The AChE inhibitory activities of the herbal extracts were evaluated using a published spectrophotometric method with slight modifications (Ellman et al., 1961). Briefly, a reaction mixture consisting of 100 μ L of 100 mM Tris-HCl buffer (pH 8.0), 20 μ L AChE enzyme solution (0.5 U/mL) and 20 μ L of test samples (concentration ranging from 25 - 300 μ g/mL in 100 mM Tris-HCl buffer containing 50% methanol) was co-incubated in the dark at 37 °C for 20 minutes in a 96-well plate. Next, 40 μ L of 0.75 mM DTNB and 20 μ L of 1.5 mM ATCI were added to the reaction mixture and incubated at room temperature for 5 min in the dark. Then the absorbance was measured at a wavelength of 405 nm using a plate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA). A 50% methanol in 100 mM Tris-HCl buffer served as the blank control and galanthamine, a known AChE inhibitor, served as the positive control. Each sample (at each concentration) was also tested without enzyme as background for the inherent color present in each sample. Inhibition of AChE was calculated as: % Inhibition = $[1 - (A_{\text{sample}} - A_{\text{background}})/A_{\text{blank}}] \times 100$.

2.10. Cell culture conditions

Murine microglial BV-2 cells were a kind gift from by Dr. Grace Y. Sun (University of Missouri at Columbia, MO, USA) and human neuronal SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, VA, USA). The cells were maintained using high glucose (4.5 g/L) DMEM/F12 supplemented with 10% heat inactivated FBS, 1% P/S (100 U/ml penicillin, 100 µg/mL streptomycin (Life Technologies, Gaithersburg, MD, USA) and incubated in 5% CO₂ at 37 °C. All test samples were dissolved in DMSO to yield a 10 mg/mL stock solution and further diluted with media to yield final solutions with DMSO < 0.1%.

2.11. Cell viability

Cellular viability was assessed using the Cell Titer Glo 2.0 (CTG 2.0) one step assay (Promega). Briefly, the murine BV-2 microglial cells were seeded at 100,000 cells/mL in a standard white walled clear bottom 96-well plate. After a 24-hour incubation period, CTG 2.0 was added in and mixed for 2 minutes on an orbital shaker prior to luminescence measurement on a plate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA).

2.12. Quantification of nitric oxide species (NOS) by the Griess assay

The murine BV-2 microglial cells, in 24-well plates (n=4) at 85% confluency (105 cells/well), were serum-starved for 4 hours prior to the treatments. The cells were then incubated with test samples (10 µg/mL) or the positive control, resveratrol (RESV; 5 µg/mL) for 1 hour after which inflammation was induced by lipopolysaccharide (LPS). Cells were incubated for 23 hours and the culture media were collected and centrifuged. The supernatants were measured for total nitric oxide species (NOS) by the Griess assay kit (Promega, Fitchburg, WI, USA) as previously reported by our laboratory (Nahar et al, 2014).

2.13. In vivo *Caenorhabditis elegans* assay

The in vivo neuroprotective effects of the extracts were evaluated using a *Caenorhabditis elegans* assay as previously reported by our group with minor modifications (Yuan et al., 2015). Transgenic *Caenorhabditis elegans* (CL4176) were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). This strain expresses a heat-induced human A β ₁₋₄₂ gene in the muscle tissues and prolonged expression of the gene leads to paralysis and death. The stock culture was maintained at 18 °C on Nematode Growth Medium (NGM) plates (1.7% Agar, 0.3% NaCl, 0.25% peptone, 1 mM CaCl₂, 1 mM MgSO₄, 5 mg/L cholesterol, and 2.5 mM KPO₄). NGM plates were seeded with 50 μ L OP50 *Escherichia coli* and allowed to incubate overnight at 37 °C. Before seeding of the plates, the *E. coli* cultures were treated with the herbal extracts or vehicle control (0.05% DMSO). Once the age of the worms were synchronized to reach the L3 stage, they were placed in a 25 °C incubator for 20 hours to induce the expression of the human A β ₁₋₄₂ gene. After 20 hours the worms were counted every 2 hours for a total of 30 hours of incubation time. Dead worms were characterized by lack of pharyngeal pumping or movement and >100 worms were used for each treatment. Kaplan-Meier curves and curve comparisons statistics were generated using GraphPad Prism software.

2.14. Inhibition of cytotoxicity of murine BV-2 microglia and differentiated human SH-SY5Y neuronal cells induced by hydrogen peroxide (H₂O₂)

Cellular viability was assessed using the Cell Titer Glo 2.0 (CTG 2.0) one step assay as previously reported by our laboratory (Ma et al., 2016). Briefly, murine BV-2 microglia and differentiated human SH-SY5Y neuronal cells were seeded at 100,000 cells/mL to yield 80-85% confluency in a standard white walled clear bottom 96-well plate. The

BV-2 cells were exposed to the herbal extracts and resveratrol (RESV; used as the positive control) for 1 hour prior to exposure to 100 μ M of H₂O₂ for 6 hours. The SH-SY5Y cells were incubated with the extracts and RESV for 24 hours at 37°C prior to exposure to 100 μ M of H₂O₂ for 6 hours. Following incubation, CTG 2.0 was added in a 1:1 ratio directly to existing media and mixed for 2 minutes on an orbital shaker prior to luminescence measurement (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA).

2.15. Quantification of total tau protein levels in differentiated human SH-SY5Y neuronal cells

The protein content collected from the cell culture media of differentiated SH-SY5Y neuronal cells were analyzed for total tau protein levels by using an enzyme linked immunosorbent assay (ELISA) (Thermo Fisher, Grand Island, NY, USA). The ELISA was performed as per the manufacturer's protocol. The SH-SY5Y cells were initially plated on 100 mm dishes at a concentration of 100,000 cells/mL. Cells were then differentiated for 7 days using retinoic acid (10 μ M) prior to treatment with test samples and resveratrol (RESV; used as the positive control). Once differentiated, the SH-SY5Y cells were exposed to vehicle control (DMSO) or the herbal extracts for 24 hours. After pre-treatment, the cells were then exposed to H₂O₂ (100 μ M) for 6 hours, after which the cell culture media was collected, centrifuged at 10,000 rpm for 5 minutes and assayed for total tau protein levels.

2.16. Statistical Analysis

Unless otherwise indicated, all assays were performed in triplicate and all data were expressed as the mean $\pm$ standard deviation ($n=3$). Significance was analyzed by one-way factorial ANOVA with Tukey–Kramer post hoc comparisons. A value of $p<0.05$

were considered as significant. In the BSA-fructose, BSA-MGO, and ThT assays, the inhibition rate (% inhibition) was defined using the following equation: % inhibition = $[1 - (\text{fluorescence intensity of solution with treatment} / \text{fluorescence intensity of control solution})] \times 100\%$.

Using a similar approach as previously reported (Seeram et al., 2008), an index score from each individual assay was determined by assigning an index value of 100 to the best score for each test, and then scores for the other samples were calculated using the following formula: $[(\text{sample score} / \text{best score}) \times 100]$. The average of the chemical/biochemical assays for each extract was then taken for the initial neuroprotective potential index (**Table 2**). Similarly, the final neuroprotective potential index was determined by the average of the test scores from the in vitro (in BV-2 cells), AChE inhibitory, and in vivo *C. elegans* assays using the aforementioned formula (**Table 4**). **Figure 1** shows the algorithm used to derive the overall index scores for the medicinal plant extracts.

3.0 Results and Discussion

3.1. Total polyphenol content and antioxidant capacities

The herbal extracts were first evaluated for their total polyphenol contents and antioxidant activities (DPPH and FRAP assays) since several natural plant antioxidants, for example, resveratrol (RESV; from grapes) and curcumin (from the Indian turmeric *Curcuma longa* spice) have been reported to show promising neuroprotective effects against AD (Li et al., 2012; Lim et al., 2001). As shown in **Table 1**, the twenty-three medicinal plant extracts were evaluated for their phenolic content by the Folin-Ciocalteu method based on gallic acid equivalents (GAEs). The total polyphenol contents ranged from 1.0 to 41.2% (w/w, based GAEs) and this wide range was attributed to the diverse types (hydrophilic vs. lipophilic) of chemical constituents found in these various extracts. As expected, among the extracts, two well-known polyphenol-rich plants, *Punica granatum* (41.2%) and *Phyllanthus emblica* (38.9%) showed the highest polyphenol contents followed by *Ocimum tenuiflorum* (37.1%), *Mucuna pruriens* (37.0%) and *C. longa* (31.5%). Similarly, as expected, the extracts with highest polyphenol contents also showed the strongest antioxidant activities in both the DPPH and FRAP assays (see **Table 1**). For example, the most potent antioxidant extracts in the DPPH assay were *P. emblica*, *P. granatum* and *M. pruriens*, which had IC₅₀ values of 11.1, 13.7 and 22.4 µg/mL, respectively, followed by *Cinnamomum cassia* (IC₅₀ 68.9 µg/mL), *O. tenuiflorum* (IC₅₀ 72.5 µg/mL), *Pterocarpus marsupium* (IC₅₀ 73.9 µg/mL) and *Terminalia arjuna* (IC₅₀ 84.3 µg/mL). All of these extracts showed superior antioxidant activity compared to the positive control, BHT (butylated hydroxytoluene), a synthetic commercial antioxidant (IC₅₀ 493.6 µg/mL). The antioxidant data obtained from the FRAP assay were in agreement with the antioxidant data obtained from the

DPPH assay. For example, at a concentration of 100 µg/mL, *P. emblica* showed the highest FRAP capacity (2405.7 AAE/mg), followed by *M. pruriens* (2269.2 AAE/mg) and *P. granatum* (2032.9 AAE/mg). Other herbal extracts which showed moderate FRAP capacity were *P. marsupium* (774.9 AAE/mg), *O. tenuiflorum* (754.5 AAE/mg), *Mangifera indica* (695.5 AAE/mg), *T. arjuna* (652.2 AAE/mg), and *Syzygium cumini* (502.0 AAE/mg).

3.2. Anti-glycation and reactive carbonyl species (RCS) scavenging capacities

Next, the extracts were evaluated for their anti-glycative abilities and reactive carbonyl species (RCS) scavenging activities using three individual assays. First, the BSA-fructose assay was used to evaluate the inhibitory effects of the extracts on total AGEs formation, Second, the BSA-methylglyoxal (BSA-MGO) assay was used to evaluate the inhibitory effects of the extracts on RCS induced AGE formation, typically seen at the middle stage of glycation (Wu and Yen, 2005). Third, the extracts were evaluated for their direct scavenging capacity on methylglyoxal (MGO) using the MGO-trapping assay.

As shown in **Figure 2A**, all twenty-three extracts (100 µg/mL) showed anti-AGEs effects which ranged from 15.1 to 90.8% in the BSA-fructose assay. Among all of the extracts, *P. granatum* showed the highest inhibition of 90.8%, which was significantly higher than the positive control, AG (67.2%), a synthetic anti-glycative agent. Extracts of *P. emblica* (85.3%) and *Moringa oleifera* (75.6%) also showed anti-AGE effects superior to AG. Additionally, several other extracts showed more than 50% inhibitory effects including *O. tenuiflorum* (65.3%), *Foeniculum vulgare* (57.4%), *M. pruriens* (55.3%), *P. marsupium* (54.8%), *Centella asiatica* (51.8%) and *Tinospora cordifolia* (51.0%).

We then evaluated these herbal extracts for their inhibitory effects on MGO induced AGEs formation. As shown in **Figure 2B**, *P. emblica* and *P. granatum* extracts were the most potent inhibitors of AGE formation (i.e. 74.1% and 48.7% respectively), superior to AG at an equivalent concentration of 100 µg/mL (44.4%). These extracts were followed by *C. longa* (46.4%), *P. vulgare* (38.8%), *C. asiatica* (38.8%), *P. marsupium* (37.2%), *O. tenuiflorum* (36.9%), *T. cordifolia* (35.7%), and *T. arjuna* (34.7%) which were comparable to the positive control, AG (44.4%).

In addition to the anti-glycative effects of the extracts, their MGO scavenging capacity may also contribute to their potential neuroprotective effects. Therefore, the MGO trapping ability of the extracts were evaluated as shown in **Figure 2C**. At a concentration of 100 µg/mL, extracts of *C. longa*, *P. granatum*, and *P. emblica* scavenged MGO by 99.0, 87.3, and 78.6%, respectively. This trend was similar to the BSA-MGO assay wherein these extracts also showed the highest inhibition levels on MGO induced glycation. The extracts of *M. oleifera*, *O. tenuiflorum*, *Bacopa monnieri*, *Tamarindus indica* and *F. vulgare* also showed strong scavenging activities yielding MGO quenching levels ranging from 55.9 to 35.0%. Notably, the positive control, AG, a known MGO scavenging agent, showed 95.7% MGO trapping ability but at a much higher concentration of 1000 µg/mL.

In summary, the anti-glycation activities of the most potent AGE inhibitors were *P. granatum*, *P. emblica*, and *C. longa* which were attributed to their strong antioxidant and MGO scavenging activities. The anti-AGEs effects of *P. granatum* is in agreement with our previously reported study (Liu et al., 2014).

3.3. Inhibitory effects on A β ₁₋₄₂ fibrillation and A β ₁₋₄₂–AGEs formation

Numerous studies have shown that certain forms of A β , such as fibrillated A β and glycated A β (A β -AGEs) are more toxic than its native counterpart (Butterfield, 2002; Koo et al., 1999; Li et al., 2013). Therefore, we evaluated the twenty-three medicinal plant extracts for their inhibitory effects on the formation of fibrillated A β and glycated A β , two common targets for AD treatment.

The ThT binding assay was used to evaluate the fibrillation level of human A β_{1-42} . In the thermo-induced fibrillation assay, A β_{1-42} fibril formation was confirmed by a significant increase in ThT fluorescence after 72 hours incubation at 37 °C. The fluorescence levels of A β treated with the extracts were then compared with the control and their inhibition levels on A β fibrillation are shown in **Figure 3A**. Upon treatment with the extracts, the lowest fibrillation level was seen in A β solution treated with *B. monnieri* (63.3% inhibition), followed by *P. granatum* (61.7% inhibition), *P. marsupium* (53.7% inhibition), *P. emblica* (47.9% inhibition) then *C. longa* (47.2% inhibition). The inhibition levels of these extracts were all significantly higher than positive control resveratrol (RESV, 31.8% inhibition), a natural phenolic compound known for its anti-amyloidosis and neuroprotective effects (Feng et al., 2009; Li et al., 2012).

Next, the herbal extracts were evaluated for their inhibitory abilities on MGO-induced A β_{1-42} fibrillation using the ThT binding assay. Studies have shown that reactive carbonyl species (RCS) such as MGO can induce A β fibrillation and aggregation (Chen et al., 2007; Chen et al., 2006). Consequently, carbonyl stress conditions with elevated RCS levels play an important role in the progression of neurodegenerative diseases including AD. Therefore, the inhibitory effects of the extracts against MGO-induced A β fibril assembly may also result in neuroprotective activities. As shown in **Figure 3B**,

the highest inhibitory effect was observed in samples treated with *B. monnieri* (63.5%) and *C. asiatica* (62.6%), with effects significantly higher than the positive control, RESV (32.1%). In addition, extracts of *P. granatum* (49.9%), *P. marsupium* (39.1%), *Boswellia serrata* (37.9%) and *P. emblica* (33.7%) also showed inhibitory effects against A β ₁₋₄₂ fibrillation superior to the positive control, RESV (32.1%).

