

Development of a neuroprotective potential algorithm for medicinal plants



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ARTICLE INFO

Article history:

Received 27 April 2016

Received in revised form

22 September 2016

Accepted 28 September 2016

Available online 29 September 2016

Keywords:

Antioxidant

Alzheimer's disease (AD)

Glycation

Beta amyloid (A β)

Neuroinflammation

Caenorhabditis elegans

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 a 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.

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1. 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 Götze, 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

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Abbreviations

AD	Alzheimer's disease
ROS	reactive oxygen species
AGEs	advanced glycation end-products
RAGE	receptor for advanced glycation end-products
A β	beta amyloid
BBB	blood brain barrier
RCS	reactive carbonyl species
APP	A β precursor protein
AG	aminoguanidine
FRAP	ferric reducing antioxidant power
AChE	acetylcholinesterase
CNS	central nervous system
NOS	nitric oxide species
TCM	traditional Chinese medicine
NPA	Neuroprotective Potential Algorithm

BHT	Butylated hydroxytoluene
BSA	bovine serum albumin
MGO	methylglyoxal
RESV	resveratrol
PD	1,2-phenylenediamine
DQ	2,3-dimethylquinoxaline
TFA	trifluoroacetic acid
ThT	thioflavin T
A β _{1–42}	beta amyloid 1–42
DMSO	dimethyl sulfoxide
GAEs	gallic acid equivalents
DMEM/F12	Dulbecco's modified eagle medium: nutrient mixture F-12
FBS	fetal bovine serum
CTG 2.0	CellTiter-Glo 2.0
LPS	lipopolysaccharide
NGM	nematode growth medium

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 40–42 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; Li et al., 2013). Fibrillated A β can induce neurotoxicity by enhancing neuronal oxidative stress and neuroinflammation (Butterfield, 2002). 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, 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 Fig. 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. Material 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 and additional details (lot numbers, solvent to extract ratio/yields, etc.) are provided in the *Supplementary Content*. Each extract was dissolved in DMSO (20.0 mg/mL) and diluted to test concentrations (10–2000 μ g/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., 2016a,b). Briefly, 100 μ L serial dilutions (10–2000 μ g/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

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)

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

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, Indian ginseng	anti-ulcer	Roots	25.7 ± 0.4	279.6 ± 20.1	166.1 ± 3.1
BHT ^d	—	—	—	—	493.6 ± 8.7	—

^a (w/w % as of gallic acid equivalents).^b Value = IC₅₀ (μg/mL), n.a. = not active (IC₅₀ > 2000 μg/mL).^c Value = ascorbic acid equivalents/mg dry plant material, n.a. = not active.^d Positive control: Butylated hydroxytoluene, BHT.

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 FeCl₃·6H₂O (20 mM). Briefly, 1.5 mL of freshly prepared FRAP reagent was mixed with 50 μL of each extract (final concentration of 100 μg/mL) and the absorbance was measured at 593 nm after 5 min 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 μL of 50% aqueous methanol, and 100 μL of each sample was incubated with 50 μL of the Folin–Ciocalteu reagent for 5 min at room temperature. Then 150 μL of a 20% sodium carbonate (Na₂CO₃) solution and 250 μL 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, amino-guanidine (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). 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; Sun et al., 2016). 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 h. 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: 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 $\mu\text{g/mL}$, respectively. Resveratrol (RESV; at 100 $\mu\text{g/mL}$) 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 h 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 h. 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 μM 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 $\mu\text{g/mL}$ of each extract or 100 $\mu\text{g/mL}$ of the positive control, AG. After 72-h 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 μL of 100 mM Tris-HCl buffer (pH 8.0), 20 μL AChE enzyme solution (0.5 U/mL) and 20 μL of test samples (concentration ranging from 25 to 300 $\mu\text{g/mL}$ in 100 mM Tris-HCl buffer containing 50% methanol) was co-incubated in the dark at 37 °C for 20 min in a 96-well plate. Next, 40 μL of 0.75 mM DTNB and 20 μL of 1.5 mM ATCl 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 $\mu\text{g/mL}$ 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-h incubation period, CTG 2.0 was added in and mixed for 2 min 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 (100,000 cells/mL, were serum-starved for 4 h prior to the treatments. The cells were then incubated with test samples (10 $\mu\text{g/mL}$) or the positive control, resveratrol (RESV; 5 $\mu\text{g/mL}$) for 1 h after which inflammation was induced by lipopolysaccharide (LPS). Cells were incubated for 23 h 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 μL 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 h to induce the expression of the human A β_{1-42} gene. After 20 h the worms were counted every 2 h for a total of 30 h 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., 2016a,b). 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 h prior to exposure to 100 μM of H₂O₂ for 6 h. The SH-SY5Y cells were incubated with the extracts and RESV for 24 h at 37 °C prior to

