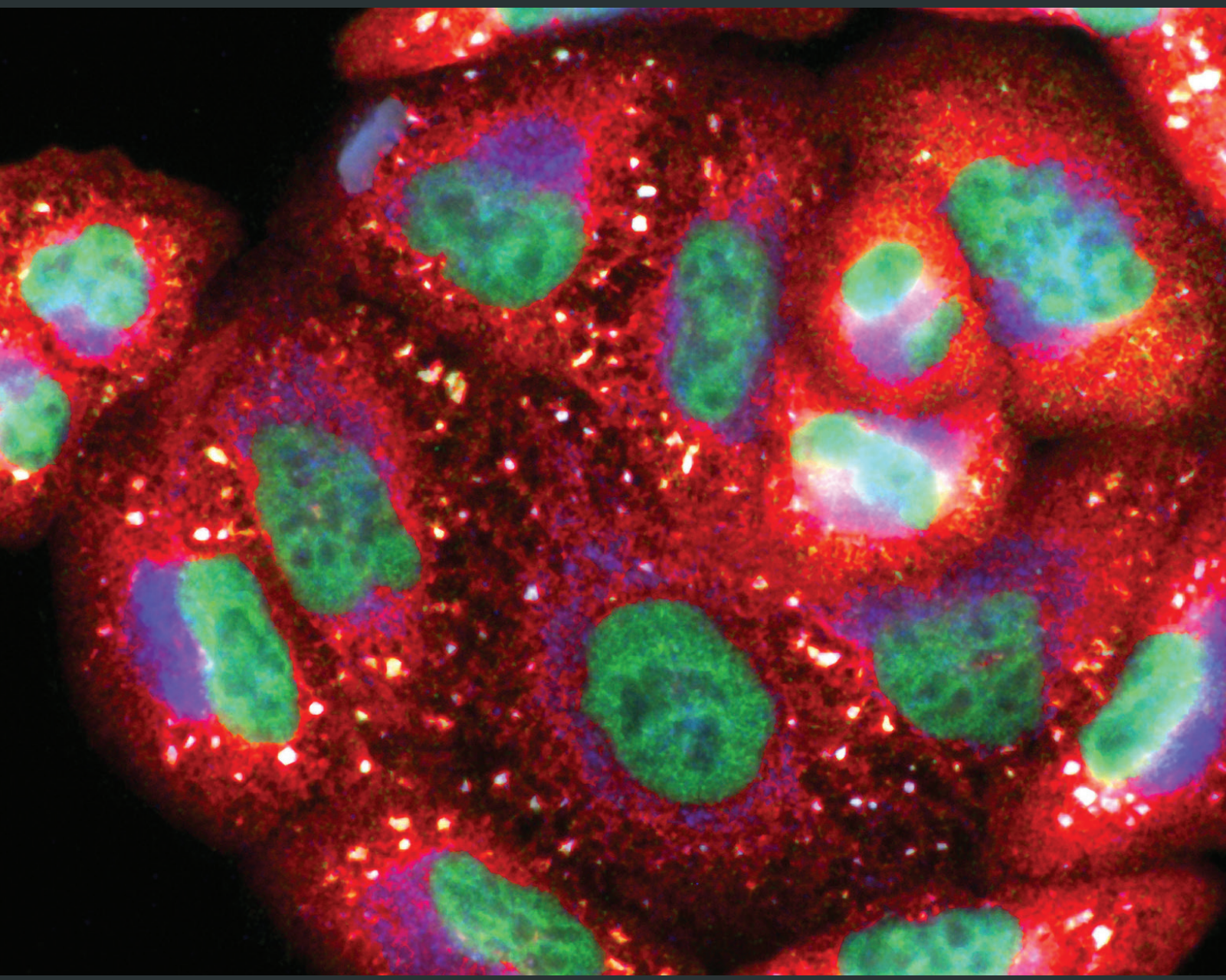
Oxidative Medicine and Cellular Longevity

Polyphenols Targeting Brain Cells Longevity, Brain's Redox Status, and Neurodegenerative Diseases

Lead Guest Editor: Anat Elmann
Guest Editors: David Vauzour and Chin-Kun Wang



Polyphenols Targeting Brain Cells Longevity, Brain's Redox Status, and Neurodegenerative Diseases

Oxidative Medicine and Cellular Longevity

Polyphenols Targeting Brain Cells Longevity, Brain's Redox Status, and Neurodegenerative Diseases

Lead Guest Editor: Anat Elmann
Guest Editors: David Vauzour and Chin-Kun Wang

Copyright © 2018 Hindawi. All rights reserved.

This is a special issue published in "Oxidative Medicine and Cellular Longevity." All articles are open access articles distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Editorial Board

Dario Acuña-Castroviejo, Spain
Fabio Altieri, Italy
Fernanda Amicarelli, Italy
José P. Andrade, Portugal
Cristina Angeloni, Italy
Antonio Ayala, Spain
Elena Azzini, Italy
Peter Backx, Canada
Damian Bailey, UK
Grzegorz Bartosz, Poland
Sander Bekeschus, Germany
Ji C. Bihl, USA
Consuelo Borrás, Spain
Nady Braidy, Australia
Darrell W. Brann, USA
Ralf Braun, Germany
Laura Bravo, Spain
Vittorio Calabrese, Italy
Amadou Camara, USA
Gianluca Carnevale, Italy
Roberto Carnevale, Italy
Angel Catalá, Argentina
Giulio Ceolotto, Italy
Shao-Yu Chen, USA
Ferdinando Chiaradonna, Italy
Zhao Zhong Chong, USA
Alin Ciobica, Romania
Ana Cipak Gasparovic, Croatia
Giuseppe Cirillo, Italy
Maria R. Ciriolo, Italy
Massimo Collino, Italy
Manuela Corte-Real, Portugal
Mark Crabtree, UK
Manuela Curcio, Italy
Andreas Daiber, Germany
Felipe Dal Pizzol, Brazil
Francesca Danesi, Italy
Domenico D'Arca, Italy
Claudio De Lucia, Italy
Yolanda de Pablo, Sweden
Sonia de Pascual-Teresa, Spain
Cinzia Domenicotti, Italy
Joël R. Drevet, France
Grégory Durand, France
Javier Egea, Spain
Ersin Fadillioglu, Turkey
Ioannis G. Fatouros, Greece
Qingping Feng, Canada
Gianna Ferretti, Italy
Giuseppe Filomeni, Italy
Swaran J. S. Flora, India
Teresa I. Fortoul, Mexico
Jeferson L. Franco, Brazil
Rodrigo Franco, USA
Joaquin Gadea, Spain
José Luís García-Giménez, Spain
Gerardo García-Rivas, Mexico
Janusz Gebicki, Australia
Alexandros Georgakilas, Greece
Husam Ghanim, USA
Eloisa Gitto, Italy
Daniela Giustarini, Italy
Saeid Golbidi, Canada
Aldrin V. Gomes, USA
Tilman Grune, Germany
Nicoletta Guaragnella, Italy
Solomon Habtemariam, UK
Eva-Maria Hanschmann, Germany
Tim Hofer, Norway
John D. Horowitz, Australia
Silvana Hrelia, Italy
Stephan Immenschuh, Germany
Maria G. Isaguliants, Sweden
Luigi Iuliano, Italy
Vladimir Jakovljevic, Serbia
Marianna Jung, USA
Peeter Karihtala, Finland
Eric E. Kelley, USA
Kum Kum Khanna, Australia
Neelam Khaper, Canada
Thomas Kietzmann, Finland
Demetrios Kouretas, Greece
Andrey V. Kozlov, Austria
Jean-Claude Lavoie, Canada
Simon Lees, Canada
Ch. Horst Lillig, Germany
Paloma B. Liton, USA
Ana Lloret, Spain
Lorenzo Loffredo, Italy
Daniel Lopez-Malo, Spain
Antonello Lorenzini, Italy
Nageswara Madamanchi, USA
Kenneth Maiese, USA
Marco Malaguti, Italy
Tullia Maraldi, Italy
Reiko Matsui, USA
Juan C. Mayo, Spain
Steven McAnulty, USA
Antonio Desmond McCarthy, Argentina
Bruno Meloni, Australia
Pedro Mena, Italy
Víctor Manuel Mendoza-Núñez, Mexico
Maria U Moreno, Spain
Trevor A. Mori, Australia
Ryuichi Morishita, Japan
Fabiana Morroni, Italy
Luciana Mosca, Italy
Ange Mouithys-Mickalad, Belgium
Iordanis Mourouzis, Greece
Danina Muntean, Romania
Colin Murdoch, UK
Pablo Muriel, Mexico
Ryoji Nagai, Japan
David Nieman, USA
Hassan Obied, Australia
Julio J. Ochoa, Spain
Pál Pacher, USA
Pasquale Pagliaro, Italy
Valentina Pallottini, Italy
Rosalba Parenti, Italy
Vassilis Paschalis, Greece
Daniela Pellegrino, Italy
Ilaria Peluso, Italy
Claudia Penna, Italy
Serafina Perrone, Italy
Tiziana Persichini, Italy
Shazib Pervaiz, Singapore
Vincent Pialoux, France
Ada Popolo, Italy
José L. Quiles, Spain
Walid Rachidi, France
Zsolt Radak, Hungary

Namakkal S. Rajasekaran, USA
Kota V. Ramana, USA
Sid D. Ray, USA
Hamid Reza Rezvani, France
Alessandra Ricelli, Italy
Paola Rizzo, Italy
Francisco J. Romero, Spain
Joan Roselló-Catafau, Spain
Gabriele Saretzki, UK
Nadja Schroder, Brazil
Sebastiano Sciarretta, Italy
Honglian Shi, USA
Cinzia Signorini, Italy
Mithun Sinha, USA
Carla Tatone, Italy
Frank Thévenod, Germany
Shane Thomas, Australia
Carlo Tocchetti, Italy
Angela Trovato Salinaro, Jamaica
Paolo Tucci, Italy
Rosa Tundis, Italy
Giuseppe Valacchi, Italy
Jeannette Vasquez-Vivar, USA
Daniele Vergara, Italy
Victor M. Victor, Spain
László Virág, Hungary
Natalie Ward, Australia
Philip Wenzel, Germany
Anthony R. White, Australia
Georg T. Wondrak, USA
Michal Wozniak, Poland
Sho-ichi Yamagishi, Japan
Liang-Jun Yan, USA
Guillermo Zalba, Spain
Jacek Zielonka, USA
Mario Zoratti, Italy
H. P. Vasantha Rupasinghe, Canada

Contents

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 7402795, 2 pages
https://doi.org/10.1155/2018/7402795

Editorial

Polyphenols Targeting Brain Cells Longevity, Brain's Redox Status, and Neurodegenerative Diseases

Anat Elmann,[1] **Chin-Kun Wang**,[2] **and David Vauzour**[3]

[1]*Department of Food Quality and Safety, Agricultural Research Organization, Volcani Center, POB 15159, Rishon LeZion 7528809, Israel*
[2]*School of Nutrition, Chung Shan Medical University, Taichung City, Taiwan*
[3]*Norwich Medical School, Faculty of Medicine and Health Sciences, University of East Anglia, Norwich NR4 7UQ, UK*

Correspondence should be addressed to Anat Elmann; aelmann@volcani.agri.gov.il

Received 11 June 2018; Accepted 11 June 2018; Published 9 August 2018

Copyright © 2018 Anat Elmann et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

It is becoming widely accepted that polyphenols may act as strong dietary strategies to combat neurodegenerative diseases. The articles in this special issue include both basic scientific studies along with review articles focused on demonstrating and understanding the underlying mechanisms by which purified polyphenols and/or polyphenols containing plant extracts affect cultured brain cells, animal models of Parkinson's or Alzheimer's diseases, and depression.

Alzheimer's disease is a proteinopathy characterised by the accumulation of hyperphosphorylated Tau and β-amyloid. Autophagy is a physiological process by which aggregated proteins and damaged organelles are eliminated through lysosomal digestion. Autophagy deficiency has been demonstrated in Alzheimer's patients. In the research article "Benefit of Oleuropein Aglycone for Alzheimer's Disease by Promoting Autophagy" by J. G. Cordero et al., the authors demonstrated that oleuropein aglycone, present in high concentration in extra virgin olive oil, is capable of inducing autophagy in both *in vitro* and *in vivo* models, and that this leads to an improvement in cognitive impairment as well as in β-amyloid and Tau aggregation. The authors propose that supplementation of diet with extra virgin olive oil may have potential benefits for Alzheimer's disease patients through the induction of autophagy by oleuropein aglycone.

The aim of the paper entitled "Mangiferin and Morin Attenuate Oxidative Stress, Mitochondrial Dysfunction, and Neurocytotoxicity, Induced by Amyloid Beta Oligomers" by E. Alberdi et al. was to investigate the neuroprotective effects of the polyphenols morin and mangiferin against Aβ oligomers and their mechanisms of action. The authors found that these polyphenols mitigated the mitochondrial dysfunction by mechanisms that regulated mitochondrial calcium homeostasis, mitochondrial membrane potential, and release to cytosol of proapoptotic cytochrome c. Moreover, morin and mangiferin treatments restored the altered redox homeostasis of antioxidant enzymes in neurons treated with oligomeric Aβ. Consequently, these polyphenols reduced protein oxidation and reestablished bioenergetic failure in Aβ-treated neurons, contributing to the substantial reduction of neuronal death.

Chlorogenic acid is a plant polyphenol, which was found to be a major bioactive constituent of *Dendropanax morbiferus* leaves extract. The contribution by S.-Y. Park et al. entitled "Aqueous Extract of *Dendropanax morbiferus* Leaves Effectively Alleviated Neuroinflammation and Behavioral Impediments in MPTP-Induced Parkinson's Mouse Model" evaluated the underlying molecular mechanism of the antineuroinflammatory activity and the neuroprotective potential of *Dendropanax morbiferus* leaves and its bioactive compound chlorogenic acid in *in vitro* and *in vivo* experimental models of Parkinson's disease. The authors demonstrate that prophylactic treatment of *Dendropanax morbiferus* leaves improved the behavioral deficits, inhibited the microglial-mediated neuroinflammation, and protected dopaminergic neuronal loss by restoring tyrosine hydroxylase levels in brain tissues of the MPTP-

induced Parkinson's mouse model. Further, polyphenols that affect Alzheimer's and Parkinson's diseases were also summarised in the systematic review article entitled "Flavonoids as Therapeutic Agents in Alzheimer's and Parkinson's Diseases: A Systematic Review of Preclinical Evidences" by R. B. de Andrade Teles et al. The results of their survey showed that flavonoids, which are the major group of polyphenols, are promising candidates for drug development, but there is a lack of translational research and clinical evidences which might facilitate their development as drugs.

Polyphenols also received growing interest due to their potential benefits in treating psychiatric disorders. Their antioxidant and anti-inflammatory activities as well as their ability to modulate synaptic plasticity contribute to their mechanism of action. The paper by J. Wang et al. entitled "An Extract of *Artemisia dracunculus* L. Promotes Psychological Resilience in a Mouse Model of Depression" aims at investigating the potential therapeutic value of this botanical extract in a model of depression. *Artemisia dracunculus* L. (Russian tarragon) extract contains flavonoids, coumarins, and phenylpropanoid acids. Using a repeated social defeat stress (RSDS) model of depression, the authors demonstrate that oral administration of *Artemisia dracunculus* L. extract promotes resilience to RSDS-mediated depression-like phenotypes. The authors also show that the behavioral improvements are associated with attenuation of stress-mediated induction of inflammatory cytokines in the periphery and alteration of synaptic plasticity in the nucleus accumbens.

We hope that this special issue would stimulate scientists from the agricultural, plant sciences, and the medical communities to promote interdisciplinary studies that will lead to the development of polyphenols as drugs or food supplements for neurodegenerative diseases.

Acknowledgments

We would like to express our appreciation and to thank all the authors and the reviewers who took part in the success of this special issue.

Anat Elmann
Chin-Kun Wang
David Vauzour

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 2856063, 13 pages
https://doi.org/10.1155/2018/2856063

Research Article

Mangiferin and Morin Attenuate Oxidative Stress, Mitochondrial Dysfunction, and Neurocytotoxicity, Induced by Amyloid Beta Oligomers

Elena Alberdi,[1,2,3] **María Victoria Sánchez-Gómez**,[1,2,3] **Asier Ruiz**,[1,2,3] **Fabio Cavaliere**,[1,2,3] **Carolina Ortiz-Sanz**,[1,2,3] **Tania Quintela-López**,[1,2,3] **Estibaliz Capetillo-Zarate**,[1,2,3,4] **Santiago Solé-Domènech**,[5] **and Carlos Matute**[1,2,3]

[1]*Departamento de Neurociencias, Universidad del País Vasco (UPV/EHU), 48940 Leioa, Spain*
[2]*Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain*
[3]*Achucarro Basque Center for Neuroscience, 48940 Leioa, Spain*
[4]*Basque Foundation for Science (Ikerbasque), 48013 Bilbao, Spain*
[5]*Department of Biochemistry, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065, USA*

Correspondence should be addressed to Elena Alberdi; elena.alberdi@ehu.eus and Carlos Matute; carlos.matute@ehu.eus

Received 23 November 2017; Revised 28 March 2018; Accepted 12 April 2018; Published 14 June 2018

Academic Editor: Anat Elmann

Copyright © 2018 Elena Alberdi et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Amyloid beta- (Aβ-) mediated ROS overproduction disrupts intraneuronal redox balance and exacerbates mitochondrial dysfunction which leads to neuronal injury. Polyphenols have been investigated as therapeutic agents that promote neuroprotective effects in experimental models of brain injury and neurodegenerative diseases. The aim of this study was to identify the neuroprotective effects of morin and mangiferin against Aβ oligomers in cultured cortical neurons and organotypic slices as well as their mechanisms of action. Cell death caused by Aβ oligomers in neuronal cultures was decreased in the presence of micromolar concentrations of mangiferin or morin, which in turn attenuated oxidative stress. The neuroprotective effects of antioxidants against Aβ were associated with the reduction of Aβ-induced calcium load to mitochondria; mitochondrial membrane depolarization; and release of cytochrome c from mitochondria, a key trigger of apoptosis. Additionally, we observed that both polyphenols activated the endogenous enzymatic antioxidant system and restored oxidized protein levels. Finally, Aβ induced an impairment of energy homeostasis due to a decreased respiratory capacity that was mitigated by morin and mangiferin. Overall, the beneficial effects of polyphenols in preventing mitochondrial dysfunction and neuronal injury in AD cell models suggest that morin and mangiferin hold promise for the treatment of this neurological disorder.

1. Introduction

Alzheimer's disease (AD) is characterized clinically by progressive cognitive decline and neuropathologically by the accumulation of amyloid β (Aβ) peptides in extracellular plaques and hyperphosphorylated tau protein in intraneuronal tangles in the brain [1]. In addition to the well-known amyloid fibrils involved in plaque formation, Aβ spontaneously forms small, soluble oligomeric assemblies [2]. These forms are described as mainly responsible for cognitive impairment in the disease [3–5]. Aβ oligomers alter the homeostasis of mitochondrial physiology since they increase mitochondrial calcium levels [6], promote the mitochondrial permeability transition pore opening and release of mitochondrial proapoptotic factors, and consequently cause mitochondrial-dependent neuronal cell death [7].

In addition, oxidative stress is another key feature in the disease [8]. Numerous studies have reported the presence of elevated DNA [9], RNA [10], lipid [11], and protein oxidation [12] in brains of subjects with AD and mild cognitive impairment, suggesting that oxidative stress is an early event in AD. Previous attempts to quench ROS have demonstrated benefits to prevent mitochondrial and neuronal injury in AD patients as well as AD animal and cell models [13–16],

suggesting that ROS scavengers hold promise for the treatment of AD. Natural polyphenolic compounds exhibit their antioxidant effect by quenching free radical species and/or promoting endogenous antioxidant capacity. Thus, the antioxidant properties certainly may contribute to their neuroprotective effects. The naturally occurring polyphenols, mangiferin, and morin are known to be powerful antioxidants. Mangiferin is a xantone which is abundantly found in fruits and in the cortex of the stalk of *Mangifera indica* L. (mango) [17], whereas morin is present at relatively high concentrations in the branches of *Morus alba* L. (white mulberry) and red wine [18].

The neuroprotective capacity of these polyfenols has been characterized in *in vitro* and *in vivo* models of ischemic neuronal death involving NMDA receptor overactivation and involves attenuation of receptor-mediated calcium influx and oxidative stress as well as apoptosis [19, 20]. Moreover, in Alzheimer's disease animal models, mangiferin and morin have exhibited neuroprotective properties. In APP/PS1 animal model, mangiferin diminished the inflammatory processes, measured by microglia and astrocyte burdens [21]. In the triple transgenic Alzheimer's disease mouse models, morin was described as a novel inhibitor of GSK3β that can reduce tau pathology in vivo [22]. In addition, morin reverses neuropathological and cognitive impairments in APPswe/PS1dE9 mice by targeting multiple pathogenic mechanisms [23].

Here, we explored novel neuroprotective mechanisms of mangiferin and morin in Aβ oligomer-induced neuronal injury. We found that natural polyphenols mitigated the mitochondrial dysfunction by mechanisms that regulated mitochondrial calcium homeostasis, mitochondrial membrane potential, and release to cytosol of proapoptotic cytochrome c. Moreover, morin and mangiferin treatments restored the altered redox homeostasis of antioxidant enzymes in neurons treated with oligomeric Aβ. Consequently, natural polyphenols reduced protein oxidation and reestablished bioenergetic failure in Aβ-treated neurons, contributing to the substantial reduction of neuronal death.

2. Materials and Methods

2.1. Drugs and Culture Medium. Neurobasal medium, B27 supplement, foetal bovine serum, horse serum, and other culture reagents were from Gibco (Invitrogen, Barcelona, Spain). Receptor antagonists MK801 was obtained from Tocris (Cookson, Bristol, UK). Morin and mangiferin (Mng) were obtained by Sigma (Sigma, St. Louis, MO, USA).

2.2. Preparation of Amyloid β Peptides. Oligomeric amyloid β (Aβ1-42) was prepared as reported previously [24]. Briefly, Aβ1-42 (ABX, Radeberg, Germany) was initially dissolved in hexafluoroisopropanol (HFIP, Sigma, St. Louis, MO, USA) to a concentration of 1 mM. For the aggregation protocol, the peptide was resuspended in dry dimethylsulfoxide (5 mM; Sigma, St. Louis, MO, USA). Hams F-12 (PromoCell, LabClinics, Barcelona, Spain) was added to adjust the final peptide concentration to 100 μM to obtain oligomers (4°C for 24 h). Monomeric Aβ was dissolved in PBS to a concentration of 100 μM.

2.3. Cortical Cell Culture and Transfection Procedures. Cortical neurons were obtained from the cortical lobes of E18 Sprague-Dawley rat embryos according to previously described procedures [19]. Neurons were resuspended in B27 Neurobasal medium plus 10% FBS and then seeded onto poly-L-ornithine-coated 48-well plates or glass coverslips (12 mm in diameter) at 1.5×10^5 cells per well. For confocal single-cell imaging experiments, cells were plated onto glass-bottom μ-dishes (Ibidi GmbH, Germany). The medium was replaced by serum-free, B27-supplemented Neurobasal medium 24 hours later. The cultures were essentially free of astrocytes and microglia and were maintained at 37°C and 5% CO_2 as was previously described [25, 26].

For transfection of cells, 4×10^6 rat neurons were transfected in suspension before plating with 3 μg of cDNA using the Rat Neuron Nucleofector kit (Lonza, Switzerland) according to the manufacturer instructions and plated and maintained as described above. Cultures were used at 8–9 days *in vitro*.

2.4. Organotypic Slice Culture of Neocortex. Cultures were prepared from coronal cerebral sections (400 μm thick with a McIlwain tissue chopper) of brains from Sprague-Dawley rat pups (5–7 days old) using a modification of the defined method [27]. Two slices were plated on each Millicell CM culture inserts (Millipore Ibérica; Madrid, Spain); maintained in 75% HME 03 (Cell Concept, Berlin, Germany), 2 mM L-glutamine (Sigma; St Louis, MO, USA), 25% horse serum, and 25 mg/ml gentamycin (Sigma; St Louis, MO, USA) for 3 days at 37°C; and then shifted in Neurobasal medium supplemented with 0.5% B27 supplement. Experiments were performed at 7–10 days *in vitro*.

2.5. Ca^{2+} Imaging in the Cytosol. For [Ca^{2+}]i measurements, neurons were loaded with Fluo-4 AM (1 μM; Molecular Probes, Invitrogen, Barcelona, Spain) in Ca^{2+}- and Mg^{2+}-free HBSS containing 20 mM HEPES, pH 7.4, 10 mM glucose, 10 μM glycine, and 2 mM $CaCl_2$ (incubation buffer) for 30 min at 37°C followed by a 20 min wash to allow de-esterification. For data analysis, a homogeneous population of 15–25 cells was selected in the field of view, and neuronal somata were selected as ROIs. Background values were always subtracted, and data are expressed as $F/F_0 \pm$ SEM (%) in which F represents the fluorescence value for a given time point and F_0 represents the mean of the resting level fluorescence.

2.6. Mitochondrial Ca^{2+} Imaging. Neurons transfected with mitochondria-targeted 2mtD4cpv Ca^{2+} indicator [28] were transferred to an incubation buffer (see above) and imaged by a TCS SP8X confocal microscope (Leica, Germany). Cells were excited at 458 nm, and cfp and yfp emissions were acquired for FRET ratio quantification at an acquisition rate of 1 frame/15 s for 5 or 10 min depending on the experiment. For data analysis, a homogeneous population of 5–12 cells was selected in the field of view and neuronal somata were selected as ROIs. Background values were always subtracted,

and data are expressed as $R/R_0 \pm$ SEM (%), in which R represents the yfp/cpf fluorescence ratio for a given time point and R_0 represents the mean of the resting FRET ratio.

2.7. Cell Viability and Toxicity Assays. Cell toxicity assays were performed as described previously with modifications [6]. Cortical neurons at 8–10 days in culture were exposed for 24 hours to Aβ oligomers. Antagonists and inhibitors were added to the cultures 30 min before Aβ oligomers. Twenty-four hours after drug application, cellular damage was estimated by measuring the level of lactate dehydrogenase released (LDH; Cytotox 96®, Promega, Madison, WI) from damaged cells into the culture media. Data were normalized to the activity of LDH released from vehicle-treated cells (100%) and calculated as a percentage of the control. Results were expressed as the means ± SEM of at least three independent experiments performed in triplicates.

Cortical slice cultures were exposed to Aβ oligomers at 5 μM for 24 h. Antagonists were added to cultures 30 min before the Aβ preparation. Cell death in organotypic cultures was evaluated by using the cellular uptake of propidium iodide (PI). Slices were stained by adding 10 μM PI into the culture for 2 h at 37°C and washed with PBS by two times for 10 min. Slices were fixed with 4% PFA in PBS for 40 min at room temperature. Afterwards, the slices were excited with 510–560 nm light and the emitted fluorescence acquired at 610 nm using a rhodamine filter on an inverted fluorescence microscope (Cell Observer Z1, Zeiss). PI fluorescence images were captured with a Plan NeoFluar 2.5x objective (Zeiss), using an EM CCD camera (Hamamatsu, C9100–13), controlled by the Axio Vision program (Zeiss). Images were analyzed with the ImageJ analysis program (NIH, MD, USA), and PI uptake was expressed as the mean gray value per area total analyzed.

2.8. Immunocytochemistry in Cultured Neurons. Cells were preincubated with antioxidants (1 μM, 30 min), treated with Aβ oligomers (5 μM; 2 h), and fixed with 4% paraformaldehyde in PBS 10 min. Immunocytochemistry, using a polyclonal antibody to cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA), was performed as described previously [29]. Cells were washed twice in PBS for 5 min at RT and permeabilized in PBS containing 0.2% Triton X-100 (TX100) for 30 min, and nonspecific binding sites were blocked in 3% BSA in PBS–0.2% TX100 for 30 min. The primary antibody was diluted 1 : 100 in PBS–0.1% TX100 and 5% NGS and applied overnight at 4°C. Cells were labeled for 2 h at RT with fluorescein-conjugated goat anti-rabbit IgG. In all cases, cells were counterstained with Hoechst 33258 to simultaneously evaluate viable cells. Fluorescence intensity quantification of micrographs was measured using Image J software.

2.9. Detection of Protein Carbonyls by Oxyblot. The carbonyl content was determined by the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) leading to the formation of stable dinitrophenyl (DNP) hydrazone adducts, which can be detected by Western blot analysis using anti-DNP antibody. The DNPH derivatization was carried out on 12 μg of neuronal protein extracts for 15 min using Oxyblot Kit (Millipore-Chemicon) following the manufacturer's instruction. The derivatives were then resolved by a broad range SDS-PAGE and transferred to a nitrocellulose membrane (Biorad). After blocking in PBST containing 1% BSA at RT for 60 min, the membranes were washed with PBST and incubated overnight with anti-DNP (1 : 200) at 4°C. The membranes were washed with PBST and incubated with horseradish peroxidase- (HRP-) conjugated anti-rabbit IgG at RT for 45 min. The membranes were washed again with PBST, and the proteins were visualized with the ECL chemiluminescence kit (SuperSignal® West Dura, Thermo Fisher Scientific, Inc, Barcelona, Spain) according to the manufacturer's protocol. Quantification of protein carbonyl content on blots was performed using the automatic band detection and band volume analysis applications of ImageLab 4.1 software.

2.10. Measurement of Catalase and Superoxide Dismutase Activity. Neurons were collected by centrifugation, and the cell pellet was sonicated on ice in 70 μl of cold PBS, 1 mM EDTA. Cell lysate was centrifugated at 10,000 ×g for 15 min at 4°C, and the supernatant was assayed for catalase and SOD activity by colorimetric assay procedures following the manufacturer's instructions (OxySelect™ catalase and SOD activity assays kits; Cell Biolabs, Inc, San Diego, CA, USA).

2.11. Measurement of Intracellular Reactive Oxygen Species. Neurons were exposed to Aβ oligomers alone or with antioxidants as described. Cells were loaded with CM-H2DCFDA at 30 μM to assay the ROS levels. Calcein-AM (1 μM; Molecular Probes, Invitrogen, Barcelona, Spain) was used to quantify the number of cells within the reading field. Fluorescence was measured using a Synergy-HT fluorimeter (Bio-Tek Instruments Incl, Beverly, MA, USA; excitation at 485 nm, emission at 527 nm). All experiments ($n \geq 3$) were performed at least in quadruplicate and plotted as means ± SEM.

2.12. Analysis of Mitochondrial Membrane Potential. Neurons were exposed to Aβ oligomers alone or in the presence of antioxidants, and changes in mitochondrial membrane potential were monitored by the reduction of JC-1 (Molecular Probes, Invitrogen, Barcelona, Spain), according to the manufacturer protocol. Briefly, after drug treatment, cells were loaded with 3 μM JC-1 for 15 min at 37°C and were washed with HBSS without phenol red two times to eliminate the excess dye. In the cytosol, the monomeric form of this dye fluoresces green (excitation at 485 nm, emission at 527 nm), whereas within the mitochondrial matrix, highly concentrated JC-1 forms aggregates that fluoresce red (excitation at 485 nm, emission at 590 nm). Both JC-1 monomers and aggregates were detectable using a Synergy-HT fluorimeter (Bio-Tek Instruments Incl, Beverly, MA, USA), and the changes in mitochondrial potential were calculated as the red/green ratio in each condition. All experiments ($n \geq 3$) were performed at least in triplicate and plotted as mean ± SEM.

2.13. Measurement of Oxygen Consumption Rate. The oxygen consumption rate (OCR) was analyzed by an XF96 Extracellular Flux Analyzer and XF Cell Mito Stress Test kit

(Seahorse Bioscience, Agilent Technologies, Santa Clara, CA, USA) following manufacturer instructions. OCR is measured before and after the addition of inhibitors to derive several parameters of mitochondrial respiration. Initially, the baseline cellular OCR is measured, from which basal respiration can be derived by subtracting nonmitochondrial respiration. Next oligomycin, a complex V inhibitor, is added, and the resulting OCR is used to derive ATP-linked respiration (by subtracting the oligomycin rate from baseline cellular OCR) and proton leak respiration (by subtracting nonmitochondrial respiration from the oligomycin rate). Next carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP), a protonophore, is added to collapse the inner membrane gradient, allowing the ETC to function at its maximal rate, and maximal respiratory capacity is derived by subtracting nonmitochondrial respiration from the FCCP rate. Lastly, antimycin A and rotenone, inhibitors of complexes III and I, are added to shut down ETC function, revealing nonmitochondrial respiration. Neurons (3×10^4 per well) were seeded on a poly-L-ornithine-coated XF96 plate and cultured for 9 days according to protocol as above. One hour before starting experiments, cells were incubated in an XF Base medium (Seahorse Bioscience), containing 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. Cells were treated with either vehicle, Aβ oligomers (5 μM), morin (1 μM), or mangiferin (1 μM), alone and in combination. For the determination of basal respiration, ATP-linked OCR, and maximal respiration, three baseline recordings were made, followed by the sequential addition of oligomycin (2 μM), FCCP (1 μM), and rotenone/antimycin A (500 nM). Values were normalized to cell viability by calcein measurement per well after XF assay completion.

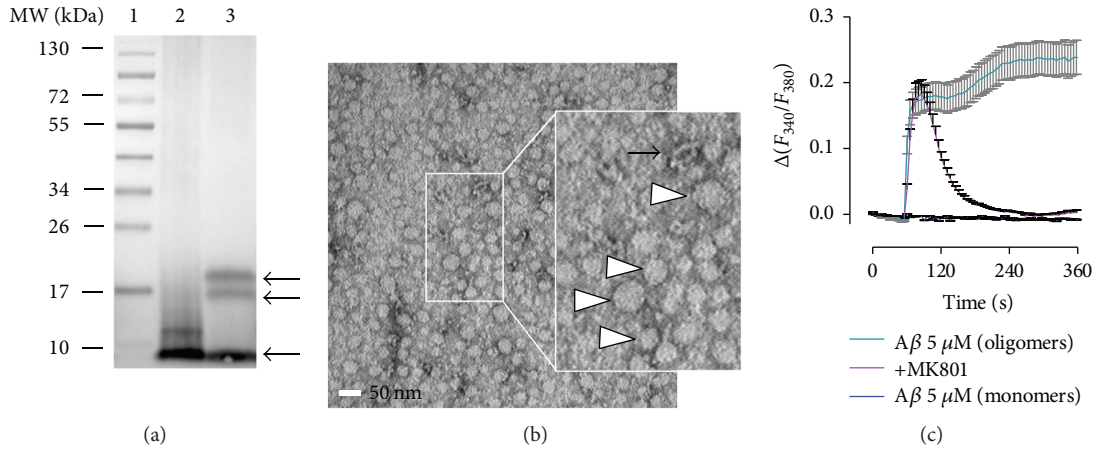
2.14. Data Analysis. All data are expressed as mean ± SEM(n), where n refers to the number of cultures assayed, each obtained from a different group of animals. In single live-cell imaging experiments, n refers to number of cells recorded from at least three independent cultures obtained from different groups of animals. For statistical analysis of the $[Ca^{2+}]_{cyt,}$ $[Ca^{2+}]_{mit}$, basal line-extracted area under curve was calculated from single-cell imaging time lapse curves. One-way analyses of variance followed by Bonferroni post hoc tests and one-tailed Student's t-tests were used unless otherwise indicated. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Characterization of Aβ Oligomers. Aβ1-42 peptide rapidly aggregates to form oligomers, protofibrils, and fibrils. To confirm the aggregation state of peptide preparation, a synthetic Aβ sample was subjected to Western blot processing and immunolabeling with 6E10 antibody, which displayed discrete bands corresponding to Aβ monomer, trimer, and tetramer sizes (Figure 1(a)). In addition, transmission electron microscopy (TEM) revealed that synthetic peptide preparation showed a nearly homogenous distribution of round particles which were identified as Aβ1-42 oligomers [30] (Figure 1(b)). Moreover, the differential activity of stable Aβ1-42 oligomers and monomers was found in the dysregulation of neuronal calcium homeostasis. Thus, oligomers, but not monomers, triggered a robust NMDA receptor-dependent calcium response in primary neuronal cultures (Figure 1(c)), as reported earlier [6]. Overall, synthetic Aβ preparation consistently yielded oligomeric forms that selectively activated NMDA receptors expressed in neurons.

3.2. Antioxidants Attenuate Aβ-Induced ROS Production and Cell Death in Neurons. Excessive production of oxygen-free radicals and other radical species plays an important role in neuronal pathology resulting from Aβ oligomer activity [6, 31]. We examined whether the pharmacological inhibition of ROS could reverse the Aβ-induced neuronal cell death. We found by real-time fluorescence measurements that Aβ oligomers induced ROS generation after 1 hour, which was blocked by compounds that prevent the assembly of NADPH oxidase, apocynin, and diphenyleneiodonium (DPI) (Figure 2(a); 141 ± 8.4%, 110 ± 12%, and 100 ± 4%, respectively; ANOVA, $P < 0.001$; Bonferroni post hoc Aβ versus control, $P < 0.001$; Aβ versus Aβ + apocynin, $P < 0.001$; Aβ versus Aβ + DPI, $P < 0.001$, $n = 6$ cultures). In addition, neurons treated with EUK-134, a mitoprotective antioxidant with superoxide dismutase and catalase activity, showed a robust attenuated oligomeric Aβ-induced signal (102 ± 7%; ANOVA, $P < 0.001$; Bonferroni post hoc Aβ versus Aβ + EUK-134, $P < 0.05$). Treatments of apocynin, DPI, and EUK-134 alone did not modify ROS levels as were compared to vehicle control (Figure 2(a)). Moreover, the DPI rescued cortical neurons from Aβ-induced toxicity (Figure 2(b), 7.5 ± 1% versus 15.7 ± 2.4%, respectively, $n = 4$ cultures; ANOVA, $P < 0.001$; Bonferroni post hoc Aβ versus Aβ + DPI, $P < 0.05$; Aβ versus control, $P < 0.001$). Overall, these results suggest that the reduction of oxidative stress by NADPH oxidase inhibitors is neuroprotective against Aβ-mediated neuronal cell death.

3.3. Antioxidant Polyphenols Reduce Aβ-Produced ROS and Neuronal Death in Cultured Neurons. Mng and morin, two natural antioxidant polyphenols, have a wide spectrum of antioxidant and antiapoptotic properties, which can reduce the mitochondrial dysfunction and neuronal damage associated with the sustained overactivation of glutamate receptor *in vivo* [19] and *in vitro* [20]. Here, we asked whether these two antioxidants might attenuate neuronal oxidative stress and cell death in Aβ oligomer-treated neurons *in vitro* and in cerebral cortex organotypic slices. First, ROS production by Aβ oligomers increased to reach a plateau after 1 hour, which was stayed at the same level for 1 more hour (132 ± 6% and 138 ± 10%, resp., $n = 4$ cultures; ANOVA, $P < 0.01$; Bonferroni post hoc Aβ versus control, $P < 0.05$). ROS levels were attenuated by morin 1 μM and Mng 1 μM (107 ± 3 and 110 ± 4%, resp.; ANOVA, $P < 0.01$; Bonferroni post hoc Aβ versus Aβ + morin, $P < 0.01$; Aβ versus Aβ + Mng $P < 0.05$), showing an effective antioxidant capacity against Aβ-generated oxidative stress (Figure 3(a)). Accordingly, both morin and Mng greatly reduced



(a) (b) (c)

Figure 1: Characterization of oligomeric forms of Aβ1-42 peptide. (a) Representative Western blot showing a broad range of molecular weight protein markers (lane 1), Aβ monomers (lane 2), and Aβ oligomers incubated at 4°C for 24 h (lane 3). Aβ was detected using the monoclonal 6E10 anti-Aβ antibody. Arrows show monomers and oligomers migrating at ~18 kDa. (b) Characterization of Aβ preparation (4°C for 24 h) by TEM showed mainly Aβ oligomers (white arrowheads) and very few protofibrils (black arrow). (c) Ca^{2+} recordings of cultured neurons showed a robust increase in the cytosolic Ca^{2+} concentration induced by Aβ oligomers, but not monomers, both at 5 μM, which is reduced by the NMDA antagonist MK801 (50 μM).

significantly the Aβ cytotoxicity (5 μM, 24 h) as was revealed by the DNA-binding dye propidium iodide staining (PI; Figure 3(b)) and by lactate dehydrogenase release assays (Figure 3(c); ANOVA, $P < 0.01$; Bonferroni post hoc Aβ versus Aβ + morin and Aβ versus Ab + Mng, $P < 0.05$). Treatments of morin and mangiferin alone did not modify ROS levels and cell viability observed in vehicle control samples (Figure 3(c)).

To further characterize the neuroprotective effects of these antioxidants, we used a more integral preparation, organotypic cultures from the cerebral cortex, to analyze Aβ oligomer-induced ROS and cell death. Slices were prepared to preserve neuronal structural integrity as shown by intense MAP2 staining in the somato-dendritic regions (Figure 3(e)). ROS generation by a short incubation of Aβ for 30 min (157 ± 19, $n = 4$ cultures; ANOVA, $P < 0.05$; Bonferroni post hoc Aβ versus vehicle, $P < 0.05$) were detected in cultured slices. Incubation of slices with morin and Mng reduced the Aβ-induced ROS to control levels (Figure 3(d); 100 ± 10% and 104 ± 2%, $n = 4$ cultures; ANOVA, $P < 0.05$; Bonferroni post hoc Aβ versus Aβ + morin and Aβ versus Aβ + Mng, $P < 0.05$). Moreover, exposure of rat cortical slices to Aβ oligomers for 24 h caused a significant increase of PI uptake by damaged/dead cells, which was strongly reduced by morin and Mng treatment (Figures 3(e) and 3(f); ANOVA $P < 0.001$, Bonferroni post hoc Aβ versus control $P < 0.001$, Aβ versus Aβ + morin, $P < 0.05$; Aβ versus Aβ + Mng, $P < 0.001$, $n = 4$ cultures). The localization of PI staining on Figure 3(e) suggests pyramidal neurons as the most vulnerable cells to Aβ oligomer treatment in cortical slices. In turn, the blockade of NMDA receptors with MK801, used as a positive control, was highly protective as well. Treatments of morin and Mng did not modify the PI uptake observed in vehicle-treatment slices (Figure 3(f)).

These results indicate that the two polyphenol antioxidants protect neurons from Aβ oligomer-induced oxidative stress and toxicity in dissociated cortical neurons as well as in cortical organotypic cultures.

3.4. Antioxidant Polyphenols Mitigate Aβ-Induced Mitochondrial Dysfunction in Neurons. Next, we asked whether these two antioxidants might attenuate mitochondrial damage in Aβ oligomer-treated neurons. First, we observed that neuronal $[Ca^{2+}]_{mit}$ increased to 2.35-fold the basal levels following application of Aβ 5 μM. The preincubation of morin 1 μM, but not of mangiferin, reduced significantly the $[Ca^{2+}]_{mit}$ overload to twofold (Figures 4(a) and 4(c); ANOVA, $P < 0.01$; Bonferroni post hoc Aβ versus Aβ + morin, $P > 0.05$,). In turn, mangiferin increased the $[Ca^{2+}]_{cyt}$ overload induced by Aβ from 2 to 2.3-fold of the basal levels (Figures 4(b) and 4(c); ANOVA, $P < 0.05$; Bonferroni post hoc Aβ versus Aβ + Mng, $P < 0.05$, $n = 4$ cultures). Moreover, morin and mangiferin prevented the loss of mitochondrial membrane potential (Figure 4(d)) and of cytochrome c release to cytosol (Figures 4(e) and 4(f)) induced by Aβ oligomers. The mitochondrial potential was monitored with the fluorescent probe JC-1 and quantified at 1 h of peptide poststimulus. Aβ oligomers induced a reduction in JC-1 fluorescence in neurons (74.9 ± 5%), which was restored by the coincubation of peptides with morin (84.8 ± 5%) and Mng (81.4 ± 3%). Treatments of morin and mangiferin did not modify the mitochondrial potential observed in vehicle-treated cells (Figure 4(d); ANOVA, $P < 0.01$; Bonferroni post hoc Aβ versus vehicle, $P < 0.001$; Aβ versus Aβ + morin, $P < 0.01$; Aβ versus Aβ + Mng, $P < 0.05$, $n = 4$ cultures).