Besides fibrillated A β , glycated A β (A β -AGEs) produced from the modification of A β by sugars and sugar metabolites also exacerbates the neurotoxicity of native A β (Li et al., 2013). Therefore, we next evaluated the inhibitory effects of the herbal extracts on the formation of glycated A β (A β -AGEs). As shown in **Figure 3C**, the positive control, AG, at a concentration of 100 μ g/mL, reduced glycated A β formation by 58.9%. Among the extracts, *T. arjuna* showed the highest inhibition (83.8%) followed by *P. emblica* (83.2%), and *P. granatum* (77.7%). Several other extracts also showed inhibition levels superior to the positive control, AG, including *T. indica* (77.1%), *S. cumini* (76.2%), and *Salacia reticulata* (75.5%) extracts.

Based on the data obtained from these six bioassays, namely, total polyphenol content, anti-oxidant capacity (DPPH and FRAP assays), anti-glycation effects, MGO trapping activity, A β fibrillation inhibition, and A β -AGEs inhibition, index scores were generated for each extract as previously reported (Seeram et al., 2008) and these results are summarized in **Table 2**. Extracts of *P. granatum* and *P. emblica* showed the highest score (91 and 90, respectively), followed by *M. pruriens* (70), *C. longa* (59) and *C. asiatica* (57). The medicinal plant extracts with a neuroprotective potential index score ≥ 40 , namely, the top twelve candidates, were selected for further evaluations for their neuroprotective potential using in vitro (BV-2 cells), AChE inhibitory, and in vivo (*Caenorhabditis elegans*) bioassays.

3.4. Inhibition of acetylcholinesterase (AChE) enzyme activity

Inhibitors of the acetylcholinesterase (AChE) enzyme are currently used as treatments for mild to moderate AD in human subjects (Birks et al., 2000) and this assay is widely used to screen for candidates with anti-AD potential (Birks, 2006). Therefore, the medicinal plant extracts (see **Table 2**) which had a neuroprotective potential index score ≥ 40 (from the initial set of bioassays described in Sec. 3.1 -3.3) were next evaluated for their inhibitory effects on the AChE enzyme. As shown in **Table 3**, the top four candidates namely, *M. pruriens*, *P. emblica*, *P. granatum*, and *C. longa* showed AChE inhibitory effects of 48.1, 43.1, 35.1, and 11.3% at 100 $\mu\text{g/mL}$, respectively, with IC_{50} values ranging from 91.3 to 304.6 $\mu\text{g/mL}$. In addition, among the twelve herbal extracts, *T. arjuna* also showed inhibitory effects against the AChE enzyme with an IC_{50} value of 313.6 $\mu\text{g/mL}$ (**Table 3**).

3.5. Inhibition of lipopolysaccharide (LPS)-induced nitric oxide species (NOS) production in murine BV-2 microglial cells

The medicinal plant extracts (see **Table 2**) which had a neuroprotective potential index score ≥ 40 (from the initial set of bioassays described in Sec. 3.1 -3.3) were next evaluated for their effects on LPS-induced neuroinflammation in an established in vitro murine BV-2 microglia cell model. As shown in **Figure 4**, seven of these extracts inhibited NOS levels at 10 $\mu\text{g/mL}$. The most active extracts were *P. marsupium* and *C. cassia*, which reduced NOS production by 23.6 and 22.2%, respectively. Also, *C. asiatica*, *C. longa*, *P. emblica*, *P. granatum*, and *B. monnieri* suppressed NOS production by 20.8, 19.4, 19.0, 16.7 and 15.0%, respectively. The extracts of *M. pruriens*, *O. tenuiflorum*, and *M. oleifera* did not significantly reduce NOS production while *T. arjuna* and *S. reticulata* extracts increased the levels of NOS. The positive

control, RESV (10 µg/mL), showed a 38.6% reduction of NOS production in the LPS-stimulated BV2 cells.

3.6. Reduction on A β ₁₋₄₂ induced neurotoxicity and paralysis in *Caenorhabditis elegans*

Next, the twelve extracts (which had a neuroprotective potential index score ≥ 40 as described above) were evaluated using an in vivo model using a transgenic *C. elegans* strain (CL4176) as previously reported by our group (Yuan et al., 2015). The CL4176 strain has a mutation of human A β ₁₋₄₂ expression in the muscle of the worms and the deposition of A β ₁₋₄₂ leads to neurotoxicity and paralysis. As shown in **Figure 5**, the heat shock induced A β ₁₋₄₂ neurotoxicity decreased the survival rate of *C. elegans* to 15.3% at 30 hours. However, among the extracts, *P. emblica*, *P. granatum*, *M. pruriens*, and *C. longa* significantly increased the survival rate of *C. elegans* to 47.8, 45.8, 44.1, and 39.1%, respectively. Several of the other extracts including *P. marsupium*, *C. cassia*, *M. oleifera*, *B. monnieri*, and *O. tenuiflorum* increased the survival rate of *C. elegans* from 33.3 to 16.7% while *S. reticulata* and *T. arjuna* did not show any neuroprotective effects with a survival rate of *C. elegans* of 6.7 and 4.8%, respectively. Based on the results from the *C. elegans* lifespan assay, *P. emblica*, *P. granatum*, *M. pruriens*, and *C. longa* were ranked among the top extracts which is in agreement with the ranking obtained from the in vitro BV-2 cell assay, wherein *C. longa*, *P. emblica*, *P. granatum*, and *M. pruriens* were also the most active extracts.

3.7. Evaluation of herbal extracts against H₂O₂ induced cytotoxicity in murine BV2 microglia and human neuronal SH-SY5Y cells

It is well established that oxidative stress is linked with the pathology of several neurodegenerative diseases including AD (Butterfield, 2002; Christen, 2000).

Therefore, based on the bioassays described in Sec. 3.4 -3.6, a final neuroprotective potential index score was calculated (see **Table 4**) and the extracts which had an index score ≥ 60 , namely, *P. granatum*, *P. emblica*, *M. pruriens* and *C. longa*, were selected for further evaluation of their neuroprotective effects against H₂O₂ induced cytotoxicity in murine BV-2 microglia and differentiated human SH-SY5Y neuronal cells. As shown in **Figure 6A**, the cell viability of the H₂O₂-treated murine microglial BV-2 cells decreased by 31.7% as compared to the control group. When the H₂O₂-exposed cells were subsequently treated with the herbal extracts (concentration of 10 μ g/mL), *M. pruriens*, *P. emblica*, *P. granatum*, and *C. longa* increased cell viability by 30.2, 22.3, 12.3, and 5.6%, respectively, as compared to the cells exposed to H₂O₂ alone. Similarly, as shown in **Figure 6B**, the cellular viability of H₂O₂-treated human neuronal SH-SY5Y cells decreased by 48.2% as compared to the control group. When the H₂O₂-exposed cells were subsequently treated with the herbal extracts (each at a concentration of 10 μ g/mL), *C. longa*, *M. pruriens*, and *P. emblica* significantly increased the cell viability by 109.1, 90.6, and 76.9%, respectively. Resveratrol (RESV), which served as the positive control, also increased cell viability of H₂O₂-treated murine BV-2 microglia (19.5%) and differentiated human SH-SY5Y neuronal (79.3%) cells (**Figure 6**).

3.8. Evaluation of herbal extracts on tau protein production in differentiated human SH-SY5Y neuronal cells

Studies have shown that in neurodegenerative models, including AD, neurons undergoing cell stress release tau proteins which on hyperphosphorylation form neurofibrillary tangles which can ultimately lead to cell death (Gomez-Ramos et al., 2006). Therefore, as described above (see Section 3.7), the extracts which had an index score ≥ 60 , namely, *P. granatum*, *P. emblica*, *M. pruriens* and *C. longa*, were selected

for further evaluation of their inhibitory effects on tau protein production in human neuronal SH-SY5Y cells. As shown in **Figure 6C**, extracts of *C. longa*, *P. granatum*, *M. pruriens*, and *P. emblica* reduced extracellular tau protein levels by 83.7, 82.6, 66.4, and 52.4%, respectively.

4.0 Conclusions

In summary, using bioassays with established links between AD and oxidative stress, carbonyl stress, glycation, A β fibrillation, A β -AGE formation, AChE inhibition, and neuroinflammation, an AD Neuroprotective Potential Algorithm (NPA) was developed as part of a strategy to help guide the selection and evaluation of medicinal plant candidates for future AD based research. From the current study, four extracts identified with a cumulative neuroprotective potential index score ≥ 60 were *Phyllanthus emblica* (amla; Indian gooseberry), *Mucuna pruriens* (velvet bean), *Punica granatum* (pomegranate) and *Curcuma longa* (curcumin; turmeric). Interestingly, published animal data support the neuroprotective effects of two of these extracts namely, *P. granatum* (Yuan et al., 2015; Ahmed et al., 2014) and *C. longa* (Ringman et al., 2005) against AD. While the other two extracts remain underexplored for their anti-AD potential, *P. emblica* has been shown to improve memory deficits in mice (Ashwlayan and Singh, 2011) and *M. pruriens* has been shown to have protective effects against Parkinson's disease in human subjects (Katzenschlager et al., 2004).

There are several limitations to the NPA developed herein, primarily, due to the fact that botanical extracts are complicated mixtures containing multiple 'multi-targeting' phytochemicals which undergo complex metabolic processes in vivo (including metabolism by the liver enzymes and gut microflora) which influence their bioavailability, metabolism, excretion, and generation of 'further' bioactive metabolites (Wang et al., 2015; Yuan et al., 2015; Wang et al., 2014). For example, our group has recently reported that the gut microbial metabolites produced from the colonic microflora metabolism of the natural ellagitannins present in the pomegranate (*P. granatum*) fruit are potentially brain absorbable and may contribute to the anti-AD

effects reported for this natural product (Yuan et al., 2015). Therefore, a major limitation of the NPA developed here is the lack of critical in vivo data (accumulated from animal models) which would account for the aforementioned important physiological considerations (Singh et al., 2008; Ebrahimi and Hermann, 2012). Other limitations of the NPA include utilizing additional AD-targeted bioassays and its validation with an increased sample size of medicinal plant extracts and their combinations thereof. Nevertheless, given all of these limitations, the NPA may be utilized in consideration with published literature data (when available) of animal and human studies, traditional and ethnomedicinal use, etc., as part of a more comprehensive research strategy to guide the selection of promising medicinal plant candidates for future AD based research using in vivo models.

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STEP 1: Measure antioxidant capacity (Table 1)

- Total polyphenol content (Folin-Ciocalteu assay)
- Free radical scavenging (DPPH assay)
- Ferric reducing antioxidant power (FRAP assay)

STEP 2: Measure inhibitory effects on glycation (Figure 2)

- Fructose-induced AGEs formation
- Carbonyl species (MGO)-induced AGEs formation
- MGO trapping ability

STEP 3: Measure inhibitory effects on A β fibrillation (Figure 3)

- Thermo-induced A β fibrillation
- Carbonyl species (MGO)-induced A β fibrillation
- A β -AGEs formation

- Obtain initial neuroprotective potential index (Table 2)
- Select candidates with score ≥ 40 (modifiable)

STEP 4: Measure inhibitory effects on AChE activity (Table 3)

STEP 5: Measure in vitro inhibitory effects on neuroinflammation (Figure 4)

- Nitric oxide species (NOS) production in murine BV-2 microglia cells

STEP 6: Measure in vivo neuroprotective effects (Figure 5)

- A β induced paralysis in *Caenorhabditis elegans*

- Obtain final neuroprotective potential index (Table 4)
- Select candidates with score ≥ 60 (modifiable)

Figure 1. Neuroprotective Potential Algorithm (NPA) for selecting and evaluating medicinal plants as potential candidates for Alzheimer's disease based research.

Figure 2. Inhibitory effects of medicinal plant extracts (100 $\mu\text{g/mL}$) on D-fructose induced BSA AGEs formation (A), MGO induced BSA AGEs formation (B) and MGO scavenging capacity (C). Aminoguanidine at 100 $\mu\text{g/mL}$ served as the positive control.

Figure 3. Inhibitory effects of medicinal plant extracts (100 µg/mL) on thermo-induced Aβ fibrillation (A), MGO induced Aβ fibrillation (B) and MGO induced Aβ-AGEs formation (C). Resveratrol at 100 µg/mL served as the positive control for Aβ fibrillation studies (A and B) while aminoguanidine at 100 µg/mL served as the positive control for Aβ-AGEs study (C).

Figure 4. Anti-neuro-inflammatory effects of medicinal plant extracts on murine BV-2 cells microglial cells by measuring NOS production induced by LPS. BV-2 cells were treated with each extracts (10 µg/mL) for 1 h followed by exposure to LPS (1 µg/mL) for 24 h. The cell culture media were used to assay the amount of NOS production by the Griess assay. Data are presented as means $\pm$ SDs of three independent experiments.

Figure 5. Protective effects of medicinal plant extracts against neurotoxicity and paralysis in *Caenorhabditis elegans* in vivo. Mobility curves of transgenic (CL4176) *C. elegans* 30 hours post $A\beta_{1-42}$ induction of muscular paralysis at 25 °C. Kaplan-Meier mobility plots of *C. elegans* worms fed on twelve extracts [Control (NGM), in dot line; (A-L) test samples: (NGM + 10 µg/mL test sample), in solid line]

Figure 6. Protective effects of medicinal plant extracts (10 μ g/mL) on H₂O₂ induced cytotoxicity in murine BV-2 microglia (A) and differentiated human SH-SY5Y neuronal cells (B). Cellular viability was assessed using Cell Titer Glo 2.0 (CTG 2.0) one step assay. C: Inhibitory effects of medicinal plant extracts (10 μ g/mL) on tau protein levels in differentiated SH-SY5Y neurons (C). All data are presented as mean $\pm$ SDs of three independent experiments.