exposure to 100 μ M of H₂O₂ for 6 h. Following incubation, CTG 2.0 was added in a 1:1 ratio directly to existing media and mixed for 2 min 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 all-trans retinoic acid (Sigma Aldrich) (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 h. After pre-treatment, the cells were then exposed to H₂O₂ (100 μ M) for 6 h, after which the cell culture media was collected, centrifuged at 10,000 rpm for 5 min 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). Fig. 1 shows the algorithm used to derive the overall index scores for the medicinal plant extracts.

3. 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 μ g/mL,

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	Neuroprotective 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

^a BSA AGEs formation induced by D-fructose.

^b BSA AGEs formation induced by methylglyoxal (MGO).

^c Thermo induced A β fibrillation (37 °C).

^d Methylglyoxal (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 µg/mL	AChE IC ₅₀ (µg/mL)
<i>P. granatum</i>	35.06 ± 2.93	304.62 ± 1.27
<i>P. emblica</i>	43.11 ± 1.72	163.92 ± 3.55
<i>M. pruriens</i>	48.11 ± 2.78	91.35 ± 2.80
<i>C. longa</i>	11.31 ± 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 ± 2.58	313.58 ± 6.21
<i>S. reticulata</i>	7.64 ± 1.43	—
Galanthamine ^c	68.50 ± 1.10	5.37 ± 3.79

^a n.a. = not active; shown as mean values ± SD (n ≥ 3).

^b —, >600 µg/mL.

^c Used as positive control at 12 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.

Table 4

Final screening for medicinal plant extracts for neuroprotective potential index based on the AChE inhibitory activity assay, in vitro anti-neuroinflammatory effects in murine BV-2 microglial cells, and in vivo neuroprotective effects against Aβ₁₋₄₂ 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

As shown in Fig. 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 Fig. 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 Fig. 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 Aβ₁₋₄₂ fibrillation and Aβ₁₋₄₂–AGEs formation