Similarly, neurons were incubated with Aβ oligomers in the presence or absence of polyphenols as above, and then cytochrome c was visualized by immunocytochemistry. We found that Aβ-treated cells demonstrate a more diffuse and significantly decreased intensity of cytochrome c staining than vehicle-treated cells, suggesting that Aβ oligomers induce cytochrome c release from mitochondria

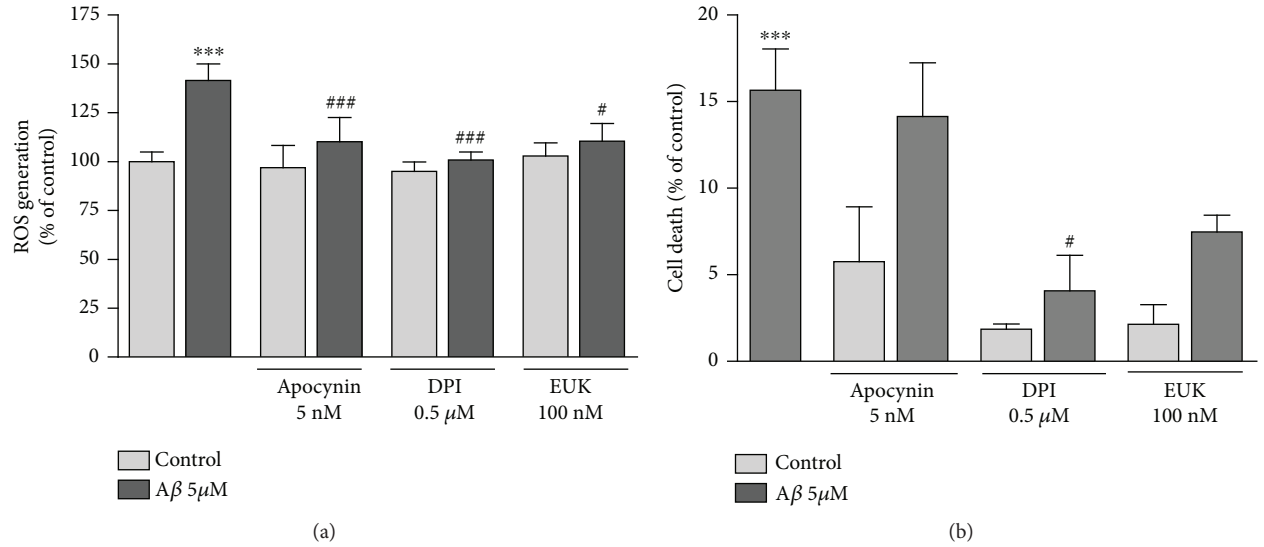


Figure 2: NADPH oxidase inhibitors are neuroprotective against Aβ oligomers. (a) Neurons were treated with $A\beta$ (5 μM) for 60 min, and ROS generation was monitored with CM-H2DCFDA (30 μM). Apocynin (5 nM), DPI (0.5 μM), and EUK-134 (100 nM) reduced Aβ-induced ROS generation in neurons. (b) The toxicity of Aβ oligomers (5 μM) in cultured cortical neurons as measured 24 h later with the LDH viability assay is prevented by the coapplication of Aβ oligomers with NOX inhibitor DPI. Data represent mean ± SEM of the CM-H2DCFDA/calcein and LDH signals in $n = 4$ cultures, expressed as a percentage of control untreated levels (100%). $^{***}P < 0.001$ compared with untreated cells; $^{###}P < 0.001$, $^{#}P < 0.05$ compared with Aβ-treated cells.

[32]. Treatments with Aβ together morin or Mng restored significantly the cytochrome c fluorescence intensity and the punctuate pattern throughout the cytoplasm suggestive of a mitochondria localization (Figures 4(e) and 4(f); ANOVA, $P < 0.01$; Bonferroni post hoc Aβ versus vehicle, $P < 0.01$; Aβ versus Aβ + morin, $P < 0.05$; Aβ versus Aβ + Mng, $P < 0.05$, $n = 3$ cultures).

Taken together, these results indicate that polyphenol antioxidants reduce $[Ca^{2+}]_{mit}$ overload, restore mitochondrial membrane potential, and inhibit cytochrome c release to the cytosol induced by Aβ in neurons.

3.5. Morin and Magiferin Restore the Aβ-Reduced Enzymatic Antioxidant Activities and Protein Carbonylation. The antioxidant activity of EUK134 against Aβ oligomer-induced oxidative stress observed in neurons (Figure 2), suggested that a reduced activity of antioxidant enzymes in these cells may underlie Aβ-caused neuronal damage.

Because of that, we verified the effect of Aβ oligomers on the antioxidant capability of neurons. SOD and catalase activities on neurons were significantly weaker after Aβ treatment for 2 h (Figure 5(a); 89 ± 1% and 86 ± 4%, respectively; ANOVA, $P < 0.001$; Bonferroni post hoc Aβ versus vehicle, $P < 0.05$ for both SOD and catalase analysis) than those of vehicle-treated cells. Morin and Mng restored significantly the decreased SOD to 107 ± 2% and 113 ± 5%, respectively (ANOVA, $P < 0.001$; Bonferroni post hoc Aβ versus Aβ + morin, $P < 0.05$; Aβ versus Aβ + Mng, $P < 0.01$), and the catalase activity was significantly restored to 114 ± 5% for Mng (Figure 5(a); ANOVA, $P < .001$; Bonferroni post hoc Aβ versus Aβ + Mng $P < 0.001$).

Among the various oxidative insults to proteins, carbonylation is the most common and severe because of its irreversible and irreparable nature. These derivatives are chemically stable and serve as markers of oxidative stress for most types of ROS. Accordingly, we performed immunoblot analysis using anti-DNP antibody to test protein carbonylation (Figure 5(b), see Methods). Neuronal samples (12 μg) after Aβ treatment (5 μM, 2 h) showed higher levels of total carbonyl content, and morin and Mng antioxidants totally prevented them (153 ± 24%, 89 ± 8%, and 91 ± 8%, respectively; ANOVA, $P < 0.01$; Bonferroni post hoc Aβ versus vehicle, Aβ versus Aβ + morin and Aβ versus Aβ + Mng, $P < 0.05$, $n = 5$ cultures). Morin and Mng alone increased slightly the total carbonyl content of neuronal proteins when levels were compared to them from vehicle-treated cells (100%, Figures 5(b) and 5(c))

Overall, these results showed that morin and Mng may protect neurons against oxidative stress caused by Aβ oligomers by mechanisms which restore the activity of antioxidant enzymes and consequently reduce protein oxidation of neurons.

3.6. Aβ-Induced Respiratory Inhibition Is Rescued by Morin and Mangiferin. The regulation of ATP production depends on calcium concentration and respiratory state of mitochondria [33]. Since Aβ oligomers increased the basal levels of $[Ca^{2+}]_{mit}$, we next analyzed the impact of Aβ on bioenergetics. O_2 consumption rate (OCR, see Methods) was measured in vehicle- and Aβ-treated cells before and after the addition of oligomycin to calculate basal respiration (BR) and ATP-linked respiration (ATP) of cells, respectively. Next, FCCP was added to calculate the maximal respiratory capacity (MUR). In all cases, neuronal OCR was inhibited after the addition of Aβ oligomers (Figures 6(a) and 6(b), $^{*}P < 0.05$ compared to control

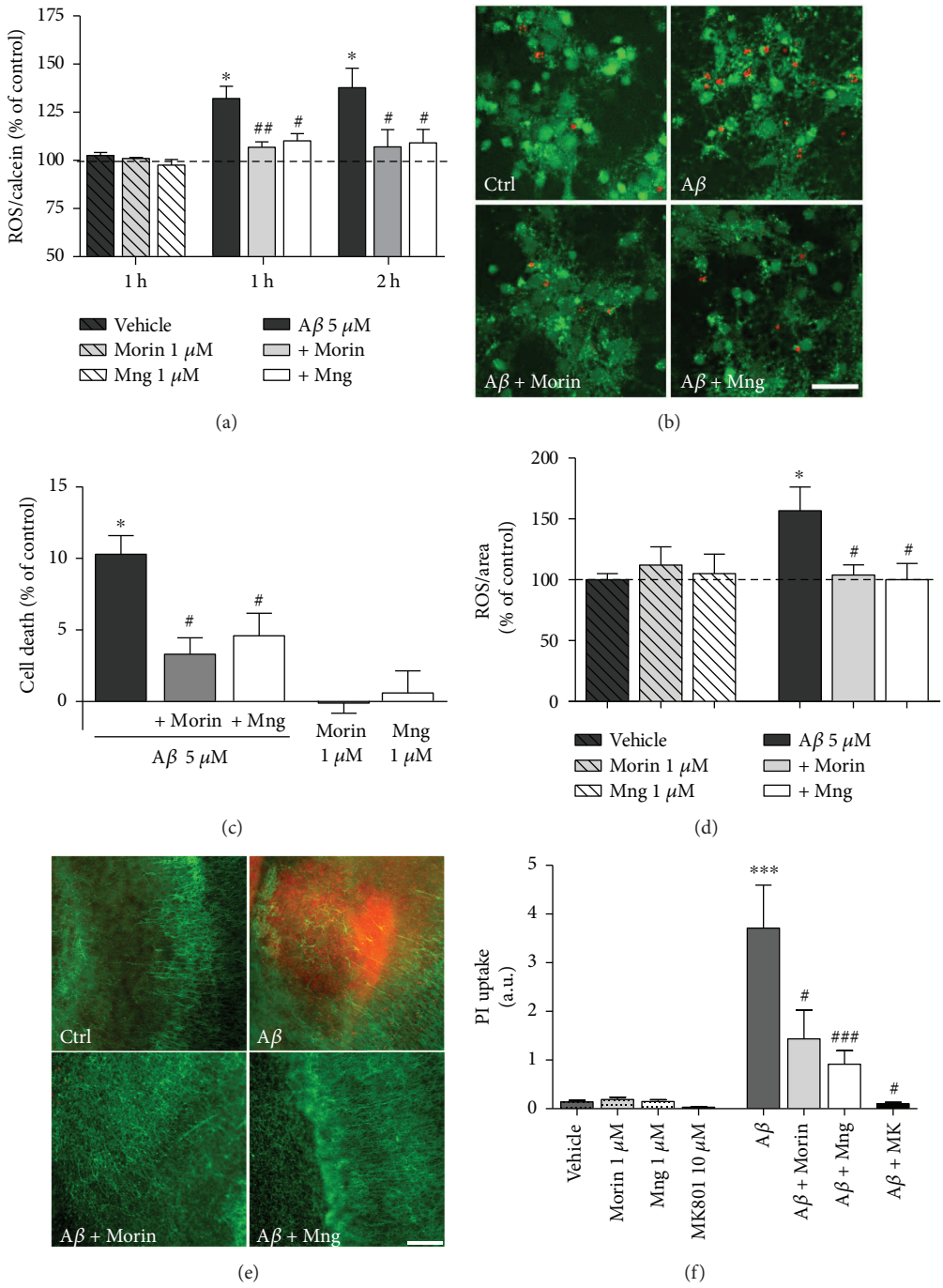


Figure 3: Mangiferin and morin prevent Aβ-induced ROS and neuronal death in dissociated neurons and in cortical organotypic cultures. Cultured neurons (A–C) or cortical organotypic slices (D–F) were incubated with Aβ 5 μM in the presence or absence of polyphenols (1 μM). (a) Mangiferin and morin reduce ROS generation after Aβ stimulus for 1 and 2 h. (b) Photographs show representative fields of calcein and PI fluorescence in cultured neurons displaying cell viability and death, respectively. Scale bar = 50 μM. (c) The toxicity of Aβ oligomers was measured 24 h later with the LDH viability assay. (d) ROS levels in slices, monitored with CM-H2DCFDA after Aβ treatment (30 min) coincubated with morin and mangiferin (1 μM), are shown. (E and F) Representative fields (green MAP-2 and red IP) of cortical organotypic slices and histogram showing Aβ (5 μM, 24 h) toxicity in cultures and protection when oligomers are applied in conjunction with morin (1 μM), mangiferin (1 μM), or MK801 (10 μM). Scale bar in (E) represents 100 μm. Bars represent the mean ± SEM of IP uptake from at least 4–7 cultures per graph, expressed as arbitrary units of mean grey intensity value. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle-treated cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with Aβ-treated cells.

cells) The coincubation of Aβ oligomers together morin and Mng antioxidants prevented significantly the inhibition of Aβ-induced OCR as is shown in Figure 6(c) (ANOVA, $P < 0.05$; Bonferroni post hoc $^{*}P < 0.05$, $^{**}P < 0.01$). The treatment of neurons with morin and Mng alone did not significantly change BR, ATP, and MUR (Figure 6(d)).

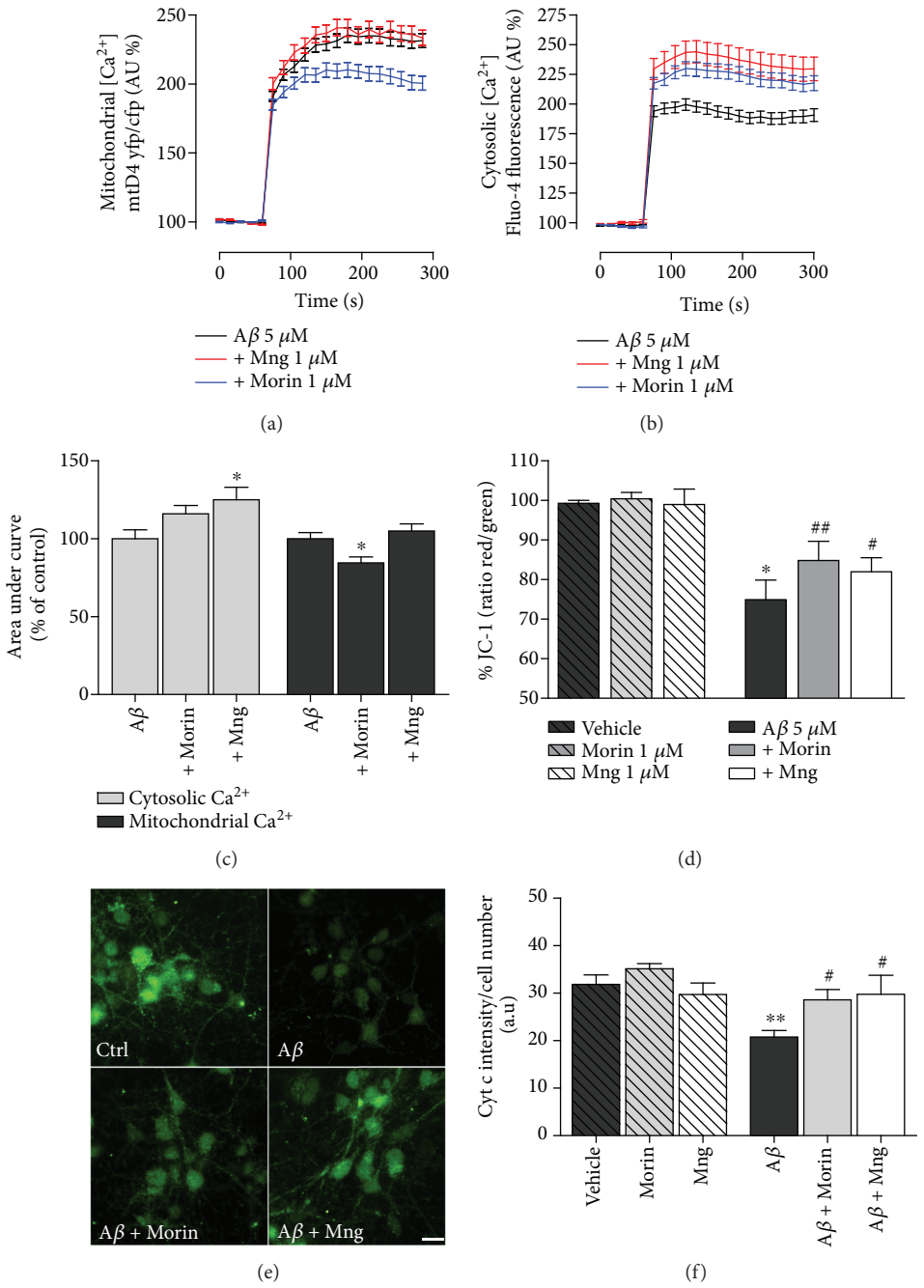


Figure 4: Mangiferin and morin attenuate Aβ-induced mitochondrial dyshomeostasis. (a) Aβ oligomers induce an accumulation of Ca^{2+} in mitochondria of neurons. Cells were transfected with the genetically encoded Ca^{2+} indicator 2mtD4cpv at DIV0, and $[Ca^{2+}]_{mit}$ was recorded after 8–10 days in culture. Morin, but not mangiferin, reduces significantly the mitochondrial Ca^{2+} overload. (b) Neurons were loaded with Fluo-4 fluorescence dye and cytosolic $[Ca^{2+}]$ changes measured upon the addition of Aβ oligomers. Mangiferin increases the cytosolic calcium levels observed with Aβ oligomers. (A, B) Traces represent the time course of normalized average of fluorescence ± SEM. (c) Graphs illustrate average ± SEM responses of 263 (A) and 187 (B) cells from at least 5 experiments. $^{*}P < 0.05$, compared to Aβ-treated cells. (d) Morin and mangiferin attenuate mitochondrial membrane depolarization during Aβ stimulation. Cells were treated with Aβ (5 μM, 1 h) after the addition of polyphenols, and the mitochondrial membrane potential was measured using JC-1 fluorescent dye 45 min after Aβ application. Data represent normalized mean ± SEM of the JC-1 red/green fluorescence ratio. $^{*}P < 0.05$ compared with vehicle-treated cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with Aβ-treated cells. (e) Micrographs illustrate cytochrome c immunolabeling in cultured neurons after Aβ treatment (5 μM, 2 h) alone or together with morin and Mng (1 μM). Graph bars represent the intensities of cytochrome c fluorescence normalized to cell number values (average ± SEM, $n = 3$ cultures) displayed as arbitrary units of fluorescence. $^{**}P < 0.01$ compared with vehicle-treated cells; $^{\#}P < 0.05$ compared with Aβ-treated cells.

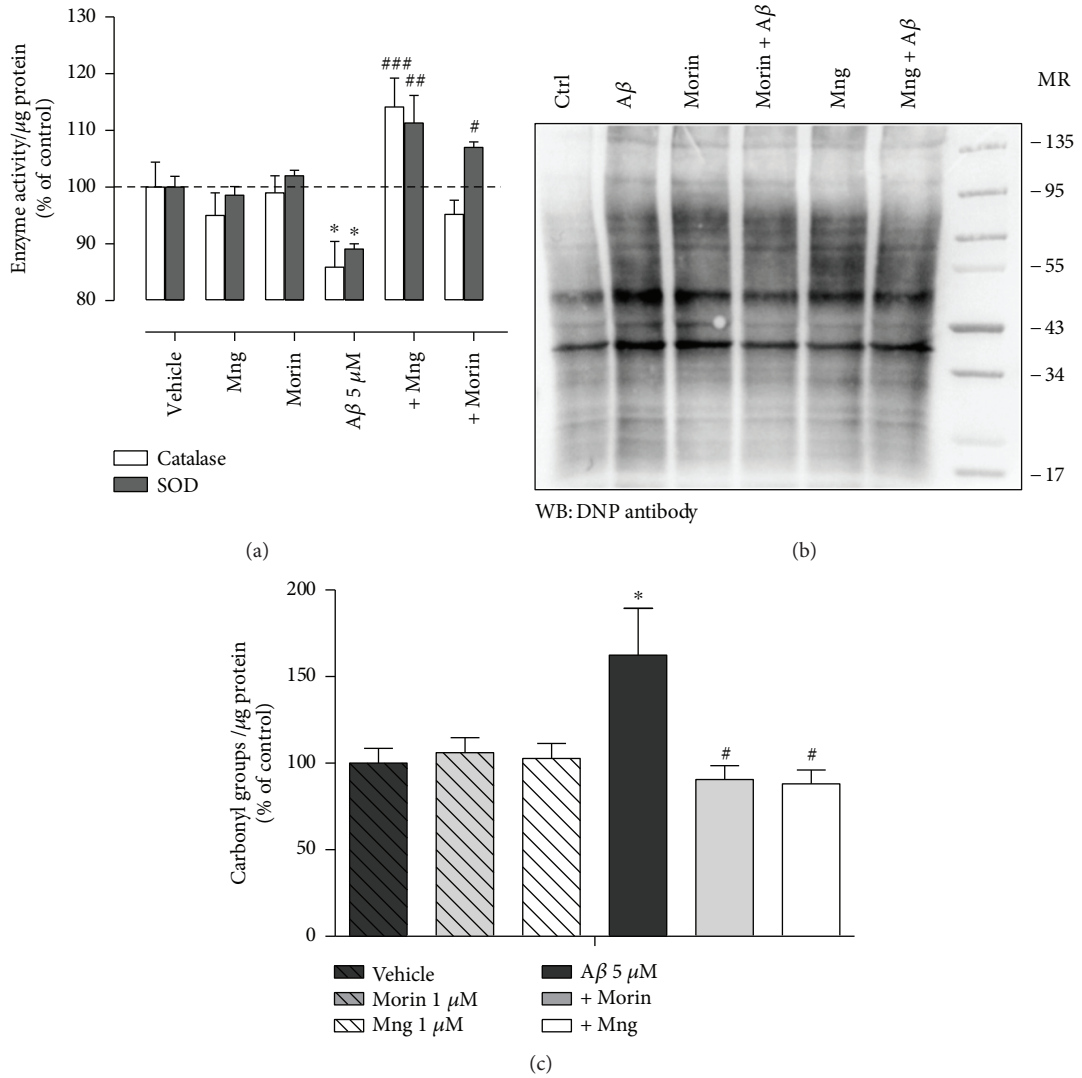


Figure 5: Effects of morin and mangiferin on antioxidant enzyme activities and protein oxidation in Aβ-treated neurons. (a) Neurons were pretreated with morin and mangiferin (1 μM, 30 min) and then exposed to 5 μM Aβ for 2 h. Cells were then harvested and lysed for the determination of levels of catalase and SOD. Data (average ± SEM) from 4 experiments were normalized to the enzyme activity of vehicle-treated cells. $^{*}P < 0.05$ compared with vehicle-treated cells, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with Aβ-treated cells. (b, c) Detection of protein carbonyls on neuronal protein extracts was performed by immunoblotting assays based on the use of antidinitrophenyl (DNP) antibody. The Western blot analysis of dinitrophenyl content of 12 μg of proteins illustrates the effects of Aβ (5 μg, 2 h) together with antioxidants in protein carbonyl levels. Graph bars represent the intensities of bands normalized to total protein load (average ± SEM, $n = 6$ cultures) displayed as a percentage of untreated cells. $^{*}P < 0.05$ compared with untreated cells; $^{\#}P < 0.05$ compared with Aβ-treated cells.

Overall, the Aβ-induced impairment of energy homeostasis due to a decreased respiratory capacity is blocked by antioxidants morin and mangiferin, a feature which, in turn, may reduce neuron demise.

4. Discussion

Under physiological conditions, low levels of ROS are necessary components of signal transduction cascades in a number of functions [34]. However, high levels of ROS, generated when their rate of production exceeds cellular scavenging capacity, are harmful in AD. In addition to oxidative stress, AD is characterized by the disruption of Ca^{2+} homeostasis, mitochondrial dysfunction, and increased sensitivity to apoptosis. All these alterations are involved in Aβ neurotoxicity.

Here, we provided evidence that two antioxidant polyphenols, mangiferin and morin, attenuate oxidative stress, mitochondrial dysfunction, and cell death caused by *Aβ* peptide oligomers in neurons in culture and in cortical organotypic slices. In addition, we showed that both antioxidants restore enzymatic antioxidant activities and consequently mitigate the protein oxidation levels and, importantly, attenuate the impairment of energy homeostasis after Aβ oligomer treatment. These natural polyphenolic compounds could be therefore promising therapeutic tools in AD.

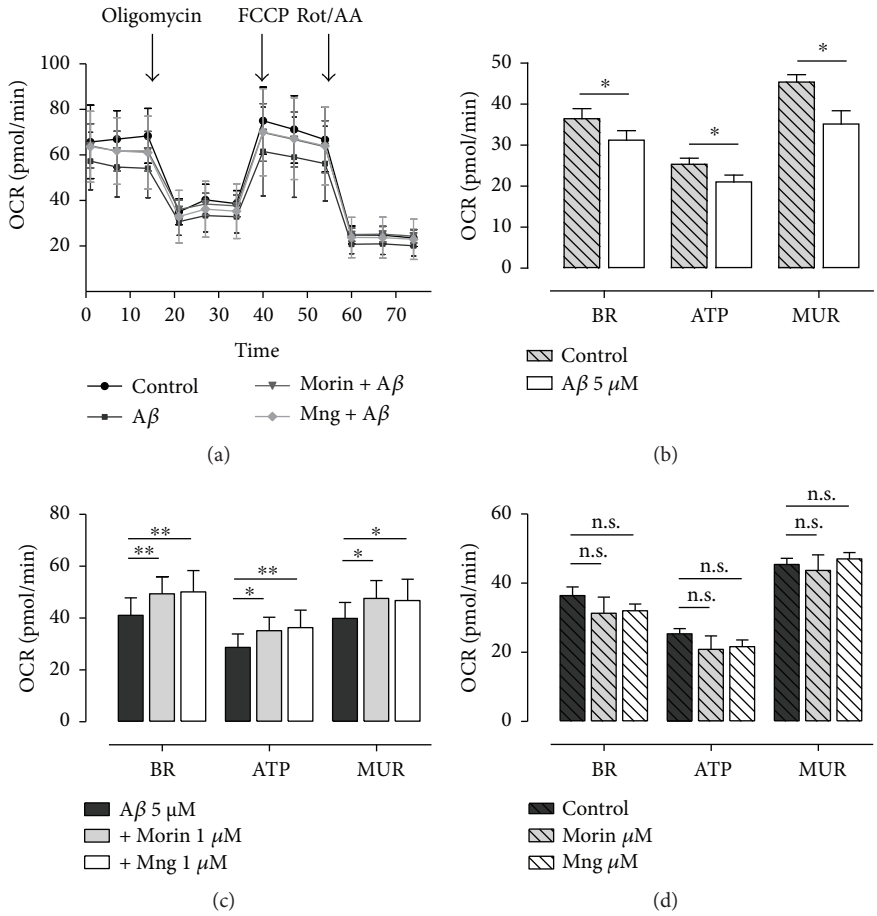


Figure 6: Aβ-induced respiratory inhibition is rescued by morin and mangiferin. (a) Primary neurons in the presence or absence of morin or mangiferin (1 μM) were exposed to vehicle or Aβ (5 μM, 1 h) in XF Base medium (Seahorse Bioscience) containing 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose, and mitochondrial oxygen consumption rate (OCR) was measured using a extracellular flux analyzer (Seahorse XFe96 analyzer). Mitochondrial function in neurons was determined through sequential addition of 2 μM oligomycin, 1 μM FCCP, and rotenone plus antimycin A (both 0.5 μM). This allowed the determination of basal oxygen consumption (BR), oxygen consumption linked to ATP synthesis (ATP) and mitochondrial uncoupled respiration (MUR) (see methods for OCR calculation). (b) Aβ oligomers caused mitochondrial dysfunction. Graph bars represent the average ± SEM of BR, ATP, and MUR OCR in vehicle-treated cells (37 ± 2.5, 25.3 ± 1.5 and 45.4 ± 1.8 pmol/min) versus Aβ-treated cells (31 ± 2, 21 ± 1.5 and 35.2 ± 3 pmol/min), respectively. $^{*}P < 0.05$, $n = 5$ cultures. (c) Morin and Mng rescued the Aβ-induced mitochondrial respiration inhibition. Graph bars represent the average ± SEM of BR, ATP, and MUR OCR in Aβ-treated, Aβ + morin-treated, and Aβ + Mng-treated cells. $^{*}P < 0.05$, $P < 0.01$ comparing antioxidant-treated cells with Aβ-treated cells. (d) Morin and Mng treatments did not change significantly the OCR in any parameters of mitochondrial respiration BR, ATP, and MUR (n.s.: not significant).

The main cellular sources of ROS are the mitochondria and NADPH oxidases which contribute to rapid ROS generation that in turn is dependent on cytosolic Ca^{2+} load in cortical neurons [35]. Furthermore, crosstalk between the mitochondria and NOX enzymes may represent a feedforward vicious circle of ROS production, which can be pharmacologically targeted under conditions of oxidative stress [36]. In our study, we showed that ROS quenching and neuronal cell death is reduced by NOX enzyme inhibitors, as DPI and apocinin, thus confirming the production of ROS through this enzymatic complex after Aβ stimulation. Previous findings have demonstrated a role for NOX-mediated ROS production in the cytotoxic effects of Aβ on cortical neurons since a specific peptide inhibitor of NOX, Gp91ds-tat, effectively abrogated Aβ-induced ROS production. In this context, a major polyphenolic component of green tea, EGCG [(−)-epigallocatechin-3-gallate], prevented ROS production by NOX and mitochondrial dysfunction demonstrating the therapeutic potential of dietary polyphenols on Aβ toxicity [37]. Furthermore, our results showed that the mitoprotective antioxidant EUK134 robustly reduced ROS levels in neurons treated with Aβ oligomeric, suggesting a causal relationship between mitochondrial ROS imbalance and Aβ-induced impairments. In a similar scenario, the naturally occurring polyphenols morin and mangiferin with oxygen radical scavenging activity [25] reduced significantly the increase in ROS and prevented neuronal demise, which suggests that both flavonoids, in a similar way as EGCG, are potent scavengers of ROS generated by both NOX enzyme activity and mitochondria.

In addition, we speculated that our findings may be related to the capacity of morin and mangiferin to protect

the mitochondria from damage that was caused by Aβ oligomers and, in this manner, to break a vicious circle between the ROS sources, described in pathophysiological processes [36]. Indeed, a sustained overactivation of glutamate receptors by Aβ oligomers markedly produces mitochondrial Ca^{2+} overload causing the depolarization of the mitochondrial membrane, ROS generation, and apoptotic neuronal death [6, 38, 39]. The results of the current study show that polyphenols attenuate mitochondrial calcium uptake and depolarization produced by Aβ oligomers. These findings may be related to the capacity of morin to reduce calcium entry via the mitochondria without changes on the Ca^{2+} permeability of glutamate receptor, as was previously showed after excitotoxic insults in cultured neurons with NMDA and AMPA agonists [19, 25]. Previous data have demonstrated that polyphenols may facilitate the balance of cellular Ca^{2+} by modulation Ca^{2+} channel and pump activities on pathophysiological conditions. Specifically, mangiferin prevented methylmercury-mediated Ca^{2+} influx in a human neuroblastoma cell line, showing a neuroprotective potential activity [40]. According to these findings, Roselle polyphenols elicited a negative ionotropic response of agonists for L-type Ca^{2+} channels by possibly modulating calcium entry in cardiac cells [41]. However, in cancer cells, resveratrol and picetannol strongly enhanced the mitochondrial Ca^{2+} uptake by mechanisms involving SERCA activity reduction [42]. Therefore, polyphenol effects on controlling Ca^{2+} mechanisms that are associated with mitochondrial injury may prevent Aβ-induced neuronal demise.

Antiapoptotic activities for antioxidants morin and mangiferin were previously described in neuronal and oligodendroglial excitotoxic cell deaths [19, 25]. Both antioxidants showed the capacity to reduce caspase-3 activation, a cell death effector related to cytosolic cytochrome c. Here, we showed that both antioxidants reduced cytochrome c release from mitochondria membranes to cytosol. The release of cytochrome c from the mitochondria is a key initiative step in the apoptotic process, although the mechanisms regulating the permeabilization of the outer mitochondrial membrane and the release of intermembrane space proteins remain controversial [43]. Cytochrome c is normally bound to the inner mitochondrial membrane by an association with the anionic phospholipid cardiolipin. It seems that the dissociation of cytochrome c from peroxidated cardiolipin might be a critical first step for cytochrome c release into the cytosol and activation of the caspase cascade [44, 45]. A plausible explanation for the antiapoptotic effects reported here for morin and mangiferin is the ability to maintain the homeostasis of the enzymatic antioxidant system after Aβ-oligomeric neuronal injury (Figure 5(a)), a feature that was also described in excitotoxic events [20]. Additionally, mangiferin is capable of chelating iron, avoiding its participation in the Fenton reaction, and preventing lipid peroxidation induced by iron more efficiently than that induced by peroxide [46]. In contrast, morin can inhibit xanthine oxidase, reducing the production of ROS [18] and radicals derived from nitrogen [47]. Thus, antioxidant properties and restoration of enzymatic antioxidant activities by morin and mangiferin might explain their antiapoptotic effects.

Among a wide range of ROS-derived modifications, biomolecule carbonylation is known to be a major hallmark of oxidative stress [48]. Carbonyl stress, characterized by the accumulation of reactive carbonylated species and their reactivity toward nucleophilic substrates, results in biomolecule malfunctions and increased toxicity and can finally lead to apoptotic cell death [49]. Further evidence to support the role of protein carbonylation in the pathogenesis of human disorders has provided a strong link between disease onset/progression and oxidative stress. In our study, we found that the acute treatment of neurons with Aβ oligomers increased levels of protein carbonyls of cultured neurons and that morin and mangiferin prevented them. Furthermore, protein carbonyls were observed in the hippocampi of the triple transgenic Alzheimer's disease mice as compared to the nontransgenic controls [50]. In addition, the majority of carbonylated proteins identified by redox proteomics were found in CSF at early stages of AD. Thus, oxidatively modified CSF proteins are already present in mild cognitive impairment compared with controls and remain oxidized in late AD, thus suggesting that the dysfunction of selected proteins initiate many years before severe dementia occurs [51]. Therefore, antioxidants such morin and mangiferin, which reduced carbonyl stress in the Alzheimer's disease, hold promise for early treatment of the disease.

Another key finding of this study was that Aβ oligomers promote a functional energetic decline affecting the mitochondrial basal respiration; oligomycin-sensitive respiration or ATP turnover; and the maximal respiration in the presence of FCCP, resulting in neuronal energy deficits. In this scenario, morin and mangiferin restored the cell respiratory control, the predominant physiological function of mitochondria. According to these findings, polyphenolic compounds EGCG and resveratrol reversed severe impairments of mitochondrial bioenergetics of hippocampal progenitor cells in Ts65Dn mice, a severe trisomic Down Syndrome mouse model, promoting neuronal progenitors cell proliferation [52]. Overall, the neuroprotective role of morin and mangiferin may derive essentially from its ability to reactivate mitochondrial bioenergetics.

5. Conclusion

In summary, we described in this study that morin and mangiferin strongly protect against Aβ-induced mitochondrial dysfunction and neuronal cell death. Specifically, we provided evidence showing clearly that these two natural antioxidants preserve cell respiration, promote detoxification of reactive oxygen species, protect from some forms of apoptosis, and regulate mitochondrial matrix calcium in neurons exposed to Aβ. Together, these results strongly suggest that morin and mangiferin are promising therapeutic tools to restore mitochondrial functions and redox homeostasis in AD.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Saioa Marcos, Ainara Martínez, and Laura Escobar for technical assistance. This study was supported by CIBERNED and by grants from Ministerio de Economía y Competitividad (SAF2016-75292-R); Gobierno Vasco; Ikerbasque, Basque Foundation for Science; and UPV/EHU. Carolina Ortiz-Sanz held a fellowship from UPV/EHU and Tania Quintela-López from Gobierno Vasco. Santiago Solé-Domènech was supported by the Swedish Research Council (VR) International postdoctoral grant DNR (637-2013-503).

References

[1] A. Serrano-Pozo, M. P. Frosch, E. Masliah, and B. T. Hyman, "Neuropathological alterations in Alzheimer disease," *Cold Spring Harbor Perspectives in Medicine*, vol. 1, no. 1, article a006189, 2011.

[2] M. P. Murphy and H. LeVine, "Alzheimer's disease and the amyloid-β peptide," *Journal of Alzheimer's Disease*, vol. 19, no. 1, pp. 311–323, 2010.

[3] L. F. Lue, Y. M. Kuo, A. E. Roher et al., "Soluble amyloid β peptide concentration as a predictor of synaptic change in Alzheimer's disease," *American Journal of Pathology*, vol. 155, no. 3, pp. 853–862, 1999.

[4] C. A. McLean, R. A. Cherny, F. W. Fraser et al., "Soluble pool of Aβ amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease," *Annals of Neurology*, vol. 46, no. 6, pp. 860–866, 1999.

[5] J. Näslund, V. Haroutunian, R. Mohs et al., "Correlation between elevated levels of amyloid β-peptide in the brain and cognitive decline," *JAMA*, vol. 283, no. 12, pp. 1571–1577, 2000.

[6] E. Alberdi, M. V. Sánchez-Gómez, F. Cavaliere et al., "Amyloid β oligomers induce Ca^{2+} dysregulation and neuronal death through activation of ionotropic glutamate receptors," *Cell Calcium*, vol. 47, no. 3, pp. 264–272, 2010.

[7] H. Du, L. Guo, F. Fang et al., "Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease," *Nature Medicine*, vol. 14, no. 10, pp. 1097–1105, 2008.

[8] A. Nunomura, K. Honda, A. Takeda et al., "Oxidative damage to RNA in neurodegenerative diseases," *Journal of Biomedicine and Biotechnology*, vol. 2006, Article ID 82323, 6 pages, 2006.

[9] F. Coppedè and L. Migliore, "DNA damage and repair in Alzheimer's disease," *Current Alzheimer Research*, vol. 6, no. 1, pp. 36–47, 2009.

[10] M. A. Lovell and W. R. Markesbery, "Oxidatively modified RNA in mild cognitive impairment," *Neurobiology of Disease*, vol. 29, no. 2, pp. 169–175, 2008.

[11] W. R. Markesbery, R. J. Kryscio, M. A. Lovell, and J. D. Morrow, "Lipid peroxidation is an early event in the brain in amnestic mild cognitive impairment," *Annals of Neurology*, vol. 58, no. 5, pp. 730–735, 2005.

[12] R. Sultana, M. Perluigi, and D. A. Butterfield, "Oxidatively modified proteins in Alzheimer's disease (AD), mild cognitive impairment and animal models of AD: role of Abeta in pathogenesis," *Acta Neuropathologica*, vol. 118, no. 1, pp. 131–150, 2009.

[13] M. J. McManus, M. P. Murphy, and J. L. Franklin, "The mitochondria-targeted antioxidant MitoQ prevents loss of spatial memory retention and early neuropathology in a transgenic mouse model of Alzheimer's disease," *Journal of Neuroscience*, vol. 31, no. 44, pp. 15703–15715, 2011.

[14] M. Calkins, M. Manczak, and P. Reddy, "Mitochondria-targeted antioxidant SS31 prevents amyloid Beta-induced mitochondrial abnormalities and synaptic degeneration in Alzheimer's disease," *Pharmaceuticals*, vol. 5, no. 10, pp. 1103–1119, 2012.

[15] M. C. Polidori and G. Nelles, "Antioxidant clinical trials in mild cognitive impairment and Alzheimer's disease—challenges and perspectives," *Current Pharmaceutical Design*, vol. 20, no. 18, pp. 3083–3092, 2014.

[16] C. Behl and B. Moosmann, "Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach," *Free Radical Biology and Medicine*, vol. 33, no. 2, pp. 182–191, 2002.

[17] A. J. Núñez Sellés, H. T. Vélez Castro, J. Agüero-Agüero et al., "Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (Mangifera indica L.) stem bark aqueous decoction used in Cuba as a nutritional supplement," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 4, pp. 762–766, 2002.

[18] Z. Yu, W. P. Fong, and C. H. K. Cheng, "The dual actions of morin (3,5,7,2′,4′-pentahydroxyflavone) as a hypouricemic agent: uricosuric effect and xanthine oxidase inhibitory activity," *Journal of Pharmacology and Experimental Therapeutics*, vol. 316, no. 1, pp. 169–175, 2005.

[19] M. Gottlieb, R. Leal-Campanario, M. R. Campos-Esparza et al., "Neuroprotection by two polyphenols following excitotoxicity and experimental ischemia," *Neurobiology of Disease*, vol. 23, no. 2, pp. 374–386, 2006.

[20] M. R. Campos-Esparza, M. V. Sánchez-Gómez, and C. Matute, "Molecular mechanisms of neuroprotection by two natural antioxidant polyphenols," *Cell Calcium*, vol. 45, no. 4, pp. 358–368, 2009.

[21] C. Infante-Garcia, J. J. Ramos-Rodriguez, I. Delgado-Olmos et al., "long-term Mangiferin extract treatment improves central pathology and cognitive deficits in APP/PS1 mice," *Molecular Neurobiology*, vol. 54, no. 6, pp. 4696–4704, 2017.

[22] E. J. Gong, H. R. Park, M. E. Kim et al., "Morin attenuates tau hyperphosphorylation by inhibiting GSK3β," *Neurobiology of Disease*, vol. 44, no. 2, pp. 223–230, 2011.

[23] Y. Du, J. Qu, W. Zhang et al., "Morin reverses neuropathological and cognitive impairments in APPswe/PS1dE9 mice by targeting multiple pathogenic mechanisms," *Neuropharmacology*, vol. 108, pp. 1–13, 2016.

[24] K. N. Dahlgren, A. M. Manelli, W. B. Stine Jr, L. K. Baker, G. A. Krafft, and M. J. LaDu, "Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability," *Journal of Biological Chemistry*, vol. 277, no. 35, pp. 32046–32053, 2002.

[25] G. Ibarretxe, M. V. Sánchez-Gómez, M. R. Campos-Esparza, E. Alberdi, and C. Matute, "Differential oxidative stress in oligodendrocytes and neurons after excitotoxic insults and protection by natural polyphenols," *Glia*, vol. 53, no. 2, pp. 201–211, 2006.

[26] G. J. Brewer, J. R. Torricelli, E. K. Evege, and P. J. Price, "Optimized survival of hippocampal neurons in B27-supplemented neurobasal™, a new serum-free medium combination," *Journal of Neuroscience Research*, vol. 35, no. 5, pp. 567–576, 1993.

[27] F. Cavaliere, K. Dinkel, and K. Reymann, "Microglia response and P2 receptor participation in oxygen/glucose deprivation-induced cortical damage," *Neuroscience*, vol. 136, no. 3, pp. 615–623, 2005.

[28] A. Ruiz, E. Alberdi, and C. Matute, "CGP37157, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger, protects neurons from excitotoxicity by blocking voltage-gated Ca^{2+} channels," *Cell Death & Disease*, vol. 5, no. 4, article e1156, 2014.

[29] M. V. Sánchez-Gómez, E. Alberdi, G. Ibarretxe, I. Torre, and C. Matute, "Caspase-dependent and caspase-independent oligodendrocyte death mediated by AMPA and kainate receptors," *Journal of Neuroscience*, vol. 23, no. 29, pp. 9519–9528, 2003.

[30] M. Ahmed, J. Davis, D. Aucoin et al., "Structural conversion of neurotoxic amyloid-β1–42 oligomers to fibrils," *Nature Structural & Molecular Biology*, vol. 17, no. 5, pp. 561–567, 2010.

[31] F. G. De Felice, P. T. Velasco, M. P. Lambert et al., "Aβ oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine," *Journal of Biological Chemistry*, vol. 282, no. 15, pp. 11590–11601, 2007.

[32] X. Wang, S. Zhu, Z. Pei et al., "Inhibitors of cytochrome *c* release with therapeutic potential for Huntington's disease," *Journal of Neuroscience*, vol. 28, no. 38, pp. 9473–9485, 2008.

[33] B. D. Fink, F. Bai, L. Yu, and W. I. Sivitz, "Regulation of ATP production: dependence on calcium concentration and respiratory state," *American Journal of Physiology-Cell Physiology*, vol. 313, no. 2, pp. C146–C153, 2017.

[34] M. Schieber and N. S. Chandel, "ROS function in redox signaling and oxidative stress," *Current Biology*, vol. 24, no. 10, pp. R453–R462, 2014.