Table 1. Medicinal plants and their total polyphenol contents and antioxidant activity (DPPH and FRAP assays)

Species	Common name	Traditional use	Plant part	Total polyphenol content% ^a	DPPH activity ^b	FRAP activity ^c
<i>Azadirachta indica</i>	Neem	antidiabetic	Leaves	12.9 ± 0.4	781.3 ± 49.0	203.8 ± 9.7
<i>Bacopa monnieri</i>	Waterhyssop	enhance memory	Whole herb	3.4 ± 1.9	n.a.	105.6 ± 5.8
<i>Boswellia serrata</i>	Indian olibanum	joint health	Gum resin	2.4 ± 0.1	n.a.	11.8 ± 1.8
<i>Elettaria cardamomum</i>	Cardamom	digestive disorders	Fruit	2.9 ± 0.7	n.a.	n.a.
<i>Centella asiatica</i>	Gotu kola	antidiabetic	Whole herb	27.0 ± 1.6	263.1 ± 25.4	224.2 ± 10.9
<i>Cinnamomum cassia</i>	Cinnamon	antidiabetic	Bark	22.4 ± 1.0	68.0 ± 10.3	324.2 ± 21.1
<i>Curcuma longa</i>	Turmeric, Curcumin	anti-inflammatory	Rhizomes	31.5 ± 1.8	111.4 ± 25.2	407.6 ± 12.8
<i>Foeniculum vulgare</i>	Fennel	anti-inflammatory	Seeds	24.0 ± 1.9	544.0 ± 14.6	179.3 ± 6.2
<i>Gymnema sylvestre</i>	Gymnema	antidiabetic	Leaves	21.0 ± 1.2	468.0 ± 9.4	253.4 ± 8.0
<i>Mangifera indica</i>	Mango	clearing digestion	Leaves	27.9 ± 2.8	60.6 ± 5.7	695.5 ± 21.7
<i>Moringa oleifera</i>	Moringa	joint health	Fruit	19.9 ± 2.5	758.9 ± 23.8	123.6 ± 7.1
<i>Mucuna pruriens</i>	Velvet bean	neurodegenerative	Seeds	37.0 ± 2.4	22.4 ± 1.8	2269.2 ± 61.3
<i>Ocimum tenuiflorum</i>	Holy basil	spice	Leaves	37.1 ± 1.7	72.5 ± 4.2	754.5 ± 16.8
<i>Phyllanthus emblica</i>	Amla	anti-inflammatory	Juice	38.9 ± 2.3	11.1 ± 1.7	2405.7 ± 5.9
<i>Pterocarpus marsupium</i>	Indian Kino	antidiabetic	Bark	20.6 ± 1.4	73.9 ± 11.0	774.9 ± 17.3
<i>Punica granatum</i>	Pomegranate	antidiabetic	Fruit Peel	41.2 ± 0.7	13.7 ± 0.7	2032.9 ± 57.5
<i>Salacia reticulata</i>	Salacia	antidiabetic	Roots	22.9 ± 1.4	791.5 ± 6.1	400.9 ± 13.3
<i>Sesamum indicum</i>	Sesame	spice	Seeds	1.0 ± 0.1	n.a.	64.9 ± 0.5
<i>Syzygium cumini</i>	Jamun, Black plum	antidiabetic	Fruit pulp	21.3 ± 3.2	129.9 ± 2.7	502.0 ± 4.5
<i>Tamarindus indica</i>	Indian date	fever	Fruit	3.6 ± 1.6	614.8 ± 12.7	75.2 ± 2.7
<i>Terminalia arjuna</i>	Arjuna	heart disease	Bark	24.6 ± 0.9	84.3 ± 19.2	652.2 ± 19.9
<i>Tinospora cordifolia</i>	Guduchi	anticancer	Stem	8.8 ± 0.5	571.1 ± 212.9	241.6 ± 2.7

<i>Withania somnifera</i>	Ashwagandha, ginseng	Indian	anti-ulcer	Roots	25.7 ± 0.4	279.6 ± 20.1	166.1 ± 3.1
BHT*	—		—	—	—	493.6 ± 8.7	—

^a(w/w % as of gallic acid equivalents); ^bvalue = IC₅₀ (µg/mL), n.a. = not active (IC₅₀ >2000 µg/mL); ^cn.d. = not detected; ^cvalue = ascorbic acid equivalents/mg dry plant material, n.a. = not active; *Positive control: Butylated hydroxytoluene, BHT

Table 2. Initial screening for neuroprotective potential index based on total polyphenol content, antioxidant, anti-glycation, and anti-A β fibrillation activities

Ranking	Plant species	Total phenolic content	DPPH	FRAP	MGO trapping	Anti-AGEs ^a	Anti-AGEs ^b	Anti-A β -AGEs	Anti-A β Agg. ^c	Anti-A β Agg. ^d	Neuro-protective potential index
1	<i>P. granatum</i>	100	99	85	100	100	66	93	79	97	91
2	<i>P. emblica</i>	95	100	100	90	94	100	99	53	76	90
3	<i>M. pruriens</i>	90	95	94	85	61	39	63	43	63	70
4	<i>C. longa</i>	77	52	17	99	41	63	71	37	75	59
5	<i>C. asiatica</i>	66	67	9	23	57	52	74	99	63	57
6	<i>P. marsupium</i>	50	59	32	3	60	50	76	62	83	53
7	<i>C. cassia</i>	55	87	13	12	43	42	56	45	57	46
8	<i>O. tenuiflorum</i>	90	63	31	45	72	50	50	0	1	45
9	<i>T. arjuna</i>	60	56	27	22	47	47	100	25	8	44
10	<i>B. monnieri</i>	8	3	4	45	35	37	49	100	100	42
11	<i>M. oleifera</i>	49	32	5	64	83	40	7	49	43	41
12	<i>S. reticulata</i>	56	22	17	11	42	30	90	41	64	41
13	<i>S. cumini</i>	52	53	21	0	21	18	91	30	26	35
14	<i>F. vulgare</i>	59	35	7	40	63	52	40	0	0	33
15	<i>T. indica</i>	9	41	3	43	48	42	92	11	0	32
16	<i>W. somnifera</i>	63	36	7	10	38	41	81	7	0	31
17	<i>T. cordifolia</i>	21	38	10	16	56	48	89	0	0	31
18	<i>M. indica</i>	68	68	29	11	42	44	0	19	0	31
19	<i>G. sylvestre</i>	51	43	11	13	26	23	38	28	8	27
20	<i>A. indica</i>	31	21	8	0	26	23	79	13	32	26
21	<i>S. indicum</i>	2	1	3	25	47	40	46	30	17	23
22	<i>B. serrata</i>	6	7	0	14	19	18	34	60	46	23
23	<i>E. cardamomum</i>	7	2	0	12	48	34	40	0	0	16

^aBSA AGEs formation induced by D-fructose; ^bBSA AGEs formation induced by methylglyoxal (MGO); ^cthermo induced A β fibrillation (37 °C); ^dMethylglyoxal (MGO) induced A β fibrillation

Table 3. Inhibitory effects on AChE activity of selected medicinal herbal extracts (top 12 based on initial screening)

Plant Species	AChE inhibition (%) at 100 $\mu\text{g/mL}$	AChE IC ₅₀ ($\mu\text{g/mL}$)
<i>P. granatum</i>	35.06 $\pm$ 2.93	304.62 $\pm$ 1.27
<i>P. emblica</i>	43.11 $\pm$ 1.72	163.92 $\pm$ 3.55
<i>M. pruriens</i>	48.11 $\pm$ 2.78	91.35 $\pm$ 2.80
<i>C. longa</i>	11.31 $\pm$ 1.81	— ^b
<i>C. asiatica</i>	n.a. ^a	—
<i>P. marsupium</i>	n.a.	—
<i>C. cassia</i>	n.a.	—
<i>B. monnieri</i>	n.a.	—
<i>O. tenuiflorum</i>	n.a.	—
<i>M. oleifera</i>	n.a.	—
<i>T. arjuna</i>	12.88 $\pm$ 2.58	313.58 $\pm$ 6.21
<i>S. reticulata</i>	7.64 $\pm$ 1.43	—
galanthamine ^c	68.50 $\pm$ 1.10	5.37 $\pm$ 3.79

^a n.a. = not active; shown as mean values $\pm$ SD ($n \geq 3$). ^b —, > 600 $\mu\text{g/mL}$. ^c Used as positive control at 12 $\mu\text{g/mL}$.

Table 4. Final screening for medicinal plant extracts for neuroprotective potential index based on the AChE inhibitory activity assay, in vitro anti-neuro-inflammatory effects in murine BV-2 microglial cells, and in vivo neuroprotective effects against A β ₁₋₄₂ induced neurotoxicity and paralysis in *C. elegans*

Ranking	Plant species	AChE inhibitory activity assay	Neuroinflammation assay	in vivo <i>C. elegans</i> assay	Neuroprotective potential index
1	<i>P. emblica</i>	90	80	100	90
2	<i>M. pruriens</i>	100	49	96	82
3	<i>P. granatum</i>	73	71	96	80
4	<i>C. longa</i>	23	82	82	62
5	<i>P. marsupium</i>	0	100	60	53
6	<i>C. asiatica</i>	0	88	70	53
7	<i>C. cassia</i>	0	94	42	45
8	<i>B. monnieri</i>	0	64	38	34
9	<i>O. tenuiflorum</i>	0	27	35	21
10	<i>M. oleifera</i>	0	19	42	20
11	<i>S. reticulata</i>	8	0	14	7
12	<i>T. arjuna</i>	12	0	10	7

MANUSCRIPT 3

Manuscript 3 appears as published in Natural Product Communications.

Phenolic compounds isolated and identified from Amla (*Phyllanthus emblica*) juice powder and their antioxidant and neuroprotective activities. Rose, Kenneth N.; Wan, Chunpeng; Thomas, A; Seeram, Navindra P.; Ma, Hang. (2018). Natural Product Communications. 13, 1309-1311.

Abstract

The edible fruit of *Phyllanthus emblica* (known as amla and Indian gooseberry) is widely used in Eastern traditional medicinal systems for a variety of ailments. Our group has previously reported that an amla juice powder shows neuroprotective effects in several in vitro and in vivo assay models but its chemical constituents and their neuroprotective activity remain unknown. Therefore, we conducted a phytochemical investigation of amla juice powder and evaluated the antioxidant and neuroprotective effects of the isolates. Ten phenolics (**1-10**), including gallic acid (**1**), five gallic acid derivatives (**2-6**), ellagic acid (**7**), and three ellagic acid derivatives (**8-10**), were isolated and identified with compounds **8-10** being reported from amla for the first time. All of the isolates showed antioxidant effects in the DPPH assay with IC₅₀ values ranging from 6-158.9 μ M superior to the synthetic commercial antioxidant, butylated hydroxytoluene (IC₅₀ = 371.4 μ M). In addition, compound **8** reduced β -amyloid-induced neurotoxicity in a transgenic *Caenorhabditis elegans* model by increasing their survival rate by 28.3% compared to the control group. This study adds to the growing body of evidence supporting the potential health benefits of amla and supports the functional food and nutraceutical applications of amla juice powder.

1.0 Introduction

Amla (*Phyllanthus emblica*), also known as Indian gooseberry, is a deciduous tree that commonly grows in the subtropical and tropical regions of Southeast Asia including southern India and China. Various parts of the amla plant, especially its fruit, are traditionally used as folk remedies for numerous ailments in traditional Chinese and Indian (Ayurvedic) medicinal systems [1, 2]. Previous phytochemical studies reported that the major bioactive compounds present in amla fruits are phenolics including hydrolyzable tannins (both ellagitannins and gallotannins), anthocyanins, flavonoids, flavonols, and phenolic acids [2-6]. While amla fruits are regionally consumed as a food and spice, because of seasonal factors and perishability, the fruits are only available for a short period. Therefore, an amla juice powder is a viable option to preserve the fruit's bioactive constituents for nutraceutical and functional beverage applications. Our laboratory has reported on the development of a neuroprotective potential algorithm (NPA) to screen medicinal plant extracts in which an amla juice powder ranked top among twenty Ayurvedic medicinal plants [7]. However, whether the bioactive compounds present in this particular amla juice powder are similar or different from amla fruits remain unknown.

In our continued efforts to further investigate the biological activities of the top ranking medicinal plants using the NPA [7], herein, we sought to isolate and identify the bioactive compounds from the aforementioned amla juice powder and evaluate their antioxidant and neuroprotective effects using assays adopted from the NPA. In the current study, ten phenolic compounds (1-10) were isolated from the amla juice powder and their chemical structures (shown in Figure 1) were identified by comparing their NMR and mass spectroscopic data with published data (see

References 8-14). Ten isolates were identified as gallic acid(1) [8], methyl gallate (2) [9], 1-*O*-galloyl-glucoside (3) [10], mucic acid 3-*O*-gallate (4) [11], corilagin (5) [12], 1,6-di-*O*-galloyl- glucoside (6) [10], ellagic acid (7) [8], ellagic acid-4-*O*-glucoside (8) [8], ellagic acid-4-*O*-xyloside (9) [13], and ellagic acid-4-*O*- rhamnoside (10) [14]. While compounds 1-7 have been previously reported from amla fruit [2-6], to the best of our knowledge, compounds 8-10 are being reported from this plant for the first time. The amla juice powder and its isolates were evaluated for antioxidant and anti- β -amyloid effects in *Caenorhabditis elegans*. Amla juice powder had an IC₅₀ value of 28.9 μ g/mL in the antioxidant (DPPH) assay and compounds 1-10 showed superior antioxidant activities (IC₅₀ values = 6.0, 3.1, 2.7, 1.6, 4.9, 3.0, 2.6, 73.4, 26.3, and 158.9 μ M, respectively) compared to the positive control, butylated hydroxytoluene (a synthetic commercial antioxidant; IC₅₀ = 371.4 μ M). The neuroprotective effects of the amla juice powder and compounds 1-10 were evaluated using a transgenic *C. elegans* nematode model (CL1476). Amla juice powder (at 10 μ g/mL) significantly reduced the β -amyloid-induced neurotoxicity in the nematodes and increased their survival rate by 28.2% as compared to the control group (Figure 2A). Compound 8 (at 10 μ M) increased the survival rate of nematodes by 28.3% as compared to the control group (Figure 2B).

None of the other isolates showed significant activity in this assay. However, further studies would be required to evaluate the neuroprotective effects of this amla juice powder and its isolated compounds. In summary, ten phenolic compounds were isolated and identified from an amla juice powder and their antioxidant and neuroprotective effects were evaluated. This study adds to the growing body of

evidence supporting the potential health benefits of amla and supports the functional food and nutraceutical applications of the fruit juice powder.

2.0 Experimental

2.1 General

The ^1H and ^{13}C nuclear magnetic resonance spectroscopic experiments were conducted in methanol (CD_3OD) on a Varian 500 MHz instrument. HR-ESIMS data were acquired on Waters SYNAPT G2-S QTOFMS system. Solvents were either ACS or HPLC grade purchased from Wilkem Scientific (Pawtucket, RI, USA). High performance liquid chromatography (HPLC) was performed on a Hitachi Elite LaChrom system (Pleasanton, CA, USA) consisting of a L-2130 pump, L-2200 autosampler, and a L-2455 diode array detector.

2.2 Plant material

Amla juice powder was kindly provided by Verdure Sciences (Noblesville, IN, USA) and voucher specimens (LEO2JP110210) are deposited in the Heber Youngken Herbarium and Greenhouse at the College of Pharmacy, the University of Rhode Island, RI, USA.