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

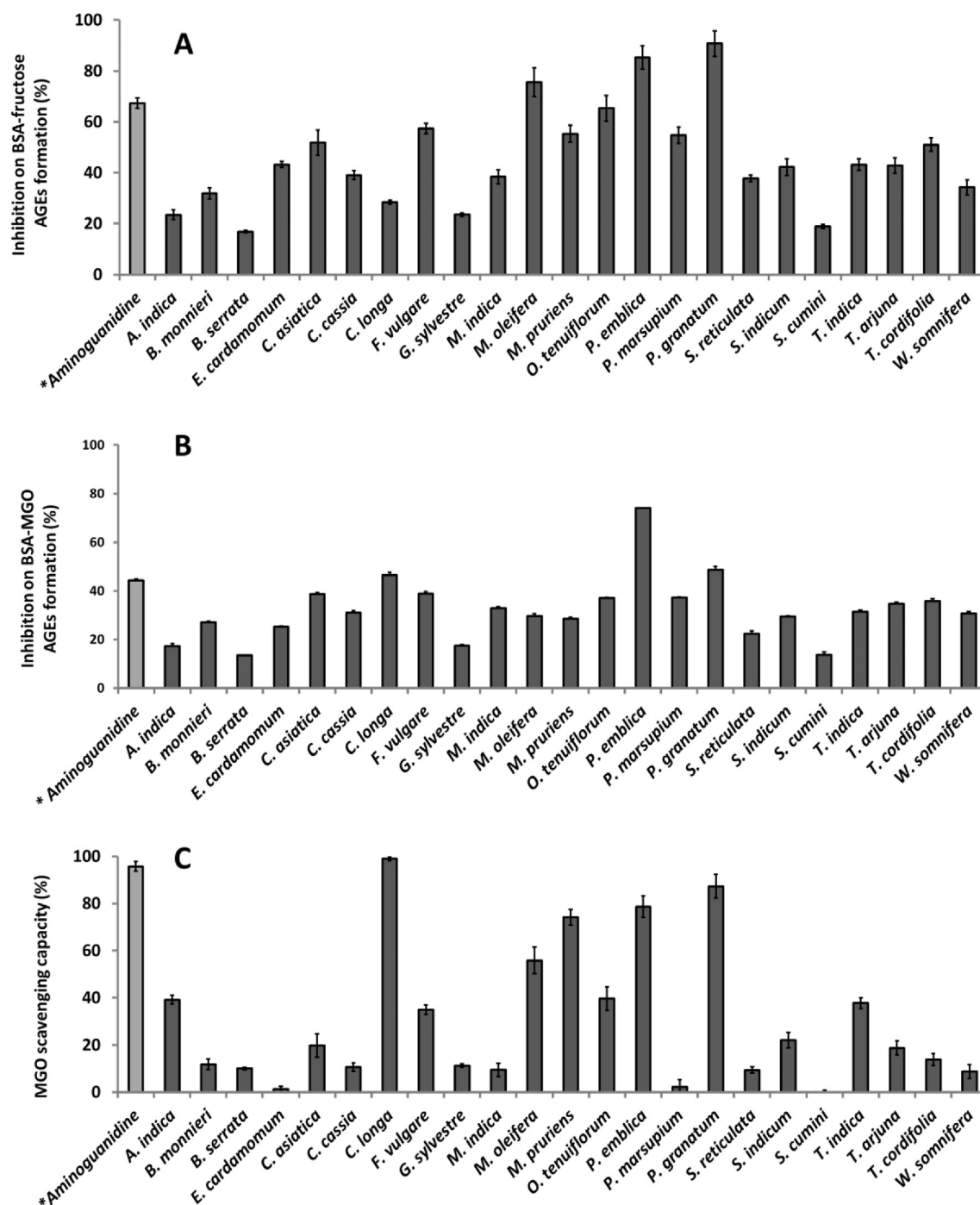


Fig. 2. Inhibitory effects of medicinal plant extracts (100 µg/mL) on D-fructose induced BSA AGEs formation (A), MGO induced BSA AGEs formation (B) and MGO scavenging capacity (C). Aminoguanidine at 100 µg/mL served as the positive control.

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 h 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 Fig. 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.,