[35] A. Clausen, T. McClanahan, S. G. Ji, and J. H. Weiss, "Mechanisms of rapid reactive oxygen species generation in response to cytosolic Ca^{2+} or Zn^{2+} loads in cortical neurons," *PLoS One*, vol. 8, no. 12, article e83347, 2013.

[36] S. Dikalov, "Cross talk between mitochondria and NADPH oxidases," *Free Radical Biology and Medicine*, vol. 51, no. 7, pp. 1289–1301, 2011.

[37] Y. He, J. Cui, J. C.-M. Lee et al., "Prolonged exposure of cortical neurons to oligomeric amyloid-β impairs NMDA receptor function via NADPH oxidase-mediated ROS production: protective effect of green tea (−)-Epigallocatechin-3-Gallate," *ASN Neuro*, vol. 3, no. 1, article AN20100025, 2010.

[38] L. Texidó, M. Martín-Satué, E. Alberdi, C. Solsona, and C. Matute, "Amyloid β peptide oligomers directly activate NMDA receptors," *Cell Calcium*, vol. 49, no. 3, pp. 184–190, 2011.

[39] S. T. Ferreira, M. V. Lourenco, M. M. Oliveira, and F. G. De Felice, "Soluble amyloid-β oligomers as synaptotoxins leading to cognitive impairment in Alzheimer's disease," *Frontiers in Cellular Neuroscience*, vol. 9, article 191, 2015.

[40] S. Das, B. Nageshwar Rao, and B. S. Satish Rao, "Mangiferin attenuates methylmercury induced cytotoxicity against IMR-32, human neuroblastoma cells by the inhibition of oxidative stress and free radical scavenging potential," *Chemico-Biological Interactions*, vol. 193, no. 2, pp. 129–140, 2011.

[41] Y.-C. Lim, S. B. Budin, F. Othman, J. Latip, and S. Zainalabidin, "Roselle polyphenols exert potent negative inotropic effects via modulation of intracellular calcium regulatory channels in isolated rat heart," *Cardiovascular Toxicology*, vol. 17, no. 3, pp. 251–259, 2017.

[42] C. T. Madreiter-Sokolowski, B. Gottschalk, W. Parichatikanond et al., "Resveratrol specifically kills Cancer cells by a devastating increase in the Ca^{2+}coupling between the greatly tethered endoplasmic reticulum and mitochondria," *Cellular Physiology and Biochemistry*, vol. 39, no. 4, pp. 1404–1420, 2016.

[43] V. Gogvadze, S. Orrenius, and B. Zhivotovsky, "Multiple pathways of cytochrome c release from mitochondria in apoptosis," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1757, no. 5-6, pp. 639–647, 2006.

[44] G. Petrosillo, F. M. Ruggiero, M. Pistolese, and G. Paradies, "Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation: possible role in the apoptosis," *FEBS Letters*, vol. 509, no. 3, pp. 435–438, 2001.

[45] V. E. Kagan, V. A. Tyurin, J. Jiang et al., "Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors," *Nature Chemical Biology*, vol. 1, no. 4, pp. 223–232, 2005.

[46] G. Pardo Andreu, R. Delgado, J. Velho, N. M. Inada, C. Curti, and A. E. Vercesi, "Mangifera indica L. extract (Vimang) inhibits Fe^{2+}-citrate-induced lipoperoxidation in isolated rat liver mitochondria," *Pharmacological Research*, vol. 51, no. 5, pp. 427–435, 2005.

[47] L. H. Zeng, D. S. Rootman, A. Burnstein, J. Wu, and T. W. Wu, "Morin hydrate: a better protector than purpurogallin of corneal endothelial cell damage induced by xanthine oxidase and SIN-1," *Current Eye Research*, vol. 17, no. 2, pp. 149–152, 1998.

[48] I. Dalle-Donne, R. Rossi, R. Colombo, D. Giustarini, and A. Milzani, "Biomarkers of oxidative damage in human disease," *Clinical Chemistry*, vol. 52, no. 4, pp. 601–623, 2006.

[49] J. M. Curtis, W. S. Hahn, E. K. Long, J. S. Burrill, E. A. Arriaga, and D. A. Bernlohr, "Protein carbonylation and metabolic control systems," *Trends in Endocrinology & Metabolism*, vol. 23, no. 8, pp. 399–406, 2012.

[50] L. Shen, C. Chen, A. Yang, Y. Chen, Q. Liu, and J. Ni, "Redox proteomics identification of specifically carbonylated proteins in the hippocampi of triple transgenic Alzheimer's disease mice at Its earliest pathological stage," *Journal of Proteomics*, vol. 123, pp. 101–113, 2015.

[51] F. Di Domenico, G. Pupo, E. Giraldo et al., "Oxidative signature of cerebrospinal fluid from mild cognitive impairment and Alzheimer disease patients," *Free Radical Biology and Medicine*, vol. 91, pp. 1–9, 2016.

[52] D. Valenti, L. De Bari, D. De Rasmo et al., "The polyphenols resveratrol and epigallocatechin-3-gallate restore the severe impairment of mitochondria in hippocampal progenitor cells from a down syndrome mouse model," *Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease*, vol. 1862, no. 6, pp. 1093–1104, 2016.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 7418681, 9 pages
https://doi.org/10.1155/2018/7418681

Research Article

An Extract of *Artemisia dracunculus* L. Promotes Psychological Resilience in a Mouse Model of Depression

Jun Wang,[1,2] Adelaida Esteban Fernández,[1] Simoni Tiano,[1] Jing Huang,[1] Elizabeth Floyd,[3] Alexander Poulev,[4] David Ribnicky,[4] and Giulio M. Pasinetti[1,2]

[1]*Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA*
[2]*Geriatric Research, Education and Clinical Center, James J. Peters Veterans Affairs Medical Center, Bronx, NY 10468, USA*
[3]*Pennington Biomedical Research Center, Baton Rouge, LA 70808, USA*
[4]*Department of Plant Biology, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901, USA*

Correspondence should be addressed to Giulio M. Pasinetti; giulio.pasinetti@mssm.edu

Received 7 February 2018; Revised 6 April 2018; Accepted 24 April 2018; Published 13 May 2018

Academic Editor: Anat Elmann

Copyright © 2018 Jun Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Stress-induced peripheral inflammation contributes to depression-like behaviors in both human and experimental models. PMI 5011, a botanical extract of *Artemisia dracunculus* L., was previously shown to have multiple bioactivities, including anti-inflammatory activity. In this work, using a repeated social defeat stress (RSDS) model of depression, we demonstrate that oral administration of the botanical extract PMI 5011 promotes resilience to RSDS-mediated depression-like phenotypes. We also show that the behavioral improvements are associated with attenuation of stress-mediated induction of inflammatory cytokines in the periphery and alteration of synaptic plasticity in the nucleus accumbens (NAc). Our studies provide experimental evidence that botanical extracts such as PMI 5011, which target pathological mechanisms (i.e., peripheral inflammation) not addressed by currently available antidepressants, could be further developed as novel therapeutics for the treatment of stress disorders and anxiety in humans.

1. Introduction

Depression and anxiety are widespread psychological conditions with broad health implications. Currently available antidepressant treatments are mainly designed to target the serotonergic and/or the noradrenergic system in the brain. Approximately half of the patients, however, do not fully respond to the approved antidepressants [1], and these treatments are often associated with therapeutic time lag and a wide range of undesirable "adverse" events [2]. This may reflect the heterogeneity of the mechanisms underlying depression, highlighting an urgent need for new therapeutic targets that are not addressed by standard antidepressants.

Depression is a multicausal disorder and the underlying etiology and pathophysiology are not completely understood. Peripheral inflammation has received increasing attention in the past two decades. Many neuroimmune factors have been implicated in depressive disorders. Clinical studies report higher levels of circulating proinflammatory cytokines, such as interleukin-1β, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), in patients with major depressive disorder (MDD) [3–5]. How peripheral inflammation may modulate depression phenotypes is currently under intense investigation. More recently, it was reported that in both human and rodent models of depression, chronic social stress alters blood vessel ultrastructure and, in combination with stress-induced peripheral inflammation, increases blood brain barrier (BBB) leakiness that allows the infiltration of inflammatory molecules into the brain [6]. These inflammatory molecules, once in the brain, can act directly on neurons or indirectly through modulation of microglia and/or other CNS immune cells leading to alteration of neuroplasticity and the development of depression-like behaviors [6, 7]. This is further supported by the observation that intracranial infusion of proinflammatory cytokine IL-6 increases depression-associated behavior [8], and systemic treatment

with monoclonal IL-6 antibody can effectively reduce circulating IL-6 and promote resilience to chronic social stress-induced depression-like behaviors [9]. These studies suggest that modulations of peripheral inflammation and associated immune signaling pathways may provide novel therapeutic strategies to prevent and/or treat depression.

Therapeutic interventions derived from natural origin are receiving increased attention due to their lack of adverse secondary effects and multitargeting mechanisms of action which may increase the likelihood of therapeutic efficacy [10]. *Artemisia dracunculus* L. (Russian tarragon) is a culinary herb that has many healthy properties. Several bioactive constituents have been described in *Artemisia dracunculus*, including flavonoids (flavones, flavanones, dihydroflavanols, and chalcones) and phenolic acids (hydroxybenzoic, caffeic, or 5-*O*-caffeoylquinic acids, among others), as well as small amounts of sesquiterpenoids or vitamins. However, its composition widely varies depending on the plant phenotype and geographic origin [11]. Various bioactivities have been described in rodents, including strong anti-inflammatory, hepatoprotective, antihyperglycemic, antihyperlipidaemic, and antioxidant activities.

PMI-5011 is a well-characterized ethanol extract of *Artemisia dracunculus* L. that has been studied for at least 18 years and is the subject of many published research articles. Some of the earlier studies were focused on the identification of specific bioactive compounds using bioactivity-guided fractionation. The characterization of the extract was published over a series of research articles [12] and was summarized very succinctly in a review article by Schmidt et al. [13]. Three distinct assays were used independently to identify 6 bioactive compounds including 4,5-di-*O*-caffeoylquinic acid, davidigenin, 6-demethoxycapillarisin, 2′,4′-dihydroxy-4-methoxydihydrochalcone, 2′,4-dihydroxy-4′-methoxydihydrochalcone, and sakuranetin. The structures of these compounds were confirmed using LC-MS and 2D NMR, and their potential antidiabetic activities were tested both *in vitro* [14, 15] and *in vivo* in rodent models of type 2 diabetes [14, 16, 17]. For example, 2′,4′-dihydroxy-4-methoxydihydrochalcone is bioactive *in vitro* to inhibit (1) aldose reductase enzyme, (2) protein tyrosine phosphatase 1B activity and expression, and (3) phosphoenolpyruvate carboxykinase overexpression and its activities, validated *in vivo* by demonstrating acute glucose-lowering effects in mice [14]. The exact molecular mechanisms underlying the effect of PMI 5011 on diabetes are still under active investigation. However, *in vitro* studies have demonstrated that PMI 5011, at 10 μg/ml, can significantly improve insulin and insulin receptor signaling in primary human skeletal muscle cells [16] whereas in cultured human primary skeletal muscle myoblasts, at 5 μg/ml, PMI 5011 notably attenuates inflammatory response to cytokine stimuli through inhibition of nuclear factor-κB (NF-κB) signaling [17]. The anti-inflammatory activity of PMI 5011 was demonstrated in mouse and human pancreatic cells, whereas at 5-10 μg/ml, it reduces nitric oxide (NO), NO synthase activity (iNOS), and IL-6 [18]. *In vivo* efficacies of PMI 5011 were also reported in several studies. For example, the administration of PMI 5011 (500 mg/kg/day) to mice fed on a high-fat diet showed antidiabetic effects comparable to conventional drug treatment, such as metformin ($P < 0.05$) [14]; the treatment with 500 mg/kg/day PMI 5011 for 7 weeks normalized glycemia ($P < 0.01$), alleviated nerve conduction slowing and sensory neuropathy ($P < 0.05$), and decreased lipoxygenase and nitrated protein accumulation in a mouse model of prediabetic neuropathy [19]. In a mouse model of diabetes, diet supplemented with 1% PMI 5011 for 8 weeks improved insulin signaling via Akt and IRS-associated PI3 kinase ($P < 0.001$) [20].

Table 1: Relative concentrations of the bioactive compounds from PMI 5011. The relative concentrations of davidigenin, 6-demethoxycapillarisin, DMC-1, and sakuranetin were quantified based on the equivalents of DMC-2 as measured by LC-MS. The standard for DMC-2 was commercially synthesized. The fingerprint of PMI-5011 is consistently comprised of these compounds in approximately these ratios.

Bioactive components of PMI 5011	% of PMI-5011 extract (*w*/*w*)
Davidigenin	1.2%
6-Demethoxycapillarisin	0.74%
2′,4-Dihydroxy-4′-methoxydihydrochalcone (DMC-1)	0.63%
2′,4′-Dihydroxy-4-methoxydihydrochalcone (DMC-2)	2.5%
Sakuranetin	2.8%

The extraction procedure for PMI 5011 from *Artemisia dracunculus* L. has been standardized and provides a consistent fingerprint of these bioactive compounds (Table 1).

Acute and chronic stress has long been used to model mood and anxiety disorders. In mice, a variety of physiological and psychological stressors have been shown to produce behaviors resembling depression-like symptoms [21–25], among which the repeated social defeat stress (RSDS) model recapitulates many key behavioral features associated with psychosocial stress in humans. The RSDS paradigm consists of repeated subordinations of an experimental C57BL/6 mouse by an aggressive dominant CD-1 mouse, leading to long-lasting behavioral consequences. As in humans, chronic social subordination of susceptible mice leads to a spectrum of depression-like behaviors, among which social avoidance and anhedonia are most relevant to human depression. Similar to human psychopathology, in which some individuals develop depression while others do not, a subset of resilient mice resist the development of such behaviors following RSDS [26]. Depression and anxiety are associated with functional abnormalities in brain regions involved in fear conditioning and emotion regulation [27], and these pathogenic alterations likely contribute to the vulnerability of certain individuals for developing depression/anxiety. Similarly, RSDS mice exhibit stress-induced abnormalities in synaptic remodeling, which include altered synaptic strength and connectivity [28–31] in the nucleus accumbens (NAc), a brain structure important for the development of anxiety/depression in response to trauma-related stimuli. Moreover, in the RSDS model, leukocyte-derived IL-6 regulates susceptibility

Table 2: Diet formulation used in the study.

Product number	D11112201 *OpenStandard Diet*		D17020901 *OpenStandard Diet*	
	gm%	kcal%	gm%	kcal%
Protein	19	*20*	19	20
Carbohydrate	63	65	62	65
Fat	7	*15*	6	15
Total		*100*		*100*
kcal/gm	3.81		3.77	
Ingredient	gm	kcal	gm	kcal
Casein	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*
Cornstarch	381	*1524*	381	*1524*
Maltodextrin 10	110	*440*	110	*440*
Dextrose	150	*600*	150	*600*
Cellulose, BW200	75	*0*	75	*0*
Inulin	25	*37.5*	25	*37.5*
Soybean oil	70	*630*	70	*630*
Mineral mix S10026	10	*0*	10	*0*
Dicalcium phosphate	13	*0*	13	*0*
Calcium carbonate	5.5	*0*	5.5	*0*
Potassium citrate, 1 H2O	16.5	*0*	16.5	*0*
Vitamin mix V10001	10	*40*	10	*40*
Choline bitartrate	2	*0*	2	*0*
PMI 5011	**0**	**0**	**10.9**	**0**
Yellow dye number 5, FD&C	0.025	*0*	*0*	*0*
Red dye number 40, FD&C	0	*0*	0.025	*0*
Blue dye number 1, FD&C	0.025	*0*	0.025	*0*
Total	**1071.05**	**4084**	**1082**	**4084**
PMI 5011 (%)	**0**	**0**	**1.0074**	**0**

versus resilience to stress, emphasizing the key role of peripheral IL-6 in depression [9, 32].

Based on the important contribution of peripheral inflammation in the pathophysiology of depression and the established anti-inflammatory activity of PMI 5011, we hypothesized that the administration of PMI 5011 may be able to attenuate depression-like phenotypes through modulation of stress-induced peripheral inflammation. In this study, we tested the preclinical efficacy of PMI 5011 in modulating depression-like behavior in the repeated social defeat stress (RSDS) mouse model of depression.

2. Materials and Methods

2.1. Materials. PMI 5011, a dried ethanolic extract of *Artemisia dracunculus* L., was prepared and analyzed as previously described [14]. Briefly, the harvested shoots were heated with 80% ethanol (*v*/*v*) to 80°C for 2 h. The extraction continued for an additional 10 h at 20°C. The extract was then filtered through cheese cloth and evaporated with a rotary evaporator. The aqueous extract was freeze-dried for 48 h, and the dried extract was homogenized with a mortar and pestle. The composition of bioactive components of PMI 5011 used in this study is shown in Table 1.

2.2. Animals. All C57BL/6J male mice were purchased from the Jackson Laboratory (Stock number 000664). Retired breeder CD-1 mice were purchased from Charles River Laboratory. All animals had access to regular chow ad lib and were maintained on a 12 : 12 h light/dark cycle with lights on at 07:00 h in a temperature-controlled (20 ± 2°C) vivarium, and all procedures were approved by the Institutional Animal Care and Use Committee (Protocol number IACUC-2014-0081).

2.3. Treatment. The male C57BL/6J mice ($n = 22$ per group) were group housed ($n = 4$-5 per cage) until the initiation of RSDS. The number of mice was calculated based on our previous social interaction studies employing RSDS. Power calculation found that 15 mice/group will have 90% statistical power to detect 25% (0.32 log2) fold change. Due to the nature of the defeat and associated injury, we used a larger number of mice ($n = 22$/group) to ensure that we will have sufficient statistical power to identify the behavioral changes in the event that not all mice complete the study. All mice were fed with a polyphenol-free diet for 10 days and were then randomly grouped into two groups: one group received a regular diet (OpenStandard Diet, D11112201, Research Diets) and the other group was treated with the same diet with 1% PMI 5011 incorporated (OpenStandard Diet, D17020901, Research Diets, Table 2), starting 2 weeks prior to RSDS and throughout RSDS and SI testing. The dose we use is a standard dose we used in all the preclinical studies conducted in diabetes research. The dose is well tolerated and has consistently produced improvement in glucose metabolism and inflammation [14, 20, 33–36]. Potential toxicity of PMI 5011 has been thoroughly tested, and we have established that dosage up to 1000 mg/kg/day for 90 days appears to be safe and nontoxic [37]. The treatment duration was based on our previous study demonstrating the efficacy of a bioactive dietary polyphenol-rich preparation in the same RSDS model [32]. One mouse from the vehicle-treated control group had to be euthanized due to the injury sustained from the RSDS. All remaining mice were subjected to SI. Twenty-four hours after the SI, one set of mice ($n = 8$ per group) was sacrificed for plasma cytokine and brain synaptic protein expression analysis without other behavioral testing, as splash testing and sucrose preference testing can potentially influence the reward circuit, which will affect synaptic gene expression. The other set of mice ($n = 13$ for the control group and $n = 14$ for the treatment group) was subjected to sucrose preference and splash testing.

2.4. Behavioral Testing

2.4.1. RSDS. RSDS was performed as previously described [26, 38]. CD-1 mice were screened for aggressive characteristics prior to the start of social defeat experiments based on previously described criteria [26]. Specifically, CD-1 mice were individually caged, and on the day of screening, a C57BL/6J mouse was placed directly into the home cage of

the CD-1 mouse for 180 seconds. The latency to aggression was noted and the same procedure repeated for two more times in the next two days, each time with a different C57BL/6J mouse as screener. The CD-1 mouse that (1) successfully attacked in at least two consecutive sessions and (2) has the latency to initial aggression less than 60 seconds was chosen and housed within the social defeat cage (26.7 w × 48.3 d × 15.2 h cm; Allentown Inc.) 24 hours prior to the start of defeats on one side of a clear, perforated plexiglass divider (0.6 × 45.7 × 15.2 cm; Nationwide Plastics). The RSDS was conducted every day under regular house light. Briefly, the mice subjected to RSDS were exposed to a novel CD-1 aggressor mouse for 10 minutes once per day, over 10 consecutive days. Following the 10 minutes of interaction, the experimental C57/BL6J mice were removed to the opposite side of the social defeat cage, and sensory contact during the following 24-hour period was allowed. The C57BL/6 mice were returned to a single house following the last defeat and before the social avoidance testing.

2.4.2. Social Avoidance Test (Social Interaction Test). Social interaction (SI) testing was performed as previously described [26]. All SI tests were performed under red light conditions. The mice were placed in a novel interaction open-field arena custom-crafted from opaque plexiglass (42 × 42 × 42 cm; Nationwide Plastics) with a small animal cage placed at one end. Their movements were then automatically monitored and recorded (Ethovision 3.0; Noldus Information Technology) for 2.5 minutes in the absence (target absent phase) of a novel CD-1 mouse. This phase is used to determine baseline exploratory behavior. We then immediately measured 2.5 minutes of exploratory behavior in the presence of a caged CD-1 mouse (target present phase), again recording the total distance travelled and the duration of time spent in the interaction and corner zones. SI behavior was then calculated as total time spent in each zone or as a ratio of the time spent in the interaction with the target present divided by the time spent in the interaction zone with the target absent. All mice with a ratio above 1.0 were classified as resilient whereas below 1.0 were classified as susceptible.

2.4.3. Splash Test. Following the SI testing, a sucrose splash test was carried out in the home cage under a red light [39]. Briefly, the mice were sprayed with 200 μl of a 10% (wt/vol) sucrose solution directly onto the animal's back using a small atomizer to induce grooming behavior. The grooming frequency and latency were recorded for 5 minutes and manually scored.

2.4.4. Sucrose Preference Testing. Following the last defeat, the mice were habituated to 50 ml tubes with a sipper top (a two-bottle choice) filled with drinking water. After the splash testing, the mice were given access to a two-bottle choice of water or 1% sucrose solution, and the consumption of each solution was recorded once every 24 hours for 48 hours. Sucrose preference was calculated as a percentage of sucrose consumption over total liquid consumption.

2.5. RNA Isolation and Gene Expression Assessment. One set of mice ($n = 8$) was sacrificed by decapitation without anesthesia 24 hours following the SI test. Trunk blood was collected from each mouse in EDTA-coated tubes, and plasma was collected following centrifugation at 2000g for 15 minutes. Total RNA from brain NAc of each mouse was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed. Gene expression was measured in 4 replicates by quantitative RT-PCR using Maxima SYBR Green Master Mix (Fermentas) in ABI Prism 7900HT. The following are primer sequences: mouse hypoxanthine phosphoribosyltransferase (HPRT) forward: CCCCAAAATGGTTAAGGTTGC, HPRT reverse: CCCCAAAATGGTTAAGGTTGC, Rac1 forward: GGTAGGTGATGGGAGTCAGC, Rac1 reverse: CTGAAGTGCGACACCACTGT, vGlut2 forward: GCTCACCTCTACCCTCAATATG, vGlut2 reverse: CCACTTGCTCCATATCCCATG, PSD95 forward: CGGGAGAAAATGGAGAAGGAC, PSD95 reverse: GCATTGGCTGAGACATCAAG, VGAT forward: ACGACAAACCCAAGATCACG, and VGAT reverse: AAGATGATGAGGAACAACCCC. HPRT expression level was used as an internal control. Data were normalized using the $2^{-\Delta\Delta Ct}$ method as previously described [40, 41]. Levels of target gene mRNAs were expressed relative to those in control mice and plotted in GraphPad Prism.

2.6. Plasma Collection and Multiplex ELISA Assay for Peripheral Cytokines. The plasma collected from the trunk blood ($n = 8$ per group, see above) was assayed for cytokine levels 24 hours after the SI test. Multiplex MAP mouse cytokine/chemokine panel (EMD Millipore) was used to measure the levels of 32 cytokines/chemokines following the manufacturer's instruction. Briefly, 12.5 μl of plasma was incubated with the mouse cytokine/chemokine magnetic premixed beads at 4°C overnight and washed three times with the washing buffer, followed by incubation with mouse cytokine/chemokine detection antibodies for 1 hour at room temperature (RT). Streptavidin-phycoerythrin was then added and incubated for 30 minutes at RT followed by three times washing and subjected to analysis on Luminex 200® Instrument xPONENT3.1 (Luminex, Austin, TX).

2.7. Overall Statistics. All values are expressed as mean and standard error of the mean (SEM). Unpaired two-tailed Student's t-tests with Welch's correction were used. In all studies, outliers are defined as 2 standard deviations (SD) from the mean and were excluded. The null hypothesis was rejected at the 0.05 level. All statistical analyses were performed using Prism Stat program (GraphPad Software Inc.).

3. Results

3.1. Prophylactic Treatment with PMI 5011 Promotes Resilience to RSDS-Mediated Depression Phenotypes. To test the efficacy of PMI 5011 in stress-mediated depression, we treated C57BL/6 male mice with PMI 5011 or vehicle delivered through their diet for 2 weeks prior to and throughout RSDS and then performed social avoidance/interaction (SI) testing (Figure 1(a)). We found that treatment with PMI

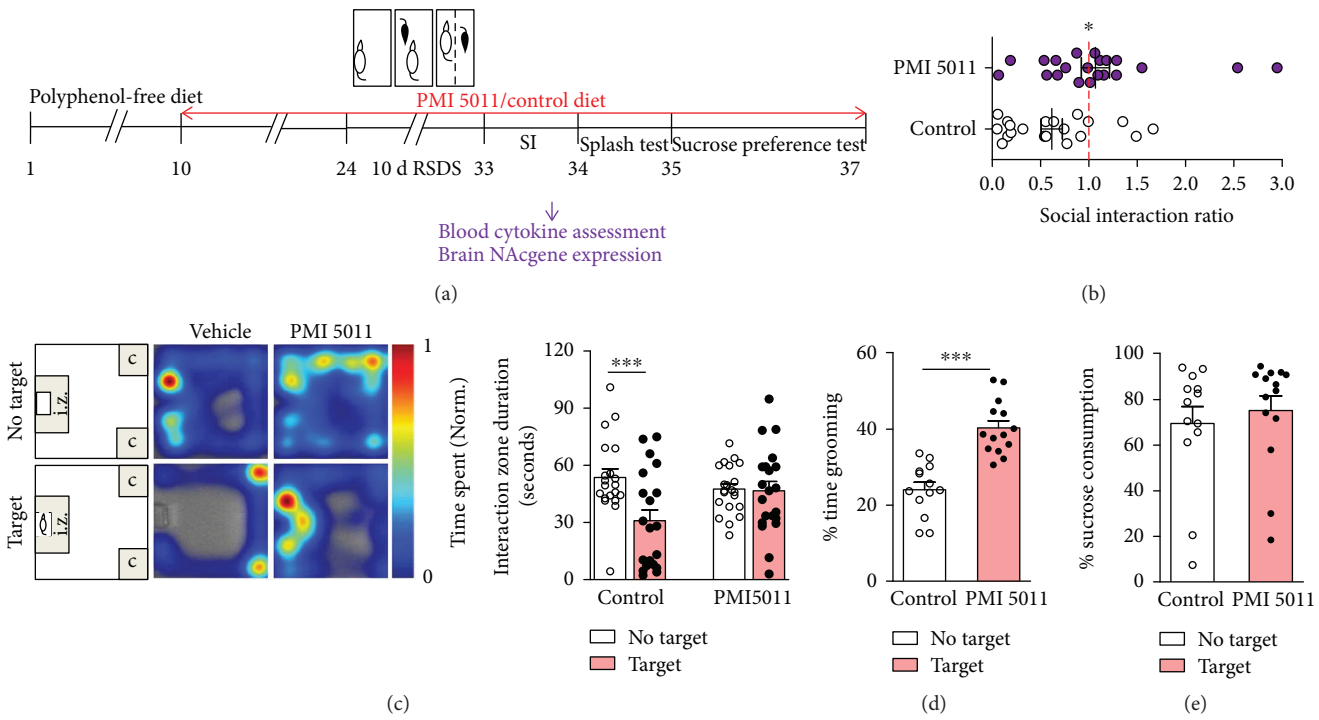


Figure 1: Oral administration of PMI 5011 promotes resilience to RSDS. (a) Schematic design of the experiment. (b) Treatment with PMI 5011 that increases the proportion of mice showing a resilient phenotype, as measured by the social interaction ratio (two-tailed unpaired t-test, $t_{39} = 2.786$, $P = 0.018$, $n = 20$, 21 mice; one mouse from each group was excluded as outlier). (c) Representative heat maps (left) and bar graph (right) of the social avoidance behavioral test of duration spent in interaction zone (seconds) in the absence or presence of social target in vehicle- and PMI 5011-treated mice (vehicle group: two-tailed paired t-test, $t_{19} = 4.552$, $P = 0.0002$; PMI 5011 group: two-tailed paired t-test, $t_{20} = 0.166$, $P = 0.870$). (d) Splash test (two-tailed unpaired t-test, $t_{25} = 6.031$, $P < 0.0001$, $n = 13$, 14 mice). (e) Sucrose preference test (two-tailed unpaired t-test, $t_{25} = 0.584$, $P = 0.565$, $n = 13$, 14 mice). All bar graphs represent mean ± SEM, $^{*}P < 0.05$, $^{***}P < 0.001$.

5011 greatly increased the proportion of mice resilient to stress compared to the vehicle-treated animals (Figure 1(b), $P < 0.05$). Overall, over 50% of mice receiving PMI 5011 showed a resilient behavioral phenotype, whereas ~20% were resilient in the vehicle control group. Moreover, we found that there was a significant reduction of duration of time spent in the interaction zone (i.z.) in the presence of an interactive mouse (target) in the vehicle-treated group compared to that in the absence of the interactive mouse following RSDS (Figure 1(c), $P < 0.001$), while there was no difference in the time spent in the i.z. in the absence of the interactive mouse in the PMI 5011 group (Figure 1(c)).

We next conducted the splash test, a measure of stress-induced decreased self-care that is only reversible by chronic standard antidepressant treatment [9]. We found that mice from the PMI 5011-treated group spent significantly increased time grooming following aerosol delivery of a 10% sucrose solution to the fur compared to the vehicle-treated group (Figure 1(d), $P < 0.001$), suggesting PMI 5011 treatment attenuates stress-induced self-neglect behavior. Following the splash test, we then conducted a sucrose preference test to evaluate the effect of PMI 5011 on stress-induced anhedonia behavior. We found that both groups had similar average sucrose consumption implicating PMI 5011 treatment does not attenuate stress-induced anhedonia phenotypes (Figure 1(e)).

3.2. Effect of PMI 5011 on Stress-Mediated Peripheral Inflammation. As peripheral inflammation is our potential target for PMI 5011, we next measured the plasma level of cytokines 24 hours after the defeat. We found that, compared to the vehicle-treated group, PMI 5011-treated mice had significantly lower levels of IL-6, TNF-α, MCP-1, G-CSF, GM-CSF, IL-17, IP-10, MIP-1α, and MIP-β (Figure 2(a)). We also found that, compared to the vehicle-treated group, PMI 5011 treatment led to an increased level of eotaxin, LIX, and M-CSF (Figure 2(b)).

3.3. Effect of PMI 5011 on Stress-Mediated Synaptic Plasticity. We previously found that, in both humans and rodents, chronic stress reduces the expression of RAS-related C3 botulinum toxin substrate 1 (*Rac1*) in the NAc and stress-mediated downregulation of Rac1 in the NAc correlates with social avoidance behavior in the RSDS model of depression [28]. We showed that downregulation of Rac1 is necessary and sufficient for social avoidance behavior and that pharmacological modulation of Rac1 attenuated stress-induced depression phenotypes [28, 32]. Moreover, Rac1 can also influence excitatory synapses, such as postsynaptic density protein 95 (PSD95) and vesicular glutamate transporter 2 (vGlut2) both *in vivo* and *in vitro* [28, 32]. We also demonstrated that peripheral inflammation can causally influence the expression of genes that are important

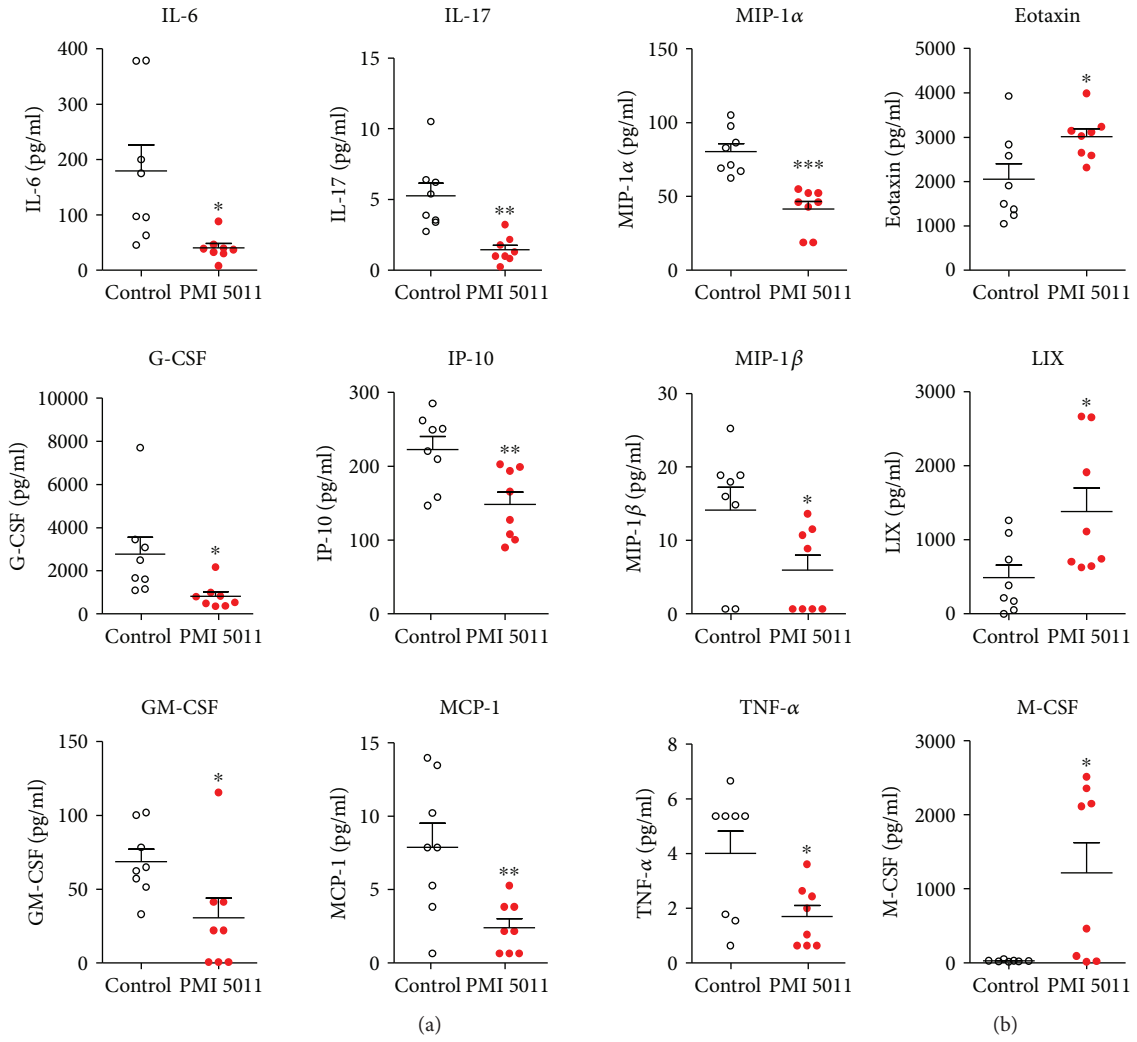


Figure 2: PMI 5011 treatment attenuates RSDS-induced inflammation in the periphery. (a) Plasma level of cytokines that was significantly reduced following PMI 5011 treatment 24 hours after last defeat (two-tailed unpaired t-test, $t_{14} = 2.786$, $P = 0.0116$ for IL-6; $t_{14} = 4.019$, $P = 0.0013$ for IL-17; $t_{14} = 5.181$, $P = 0.0001$ for MIP-1α; $t_{14} = 2.469$, $P = 0.027$ for G-CSF; $t_{14} = 3.080$, $P = 0.0082$ for IP-10; $t_{14} = 2.196$, $P = 000455$ for MIP-1β; $t_{14} = 2.393$, $P = 0.0313$ for GM-CSF; $t_{14} = 3.146$, $P = 0.0072$ for MCP-1 and $t_{14} = 2.549$, $P = 0.0231$ for TNF-α; $n = 8$ per group). (b) Plasma level of cytokines that was significantly increased following PMI 5011 treatment 24 hours after last defeat (two-tailed unpaired t-test, $t_{14} = 2.429$, $P = 0.0292$ for eotaxin; $t_{14} = 2.487$, $P = 0.0261$ for LIX; $t_{14} = 2.706$, $P = 0.0180$ for M-CSF, $n = 8$ per group). All bar graphs represent mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

for synaptic function in the NAc [32]. Therefore, we next measured the expression of synaptic protein in the NAc of mice following RSDS by real-time PCR. We found that there was an ~25% significant increase in the expression of Rac1 in the NAc in the PMI 5011-treated group compared to vehicle-treated mice (Figure 3(a), $P < 0.050$). Moreover, we found that treatment with PMI 5011 led to a significant reduction of >60% in the expression of vGlut2 ($P < 0.050$) and an ~25% reduction of PSD-95, however, did not reach statistical significance. Both vGlut 2 and PSD95 are markers of excitatory neurons that are shown to be increased in the NAc following RSDS. Consistent with our previous findings, there were no differences in the expression of GABAergic vesicular GABA transporter (VGAT) (Figures 3(b)–3(d)).

4. Discussion

Major depressive disorder is a psychiatric disease that is the fourth most common cause of disability worldwide. Molecular mechanisms underlying the pathophysiology of major depressive disorders (MDD) are very complex and are affected by genetic, environmental, and biological processes. Currently, three major aspects of depression are being actively investigated. First of all, depression is influenced by an imbalance of neurotransmitters and receptors, including serotonin, adrenaline, dopamine, and glutamate [42]. Secondly, depression is associated with the hyperactivity of immune inflammatory responses as manifested by elevated expression of proinflammatory molecules, such as IL-6 and TNF-α. The overall elevated status of inflammation together

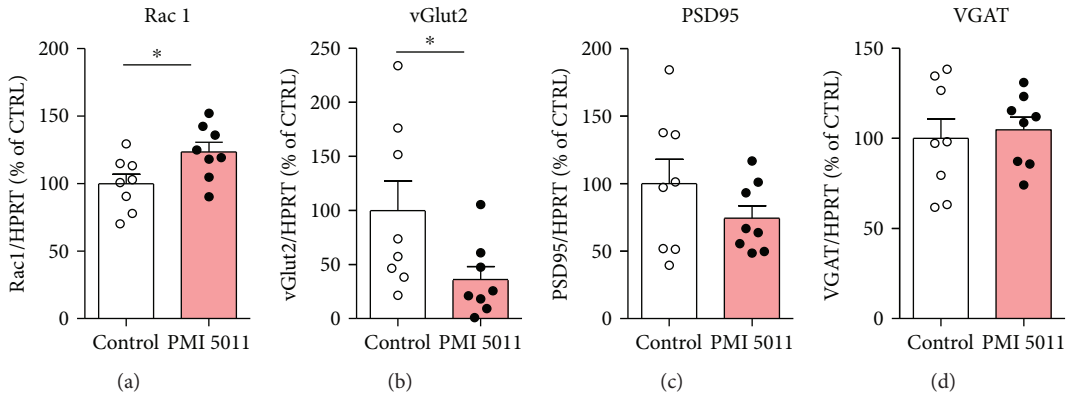


Figure 3: PMI 5011 treatment normalizes the expression of stress-induced excitatory synaptic protein in the NAc (a) Expression of Rac1 in the NAc ($t_{14} = 2.354$, $P = 0.0337$, $n = 8$ per group). (b, c) Expression of glutamatergic neuron markers vGlut2 and PSD95 ($t_{14} = 2.137$, $P = 0.050$ for vGlut2; $t_{14} = 1.265$, $P = 0.2264$ for PSD95, $n = 8$ per group). (d) Expression of GABAergic neuron marker VGAT ($t_{14} = 0.3679$, $P = 0.7184$, $n = 8$ per group). All bar graphs represent mean ± SEM, $^*P < 0.05$.

with neurovasculature pathology and impaired BBB structural function leads to malfunction of the brain circuits related to mood and anxiety [5]. Lastly, stress-induced depression causes a disruption in the normal synaptic plasticity and induces changes in brain architecture [43]. Conventional antidepressant therapies mainly target neurotransmitters and are associated with low overall treatment efficacy and various unwanted side effects. Therefore, therapy targeting inflammation and brain synaptic plasticity may provide novel treatment strategies for MDD.

In recent years, natural products, especially polyphenols, have received growing interest due to their potential benefits in treating psychiatric disorders. It is believed that their strong antioxidant and anti-inflammatory activities and their ability to modulate synaptic plasticity may contribute to their mechanisms of action [10, 42, 44]. PMI 5011 is an ethanol extract from *A. drancunculus*, characterized by a high content of secondary metabolites, including coumarins, flavonoids, and phenylpropanoid acids [11]. In this study, we demonstrated that treatment with PMI 5011 significantly attenuated stress-induced social avoidance and self-neglect behaviors in a mouse model of depression. Moreover, we found that the improvement of behavior was associated with significant reduction of inflammatory cytokines in the blood.

Previous studies demonstrated that increased glutamatergic transmission on ventral striatum medium spiny neurons (MSNs) mediates stress-induced susceptibility following RSDS [45, 46]. More recently, we showed a cause-effect relationship among leukocyte-derived proinflammatory responses, brain reward circuitry synaptic remodeling, and the manifestation of depression-like behavioral phenotypes [32]. Here, we demonstrated that PMI 5011 treatment also reduced the expression of excitatory markers in the NAc, which may contribute to the phenotypes we observed. This modulation of glutamatergic synapses could be a result of PMI 5011-mediated downregulation of peripheral inflammation. It is also possible that selected metabolites derived from PMI 5011 may pass the BBB and reach the brain to directly modulate synaptic plasticity.

We demonstrated that treatment with PMI 5011 protects against susceptibility to stress-mediated depression phenotypes by reducing peripheral inflammation and preserving synaptic plasticity in the NAc. Our observation is consistent with clinical and preclinical evidence that overly active peripheral inflammation processes involving inflammatory cytokines and disruptions in the normal synaptic plasticity responses in the NAc are two key pathological mechanisms underlying depression and anxiety. The efficacy of PMI 5011 in alleviating depression-like symptoms may also be suitable for treating other neuropsychological disorders such as posttraumatic stress disorder, traumatic brain injury-induced mood disorder, and bipolar depression, which share similar symptoms with MDD. Our evidence supports the development of PMI 5011 as a novel therapeutic agent to treat patients with treatment-resistant MDD, particularly among the majority of patients who are characterized as having high plasma levels of inflammatory cytokines [9]. Given the excellent safety profile of PMI 5011 [11] and its noted anti-inflammatory potential [17], it can be readily tested in clinical studies for the treatment of stress disorders and depression either alone or in combination with currently available antidepressants.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Dr. Giulio M Pasinetti holds a Senior VA Career Scientist Award. The authors acknowledge that the contents of this study do not represent the views of the NCCIH, the ODS, the NIH, the U.S. Department of Veterans Affairs, or the United States Government.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by the National Center for Complementary and Integrative Health (Grant no. P50 AT008661-01) and the Office of Dietary Supplements.

References

[1] V. Krishnan and E. J. Nestler, "The molecular neurobiology of depression," *Nature*, vol. 455, no. 7215, pp. 894–902, 2008.

[2] J. M. Ferguson, "SSRI antidepressant medications: adverse effects and tolerability," *The Primary Care Companion to The Journal of Clinical Psychiatry*, vol. 3, no. 1, pp. 22–27, 2001.

[3] Y. Dowlati, N. Herrmann, W. Swardfager et al., "A meta-analysis of cytokines in major depression," *Biological Psychiatry*, vol. 67, no. 5, pp. 446–457, 2010.

[4] M. Maes, "Evidence for an immune response in major depression: a review and hypothesis," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 19, no. 1, pp. 11–38, 1995.

[5] A. H. Miller and C. L. Raison, "The role of inflammation in depression: from evolutionary imperative to modern treatment target," *Nature Reviews Immunology*, vol. 16, no. 1, pp. 22–34, 2016.