2.3 Extraction and isolation

Amla juice powder (20 g) was dissolved in 50 mL DI water and chromatographed on a XAD-16 resin column (3×10 inch) eluted with a gradient system of MeOH/ H_2O (0/100, 50/50 and 100/0; v/v) to afford three sub-fractions (A_1 - A_3). Sub-fraction A_2 was separated by medium-pressure liquid chromatographic (MPLC) column (2×15 cm) with C18 resin eluted with a gradient system of MeOH/ H_2O (10/90 to 40/60; v/v) to afford four sub-fractions (B_1 - B_4). Sub-fraction B_1 was separated by semi-preparative HPLC eluted with MeOH/ H_2O (11/89; v/v) to yield compounds 1 (229 mg, $t_R = 13.0$ min), 3 (18.6 mg, $t_R = 8.0$ min), and 4 (12.6 mg, $t_R = 12.0$ min). Sub-fraction B_2 was separated by semi-preparative HPLC eluted with a gradient system

of MeOH/H₂O (18/82 to 35/65; v/v) to yield compounds 2 (16.0 mg, t_R = 15.16 min), and 6 (8.6 mg, t_R = 12.58 min). Sub-fraction B₃ was separated by semi-preparative HPLC eluted with a gradient system of MeOH/H₂O (18/82 to 40/60; v/v) to yield compound 5 (13.1 mg, t_R = 14.24 min). Fraction A₃ was separated on the C18 MPLC column (2 × 15 cm) eluted with a gradient system of MeOH/H₂O (3:7 to 8:2, v/v) to afford 4 sub-fractions (C₁-C₄). Sub-fractions C₄ was separated by a Sephadex LH-20 resin column (2 × 58 cm) eluted with MeOH to afford 3 sub-fractions (D₁-D₃). Sub-fractions D₂ was separated by semi-preparative HPLC eluted with a gradient system of MeOH/H₂O (45/55 to 68/32; v/v) to yield compounds 7 (62.9 mg, t_R = 17.8 min), 8 (4.5 mg, t_R = 11.6 min), 9 (3.9 mg, t_R = 15.3 min) and 10 (4.1 mg, t_R = 16.3 min).

2.4 Antioxidant activity

The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [15, 16]. Briefly, 100 µL of test sample (1-1000 µg/mL for amla juice powder and 1- 500 µM for the isolates) was mixed with 100 µL of DPPH dissolved in methanol (0.25 mM) in a 96-well plate. The plate was incubated at room temperature in the dark for 35 min and then the absorbance of each well was read at 517 nm using a plate reader (SpectraMax M2, Molecular Devices Corp., Sunnyvale, CA, USA).

2.5 In vivo anti-neurotoxicity assay (*Caenorhabditis elegans* assay)

C. elegans (CL1476) were obtained from the University of Minnesota *Caenorhabditis* Genetics Center. Worms were grown on nematode growth agar in Petri dishes maintained at 16°C. UV-killed OP50 *E. coli* was plated as a food source before the worms were introduced. Worms were age synchronized via the standard

hypochlorite method [7, 17]. Age synchronous *C. elegans* were washed from the Petri dish with liquid nematode growth medium. Aliquots of the washed worms were placed into the wells of a 96-well plate. A microscope was used to ensure that approximately 20 worms were placed into each well with a final volume of 100 μ L diluting with liquid nematode growth medium. Amla juice powder (10 μ g/mL) or each of the phenolic compounds (10 μ M) was added to each well and DMSO (0.01%) served as vehicle control. Worms were placed at 16°C for 24 hours after treatment and then a baseline count of living worms was taken followed by incubation at 25°C for the duration of the experiment. Worms were counted every hour for 6 hours and considered dead if they were not moving or pumping their pharynx. Kaplan-Meier survival curves were created using GraphPad Prism 7 software.

3.0 Acknowledgments

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Figure 1: Chemical structures of compounds **1-10** from amla juice powder

Figure 2: Protective effects of amla juice powder (10 $\mu\text{g}/\text{mL}$; A) and compound 8 (10 μM ; B) against neurotoxicity and paralysis in *C. elegans* in vivo. Kaplan-Meier mobility plots of *C. elegans* worms treated with test sample [Control (NGM), in dotted line; test samples: (NGM + amla juice powder or compound 8), in solid line].

CHAPTER 2

MOLECULAR BIOLOGY AND MASS SPECTROMETRY TECHNIQUES FOR THE DECONCOLUTION OF POTENTIAL MECHANISMS OF ACTIONS OF ISOLATED PURE COMPOUNDS FROM TERRESTRIAL PLANT SOURCES

Manuscript 4 is prepared for submission to the Journal of Natural Products as a note

Increased complex I activity induced by methylated urolithin B treatment is a possible mechanism of action for efficacy in non-oncology disease models. Rose, Kenneth N.; Barlock, Benjamin; DaSilva, Nicholas; Johnson, Shelby L.; Fatemeh, Akhlaghi; Seeram, Navindra P.

Manuscript 5 is prepared for submission to Natural Products Communications

Methylated urolithin B identified as a poly-ADP-ribose polymerase-1 inhibitor by drug affinity responsive target stability (DARTS). Rose, Kenneth N.; Barlock, Benjamin; DaSilva, Nicholas; Johnson, Shelby L.; Fatemeh, Akhlaghi; Seeram, Navindra P.

ABSTRACT

Recently, there has been increasing interest in gut microflora's effects on human health. Gut microflora may contribute to human health by the biotransformation of compounds found in food to metabolites that may differ significantly in terms of structure and bioactivity from their parent compounds. These compounds may be beneficial to health and particularly to brain health. Gut microbiota metabolites are often smaller and less polar than their parent molecules, increasing the likelihood of passive transport across the blood-brain barrier. Urolithins are a class of compounds that result from the biotransformation of ellagitannins by gut the microbiota. Ellagitannins are common in several edible botanical sources such as walnuts, pomegranates, and oak-aged beverages. Urolithins have been studied regarding cancer cell viability, however, recent research has suggested that urolithins have a neuroprotective effect as they perform well in screening assays against amyloid- β -induced toxicity. Therefore, we hypothesized that a mechanism of action must exist to explain their neuroprotective effects. Using proteomics to evaluate the effects of urolithin treatment on protein expression and a technique known as drug affinity responsive target stability (DARTS) to assess protein targets of urolithins, we were able to demonstrate that the model compound, methylated Urolithin B, can increase the activity of complex I in a human neuroblastoma cell model and can inhibit the activity of the DNA repair protein poly ADP-ribose polymerase-1 at nanomolar concentrations. These mechanisms can help to explain the antiproliferative effects of urolithins, through PARP-1 inhibition, and possibly the neuroprotective effects through increases in complex I activity.

MANUSCRIPT 4

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Increased complex I activity induced by methylated urolithin B treatment is a possible mechanism of action for efficacy in non-oncology disease models. Rose, Kenneth N.; Barlock, Benjamin; DaSilva, Nicholas; Johnson, Shelby L.; Fatemeh, Akhlaghi; Seeram, Navindra P.

1.0 Introduction

Complex I is a group of proteins that serves a vital role in the electron transport chain as it develops the electron gradient across the mitochondrial membrane necessary for the production of ATP.¹ Of the five complexes within the mitochondria, complex I initiates the electron transport chain. Decreases in complex I activity affect the other constituents of the electron transport chain and therefore alters the respiration capacity of the mitochondria². Deficiencies in complex I function have been linked to several disorders including mitochondrial diseases, neurological disorders, and inflammatory diseases.^{3,4} Therefore, restoring complex I activity to basal levels may have a therapeutic benefit.

Urolithins are gut microbiota metabolites of ellagitannins which are found in several food sources such as walnuts, pomegranates, and oak-aged beverages.⁵ Urolithins have been studied heavily regarding their anti-cancer effects, particularly in colon cancer where urolithin concentrations are presumably at their highest concentration within the body.⁶ Several groups have identified mechanisms of action for urolithins' anti-cancer effect including p21 upregulation and inhibition of EphA2 phosphorylation.^{7,8}

Recently, urolithins have been identified to have beneficial effects in inflammatory, neurodegenerative, and mitochondrial disease models.^{9,10} The mechanisms responsible for urolithins' anti-cancer effects are unlikely to be responsible for the observed effects in other diseases models. Therefore, urolithins may be acting upon separate pathways not related to their anti-cancer activity that explains their efficacy in a variety of disease models. Recent research has demonstrated that urolithin A increases

mitochondria respiration capacity.⁹ With this knowledge, we focused our investigation for urolithins' mechanism of action to the mitochondria. Herein, we demonstrate through several *in vivo*, *in vitro*, and advanced proteomic methods that methylated urolithin B (mUB), a model compound, improves oxidative stress resistance in *C. elegans*, upregulates a core component of complex I, and restores complex I activity to normal levels in a high oxidative stress environment. Furthermore, we assert that mUB induced increases in complex I activity is responsible, in part, for urolithins' efficacy in non-oncology disease models.

To model a broad range of diseases where oxidative stress plays a role in disease progression, we use *Caenorhabditis elegans* treated with the pro-oxidant 5-hydroxy-1,4-naphthalenedione (juglone).¹¹ Treatment with juglone leads to the production of reactive oxygen species and significantly reduces the lifespan of *C. elegans*. When juglone is co-administered with mUB, *C. elegans* lifespan is significantly increased (Figure 1). Even at mUB concentrations as low as 1 μ M, the lifespan of *C. elegans* was increased by approximately 20% with maximum life extension at 5 μ M increasing lifespan by 33%. Given that this model relies on the production of ROS to be detrimental to *C. elegans* lifespan, it is likely that compounds that can scavenge free radicals would be efficacious. However, urolithins can act as both pro- and antioxidants depending on the cellular context.¹² Therefore, the extension of lifespan in *C. elegans* may be due to mechanisms unrelated to urolithins' antioxidant properties.

To determine which mechanisms are responsible for the extension of lifespan in juglone treated *C. elegans* we used LC-MS/MS^{all} with SWATH acquisition. This data independent acquisition method is on the cutting edge of proteomics research and holds several advantages over traditional LC-MS based proteomic approaches including reproducibility and enhanced quantification.¹³ Using SWATH acquisition we identified 9 of the 12 *C. elegans* mitochondrial proteins from the UniProt database and created a heat map of their relative intensities (Figure 2). The protein NADH-ubiquinone oxidoreductase core chain 4L (NU4LM) was expressed nearly 2-fold higher in *C. elegans* treated with mUB. NU4LM is a part of the minimal components necessary to the function of complex I which creates the electron gradient across the mitochondrial membrane. Mutations of complex I are correlated with the hereditary mitochondrial disease Leber's optic neuropathy and mitochondrial encephalopathy.^{14,15}

Being a core component of complex I, we hypothesized that overexpression of NU4LM would increase the activity of complex I. To study the relationship between complex I and mUB we used human neuroblastomas, SH-SY5Y, as readily available kits exist for complex I activity in humans and other mammals, but not for *C. elegans*. When cells are treated with hydrogen peroxide, complex I activity is reduced to approximately half of its basal level. Co-administering hydrogen peroxide and mUB at all tested concentrations (1, 5, and 10 μ M) returns complex I activity to near basal level (Figure 3).

Our findings are in agreement with recently published research describing urolithin A's ability to improve cellular respiration⁹. Increases in complex I function provide an explanation for this activity as short-term administration of urolithin A's does not increase the number of mitochondria within a cell, but energy output increased. Furthermore, several of the diseases in which urolithins have been identified as being beneficial for such as inflammatory and neurodegenerative are possibly due, in part, to ROS accumulation¹⁶. As demonstrated in figure 3, excessive ROS leads to decreases in complex I activity and mUB restores complex I activity to near basal levels. Reduction in complex I activity leads to the degradation of mitochondrial membrane potential which initiates the cascade of cellular events leading to mitochondria induced apoptosis¹⁷. With this in mind, we hypothesized that mUB treatment would decrease the number of cells undergoing apoptosis induced by juglone. Using the stain SYTO-12, we found that mUB at the concentration of 10 μ M significantly reduced the number of cells undergoing apoptosis (Figure 4). While the other two concentrations of urolithins were not statistically significant at reducing the number of apoptotic cells, there is a trend of apoptotic cell reduction.

In summary, urolithins have several different bioactivities described in the literature. However, molecular mechanisms responsible for their efficacy have only been well described for oncology models. Until this point, few mechanisms have been identified that explains the efficacy in non-oncology related disease models. Using various in vivo and in vitro experiments along with recent advances in proteomics, we have identified that mUB induced overexpression of NU4LM and increased complex I

activity may, in part, be responsible for the beneficial effects of mUB in a high oxidative stress environment like those found in several diseases.

2.0 Experimental

2.1 *C. elegans* Maintenance

Wild-type (N2) *C. elegans* were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. Worm cultures were maintained on Nematode Growth Medium (NGM) (1.7% Agar, 0.3% NaCl, 0.25% peptone, 1mM CaCl₂, 1 mM MgSO₄, 5mg/L cholesterol in ethanol, and 2.5 mM KPO₄) in petri dishes at 20°C with UV-killed OP50 *E. coli* as a food source. Age synchronous cultures were obtained by standard hypochlorite method¹⁸. For mUB treatment, *C. elegans* were washed from their plates with liquid nematode growth medium (0.3% NaCl, 0.25% peptone, 1 mM CaCl₂, 1 mM MgSO₄, 5 mg/L cholesterol in ethanol, and 2.5 mM KPO₄) and transferred to culture flasks containing UV-killed OP50 *E. coli* as a food source.

2.2 Cell Culture Conditions

SH-SY5Y (human neuroblastoma) cell lines were obtained from the American Type Culture Collection (ATCC) and were maintained using high glucose (4.5 g/L) DMEM/F12 supplemented with 10% heat inactivated FBS, 1% penicillin (100 U/mL) and streptomycin (100 µg/mL) and incubated in 5% CO₂ at 37°C. To harvest SH-SY5Y, cells were trypsinized (0.25% Trypsin/ EDTA), centrifuged (1500 rpm for 5 min) and re-suspended in 10% DMEM/F12. Cell concentration was determined by counting cells with a hemocytometer and viability was assessed by trypan blue staining. Prior to starting experiments, all-trans retinoic acid (RA) was used to differentiate SH-SY5Y for 7 days at a concentration of 10 µM while changing media every 48 hours to ensure proper neurite growth and cell morphology¹⁹.

2.3 *C. elegans* Stress Resistance Assay

An age synchronous culture of N2 (L2) worms in liquid NGM was split into five culture flasks. The flasks were treated with methylated urolithin B at 1, 5, and 10 μ M and 0.05% DMSO control. The cultures were placed on a rocker and incubated for 24 hours. After incubation, the cultures were transferred to small Petri-dishes containing NGM with 400 μ M Juglone. Immediately after the worms were added to their respective petri dishes, a baseline count was taken with a dissecting microscope. Then, worms were counted every hour until no surviving worms were left.²⁰ Dead worms were characterized via lack of pharyngeal pumping.

2.4 Staining for Apoptotic Cells in *C. elegans*

An age synchronous culture of N2 (L2) *C. elegans* were washed from their plate with liquid NGM. The culture was then split into five culture flasks and treated with the mUB and solvent control to a concentration of 1, 5, and 10 μ M and 0.05% DMSO. The flasks were placed on a rocker and incubated for 24 hours. The cultures were then incubated with 400 μ M juglone for one hour; this compound induces oxidative stress eventually leading to death. After which time, the cultures were washed three times with PBS to remove the *E. coli* food source. The stain, SYTO-12, was added to a final concentration of 33 μ M.²¹ The worms were incubated for an additional thirty minutes in the dark. After incubation, the worms were washed once in PBS and then anesthetized with 3% sodium azide in PBS and mounted onto microscope slides. Visualization of SYTO-12 stain was done under an EVOS microscope with an excitation filter of 518 nm. At least 20 worms were photographed per experimental group. Raw images were processed through ImageJ and pixel area was measured.