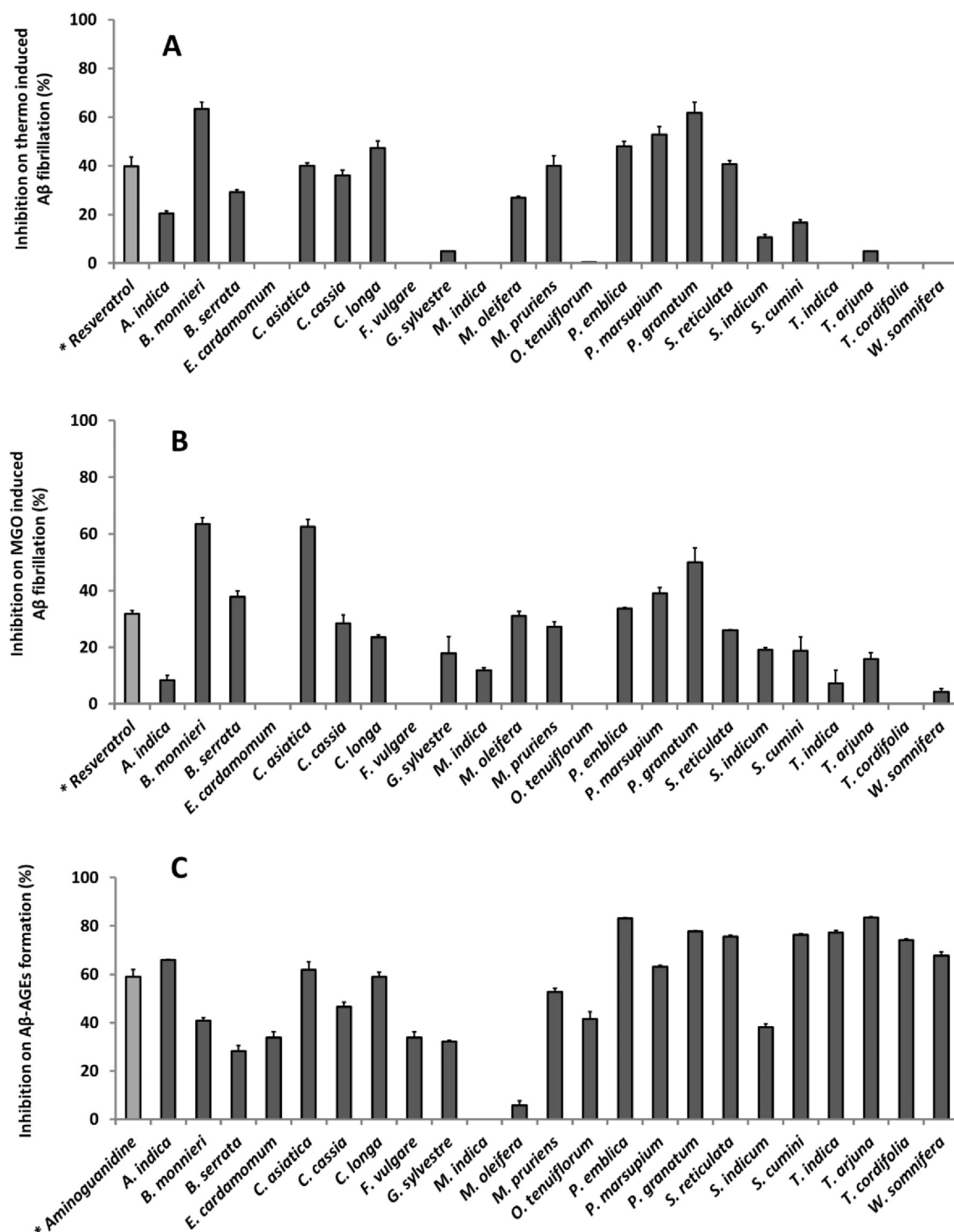


Fig. 3. Inhibitory effects of medicinal plant extracts (100 $\mu\text{g/mL}$) on thermo-induced A β fibrillation (A), MGO induced A β fibrillation (B) and MGO induced A β -AGEs formation (C). Resveratrol at 100 $\mu\text{g/mL}$ served as the positive control for A β fibrillation studies (A and B) while aminoguanidine at 100 $\mu\text{g/mL}$ served as the positive control for A β -AGEs study (C).

2006, 2007). 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 Fig. 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 , glycosylated 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 glycosylated A β (A β -AGEs). As shown in Fig. 3C, the

positive control, AG, at a concentration of 100 $\mu\text{g/mL}$, reduced glycosylated 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 IC₅₀ 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 IC₅₀ 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 Fig. 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 $\mu\text{g/mL}$), 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 Fig. 5, the heat shock induced A β _{1–42} neurotoxicity decreased the survival rate of *C. elegans* to 15.3% at 30 h. 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

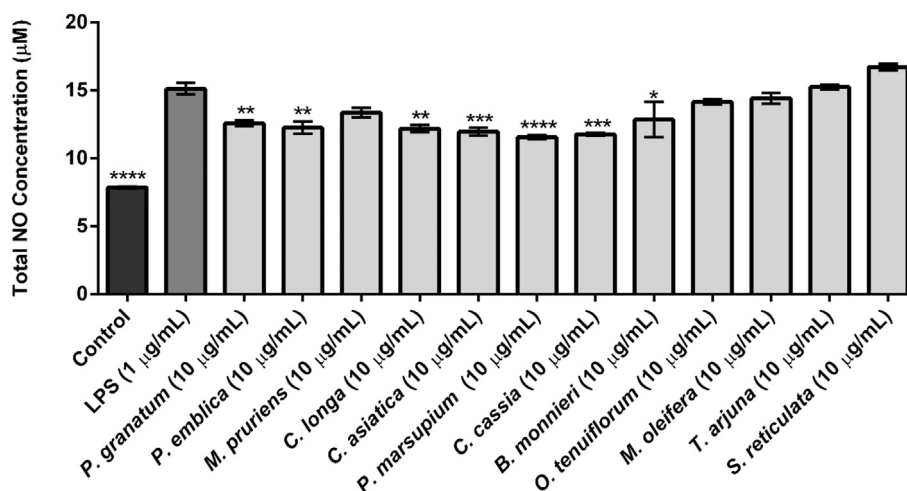


Fig. 4. Anti-neuroinflammatory effects of medicinal plant extracts on murine BV-2 cells microglial cells by measuring NOS production induced by LPS. BV-2 cells were treated with each extracts (10 $\mu\text{g/mL}$) for 1 h followed by exposure to LPS (1 $\mu\text{g/mL}$) for 24 h. The cell culture media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