[6] M. Merad, M. G. Manz, H. Karsunky et al., "Langerhans cells renew in the skin throughout life under steady-state conditions," *Nature Immunology*, vol. 3, no. 12, pp. 1135–1141, 2002.

[7] W. A. Banks, A. J. Kastin, and E. G. Gutierrez, "Penetration of interleukin-6 across the murine blood-brain barrier," *Neuroscience Letters*, vol. 179, no. 1-2, pp. 53–56, 1994.

[8] M. W. Meagher, R. R. Johnson, E. E. Young et al., "Interleukin-6 as a mechanism for the adverse effects of social stress on acute Theiler's virus infection," *Brain, Behavior, and Immunity*, vol. 21, no. 8, pp. 1083–1095, 2007.

[9] G. E. Hodes, M. L. Pfau, M. Leboeuf et al., "Individual differences in the peripheral immune system promote resilience versus susceptibility to social stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 45, pp. 16136–16141, 2014.

[10] A. Sureda and S. Tejada, "Polyphenols and depression: from chemistry to medicine," *Current Pharmaceutical Biotechnology*, vol. 16, no. 3, pp. 259–264, 2015.

[11] D. Obolskiy, I. Pischel, B. Feistel, N. Glotov, and M. Heinrich, "*Artemisia dracunculus* L. (tarragon): a critical review of its traditional use, chemical composition, pharmacology, and safety," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 21, pp. 11367–11384, 2011.

[12] S. Logendra, D. M. Ribnicky, H. Yang et al., "Bioassay-guided isolation of aldose reductase inhibitors from *Artemisia dracunculus*," *Phytochemistry*, vol. 67, no. 14, pp. 1539–1546, 2006.

[13] B. Schmidt, D. M. Ribnicky, A. Poulev, S. Logendra, W. T. Cefalu, and I. Raskin, "A natural history of botanical therapeutics," *Metabolism*, vol. 57, Supplement 1, pp. S3–S9, 2008.

[14] D. M. Ribnicky, P. Kuhn, A. Poulev et al., "Improved absorption and bioactivity of active compounds from an antidiabetic extract of *Artemisia dracunculus* L," *International Journal of Pharmaceutics*, vol. 370, no. 1-2, pp. 87–92, 2009.

[15] D. Govorko, S. Logendra, Y. Wang et al., "Polyphenolic compounds from *Artemisia dracunculus L.* inhibit PEPCK gene expression and gluconeogenesis in an H4IIE hepatoma cell line," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 293, no. 6, pp. E1503–E1510, 2007.

[16] I. Kheterpal, P. Scherp, L. Kelley et al., "Bioactives from *Artemisia dracunculus* L. enhance insulin sensitivity via modulation of skeletal muscle protein phosphorylation," *Nutrition*, vol. 30, no. 7-8, pp. S43–S51, 2014.

[17] B. Vandanmagsar, K. R. Haynie, S. E. Wicks et al., "*Artemisia dracunculus* L. extract ameliorates insulin sensitivity by attenuating inflammatory signalling in human skeletal muscle culture," *Diabetes, Obesity and Metabolism*, vol. 16, no. 8, pp. 728–738, 2014.

[18] S. Aggarwal, G. Shailendra, D. M. Ribnicky, D. Burk, N. Karki, and M. S. Qingxia Wang, "An extract of *Artemisia dracunculus* L. stimulates insulin secretion from β cells, activates AMPK and suppresses inflammation," *Journal of Ethnopharmacology*, vol. 170, pp. 98–105, 2015.

[19] P. Watcho, R. Stavniichuk, D. M. Ribnicky, I. Raskin, and I. G. Obrosova, "High-fat diet-induced neuropathy of prediabetes and obesity: effect of PMI-5011, an ethanolic extract of *Artemisia dracunculus L.*," *Mediators of Inflammation*, vol. 2010, Article ID 268547, 10 pages, 2010.

[20] Z. Q. Wang, D. Ribnicky, X. H. Zhang et al., "An extract of *Artemisia dracunculus* L. enhances insulin receptor signaling and modulates gene expression in skeletal muscle in KK-A^y mice," *The Journal of Nutritional Biochemistry*, vol. 22, no. 1, pp. 71–78, 2011.

[21] R. G. Hunter, K. J. McCarthy, T. A. Milne, D. W. Pfaff, and B. S. McEwen, "Regulation of hippocampal H3 histone methylation by acute and chronic stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 49, pp. 20912–20917, 2009.

[22] Y. Golub, C. P. Mauch, M. Dahlhoff, and C. T. Wotjak, "Consequences of extinction training on associative and non-associative fear in a mouse model of posttraumatic stress disorder (PTSD)," *Behavioural Brain Research*, vol. 205, no. 2, pp. 544–549, 2009.

[23] A. Siegmund, M. Dahlhoff, U. Habersetzer et al., "Maternal inexperience as a risk factor of innate fear and PTSD-like symptoms in mice," *Journal of Psychiatric Research*, vol. 43, no. 14, pp. 1156–1165, 2009.

[24] V. Brinks, E. R. de Kloet, and M. S. Oitzl, "Corticosterone facilitates extinction of fear memory in BALB/c mice but strengthens cue related fear in C57BL/6 mice," *Experimental Neurology*, vol. 216, no. 2, pp. 375–382, 2009.

[25] R. Hammamieh, N. Chakraborty, T. C. M. de Lima et al., "Murine model of repeated exposures to conspecific trained aggressors simulates features of post-traumatic stress disorder," *Behavioural Brain Research*, vol. 235, no. 1, pp. 55–66, 2012.

[26] S. A. Golden, H. E. Covington, O. Berton, and S. J. Russo, "A standardized protocol for repeated social defeat stress in mice," *Nature Protocols*, vol. 6, no. 8, pp. 1183–1191, 2011.

[27] L. M. Shin and K. Handwerger, "Is posttraumatic stress disorder a stress-induced fear circuitry disorder?," *Journal of Traumatic Stress*, vol. 22, no. 5, pp. 409–415, 2009.

[28] S. A. Golden, D. J. Christoffel, M. Heshmati et al., "Epigenetic regulation of RAC1 induces synaptic remodeling in stress disorders and depression," *Nature Medicine*, vol. 19, no. 3, pp. 337–344, 2013.

[29] D. J. Christoffel, S. A. Golden, M. Heshmati et al., "Effects of inhibitor of κB kinase activity in the nucleus accumbens on

emotional behavior," *Neuropsychopharmacology*, vol. 37, no. 12, pp. 2615–2623, 2012.

[30] D. J. Christoffel, S. A. Golden, and S. J. Russo, "Structural and synaptic plasticity in stress-related disorders," *Reviews in the Neurosciences*, vol. 22, no. 5, pp. 535–549, 2011.

[31] D. J. Christoffel, S. A. Golden, D. Dumitriu et al., "IκB kinase regulates social defeat stress-induced synaptic and behavioral plasticity," *Journal of Neuroscience*, vol. 31, no. 1, pp. 314–321, 2011.

[32] J. Wang, G. E. Hodes, H. Zhang et al., "Epigenetic modulation of inflammation and synaptic plasticity promotes resilience against stress in mice," *Nature Communications*, vol. 9, no. 1, p. 477, 2018.

[33] S. Wicks, C. M. Taylor, M. Luo et al., "*Artemisia* supplementation differentially affects the mucosal and luminal ileal microbiota of diet-induced obese mice," *Nutrition*, vol. 30, no. 7-8, pp. S26–S30, 2014.

[34] H. Kirk-Ballard, Z. Q. Wang, P. Acharya et al., "An extract of *Artemisia dracunculus* L. inhibits ubiquitin-proteasome activity and preserves skeletal muscle mass in a murine model of diabetes," *PLoS One*, vol. 8, no. 2, article e57112, 2013.

[35] D. M. Ribnicky, D. E. Roopchand, A. Poulev et al., "*Artemisia dracunculus* L. polyphenols complexed to soy protein show enhanced bioavailability and hypoglycemic activity in C57BL/6 mice," *Nutrition*, vol. 30, no. 7-8, pp. S4–S10, 2014.

[36] Y. Yu, T. M. Mendoza, D. M. Ribnicky et al., "An extract of Russian tarragon prevents obesity-related ectopic lipid accumulation," *Molecular Nutrition & Food Research*, vol. 62, no. 8, article e1700856, 2018.

[37] D. M. Ribnicky, A. Poulev, J. O'Neal et al., "Toxicological evaluation of the ethanolic extract of *Artemisia dracunculus* L. for use as a dietary supplement and in functional foods," *Food and Chemical Toxicology*, vol. 42, no. 4, pp. 585–598, 2004.

[38] V. Krishnan, M. H. Han, D. L. Graham et al., "Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions," *Cell*, vol. 131, no. 2, pp. 391–404, 2007.

[39] I. Yalcin, F. Aksu, and C. Belzung, "Effects of desipramine and tramadol in a chronic mild stress model in mice are altered by yohimbine but not by pindolol," *European Journal of Pharmacology*, vol. 514, no. 2-3, pp. 165–174, 2005.

[40] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[41] J. Wang, B. Gong, W. Zhao et al., "Epigenetic mechanisms linking diabetes and synaptic impairments," *Diabetes*, vol. 63, no. 2, pp. 645–654, 2014.

[42] V. Maletic, M. Robinson, T. Oakes, S. Iyengar, S. G. Ball, and J. Russell, "Neurobiology of depression: an integrated view of key findings," *International Journal of Clinical Practice*, vol. 61, no. 12, pp. 2030–2040, 2007.

[43] R. S. Duman, G. K. Aghajanian, G. Sanacora, and J. H. Krystal, "Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants," *Nature Medicine*, vol. 22, no. 3, pp. 238–249, 2016.

[44] J. Trebaticka and Z. Durackova, "Psychiatric disorders and polyphenols: can they be helpful in therapy?," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 248529, 16 pages, 2015.

[45] T. C. Francis, R. Chandra, D. M. Friend et al., "Nucleus accumbens medium spiny neuron subtypes mediate depression-related outcomes to social defeat stress," *Biological Psychiatry*, vol. 77, no. 3, pp. 212–222, 2015.

[46] D. J. Christoffel, S. A. Golden, J. J. Walsh et al., "Excitatory transmission at thalamo-striatal synapses mediates susceptibility to social stress," *Nature Neuroscience*, vol. 18, no. 7, pp. 962–964, 2015.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 7043213, 21 pages
https://doi.org/10.1155/2018/7043213

Review Article

Flavonoids as Therapeutic Agents in Alzheimer's and Parkinson's Diseases: A Systematic Review of Preclinical Evidences

Roxana Braga de Andrade Teles,[1,2] Tâmara Coimbra Diniz,[1] Tiago Coimbra Costa Pinto,[3] Raimundo Gonçalves de Oliveira Júnior,[2,4] Mariana Gama e Silva,[2] Érica Martins de Lavor,[2] Antonio Wilton Cavalcante Fernandes,[2] Ana Paula de Oliveira,[2] Fernanda Pires Rodrigues de Almeida Ribeiro,[2] Amanda Alves Marcelino da Silva,[5] Taisy Cinthia Ferro Cavalcante,[5] Lucindo José Quintans Júnior,[6] and Jackson Roberto Guedes da Silva Almeida[1,2]

[1]*Postgraduate Program in Biotechnology, State University of Feira de Santana, 44036-900 Feira de Santana, BA, Brazil*
[2]*Federal University of San Francisco Valley, 56304-205 Petrolina, PE, Brazil*
[3]*Postgraduate Program in Neuropsychiatry and Behavioural Sciences, Federal University of Pernambuco, 50740-521 Recife, PE, Brazil*
[4]*UMRi CNRS 7266 LIENSs University of La Rochelle, La Rochelle, France*
[5]*University of Pernambuco, 56328-903 Petrolina, PE, Brazil*
[6]*Department of Physiology, Federal University of Sergipe, 49100-000 São Cristóvão, SE, Brazil*

Correspondence should be addressed to Jackson Roberto Guedes da Silva Almeida; jackson.guedes@univasf.edu.br

Received 12 January 2018; Accepted 2 April 2018; Published 10 May 2018

Academic Editor: David Vauzour

Copyright © 2018 Roxana Braga de Andrade Teles et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Alzheimer's and Parkinson's diseases are considered the most common neurodegenerative disorders, representing a major focus of neuroscience research to understanding the cellular alterations and pathophysiological mechanisms involved. Several natural products, including flavonoids, are considered able to cross the blood-brain barrier and are known for their central nervous system-related activity. Therefore, studies are being conducted with these chemical constituents to analyze their activities in slowing down the progression of neurodegenerative diseases. The present systematic review summarizes the pharmacological effects of flavonoids in animal models for Alzheimer's and Parkinson's diseases. A PRISMA model for systematic review was utilized for this search. The research was conducted in the following databases: PubMed, Web of Science, BIREME, and Science Direct. Based on the inclusion criteria, 31 articles were selected and discussed in this review. The studies listed revealed that the main targets of action for Alzheimer's disease therapy were reduction of reactive oxygen species and amyloid beta-protein production, while for Parkinson's disease reduction of the cellular oxidative potential and the activation of mechanisms of neuronal death. Results showed that a variety of flavonoids is being studied and can be promising for the development of new drugs to treat neurodegenerative diseases. Moreover, it was possible to verify that there is a lack of translational research and clinical evidence of these promising compounds.

1. Introduction

For many years, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases have been a major focus of neuroscience research to understanding the cellular alterations and pathophysiological mechanisms [1]. Neurodegenerative diseases are multifactorial conditions in which many biological processes become unregularly [2] mediated by endogenous, genetic, and environmental factors [3] intimately associated with progressive brain damage [4].

The generation of free radicals and oxidative stress producing cellular impairments is often cited as an important

factor in the etiology of neurodegenerative diseases [5, 6]. Beyond the oxidative stress, the neurodegenerative disease pathogenesis has some common characteristics such as neuroinflammation, abnormal accumulation of proteins, and aging [7–9].

Alzheimer's disease (AD) is a chronic progression characterized by loss of memory and cognitive deficits such as agnosia, aphasia, and apraxia, among others, facts that cause interference in daily life and in the individual's work. The prevalence rate is about 7% for individuals aged 65 or more, with the risk doubling every 5 years [10].

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's. PD is a chronic neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra which leads to decreased levels of dopamine in the striatum and disrupted motor control [11, 12]. Its incidence is usually comprised between 10 and 50/100,000 person-years, and its prevalence between 100 and 300/100,000 population and prevalence both increase progressively after 60 years of age [13].

Recently, studies showed considerable efforts on search of antioxidant plant-derived polyphenol compounds with neuroprotective potential for the treatment of neurodegenerative diseases [14]. Dietary flavonoids have been suggested to prevent and treat neurodegenerative diseases [9].

Flavonoids are found in numerous plants, fruits, and vegetables [15] and are known as the most common phytochemicals which possess a multiple range of pharmacological effects [16]. These secondary metabolites have been described as potent antioxidant, free radical scavengers, and metal chelators [4–17], also presenting anticholinesterase [18], antiaging [7], neuroprotective [2, 19] and anti-inflammatory properties [5, 20, 21], and neurotrophic roles [22], ameliorating learning and memory [23], possessing potent antidepressant and anti-amyloidogenic effects [16], suppressing the activation of microglia, and mediating inflammatory processes in the central nervous system (CNS) [24]. Moreover, flavonoids are able to cross the blood-brain barrier with chronic or acute administration suggesting that these compounds can feasibly have a direct effect on the brain, so this chemical compounds could be used as a prophylactic, in order to slow down the progression of diseases such as AD and PD [11].

Considering that neurodegenerative diseases represent some of the greatest challenges for basic science and clinical medicine, our study consists a systematic review of pharmacological reports involving the use of flavonoids on neurodegenerative conditions, especially AD and PD.

2. Materials and Methods

2.1. Search Strategy. The present systematic review was conducted according to the guidelines for Transparent Reporting of Systematic Reviews and Meta-Analyses (PRISMA statement) [25]. In this review, the specialized databases Pubmed, Web of Science, "Biblioteca Regional de Medicina" (BIREME), and Science Direct were used for the literature search in September and October 2017, using the terms "flavonoids, bioflavonoids, flavonols, flavonoid, anthocyanin, flavone, flavones, 2-phenyl-benzopyrans, 2-phenyl-chromenes, isoflavonoids or flavonones," combined with "neurodegenerative diseases, Alzheimer disease or Parkinson disease." We did not contact investigators, nor did we attempt to identify unpublished data.

2.2. Study Selection. In this step, two independent researchers (T.C.C.P. and T.C.D.) conducted the selection, and electronic search titles, selected abstracts, and full-text articles were independently reviewed by a third researcher who conducted the analysis of the full text. Disagreements on study inclusion/exclusion were resolved with a consensus between all investigators. The following inclusion criteria were applied: studies with flavonoids acting on CNS in animal models. Studies were excluded according to the following exclusion criteria: review articles, meta-analyses, abstracts, conference proceedings, editorials/letters, case reports, studies in humans, and published over 5 years. Additional papers were included in our study after analyses of all references from the selected articles.

2.3. Data Extraction. Data were extracted by one reviewer using standardized forms and were checked by a second reviewer. Extracted information included the substance, animal models, doses and concentrations, and evaluated parameters.

2.4. Methodological Quality Assessment. The risk of bias and quality of preclinical evidences were assessed using a checklist adapted from Hooijmans et al. [26] and Siqueira-Lima et al. [27]. This analysis allowed evaluating the methodological quality of the selected manuscripts regarding the outcome measurements, randomization of treatment allocation, blinded drug administration, blinded outcome assessment, appropriate description of the doses and routes of administration used, and appropriately reported statistical analysis of data. Studies that reported randomization of animals, blinding, outcome measurements, and statistical analysis were considered to be of higher methodological quality.

3. Results and Discussion

3.1. Study Selection. The primary search identified 2456 articles, including 347 from PubMed, 1338 from BIREME, 595 from Web of Science, and 176 from Science Direct. Among these, 1157 documents were published more than five years ago and were therefore excluded from the review. In addition, 305 manuscripts were indexed in two or more databases and were considered only once, resulting in 994 eligible articles. After an initial screening of titles and abstracts, followed by a full text analysis, 31 articles were included in this systematic review, while the reminder ($n = 728$) was excluded since they did not meet the inclusion criteria. A flowchart illustrating the progressive study selection and numbers at each stage is shown in Figure 1.

3.2. Characteristics of Included Studies. In general, the studies were conducted by research groups from several countries, mainly China (10 reports, 32.3%), Republic of Korea (07 reports, 22.6%), and India (05 reports, 16.1%). These findings reflect the contribution of Oriental medicine in the search for new drugs from natural products. In fact, Ayurveda and

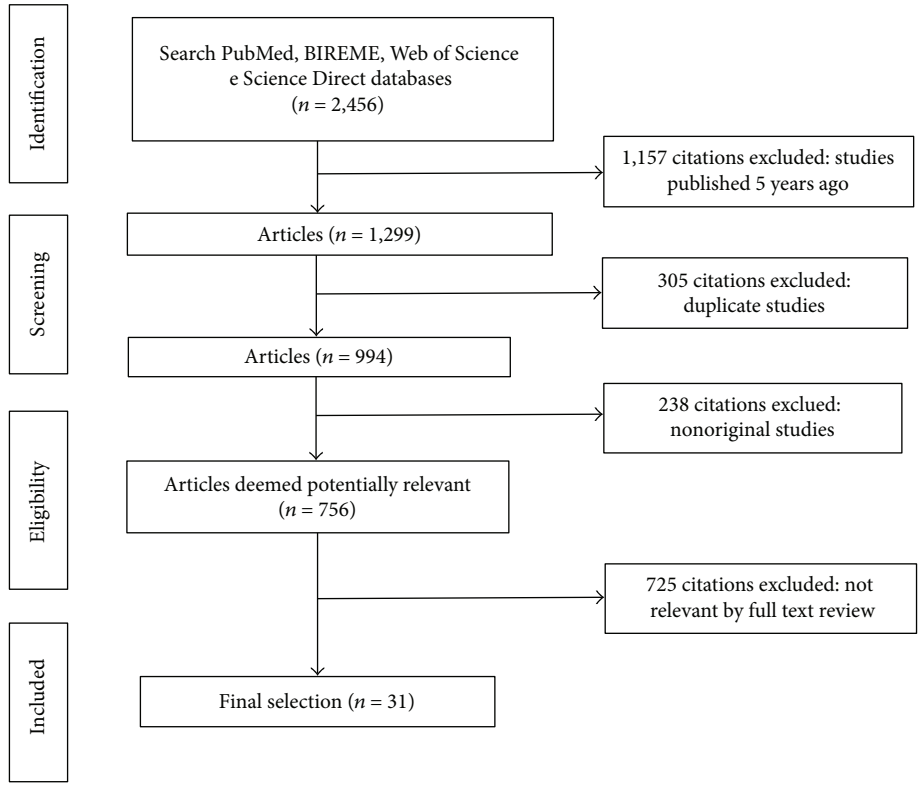


Figure 1: Flowchart for the literature searching and screening.

Traditional Chinese Medicine (TCM) are major traditional treatment systems used not only in India and China, but also in several countries [28, 29]. These systems have provided important information for the development of new pharmaceutical products based on plant extracts or even small molecules of original chemical structure and with innovative mechanisms of action. Their by-products have been applied in a variety of diseases, including central nervous system disorders such as AD and PD [30, 31].

Chinese herbal medicines have been clinically used to treat AD for a long time with significant effectiveness [32]. Recently, Jiang et al. [33] have described the progress of TCM in the treatment of AD, highlighting traditional Chinese medicinal herbs as potential AChE inhibitors for anti-AD approach. Among bioactive molecules, numerous flavonoids have been cited, including quercetin, apigenin, epigallocatechin-3-gallate, catechin, epigallocatechin, epicatechin-3-*O*-gallate, icariin, procyanidin, and silibinin. Similarly, TCM has also been reported for the treatment of PD. Several Chinese plant derivatives have been shown to be promising neuroprotective agents in PD, including resveratrol, curcumin, and ginsenoside [34, 35]. In this context, such information justifies the significant number of Chinese authorship studies included in this review.

Although most of the manuscripts was focused on AD (20 reports), a relevant number of studies involving experimental protocols in PD were also found (13 reports). Many studies have evaluated important behavioral parameters related to memory, motor functions, and cognitive activities of animals. However, biochemical and molecular targets were also verified by colorimetric and enzymatic assays; histological, biochemical, and hematological analyses; and western blot, immunohistochemical, and immunofluorescence techniques. The parameters evaluated and main outcomes are summarized in Table 1.

3.3. Methodological Quality. Concerning the methodological quality, all reports were carefully evaluated through a standard checklist. As shown in Figure 2, only one of the included articles reported sample size calculations. Only 48.4% of the studies reported randomization of animals for outcome assessment. Allocation of the animals in different treated groups was adequately reported by 64.5% of the studies. Blinding of investigators during animal treatment and outcome evaluation was found only in 16.1 and 12.9% of the studies, respectively. The remaining studies did not report any blindness strategy.

Although these parameters are most often applied in clinical trials, the need of randomization, blinding, allocation concealment, and attempt to minimize variation has been discussed and strongly recommended for preclinical studies, especially when the experimental protocols involve the evaluation of behavioral parameters. Since human studies are often justified based on results from animal studies, it is extremely important to plan preclinical protocols that follow the same rigor of clinical trials. In addition to reducing the risk of bias, this research strategy avoids obtaining distinct results between the two study designs (preclinical and clinical) [67–69]. In this context, the methodological quality of the studies included in our review was

Table 1: Characteristics of studies inserted in the review.

Authors, year, country	Substance(s)	Disease	Animals, (strain/sex) n (per group)	Doses, route, administration, period	Preclinical models	Evaluated parameters	
						Behavior	Biochemical/molecular
Wei et al., 2013 [36], China	(2S)-5, 2′, 5′-trihydroxy-7-methoxyflavanone (TMF)	Alzheimer's	Mice (Kunming/NR) ($n = 8$)	4 or 8 mg/kg, i.p., 1 week	D-galactose	(i) Spatial learning and memory (Morris water maze test)	(i) Colorimetric assay: GSH and GSSG (ii) ELISA: AP-1 and BDNF (iii) Western blot: CREB and p-CREB
Mani et al., 2013 [37], India	Naringin	Alzheimer's	Rats (W/M), $n = 6$	100 mg/kg, p.o., 21 days	Deltamethrin	(i) NR	(i) Agarose gel electrophoresis: DNA fragmentation (ii) Biochemical: LDH, CK, AChE, SOD, CAT, GPx, GR, GSH, vitamin C, vitamin E, and lipid peroxidation level (iii) Histological analysis (iv) Native gel electrophoresis: SOD and CAT
Nakajima et al., 2013 [38], Japan	Nobiletin	Alzheimer's	Rats (SAMP8/M) $n = 47$	10 or 50 mg/kg, i.p., unclear	Senescence-accelerated prone mouse (SAMP8) model	(i) Nonspatial memory (novel object test) (ii) Contextual and auditory fear memory (fear conditioning test) (iii) Emotional reactivity, anxiety (elevated plus maze test)	(i) Biochemical: MDA, protein carbonyl level, SOD, and GPx (ii) HPLC: GSH and GSSG (iii) Real-time RT-PCR: GPx1 and GPx4 (iv) Western blot: $A\beta_{1-42}$, tau, p-tau, GPx1, and GPx4
Moghbelinejad et al., 2014 [39], Iran	Rutin	Alzheimer's	Rats (W/M) $n = 30$	100 mg/kg, i.p., 3 weeks	Amyloid beta ($A\beta_{1-42}$)	(i) Memory retrieval (passive avoidance apparatus test)	(i) Biochemical: MDA and total SH groups (ii) Real-time RT-PCR: BDNF, ERK1, ERK2, and CREB1
Li et al., 2014 [40], Germany	Hesperidin	Alzheimer's	Mice (APP/PS1–21/M), $n = 12$	100 mg/kg, p.o., NR	Transgenic APP/PS1–21 mouse model	(ii) Nesting behavior (nest building assay) (iii) Social behavior, degree of interaction (social interaction assay)	(i) Double immunostaining (ii) Immunohistochemistry: $A\beta$, GFAP, TGF-β1, and Alzheimer precursor protein A4
Javed et al., 2014 [41], India	Hesperidin	Alzheimer's	Mice (S/M), $n = 10$ or $n = 12$	100 and 200 mg/kg, i.p, 15 days	Streptozotocin	(i) Spatial learning and memory (Morris water maze test)	(i) Biochemical: TBARS, GSH, AChE, ganglioside, and phospholipids (ii) Immunohistochemistry: GFAP, NF-κB, iNOS, and COX-2

Table 1: Continued.

Authors, year, country	Substance(s)	Disease	Animals, (strain/sex) n (per group)	Doses, route, administration, period	Preclinical models	Evaluated parameters	
						Behavior	Biochemical/molecular
Walker et al., 2015 [42], USA	Epigallocatechin gallate	Alzheimer's	Mice (TgCRND8 (Tg) and wild type (nTg)/M and F), $n = 10$, $n = 11$, or $n = 12$	50 mg/kg, p.o., 4 months	TgCRND8 amyloid precursor protein transgenic mice	(i) Acquisition experience (nest building) (ii) Locomotor activity and exploratory (open field test) (iii) Novelty-seeking and anxiety-like behaviors (light-dark box) (iii) Learning (Barnes maze)	(i) ELISA: Aβ_{1-42}
Kou et al., 2016 [43], China	Dihydromyricetin	Alzheimer's	Rats (SD/M), $n = 10$	100 and 200 mg/kg, p.o., 6 weeks	D-galactose	(i) Spatial learning and memory (Morris water maze test)	(i) Histological analysis (ii) Real-time PCR: miR-34a (iii) SA-β-gal staining (iv) Western blot: caspase-3, Bcl-2, SIRT1, p53, p21, Atg7, LC3-II/LC3-I, GFAP, and mTOR
Ali et al., 2016 [44], Republic of Korea	Anthocyanins and anthocyanin-loaded PEG-AuNPs	Alzheimer's	Mice (C57BL/M), $n = 15$	12 μg/g/day, p.o., 14 days	Amyloid beta (Aβ_{1-42})	(i) Spatial learning and memory (Morris water maze and Y-maze tests)	(i) Immunohistochemical Nissl and FJB staining (ii) Immunofluorescence (iii) Western blot: Aβ, BACE-1, SNAP23, synaptophysin, p-AMPARs, p-PI3K, p-Akt, p-GSK3β, p-tau, PSD95, caspase-3, Cyt c, Bax, Bcl2, and poly (ADP-ribose) polymerase-(PARP-1)
Ramalingayya et al., 2016 [45], India	Naringin and Rutin	Alzheimer's	Rats (W/F), $n = 12$	50 and 100 mg/kg, p.o., 15 days	Donepezil and scopolamine	(i) Locomotor activity and time spent in the center zone (open field test) (ii) Nonspatial memory (object recognition task)	(i) Hematological
Chen et al., 2016 [46], China	Quercetin	Alzheimer's	Mice (C57BL/6J/M), NR	30 mg/kg, p.o., NR	Cognitive disorders per high-fat diet (HFD)	(i) Spatial learning and memory (Morris water maze test)	(i) Immunohistochemisty: p-PERK, p-IRE1α, NLRP3, and p-tau (ii) Light microscopy: CA1 (iii) Western blot: AMPK, p-AMPK, IRE1α, p-IRE1α, eIF-2α, p-IF-2α, TXNIP, NLRP3, GSK-3β, p-GSK-3βser9, tau, and p-tau

Table 1: Continued.

Authors, year, country	Substance(s)	Disease	Animals, (strain/sex) n (per group)	Doses, route, administration, period	Preclinical models	Evaluated parameters	
						Behavior	Biochemical/molecular
Song et al., 2017 [47], China	Silibinin	Alzheimer's	Rats (SD/M), NR	25, 50, and 100 mg/kg, p.o., 28 days	Amyloid beta ($A\beta_{25-35}$)	(i) Anxiety and locomotor activity (Elevated plus maze test) (ii) Spatial learning and memory ability (Morris water maze test) (iii) Learning and memory (novel object-recognition test) (iv) Memory (memory flexibility test)	(i) Biochemical: MDA and GSH (ii) ELISA: IL-1β, IL-4 (iii) Flow cytometric analysis (iv) Transmission electron microscopy (v) Western blot: NF-κB, COX-2, iNOS, p53, and p-p53
Moreno et al., 2017 [48], Spain	Quercetin and quercetin-loaded nanoparticles	Alzheimer's	Rats (SAMP8/M), $n = 8$	Quercetin (25 mg/kg, p.o., 2 months) Quercetin-loaded nanoparticles (25 mg/kg every 48 hours, p.o., 2 months)	Senescence-accelerated prone mouse (SAMP8) model	(i) Locomotor activity (open field test) (ii) Motor coordination and balance (rotarod test) (ii) Exploratory motivation (marble burying test) (iii) Spatial memory and both the working and reference memory functions (Morris water maze test)	(i) Western blot: GFAP and CD11b
Godoy et al., 2016 [49], Chile	Quercetin	Alzheimer's	Rats (B6.129S7-Sod2tm1Leb/J/NR), NR	50 mg/kg, two times a week, p.o., 4 weeks	Amyloid beta ($A\beta_{25-35}$)	(i) NR	(i) Electrophysiology
Palle and Neerati, 2016 [50], India	Quercetin and quercetin nanoparticles	Alzheimer's	Rats (W/M), $n = 6$	30 mg/kg, i.p., 8 days	Scopolamine	(i) Conditioning, avoidance responses (conditioned avoidance test) (ii) Learning, memory (rectangular maze test)	(i) Biochemical: MDA, GPx, AChE, and CAT (ii) Histological analysis
Ahmad et al., 2016 [51], Republic of Korea	Fisetin	Alzheimer's	Mice (C57BL/6N/M), $n = 12$	20 mg/kg, i.p., 2 weeks	Amyloid beta $A\beta_{1-42}$	(i) Spatial memory and both the working and reference memory functions (Morris water maze test)	(i) FJB staining (ii) Immunofluorescence: Aβ (B4), synaptophysin, PSD95, p-tau, GFAP, and Iba-1 (iii) Immunohistochemistry: caspase-3 (iv) Western blot: caspase-9, SYN, p-AMPAR1, p-CREB, p-CAMKII, p-PI3K, and p-Akt

Table 1: Continued.

Authors, year, country	Substance(s)	Disease	Animals, (strain/sex) n (per group)	Doses, route, administration, period	Preclinical models	Evaluated parameters	
						Behavior	Biochemical/molecular
Rehman et al., 2017 [52], Republic of Korea	Anthocyanins	Alzheimer's	Rats (SD/M), $n = 13$	100 mg/kg, i.p., 7 weeks	D-galactose	(i) Spatial learning and memory (Morris water maze and Y-maze tests)	(i) Biochemical: ROS, MDA (ii) Immunofluorescence: Aβ, 8-OxoG, p-JNK, GFAP, and Iba-1 (iii) Western blot: Aβ, BACE-1, RAGE, 8-OxoG, TNF-α, iNOS, p-JNK, Bax, Bcl2, PARP-1, syntaxin, synaptophysin, SNAP-23, p-CREB, GFAP, and Iba-1
Kim et al., 2017 [53], Republic of Korea	Anthocyanins alone and anthocyanin-loaded poly (ethylene glycol) gold nanoparticles (PEG-AuNPs)	Alzheimer's	Mice (C57BL/6N/M), $n = 8$	10 mg/kg, i.v., 14 days	Amyloid β ($A\beta_{1-42}$)	(i) NR	(i) ICP-AES (ii) Immunofluorescence: GFAP, Iba-1, and RAGE (iii) Nissl staining (iv) TEM (v) Western blot: Aβ, BACE-1, GSK3β, CDK5, tau, NF-κB, iNOS, p-JNK, Bcl2, Bax, Cyt c, FJB, COX-2, NOS3, IL-1β, and TNF-α
Sharma et al., 2016 [54], India	Quercetin	Alzheimer's and Parkinson's	Rats (W/M), $n = 6$	10 mg/kg, p.o., 12 weeks	Aluminum	(i) NR	(i) Biochemical: ROS, MnSOD (ii) DNA isolation for DNA fragmentation (iii) Electron microscopy analysis (iv) Histological analysis (v) Immunohistochemistry: MnSOD, c-c, and caspase-3 (vi) RT-PCR: MnSOD; (vi) Western blot: MnSOD, Cyt c, Bax, Bcl-2, p53, and caspase-3
Jeong et al., 2015 [55], Republic of Korea	Naringin	Epilepsy, Parkinson's, and Alzheimer's	Mice (C57BL/6/M) NR	80 mg/kg, i.p., 7 days	Kainic acid	(i) NR	(i) Immunohistochemical: NeuN and Iba-1 (ii) Light microscopy: CA1 (iii) Western blot: LC3B and TNFα
Lee et al., 2014 [56], Republic of Korea	Baicalein	Parkinson's	Mice (C57B/6/M), $n = 8 - 14$	1 and 10 mg/kg (i.p.), 7 days	MPTP	(i) Motor coordination and balance (rotarod test)	(i) DAB immunostaining: TH (ii) Double label immunostaining: TH, GFAP, and Iba-1 (iii) Histological analysis (iv) Western blot: GFAP

Table 1: Continued.

Authors, year, country	Substance(s)	Disease	Animals, (strain/sex) n (per group)	Doses, route, administration, period	Preclinical models	Evaluated parameters	
						Behavior	Biochemical/molecular
Antunes et al., 2014 [57], Brazil	Hesperidin	Parkinson's	Mice (C57 BL/6/F), $n = 10$	50 mg/kg, p.o., 28 days	6-OHDA	(i) Depression (Tail-suspension test) (ii) Spatial learning and memory (Morris water maze) (iii) Locomotor activity and time spent in the center zone (open field test)	(i) Biochemical: GSH, ROS, TRAP, SOD, CAT, GR, GPx, GST, DA, DOPAC, and HVA
Lou et al., 2014 [58], China	Naringenin	Parkinson's	Mice (C57BL/6/F), $n = 10$	70 mg/kg, p.o., 4 days	6-OHDA	(i) Rotational behavior—numbers of rotations (apomorphine-induced circling behavior)	(i) HPLC-MS: DA, DOPAC, and HVA (ii) Immunohistochemistry: TH (iii) Western blot: Nrf2, HO-1, GCLM, GCLC, Lamin A, cleaved caspase-3, p-JNK, JNK, p-p38, and p38
Wang et al., 2015 [59], China	Tanshinone I	Parkinson's	Mice (C57BL/6/M), NR	5, 10 mg/kg, p.o., 7 days	MPTP	(i) Motor coordination and balance (rotarod test)	(i) Biochemical: ALT, AST, and ALP (ii) ELISA: TNF-α and IL-10 (iii) HPLC: DA, DOPAC, HVA, and MPP+ (iv) Immunohistochemistry: TH and Iba-1
Chen et al., 2015 [60], China	Silibilin	Parkinson's	Rats (M and F), NR	25 and 50 mg/kg, p.o. in second day	Increased neonatal iron intake	(i) Locomotor activity and time spent in the center zone (open field test) (ii) Motor coordination and balance (rotarod test)	(i) Biochemical: MDA and GSH (ii) HPLC-ECD: DA and 5-HT
Mu et al., 2016 [61], China	Quercetin	Parkinson's	Rats (SD/M), NR	100, 200, and 400 mg/kg, i.g., NR	6-OHDA	(i) Rotational behavior—numbers of rotations (apomorphine-induced circling behavior)	(i) HPLC-ECD: DA, DOPAC, HVA, 5-HT, and 5-HIAA
Lee et al., 2015 [62], Korea	Silibinin	Parkinson's	Mice (C57B/6/M), $n = 10$ or 12	1 or 10 mg/kg, i.p., 5 days	MPTP	(i) Motor coordination and balance (rotarod test)	(i) DAB immunostaining: TH (ii) Double label immunostaining: GFAP and Iba-1
Hu et al., 2016 [63], China	Baicalein	Parkinson's	Mice (C57BL/6/M), NR	100 mg/kg, i.p., 7 weeks to 12 weeks	Rotenone	(i) Motor coordination and balance (rotarod test) (ii) Motor dysfunctions (grid test)	(i) HPLC: DA, DOPAC, and HVA (ii) Immunofluorescence: α-syn, TH, and ChAT (iii) Real-time PCR: α-syn (iv) TEM (v) Western blot: α-synuclein and GAPDH

Table 1: Continued.

Authors, year, country	Substance(s)	Disease	Animals, (strain/sex) n (per group)	Doses, route, administration, period	Preclinical models	Evaluated parameters	
						Behavior	Biochemical/molecular
Zhang et al., 2017 [64], China	Baicalein	Parkinson's	Rats (SD/M), $n = 15$, or 10	100, 200, and 400 mg/kg, p.o., 28 days	Rotenone	(i) Locomotor activity (open field test) (ii) Motor coordination and balance (rotarod test) (iii) The inclined plane assessment	(i) Immunohistochemistry: TH (ii) TEM (iii) TUNEL staining (iv) Western blot: caspase-3, PGC-1α, NRF-1, and TFAM
Goes et al., 2017 [65], Brazil	Chrysin	Parkinson's	Mice (C57B/6J/M), $n = 6$	10 mg/kg, p.o., 28 days	6-OHDA	(i) Motor coordination and balance (rotarod test) (ii) Rotational behavior—numbers of rotations (apomorphine-induced circling behavior)	(i) ELISA: IFN-γ, TNF-α, IL-6, IL-10, NF-κB, S100B, BDNF, GDNF, and NGF (ii) HPLC: DA, DOPAC, and HVA (iii) TRAP and TAR (iv) Immunohistochemistry: TH^+ neurons
Ay et al., 2017 [66], USA	Quercetin and quercetin-containing formulation (QB3C)	Parkinson's	Mice (MitoPark and C57BL/6/M/F), $n = 8$ or $n = 9$	Quercetin (25 mg/kg, p.o., 6 weeks) QB3C comprising quercetin (175 mg/kg, p.o., 8 weeks)	MitoPark transgenic mouse models	(i) Locomotor activity (open field test) (ii) Motor coordination and balance (rotarod test)	(i) HPLC: DA, DOPAC, and HVA (ii) DAB immunostaining: TH

Animals: SD: Sprague-Dawley; W: Wistar; S: Swiss; SAMP8: senescence-accelerated prone mouse 8; NR: not reported. Parameters assessed: DPPH: 2,2-diphenyl-1-picrylhydrazyl radical; MDA: malonaldehyde; TBARS: thiobarbituric acid reactive substances; AAPH: 2,2′-azobis(2-methylpropionamidine) dihydrochloride; FeSO4: ferrous sulphate; 6-OHDA: 6-hydroxydopamine; MPTP: 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine; FJB: Fluoro-Jade B; GSH: reduced glutathione; GSSG: oxidized glutathione; AP-1: activator protein-1; BDNF: brain-derived neurotrophic factor; CREB: cAMP response element-binding protein; p-CREB: phosphorylated; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; LDH: lactate dehydrogenase; CK: creatine kinase; AChE: acetylcholinesterase; MDA: malondialdehyde; p-tau: phosphorylated tau; TGF-β1: transforming growth factor beta 1; SYN: synaptophysin; p-AMPAR1: phospho-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; p-CRE: phosphorylated cAMP response element binding protein; p-CAMKII: phosphorylated calcium/calmodulin-dependent protein kinase II; p-PI3K: phosphorylated phosphatidylinositol-4,5-bisphosphate 3-kinase; p-Akt: phosphorylated protein kinase B; GFAP: antiglial fibrillary acidic protein; Iba-1: anti-ionized calcium-binding adapter molecule 1; 8-OxoG: 8-oxoguanine; p-JNK: C-jun N-terminal kinase; ICP-AES: inductively coupled plasma-atomic emission spectrometer; TEM: transmission electron microscopy; Aβ: brain expression levels of amyloid beta; BACE-1: beta-site APP cleaving enzyme 1; GSK3β: glycogen synthase kinase-3β; CDK5: cyclin-dependent kinase 5; GFAP: glial fibrillary acidic protein; NF-κB: nuclear factor kappa B; iNOS: inducible nitric oxide synthase; COX-2; NOS3; IL-1β; TNF-α; p-JNK: phospho-JNK; Bcl2; Bax; Cyt c: cytochrome c; FJB. RAGE receptor for advanced glycation end products; MnSOD: mitochondrial superoxide dismutase; NeuN: neuronal nuclei; LC3B: microtubule-associated protein light chain 3 isoform B; MS: mass spectrometry; DA: dopamine; DOPAC: dihydroxyphenylacetic acid; HVA: homovanillic acid; HO-1: hemeoxygenase; Nrf2: nuclear factor E2-related factor 2; GCLC: glutathione cysteine ligase regulatory subunit; GCLM: glutathione cysteine ligase modulatory subunit; JNK: c-Jun N-terminal kinase; MPP+: 1-methyl-4-phenylpyridinium; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; HPLC: high-performance liquid chromatography; ECD: equipped with electro chemical detector; TRAP: total reactive antioxidant potential; TAR: total antioxidant reactivity; S100B: calcium-binding protein B; GDNF: glial cell line-derived neurotrophic factor; NGF: nerve growth factor; DAB: diaminobenzidine; *SA-β-Gal*: senescence-associated *β-galactosidase*.