2.5 *C. elegans* Culturing for Mass Spectrometry Analysis

C. elegans were cultured on 100 mm plates using standard laboratory procedures.

When the worms reach the L4 stage, the plates were washed with 5ml of PBS. The solution was centrifuged gently at 1,000 X g for 5 minutes to form a pellet gently. The pellet was washed three times with 1 mL of PBS to remove the *E. coli* food source.

After the final washing, the supernatant was removed, and the pellet was suspended in lysis buffer and allowed to incubate on ice for 10 minutes. Next, the solution was centrifuged at 16,000 x g for 10 minutes at 4 C to pellet genetic material and cellular debris. The supernatant was removed and kept on ice until further processing.

2.6 Protein Digestion Aided with Pressure Cycling Technology (PCT)

Protein digestion was performed on *C. elegans* lysate according to the published method with modifications¹³. After obtaining 500 µL of the lysate at 1 mg/mL, 80 µL of 100 mM DTT and 200 µL of 50 mM ABC were added and incubated for 10 min at 95°C. After, 80 µL of 200 mM IAA was added to the samples, vortexed and incubated at room temperature for 30 min in the dark. An ice-cold mixture of methanol (0.5 mL), chloroform (0.25 mL) and water (0.25 mL) was then added to the samples to concentrate the protein. Samples were vortexed and spun at 16,000x g for 5 min at 4°C followed by an ice-cold methanol wash off the pellet. The pellet was re-suspended in 145 µL of 50 mM ammonium bicarbonate (pH 7.4), and 25 µL of TPCK treated trypsin (1mg/mL) was added to achieve a 1:20 trypsin ratio. Samples were transferred to MicroTubes (Pressure BioSciences Inc., South Easton, MA) and placed into a Barocycler NEP2320-45k (Pressure BioSciences Inc., South Easton, MA) to

perform PCT-aided digestion following a published method with modifications. Pressure cycling was performed for 90 cycles at 50°C. Each cycle consisted of 50 seconds at 35,000 psi and 10 seconds at ambient pressure. Trypsin digestion was stopped by adding formic acid to achieve a final concentration of 0.1% formic acid. Samples were vortex and spun at 1000x g for 5 min at 10°C. The supernatant was collected and transferred to 150 µL micro-inserts for analysis.

2.7 LC-MS/MS Analysis

LC-MS/MS analysis was done as previously described¹³. In brief, the analysis was done on a Sciex 5600 TripleTOF™ mass spectrometer using a DuoSpray™ ion source (AB Sciex, Concord, Canada). The mass spectrometer was coupled to an Acquity UPLC HClass system (Waters Corp., Milford, MA, USA) using an Acquity UPLC Peptide BEH C18 (2.1 X 150 mm, 300 Å, 1.7 µm) with an Acquity VanGuard pre-column (2.1 X 5 mm, 300 Å, 1.7 µm). The injection volume was 10 µL and the amount of protein per injection was 30 µg. TOF detector mass calibration was monitored by injecting trypsin-digested β-galactosidase peptides before and after the samples were run. Chromatographic and mass spectrometric methods for both standard information dependent acquisition (IDA) and (SWATH-MS) data acquisitions are previously described.¹³

2. 8 Data Processing with Skyline and R Studio

SWATH-MS data was imported into Skyline (University of Washington) for analysis of proteins. Peptides were selected for each protein followed by peak visualization and curation as previously described.¹³ The area under the curves (AUCs) for each peak

were exported for comparison and heatmap generation in R-Studio using the Pretty Heatmap and Color Brewer Palettes packages.

2.9 Complex I activity in human neuroblastomas, SH-SY5Y

Complex I activity was measured in differentiated SH-SY5Y human neuroblastomas.

Cells were pre-treated with vehicle control or mUB at 1, 5, or 10 μ M for 24 hours.

After which time, the cells were treated with 400 μ M hydrogen peroxide for an additional 24 hours. Cells were harvested and washed twice in PBS. Complex I activity was then measured using a colorimetric complex I enzyme activity microplate assay kit (Abcam), per the manufacturer's instructions.

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Figure 1. Kaplan-Meier survival curves of *C. elegans* treated 400 μ M Juglone and DMSO solvent control, 1, 5, or 10 μ M of mUB.

Figure 2. Heat map of the proteins identified in the samples that belong to the mitochondria proteome. Values are relative changes about the control. Orange indicates no changes in relative protein quantity. Red indicates a decrease in relative protein quantity and green indicates an increase in relative protein quantity.

Figure 3. Complex I activity in human neuroblastoma, SH-SY5Y, treated with mUB at 1, 5, and 10 micromolar. Complex I activity is significantly decreased by hydrogen peroxide treatment. mUB treatment rescued complex I activity from hydrogen peroxide treatment.

Figure 4. Percent Fluorescent signal of apoptotic cells after incubation with juglone and treatment with mUB, or solvent control. Fluorescent microscopy images of *C. elegans*. Staining using SYTO-12 for apoptotic cells. (A) *C. elegans* treated with Juglone and 0.05% DMSO. (B) *C. elegans* treated with Juglone and 1 μ M mUB. (C) *C. elegans* treated with Juglone and 5 μ M mUB. (D) *C. elegans* treated with Juglone and 10 μ M mUB.

MANUSCRIPT 5

Manuscript 5 is prepared for submission to Natural Products Communications.

Methylated urolithin B identified as a poly-ADP-ribose polymerase-1 inhibitor by drug affinity responsive target stability (DARTS). Rose, Kenneth N.; Barlock, Benjamin; DaSilva, Nicholas; Johnson, Shelby L.; Fatemeh, Akhlaghi; Seeram, Navinda P.

ABSTRACT

The biotransformation of ellagic acid by specific species gut microflora results in the production of a group of compounds known as urolithins. The bioactivity of these compounds has been studied in the context of multiple indications. One of the most studied bioactivities is cancer where several therapeutic mechanisms of urolithins have been proposed. However, recent research has identified that urolithins may also have neuroprotective effects. As mechanisms responsible for the induction of cancerous cell death are not likely to be responsible neuroprotection, we investigated possible protein-urolithin interactions in a human neuroblastoma cell model. Using a mechanism of action deconvolution method called drug affinity responsive target stability (DARTS) we identified that the model compound, methylated urolithin B, binds to the protein poly ADP-ribose polymerase-1 (PARP-1) and inhibits its activity at nanomolar concentrations. This activity may help to explain the anti-cancer and neuroprotective effects of urolithins as PARP -1 inhibitors are currently on the market as cancer therapies and have gained attention in the current literature for potential their application to neurodegenerative indications.

1.0 Introduction

Urolithins are low molecular weight microbiota metabolites that have been studied in relation to numerous indications^{1,2}. Of these, the most notable is oncology where urolithins have been studied regarding *in vitro* cancer cell viability³. Several publications implicate molecular targets by which urolithins may exert an anti-cancer therapeutic benefit^{4,5}. However, recent reports suggest that some urolithins may have neuroprotective effects⁶. Treatment with urolithins has been demonstrated to decrease neuroinflammation and attenuate mitochondria dysfunction as induced by aging⁷. While molecular targets of urolithins' anti-cancer effects have been suggested, the same targets are not likely to be responsible for the observed neuroprotective effects. Urolithin induced cell-cycle arrest is observed at high concentrations, ~50 μM , which is unlikely to be found in the brain. Therefore, we assert that molecular mechanisms, distinctly different from those related to cancer, must exist to explain urolithin's neuroprotective properties.

Given this hypothesis, we chose to investigate molecular targets of methylated urolithin B (mUB), a model compound, within human neuroblastoma, SH-SY5Y, using drug affinity responsive target stability (DARTS). This technique is used for identifying small molecules' molecular targets. DARTS relies on the thermodynamic stabilization of protein-ligand complexes and their resistance to protease degradation⁸. After incubating SH-SY5Y cell lysate with mUB and a protease, SDS-PAGE is used to determine differences in protein digestion, indicating a complex has formed between a protein and mUB. We identified a band of protein protected from protease digestion between 75 kilodaltons and 100 kilodaltons (Figure 1).

The protein from the band of interest was extracted and eighteen peptides were identified from the band (Table 1). Seventeen of these peptides corresponded to proteins in the poly ADP-ribose family. Using the protein BLAST software tool (NCBI, Bethesda, MD) it was identified that several of these peptides correspond to conserved domains within the PARP family (Table 2). Given that PARP-1 is the most represented protein in the band of interest, the molecular weight of the band of interest most closely matches with PARP-1's known molecular weight, and several of the peptides identified are conserved within the protein family, we tentatively assigned the identity of the protein band to be PARP-1. This initial identity from mass spectrometry was then confirmed by a western blot (Figure 2). To further validate that PARP-1 is a molecular target of mUB, PARP-1 activity was measured in the presence of activated DNA, PARP-1's endogenous target, and varying concentrations of mUB (Figure 3). At low concentrations, 100 nM, PARP-1 activity was inhibited about 20% with an increasing trend of inhibition with the highest concentration tested, 500 nM, inhibiting about 30% of PARP-1 activity.

Further target validation comes from homology structure analysis. A structurally similar compound, 6(5H)-phenanthridinone (Figure 4) is a known PARP-1 inhibitor with an IC_{50} of 300 nM⁹. Despite mUB being a weaker inhibitor than 6(5H)-phenanthridinone, a significant level of PARP-1 inhibition can be reached with concentrations much lower than those commonly tested in *in vitro* and *in vivo* models. With this in mind, it is important to consider the effect of PARP-1 inhibition when investigating any efficacious effects of mUB. Given the highly conserved structure of

urolithins, it is likely that many if not all of the urolithins can modulate PARP-1 activity. However, this has yet to be established and warrants future investigation.

As many groups have reported several different bioactivities of urolithins that are fundamentally different than anti-cancer activity, we investigated alternative mechanisms of action which might explain these reports. We found that the model compound, mUB, is an inhibitor of PARP-1 which is implicated in several diseases including cancer and neurodegenerative disease. These findings help to explain the several different bioactivities of urolithins that have been reported.

2.0 Experimental

2.1 Synthesis of methylated urolithin B

A previously published synthesis route was used for the synthesis of mUB¹⁰. The identity of the compound was verified via high-performance liquid chromatography.

2.2 Cell culture and maintenance

Human neuroblastomas, SH-SY5Y, were purchased from ATCC (Manassas, VA) and cultured using standard laboratory procedures. Cells were differentiated using all-trans retinoic acid as previously described.¹¹ Cells were harvested via trypsinization (0.25% Trypsin/EDTA, Life Technologies, Grand Island, NY). After trypsinization, cells were centrifuged at 1500 rpm for 5 minutes. Cells were then stored on ice until further processing.

2.3 Drug affinity responsive target stability

DARTS was performed as described with modifications¹². Briefly, SH-SY5Y cells were lysed with M-PER (Thermo Fisher, Waltham, MA) on ice for 10 minutes followed by centrifugation (1500 rpm for 5 minutes). The lysate was split into 99 μ L aliquots in 1.5mL centrifuge tubes. To one centrifuge tube, 1 μ L of 50% DMSO was added as a solvent control. To a separate centrifuge tube, 1 μ L of 1,000 μ M methylated urolithin B in 50% DMSO was added to a final concentration of 10 μ M methylated urolithin B. The resulting mixtures were then incubated for thirty minutes at room temperature with vigorous shaking. Each tube was split into three separate aliquots in 1.5mL centrifuge tubes. To the tubes was added 2 μ L of protease in a ratio of 1:100 pronase:protein and the mixture was incubated for 15 minutes at room

temperature. At that time, 2 μ L of 20x protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added along with an equal volume of 2x Laemmli sample buffer (Bio-Rad, Hercules, CA) and the samples were boiled for ten minutes. After heating, samples were frozen at -80°C until further processing.

2.4 SDS-PAGE

Pre-cast polyacrylamide gels (4-20% polyacrylamide gradient with ten 50 μ L wells) were purchased from Bio-Rad (Hercules, CA). Gels were placed into an electrophoresis chamber and immersed in SDS running buffer (25 mM tris, 192 mM glycine, 0.1% SDS). Gels were run at 24 mA until the loading buffer dye had reached the bottom of the gel. Then, gels were fixed with a solution of 10% acetic acid, 25% isopropanol, and 65% de-ionized water for 15 minutes. After fixing, gels were stained with Coomassie Blue (10% acetic acid, 0.006% Coomassie Blue dye, 90% de-ionized water) for two hours. Gels were then de-stained with 10% acetic acid until the protein bands had become visible.

2.5 Protein Identification

Proteins were extracted from the polyacrylamide gel matrix as previously described with modifications¹³. Briefly, protein bands were excised from SDS-PAGE gels with a razor blade and placed into 1.5 mL centrifuge tubes. Bands were de-stained with 10% acetic acid, with changes every 30 minutes, until the bands were completely de-stained. Then, disulfide bonds were reduced and cystine residues were alkylated with dithiothreitol (DTT) and iodoacetamide (IAA) respectively. Gel bands were dehydrated with acetonitrile for 10 minutes, after which time the solution was

removed and the bands were lyophilized overnight. Then, gel bands were re-hydrated on ice for 1 hour with a 10 µg/mL solution of trypsin in 25 mM ammonium bicarbonate. Next, the excess solution was removed and the gel piece was covered with 25 mM ammonium bicarbonate. The protein band/trypsin mixture was then incubated overnight at 37°C. After incubation, the supernatant was collected and placed into a 1.5 mL centrifuge tube. The gel band was washed with 20% acetonitrile, 1% trifluoroacetic acid, and 79% H₂O for 1 hour while vortexing. The supernatant was removed and added to the previous step's supernatant. The combined fractions were frozen at -80°C overnight and then lyophilized to dryness. Samples were reconstituted in 50% acetonitrile/ 50% water and placed into HPLC sample vials until further processing. The protein identity was determined using peptide mass fingerprinting with a SciexTripleTOF 5600 mass spectrometer (Framingham, MA) using previously described methods¹⁴.

2.6 Protein BLAST

Peptide sequences corresponding to the PARP protein family were analyzed via the protein BLAST tool freely available from the NCBI (Bethesda, MD) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). FASTA peptides sequences were queried for *Homo sapiens* proteins in the SwissProt database. The protein-protein algorithm was used for sequence alignment.

2.7 Western blotting

SDS-PAGE gels of cell lysate processed via the DARTS method were prepared as described following standard laboratory procedures. Cell lysates were treated with pronase at pronase:protein ratios of 1:1000, 1:500, and 1:100. Proteins were transferred to a nitrocellulose membrane using a mini Trans-Blot transfer apparatus (Bio-rad, Hercules, CA) at 100mA for 16 hours at 4°C. Membranes were removed from the apparatus and blocked in 5% bovine serum albumin (BSA) for 1 hour at room temperature. Then PARP-1 antibody (Abcam, Berlin, Germany), diluted in blocking buffer, was applied to the membrane and allowed to incubate for 16 hours at 4°C. After several washes in TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris Base), primary antibody detection was carried out using a Vectastain Elite ABC kit (Vector Labs, Burlingame, CA) as per the manufacturer's instructions.

2.8 PARP-1 Inhibition

The inhibitory effect of mUB on PARP-1 activity determined with a PARP homogenous inhibition assay kit (Trevigen, Gaithersburg, MD) as per the manufacturer's instructions. All tested mUb concentrations were performed in triplicate.