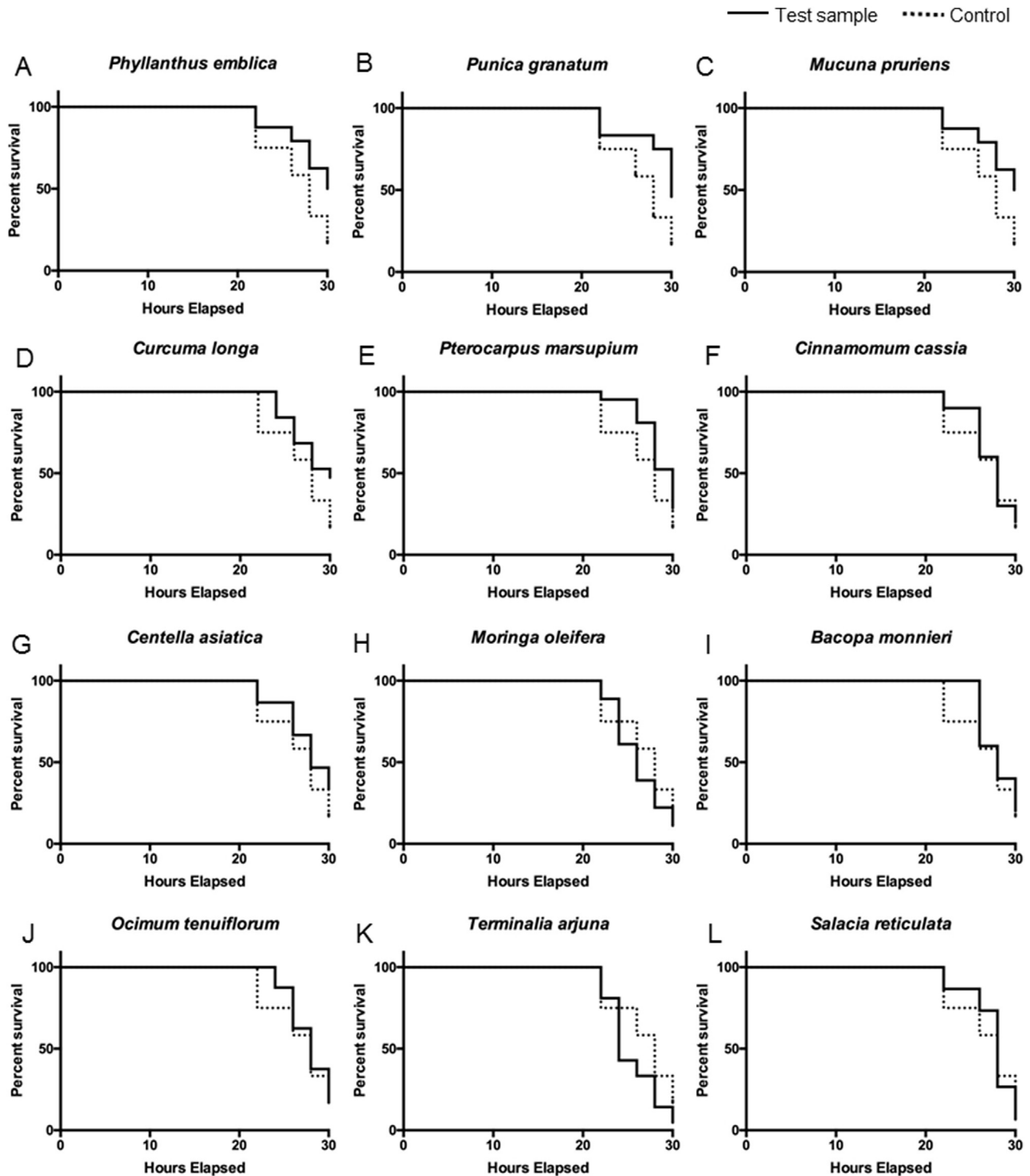


Fig. 5. Protective effects of medicinal plant extracts against neurotoxicity and paralysis in *Caenorhabditis elegans* in vivo. Mobility curves of transgenic (CL4176) *C. elegans* 30 h post A β_{1-42} induction of muscular paralysis at 25 °C. Kaplan-Meier mobility plots of *C. elegans* worms fed on twelve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

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 Fig. 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),