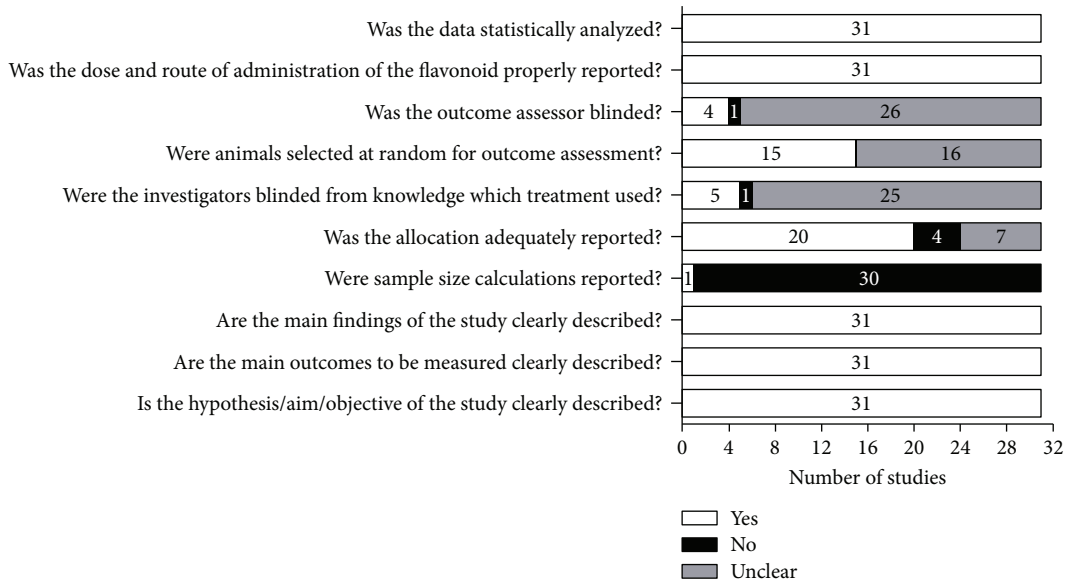


Figure 2: Methodological quality of included studies. *Light bars* indicate the proportion of articles that met each criterion; *dark bars* indicate the proportion of studies that did not; and *white gray bars* indicate the proportion of studies with unclear answers.

considered low to moderate, which limits the interpretation of the results.

3.4. Animal Models in Alzheimer's Disease. AD is a gradual and highly prevalent neurodegenerative disease and has as pathological neuroinflammation characteristic, neuronal loss, and impairment of cognitive function [47, 70]. AD is classified into two subtypes: early-onset familial outcome, related to specific mutations in genes that code for presenilin 1 (PS1), presenilin 2 (PS2), and amyloid precursor protein (APP), and late-onset sporadic disease, associated with mutations in genes that code for apolipoprotein E (ApoE), which include several environmental and genetic risk factors, yet unknown [71].

Among the pathological features, there is an amyloid dense core of β-amyloid peptide (Aβ) and intracellular neurofibrillary tangles (NFTs) composed of an abnormally phosphorylated form of the tau protein [41]. The deposition of amyloid β peptide culminates in pathological processes of synaptic and cognitive dysfunction and neuronal death [47, 70].

Animal models recapitulate pathological feature characteristic of AD in addition to several phenotypic traits simulate of the disease, acting as therapeutic targets and in their preclinical validation. According to Laurijssens et al. [72], animal models used in AD can be divided into three categories: natural (dog, mouse lemur, Octodon, and Rhesus monkey), genetic (APP mice, Tg2576 mice, and PSAPP mice), and interventional models (rat, intrahippocampal amyloid infusion model).

In the selected studies, interventional models were the most used [39, 44, 47, 49, 51, 53]. In fact, interventional models would generally be better at identifying symptomatic or corrective treatment. These models can provide important insights such as the Aβ pharmacochemical substance-induced model and the understanding of inflammation, neurotoxicity, neurodegeneration and synaptic function, specific brain lesions, and neuronal mechanism underlying memory dysfunction [72, 73].

3.5. Animal Models in Parkinson's Disease. PD is a progressive neurodegenerative disease defined by the selective loss of dopaminergic neurons in the nigrostriatal pathway [62]. A disruption of synaptic activity may represent the primary event in PD pathogenesis. Recently, studies on the neurodegeneration mechanisms in PD have revealed pathological features and important genetic influences. Common pathogenic pathways are present in PD, such as proteostatic deficits, mitochondrial dysfunctions, oxidative stress, and inflammation process [74, 75].

As well as knowledge of new pathophysiological mechanisms involved, the development of experimental models in PD has also advanced. As in other neurodegenerative disorders, PD experimental protocols have become increasingly sensitive and accurate, allowing not only the evaluation of new drugs, but also the understanding of the molecular mechanisms that play an important role in the disease [74]. However, it is important to note that animal models still cannot express the complexity of human pathological hallmarks and clinical features, each one has specific limitations, and its choice must be conditioned to what best responds to the research objective [74, 76].

The papers selected in this review reported animal models to evaluate pathogenic mechanisms, motor and nonmotor manifestations of PD by administering different neurotoxic agents such as 6-hydroxydopamine (6-OHDA) [57, 58, 61, 65], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [56, 59, 62], or rotenone [63, 64]. The most cited animal model in the selected studies was 6-OHDA. It exerts toxic effects on catecholaminergic neurons and is characterized as a neurotoxin structural analogue of catecholamines, dopamine, and noradrenaline. It is toxic at the peripheral and central levels although its toxicity in the

CNS is only possible through direct stereotaxic surgery. Its neurotoxicity involves the accumulation of toxin in the catecholaminergic neurons triggering the alteration of the cellular homeostasis and neuronal damages. 6-OHDA oxidation by MAO-A generates H_2O_2 which, besides being highly cytotoxic, triggers the production of oxygen radicals [77–79]. Another model included the alteration of dopaminergic neurotransmission by drugs, such as reserpine [80], or by genetic manipulation to study the progression of dopaminergic cell degeneration and motor signs [81].

The models that use toxins are widely used and act in the replication of most pathological and phenotypic features of the disease. However, as a limitation of this tool, the loss of approximately 70 to 80% of the dopaminergic neurons in the acutely induced neurodegeneration is difficult to achieve, making it difficult to explore dysfunction and progression. In view of the early synaptic dysfunction and neurodegeneration that are inherent to the pathology, new models are necessary to help the early stages of the disease, as well as new therapeutic strategies for the treatment of PD [76].

3.6. Flavonoids in the AD and PD Treatment. The plants use adaptation systems in extreme conditions, such as high temperatures, lighting, and water scarcity, which culminate in oxidative damages leading to excessive production of free radicals. Among the main natural antioxidant agents, we highlight carotenoids, flavonoids, anthocyanins, and phenolic derivatives that act in several mechanisms against oxidative stress [82].

Flavonoids are a class of secondary metabolites that always attract the attention of the pharmaceutical industry due to their versatility in therapeutic properties. Additionally, they are part of the human diet due to their abundance in vegetables, fruits, seeds, and beverages such as coffee, tea, and red wine, as well as in medicinal herbs. More than 9000 different flavonoids have been identified and are divided into six subclasses based on their molecular structure. These subclasses include flavonols (rutin, quercetin), flavanols (catechin, epicatechin, and epigallocatechin), isoflavones (genistein, daidzein, glycetin, and formanantine), anthocyanidins (cyanidin, malvidine, and delphinidine), flavanones (hesperetin, naringenin), and flavones (apigenin, luteolin) [83].

Studies indicate that phenolic compounds play an important role in prevention and treatment of age-associated neurodegenerative diseases. Among the neuroprotective actions of dietary flavonoids, we highlighted their potential to modulate cell signaling pathways, protect neurons against oxidative stress, inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, subsequently decrease reactive oxygen species production, and downregulate proinflammatory transcription factors, as well as its ability to suppress neuroinflammation through reduction of the release of cytokines [84].

In our systematic review, quercetin, rutin, silibinin, naringin, baicalein, hesperidin, and anthocyanins (Figure 3) were the most studied flavonoids for PD and AD treatment. In this sense, we describe below the pharmacological effects of these compounds to better understand the role of flavonoids in the treatment of AD and PD (Figures 4 and 5, resp.). In general, they act to improve the progression of the disease, but in the included reports, there is no consensus of dose, treatment duration, and route of administration of these flavonoids. However, the researchers argue that they have good bioavailability.

3.6.1. Quercetin. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a flavonoid found naturally in plants and among other foods such as onions, apples, broccoli, and red wine. It presents pharmacological, antioxidant, cardioprotective, and antiapoptotic properties [85]. Some studies relate their neuroprotective action in the prevention of neurodegenerative diseases like AD and PD [86]. Sharma et al. [54] evaluated the effect of quercetin manipulation in young rats treated with aluminum on isolated mitochondrial hippocampus. A decrease in the levels of reactive oxygen species (ROS) was observed; however, there were no significant changes in mitochondrial superoxide dismutase activity (MnSOD) [54].

Expression of the apoptotic markers in the mitochondrial fraction revealed levels of reduced Bax and increased Bcl. When the cytosolic fractions were evaluated, the expression of Bcl-2 and Cyt c was decreased, reducing the activation of caspase-3 suggesting that the administration of quercetin inhibits apoptosis. Quercetin prevents the release of Cyt c and subsequent activation of caspase-3 and decreases p53 expression. The histopathological analysis revealed no significant degeneration [54]. Thus, the study performed by Sharma et al. [54] has shown that quercetin attenuates aluminum-induced mitochondrial turgor, loss of ridge, and chromatin condensation.

Environmental factors such as hyperlipidemic diet consumption may increase the inflammatory response in the brain by increasing the release of cytokines such as TNF-α and IL-6 [87]. Studies have shown that the action of these cytokines promotes neuronal death by activating the apoptotic cascade [88]. Quercetin is also involved in the activity of AMPK, an energy sensor, which has the ability to prevent the phosphorylation of tau protein [89]. Tau protein facilitates the polymerization of tubulin in the cell, resulting in microtubule formation. In the neurofibrillary tangles, the aggregation of Tau is by irreversible phosphorylation suffered by this protein. This prevents its normal function and at the same time facilitates its aggregation in fibrils, preventing the normal functioning of the neuron [90].

Chen et al. [46] evaluated whether the activation of AMPK through the action of quercetin is able to block or not the phosphorylation of tau protein in animals submitted to a hyperlipidic diet. It was seen that quercetin was able to promote downregulation in blood levels of TNF-α and IL-6. And it especially prevented tau phosphorylation by restoring AMPK activation. Additionally, quercetin inhibits GSK3β enzyme activity by dephosphorylation, attenuates the expression of P-PERK and P-IRE1α (membrane sensors present in the endoplasmic reticulum whose activation indicates oxidative stress), and normalizes the inflammation of NLRP3 (inflammation promoter). Improvement in learning and spatial memory was also seen [46].

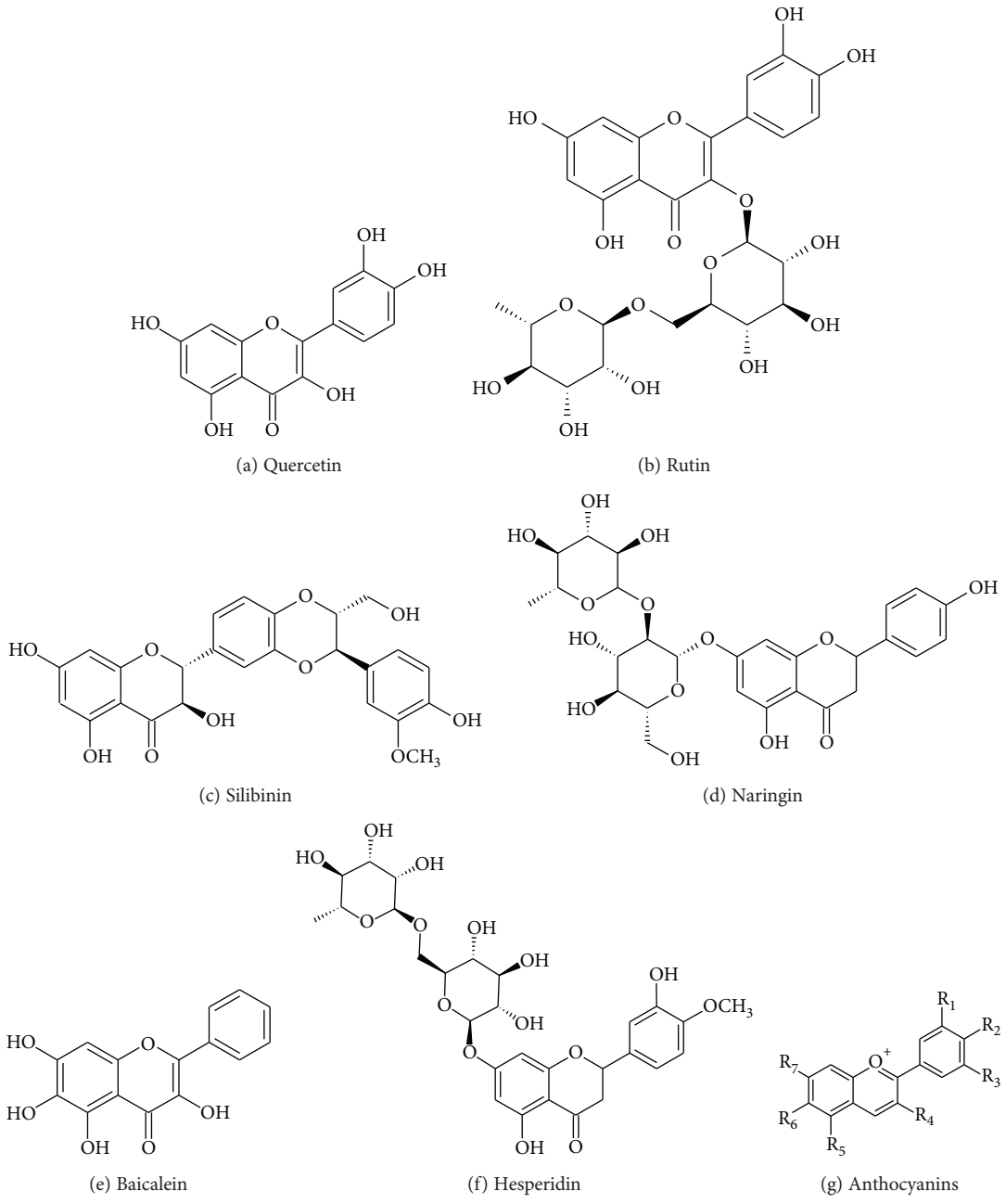


Figure 3: Chemical structures of flavonoids most cited in this review.

In a study that evaluated the manipulation of quercetin nanoparticle facilitating oral absorption, it was seen that there was no change in coordination or locomotor activity [48]. The nanoparticle treatment of quercetin is capable of promoting reversal of abnormal exploratory behavior by improving learning and memory assessed by the Morris water maze test. In addition, the expression of an inflammatory marker GFAP (glial fibrillary acidic protein) in the hippocampus was significantly reduced, which was not observed with free quercetin. On the other hand, the expression of CD11b, a member of the complement cascade, whose function is adhesion and leukocyte migration in response to inflammation, did not present significant results. Therefore, manipulation of quercetin-loaded nanoparticles has been shown to increase the concentration of this flavonoid in the brain of animals [48].

In the study performed by Palle and Neerati [50], the protective effect of quercetin nanoparticles compared to free quercetin against the induction of spatial memory deficiency promoted by scopolamine in animals was evaluated. This work highlighted a relevant role of quercetin nanoparticles on oxidative stress, revealing that these nanoparticles are able to significantly reduce malonaldehyde levels and increase levels of glutathione peroxidase and catalase in the brain. In observing the effect of treatment of quercetin nanoparticles on the pharmacological action of scopolamine, it has been

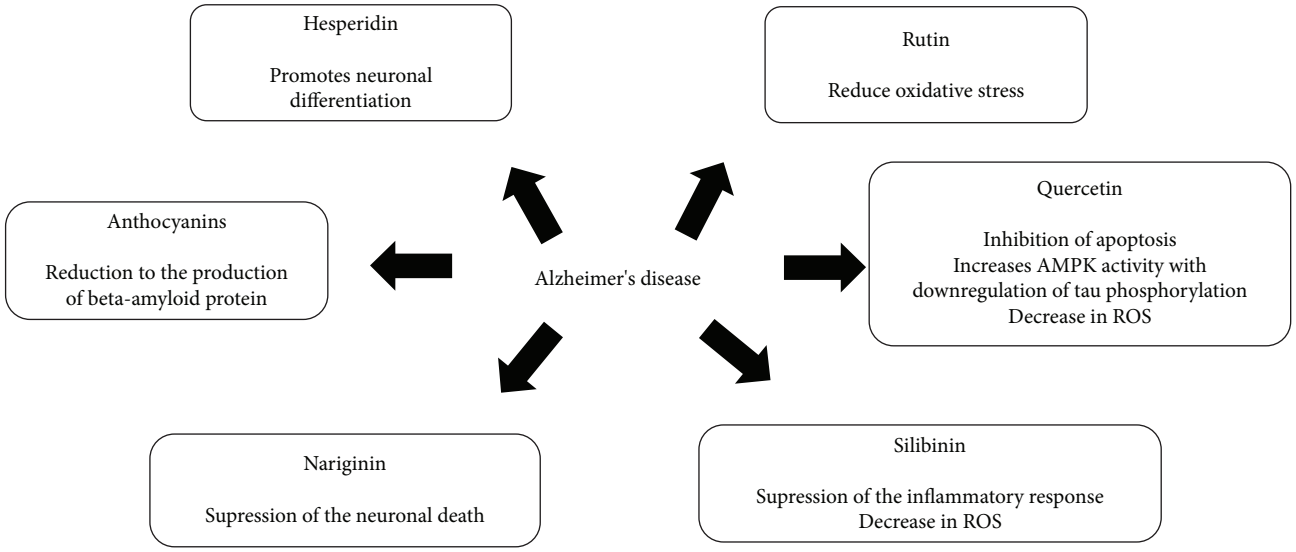


Figure 4: Possible mechanisms of action of flavonoids against Alzheimer's disease.

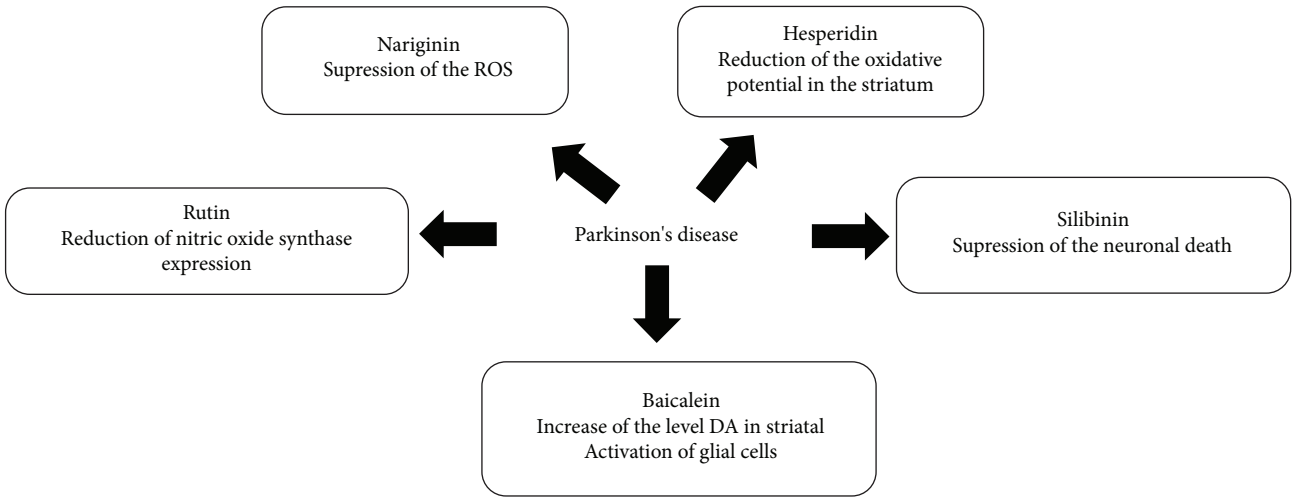


Figure 5: Possible mechanisms of action of flavonoids against Parkinson's disease.

seen that they are able to reduce the induction of elevation of scopolamine-promoted anticholinesterase activity. In addition, treatment with quercetin nanoparticles significantly reduces morphological abnormalities revealing the cellular protective effect of this flavonoid [50].

It is believed that changes in mitochondrial activity are the main cause of the occurrence of neurodegenerative disorders because they are the main producers of ROS [76]. Godoy et al. [49] have observed that quercetin exhibits antioxidant activity protecting against neuronal toxicity induced by hydrogen peroxide, although this protection has been partial in rat hippocampal neurons. Animals treated with quercetin caused a reduction in ROS levels, recovered normal mitochondrial morphology, and prevented mitochondrial dysfunction in neurons that were manipulated with hydrogen peroxide [49].

Of all the works selected in this review, only one demonstrated the role of quercetin on Parkinson's disease. Mu et al. [61] investigated the antitremor effect of quercetin in the experimental model of PD induced by the application of 6-hydroxydopamine (6-OHDA). The study demonstrated that injection of 6-OHDA into the striatum induces marked decrease in serotonin levels and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA). Therefore, it was seen that these animals when treated with quercetin had attenuated serotonin levels, which may be related to an improvement in the tremor level of these animals [61].

Quercetin seems to act by inhibiting the cell oxidative potential, in addition to exhibiting anti-inflammatory action. Both events may minimize the progression of PD and AD.

3.6.2. Silibinin. Silibinin, (2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*, 3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2- (hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydro-4*H* -chromen-4-one, is a flavonoid derived from milk thistle of the species *Silybum marianum* [91]. Recent research investigated the

effects of silibinin on the inflammatory process, oxidative stress, and autophagy [92, 93]. Following this subject, Song et al. [47] investigated the effect of silibinin treatment on locomotor activity, learning, and spatial memory. In addition to the concentration of the proinflammatory cytokines IL-1β, IL-4, and the levels of the antioxidant enzyme (GSH) and malondialdehyde (MDA), a lipid peroxidation marker, which occurs in response to excess free radicals the NF-κB (nuclear factor kappa B), a regulator of the immune response released in different situations among these oxidative stress, cyclooxygenase-2 (COX-2), i-NOS (nitric oxide synthase) are products of glial cells contributing to an inflammatory response in the brain. P-53, a tumor suppressor agent, is a critical component of the acute stress cell response, and p-p53 is its phosphorylated component. It has been seen that silibinin decreases anxiety-like behavior, reverses memory damage and spatial learning caused by the treatment of Aβ25-35, and improves the ability to recognize new objects and memory flexibility. Silibinin is able to suppress the inflammatory response and improve oxidative stress levels in the hippocampus, in addition to suppressing the expression of p-p53 and p-53 [47].

Chen et al. [60] analyzed the levels of glutathione and malondialdehyde in animals that underwent neonatal manipulation with carbonyl iron dose and its consequence in young adult and old animals as well as treatment with silibinin. It was first seen that iron consumption resulted in abnormal behavior of coordination and locomotor activity only in the old animals. Also in these animals, iron in the neonatal period also increased levels of malondialdehyde and reduced those of glutathione enzyme. Treatment with silibinin in aging animal's decrease dopamine depletion in the striatum improving motor behavior was also able to significantly reduce the content of MDA and increase the content of GSH in the nervous system. It is concluded that silibinin acts as a neuroprotective factor preventing oxidative stress; one of the consequences of neurodegenerative diseases, among them, is Parkinson's disease [60].

Using a model of dopaminergic neuronal death caused by the pharmacological agent MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin capable of inducing Parkinsonism, Lee et al. [62] investigated the neuroprotective mechanism of sibilinin. Treatment with silibinin prevented motor dysfunction in the pharmacological parkinsonism (MPTP) model, as well as neuronal loss (TH-positive neurons) in the striatum and substantia nigra. Silibinin effectively protects dopaminergic neurons in the striatum and black matter of neuronal death caused by MPTP, but does not impede the inflammatory response, since it does not act effectively on glial cell activation or modulation of oxidative stress. The latter result differs from the previous work probably due to the dose difference used in the present study, from 1 to 10 mg/kg body weight. This demonstrates that the neuroprotective action of silibinin is dose dependent [62].

It can be concluded that silibinin is a flavonoid with a neuroprotective characteristic especially acting on the inflammatory response and on the components of oxidative stress and neuronal death, thus having a potential therapeutic agent in neurodegenerative diseases.

In summary, silibinin is a flavonoid with a neuroprotective potential, especially acting on the inflammatory response, on components of oxidative stress, and on neuronal death.

3.6.3. Anthocyanins. Anthocyanins are polyphenolic flavonoids widely found in fruits, flowers, grains, and vegetables [94]. This flavonoid has antioxidant, anti-inflammatory, and antiapoptosis properties, in addition to improving memory and cognition [95]. They are characterized by the basic flavylium core (2-phenylbenzopyryl cation) consisting of two aromatic rings joined by a three-carbon unit and condensed by an oxygen. The anthocyanin molecule consists of two or three portions, an aglycone (anthocyanidin), a group of sugars, and often a group of organic acids [96].

There are several evidences that relate the role of this flavonoid in cognition and memory, being characterized with a neuroprotective factor in the dementias among them AD. Alim et al. [44] observed in genetically modified mice ($A\beta_{1-42}$ mouse model of AD) the ability of anthocyanin particles and (ethylene glycol) gold nanoparticles (PEG-AuNPs) (polyphenolic flavonoid anthocyanins for conjugation to PEG-AuNPs) to improve memory loss and synaptic deficit and neurodegeneration. The behavioral assessment showed that the latency times of there quired to reach the hidden platform in were shorter, increased the number of platform crossings and time spent in the target quadrant during the probe test in mice treated with anthocyanins and anthocyanin-loaded PEG-AuNPs. Furthermore, anthocyanins and anthocyanin-loaded PEG-AuNPs increased the spontaneous alteration behavior. Anthocyanin-loaded PEG-AuNPs reduced the levels of Aβ (β-amyloid protein), BACE-1 (a beta-secretase, a key enzyme in the formation of the β-amyloid protein), and APP (protein precursor antiamyloid). This demonstrates a potential action of this flavonoid on the production of beta-amyloid protein. The administration of free anthocyanins and anthocyanin-loaded PEG-AuNPs mitigated the effect of $A\beta_{1-42}$ and increased the expression levels of synaptophysin, PSD95, and SNAP23; these molecules are related to the synapse process between the neurons. Anthocyanins and anthocyanin-loaded PEG-AuNPs increased the phosphorylation of GluR1 at Ser845 and increased the expression level of p-CREB (Ser133), which can improve the memory process. The administration of free anthocyanins and anthocyanin-loaded PEG-AuNPs increased phosphorylation and elevated the levels of p-PI3K and p-Aktat Ser473, increased the level of p-GSK3β at Ser9, and reduced the level of p-tau at Ser413 and Ser404, which consequently may reduce the level of formation of fibrillar components. The results showed a reduction of the ratio of Bax/Bcl2 and Cyt c, but anthocyanin-loaded PEG-AuNPs were more effective than free anthocyanin. A reduction in the caspase-9, cleaved caspase-3, and PARP-1 levels in the hippocampus was also observed, and these results demonstrate the neuroprotective action of this flavonoid. Finally, an increase in the number of surviving neurons and a reduction in the $A\beta_{1-42}$-induced-degenerated neuronal cells in the hippocampus and cortex were seen [44].

In the work of Kim et al. [53], the therapeutic efficacy of anthocyanins alone and anthocyanin-loaded PEG-AuNPs in the $A\beta_{1-42}$-induced AD mouse model was also investigated. It was observed that the anthocyanin-loaded PEG-AuNPs can cross the blood-brain barrier and accumulate in the $A\beta$-injected mice. Furthermore, the anthocyanin-loaded PEG-AuNPs reduced β-amyloid and BACE-1 expressions and also prevented tau hyperphosphorylation GSK-3β/CDK5 pathway. Anthocyanin-loaded PEG-AuNPs also reduced $A\beta_{1-42}$-induced microglia and astrocyte cell activation [53].

In another study, the anthocyanins inhibited activated astrocytes and various inflammatory markers including p-NF-κB, inducible nitric oxide synthase (iNOS), and tumor necrosis factor-alpha (TNF-α) in the hippocampus and cortex regions of D-gal-treated rats [52].

Anthocyanins are able to inhibit the cascade of myeloid beta-protein production and to decrease synaptogenesis and neuronal death. They also induce microglial activation in areas important to the process of memory as hippocampus and cortex.

3.6.4. Naringin. Naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside) belongs to a family of C_6-C_3-C_6 polyphenol compounds and exists in grapefruit and other citrus fruits. This flavonoid has been shown to possess numerous biological benefits such as antioxidant and anti-inflammatory [97]. Preclinical models of atherosclerosis, cardiovascular disorders, diabetes mellitus, neurodegenerative disorders, osteoporosis, and rheumatological disorders were established in a few studies of naringin *in vitro* and *in vivo* [98]. Recently, studies demonstrated a neuroprotective effect of naringin by modulation of endogenous biomarkers and downregulation of free radical and cytokines, including tumor necrosis factor-α (TNF-α) in streptozotocin-induced painful diabetic neuropathy [99]. Mani et al. [37] investigated the effect of naringin against deltamethrin-induced neurotoxicity in male Wistar rats. The result showed that treatment leads to a significant revival of the oxidative status, which confirms the protective effect of naringin.

Behavioral analysis of the effect of naringin on memory deficit in a pharmacological model (donepezil and scopolamine) in animals has demonstrated a significant difference in the locomotor activity and confirmed that naringin has no confounding influence on locomotion on improving the potential for episodic memory, in the familiarization trial no preference or discrimination toward any of the objects used. The flavonoid reversed the time-induced episodic memory deficit increase in novel object exploration time compared with familiar object and improvement in recognition and discriminative indices. Therefore, naringin reversed the scopolamine-induced short-term episodic memory deficits and improved discrimination and recognition [45].

In a model of animal excitotoxicity by treatment with kainic acid (KA), a potent agonist of excitatory amino acids, especially glutamate, the prevention effect of autophagy and neuroinflammation of naringin was investigated [55]. Excess KA becomes a neurotoxin leading to neuronal death due to excitotoxicity. The naringin treatment significantly decreased the frequency of chronic spontaneous seizures in KA-treated mice compared with KA alone suggesting that naringin might have beneficial properties as an antiepileptic agent. Additionally, treatment with naringin attenuated the loss of hippocampal neurons in the KA-treated CA1 region, suggesting that naringin might have a property of reducing autophagic stress, which could be involved in neuronal cell death. In addition, the naringin treatment attenuated an increase in TNF-α within Iba1-positive microglia in the KA-treated hippocampus characteristic of diseases such as Parkinson's and Alzheimer's [55].

The naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-chroman-4-one) is a flavonoid of citrus fruits, predominantly found in grapefruit. The antinociceptive, anti-inflammatory, and antioxidant effects of the naringenin have already been demonstrated [100]. Lou et al. [58] investigated the effect of naringenin on PD through the pharmacological parkinsonism model (6-OHDA). Naringenin treatment resulted in an increase in nuclear factor E2-related factor 2 (Nrf2) protein (regulatory factor of the expression of antioxidant protein genes) levels and subsequent activation of antioxidant response element (ARE) pathway genes. Additionally, the pretreatment with naringenin protected mice against 6-OHDA-associated ROS damage in striatum. The 6-OHDA-induced loss of TH-positive neurons in the striatum and SNC were remarkably attenuated by naringenin treatment. Therefore, there was a profound reduction in striatal DA and its metabolites after 6-OHDA lesioning that was attenuated by naringenin treatment, which produced a significant elevation in striatal DA and the metabolites DOPAC (dihydroxyphenylacetic acid) and HVA (homovanillic acid). Furthermore, apomorphine-induced asymmetrical rotations contralateral to the 6-OHDA injection site were significantly reduced by naringenin treatment as compared to mice lesioned with 6-OHDA [58].

Narigin seems to act especially through the inhibition of oxidative cellular stress, which reflects in reduction of neuronal loss (autophagic stress). It is known that oxidative stress is a strong mechanism of neuroinflammation and has an important consequence for neurodegenerative diseases.

3.6.5. Baicalein. Baicalein (5,6,7-trihydroxyflavone) is a flavonoid originally isolated from the roots of *Scutellaria baicalensis* and *Scutellaria lateriflora* [101]. It has neuroprotective properties against PD and antioxidant and anti-inflammatory properties [102]. Hu et al. [63] noted that baicalein treatment attenuated the motor deficits, in addition to increase in striatal neurotransmitters: DA (dopamine), DOPAC (3,4-dihydroxyphenylacetic acid), and HVA (homovanillic acid). Analysis of fluorescence intensity by microscopy and the amount of α-syn in enteric nervous system was also lower and increased of the in the number of TH-positive neurons. There was a decrease of α-synoligomers, not monomers, in ileum, thoracic spinal cord, and midbrain; baicalein had no effect on α-syn mRNA expression. Therefore, it can be inferred that this flavonoid can prevent the progression of α-syn accumulation in PD, by inhibiting the formation of α-syn oligomers [63].

When investigating the therapeutic effects of baicalein on rotenone-induced PD rats and exploring whether the neuroprotective potential practices by baicalein were through intervening in mitochondrial function and mitobiogenesis, it was found that baicalein partially ameliorated the motor dysfunction and increased the number of TH+ cells in the substance nigra (SN) in rotenone-induced PD rats [64]. This flavonoid also protected neurons in the SN against rotenone-induced apoptosis. The baicalein ameliorated the dysfunction of mitochondrial complex I in the ventral midbrain that was damaged by rotenone [64]. In addition, the administration of baicalein increased the protein levels of PGC-1α (a regulator of mitochondrial mitobiogenesis), NRF-1 (transcription factor that regulates the expression of antioxidant proteins), and TFAM (mitochondrial transcription factor) in the ventral midbrain, which may improve the brain's response to oxidative stress and consequent neuronal loss observed in Parkinson's disease [64].

Lee et al. [56], through a model of pharmacological parkinsonism in animals (MPTP administration), observed that the, in low doses, baicalein improves motor ability and prevented the loss of dopaminergic neurons caused by MPTP. In addition, microglial activation and astrocyte activation were reduced in the animal with pretreated baicalein PD. This study reveals the importance of astrocyte activation for the occurrence of the neurodegenerative process. It has also been reported that baicalein reduces MPP (a toxic molecule that interferes with oxidative phosphorylation in mitochondria) that is capable of inducing the activation of NF-κB, ERK (protein kinase intracellular signaling), and JNK (c-jun N-terminal kinase) in the astrocyte leading to a mechanism of neuroinflammation considered as a potent inducer of PD [56].

These studies taken together reveal the neuroprotective action of baicalein especially on mitochondrial activity and activation of glial cells. Both processes are recognized with potential mechanisms capable of increasing the risk of neurodegenerative diseases. This flavonoid proved to be effective as a therapeutic strategy especially against PD disease.

3.6.6. Hesperidin. Hesperidin, (2*S*)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromen-7-yl 6-*O*-(6-deoxy-α-*L*-mannopyranosyl)-β-*D*-glucopyranoside, is the major flavanone glycoside present in citrus fruits. This compound has an important neuroprotective property and radical scavenging properties related to diverse neuronal insults, such as ischemia [103], stroke [104], and oxidative-induced damage [105], as well as pathology related to AD [106] and Huntington's disease [107].

Nones et al. [108] showed that hesperidin promotes neuronal differentiation and survival and also enhances the neuroprotective capacity of astrocytes, by inducing them to secrete soluble factors involved in neuronal survival *in vitro*.

Li et al. [40] investigated the potential therapeutic effect of hesperidin on behavioral dysfunction, Aβ deposition, and neuroinflammation in the transgenic APP/PS1–21 mouse model. In this research, the treatment with hesperidin caused a decrease in the nesting ability and social interaction. Furthermore, the improvement of amyloid beta accumulation and APP expression, with reduction of microglial activation, suggests that hesperidin might be a potential candidate for the treatment of AD or even of other neurodegenerative diseases.

An investigation into the anti-inflammatory potential, antioxidants, and protective effects of hesperidin was performed by Javed et al. [41] using the mouse model of sporadic dementia of Alzheimer's type (SDAT). In this model, researchers have shown that hesperidin can be used for the treatment of cognitive disorders because of its neuronal cell death modulation by inhibiting the overexpression of inflammatory markers like nuclear factor κB, coupled with the inducible nitric oxide synthase, cyclooxygenase-2, and glial fibrillary acidic protein-positive astrocytes.

Antunes et al. [57] evaluate the role of the flavonoid hesperidin in an animal model of PD induced by 6-OHDA and demonstrated that hesperidin (50 mg/kg) treatment was effective in preventing memory impairment and depression-like behavior with reduction in glutathione peroxidase and catalase activity, total reactive antioxidant potential, and the dopamine and its metabolite levels in the striatum of aged mice.

Matias et al. [109] described hesperidin as a new drug able to improve memory in healthy adult mice by two main mechanisms: by inducing synapse formation and function between hippocampal and cortical neurons. In addition, other mechanisms enhance the synaptogenic ability of cortical astrocytes by means of increased secretion of transforming growth factor beta-1 (TGF-β1) by these cells.

Hesperidin appears to present a neuroprotective role through its action on glial cells, microglia, and astrocytes, promoting reduction of neuroinflammation and oxidative stress. In addition, it may induce synapse formation in brain regions involved with memory and decision-making.

3.6.7. Rutin. Rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl 6-*O*-(6-deoxy-α-*L*-mannopyranosyl)-β-*D*-glucopyranoside) is a glycone of quercetin with a flavonol structure. This substance modifies the cognitive and various behavioral symptoms of neurodegenerative diseases due to the ability of rutin and/or its metabolites to cross the blood-brain barrier; this way causes effects on the various cellular functions under pathological conditions [110, 111].

Some reports have demonstrated that rutin scavenges superoxide radicals, increases antioxidant enzymatic activity *in vitro*, reduces lipid peroxidation and cytokine production, and prevents cognitive deficits including CNS injuries in rat models [112, 113].

In recent studies, Rodrigues et al. [114] investigate the effect of treatment with rutin after induction of focal cortical ischemia and results that shown that rutin is a putative candidate to treat stroke. Few studies have evaluated the treatment with rutin in models of global and focal brain ischemia, showing positive effects [115].

According to Hhan et al. [116], similar to other flavonoids, the main expected mechanisms of action of rutin are its anti-inflammatory and antioxidative potentials. Thus, anti-inflammatory action of rutin was demonstrated with reduction of inducible nitric oxide synthase expression in a model of PD.

Moghbelinejada et al. [39] investigated the possible effects of rutin on MAPK and BDNF gene expression and memory retrieval in β-amyloid-injected rats. Their results demonstrated improved memory impairment caused by injection of Aβ in rats through activation of MAPK and BDNF, as also reduced oxidative stress in the hippocampus of rats by reducing MDA level and increasing thiol content in the hippocampus. Further studies are necessary to clarify the effects and molecular mechanisms of this flavonoid.

Rutin has antioxidant and anti-inflammatory roles; its mechanism of action is not yet completely elucidated but may be related to the MAPK pathway and reduction of nitric oxide synthase activation.

4. Conclusion and Perspective

This systematic review suggests that the flavonoids reported have a potential for the treatment of neurodegenerative diseases such as PD and AD and are considered drug candidates in the future clinical research. The studies listed in this review revealed that the main targets of action for Alzheimer's disease therapy were reduction of reactive oxygen species and amyloid beta-protein production. In Parkinson's disease, reduction of the cellular oxidative potential and mechanisms of neuronal death are often involved in the neuroprotective potential of flavonoids.

It was observed that flavonoids had been studied using various *in vivo* animal models, including the evaluation of its mechanism of action and effects on the molecular level. However, it is essential to improve the rigor of study design and data in view of the fact that most of the included studies presented low to moderate methodological quality, which limits the interpretation of the results and the continuity of the studies.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors acknowledge the financial support from the Brazilian agencies CNPq, CAPES, FACEPE, and State University of Feira de Santana (UEFS).

References

[1] V. M. Mani, S. Asha, and A. M. M. Sadiq, "Pyrethroid deltamethrin-induced developmental neurodegenerative cerebral injury and ameliorating effect of dietary glycoside naringin in male wistar rats," *Biomedicine & Aging Pathology*, vol. 4, no. 1, pp. 1–8, 2014.

[2] A. Gomes, R. C. Pimpão, S. Fortalezas et al., "Chemical characterization and bioactivity of phytochemicals from Iberian endemic *Santolina semidentata* and strategies for *ex situ* propagation," *Industrial Crops and Products*, vol. 74, pp. 505–513, 2015.

[3] M. Relja, "Pathophysiology and classification of neurodegenerative diseases," *Journal of the International Federation of Clinical Chemistry*, vol. 15, no. 3, 2004.

[4] C. M. Nday, E. Halevas, G. E. Jackson, and A. Salifoglou, "Quercetin encapsulation in modified silica nanoparticles: potential use against Cu(II)-induced oxidative stress in neurodegeneration," *Journal of Inorganic Biochemistry*, vol. 145, pp. 51–64, 2015.

[5] M. E. Ahmed, M. M. Khan, H. Javed et al., "Amelioration of cognitive impairment and neurodegeneration by catechin hydrate in rat model of streptozotocin-induced experimental dementia of Alzheimer's type," *Neurochemistry International*, vol. 62, no. 4, pp. 492–501, 2013.

[6] Q. Zhang, J. J. Zhao, J. Xu, F. Feng, and W. Qu, "Medicinal uses, phytochemistry and pharmacology of the genus *Uncaria*," *Journal of Ethnopharmacology*, vol. 173, pp. 48–80, 2015.

[7] L. Lin, B. Ni, H. Lin et al., "Traditional usages, botany, phytochemistry, pharmacology and toxicology of *Polygonum multiflorum Thunb.*: a review," *Journal of Ethnopharmacology*, vol. 159, pp. 158–183, 2015.

[8] M. M. Khan, D. Kempuraj, R. Thangavel, and A. Zaheer, "Protection of MPTP-induced neuroinflammation and neurodegeneration by pycnogenol," *Neurochemistry International*, vol. 62, no. 4, pp. 379–388, 2013.

[9] C. Grosso, P. Valentão, F. Ferreres, and P. Andrade, "The use of flavonoids in central nervous system disorders," *Current Medicinal Chemistry*, vol. 20, no. 37, pp. 4694–4719, 2013.

[10] R. C. Brown, A. H. Lockwood, and B. R. Sonawane, "Neurodegenerative diseases: an overview of environmental risk factors," *Environmental Health Perspectives*, vol. 113, no. 9, pp. 1250–1256, 2005.

[11] A. Elbaz, L. Carcaillon, S. Kab, and F. Moisan, "Epidemiology of Parkinson's disease," *Revue Neurologique*, vol. 172, no. 1, pp. 14–26, 2016.

[12] O. B. Tysnes and A. Storstein, "Epidemiology of Parkinson's disease," *Journal of Neural Transmission*, vol. 124, no. 8, pp. 901–905, 2017.

[13] T. Pringsheim, N. Jette, A. Frolkis, and T. D. Steeves, "The prevalence of Parkinson's disease: a systematic review and meta-analysis," *Movement Disorders*, vol. 29, no. 13, pp. 1583–1590, 2014.

[14] N. A. Kelsey, H. M. Wilkins, and D. A. Linseman, "Nutraceutical antioxidants as novel neuroprotective agents," *Molecules*, vol. 15, no. 11, pp. 7792–7814, 2010.

[15] C. M. Liu, J. Q. Ma, S. S. Liu, G. H. Zheng, Z. J. Feng, and J. M. Sun, "Proanthocyanidins improves lead-induced cognitive impairments by blocking endoplasmic reticulum stress and nuclear factor-κB-mediated inflammatory pathways in rats," *Food and Chemical Toxicology*, vol. 72, pp. 295–302, 2014.

[16] S. F. Nabavi, N. Braidy, S. Habtemariam et al., "Neuroprotective effects of chrysin: from chemistry to medicine," *Neurochemistry International*, vol. 90, pp. 224–231, 2015.

[17] J. K. Li, Z. T. Jiang, R. Li, and J. Tan, "Investigation of antioxidant activities and free radical scavenging of flavonoids in leaves of *Polygonum multiflorum* Thumb," *China Food Additives*, vol. 2, pp. 69–74, 2012.

[18] B. C. Adedayo, G. Oboh, S. Oyeleye, I. I. Ejakpovi, A. A. Boligon, and M. L. Athayde, "Blanching alters the phenolic constituents and in vitro antioxidant and anticholinesterases

properties of fireweed (Crassocephalum crepidioides)," *Journal of Taibah University Medical Sciences*, vol. 10, no. 4, pp. 419–426, 2015.

[19] D. Prakash and G. Sudhandiran, "Dietary flavonoid fisetin regulates aluminium chloride-induced neuronal apoptosis in cortex and hippocampus of mice brain," *The Journal of Nutritional Biochemistry*, vol. 26, no. 12, pp. 1527–1539, 2015.

[20] Q. Li, H. Zhao, M. Zhao, Z. Zhang, and Y. Li, "Chronic green tea catechins administration prevents oxidative stress-related brain aging in C57BL/6J mice," *Brain Research*, vol. 1353, pp. 28–35, 2010.