3.0 Acknowledgements

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Figure 1. SDS-PAGE gel of SH-SY5Y cell lysate processed with the DARTS method and stained with Coomassie Blue. Asterisks flank bands protected from digestion which suggests a complex has been formed between mUB and the protein.

Table 1. List of peptides identified in the protein gel band protected by mUB.

Peptide Sequence	Protein Name	Peptide Retention Time (Minutes)	Quantification
EMVPPIAQYGDVLVR	sp P08684 CP3A4_HUMAN	40.32	Normalized Area: 3.1245E+3
MAIMVQSPMFDGK	sp P09874 PARP1_HUMAN	33.21	Normalized Area: 7.3108E+5
WYHPGCFVK	sp P09874 PARP1_HUMAN	35	Normalized Area: 1.0171E+4
EDAIEHFMK	sp P09874 PARP1_HUMAN	33.2	Normalized Area: 4.9199E+3
FYTLIPHDFGMK	sp P09874 PARP1_HUMAN	33.21	Normalized Area: 5.3261E+4
IAPPEAPVTGYMFGK	sp P09874 PARP1_HUMAN	37.67	Normalized Area: 2.5823E+3
APVDPECTAK	sp Q9UGN5 PARP2_HUMAN	33.24	Normalized Area: 6.1902E+3
SANYCFASR	sp Q9UGN5 PARP2_HUMAN	31.49	Normalized Area: 1.7080E+3
QLLGLQPISTVSPLHR	sp Q9UUK3 PARP4_HUMAN	38.38	Normalized Area: 1.5883E+4
AGGFELQEEESK	sp Q8IXQ6 PARP9_HUMAN	35.12	Normalized Area: 3.1989E+3
TELEIEGAR	sp Q8IXQ6 PARP9_HUMAN	33.55	Normalized Area: 1.9720E+3
AFYDTLDAAR	sp Q53GL7 PAR10_HUMAN	36.33	Normalized Area: 6.1815E+3
VPPLFVPQGTSER	sp Q9H0J9 PAR12_HUMAN	44.56	Normalized Area: 4.2186E+3
LQMYFQSPK	sp Q460N5 PAR14_HUMAN	32.62	Normalized Area: 1.4559E+4
ELIESTTQK	sp Q460N5 PAR14_HUMAN	32.38	Normalized Area: 3.4215E+3
LPVDVVVNASNEDLK	sp Q460N5 PAR14_HUMAN	37.67	Normalized Area: 2.7251E+3
AAGPELQADCDQIVK	sp Q460N5 PAR14_HUMAN	37.67	Normalized Area: 4.7324E+3
YFVVTNNQLLR	sp Q8N5Y8 PAR16_HUMAN	33.56	Normalized Area: 6.5868E+2

Table 2. List of conserved protein regions for the 17 peptides identified as belonging to the PARP family.

Name	Accession	Description	E-value
PLN03124	PLN03124	PARP; Provisional	7.77E-12
PARP_Like	cd01437	PARP Catalytic Domain	1.03E-11
PARP	pfam00644	PARP Catalytic Domain	7.68E-10
zf-PARP	pfam00645	PARP Zinc Finger Region	2.70E-04
PARP_reg	pfam02877	PARP Regulatory Domain	2.84E-04

Figure 2. Western blot confirming that the identity of the protected protein band to be PARP-1. The “-” indicates that pronase or mUB was not added to the reaction mixture. The “+” indicates the addition of pronase or mUB to the reaction mixture. A box surrounds the two bands which demonstrate that the addition of mUB protects PARP-1 from pronase digestion. “+” = 1:1000, “++” = 1:500, “+++” = 1:100 pronase:protein ratio.

Figure 3. Inhibition curve of mUB on PARP-1 activity.

Figure 4. Chemical structure of mUB (Top) and 6(5H)-phenanthridinone (Bottom).

CHAPTER 3

EFFICACY OF A PHENOLIC-ENRICHED EXTRACT OF MAPLE SYRUP IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

Manuscript 6 has been submitted to the journal of Nutritional Neuroscience

Anti-inflammatory effects of a food-grade phenolic-enriched maple syrup extract in a mouse model of Alzheimer's disease. Rose, Kenneth N.; Barlock, Benjamin; Liu, Chang; DaSilva, Nicholas; Johnson, Shelby L.; Nelson, Robert; Fatemeh, Akhlaghi; Seeram, Navindra P.

ABSTRACT

In recent years, the role of inflammation in Alzheimer's Disease pathogenesis and progression has come to the forefront in the field. Several studies have suggested that chronic inflammation, as a result of Alzheimer's Disease pathology, can lead to exacerbated disease progression. Therefore, targeting inflammation is a likely therapeutic option. The sugar free, phenolic-enriched extract of maple syrup, known as MSX, is a mixture of polyphenols and has been studied for safety, tolerability, and a variety of different biological effects. Of these, MSX has shown to reduce neuroinflammation in the human neuroblastoma cell line, SH-SY5Y. For these reasons, this study investigated the efficacy of MSX in a transgenic mouse model of Alzheimer's disease and examine neuroinflammation as outcomes measure to determine efficacy. We found that two doses of MSX were able to significantly decrease the expression of several proteins related to inflammatory cascades and phenotypic markers of innate immune cells that are thought to specifically respond to Alzheimer's disease pathology.

MANUSCRIPT 6

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Anti-inflammatory effects of a food-grade phenolic-enriched maple syrup extract in a mouse model of Alzheimer's disease. Rose, Kenneth N.; Barlock, Benjamin; Liu, Chang; DaSilva, Nicholas; Johnson, Shelby L.; Nelson, Robert; Fatemeh, Akhlaghi; Seeram, Navindra P.

Abstract

Alzheimer's disease (AD) is a growing global health crisis exacerbated by increasing life span and an aging demographic. Convergent lines of evidence, including genome-wide association studies, strongly implicate neuroinflammation in the pathogenesis of AD. Several dietary agents, including (poly)phenolic-rich foods, show promise for the prevention and/or management of AD, which in large part, has been attributed to their anti-inflammatory effects. We previously reported that a food-grade phenolic-enriched maple syrup extract (MSX) inhibit neuroinflammation *in vitro* but whether these effects were translatable *in vivo* remain unknown. Here, we report that oral administration of MSX (at doses of 100 and 200 mg/kg for 30 days) attenuates several neuroinflammatory markers in 3xTg-AD mice, a murine model of amyloid and tau pathology occurring in AD. Proteomics analysis by LC-MS/MS with SWATH acquisition showed that 3xTg-AD mice dosed orally with MSX have decreased expression of several inflammatory proteins, including, most notably, the AD risk-associated protein "triggering receptor expressed on myeloid cells-2" (TREM2), as well as stimulator of interferon genes TMEM173, and suppressor of cytokine signaling-6 (SOCS6). These data demonstrate that oral administration of this maple syrup derived natural product reduces key neuroinflammatory indices of AD in the 3xTg-AD model of amyloid and tau pathology. Therefore, further studies to investigate MSX's potential as a dietary intervention strategy for AD prevention and/or management are warranted.

1.0 Introduction

The immune system is integral to the central nervous system (CNS). Having a major role in brain homeostasis and brain development, the immune system, particularly innate immunity, is necessary for proper brain function over an individual's lifetime. Under homeostatic conditions, many cell types with several different phenotypes work in concert to survey the brain and regulate inflammatory responses. However, it is thought that chronic diseases affecting the CNS dysregulate the immune system function, which may in turn lead to exacerbated pathology^{1,2}. For this reason, inflammation has become an important target for neurodegenerative disease drug discovery research.

Alzheimer's disease (AD) is a chronic, neurodegenerative disease with a well-established connection to the inflammatory response³. The hallmark pathological lesions in AD, amyloid- β plaques and neurofibrillary tangles, are associated with dysregulation of central immune system function⁴. Recently, several groups have reported that AD pathology involves a phenotypic switch of innate immune cells from a homeostatic state to a neurodegenerative state^{5,6}. Interestingly, after switching to a neurodegenerative disease state, these cells lose their ability to regulate brain homeostasis, a possible mechanism for how dysregulation of the innate immune system can exacerbate pathology⁶. Studies investigating a potential molecular switch for the shift between "homeostatic" and "neurodegenerative" phenotypes have identified a signaling pathway that includes the proteins triggering receptor expressed on myeloid cells-2 (TREM2) and apolipoprotein E (APOE) as key participants⁶. By suppressing this signaling pathway, the investigators reported that a homeostatic

phenotype could be restored. These findings suggest that suppressing neuroinflammation and the TREM2-APOE pathway may have a beneficial therapeutic effect against AD.

Evidence from genome-wide association studies (GWAS) has implicated variants in the APOE and TREM2 genes with risk for AD. While the association between APOE and AD has been established since the early 1990's, variants of TREM2 and AD risk have recently emerged. A clear genetic relationship exists between TREM2 and AD, but the biological mechanism by which an increase in risk or protective effect occurs is still unknown. With regard to other types of dementia, mutations in TREM2 leading to its complete loss of function result in Nasu-Hakola disease (NHD), a rare disorder leading to presenile dementia. There are many similarities between the brains of patients with AD and NHD such as astrogliosis and immune cells in pro-inflammatory states⁷. Furthermore, patients with NHD can develop AD-like pathology with amyloid- β plaques and neurofibrillary tangles⁸. These findings demonstrate an important connection between TREM2 induced dysregulation of the immune system and the effects this can have on the brain.

Several attempts have been made to treat AD by regulating inflammation. For instance, the non-steroidal anti-inflammatory drugs (NSAIDs), celecoxib and naproxen, were evaluated for their effects on improving cognitive performance in humans with AD. However, no statistically significant effects were observed with NSAID treatment⁹. While GWASs have clearly identified the immune system as a component contributing to pathology, the mechanisms by which this occurs are not

fully understood. This lack of mechanistic understanding of AD has made drug discovery efforts difficult. A potential method to circumvent this hurdle is to utilize medicinal plant foods and their derived products, especially those rich in (poly)phenolics which are known for their anti-inflammatory properties, as dietary intervention strategies for AD prevention and/or management. In fact, several phenolic-rich natural products including certain fruits, berries, spices, and green tea have been reported to have beneficial effects against AD¹⁰⁻¹². Recently, it has been reported that polyphenol dietary supplements can improve deficits in cognition and decrease arachidonic acid levels in the mouse model of AD, 3xTg-AD¹³⁻¹⁵.

Our group has conducted extensive chemical compositional studies on maple syrup, a natural sweetener produced by boiling the sap of sugar maple (*Acer saccharum*) trees, leading to the identification of over 60 phenolic constituents¹⁶⁻¹⁸. A phenolic-enriched maple syrup extract was reported to decrease oligomerization and aggregation of both A β 1-42 and tau peptides¹⁹. In addition, our group reported on the anti-neuroinflammatory effects and anti-AD effects of a phenolic-enriched maple syrup extract (MSX) *in vitro* and in *Caenorhabditis elegans*²⁰. However, to date, the effects of MSX against AD has not been studied in any rodent models. Here we sought to investigate whether MSX exerts anti-inflammatory effects using the 3xTg-AD mouse model of AD.

GWAS and mRNA screening techniques have led to a deeper understanding of the interplay between the immune system and AD. However, these techniques suffer from

limited interpretation as genetic variants and mRNA do not perfectly correlate with protein expression. As the functional component, monitoring protein expression is preferred but often impractical. Here, we utilized sequential window acquisition of all theoretical mass spectra (SWATH-MS), a data-independent proteomics method which greatly improves upon the sensitivity and reproducibility of more traditional proteomic approaches. SWATH-MS allows for the efficient and comprehensive characterization of the inflammatory response at a protein level in response to pathology.

2.0 Methods

2.1 Production of MSX

Details of the preparation and chemical standardization of a food grade phenolic-enriched maple syrup extract (MSX) has been previously reported¹⁷. MSX contains over 60 phenolic constituents present in the whole maple syrup food but with reduced sucrose content. The extract is well tolerated with no toxicity observed at 1000 mg/kg for 7 days in Sprague-Dawley rats¹⁷.

2.2 Mice

All female 4-week-old transgenic 3xTg-AD mice (The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. These mice harbor three familial mutations of AD: (1) amyloid precursor protein (APP) KM670/671NL (Swedish). (2) Microtubule associated protein tau (MAPT) P301L. (3) Presenillin-1 M146V. These particular mice were chosen because they display intra-neuronal amyloid deposits early in age, similar to the early stages of AD.

Mice were maintained in the University of Rhode Island's mouse vivarium with a 12-hour light/dark cycle with free access to food and water. Prior to treatment, mice were acclimated for one week. Following acclimation, mice were treated for thirty days with 100 mg/kg/day or 200 mg/kg/day MSX, or an equal volume of 0.5% carboxymethylcellulose (CMC) as a solvent control via oral gavage five times per week. Allometric scaling was used to choose doses of MSX which translate to a reasonable dose in a human²¹. Based on this scaling method, the low and high doses translate to 487.8 mg/day and 975.6 mg/day, respectively, for a 60 kg human. Each

treatment groups consisted of six mice. One mouse in the 100 mg/kg/day MSX group had to be euthanized per order of the attending veterinary due to hydrocephalus. An additional five female C57BL/6 mouse brains, of the same age, were purchased from BioIVT (Westbury, NY) to act as genetic background expression controls.

After thirty days of treatment, mice were anesthetized using an isoflurane chamber (Patterson Scientific, Waukesha, WI). Mice were then euthanized via decapitation and their brains removed and immediately stored on ice. Following removal, brains were snap frozen in liquid nitrogen. After freezing, brains were stored at -80°C until further processing. All protocols and procedures were approved by the University of Rhode Island's Institutional Animal Care and Use Committee.

2.3 Quantification of amyloid species

Sections of cortex or hippocampus were weighed out and placed into 2 mL screw cap vials containing 1.4 mm ceramic beads (Omni International, Kennesaw, GA). Eight volumes of 5 M guanidine-HCl in 50 mM Tris (pH 8.0) was added to the brain sections. Brains were homogenized in a Bead Ruptor 24 (Omni International, Kennesaw, GA). After which time the homogenate was shaken at room temperature for three hours. Before amyloid analysis, the homogenate was diluted 10-fold with PBS containing 1x protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO).

Quantification of amyloid was performed using an Amyloid beta 42 Mouse ELISA Kit and Amyloid beta 40 Mouse Kit (ThermoFisher, Waltham, MA) following the manufacturer's instructions. Absorbance was read using a SpectraMax microplate reader (Molecular Devices, San Jose, CA).

2.4 Lipid Peroxidation Analysis

Brain lipid peroxidation was analyzed using a fluorometric lipid peroxidation (MDA) assay kit (Abcam, Cambridge, UK). Brains sections from cortex and hippocampus were weighed and homogenized in 150 μ L of water and three μ L BHT (supplied in the kit). Samples were prepared in 2 mL screw cap vials containing 1.4 mm ceramic beads and homogenized with a Bead Ruptor 24 (Omni International). Proteins were precipitated with 153 μ L of 2 N perchloric acid. The quantification of lipid peroxidation from this prepared homogenate was performed following the manufacturer's instructions. Fluorescence was read using a SpectraMax microplate reader (Molecular Devices, San Jose, CA).