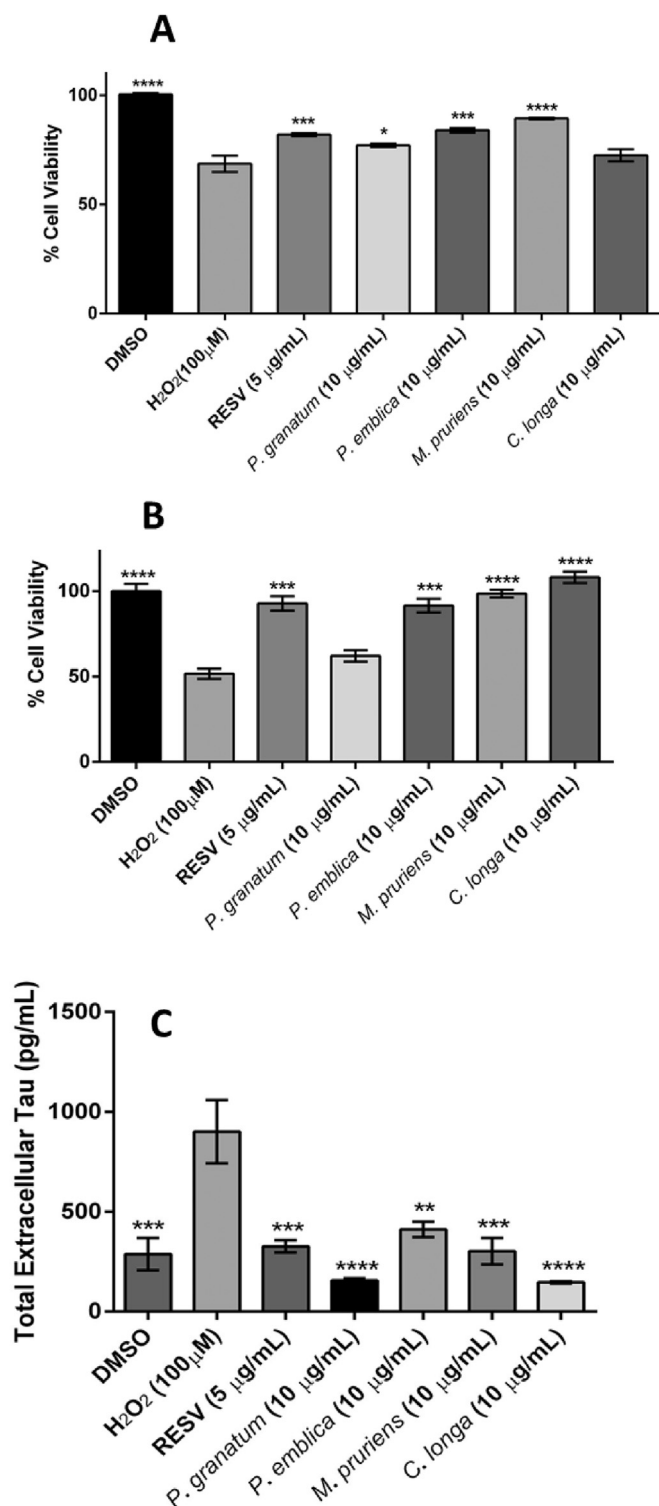


Fig. 6. Protective effects of medicinal plant extracts (10 μ g/mL) on H₂O₂ induced cytotoxicity in murine BV-2 microglia (A) and differentiated human SH-SY5Y neuronal cells (B). Cellular viability was assessed using Cell Titer Glo 2.0 (CTG 2.0) one step assay. C: Inhibitory effects of medicinal plant 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.

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 Fig. 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 (Fig. 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 (Gómez-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 Fig. 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. Conclusion

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, a Neuroprotective Potential Algorithm (NPA) was developed as part of a strategy to help guide the selection and evaluation of medicinal plant candidates for their neuroprotective potential. 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., 2014, 2015; Yuan et al., 2015). 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 Schluesener, 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 extracts for further research using *in vivo* models.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

HM was supported by a scholarship from the Omar Magnate Family Foundation. The herbal extracts were kindly provided by Verdure Sciences (Noblesville, IN, USA) courtesy of Mr. Ajay Patel. Spectrophotometric data were acquired from instruments in the RI-INBRE core facility located at the University of Rhode Island (Kingston, RI, USA) supported by grant # 5P20GM103430-13 from the National Institute of General Medical Sciences of the National Institutes of Health.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuint.2016.09.014>.

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