[21] M. Ashafaq, S. S. Raza, M. M. Khan et al., "Catechin hydrate ameliorates redox imbalance and limits inflammatory response in focal cerebral ischemia," *Neurochemical Research*, vol. 37, no. 8, pp. 1747–1760, 2012.

[22] K. Matsuzaki, K. Miyazaki, S. Sakai et al., "Nobiletin, a citrus flavonoid with neurotrophic action, augments protein kinase A-mediated phosphorylation of the AMPA receptor subunit, GluR1, and the postsynaptic receptor response to glutamate in murine hippocampus," *European Journal of Pharmacology*, vol. 578, no. 2-3, pp. 194–200, 2008.

[23] D. H. Kim, S. Kim, S. J. Jeon et al., "Tanshinone I enhances learning and memory, and ameliorates memory impairment in mice via the extracellular signal-regulated kinase signalling pathway," *British Journal of Pharmacology*, vol. 158, no. 4, pp. 1131–1142, 2009.

[24] J. P. Spencer, K. Vafeiadou, R. J. Williams, and D. Vauzour, "Neuroinflammation: modulation by flavonoids and mechanisms of action," *Molecular Aspects of Medicine*, vol. 33, no. 1, pp. 83–97, 2012.

[25] D. Moher, A. Liberati, J. Tetzlaff, D. G. Altman, and The PRISMA Group, "Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement," *PLoS Medicine*, vol. 6, no. 7, article e1000097, 2009.

[26] C. R. Hooijmans, M. M. Rovers, R. B. Vries, M. Leenaars, M. Ritskes-Hoitinga, and M. W. Langendam, "SYRCLE's risk of bias tool for animal studies," *BMC Medical Research Methodology*, vol. 14, no. 1, p. 43, 2014.

[27] P. S. Siqueira-Lima, J. C. Silva, J. S. S. Quintans et al., "Natural products assessed in animal models for orofacial pain – a systematic review," *Revista Brasileira de Farmacognosia*, vol. 27, no. 1, pp. 124–134, 2017.

[28] P. Balachandran and R. Govindarajan, "Ayurvedic drug discovery," *Expert Opinion on Drug Discovery*, vol. 2, no. 12, pp. 1631–1652, 2007.

[29] W. Y. Wu, J. J. Hou, H. L. Long, W.-Z. Yang, J. Liang, and D.-A. Guo, "TCM-based new drug discovery and development in China," *Chinese Journal of Natural Medicines*, vol. 12, no. 4, pp. 241–250, 2014.

[30] Z. L. Ren and P. P. Zuo, "Neural regeneration: role of traditional Chinese medicine in neurological diseases treatment," *Journal of Pharmacological Sciences*, vol. 120, no. 3, pp. 139–145, 2012.

[31] Z. Wang, H. Wan, J. Li, H. Zhang, and M. Tian, "Molecular imaging in traditional Chinese medicine therapy for neurological diseases," *BioMed Research International*, vol. 2013, Article ID 608430, 11 pages, 2013.

[32] Y. Su, Q. Wang, C. Wang, K. Chan, Y. Sun, and H. Kuang, "The treatment of Alzheimer's disease using Chinese medicinal plants: from disease models to potential clinical applications," *Journal of Ethnopharmacology*, vol. 152, no. 3, pp. 403–423, 2014.

[33] Y. Jiang, H. Gao, and G. Turdu, "Traditional Chinese medicinal herbs as potential AChE inhibitors for anti-Alzheimer's disease: a review," *Bioorganic Chemistry*, vol. 75, pp. 50–61, 2017.

[34] W. F. Kum, S. S. Durairajan, Z. X. Bian et al., "Treatment of idiopathic Parkinson's disease with traditional Chinese herbal medicine: a randomized placebo-controlled pilot clinical study," *Evidence-based Complementary and Alternative Medicine*, vol. 2011, Article ID 724353, 8 pages, 2011.

[35] W. Fu, W. Zhuang, S. Zhou, and X. Wang, "Plant-derived neuroprotective agents in Parkinson's disease," *American Journal of Translational Research*, vol. 7, no. 7, pp. 1189–1202, 2015.

[36] H. Wei, G. Wu, J. Chen et al., "(2*S*)-5, 2′, 5′-trihydroxy-7-methoxyflavanone, a natural product from *Abacopterispenangiana*, presents neuroprotective effects *in vitro* and *in vivo*," *Neurochemical Research*, vol. 38, no. 8, pp. 1686–1694, 2013.

[37] V. M. Mani, S. Asha, and A. M. M. Sadiq, "Pyrethroid deltamethrin-induced developmental neurodegenerative cerebral injury and ameliorating effect of dietary glycoside naringin in male wistar rats," *Biomedicine & Aging Pathology*, vol. 4, no. 1, pp. 1–8, 2014.

[38] A. Nakajima, Y. Aoyama, T. T. Nguyen et al., "Nobiletin, a citrus flavonoid, ameliorates cognitive impairment, oxidative burden, and hyperphosphorylation of tau in senescence-accelerated mouse," *Behavioural Brain Research*, vol. 250, pp. 351–360, 2013.

[39] S. Moghbelinejada, M. Nassiri-Asla, T. N. Farivar et al., "Rutin activates the MAPK pathway and BDNF gene expression on beta-amyloid induced neurotoxicity in rats," *Toxicology Letters*, vol. 224, no. 1, pp. 108–113, 2014.

[40] C. Li, C. Zug, H. Qu, H. Schluesener, and Z. Zhang, "Hesperidin ameliorates behavioral impairments and neuropathology of transgenic APP/PS1 mice," *Behavioural Brain Research*, vol. 281, no. 15, pp. 32–42, 2014.

[41] H. Javed, K. Vaibhav, M. E. Ahmed et al., "Effect of hesperidin on neurobehavioral, neuroinflammation, oxidative stress and lipid alteration in intracerebroventricular streptozotocin induced cognitive impairment in mice," *Journal of the Neurological Sciences*, vol. 348, no. 1-2, pp. 51–59, 2015.

[42] J. M. Walker, D. Klakotskaia, D. Ajit et al., "Beneficial effects of dietary EGCG and voluntary exercise on behavior in an Alzheimer's disease mouse model," *Journal of Alzheimer's Disease*, vol. 44, no. 2, pp. 561–572, 2015.

[43] X. Kou, X. Liu, X. Chen, J. Li, X. Yang, and N. Chen, "Ampelopsin attenuates brain aging of D-gal-induced rats through miR-34a-mediated SIRT1/mTOR signal pathway," *Oncotarget*, vol. 7, no. 46, pp. 74484–74495, 2016.

[44] T. Alim, M. J. Kim, S. U. Rehman, A. Ahmad, and M. O. Kim, "Anthocyanin-loaded PEG-gold nanoparticles enhanced the neuroprotection of anthocyanins in an $A\beta_{1\text{-}42}$ mouse model of Alzheimer's disease," *Molecular Neurobiology*, vol. 54, no. 8, pp. 6490–6506, 2016.

[45] G. V. Ramalingayya, M. Nampoothiri, P. G. Nayak et al., "Naringin and Rutin alleviates episodic memory deficits in two differentially challenged object recognition tasks," *Pharmacognosy Magazine*, vol. 12, no. 45, pp. 63–70, 2016.

[46] J. Chen, X. Deng, N. Liu et al., "Quercetin attenuates tau hyperphosphorylation and improves cognitive disorder via suppression of ER stress in a manner dependent on AMPK pathway," *Journal of Functional Foods*, vol. 22, pp. 463–476, 2016.

[47] X. Song, B. Zhou, L. Cui et al., "Silibinin ameliorates A$\beta_{25\text{-}35}$-induced memory deficits in rats by modulating autophagy and attenuating neuroinflammation as well as oxidative stress," *Neurochemical Research*, vol. 42, no. 4, pp. 1073–1083, 2017.

[48] L. C. G. E. I. Moreno, E. Puerta, J. E. Suárez-Santiago, N. S. Santos-Magalhães, M. J. Ramirez, and J. M. Irache, "Effect of the oral administration of nanoencapsulated quercetin on a mouse model of Alzheimer's disease," *International Journal of Pharmaceutics*, vol. 517, no. 1-2, pp. 50–57, 2017.

[49] J. A. Godoy, C. B. Lindsay, R. A. Quintanilla, F. J. Carvajal, W. Cerpa, and N. C. Inestrosa, "Quercetin exerts differential neuroprotective effects against H_2O_2 and Aβ aggregates in hippocampal neurons: the role of mitochondria," *Molecular Neurobiology*, vol. 54, no. 9, pp. 7116–7128, 2017.

[50] S. Palle and P. Neerati, "Quercetin nanoparticles attenuates scopolamine induced spatial memory deficits and pathological damages in rats," *Bulletin of Faculty of Pharmacy, Cairo University*, vol. 55, no. 1, pp. 101–106, 2017.

[51] A. Ahmad, T. Ali, H. Y. Park, H. Badshah, S. U. Rehman, and M. O. Kim, "Neuroprotective effect of Fisetin against amyloid-beta-induced cognitive/synaptic dysfunction, neuroinflammation, and neurodegeneration in adult mice," *Molecular Neurobiology*, vol. 54, no. 3, pp. 2269–2285, 2017.

[52] S. U. Rehman, S. A. Shah, T. Ali, J. I. Chung, and M. O. Kim, "Anthocyanins reversed D-galactose-induced oxidative stress and neuroinflammation mediated cognitive impairment in adult rats," *Molecular Neurobiology*, vol. 54, no. 1, pp. 255–271, 2017.

[53] M. J. Kim, S. U. Rehman, F. U. Amin, and M. O. Kim, "Enhanced neuroprotection of anthocyanin-loaded PEG-gold nanoparticles against A$\beta_{1\text{-}42}$-induced neuroinflammation and neurodegeneration via the NF-$_K$B/JNK/GSK3β signaling pathway," *Nanomedicine: Nanotechnology, Biology, and Medicine*, vol. 13, no. 8, pp. 2533–2544, 2017.

[54] D. R. Sharma, W. Y. Wani, A. Sunkaria et al., "Quercetin attenuates neuronal death against aluminum-induced neurodegeneration in the rat hippocampus," *Neuroscience*, vol. 324, pp. 163–176, 2016.

[55] K. H. Jeong, U. J. Jung, and S. R. Kim, "Naringin attenuates autophagic stress and neuroinflammation in kainic acid-treated hippocampus *in vivo*," *Evidence-based Complementary and Alternative Medicine*, vol. 2015, Article ID 354326, 9 pages, 2015.

[56] E. Lee, H. R. Park, S. T. Ji, Y. Lee, and J. Lee, "Baicalein attenuates astroglial activation in the 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine-induced Parkinson's disease model by downregulating the activations of nuclear factor-κB, ERK, and JNK," *Journal of Neuroscience Research*, vol. 92, no. 1, pp. 130–139, 2014.

[57] M. S. Antunes, A. T. R. Goes, S. P. Boeira, M. Prigol, and C. R. Jesse, "Protective effect of hesperidin in a model of Parkinson's disease induced by 6-hydroxydopamine in aged mice," *Nutrition*, vol. 30, no. 11-12, pp. 1415–1422, 2014.

[58] H. Lou, X. Jing, X. Wei, H. Shi, D. Ren, and X. Zhang, "Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signaling pathway," *Neuropharmacology*, vol. 79, pp. 380–388, 2014.

[59] S. Wang, H. Jing, H. Yang et al., "Tanshinone I selectively suppresses pro-inflammatory genes expression in activated microglia and prevents nigrostriatal dopaminergic neurodegeneration in a mouse model of Parkinson's disease," *Journal of Ethnopharmacology*, vol. 164, pp. 247–255, 2015.

[60] H. Chen, X. Wang, M. Wang et al., "Behavioral and neurochemical deficits in aging rats with increased neonatal iron intake: silibinin's neuroprotection by maintaining redox balance," *Frontiers in Aging Neuroscience*, vol. 7, p. 206, 2015.

[61] X. Mu, X. Yuan, L. D. Du, G. R. He, and G. H. Du, "Antagonism of quercetin against tremor induced by unilateral striatal lesion of 6-OHDA in rats," *Journal of Asian Natural Products Research*, vol. 18, no. 1, pp. 65–71, 2016.

[62] Y. Lee, H. R. Park, H. J. Chun, and J. Lee, "Silibinin prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease via mitochondrial stabilization," *Journal of Neuroscience Research*, vol. 93, no. 5, pp. 755–765, 2015.

[63] Q. Hu, V. N. Uversky, M. Huang et al., "Baicalein inhibits α-synuclein oligomer formation and prevents progression of α-synuclein accumulation in a rotenone mouse model of Parkinson's disease," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1862, no. 10, pp. 1883–1890, 2016.

[64] X. Zhang, L. Du, W. Zhang, Y. Yang, Q. Zhou, and G. Du, "Therapeutic effects of baicalein on rotenone-induced Parkinson's disease through protecting mitochondrial function and biogenesis," *Scientific Reports*, vol. 7, no. 1, article 9968, 2017.

[65] A. T. R. Goes, C. R. Jesse, M. S. Antunes et al., "Protective role of chrysin on 6-hydroxydopamine-induced neurodegeneration a mouse model of Parkinson's disease: involvement of neuroinflammation and neurotrophins," *Chemico-Biological Interactions*, vol. 279, pp. 111–120, 2018.

[66] M. Ay, J. Luo, M. Langley et al., "Molecular mechanisms underlying protective effects of quercetin against mitochondrial dysfunction and progressive dopaminergic neurodegeneration in cell culture and MitoPark transgenic mouse models of Parkinson's disease," *Journal of Neurochemistry*, vol. 141, no. 5, pp. 766–782, 2017.

[67] J. A. Hirst, J. Howick, J. K. Aronson et al., "The need for randomization in animal trials: an overview of systematic reviews," *PLoS One*, vol. 9, no. 6, article e98856, 2014.

[68] P. J. Karanicolas, F. Farrokhyar, and M. Bhandari, "Blinding: who, what, when, why, how?," *Canadian Journal of Surgery*, vol. 53, no. 5, pp. 345–348, 2010.

[69] J. E. Aguilar-Nascimento, "Fundamental steps in experimental design for animal studies," *Acta Cirurgica Brasileira*, vol. 20, no. 1, pp. 2-3, 2005.

[70] C. Tapia-Rojas, C. B. Lindsay, C. Montecinos-Oliva et al., "Is L-methionine a trigger factor for Alzheimer's-like neurodegeneration?: changes in Aβ oligomers, *tau* phosphorylation, synaptic proteins, *Wnt* signaling and behavioral impairment in wild-type mice," *Molecular Neurodegeneration*, vol. 10, no. 1, p. 62, 2015.

[71] J. C. Corrêa-Velloso, M. C. B. Gonçalves, Y. Naaldijk, A. Oliveira-Giacomelli, M. M. Pillat, and H. Ulrich, "Pathophysiology in the comorbidity of bipolar disorder and Alzheimer's disease: pharmacological and stem cell approaches," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 80, Part A, pp. 34–53, 2018.

[72] B. Laurijssens, F. Aujard, and A. Rahman, "Animal models of Alzheimer's disease and drug development," *Drug Discovery Today: Technologies*, vol. 10, no. 3, pp. e319–e327, 2013.

[73] A. Castañé, D. E. Theobald, and T. W. Robbins, "Selective lesions of the dorsomedial striatum impair serial spatial reversal learning in rats," *Behavioural Brain Research*, vol. 210, no. 1, pp. 74–83, 2010.

[74] V. Francardo, "Modeling Parkinson's disease and treatment complications in rodents: potentials and pitfalls of the current options," *Behavioural Brain Research*, vol. 17, pp. 31471–31477, 2017.

[75] D. N. Hauser and T. G. Hastings, "Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism," *Neurobiology of Disease*, vol. 51, pp. 35–42, 2013.

[76] P. Imbriani, G. Sciamanna, M. Santoro, T. Schirinzi, and A. Pisani, "Promising rodent models in Parkinson's disease," *Parkinsonism & Related Disorders*, vol. 46, pp. S10–S14, 2018.

[77] G. Cohen, "Oxy-radical toxicity in catecholamine neurons," *Neurotoxicology*, vol. 5, no. 1, pp. 77–82, 1984.

[78] J. Luthman, A. Fredriksson, E. Sundstrom, G. Jonsson, and T. Archer, "Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: motor behavior and monoamine alterations at adult stage," *Behavioural Brain Research*, vol. 33, no. 3, pp. 267–277, 1989.

[79] A. Wąsik, D. Polak, I. Romańska, J. Michaluk, and L. Antkiewicz-Michaluk, "The impact of 1MeTIQ on the dopaminergic system function in the 6-OHDA model of Parkinson's disease," *Pharmacological Reports*, vol. 68, no. 6, pp. 1205–1213, 2016.

[80] E. Shireen, S. Pervez, M. Masroor et al., "Reversal of haloperidol induced motor deficits in rats exposed to repeated immobilization stress," *Pakistan Journal of Pharmaceutical Sciences*, vol. 27, no. 5, pp. 1459–1466, 2014.

[81] A. Quiroga-Varela, E. Aguilar, E. Iglesias, J. A. Obeso, and C. Marin, "Short- and long-term effects induced by repeated 6-OHDA intraventricular administration: a new progressive and bilateral rodent model of Parkinson's disease," *Neuroscience*, vol. 361, pp. 144–156, 2017.

[82] E. Kelly, P. Vyas, and J. T. Weber, "Biochemical properties and neuroprotective effects of compounds in various species of berries," *Molecules*, vol. 23, no. 1, p. 26, 2018.

[83] M. L. F. Ferreyra, S. P. Rius, and P. Casati, "Flavonoids: biosynthesis, biological functions, and biotechnological applications," *Frontiers in Plant Science*, vol. 3, p. 222, 2012.

[84] I. Solanki, P. Parihar, and M. S. Parihar, "Neurodegenerative diseases: from available treatments to prospective herbal therapy," *Neurochemistry International*, vol. 95, pp. 100–108, 2016.

[85] Y. Li, J. Yao, C. Han et al., "Quercetin, inflammation and immunity," *Nutrients*, vol. 8, no. 3, p. 167, 2016.

[86] C. Spagnuolo, S. Moccia, and G. L. Russo, "Anti-inflammatory effects of flavonoids in neurodegenerative disorders," *European Journal of Medicinal Chemistry*, vol. 17, pp. 30683–30689, 2017.

[87] M. C. Morris and C. C. Tangney, "Dietary fat composition and dementia risk," *Neurobiology of Aging*, vol. 35, pp. S59–S64, 2014.

[88] E. Bahar, J. Y. Kim, and Y. Yoon, "Quercetin attenuates manganese-induced neuroinflammation by alleviating oxidative stress through regulation of apoptosis, iNOS/NF-κB and HO-1/Nrf2 pathways," *International Journal of Molecular Sciences*, vol. 18, no. 9, p. 1989, 2017.

[89] D. M. Wang, S. Q. Li, W. L. Wu, X. Y. Zhu, Y. Wang, and H. Y. Yuan, "Effects of long-term treatment with quercetin on cognition and mitochondrial function in a mouse model of Alzheimer's disease," *Neurochemical Research*, vol. 39, no. 8, pp. 1533–1543, 2014.

[90] C. Li and J. Gotz, "Tau-based therapies in neurodegeneration: opportunities and challenges," *Nature Reviews Drug Discovery*, vol. 16, no. 12, pp. 863–883, 2017.

[91] Z. Švagera, N. Škottová, P. Váňa et al., "Plasma lipoproteins in transport of silibinin, an antioxidant flavonolignan from *Silybum marianum*," *Phytotherapy Research*, vol. 17, no. 5, pp. 524–530, 2003.

[92] R. Lim, C. J. Morwood, G. Barker, and M. Lappas, "Effect of silibinin in reducing inflammatory pathways in in vitro and in vivo models of infection-induced preterm birth," *PLoS One*, vol. 9, no. 3, article e92505, 2014.

[93] M. Wang, Y. J. Li, Y. Ding et al., "Silibinin prevents autophagic cell death upon oxidative stress in cortical neurons and cerebral ischemia-reperfusion injury," *Molecular Neurobiology*, vol. 53, no. 2, pp. 932–943, 2016.

[94] S. Y. Wang, C. T. Chen, W. Sciarappa, C. Y. Wang, and M. J. Camp, "Fruit quality, antioxidant capacity, and flavonoid content of organically and conventionally grown blueberries," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 14, pp. 5788–5794, 2008.

[95] A. N. Winter, E. K. Ross, H. M. Wilkins et al., "An anthocyanin-enriched extract from strawberries delays disease onset and extends survival in the hSOD1^{G93A} mouse model of amyotrophic lateral sclerosis," *Nutritional Neuroscience*, vol. 9, pp. 1–13, 2017.

[96] A. Smeriglio, D. Barreca, E. Bellocco, and D. Trombetta, "Chemistry, pharmacology and health benefits of anthocyanins," *Phytotherapy Research*, vol. 30, no. 8, pp. 1265–1286, 2016.

[97] S. Qin, Q. Chen, H. Wu et al., "Effects of naringin on learning and memory dysfunction induced by gp120 in rats," *Brain Research Bulletin*, vol. 124, pp. 164–171, 2016.

[98] S. Bharti, N. Rani, B. Krishnamurthy, and D. S. Arya, "Preclinical evidence for the pharmacological actions of naringin: a review," *Planta Medica*, vol. 80, no. 6, pp. 437–451, 2014.

[99] A. D. Kandhare, K. S. Raygude, P. Ghosh, A. E. Ghule, and S. L. Bodhankar, "Neuroprotective effect of naringin by modulation of endogenous biomarkers in streptozotocin induced painful diabetic neuropathy," *Fitoterapia*, vol. 83, no. 4, pp. 650–659, 2012.

[100] M. F. Manchope, C. Calixto-Campos, L. Coelho-Silva et al., "Naringenin inhibits superoxide anion-induced inflammatory pain: role of oxidative stress, cytokines, Nrf-2 and the NO–cGMP–PKG–$_{KATP}$ channel signaling pathway," *PloS One*, vol. 11, no. 4, article e0153015, 2016.

[101] T. Makino, A. Hishida, Y. Goda, and H. Mizukami, "Comparison of the major flavonoid content of *S. Baicalensis*, *S. Lateriflora*, and their commercial products," *Journal of Natural Medicines*, vol. 62, no. 3, pp. 294–299, 2008.

[102] F. Q. Li, T. Wang, Z. Pei, B. Liu, and J. S. Hong, "Inhibition of microglial activation by the herbal flavonoid baicalein

attenuates inflammation-mediated degeneration of dopaminergic neurons," *Journal of Neural Transmission*, vol. 112, no. 3, pp. 331–347, 2005.

[103] V. Gaur and A. Kumar, "Hesperidin pre-treatment attenuates NO-mediated cerebral ischemic reperfusion injury and memory dysfunction," *Pharmacological Reports*, vol. 62, no. 4, pp. 635–648, 2010.

[104] S. S. Raza, M. M. Khan, A. Ahmad et al., "Hesperidin ameliorates functional and histological outcome and reduces neuroinflammation in experimental stroke," *Brain Research*, vol. 1420, pp. 93–105, 2011.

[105] M. Chen, H. Gu, Y. Ye et al., "Protective effects of hesperidin against oxidative stress of *tert*-butyl hydroperoxide in human hepatocytes," *Food and Chemical Toxicology*, vol. 48, no. 10, pp. 2980–2987, 2010.

[106] S. M. Huang, S. Y. Tsai, J. A. Lin, C. H. Wu, and G. C. Yen, "Cytoprotective effects of hesperetin and hesperidin against amyloid β-induced impairment of glucose transport through downregulation of neuronal autophagy," *Molecular Nutrition & Food Research*, vol. 56, no. 4, pp. 601–609, 2012.

[107] E. T. Menze, M. G. Tadros, A. M. Abdel-Tawab, and A. E. Khalifa, "Potential neuroprotective effects of hesperidin on 3-nitropropionic acid-induced neurotoxicity in rats," *Neurotoxicology*, vol. 33, no. 5, pp. 1265–1275, 2012.

[108] J. Nones, A. P. Costa, R. B. Leal, F. C. A. Gomes, and A. G. Trentin, "The flavonoids hesperidin and rutin promote neural crest cell survival," *Cell and Tissue Research*, vol. 350, no. 2, pp. 305–315, 2012.

[109] I. Matias, L. P. Diniz, A. Buosi, G. Neves, J. Stipursky, and F. C. A. Gomes, "Flavonoid hesperidin induces synapse formation and improves memory performance through the astrocytic TGF-β1," *Frontiers in Aging Neuroscience*, vol. 9, p. 184, 2017.

[110] W. Arjumand, A. Seth, and S. Sultana, "Rutin attenuates cisplatin induced renal inflammation and apoptosis by reducing NFκB, TNF-α and caspase-3 expression in wistar rats," *Food and Chemical Toxicology*, vol. 49, no. 9, pp. 2013–2021, 2011.

[111] S. Habtemariam, "Rutin as a natural therapy for Alzheimer's disease: insights into its mechanisms of action," *Current Medicinal Chemistry*, vol. 23, no. 9, pp. 860–873, 2016.

[112] H. Javed, M. M. Khan, A. Ahmad et al., "Rutin prevents cognitive impairments by ameliorating oxidative stress and neuroinflammation in rat model of sporadic dementia of Alzheimer type," *Neuroscience*, vol. 210, pp. 340–352, 2012.

[113] P. X. Xu, S. W. Wang, X. L. Yu et al., "Rutin improves spatial memory in Alzheimer's disease transgenic mice by reducing Aβ oligomer level and attenuating oxidative stress and neuroinflammation," *Behavioural Brain Research*, vol. 264, pp. 173–180, 2014.

[114] A. M. G. Rodrigues, F. S. Marcilio, M. F. Muzitano, and A. Giraldi-Guimarães, "Therapeutic potential of treatment with the flavonoid rutin after cortical focal ischemia in rats," *Brain Research*, vol. 1503, pp. 53–61, 2013.

[115] M. M. Khan, A. Ahmad, T. Ishrat et al., "Rutin protects the neural damage induced by transient focal ischemia in rats," *Brain Research*, vol. 1292, pp. 123–135, 2009.

[116] M. M. Khan, S. S. Raza, H. Javed et al., "Rutin protects dopaminergic neurons from oxidative stress in an animal model of Parkinson's disease," *Neurotoxicity Research*, vol. 22, no. 1, pp. 1–15, 2012.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 3175214, 14 pages
https://doi.org/10.1155/2018/3175214

Research Article

Aqueous Extract of *Dendropanax morbiferus* Leaves Effectively Alleviated Neuroinflammation and Behavioral Impediments in MPTP-Induced Parkinson's Mouse Model

Shin-Young Park,[1] Govindarajan Karthivashan,[2] Hyun Myung Ko,[3] Duk-Yeon Cho,[1] Joonsoo Kim,[1] Dae Jun Cho,[4] Palanivel Ganesan,[2,5] In Su-Kim,[2] and Dong-Kug Choi[1,2]

[1]*Department of Applied Life Sciences, Graduate school of Konkuk University, Chungju 27478, Republic of Korea*
[2]*Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, Chungju 27478, Republic of Korea*
[3]*Department of Eco-Biological Science, College of Science and Technology, Woosuk University, 66 Daehak-ro, Jincheon-eup, Chungcheongbuk-do 27841, Republic of Korea*
[4]*Department of Quality Control, Namyang F&B Co. Ltd., Eumseong, Republic of Korea*
[5]*Nanotechnology Research Center and Department of Applied Life Science, College of Biomedical and Health Science, Konkuk University, Chungju 27478, Republic of Korea*

Correspondence should be addressed to Dong-Kug Choi; choidk@kku.ac.kr

Received 24 November 2017; Revised 14 February 2018; Accepted 18 February 2018; Published 15 April 2018

Academic Editor: Anat Elmann

Copyright © 2018 Shin-Young Park et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Parkinson's disease (PD) is a commonly reported age-related neurodegenerative disorder. Microglial-mediated neuroinflammation is one of the cardinal hallmarks of various neurodegenerative disorders, including PD progression. Inadequate therapeutic strategies and substantial adverse effects of well-established drug candidates demand new therapeutic leads to treat PD. *Dendropanax morbifera* (DM) is an endemic plant species of South Korea, and it has been used extensively as traditional medicine to treat numerous clinical complications. In this study, we conducted an initial profiling of the few major phytoconstituents of aqueous DM leaf extracts (DML) and quantified the same using high-performance liquid chromatography tandem mass spectrometry with electrospray ionization (HPLC-ESI-MS/MS). We subsequently evaluated the antineuroinflammatory activity and ameliorative potential of DML in both *in vitro* and *in vivo* experimental PD models. The prophylactic treatment of DML effectually improved the behavioral deficits, curbed the microglial-mediated neuroinflammation, and protected dopaminergic (DA) neuronal loss by restoring tyrosine hydroxylase (TH) levels in brain tissue of the MPTP-induced PD mouse model. We conducted chromatographic profiling and identified chlorogenic acid (CA) as a major constituent (19.5 mg/g of BuOH fraction), which has been well documented as an antioxidant and anti-inflammatory agent. This was found to be in harmony with our *in vitro* results, where DML suppressed the level of inflammatory mediators and allied the signaling pathway in LPS-stimulated microglial cells. The results of our study indicate that DML and its bioactive constituents can be developed as potential therapeutic candidates against progressive PD complications.

1. Introduction

Parkinson's disease (PD) is the second most commonly reported age-related neurodegenerative disorder, and it is substantially characterized by progressive dopaminergic (DA) neuronal loss in the substantia nigra pars compacta (SNpc) of the nigrostriatal system, functional impairment of microglial cells, and motor dysfunction (i.e., resting tremor, bradykinesia, and postural instability) [1, 2]. Microglia, the resident macrophages of the central nervous system (CNS), play a major role in retaining the homeostasis in the brain milieu by providing a first-line defense against various exogenous and endogenous contaminants, curbing the potential spread of injury inflicted by clinical complications

[3]. Previous studies suggested that the microglia involved in the phagocytosing of dead neuronal cells in the brain milieu also aid in the survival of nerve cells by releasing varied neurotrophic factors [4, 5]. Increasing evidence suggests that the activation of resting glial cells was attained either by direct factors, that is, toxins, pathogens, or endogenous proteins, or by indirect factors due to neuronal death cascades [6, 7]. Precisely, activated glial cells trigger the inflammatory cascade by releasing proinflammatory cytokines (tumor necrosis factor-alpha (TNF-α) and interleukin (IL-1β and IL-6)) and inflammatory mediators (nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2)) by activating nuclear factor kappa-light-chain-enhancer (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [8, 9]. These cascades lead to NO accumulation and exhaustive DA neuronal loss, as clinically observed in the autopsy reports of several PD patients [10–13]. The exogenous activation of microglial cells was achieved both *in vitro* and *in vivo* via exposure of lipopolysaccharide (LPS), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and other neurotoxins [14–16]. In particular, the MPTP-induced mouse model is the most commonly used PD model, and it depicts significant clinical hallmarks of PD pathogenesis, that is, neuroinflammation and DA neuronal cell death more proximal to human brains [17]. MPTP was also reported to reduce the ratio of tyrosine hydroxylase (TH) positive in the SNpc and striatum (STR) regions compared to wild-type control, which subsequently contributes to the striatal DA deficiency and results in behavioral defects. In addition to the histopathological alterations, MPTP-treated mice exhibit substantial motor deficits, which shall be associated to tremors, rigidity, and posture imbalance of mice. However, experimentally, the behavioral alternation of the animals was measured using pole and rotarod tests, which shall relatively measure the motor skills of the animals (i.e., bradykinesia and hypokinesia). Current therapeutic strategies for PD provide inadequate benefits with substantial adverse effects; so to overcome this limitation, a safe and effective alternative should be developed. In recent years, phytotherapeutic leads have been in the limelight due to their improved potential with minimal or null toxicity in PD.

Dendropanax morbiferus (DM) is an endemic plant species that is widely distributed in the southern parts of Korea [18]. The leaves of this plant were used as prebiotic, probiotic, and antibacterial agents against various pathogens [19]. Various other parts of the plant have been documented as alternative, folkloric medicine against dermal complications and other infectious diseases [18–20]. Numerous scientific studies have reported the antioxidant, antidiabetic, anticarcinogenic, and nephroprotective potential of this plant species [21–23]. DM has also been recently reported to effectively alleviate the hippocampal function in mercury-induced neurotoxic rats by improving the endogenous antioxidant levels [24]. Though several studies reported broad medicinal properties of DML, its mechanism of action in the PD model has been poorly understood. In this study, we evaluated the underlying molecular mechanism of the antineuroinflammatory activity and the neuroprotective potential of DML and its bioactive compound (CA) in *in vitro* and *in vivo* experimental models of PD.

2. Materials and Methods

2.1. Reagents. *Dendropanax morbiferus* leaves were procured from Hanna arboretum (Jeonnam, Korea). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), 3-(3,4-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), chloroform, quercetin, kaempferol, rutin, vitexin, luteolin, tricin, ferulic acid, caffeic acid, chlorogenic acid, and N-1(1-naphthyl)ethylene-diamine dihydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). A 10x RIPA buffer was obtained from Millipore (Milford, MA, USA). Protease inhibitor and phosphatase inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN, USA). Plastic wares (6-well, 12-well, and 24-well tissue culture plates and 100 mm culture dishes) were purchased from SPL (Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 1x trypsin-EDTA (TE), and 100,000 U/ml penicillin-streptomycin (P-S) were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). Primary antibodies anti-ERK (1 : 2000), anti-p-ERK (1 : 2000), anti-JNK (1 : 1000), anti-p-JNK (1 : 1000), anti-p38 (1 : 2000), anti-p-p38 (1 : 2000), and anti-TH (1 : 1000) were obtained from Cell Signaling Technology (Danvers, MA, USA); anti-COX-2 (1 : 1000) and anti-TH (1 : 200) for IHC were obtained from Abcam (Cambridge, UK) and Calbiochem (San Diego, CA, USA); anti-Iba-1 (1 : 1000) and anti-iNOS (1 : 1000) were procured from Wako-chem (Chuo-ku, Osaka, Japan) and BD Biosciences (San Jose, CA, USA), respectively. Secondary antibodies antimouse (1 : 2000) and antirabbit (1 : 2000) were obtained from Cell Signaling Technology (Danvers, MA, USA) and Bio-Rad (Hercules, CA, USA), respectively.

2.2. Plant Leaf Extraction of Dendropanax morbiferus Leaves. DM leaves were purchased from Hanna arboretum, Republic of Korea. The obtained fresh DM leaves were washed in running tap water, oven-dried (50–60°C) for 3–5 days, and it is crushed manually to obtain the leaf flakes. The flakes were subjected to heat maceration in 1 l of distilled water at 100°C for 2 h, and the extract was filtered through Whatman® filter paper number 2. The obtained filtrate was further concentrated using rotary evaporator (EYELA N-1000, Tokyo), for 2 h, 3 times. The DM leaf extract (DML) residual was freeze-dried for 7 days and stored in an airtight container at −20°C.

2.3. Cell Culture Treatment. The BV-2 microglial cells were generously provided by Dr. K. Suk (Kyung-Pook National University, Daegu, Korea). As previously reported [25], the cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS and 50 μg/ml penicillin-streptomycin and maintained in a humidified incubator supplied with 5% CO_2 and 95% O_2. The cells were seeded at a density of 5×10^4 cells/ml and were pretreated for 1 h with varying concentrations of DML (100, 250, and 500 μg/ml), followed by LPS incubation

(200 ng/ml) at the respective time points (30 min, 6 h, and 24 h).

2.4. Animal Experimental Design. Six-week-old male C57BL/6N mice were obtained from DBL (Chungbuk, Korea) and acclimatized for 1 week before the start of the experiment. The 7-8-week-old (25–27 g) mice were used in this study. All experiments were performed in accordance with the principles of laboratory animal care (NIH publication number 85-23, revised 1985) and were approved by Konkuk University Institutional Animal Care and Use Committee (KU 17009). The animals were housed in a controlled environment ($23 \pm 1^{\circ}C$ and $50\% \pm 5\%$ humidity; 12 h dark-light cycle) and allowed food and water ad libitum. The animals ($n = 24$) were divided into three groups ($n = 8$ per group). The groups included a vehicle group (untreated), MPTP group (20 mg/kg of bw—four times, day 7 at 2 h intervals, i.p.), and DML group (200 mg/kg of bw—single dose/day until day 7, p.o.). MPTP and DML were dissolved in saline and prepared just prior to dosing.

2.5. Cell Cytotoxicity and NO Release. BV-2 cells were seeded at a density of 5×10^4 cells/well and were pretreated with various concentrations of DM (100, 250, and 500 μg/ml) for 1 h, followed by LPS (200 ng/ml) induction for 24 h. 20 μl of MTT (2.0 mg/ml) was added to each well, and after 2 h of incubation at 37°C in 5% CO_2, the supernatants were removed from each well, and the formed formazan crystals in viable cells were dissolved in DMSO. The absorbance was determined at 540 nm using a microplate reader (Tecan Trading AG, Basel, Switzerland). The inhibitory effect of DM on NO production was determined as previously described [25]. BV-2 microglial cells (5×10^4 cells/well) were incubated with LPS (200 ng/ml) in the presence or absence of DM (100, 250, and 500 μg/ml) for 24 h. After 24 h, 100 μl of supernatants was initially collected and assayed for NO release using commercially available Griess reagent (1 vol. 0.1% naphthylethylenediamine and 1 vol. 1% sulfanilamide in 5% H_3PO_4). Absorbance was determined at 540 nm using a microplate reader (Tecan Trading AG).

2.6. RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was isolated from BV-2 microglial cells (5×10^4 cells/well) treated for 6 h, using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was primarily performed using 2500 ng of total RNA and GoScript™ Reverse Transcription System (Promega, USA). Polymerase chain reaction was performed using an initial step of denaturation (2 min at 95°C), 24–30 cycles of amplification (95°C for 30 s, 55–58°C for 45 s, and 72°C for 1 min), and extension at 72°C for 5 min. The primer sequences used for inflammatory genes are represented in Table 1. PCR products were analyzed in 1% agarose gels. For quantification, the gels were photographed and the pixel intensity for each band was determined using the ImageJ (NIH) software and was normalized to the band intensity of GAPDH mRNA. The results are representative of three independent experiments.

Table 1: Quantification of phytophenolic constituents in DML-BuOH fraction.

Compounds	Contents (mg) (per 1 g aqueous DML)
Quercetin	0.1
Kaempferol	0.02
Rutin	6.38
Vitexin	0.23
Luteolin	0.02
Tricin	0.06
Ferulic acid	0.03
Chlorogenic acid	19.5
Caffeic acid	0.10

2.7. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis. Real-time PCRs (RT-PCRs) were performed in a Roche LightCycler 96 Real-Time System using Power SYBR green master mix (Life Technologies), according to the manufacturer's instructions, in a final volume of 20 μl reactions. The PCR conditions were as follows: initially incubated for 10 minutes at 95°C, followed by 15 sec incubation at 95°C (40 cycles) and a final 60 sec incubation at 60°C. Further, the specificity of each primer was verified by melting curve analysis (at 65–95°C, with fluorescence recording at every 0.5°C).

2.8. Immunocytochemistry (ICC) Analysis. BV-2 microglia cells (1×10^5 cells/well in a 12-well plate) were cultured on sterile 12 mm cover slips in 24-well plates and treated with DM (100, 250, and 500 μg/ml) and LPS (200 ng/ml) to detect the intracellular location of the nuclear factor kappa-B (NF-κB) p65 subunit. A fluorescence immunocytochemistry assay was performed, and the representative images were obtained using a fluorescence microscope (Carl Zeiss Inc., Oberkochen, Germany) as previously described [25, 26].

2.9. Behavioral Studies

2.9.1. Pole Test. The pole test for bradykinesia was conducted as previously described [27]. The mice were placed at the top of a rough-surfaced pole (8 mm diameter and 55 height) with the head-up posture, and the total locomotor activity (TLA) was measured. The TLA is the time taken by the mouse to reach the floor. The duration of these parameters reflects bradykinesia in PD. This test was performed successively five times for each mouse, and the average was analyzed.

2.9.2. Rotarod Test. The rotarod test was conducted to evaluate the motor deficits, as previously described with slight modification [28]. A computerized, automated rotarod 5-unit lane (DBL-02-MA5, Korea) machine was used for each mouse per lane. The rotarod machine was set with a preprogrammed protocol and was allowed to rotate at selective speed and time limits. The machine detects the mouse fall and records the time and distance covered by each mouse on their corresponding lanes. The mice from all groups were pretrained in the rotarod machine, at 10 rpm (5 min), once per day for three consecutive days (days 5–7), prior to MPTP

injection. On day 14, the experiment was started at a low speed limit of 4 rpm, with a gradual increase until reaching a high speed limit of 40 rpm. The time taken to fall—latency of fall (LTF) and total distance (TD) covered by each mouse during their respective sessions—was recorded and analyzed.

2.10. Western Blot Analysis. Treated BV-2 cells (5×10^5 cells/well) and animal brain tissue were washed twice with PBS and lysed for 10 min using 1x RIPA lysis buffer (4°C), respectively. Cell and tissue lysates were centrifuged at 14,000 rpm, 4°C, and the corresponding supernatants were collected and separately stored for further analysis. The protein concentration of each sample was obtained using a DC Protein Assay kit (Bio-Rad). Equal amounts of protein (20–40 μg for cells/600 μg for animal) were separated electrophoretically by 10% sodium dodecyl sulfate-polyacrylamide electrophoresis, and the resolved proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h with 3% BSA in TBS buffer to block nonspecific binding. The membranes were then incubated with primary antibodies to anti-inducible nitric oxide synthase, antityrosine hydroxylase (TH), anti-IκB-α, anti-phospho-IκB-α, anti-p65, anti-cyclooxygenase-2, anti-β-actin, and anti-ionized calcium-binding adapter molecule 1 (Iba-1), followed by incubation for 1 h with horseradish peroxidase-conjugated-specific secondary antibodies (1 : 2000; Cell Signaling, MA). The blots were visualized by a PowerOpti-ECL kit obtained from the Animal Genetics Inc. (Gyeonggi-do, Korea) detection system according to the recommended procedures.

2.11. Immunohistochemistry (IHC) Analysis. Following the behavioral tests, the mice were anesthetized using 23% urethane (i.p.) for immunohistochemical investigations. The brains of each mice were perfusion fixed via cardiac puncture with 4% paraformaldehyde, followed by a saline flush (Biosesang, Korea). The brains were removed after perfusion fixation at 4°C, immersed in the same fixative, and dehydrated in 30% sucrose solution. Subsequently, the brain was frozen-embedded using tissue freezing medium (Leica, GmbH Heidelberger, Germany). The fixed frozen brains were sectioned (30 μm) coronally to obtain the striatum and substantia nigra ($n = 3$/group). The free-floating brain sections (30 μm) were then incubated with specific anti-TH (1 : 200; Calbiochem; Merck KGaA, Darmstadt, Germany), VECTASTAIN ABC kit, and biotinylated secondary antibodies. The samples were visualized using DAB peroxidase (HRP) substrate kit (Vector Laboratories, CA, USA) by following the manufacturer's protocol.

2.12. Fractionation and Chromatographic Analysis. To identify the potential bioactive compounds responsible for the therapeutic efficacy of DML, we further obtained phenol-enriched ethyl acetate and butanol fractions of DML. The HPLC analysis of DML and its fractions were conducted using Agilent Technologies 6410 Triple Quad LC-MS/MS (Agilent, Santa Clara, CA, USA) with C18, 2.1×100 mm, 2.7 μm column. The mobile phases—solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile)—were used at a flow rate of 400 μl/min. The sample injection volume was 3 μl/sample. The gradient program set for the analysis is as follows: 95% solvent A : 5% solvent B, 0–30 min; 50% solvent A : 50% solvent B, 30–40 min. Compound identification was achieved using a coupled mass spectrometry system with the following source parameters: gas temp 350°C; capillary volt. 4000 V; nebulizer 40 psi; fragmentor 190 V allied with a MassHunter Software system. To quantify the identified compounds, commercially acquired authentic standards of quercetin, kaempferol, rutin, vitexin, luteolin, tricin, ferulic acid, chlorogenic acid, and caffeic acid dissolved in MeOH were used for the analysis.