2.5 Brain Tissue Preparation and Protein Digestion

Pre-weighed brain tissue was transferred to 2 mL screw cap vials filled with 1.4 mm ceramic beads. Vials contained 7 volumes of pre-chilled homogenization buffer (50 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.25 M sucrose, and 20 μ M BHT). Brain tissue was then homogenized using a Bead Ruptor 24 (Omni International, Kennesaw, GA). The resulting homogenate was centrifuged at 10,000 x g for 20 minutes at 4°C. Homogenate protein concentration was determined using a Pierce BCA protein kit (ThermoFisher, Waltham, MA). Homogenate was diluted to 2.5 mg/mL using 50 mM ammonium bicarbonate. Proper dilution was confirmed using a NanoDrop One/One^C microvolume 9UV-Vis spectrophotometer to conserve sample (ThermoFisher, Waltham, MA). Then, proteins were digested using a protein preparation kit and

trypsin (SCIEX, Framingham, MA) along with pressure cycling technology (PCT) as previously reported²².

2.6 Mass Spectrometry Data Acquisition with SWATH-MS

Mass spectrometry was performed as previously described with modifications²². Data was acquired on a SCIEX TripleTOF® 5600 mass spectrometer using a DuoSpray™ ion source (SCIEX, Framingham, MA) coupled to an Acquity HClass UHPLC system (Waters Corp., Milford, MA). An Acquity VanGuard pre-column (2.1 x 5 mm², 300 Å, 1.7 µm) with an Acquity UPLC Peptide BEH C18 column (2.1 x 150 mm², 300 Å, 1.7 µm) was used to achieve separation. The column temperature was set to 40°C and the autosampler was set to 10°C. A linear gradient was used with a flow rate of 100 µL/min for 90 min. Mobile phase A consisted of 98% acetonitrile, 2% water and 0.1% formic acid. Mobile phase B consisted of 98% water, 2% acetonitrile and 0.1% formic acid. The gradient was as follows: 98% A from 0 to 5 min, 98% to 75% from 5 to 55 min, 75% to 50% A from 55 to 60 min, 50% to 20% from 60 to 70 min. Mobile phase A was held at 20% from 70 to 75 min and returned to 98% A at 80 min. The column was held at 98% A for 10 min to equilibrate prior to the next sample. A mixture of trypsin-digested β-galactosidase peptides were used between every 10 samples to calibrate masses and monitor the QTOF detector.

Positive ionization mode was used for SWATH data acquisition. The mass spectrometer parameters are as follows: gas 1 60 psi, gas 2 60 psi, curtain gas 25 psi, temperature 450°C, ion spray voltage floating 5500 V, declustering potential 120, collision energy 10 and collision energy spread 5. For data acquisition, a maximum of 50 candidate ions were monitored for each survey scan. All ions had a charge state

from 2 to 4. A range of m/z 300-1500 was used for exclusion criteria and all ions that had an intensity greater than 25 cps were chosen for MS/MS analysis. The temperature was set at 400°C and the total cycle time was 3.95 sec. Seventy SWATH windows per cycle were collected over m/z 400-1100 with each window size being m/z 10.

2.7 Mass Spectrometry Data Analysis

SWATH data was used for peptide and transition selections in Skyline (MacCoss lab, University of Washington). The FASTA file for each protein to be analyzed (Table 1) was imported into Skyline. Three surrogate, flyable peptides containing at least 3 transitions were selected and correlated with the other chosen peptides for each respective protein. Peptide cutoff criteria included a minimum length of 6 and maximum length of 25 amino acids and did not occur in the transmembrane domain, did not contain posttranslational modifications or polymorphic variations. Peptide selections were filtered down to two peptides per protein wherever possible. The program MPPreport was used to calculate the total area under the curve for each protein analyzed. Subsequently, transgenic animal protein expression was normalized to wild-type animal protein expression.

2.8 Statistics

The statistical program Prism 7 (Graph Pad, San Diego, CA) was used for statistical analysis and generation of figures. Comparing wild-type and untreated 3x-Tg-AD protein expression was done with multiple, two-tailed t-tests with a α level of 0.05. Two-way ANOVAs and Dunnett's *post hoc* test were used to compare protein expression across treated and untreated transgenic groups.

3.0 Results

3.1 Early stage AD pathology induces changes in the expression of inflammatory proteins in the brains of 3xTg-AD mice.

To better understand the nature of the inflammatory response at this stage of pathology, the expression of inflammatory and AD-related proteins (Table 1) were analyzed by SWATH-MS. Several inflammatory mediators were differentially expressed in the cortex of 3xTg-AD mice as compared to wild-type mice (Table 2). However, only two proteins were differentially expressed in the hippocampus (Table 3). These alterations in protein expression of inflammatory mediators and AD-related proteins were then used as outcome measure for the possible efficacious benefit of MSX supplementation.

3.2 Oral supplementation of MSX does not alter the generation of amyloid species or the abundance of lipid peroxides

In addition to the expression of inflammatory mediators, lipid peroxidation and pathogenic generation of amyloid species were evaluated as outcome measures to quantify the potential efficacious benefits of MSX. No statistically significant differences in lipid peroxidation levels were found between the cortex of untreated or treated animals (untreated vs. low dose $p=0.9998$, untreated vs. high dose $p=0.9993$). The same trend was identified in the hippocampus (untreated vs. low dose $p=0.9821$, untreated vs. high dose $p=0.2245$) (Figure 1). The generation of $A\beta_{42}$ in the cortex was not found to be statistically significant across groups (untreated vs. low dose $p=0.9999$, untreated vs. high dose $p=0.9978$). Generation of $A\beta_{42}$ in the hippocampus was slightly lower than the cortex, but generation did not change across treatment groups (untreated vs. low dose $p=0.1259$, untreated vs. high dose $p=0.1350$) (Figure 2). Generation of $A\beta_{40}$ was also unchanged by MSX treatment (Figure 3) (Cortex:

untreated vs. low dose $p=0.9065$, untreated vs. high dose $p=0.9978$) (Hippocampus: untreated vs. low dose $p=0.9995$, untreated vs. high dose $p=0.9942$).

3.3 Oral MSX supplementation significantly reduces the expression of several pro-inflammatory proteins correlated with AD in the cortex of 3xTg-AD mice

With MSX supplementations, several proteins related to AD were downregulated to near wild-type expression levels (Figure 4). TREM2 ($p<0.0001$), CD36 ($p=0.0017$), ITGAX ($p<0.0001$), and CSF1R ($p=0.0005$) were overexpressed in transgenic animals compared to wild-type, and high dose (200 mg/kg/day) MSX reduced expression to near baseline expression. Low dose (100 mg/kg/day) of MSX has a similar trend to the high dose, with TREM2 ($p<0.0001$) and CSF1R ($p=0.0264$) showing reduced expression. In both the high and low dose groups, APOE expression was unchanged ($p=0.3798$, $p=0.3798$ respectively).

The inflammatory mediators TMEM173 and SOCS6 were highly overexpressed in the control treated 3xTg-AD mice compared to wild-type expression. Both the high and low dose MSX treatments reduced TMEM173 ($p<0.0001$, $p=0.0036$ respectively) and SOCS6 ($p<0.0001$, $p<0.001$ respectively) expression to near baseline expression (Figure 5).

The higher dose of MSX reduced the expression of proteins associated with AD and inflammation associated innate immune cells. The expression of C1qa, while unaffected by low dose MSX ($p=0.2996$) was significantly reduced by high dose MSX ($p<0.0001$). Furthermore, CTSB ($p<0.0001$), CTSL ($p=0.0021$), and LPL

($p < 0.0001$) were also downregulated toward baseline expression with high dose MSX treatment (Figure 6).

Several inflammatory mediators and AD related proteins analyzed were not shown to have differential expression with either high or low MSX treatment. However, TNFA ($p = 0.0007$), SOD1 ($p = 0.0378$), TLR4 ($p = 0.0217$), and were upregulated with low dose MSX treatment but not with high dose MSX treatment ($p = 0.0694$, $p = 0.5334$, $p = 0.8886$, $p = 0.9959$ respectively) (Figure 7). High dose MSX reduced TIMP2 ($p = 0.0484$) expression but these differences were not seen in low dose MSX treatment. BACE expression was increased in both the high ($p < 0.0001$) and low ($p = 0.0044$) MSX groups.

3.4 Oral supplementation of MSX does not significantly alter the expression of pro-inflammatory proteins in the hippocampus of 3xTg-AD mice

The hippocampus had minimal changes in protein expression across all of the proteins analyzed (Table 4). However, SOCS6 was marginally overexpressed in wild-type mice and high dose of MSX reduced its expression to below baseline expression ($p = 0.0424$). High dose MSX also reduced TIMP2 ($p = 0.0006$) and FADD ($p = 0.0255$) expression in the hippocampus, but low dose MSX did not affect the expression of any of these proteins (Figures 8-11).

4.0 Discussion

There is a clear relationship between neuroinflammation and AD^{23–27}. Evidence from genome-wide association studies (GWAS), genome-wide expression studies (GWES), and immunohistochemistry (IHC) on human AD tissue suggest that the immune system is dysregulated in patients with AD^{28–32}. Furthermore, dysregulation of the innate immune system may be contributing to the pathology or progression of AD^{33,34}. Therefore, we sought to characterize the inflammatory response of the 3xTg-AD mouse model using the proteomics technique SWATH-MS. Then, we evaluated the effects of a phenolic-enriched extract of maple syrup, MSX, on the model's specific inflammatory response.

Dietary sources of anti-inflammatory compounds which may alter neuroinflammation are plant polyphenols. Several phenolic-enriched plant extracts and isolated pure compounds have been studied in the context of inflammation and AD^{35–38}. However, *in vitro* research often induces inflammation with LPS, a ligand of toll-like receptor-4 (TLR4). While LPS produces robust and repeatable results, recent studies investigating the immune response to AD pathology have demonstrated the complexity and uniqueness of this response⁵. MSX has shown anti-inflammatory and neuroprotective properties *in vitro* with LPS stimulation¹⁹. Therefore, we sought out to evaluate these effects in an *in vivo* model of AD.

Early in pathology, the 3xTg-AD has several changes in the expression of inflammatory mediators. At this stage of pathology, the cortex is the most affected while the hippocampus had little to moderate changes in protein expression. We used

a custom-made list of inflammatory mediators (Table 1) that have been implicated in AD pathology and related inflammatory responses. The largest differences were seen with TREM2, TMEM173, and SOCS6. Alterations in the expression of CSF1R, ITGAX, and CD36 were also observed which was expected as these proteins have been previously implicated in the AD immune response. Surprisingly, APOE was not upregulated by pathology. Previous research suggests that APOE works in concert with TREM2 to regulate innate immune cell phenotype fate, however we did not replicate these findings. As expected, increases in pro-inflammatory proteins were also observed. The complement protein C1qA, NOS2, MAPK8, LPL, and the cathepsins CTSB and CTSL were all upregulated by 3xTg-AD pathology (Table 2, 3). These changes in inflammatory mediators clearly establish that the 3xTg-AD model, even at this early stage of pathology, has a significant inflammatory response in the brain.

While there were several significant changes to inflammatory mediators, alteration to lipid peroxidation levels were not observed (Figure 1). Often used as a marker of cell damage, lipid peroxidation levels correlate with pathology and age^{39,40}. In these relatively young animals, differences in lipid peroxidation may be too small to notice significant differences. However, as expected with this model, A β ₄₂ generation was significantly higher than A β ₄₀. Treatment with MSX did not alter generation of either of these amyloid species (Figure 2,3). This suggests that any anti-inflammatory effects of MSX are not due to differences in pathological generation of amyloid species.

Once the inflammatory response in the 3xTg-AD model was established with SWATH-MS, we were able to evaluate the effects of MSX using alterations in the

inflammatory response as outcome measures. The anti-inflammatory effects of MSX were shown to be specific to the cortex. Given the age of the animals used and that in this animal model, pathology begins in the cortex and appears in the hippocampus later in life, therefore we expected minimal changes to hippocampal protein expression⁴¹. After MSX treatment, the expression of several proteins that have been implicated in AD pathogenesis were reduced (Figure 4). Particularly, CSF1R, a promotor of microglia proliferation, was reduced to near basal expression. CSF1R serves as an interesting topic as pharmacological inhibition of CSF1R has been shown to reduce AD-like pathology in vivo⁴². Furthermore, the expression of CD36 was also significantly reduced. CD36 has been identified as a risk factor for the development of AD and has been demonstrated to associate with amyloid plaques and stimulate the release of pro-inflammatory cytokines^{43,44}. Similarly, the expression of ITGAX and TREM2, markers of innate immune cells in a pro-inflammatory phenotype, were reduced^{5,45}. Together, these findings suggest that the phenotype of innate immune cells in the brain has changed with MSX treatment. Given that these markers are associated with a pro-inflammatory response, it is likely that the immune cell phenotype induced by MSX treatment is associated with a reduction in inflammation. Interestingly, the expression of APOE was unaltered by MSX treatment. Given previous findings that suggest APOE and TREM2 work in concert to alter inflammatory phenotypes, this result was unexpected. However, the animals used in this study are relatively young and of a different genotype than previously researched models. Our data suggests that at the endpoint of this study, APOE expression is not drastically overexpressed. It may be possible that the pathology present in the 3xTg-AD mice at the endpoint of this study do not elicit significant increases in APOE expression to notice differences in

pharmacological modulation. Future research that analyses a longer time course of MSX treatment and AD pathology progression is necessary.

The expression of two inflammatory mediators, TMEM173 and SOCS6, were profoundly increased in control treated animals as compared to wild-type animals (Figure 5). MSX treatment was able to reduce the expression of these proteins to near baseline levels. TMEM173, also known as the stimulator of interferon genes, is a potent initiator of an inflammatory response⁴⁶. TMEM173 signaling leads to the expression of type I interferons which further orchestrates an inflammatory response⁴⁷. Genetic knockouts of TMEM173 in models of murine prion disease reduced the expression of inflammatory markers in the brain. While knockouts of type I interferon slowed disease progression suggesting that limiting this signaling pathway may be beneficial to attenuate neurodegenerative processes⁴⁸. SOCS6 is a negative regulator of receptor tyrosine kinase signaling that is overexpressed in the brains of patients with AD⁴⁹. Expression of SOCS6 can lead to mitochondrial dysfunction and apoptosis through the BAX pathway^{50,51}. Mitochondrial dysfunction is thought to play a role in AD pathogenesis and/or progression^{52,53}. Taken together, the profound increase in TMEM173 and SOCS6 in 3xTg-AD mice as compared to wild-type mice, and then the dose dependent reduction in expression with MSX treatment suggest that MSX alters inflammatory signaling. This provides further support that MSX alters the inflammatory phenotype of innate immune cells in the brains in 3xTg-AD mice.

Recent work has suggested that a pro-inflammatory phenotype exist in the brains of AD animal models which restrict homeostatic regulation. Therefore, limiting this

phenotype may also confer a protective or therapeutic benefit to AD pathology. The expression of several markers of these cells were significantly increased in the 3xTg-AD mice which were then decreased by MSX treatment (Figure 6). In particular, several cathepsins (CTSB, CTSL), C1qA, and LPL were shown to increase in response to 3xTg-AD pathology. MSX treatment significantly reduced the expression of these markers. These findings further provide evidence that MSX treatment shifts the innate immune cell phenotype away from a pro-inflammatory state.

A select few inflammatory markers were upregulated with MSX treatment. The expression of TNF- α and TLR4 was increased with low-dose MSX treatment but not with high-dose MSX. In vitro experiments in murine microglia suggest that MSX treatment reduces TNF- α levels, however our results do not replicate these finding²⁰. In addition to increases in TNF- α , the cell surface receptor, TLR4, was also increased. This finding is not unexpected as TLR4 and TNF- α are part of the same signaling pathway. TLR4 has been demonstrated to help coordinate an inflammatory response against amyloid pathology⁵⁴. It is possible that MSX may not act on pathways leading to TLR4 expression resulting in increased TLR4 and through its signaling, increases in TNF- α expression. Another unexpected result was a modest increase in BACE1 expression. BACE1 is a secretase responsible for the production of C99, the precursor to beta-amyloid. However, this increase did not result in an increase in amyloid generation of any species. More research is required to determine if this increase has an effect on plaque pathology over the time course of the disease.

Understanding the mechanism of MSX's anti-inflammatory effects is complex. As an extract, MSX contains several compounds, more than sixty of which have been identified by our laboratory^{16-18, 55-57}. The majority of compounds are lignans which can act as phytoestrogens and may play a role in estrogen receptor signaling^{58,59}. Several other classes of polyphenols are also present which may contribute to the reduction in inflammation through sequestration of radical oxygen species⁶⁰. Our lab group has isolated sufficient quantities of more than twenty of the compounds found in MSX. When used in a *C. elegans* model of oxidative stress, only a few of the isolated compounds were able to protect the worms from oxidative stress¹⁶. This suggests that not all compounds found in MSX are active and that a synergy may exist between several compounds which leads to the observed effects in this study. The use of bio-assay guided fractionation or cheminformatics may be useful to better understand the chemical constituents of MSX that leads to the reduction of AD neuroinflammation *in vivo*. This topic is under current investigation by our group.

5.0 Conclusion

The immune system has gained recent attention in the neurodegenerative research field. Particularly in AD as several clinical trials with therapies aimed at the amyloid hypothesis have failed to reach their clinical endpoints. The chronic inflammation as seen in the human condition and recapitulated by animal models is thought to enhance disease progression and may even be part of AD pathogenesis. Therefore, identifying pharmaceutical agents that limit inflammation in the brain through practical routes, such as oral administration, is of great importance and may lead to future clinical trials. Herein, we have described an orally dosed extract of maple syrup (MSX) that reduces neuroinflammation in the early stages of pathology in the 3xTg-AD model of AD. By using advanced proteomic techniques, we monitored several proteins to characterize the inflammatory phenotype in the brains of wild-type, untreated transgenic, and treated transgenic mice with two different doses of MSX. Our results demonstrate that MSX treatment may shift the innate immune system's phenotype away from an inflammatory state, potentially to a homeostatic state. However, more research is required to rigorously phenotype these cells and how this change may affect progression over the course of the AD.

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Table 1. List of proteins analyzed by LC-MS/MS with SWATH acquisition along with their abbreviations

Protein Abbreviation	Protein Name
CHIT1	Chitotriosidase-1
CH3L1	Chirinase-2-like protein 1
CCL2	C-C motif chemokine 2
CD14	Monocyte differentiation antigen CD14
ITGAM	Integrin alpha-M
TNFA	Tumor Necrosis Factor-alpha
IL1B	Interleukin-1 beta
IL15	Interleukin-15
TNF10	Tumor necrosis factor ligand superfamily member 10
CD36	Platelet glycoprotein 4
BACE1	Beta-secretase 1
TNFR11	Tumor necrosis factor receptor superfamily member 11a
TLR4	Toll-like receptor 4
CD47	Leukocyte surface antigen CD47
NLRP3	NACHT, LRR and PYD domains-containing protein 3
IRAK4	Interleukin-1 receptor associated kinase 4
NOS2	Nitric oxide synthase, inducible
SOD1	Superoxide dismutase [Cu-Zn]
TMEM173	Stimulator of interferon genes protein
FADD	FAS-associated death domain protein
SHARPIN	Sharpin
SOCS6	Suppressor of cytokine signaling 6
APP	Amyloid precursor protein
P2RY12	P2Y purinoceptor 12
APOE	Apolipoprotein E
TCF4	Transcription factor 4
TREM2	Triggering receptor expressed on myeloid cells 2
CST3	Cystatin-C
CSF1R	Macrophage colony-stimulating factor 1 receptor
C1QA	Complement C1q subunit A
TMEM119	Transmembrane protein 119
CTSB	Cathepsin B
UFO	Tyrosine-protein kinase receptor UFO
CTS7	Cathepsin 7
CTSL	Cathepsin L1
LPL	Lipoprotein lipase
CSF1	Macrophage colony-stimulating factor 1
LIRB4	Leukocyte immunoglobulin-like receptor subfamily B member 4
TIMP2	Metalloproteinase inhibitor 2
ITGAX	Integrin alpha-x

Table 2. Comparison of protein expression between wildtype mice and 3xTg-AD mice in the cortex. Individual *t*-tests were used to determine statically significant differences in protein expression between groups.

Protein Abbreviation	<i>t</i> ratio	Statistically Significant	Adjusted P-Value
CHIT1	2.805	No	0.2767
CH3L1	3.01	No	0.2229
CCL2	2.82	No	0.2767
CD14	1.618	No	0.7013
ITAM	5.821	Yes	0.0088
TNFA	4.604	Yes	0.0316
IL1B	4.046	No	0.0647
IL15	1.604	No	0.7013
TNF10	2.164	No	0.4861
CD36	5.572	Yes	0.0102
BACE1	3.477	No	0.1245
TNR11	1.25	No	0.7013
TLR4	1.862	No	0.5948
CD47	2.753	No	0.2767
NLRP3	2.772	No	0.2767
IRAK4	5.336	Yes	0.0131
NOS2	5.585	Yes	0.0102
SOD1	1.402	No	0.7013
TMEM173	5.638	Yes	0.0101
FADD	3.094	No	0.2077
SHARPIN	1.975	No	0.5642
SOCS6	7.479	Yes	0.0015
APP	3.548	No	0.1177
P2RY12	4.209	No	0.0532
APOE	1.258	No	0.7013
TCF4	2.437	No	0.3683
TREM2	8.696	Yes	0.0005
CST3	1.205	No	0.7013
CSF1R	8.209	Yes	0.0007
C1QA	4.697	Yes	0.0288
TMEM119	1.435	No	0.7013
CTSB	7.463	Yes	0.0015
UFO	6.644	Yes	0.0035
CTS7	5.636	Yes	0.0101
CTSL	7.261	Yes	0.0018
LPL	3.661	No	0.1042
CSF1	4.752	Yes	0.0277
LIRB4	1.592	No	0.7013
TIMP2	6.283	Yes	0.0052
ITGAX	5.673	Yes	0.0100

Table 3. Comparison of protein expression between wildtype mice and 3xTg-AD mice in the hippocampus. Individual *t*-tests were used to determine statically significant differences in protein expression between groups.

Protein Abbreviation	<i>t</i> ratio	Statistically Significant	Adjusted P-Value
CHIT1	2.699	No	0.5356
CH3L1	1.221	No	0.9769
CCL2	2.411	No	0.6737
CD14	1.373	No	0.9734
ITAM	3.725	No	0.1571
TNFA	3.527	No	0.2026
IL1B	3.489	No	0.2081
IL15	0.01164	No	0.9986
TNF10	0.8633	No	0.9906
CD36	1.904	No	0.8841
BACE1	7.581	Yes	0.0014
TNR11	1.225	No	0.9769
TLR4	1.416	No	0.9724
CD47	5.23	Yes	0.0220
NLRP3	1.464	No	0.9701
IRAK4	0.3198	No	0.9974
NOS2	1.078	No	0.9769
SOD1	3.796	No	0.1456
TMEM173	1.557	No	0.9646
FADD	0.2949	No	0.9974
SHARPIN	2.097	No	0.8156
SOCS6	2.26	No	0.7379
APP	2.538	No	0.5956
P2RY12	0.8742	No	0.9842
APOE	1.928	No	0.8732
TCF4	3.888	No	0.1309
TREM2	0.04763	No	0.9986
CST3	2.798	No	0.4782
CSF1R	1.306	No	0.9690
C1QA	2.09	No	0.8156
TMEM119	4.004	No	0.1138
CTSB	0.8642	No	0.9842
UFO	1.871	No	0.8745
CTS7	1.547	No	0.9582
CTSL	1.319	No	0.9690
LPL	2.642	No	0.5455
CSF1	3.813	No	0.1421
LIRB4	0.6078	No	0.9842
TIMP2	1.141	No	0.9691
ITGAX	1.754	No	0.9099

Figure 1. Abundance of lipid peroxidation is unaltered in the brains of 3xTg-AD mice treated with MSX.

Figure 2. Generation of A β_{42} in the cortex and hippocampus is unaltered by MSX treatment.

Figure 3. Generation of A β_{40} in the cortex and hippocampus is unaltered by MSX treatment.

Figure 4. Differential expression of AD related proteins in the cortex of 3xTg-AD mice with MSX treatment. The dietary supplementation of MSX results in a dose-dependent decrease in the expression of inflammatory mediators specifically implicated in AD. Data shown as mean of all biological replicates with standard deviation, *= p<0.05.

Figure 5. Proteins highly overexpressed in untreated 3xTg-AD mice were significantly reduced in the cortex of 3xTg-AD mice with MSX treatment. The dietary supplementation of MSX results in a dose-dependent decrease in the expression of inflammatory mediators which were drastically overexpressed as compared to control. Data shown as mean of all biological replicates with standard deviation, *= p<0.05.

Figure 6. MSX treatment results in the decrease of inflammatory mediators associated with AD related pro-inflammatory immune cells. Markers of disease associated immune cells were significantly reduced in a dose dependent manner with dietary supplementation of MSX. Data shown as mean of all biological replicates with standard deviation, *= p<0.05.

Figure 7. Treatment with MSX resulted in minimal changes to several inflammatory mediators. Several of the proteins analyzed were unaltered or minimally altered by MSX treatment. Data shown as mean of all biological replicates with standard deviation, *= p<0.05.

Figure 8. Treatment with MSX did not alter the expression of AD related inflammatory mediators in the cortex. Supplementation of MSX did not result in differential expression of inflammatory mediators. Data shown as mean of all biological replicates with standard deviation, *= p<0.05.

Figure 9. Proteins highly overexpressed in the cortex showed no changes in expression as compared to wild-type mice or with MSX treatment. Data shown as mean of all biological replicates with standard deviation, *= p<0.05.

Figure 10. Treatment with MSX did not reduce the expression of pro-inflammatory immune cell signatures in the hippocampus. MSX treatment did not result in differential expression of inflammatory mediators associated with pro-inflammatory immune cells. Data shown as mean of all biological replicates with standard deviation, *= p<0.05.

Figure 11. Dietary supplementation with MSX resulted in minimal alterations to the expression pattern of several inflammatory mediators in the hippocampus. At this age, wild-type mice and 3xTg-AD mice have similar expression patterns in the cortex. MSX treatment had minimal effect on this expression pattern. Data shown as mean of all biological replicates with standard deviation, $\ast = p < 0.05$.

CONCLUSIONARY REMARKS

Humanity has been exploiting the therapeutic and psychoactive effects of natural products for thousands of years, however, the idea of *drug hunting* in natural products is a relatively modern idea. Since the conceptualization of modern drug-discovery in the early 19th century, natural products have served as the foundation on which many medicines are built upon. In the case of Alzheimer's disease, botanical natural products may be particularly helpful in drug discovery efforts. Epidemiological evidence suggests that higher dietary intake of botanical natural products, like those from fruits, vegetables, and spices, is correlated with lower risk of developing the disease. These findings suggest that dietary natural products have compounds intrinsic to them which may be useful for the treatment or prevention of Alzheimer's disease.

However, despite the strong epidemiological evidence, clinical trials aimed at treating Alzheimer's disease with natural products have been unsuccessful. These failures are due, in part, to the difficulties of studying the disease. With a long pre-clinical stage, rapid cognitive decline in the clinical stage, and poor methods for early detection and diagnosis, establishing suitable inclusion/exclusion criteria and outcome measures to evaluate a particular drug is a significant challenge. Compounding the difficulty of clinical trials is the lack of understanding surrounding the disease biology. For decades the main targets of drug discovery efforts were the pathological hallmarks of the disease, amyloid plaques, and tau tangles. Despite the strong connection between the appearance of these hallmarks and Alzheimer's disease, therapeutics aimed at these targets have found little success in the clinic. To overcome these challenges, a

paradigm shift in the way Alzheimer's disease is researched, and thereby treated, is needed.

Currently, the Alzheimer's disease research community is on the brink of this paradigm shift. Instead of focusing on targeting and removing the pathological hallmarks, the focus is starting to shift to systems ancillary to plaque and tangle pathology. One of the main targets is neuroinflammation as it is thought to play a role in disease progression. However, the neuroinflammatory response to disease is very complex and it is not yet clear which mechanisms should be targeted to produce a therapeutic benefit. To overcome this hurdle, a bottom-up approach can be used. Instead of taking a critical part of disease biology, identifying a therapeutic mechanism, and then exploit that mechanism pharmacologically, the bottom-up approach is agnostic to therapeutic mechanism and may in turn discover therapeutic mechanisms. One way to go about this approach is to treat animal models of the disease that recapitulate the neuroinflammation that has been observed in the human condition with extracts of natural products. These extracts would have a diverse chemical makeup and therefore, likely act on multiple systems. Changes in neuroinflammation can be monitored as outcome measures. Extracts that alter the neuroinflammatory phenotype can then be further tested to elucidate the compound or compounds responsible for the therapeutic effect, then the mechanisms of action of these compounds can be identified. This method allows for the relatively efficient screening of dozens of extracts containing several hundreds to even thousands of individual constituents. Furthermore, this method may lead to new insights in disease biology which may change how Alzheimer's disease is treated.

This dissertation is the beginning of the bottom-up approach that has established the tools and methods required for this approach to be successful. The screening of a large library of natural product extracts and pure compounds using wild-type and transgenic *Caenorhabditis elegans* concluded in the identification of several extracts and natural products that prolong the lifespan of these models when undergoing stress from a toxin or one of the pathological hallmarks of Alzheimer's disease. Investigations into one of these compounds, a gut microbiota metabolite of a natural product, led to the discovery of two mechanisms of action which potentially explain the observed protective effects against Alzheimer's disease pathology. And finally, investigations into a natural product extract orally dosed in a transgenic mouse model of Alzheimer's disease led to the identification of two efficacious doses that significantly reduce early neuroinflammation.

While Alzheimer disease drug discovery has been one of the greatest medical challenges to date, recent work has given the research community a more refined understanding of disease biology. With these revelations and the failures of many major clinical trials focusing on pathological hallmarks of the disease, the field is shifting to new hypotheses that may provide more insight into the disease and tractable therapeutic routes. Botanical natural products and the gut microbiota metabolites of natural products may provide a source of compounds which can be used to evaluate several hypotheses and, with more research, be used therapeutically.