2.13. Statistical Analysis. All the data were analyzed using GraphPad Prism software ver. 5.01 (GraphPad Inc., La Jolla, CA, USA). All data are expressed as mean ± standard deviation of at least three independent experiments. The statistical analysis was performed with a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The P values < 0.05 were considered to be significant.

3. Results

3.1. Effects of DML on the Cell Viability and Nitric Oxide Production in LPS-Stimulated BV-2 Microglial Cells. In this preliminarily study, we investigated the cytotoxic nature of DML in BV-2 microglial cells, to observe the toxic traits of the extract. The cells were treated with various concentrations of DML (100, 250, and 500 μg/ml) alone or with LPS (200 ng/ml), and the cytotoxicity was assayed using MTT. The results of our study indicated that at selected concentrations, LPS alone or with DML-treated cells did not show any significant toxicity. Interestingly, we observed that the DML alone at the chosen higher concentration of 500 μg/ml showed no toxic effects in BV-2 cells (Figure 1(a)). To further evaluate the inhibitory effect of DML in LPS-induced inflammatory responses, NO released from the DML-pretreated (100, 250, and 500 μg/ml) LPS-inflicted cells or DML-treated cells alone was analyzed. Cells treated with DML alone did not exhibit any changes in NO levels, similar to the control cells. On the other hand, cells incubated with LPS (200 ng/ml) significantly elevated the NO release (28 ± 2.2 μM, $P < 0.05$), which was dose dependently suppressed by DML treatment at described doses with values of 16.5 ± 0.3 μM, 11.7 ± 0.2 μM, and 4.9 ± 0.7 μM, respectively (Figure 1(b)).

3.2. DML Attenuates the Proinflammatory Mediators in LPS-Stimulated BV-2 Cells. To examine the impact of DML in modulating the proinflammatory mediators, the BV-2 microglial cells were stimulated with LPS (200 ng/ml) and treated with or without DML at indicated concentrations (100, 250, and 500 μg/ml). The alterations in the mRNA levels of the proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and associated mediator (iNOS, COX-2) genes were observed after 6 h followed by LPS induction, using RT-PCR analysis. The densitometric analysis of the bands showed that LPS-stimulated BV-2 cells significantly upregulated the proinflammatory cytokine level with a ninefold increase in TNF-α, sixfold increase in both IL-1β and IL-6

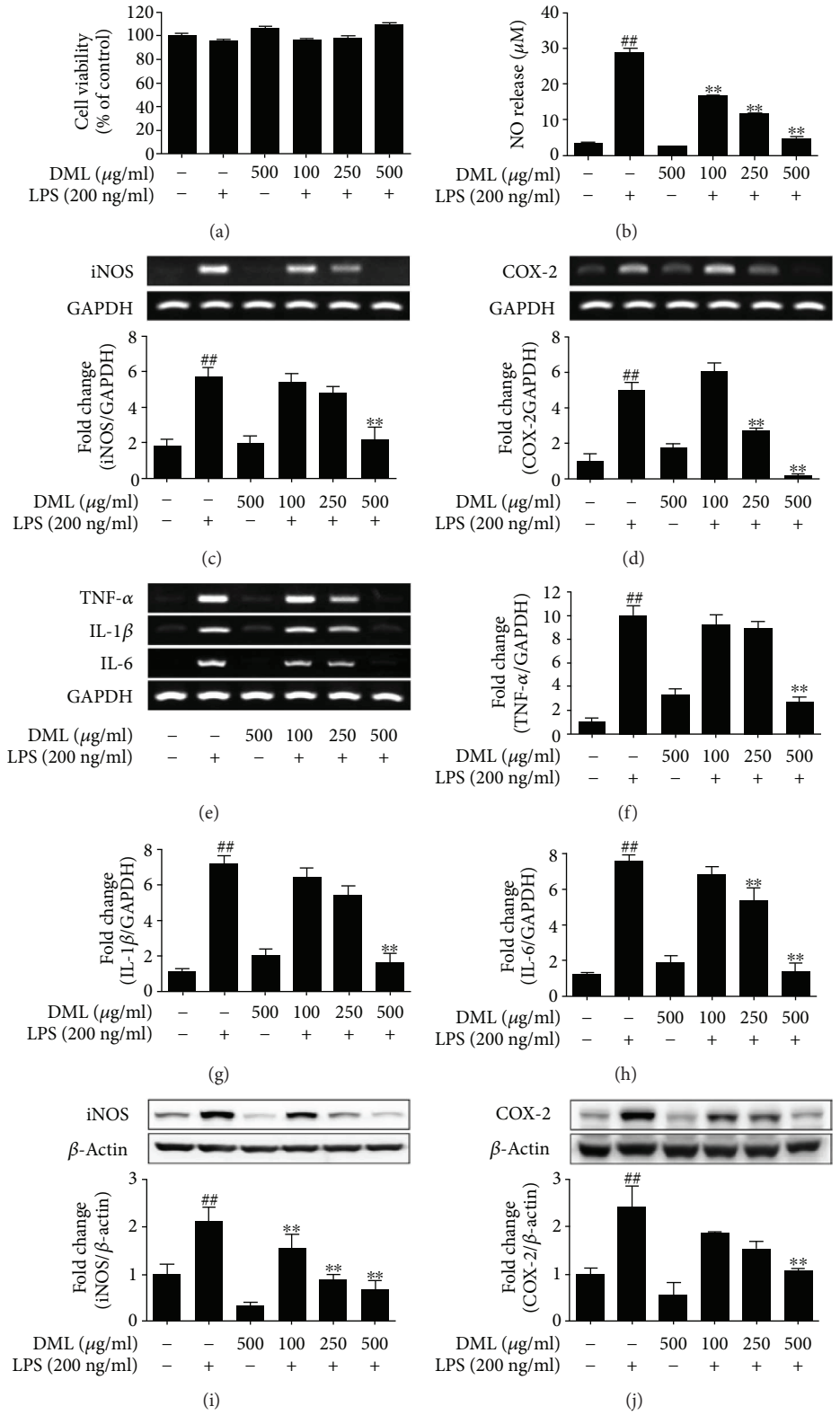


Figure 1: DML attenuates nitric oxide production, cell viability, and proinflammatory mediators in LPS-stimulated BV-2 cells. The cells were incubated with DML in the presence or absence of LPS (200 ng/ml) for 6 h (RNA levels) and 24 h (NO assay, MTT assay, and protein levels). The cytotoxicity and NO release results were displayed as percentage of control (a) and released NO in μM (b), respectively. The RT-PCR results of the inflammatory cytokines were expressed as bands and fold-change quantification with respect to GAPDH ratio of iNOS (c), COX-2 (d), TNF-α, IL-1β, and IL-6 (e–h). The immunoblot results of inflammatory mediators iNOS (i) and COX-2 (j) were expressed as blots and fold-change quantification with respect to β-actin ratio. Data are presented as mean ± standard error ($n = 3$) of three independent experiments. The values are mean ± standard error ($^{\#\#}P < 0.05$ versus control group and $^{**}P < 0.05$ versus LPS-treated group).

level, and around fourfold increase in both iNOS and COX-2 level compared to the control group. Meanwhile, pretreatment of LPS-stimulated BV-2 cells with DML for 1 h effectively alleviated the upregulation of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and associated mediator (iNOS, COX-2) (Figures 1(c)–1(h)) genes in a dose-dependent manner. These results indicate that DML substantially suppress the proinflammatory mediators at the transcriptional level.

3.3. DML Attenuates the Inflammatory Protein Expression Level in LPS-Stimulated BV-2 Cells. To evaluate the effect of DML on the inflammatory protein expression in LPS-stimulated BV-2 microglia, the cells were pretreated with DML at described concentrations (100, 250, and 500 μg/ml) and after 1 h incubated with LPS (200 ng/ml) for 24 h, followed by an evaluation of the protein markers using a Western blot analysis. The LPS-induced BV-2 cells significantly upregulated the iNOS and COX-2 protein levels with around a onefold increase compared to the control groups, and these have been substantially alleviated by DML treatment in a dose-dependent manner (Figures 1(i) and 1(j)).

3.4. DML Inhibits Inflammatory Response in LPS-Stimulated BV-2 Microglial Cells via Regulation of NF-κB and JNK Pathways. To explore the underlying molecular mechanism through which DML inhibits the inflammatory response in LPS-stimulated BV-2 microglia, we evaluated the protein expression level of the biomarkers associated with the NF-κB pathway (p-IκB-α, p-p65) and MAPK pathway biomarkers (p-ERK, p-p38, and p-JNK). BV-2 microglia cells were pretreated with DML at described concentrations (100, 250, and 500 μg/ml) for one hour, incubated with LPS (200 ng/ml) for 30 min, and then evaluated for protein markers via Western blot. Our results indicate that the LPS stimulation significantly improved the IκB-α phosphorylation, nuclear translocation, and phosphorylation of the NF-κB p65 subunit and also involved in the upregulated phosphorylation of ERK, p38, and JNK protein expression in BV-2 cells. In contrast, the DML dose dependently suppressed the phosphorylation of both the IκB-α and NF-κB p65 subunit (Figures 2(e)–2(h)). Interestingly, among the evaluated MAPK biomarkers, DML suppressed only the phosphorylation of JNK and showed no effects for ERK and p38 (Figures 2(a)–2(d)). Thus, from this data, we understand that the DML effectively suppressed the inflammatory response in BV-2 cells by regulating both NF-κB and JNK pathways.

3.5. DML Alleviated the Tyrosine Hydroxylase (TH) Depletion in MPTP-Intoxicated Mouse Model of PD. To evaluate the protective effects of DML against MPTP-inflicted TH depletion in mice, the protein expression of TH levels in the ventral midbrain (VM) was evaluated via Western blot (Figure 3(b)) and the immunoreactivity of the TH-positive cells in SNpc and STR was obtained through IHC (Figure 3(a)). As expected, MPTP intoxication significantly reduced the TH protein level expression in VM ($P < 0.05$), with a relative loss of TH-immunopositive fibers in striatum and SNpc compared to the control group, whereas DML-treated mice showed significant ($P < 0.05$) elevation in TH protein levels with substantial protection of TH-immunopositive fibers in striatum and SNpc as in the control group.

3.6. DML Suppress the Inflammatory Protein Expression and Microglial Activation Markers in MPTP-Intoxicated Mouse Model of PD. Neuronal inflammation mediated by microglial cell activation is a clinical hallmark of PD. To evaluate the neuroprotective effect of DML, mice were intoxicated with MPTP (20 mg/kg of bw, i.p.) followed by DML pretreatment (200 mg/kg of bw, p.o.) or saline treated or untreated (control), and the characteristic inflammatory (iNOS, COX-2) and microglial activation (Iba-1) protein expressions of the brain procured from respective groups were analyzed via Western blot (Figure 3(c)). MPTP-intoxicated mice substantially upregulated the expression of inflammatory proteins (iNOS: sixfold/COX-2: twofold) (Figures 3(d) and 3(e)) and microglial activation (Iba-1: onefold) protein (Figure 3(f)) compared to the control group. This upregulation of the characteristic protein expression was significantly suppressed by DML treatment, and it is quite equivalent to that of control.

3.7. DML Improved the Behavioral Deficits in MPTP-Intoxicated Mouse Model of PD. To evaluate the protective effects of DML in terms of behavioral deficits, the mice were intoxicated with MPTP (20 mg/kg of bw, i.p.) followed by DML pretreatment (200 mg/kg of bw, p.o.) or saline treated or untreated (control), and their behavioral modulations were evaluated by performing a pole test and a rotarod test. The MPTP-intoxicated mice significantly reduced the latency to fall (LTF) (85.7 ± 20.9 s) (Figure 4(a)) and total distance (TD) covered (1.7 ± 0.7 m) (Figure 4(b)) in the rotarod test with an increased total locomotor activity (TLA) (45.4 ± 17.8 s) (Figure 4(c)) in the pole test, compared to the control group, whereas pretreatment with DML significantly ameliorated the behavioral deficit with improved LTF (139.7 ± 30.8 s) (Figure 4(a)), TD (4.3 ± 1.5 m) (Figure 4(b)), and reduced TLA (8.7 ± 3.1 s) (Figure 4(c)). This data suggests that DML improves the behavioral deficits exerted by MPTP intoxication in a mouse model of PD.

3.8. Chromatographic Analysis of DML/Fractions and Quantification of Its Phenolic Constituents. Among the chromatographic profiles of DML and its fractions, the butanol fraction of DML exhibited a relatively higher quantity of phenolic constituents, which have been quantified and enlisted in Table 1. Despite this, the chlorogenic acid (CA) was reported to be present in a higher amount (19.5 mg/g of fraction) in the butanol fraction compared to other quantified phenolic constituents. The chromatographic profiles of the standard mixtures, DML-BuOH fraction, and CA are represented in Figures 5(a)–5(c), respectively.

3.9. Chlorogenic Acid Alleviates the Proinflammatory Mediators in LPS-Stimulated BV-2 Cells. In accordance with our chromatographic results, CA was found to be a major

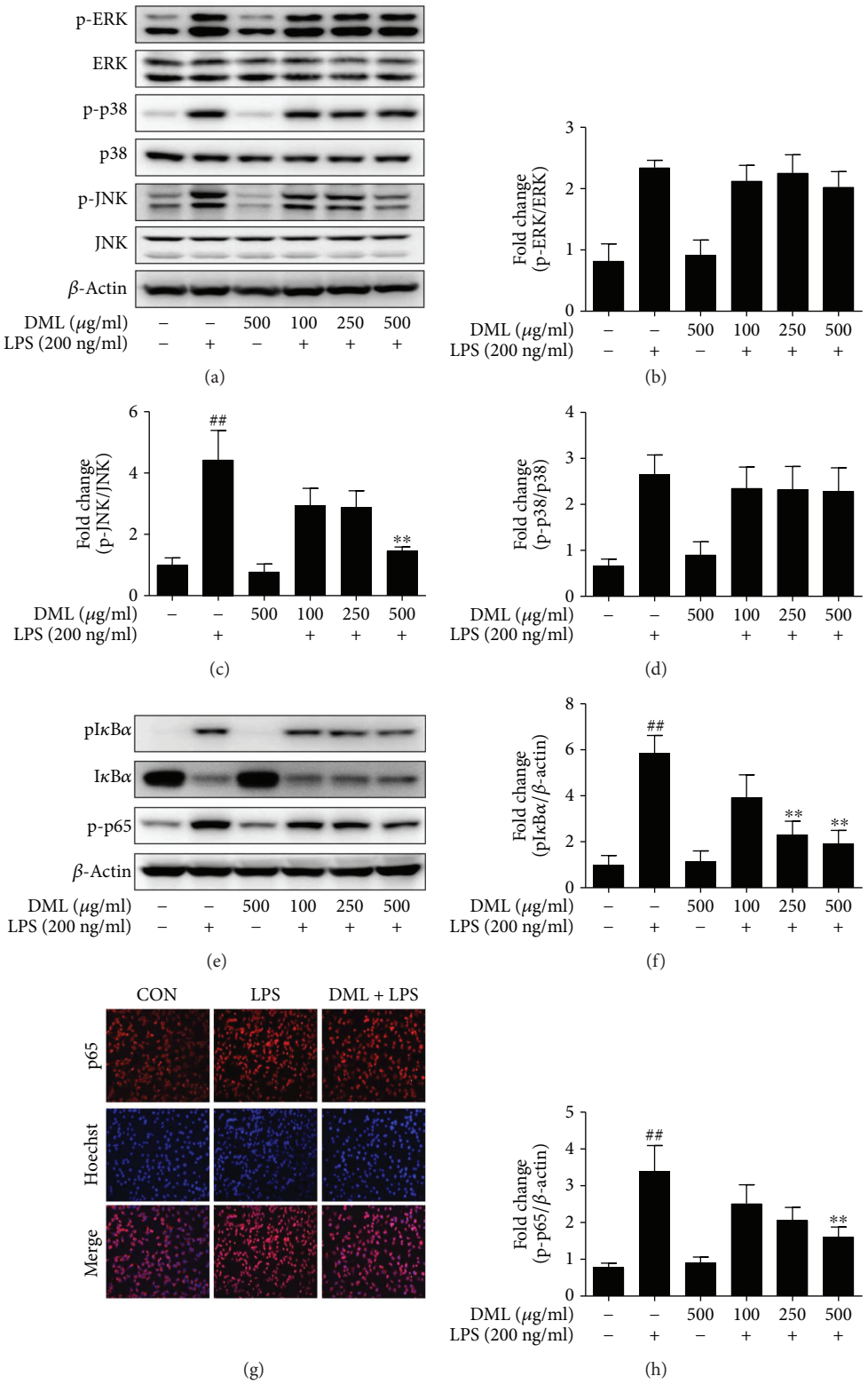


Figure 2: DML modulates the protein levels of MAPKs, NF-κB, and IκB-α in LPS-stimulated BV-2 cells. The protein expressions of MAPKs (a–d) were represented as blots and fold-change quantification with respect to their corresponding phosphorylated protein ratio. The IκB-α, phospho-IκB-α, and phospho-p65 (e, f, and h) were represented with respect to β-actin ratio. Data were mean ± standard error ($n = 3$) of three independent experiments. Values are mean ± standard error ($^{\#\#}P < 0.05$ versus control group and $^{**}P < 0.05$ versus LPS-treated group). The subcellular location of NF-κB p65 subunit was determined by immunofluorescence assay (g), using Alexa Fluor® 568 red with Hoechst background staining.

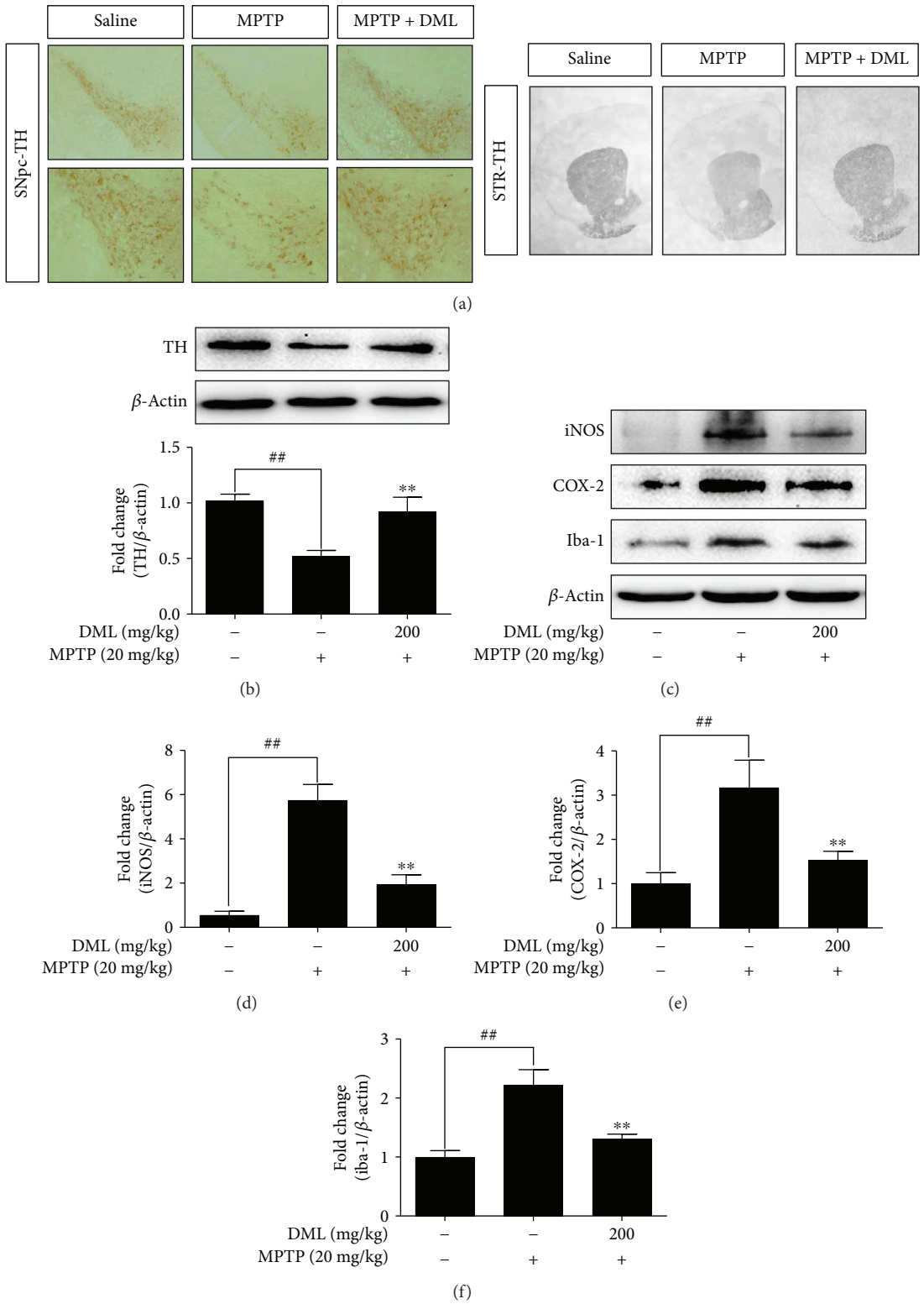


Figure 3: Defensive effect of DML on MPTP-induced loss of TH and elevated inflammatory mediator expression in substantia nigra pars compacta (SNpc). (a) Representative image of TH-positive cell immunoreactivity (IR) in substantia nigra pars compacta (SNpc) section and optical density (OD) analysis for TH-positive fibers in striatum (STR) section. The protein expressions of TH (b), iNOS, COX-2, and Iba-1 (c–f) in ventral midbrain (VM) were represented as blots and fold-change quantification with respect to β-actin ratio for three independent experiments. The values were mean ± standard error ($^{\#\#}P < 0.05$ versus control group and $^{**}P < 0.05$ versus MPTP-treated group).

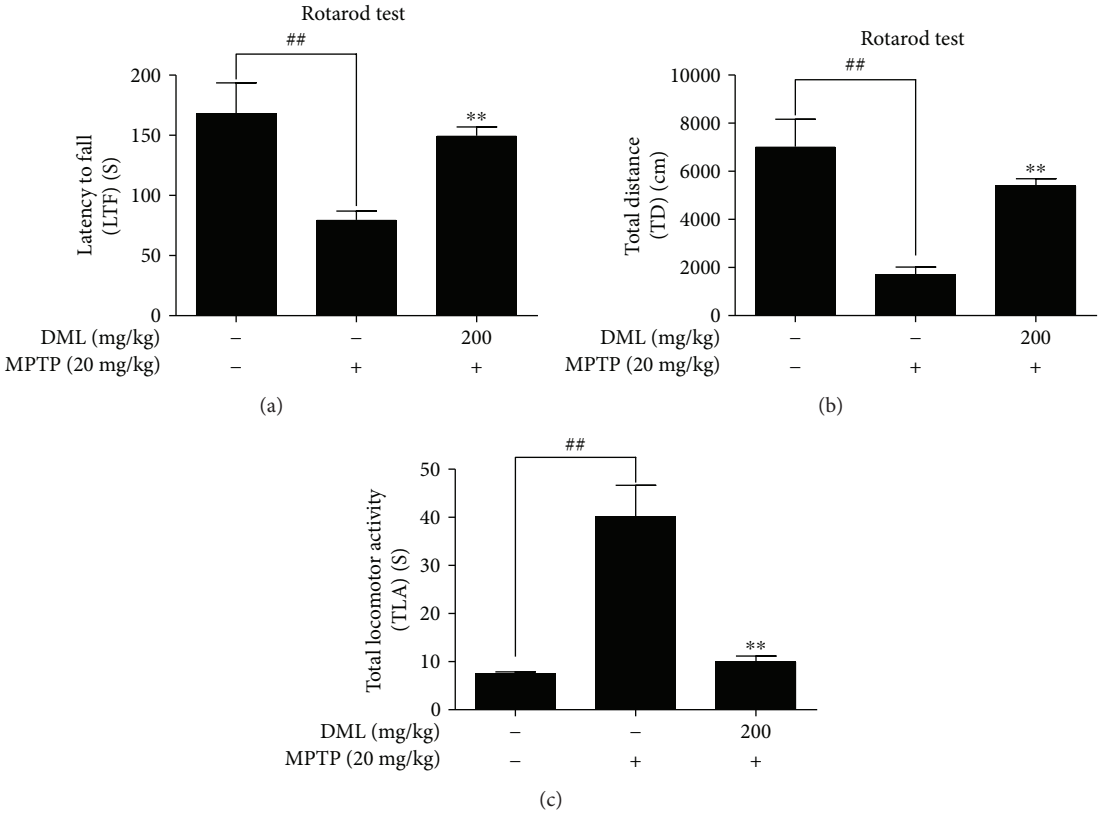
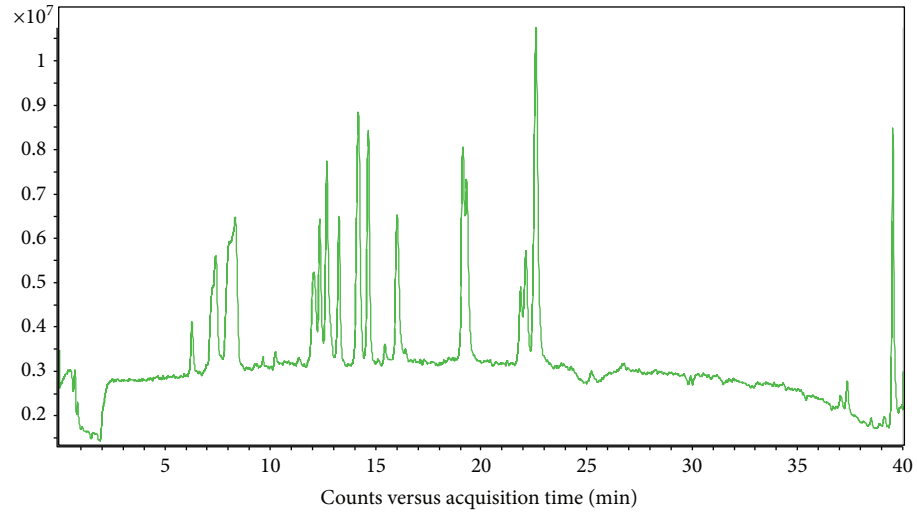


Figure 4: DML ameliorated the behavioral deficits in MPTP-intoxicated mouse. The rotarod and pole test were conducted on day 14. The latency of fall (LTF) time, total distance (TD), and total locomotor activity (TLA) of the animals were recorded and graphically represented in a, b, and c, respectively. Values shown were mean ± standard error for five mice (each group) ($^{\#\#}P < 0.05$ versus control group and $^{**}P < 0.05$ versus MPTP-treated group).

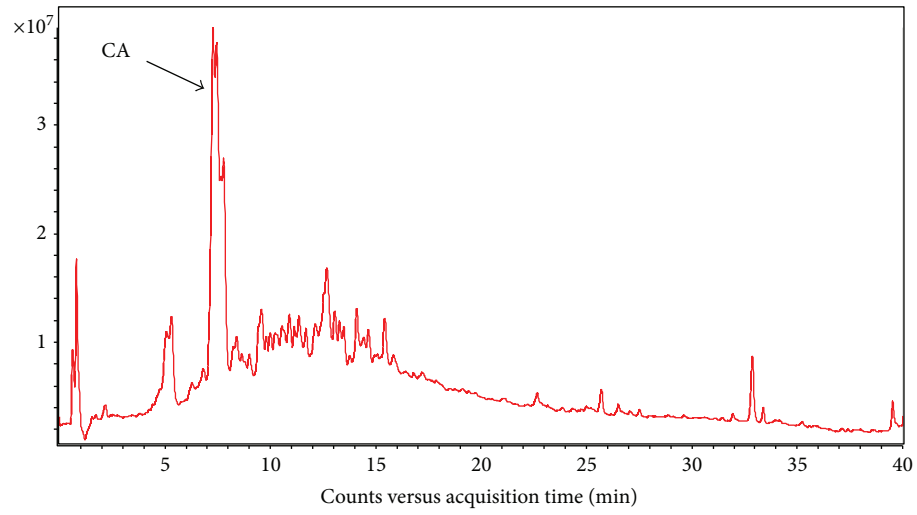
bioactive constituent of DML-butanol fraction. Thus, we further extended our study to evaluate the antineuroinflammatory potential of CA. The qRT-PCR and Western blot results of our study indicated that CA substantially downregulated the gene (Figures 6(a)–6(d)) as well as the protein expressions (Figures 6(e) and 6(f)) of proinflammatory cytokines and associated inflammatory mediators in a dose-dependent manner in LPS-induced BV-2 microglial cells. This result indicates that CA could possibly contribute to the antineuroinflammatory and ameliorative potential of DML in the MPTP-induced PD model.

4. Discussion

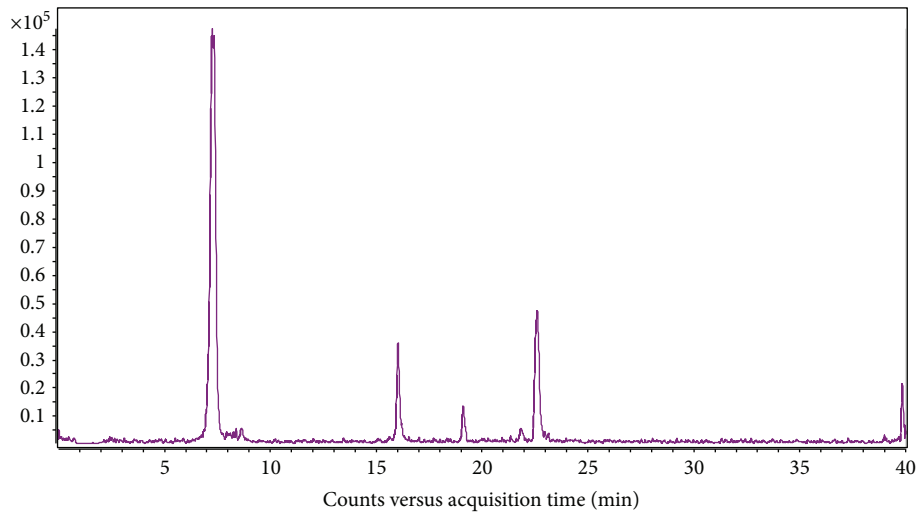
Neuroinflammation is a major hallmark of various progressive neurodegenerative disorders, including PD [29, 30]. An increase in *in vitro* and *in vivo* evidence suggests that inhibiting inflammatory cascades mediated by microglial activation is an effective therapeutic strategy to curb the progression of PD [31–34]. Lately, DML was also reported to show remedial effects on few neuronal-associated complications, that is, paralysis, stroke, and migraines [35]. Thus, in this study, we investigated the antineuroinflammatory and neuroprotective role of DML in the MPTP model. DML substantially curbed the advancement of microglia-mediated neuroinflammatory cascades both *in vitro* and *in vivo* and effectively alleviated the behavioral deficiencies detected in an MPTP-intoxicated PD mouse model. The resting microglia can be evoked by various inflammatory insults, such as LPS/MPTP-induced toxicity, which in turn leads to an upsurge in proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and inflammatory mediators (COX-2 and inducible nitric oxide synthase (iNOS)), generating a ROS/RNS environment [14, 36, 37]. Jiang et al. described the antineuroinflammatory role of *Acorus gramineus* leaf aqueous extract by elucidating its potential modulating role on inflammatory mediators in MPTP-induced mouse model of Parkinson's disease [38]. A recent study reported that the aqueous extract of *Withania somnifera* leaves effectively suppressed the LPS-induced microglial inflammatory action by modulating the reported inflammatory mediators [39]. Accordingly, in our study, DML evidently alleviated the inflammatory effects inflicted by LPS- and MPTP-mediated glial cell activation both *in vitro* and *in vivo*, respectively, with a significant downregulation of proinflammatory cytokine (TNF-α, IL-1β, and IL-6) levels and inflammatory mediators (iNOS and COX-2). The underlying neuroinflammation event has been generally reported to be interlinked with several molecular pathways, among them are NF-κB and MAPK signaling pathways, which are well documented [8, 40]. Phosphorylated IκB-α-mediated nuclear translocation of NF-κB and subsequent phosphorylation of mitogen-activated protein



(a) STD mixture



(b) Sample (BuOH fr.)



(c) Chlorogenic acid (M.W. = 354)

Figure 5: HPLC chromatograms of butanol fractions from aqueous extract of DML: (a) standard mixture, (b) butanol fraction of DML extract, and (c) chlorogenic acid standard.

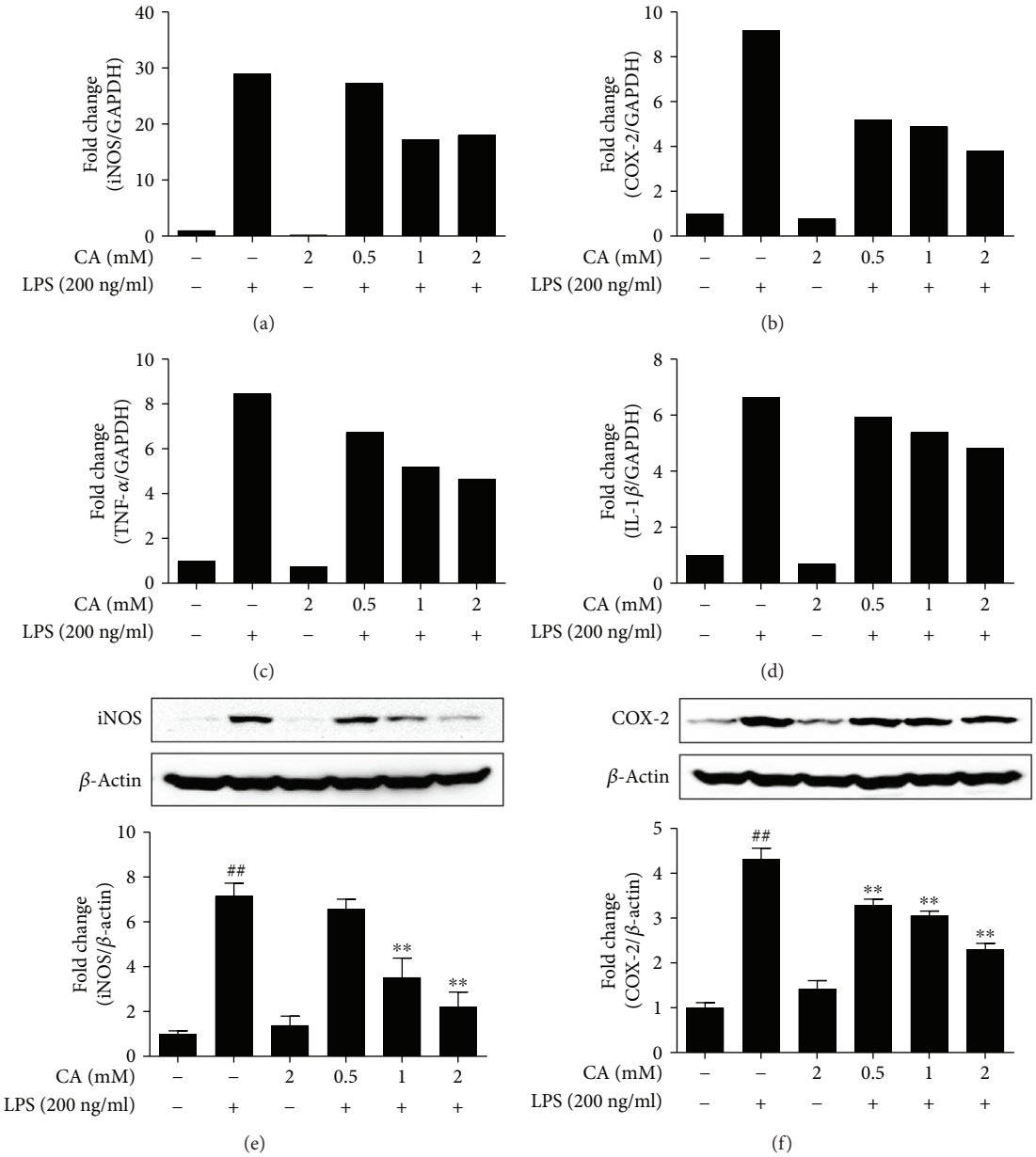


Figure 6: Modulatory effects of chlorogenic acid (CA) on proinflammatory cytokines and inflammatory mediators in LPS-activated BV-2 microglial cells. Cells were incubated with various concentrations of CA (0.5, 1, and 2 mM) in the presence or absence of LPS (200 ng/ml) for 6 h (RNA levels) and 24 h (protein levels). The qPCR results of the inflammatory cytokines were expressed as bands and fold-change quantification with respect to GAPDH ratio for iNOS (a), COX-2 (b), TNF-α (c), and IL-1β (d). The immunoblot results of inflammatory mediators iNOS (e) and COX-2 (f) were expressed as blots and fold-change quantification with respect to β-actin ratio. Data were mean ± standard error ($n = 3$) of three independent experiments. Values were mean ± standard error ($^{\#\#}P < 0.05$ versus control group and $^{**}P < 0.05$ versus LPS-treated group).

kinases (MAPKs, i.e., p-ERK, p-p38, and p-JNK) were reported to play an essential role in regulating transcriptional genes for iNOS and COX-2 production [40–42]. In this study, DML effectively suppressed the activation of NF-κB by inhibiting the phosphorylation of IκB-α and mediating the nuclear translocation of NF-κB p65 subunit. Consequently, this inhibits iNOS and COX-2 production. Interestingly, our results also indicated that DML achieved the same results by suppressing the phosphorylation of JNK, but not other MAPKs. Earlier studies employed Iba-1 as an effective biomarker to positively stain the activated microglia, immunohistochemically [43]. In this study, the microgliosis inhibitory action of DML was double confirmed by capturing its suppressive role on microglial activation, with a decreased Iba-1 immunoreactivity in DML-treated MPTP-intoxicated mouse brains. Since DML inhibits neuroinflammation by targeting multiple molecular targets, it can be further developed as a potential therapeutic agent to halt the neuroinflammation in PD progression. MPTP intoxication inflicts motor deficits and bradykinesia in mice [44–46]. Ren et al. reported

that dihydromyricetin, a natural flavonoid from *Ampelopsis grossedentata* sp., effectively alleviated motor impairments inflicted by MPTP-intoxicated PD mouse model, as observed in climbing pole and rotarod test results [47]. Accordingly, the results of our study indicated that MPTP intoxication exhibited severe behavioral deficits, including prolonged TLA in the pole test with reduced LTF and TD in the rotarod test. This was significantly alleviated by the DML pretreatment and remarkably improved the behavioral motor function. Numerous studies documented the reduction of dopamine levels associated with enormous loss of tyrosine hydroxylase- (TH-) positive fibers in the striatum and SNpc as a characteristic event of PD progression [44, 48–51]. In accordance, TH-positive (TH^+) stains as a potential marker to immunohistochemically depict the dopaminergic neuron status [52, 53]. In this study, the MPTP intoxication inflicted substantial DA neuronal loss at STR and SNpc, with a decline in TH staining. In contrast, DML-treated animals showed significant positive TH staining, which indicates the neuroprotective activity of DML by shielding the loss of DA neurons in the PD pathogenesis. The chromatographic profiling of the DML/fraction showed that the butanol fraction of DML exhibited a relatively higher concentration of phenolic-flavonoids. Previous reports indicated that phenolic-flavonoids, such as kaempferol, quercetin, apigenin, coumaric acid, and caffeic acid, were reported to possess anti-inflammatory potential. In accordance, in this study, among several identified phenolic constituents of DML-butanol fraction, CA was found to be present at a higher concentration, and it exhibited substantial antineuroinflammatory potential in LPS-induced microglia cells. It is also worth noting that CA has been earlier reported to have significant antioxidant, anti-inflammation, antidiabetic, anticarcinogenic, and antiobesity activities, thereby facilitating a nonpharmacological and noninvasive approach for treatment or prevention of some chronic disease [54]. Taken together, we report that CA is a potential therapeutic candidate that contributes to the enhanced neuroprotective potential of DML.

5. Conclusion

In conclusion, our study showed that DML significantly attenuated neuroinflammatory cascades in activated microglia and restored the behavioral motor deficits in PD progression. The underlying molecular mechanism of DML can be explained as it effectively curbs the microglia-stimulated neuroinflammation by modulating the NF-κB/IκB-α and JNK-MAPK signaling pathways. However, further preclinical/clinical investigations of DML/bioactive compound on the inflammation trail shall pave the way to produce an effective therapeutic candidate to treat various neurodegenerative disorders.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

This research work was supported by the Business for Cooperative R&D between Industry, Academy, and Research Institute, funded by the Korea Small and Medium Business Administration in 2016 (Grant no. C0395596). This research was also supported by The Leading Human Resource Training Program of Regional Neo Industry through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT and Future Planning (Grant no. NRF-2016H1D5A1909610).

References

[1] S. Vivekanantham, S. Shah, R. Dewji, A. Dewji, C. Khatri, and R. Ologunde, "Neuroinflammation in Parkinson's disease: role in neurodegeneration and tissue repair," *International Journal of Neuroscience*, vol. 125, no. 10, pp. 717–725, 2015.

[2] V. Licker, N. Turck, E. Kovari et al., "Proteomic analysis of human substantia nigra identifies novel candidates involved in Parkinson's disease pathogenesis," *Proteomics*, vol. 14, no. 6, pp. 784–794, 2014.

[3] R. A. Rice, J. Pham, R. J. Lee, A. R. Najafi, B. L. West, and K. N. Green, "Microglial repopulation resolves inflammation and promotes brain recovery after injury," *Glia*, vol. 65, no. 6, pp. 931–944, 2017.

[4] R. Fu, Q. Shen, P. Xu, J. J. Luo, and Y. Tang, "Phagocytosis of microglia in the central nervous system diseases," *Molecular Neurobiology*, vol. 49, no. 3, pp. 1422–1434, 2014.

[5] A. Suzumura, H. Takeuchi, G. Zhang, R. Kuno, and T. Mizuno, "Roles of glia-derived cytokines on neuronal degeneration and regeneration," *Annals of the New York Academy of Sciences*, vol. 1088, no. 1, pp. 219–229, 2006.

[6] M. E. Lull and M. L. Block, "Microglial activation and chronic neurodegeneration," *Neurotherapeutics*, vol. 7, no. 4, pp. 354–365, 2010.

[7] J. Ciesielski-Treska, G. Ulrich, S. Chasserot-Golaz et al., "Mechanisms underlying neuronal death induced by chromogranin A-activated microglia," *Journal of Biological Chemistry*, vol. 276, no. 16, pp. 13113–13120, 2001.

[8] Y. S. Kim and T. H. Joh, "Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease," *Experimental & Molecular Medicine*, vol. 38, no. 4, pp. 333–347, 2006.

[9] I. C. M. Hoogland, C. Houbolt, D. J. van Westerloo, W. A. van Gool, and D. van de Beek, "Systemic inflammation and microglial activation: systematic review of animal experiments," *Journal of Neuroinflammation*, vol. 12, no. 1, p. 114, 2015.

[10] X. Su, K. A. Maguire-Zeiss, R. Giuliano, L. Prifti, K. Venkatesh, and H. J. Federoff, "Synuclein activates microglia in a model of Parkinson's disease," *Neurobiology of Aging*, vol. 29, no. 11, pp. 1690–1701, 2008.

[11] T. Shabab, R. Khanabdali, S. Z. Moghadamtousi, H. A. Kadir, and G. Mohan, "Neuroinflammation pathways: a general review," *International Journal of Neuroscience*, vol. 127, no. 7, pp. 624–633, 2017.

[12] R. von Bernhardi, L. E.-v. Bernhardi, and J. Eugenín, "Microglial cell dysregulation in brain aging and neurodegeneration," *Frontiers in Aging Neuroscience*, vol. 7, no. 124, 2015.

[13] B.-W. Kim, S. Koppula, H. Kumar et al., "α-Asarone attenuates microglia-mediated neuroinflammation by inhibiting

NF kappa B activation and mitigates MPTP-induced behavioral deficits in a mouse model of Parkinson's disease," *Neuropharmacology*, vol. 97, pp. 46–57, 2015.

[14] K. U. Tufekci, R. Meuwissen, S. Genc, and K. Genc, "Inflammation in Parkinson's disease," *Advances in Protein Chemistry and Structural Biology*, vol. 88, pp. 69–132, 2012.

[15] D. K. Andrew and G. J. Harry, "Features of Microglia and Neuroinflammation Relevant to Environmental Exposure and Neurotoxicity," *International Journal of Environmental Research and Public Health*, vol. 8, no. 7, pp. 2980–3018, 2011.

[16] J. Emerit, M. Edeas, and F. Bricaire, "Neurodegenerative diseases and oxidative stress," *Biomedicine & Pharmacotherapy*, vol. 58, no. 1, pp. 39–46, 2004.

[17] G. E. Meredith and D. J. Rademacher, "MPTP mouse models of Parkinson's disease: an update," *Journal of Parkinson's Disease*, vol. 1, no. 1, pp. 19–33, 2011.

[18] B.-Y. Park, B.-S. Min, S.-R. Oh et al., "Isolation and anticomplement activity of compounds from *Dendropanax morbifera*," *Journal of Ethnopharmacology*, vol. 90, no. 2-3, pp. 403–408, 2004.

[19] Y.-H. Lee, J. Y. Lee, J. W. Kim, and Y. J. An, "Effect of leaf extracts of *Dendropanax morbifera* on selected probiotics and pathogenic bacteria," *The FASEB Journal*, vol. 31, article lb407, 1 Supplement, 2017.

[20] Y. M. Park and J. S. Han, "A study on the utilization of *Dendropanax morbifera* Lev. leaf extract for material of functional cosmetics and hair growth products," *Asian Journal of Beauty and Cosmetology*, vol. 14, no. 3, pp. 277–288, 2016.

[21] T. K. Hyun, M.-o. Kim, H. Lee, Y. Kim, E. Kim, and J.-S. Kim, "Evaluation of anti-oxidant and anti-cancer properties of *Dendropanax morbifera* Léveille," *Food Chemistry*, vol. 141, no. 3, pp. 1947–1955, 2013.

[22] H.-I. Moon, "Antidiabetic effects of dendropanoxide from leaves of Dendropanax morbifera Leveille in normal and streptozotocin-induced diabetic rats," *Human & Experimental Toxicology*, vol. 30, no. 8, pp. 870–875, 2010.

[23] E. S. Kim, J. S. Lee, M. Akram et al., "Protective activity of *Dendropanax morbifera* against cisplatin-induced acute kidney injury," *Kidney and Blood Pressure Research*, vol. 40, no. 1, pp. 1–12, 2015.

[24] W. Kim, D. W. Kim, D. Y. Yoo et al., "Antioxidant effects of *Dendropanax morbifera* Léveille extract in the hippocampus of mercury-exposed rats," *BMC Complementary and Alternative Medicine*, vol. 15, no. 1, p. 247, 2015.

[25] B.-W. Kim, S. Koppula, I. S. Kim et al., "Anti-neuroinflammatory activity of kamebakaurin from *Isodon japonicus* via inhibition of c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase pathway in activated microglial cells," *Journal of Pharmacological Sciences*, vol. 116, no. 3, pp. 296–308, 2011.

[26] B.-W. Kim, S. Koppula, S.-S. Hong et al., "Regulation of microglia activity by glaucocalyxin-A: attenuation of lipopolysaccharide-stimulated neuroinflammation through NF-κB and p38 MAPK signaling pathways," *PLoS One*, vol. 8, no. 2, article e55792, 2013.

[27] N. Ogawa, Y. Hirose, S. Ohara, T. Ono, and Y. Watanabe, "A simple quantitative bradykinesia test in MPTP-treated mice," *Research Communications in Chemical Pathology and Pharmacology*, vol. 50, no. 3, pp. 435–441, 1985.

[28] F. Bergquist, H. N. Shahabi, and H. Nissbrandt, "Somatodendritic dopamine release in rat substantia nigra influences motor performance on the accelerating rod," *Brain Research*, vol. 973, no. 1, pp. 81–91, 2003.

[29] Q. Wang, Y. Liu, and J. Zhou, "Neuroinflammation in Parkinson's disease and its potential as therapeutic target," *Translational Neurodegeneration*, vol. 4, no. 1, p. 19, 2015.

[30] M. G. Tansey and M. S. Goldberg, "Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention," *Neurobiology of Disease*, vol. 37, no. 3, pp. 510–518, 2010.

[31] U. K. Hanisch and H. Kettenmann, "Microglia: active sensor and versatile effector cells in the normal and pathologic brain," *Nature Neuroscience*, vol. 10, no. 11, pp. 1387–1394, 2007.

[32] R. J. Horvath, N. Nutile-McMenemy, M. S. Alkaitis, and J. A. DeLeo, "Differential migration, LPS-induced cytokine, chemokine, and NO expression in immortalized BV-2 and HAPI cell lines and primary microglial cultures," *Journal of Neurochemistry*, vol. 107, no. 2, pp. 557–569, 2008.

[33] G. Porras, Q. Li, and E. Bezard, "Modeling Parkinson's disease in primates: the MPTP model," *Cold Spring Harbor Perspectives in Medicine*, vol. 2, no. 3, article a009308, 2012.

[34] V. Sanchez-Guajardo, C. J. Barnum, M. G. Tansey, and M. Romero-Ramos, "Neuroimmunological processes in Parkinson's disease and their relation to α-synuclein: microglia as the referee between neuronal processes and peripheral immunity," *ASN Neuro*, vol. 5, no. 2, article AN20120139, p. AN20120066, 2013.

[35] H.-J. Shim, S. Park, J.-W. Lee et al., "Extracts from *Dendropanax morbifera* leaves have modulatory effects on neuroinflammation in microglia," *The American Journal of Chinese Medicine*, vol. 44, no. 1, pp. 119–132, 2016.

[36] V. Chhor, T. Le Charpentier, S. Lebon et al., "Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia *in vitro*," *Brain, Behavior, and Immunity*, vol. 32, pp. 70–85, 2013.

[37] S.-c. Zhao, L.-s. Ma, Z.-h. Chu, H. Xu, W.-q. Wu, and F. Liu, "Regulation of microglial activation in stroke," *Acta Pharmacologica Sinica*, vol. 38, no. 4, pp. 445–458, 2017.

[38] J. Jiang, J.-J. Kim, D.-Y. Kim et al., "*Acorus gramineus* inhibits microglia mediated neuroinflammation and prevents neurotoxicity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of Parkinson's disease," *Journal of Ethnopharmacology*, vol. 144, no. 3, pp. 506–513, 2012.

[39] M. Gupta and G. Kaur, "Aqueous extract from the *Withania somnifera* leaves as a potential anti-neuroinflammatory agent: a mechanistic study," *Journal of Neuroinflammation*, vol. 13, no. 1, p. 193, 2016.

[40] B. Kaminska, A. Gozdz, M. Zawadzka, A. Ellert-Miklaszewska, and M. Lipko, "MAPK signal transduction underlying brain inflammation and gliosis as therapeutic target," *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, vol. 292, no. 12, pp. 1902–1913, 2009.

[41] R.-H. Shih, C.-Y. Wang, and C.-M. Yang, "NF-kappaB signaling pathways in neurological inflammation: a mini review," *Frontiers in Molecular Neuroscience*, vol. 8, p. 77, 2015.

[42] E. K. Kim and E.-J. Choi, "Pathological roles of MAPK signaling pathways in human diseases," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1802, no. 4, pp. 396–405, 2010.

[43] R. J. Smeyne, C. B. Breckenridge, M. Beck et al., "Assessment of the effects of MPTP and paraquat on dopaminergic neurons and microglia in the substantia nigra pars compacta of C57BL/6 mice," *PLoS One*, vol. 11, no. 10, article e0164094, 2016.

[44] W. Dauer and S. Przedborski, "Parkinson's disease: mechanisms and models," *Neuron*, vol. 39, no. 6, pp. 889–909, 2003.

[45] H. Braak, K. Del Tredici, U. Rüb, R. A. I. de Vos, E. N. H. Jansen Steur, and E. Braak, "Staging of brain pathology related to sporadic Parkinson's disease," *Neurobiology of Aging*, vol. 24, no. 2, pp. 197–211, 2003.

[46] J. Lotharius and P. Brundin, "Pathogenesis of Parkinson's disease: dopamine, vesicles and α-synuclein," *Nature Reviews Neuroscience*, vol. 3, no. 12, pp. 932–942, 2002.

[47] Z.-x. Ren, Y.-f. Zhao, T. Cao, and X.-c. Zhen, "Dihydromyricetin protects neurons in an MPTP-induced model of Parkinson's disease by suppressing glycogen synthase kinase-3 beta activity," *Acta Pharmacologica Sinica*, vol. 37, no. 10, pp. 1315–1324, 2016.

[48] S. C. Daubner, T. Le, and S. Wang, "Tyrosine hydroxylase and regulation of dopamine synthesis," *Archives of Biochemistry and Biophysics*, vol. 508, no. 1, pp. 1–12, 2011.

[49] M. Cossette, A. Parent, and D. Levesque, "Tyrosine hydroxylase-positive neurons intrinsic to the human striatum express the transcription factor *Nurr1*," *European Journal of Neuroscience*, vol. 20, no. 8, pp. 2089–2095, 2004.

[50] J. L. George, S. Mok, D. Moses et al., "Targeting the progression of Parkinson's disease," *Current Neuropharmacology*, vol. 7, no. 1, pp. 9–36, 2009.

[51] H.-C. Cheng, C. M. Ulane, and R. E. Burke, "Clinical progression in Parkinson disease and the neurobiology of axons," *Annals of Neurology*, vol. 67, no. 6, pp. 715–725, 2010.

[52] Y. Bi, P. C. Qu, Q. S. Wang et al., "Neuroprotective effects of alkaloids from *Piper longum* in a MPTP-induced mouse model of Parkinson's disease," *Pharmaceutical Biology*, vol. 53, no. 10, pp. 1516–1524, 2015.

[53] B.-W. Kim, S. Koppula, S.-Y. Park et al., "Attenuation of neuroinflammatory responses and behavioral deficits by *Ligusticum officinale* (Makino) Kitag in stimulated microglia and MPTP-induced mouse model of Parkinson's disease," *Journal of Ethnopharmacology*, vol. 164, pp. 388–397, 2015.

[54] N. Tajik, M. Tajik, I. Mack, and P. Enck, "The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature," *European Journal of Nutrition*, vol. 56, no. 7, pp. 2215–2244, 2017.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 5010741, 12 pages
https://doi.org/10.1155/2018/5010741

Review Article

Benefit of Oleuropein Aglycone for Alzheimer's Disease by Promoting Autophagy

Joaquín G. Cordero,[1] Ramón García-Escudero,[2,3,4] Jesús Avila,[5,6] Ricardo Gargini,[5,7] and Vega García-Escudero[1,5,6]

[1]*Departamento de Anatomía, Histología y Neurociencia, Facultad de Medicina, UAM, Arzobispo Morcillo 4, 28029 Madrid, Spain*
[2]*Molecular Oncology Unit, CIEMAT, Complutense 40, 28040 Madrid, Spain*
[3]*Biomedical Research Institute I+12, University Hospital 12 de Octubre, Avenida de Córdoba s/n, 28041 Madrid, Spain*
[4]*Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain*
[5]*Centro de Biología Molecular "Severo Ochoa" (UAM-CSIC), Nicolás Cabrera 1, Cantoblanco, 28049 Madrid, Spain*
[6]*Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Valderrebollo 5, 28031 Madrid, Spain*
[7]*Neuro-oncology Unit, Instituto de Salud Carlos III-UFIEC, Crtra, Pozuelo Km 2, Majadahonda, 28220 Madrid, Spain*

Correspondence should be addressed to Ricardo Gargini; ragargini@cbm.csic.es
and Vega García-Escudero; v.garcia-escudero@uam.es

Received 3 December 2017; Accepted 21 January 2018; Published 20 February 2018

Academic Editor: David Vauzour

Copyright © 2018 Joaquín G. Cordero et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Alzheimer's disease is a proteinopathy characterized by accumulation of hyperphosphorylated Tau and β-amyloid. Autophagy is a physiological process by which aggregated proteins and damaged organelles are eliminated through lysosomal digestion. Autophagy deficiency has been demonstrated in Alzheimer's patients impairing effective elimination of aggregates and damaged mitochondria, leading to their accumulation, increasing their toxicity and oxidative stress. In the present study, we demonstrated by microarray analysis the downregulation of fundamental autophagy and mitophagy pathways in Alzheimer's patients. The benefits of the Mediterranean diet on Alzheimer's disease and cognitive impairment are well known, attributing this effect to several polyphenols, such as oleuropein aglycone (OLE), present in extra virgin olive oil. OLE is able to induce autophagy, achieving a decrease of aggregated proteins and a reduction of cognitive impairment in vivo. This effect is caused by the modulation of several pathways including the AMPK/mTOR axis and the activation of autophagy gene expression mediated by sirtuins and histone acetylation or EB transcription factor. We propose that supplementation of diet with extra virgin olive oil might have potential benefits for Alzheimer's patients by the induction of autophagy by OLE.

1. Introduction

1.1. Alzheimer's Disease. Alzheimer's disease (AD) is a progressive, fatal, and currently incurable neurodegenerative disease. It is clinically characterized by a gradual loss of cognitive function, including slow deterioration of memory, reasoning, abstraction, language, and emotional stability [1]. As a consequence, in the final stages of the disease, the patient is unable to perform any daily task without adequate assistance from family members or social services [1]. AD is the most common cause of dementia worldwide, accounting for between 50% and 70% of the cases recorded among people over 65 years old. The aging population presents the highest risk of the disease, especially in developed countries, therefore the number of affected people is expected to increase dramatically up to 115 million in 2050 [2].

The causes of the disease have not been fully clarified, but several risk factors have been associated with AD. These are genetic factors (presenilin 1 and 2, apolipoprotein Eε4 allele), vascular events, history of traumatic brain injury, oxidative stress, decreased endothelial nitric oxide production and consequent inflammation, hypertension,

hyperhomocysteinemia, diabetes, insulin resistance, hypercholesterolemia, obesity, hormonal alterations, lifestyle factors (saturated fat intake, vitamin E intake, low physical activity, smoking, etc.), and psychological factors [3].

The main histologic sign confirming the AD diagnosis is the presence of intracellular neurofibrillary tangles of hyperphosphorylated Tau protein and extracellular deposits of beta-amyloid (Aβ) peptide (senile plaques) in certain areas of the brain. Tau belongs to the microtubule-associated proteins (MAPs) family that participates in the assembly and stabilization of microtubules. This process is necessary for the maintenance of cellular shape and transport of proteins, organelles, and other biological components through axons [4]. Additionally, it is important in the connections between microtubules and other elements of the cytoskeleton such as neurofilaments, spectrin, or actin filaments [4]. The Aβ is a peptide that contains between 39 and 43 amino acids, being a natural product of proteolytic processing of the amyloid precursor protein (APP), due to the sequential action of enzymes β- and γ-secretase [5].

For causes not entirely clarified, in AD, both proteins tend to generate cytotoxic aggregates. These trigger series of neuronal alterations such as loss of synaptic transmission, gliosis or proliferation and abnormal activation of glial cells (astrocytes and microglia) [6], vascular dysfunction due to fibrillary amyloid deposition at cerebral vessel walls [7], increased oxidative stress, augmented inflammatory response, and deregulation of calcium homeostasis [8].

1.2. Autophagy Pathway. Within eukaryotic cells, there are two systems responsible for the degradation of cytoplasmic proteins: the ubiquitin-proteasome system (UPS) and the autophagy. In the case of poorly folded protein aggregates, it is suggested that autophagy (specifically macroautophagy) is more efficient in degrading them than UPS. This is due to the small pore size of proteasomes, which hinders the entry of proaggregating oligomers and their subsequent elimination [9].

Autophagy is a process by which the different cellular components and organelles are transferred to lysosomes to be degraded by their hydrolytic enzymes [10]. It is a key process for the correct cell function, acting as a recycling system in energy restriction conditions and allowing the cell to degrade nonessential organelles and proteins to reuse their components. In addition, it functions as a system for eliminating aggregated protein and cytotoxic damaged organelles [11].

Macroautophagy process (or "autophagy" as we will call it from now onwards for simplicity) can be divided into three phases [11]: autophagosome formation, substrate recognition, and autophagosome trafficking and degradation.

1.2.1. Formation of the Autophagosome. First, cellular materials to be degraded are isolated into a double membrane vesicle known as autophagosome. Its formation requires the fusion of several smaller membranous vesicles to form a flattened structure called phagophore. This fusion process continues until the autophagosome with a double membrane is formed enveloping small fragments of the cytoplasm and organelles. Initiation of phagosome formation requires activation of the quaternary complex ULK1-ATG13-ATG101-FIP200 [12]. This complex is controlled by two signaling pathways: the *mTORC1 pathway* which acts as an autophagy blocker by inhibiting ULK1 phosphorylation and the *adenosine monophosphate activated protein kinase* (AMPK) which activates ULK1 by phosphorylation of mTORC1 different residues [13]. Once activated ULK1 phosphorylates and activates Beclin-1, which in turn triggers the activation of the VPS34 complex. The latter produces an accumulation of phosphatidylinositol-3-phosphate (PI3P) in phagophore, allowing the recruitment of numerous binding proteins among which the ATG5-ATG12-ATG16L1 complex stands out. This complex is necessary for phagophore elongation through the binding to microtubule-associated protein 1 light chain 3 (LC3), which after several conjugations will result in the active form LC3-II [14]. Finally, the ATG5-ATG12-ATG16L1 complex is dissociated from the phagophore membrane and it ends up closing, creating the autophagosome and leaving LC3-II associated with the two faces (external and internal) of autophagosomes [14].

1.2.2. Substrate Recognition and Selective Autophagy. There are several adaptor proteins that recognize cargo to be engulfed into autophagosomes such as p62 (sequestosome 1), Next to BRCA1 gene 1 protein (NBR1), Nuclear Domain 10 Protein 52 (NDP52; also known as CALCOCO2), and optineurin. These proteins recognize substrates specifically labeled for degradation by ubiquitination, for example, and bind to LC3 family proteins of phagosome mediating the recruitment of cargoes [15]. Selective targeting can also be mediated by autophagy receptors that form a bridge between the cargo-autophagy receptor complex and components of the autophagosome membrane such as ATG5 and PI3P [16]. This is the case of autophagy-linked FYVE protein (ALFY) that serves as a scaffold protein for p62 mediated labeling [16]. Additionally, BNIP3L is a mitochondrial membrane protein containing a LC3-interacting region motif involved in the targeted clearance of damaged mitochondria [17].

1.2.3. Autophagosome Trafficking and Degradation. Once the autophagosome is formed, it is transported by dynein engines through microtubules to the perinuclear region. In this pathway, autophagosome may fuse with vesicles from the endocytic pathway to form amphysomes. Finally, autophagosomes and amphysomes fuse with lysosomes forming autolysosomes [18]. In them, the degradation of the compounds will be achieved, thanks to the combined action of acid pH and lysosomal enzymes. The macromolecules resulting from lysosomal digestion are released to the cytosol by permeases [19]. Transcription factor EB (TFEB) is as a master regulator for lysosomal biogenesis, therefore its activation favors the degradation phase of autophagy [20].

1.3. Autophagy Deficiency in AD. The accumulation of extracellular protein aggregates, mainly composed by polymeric Aβ42 peptide, a product of proteolysis of APP, is one of the main responsible for neurological damage and cognitive

deficit. Under normal conditions, protein aggregates and damaged organelles are eliminated through autophagy system, avoiding their cytotoxic effect. But, as a consequence of age and oxidative stress, the efficacy of this system is reduced. This results in the accumulation of poorly digested proteins in autophagic vacuoles and damaged mitochondria, which cause an increase in oxidative stress and neuronal death [21].

Several key regulatory proteins in autophagy are reduced in AD, such as Beclin-1, PARK2/parkin and Nuclear Receptor Binding Factor 2 (NRBF2). Beclin-1 is a fundamental protein for autophagy regulation and cell death that is found decreased in brain samples from early-stage Alzheimer's patients [22]. In addition, inhibition of Beclin-1 gene expression in a mice model that expresses human APP produces intraneuronal accumulation of Aβ, extracellular Aβ deposition, and neurodegeneration [22]. PARK2 is one of the proteins involved in mitophagy, a specialized form of autophagy by which mitochondria are selectively degraded and recycled. PARK2 labels damaged mitochondria for their subsequent degradation by its E3 ubiquitin ligase activity. PARK2 is found reduced in the cerebral cortex of AD patients, leading to a pathological increase of oxidative stress [23]. NRBF2 is a Beclin-1-Vps34-binding protein that modulates autophagy via Atg14L-linked Vps34 activity regulation [24]. NRBF2 expression is found reduced in the hippocampus of transgenic mice model that reproduces amyloid pathology characteristic of AD in humans [25].

It has been also proposed that Aβ can cause a pathological accumulation of autophagic vacuoles. Intracellular Aβ can also modulate the autophagy process by RAGE-calcium-CaMKKβ-AMPK pathway [26] or by generating ROS by mitochondrial damage [27].

2. Gene Expression Profiling Demonstrates Autophagy Dysfunction in AD

In the last years, the advances of high-throughput genomic analysis have generated data from patients that allow us the integration of gene deregulations and the determination of what biological processes are affected in different pathologies such as neurodegenerations. Particularly in AD, these gene expression datasets have demonstrated a significant deregulation of biological processes associated with calcium signaling, inflammation, and mitochondrial dysfunction [28]. Several of these processes are known to have a deep implication in AD pathology. However, these are not related to one of the main defects associated to AD, as it is proteotoxicity [28, 29]. Trying to shed light on this issue, we have analyzed a microarray dataset of brain samples of AD patients compared to healthy subjects, in which we focused in protein degradation-associated processes. The row data of this microarray dataset were previously published by the group of Berchtold [30]. Using gene enrichment analysis of main genes down or upregulated in AD versus healthy with significant differences (p value <0.01), we analyzed what biological processes were altered by Gene Ontology. We could observe that several metabolism and catabolism linked processes such as mTORC1 pathway, autophagy, and mitophagy were affected in AD with respect to healthy individuals. These results are shown in Figure 1, in which the principal modified genes were represented as well as the gene enrichment p values for each pathway. Multiple genes from mTORC1 pathway were increased in AD patients which generates an inhibitory effect over autophagy route, as we mentioned before. Accordingly, autophagy and mitophagy pathways were downregulated in these patients' brain samples. These effects together determine that the autophagy process was impaired in AD. Due to the fact that our data is derived from a large number of patients and different brain zones, this kind of study by global genomic analysis allows unraveling how specific biological processes with deep clinic and therapeutic involvement are affected. These analyses as well as multiple other studies that demonstrate an alteration of metabolic/catabolic pathways that converge in autophagy deficiency show us an evident necessity to evaluate new compounds that modulate these routes.

3. Activation of Autophagy as a Therapeutic Strategy for AD

If the autophagy pathway is altered as part of the pathological process of AD, autophagy activation may be effective in combating the cellular aggregate characteristic of this pathology. Numerous studies have shown that the pharmacological activation of autophagy might be beneficial for AD pathology. For example, the effect of 10-week treatment with rapamycin, an mTOR inhibitor, was studied in a transgenic mice model that reproduces amyloid and Tau pathology characteristic of AD in humans. They could achieve a reduction of Tau and Aβ accumulation as well as cognitive and memory improvement measured by the spatial reference version of Morris's water maze associated to increased autophagy [31]. However, in a later study, it was demonstrated that rapamycin accelerated the degeneration of motor neurons and reduced life expectancy in transgenic mice models. These results suggested that the pharmacological activation of rapamycin might affect other pathways that restricted neuron survival [32].

Other studies have demonstrated the beneficial effect of promoting autophagy by increasing PARK2 levels. These works use viral vectors to transfer PARK2 to a triple transgenic AD mice model demonstrating the enhancement of Aβ and phospho-Tau clearance by autophagy activation that improved mitochondrial function and restored synaptic function [33, 34]. This mitophagy improvement was further demonstrated in a human cell model of sporadic AD [23].

On the other hand, the activation of degradation phase of autophagy has also demonstrated its therapeutic potential in AD. The deletion of cystatin B, an endogenous inhibitor of cysteine proteases, to relieve cathepsins inhibition improving lysosomal function demonstrated to decrease extracellular amyloid pathology. This change prevented the development of learning and memory deficits of a transgenic model of AD (TgCRND8 mice that overexpress APP695 version including Swe and Ind mutations) [35].

Although the activation of autophagy has demonstrated a proven effect in the early stages and as a preventive, it is still

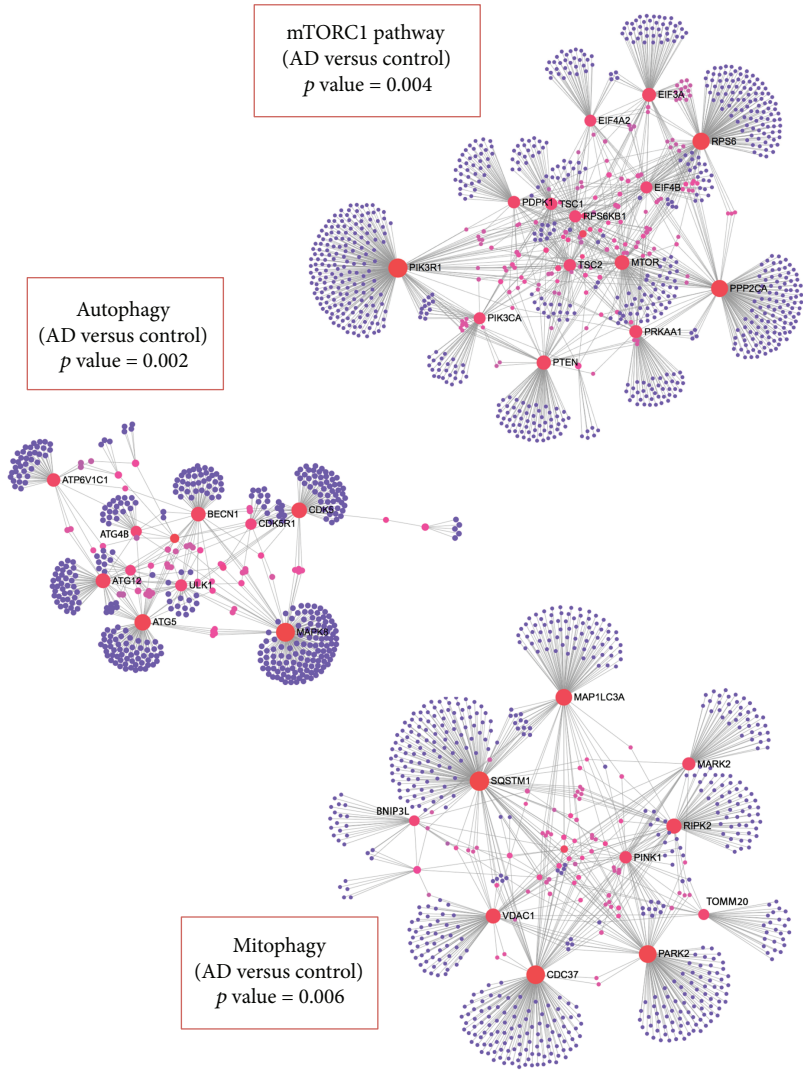


Figure 1: Gene expression profiling revealed dysregulation of mTOR, autophagy, and mitophagy pathways in Alzheimer's disease patients. Genes having significant differential expression between brain samples from normal controls subjects and AD patients were extracted. The analysis was done from a microarray dataset having 253 samples from 84 patients ($n = 56$ normal; $n = 28$ AD patients [30]). Samples were collected from four brain regions: hippocampus, entorhinal cortex, superior frontal cortex, postcentral gyrus. Enrichment in pathways and biological processes of deregulated genes was done using GSEA and Gene Ontology analyses, and the significance of the results (p value) for each pathway was represented in the graph. We could observe that mTOR pathway was significantly upregulated, whereas autophagy and mitophagy were significantly downregulated in AD. Genes that exhibited up or downregulation with p value <0.01 using two-tailed Student's t-test were selected for representation. Graphs show nodes plots of these altered genes in AD versus controls in each of these pathways using NetworkAnalyst software (http://www.networkanalyst.ca/faces/home.xhtml).

under debate whether this may have poor effect in the advanced stages of the disease [36].

All these works are the proof of concept that activating autophagy may be used as a therapeutic approach for AD. Therefore, it is necessary to find new substances to induce autophagy minimizing collateral effects.

4. Neuroprotective Role of Mediterranean Diet and Extra Virgin Olive Oil

Alzheimer's disease is a multifactorial pathology in which both genetic and environmental factors are involved, highlighting among the latter certain aspects of lifestyle such as nutrition. Diet is a modifiable risk factor for dementia; thus, increasing efforts have been done to find nutrients that help to fight cognitive impairment. Some diets have been associated to reduced risk of AD; therefore, they may be helpful to find new compounds that may be beneficial for AD patients. LipiDiDiet is a research consortium, which studies the preclinical and clinical impact of nutrition in Alzheimer's disease (http://www.lipididiet.eu/). A recent study has demonstrated that the multinutrient combination Fortasyn Connect was able to slow hippocampal atrophy and functional decline by supplying rate-limiting compounds for brain phospholipid synthesis [37].

The Mediterranean diet (MD) has already demonstrated on multiple occasions its beneficial effects in preventing age-specific defects, including attenuating and preventing AD and cognitive impairment [38, 39]. For example, the 4-year study in a population of 2258 New York City residents found that increased adherence to MD reduced the risk of developing AD and was especially effective in preventing the conversion of mild cognitive impairment to AD [40]. This study showed that individuals with high adherence to MD had a 40% lower risk of developing AD and a 48% lower risk of progressing from mild cognitive impairment to AD [40]. In another study using a population of 1410 Bordeaux citizens over 65 years of age, high adherence to MD was associated with a reduction of cognitive impairment demonstrated by the Mini-Mental State Examination [41].

This neuroprotective effect has been associated with several foods found in MD, including wine and extra virgin olive oil (EVOO) standing out, the latter being one of the most internationally recognized due to its multiple beneficial properties. Among them, it is worth mentioning its role as a mental health promoter and in slowing cognitive decline in neurodegenerative diseases in the elderly population. This was demonstrated by the "three-cities study" on an elderly population of more than 8000 subjects, being the first report relating olive oil consumption with lower reduction of visual memory in a population over 65 years old [42].

Similar beneficial effect has been observed in both in vitro and in vivo studies where supplementation with EVOO is able to reduce the advance of Tau and Aβ pathology and cognitive deterioration [43–45]. The most recent study concludes that consumption of EVOO in early ages and for a long time could provide a protective effect against AD pathology and cognitive deterioration [45].

Several studies in animal models have shown that this beneficial action is due to a series of substances known as polyphenols [46], including oleuropein aglycone (OLE) present in EVOO [47].

5. Oleuropein Aglycone as an Autophagy Inducer

5.1. Polyphenols and Autophagy. Since the discovery of the benefits of moderate wine consumption for the prevention of dementia thanks to the presence of a substance called resveratrol (and not alcohol as initially suggested), numerous studies have attributed the beneficial effects of polyphenols on health. They are considered the substances responsible for the multiple mental health benefits attributed to the Mediterranean and Oriental diet [48].

Polyphenols are an extensive group of nonenergetic substances present in plant-based foods characterized by the presence of one or more phenolic rings. They can be classified according to their number of phenolic rings and the structural elements present these rings. The main groups are phenolic acids (derived from hydroxybenzoic acid or hydroxycinnamic acid), stilbenes, lignans, phenolic alcohols, and flavonoids [49].

An interesting fact showed by the different studies is that polyphenols do not act in a single pathway or modulating a certain aspect of AD, such as inhibition of Aβ aggregation. Their neuroprotective function comes from the modulation of different cellular and tissue processes that together are responsible for the reduction of cognitive impairment [35]. The Aβ toxic aggregates inhibition and the decrease in ROS produced by mitochondria and neuroinflammation are the main positive effects of polyphenols. But some of these effects may be in part due to another not so well studied action of these substances, and that is the induction of autophagy [50].

As we have previously described, autophagy plays a crucial role in AD, whose alterations have been considered preclinical events to pathology. In addition, induction of this process has proven to be a promising target for pharmacological action. Hence, it would be very helpful to look for new substances that have a positive effect on this detoxification system, and this is where polyphenols come in.

There are multiple pathways by which polyphenols can modulate autophagy, and not all of them act through the same route. See Hasima and Ozpolat [51] for a summary of involved pathways.

Polyphenols are able to modulate autophagy through canonical (Beclin-1 dependent) and noncanonical (Beclin-1 independent) signaling pathways. In the canonical pathway, Beclin-1 together with the regulatory subunits Vps34 and Vps15 form a protein complex known as Class III phosphatidylinositol 3-kinase (PI3K), inducing autophagy by modulating the autophagosomal nucleation. While in noncanonical or Beclin-1 independent, two activation mechanisms can be given, Atg5/Atg7 dependent or independent [51, 52].

For example, genistein is able to induce PI3K/Akt-dependent autophagy by reducing Akt phosphorylation levels, leading to autophagy induction as a false signal of calorie restriction [53]. Curcumin performs its function through inhibition of Akt/mTOR/p70S6K protein complex and Erk1/2 protein kinase activation, demonstrating an increase of autophagy in glioblastoma cells [54]. Rottlerin, a polyphenol extracted from the Asian *Mallotus philippensis* tree, has demonstrated its effect as an autophagy inducer through its antioxidant function. Rottlerin blocks the generation of free radicals and triggers inhibition of NF-κB and activation of AMPK, which is associated with a decrease of ATP levels. The cell translates this ATP drop as a state of caloric restriction, so it activates autophagy as a regulatory mechanism [55]. Rottlerin is also able to activate autophagy through inhibition of PKCδ, a protein kinase that blocks autophagy through activation of tissue transglutaminase 2 (TG2) [56].

One of the most studied mechanisms of action of polyphenols is through sirtuins, a group of deacetylases that regulate cellular functions as important as metabolism, immunity, inflammation, and cell survival. Sirtuins act as modulators of autophagy both directly by promoting the deacetylation of key components such as autophagic gene products Atg5, Atg7, and Atg8 and indirectly by regulating FOXO3a transcription factor [52]. Once activated, FOXO3a induces autophagy by synthesis of glutamine synthetase, an enzyme that increases glutamine levels. This increase in glutamine causes inhibition of mTOR and the consequent activation of autophagy [57]. Resveratrol, genistein, and

quercitin have been shown to be promising activators of sirtuins [38, 52].

5.2. Mechanisms of Autophagy Induction by Oleuropein Aglycone. One of the polyphenols better characterized as an autophagy inducer is oleuropein aglycone (OLE), which is found in EVOO. Oleuropein is a secoiridoid glycoside with a phenylpropanoid alcohol obtained from the biosynthesis of mevalonic acid. From the chemical point of view, it is an 11-methyl-ester (elenolic acid glucoside) ester combined with 3,4-dihydroxyphenylethanol (3,4-DHPEA). It is the major phenolic component of the pulp of the green olives and leaves of the *Gentianaceae*, *Cornaceae*, and *Oleaceae* families, in the latter family being especially relevant in the variety *Olea europaea* L. (which gives its name to the glycoside) [58]. After the process of maturation and extraction of the olive juice, in the EVOO, due to the action of the enzyme β-glucosidase, we can only find this secoiridoid in its aglyconic form as OLE (3,4-DHPEA-EA), being aldehydic form of OLE, the main responsible for its bitter taste [59, 60]. The relative amount of OLE in EVOO depends on the variety of olive fruit used, being in many of them one of the most abundant polyphenols [61].

Several in vitro and in vivo studies have demonstrated the multiple benefits of oleuropein and its derivatives associated to their antioxidant, antidiabetic, antimicrobial, antiviral, antitumor, hepatoprotective, cardioprotective, neuroprotective, antiaging, and anti-inflammatory properties [62–65]. In terms of neuroprotective activity, the most recent experimental studies have shown that OLE reduces cognitive impairment and improves synaptic function in animal models. This is due to the inhibition of the aggregation and toxicity of Tau [66] and Aβ [67], the epigenetic modulation by histone acetylation [68], the reduction of astrocytosis and modulation of astroglia activity, and the induction of autophagy [69].

The effects of OLE on Tau aggregation was demonstrated by studying the antiaggregant capacity of oleuropein and its derivatives (OLE and hydroxytyrosol) over wild-type and P301L Tau protein in vitro. They were able to obtain results similar to methylene blue in inhibiting fibrillization at low micromolar concentrations of Tau, demonstrating a higher inhibitory capacity of OLE related to the presence of aldehyde groups in its structure [66]. A posterior study demonstrated the inhibition of Aβ aggregation using the transgenic strain CL2006 of *Caenorhabditis elegans*, a simplified model of AD expressing human Aβ peptide in the cytoplasm of muscle cells of the body wall. They found that larvae fed with OLE showed a reduction of Aβ plaque deposits, a lower content of toxic Aβ oligomers, a marked decrease of paralysis, and an increase of life expectancy with respect to untreated animals [67].

On the other hand, several reports have associated the effect of OLE to a positive modulation of autophagy pathway that leads to cognitive improvement in animal models. One of the first works that demonstrated this hypothesis was the study carried out by Grossi and collaborators using wild-type and TgCRND8 transgenic mice, as mentioned before, a model for human Aβ pathology [35, 70]. In this study, mice whose diet was supplemented with OLE (50 mg/kg of food) exhibited an increase of autophagic vesicles. This was demonstrated by the augmented levels of Beclin-1 and LC3 in the soma and dendrites of neurons from different parts of the somatosensory/parietal and entorhinal/piriform cerebral cortex correlating with increased LC3II/LC3I ratio. This induction was more significant in TgCRND8 transgenic mice rather than in wild-type. Additionally, these authors demonstrated that OLE improved the autophagosome-lysosome fusion measured as the increase of p62 and cathepsin B levels in OLE supplemented TgCRND8 mice up to the levels found in wild-type mice. They also reported the colocalization of both p62 and cathepsin B labels suggesting a proper fusion of lysosomes to autophagic vesicles and, therefore, functional degradation phase of autophagy. They proposed that the mechanism of autophagy activation might be due to the inhibition of mTOR pathway reflected in the decrease of phosphorylation of its target p70S6 protein kinase in cell culture.

5.2.1. OLE as Modulator of Ca^{+2}-CaMKK-AMPK-mTOR Axis. Initially, it was proposed that the mechanism by which OLE induced autophagy would be through the increase of cytosolic levels of Ca^{+2} and the subsequent activation of the enzyme complex AMPK through Ca^{2+}/Calmodulin Protein Kinase Kinase β (CaMKKβ). This complex facilitates mTORC1 inhibition and ULK1 activation to generate autophagic vacuole induction [71] (Figure 2). This is also the case of other polyphenols such as resveratrol [72] and epigallocatechin gallate (EGCG) [73]. Accordingly, they demonstrated a biphasic phosphorylation of the regulatory residue Thr172 of AMPK correlating with elevated Beclin-1 levels in SH-SY5Y cells treated with 50 μM OLE [71]. This was mediated by the biphasic increase of intracellular Ca^{2+} levels that come from the endoplasmic reticulum that induce CaMKKβ activation. This correlates with a fast increase of Beclin-1 levels that was proposed to arise from the Beclin-1 fraction complexed with Bcl-2/Bcl-xL in the cytoplasm rather than to new synthesis. The release of Beclin-1 from this complex is critical for inducing autophagy because free Beclin-1 interaction with VPS34 is needed to initiate autophagosome formation [74]. The activation of autophagy by OLE was partially inhibited by STO-609 and component C, inhibitors of CaMKKβ and AMPK, respectively, suggesting that autophagy activation by OLE occurs mainly through the Ca^{2+} increase that induces CaMKKβ activation and the subsequent AMPK phosphorylation [71].

All these results together indicate that OLE activates AMPK, which can allow the formation of ULK1 quaternary complex directly or indirectly by the inhibition of mTOR that inhibits ULK1 (Figure 2). ULK1 promotes autophagy by Beclin-1 phosphorylation and VPS34 lipid kinase activation that produces phosphatidylinositol 3-phosphate, necessary for the formation of the early autophagosomal membrane [75].

5.2.2. OLE as Modulator of PARP1-SIRT1 Axis. Multiple polyphenols are known to induce autophagy by the activation of sirtuins (SIRT) [52]. SIRT1 deacetylates many

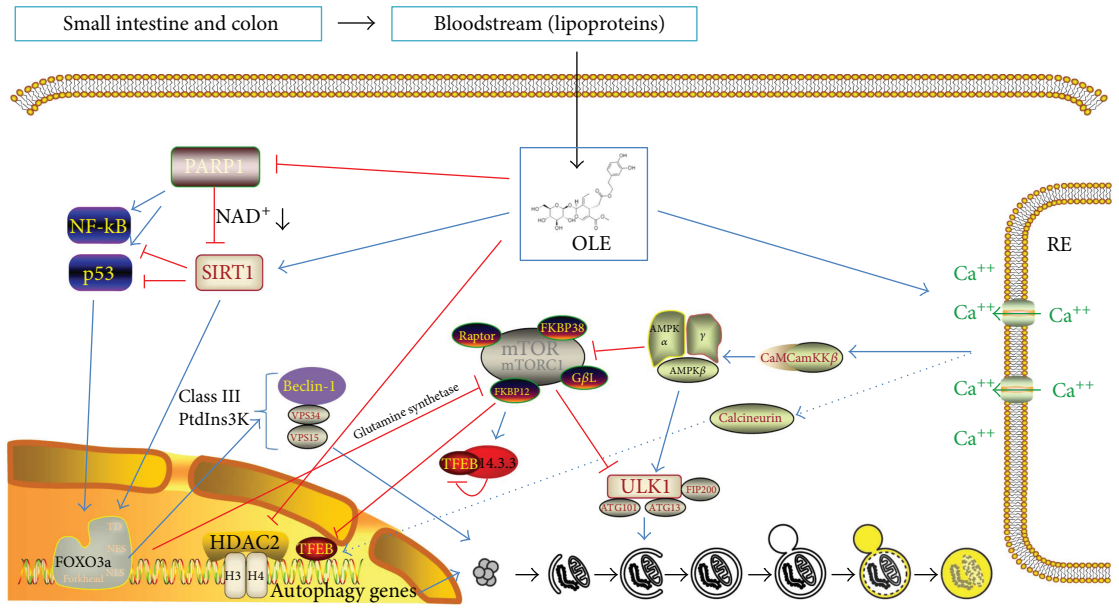


FIGURE 2: Pleiotropic action of OLE over autophagy induction. Summary of autophagy pathways in which OLE has demonstrated an effect.

transcription factors such as p53, NF-κB, and FOXO, a mediator of autophagy. SIRT1 could influence autophagy directly via deacetylation of key components of the autophagy induction network, such as the products of autophagy genes Atg5, Atg7, and Atg8 [76]. The treatment of TgCRND8 mice with OLE (50 mg/Kg of diet) was able to reduce the activation of Poly (ADP-ribose) polymerase-1 (PARP1) at both RNA and protein levels as well as the subsequent accumulation of PAR polymers up to the levels found in wild-type mice [77]. Moreover, OLE was able to abolish the increase of the apoptotic mediators phospho-NF-κB and phospho-p53 Ser46 in these animals. PARP1 activation causes a reduction of NAD^+ levels that result in SIRT1 inhibition [78]. Therefore, OLE-mediated reduction of PARP1 increased NAD^+ levels that were able to induce SIRT1 in TgCRND8 mice [77] (Figure 2). Accordingly, the treatment of N2a cells with OLE 100 μM for 24 h was able to reverse the PARP1 activation caused by methylnitronitrosoguanidine (MNNG), a mutagen that activates PARP1 expression, as well as increased SIRT1 and Beclin-1 [77].

5.2.3. OLE as Epigenetic Modulator. It is also worth mentioning the action of OLE as an epigenetic modulator. It is known that abnormal acetylation takes place in memory and learning disorders such as AD, where significant increase of histone deacetylase 2 (HDAC2) inhibits gene expression of specific locus, such as autophagy markers [79]. Moreover, histone acetylation has been shown to ameliorate cognitive deficits in AD animal models suggesting its targeting as a promising therapeutic strategy for this disease [79]. Polyphenols have shown to regulate gene expression by modulating histone acetylation and DNA methylation, as is the case of EGCG in cancer cells [80].

Noteworthy, TgCRND8 mice showed increased levels of HDAC2 correlating with decreased levels of histone 3 acetylation on lysine 9 (H3K9) and of histone 4 acetylation on lysine 5 (H4K5) in cortex and hippocampus [68]. The treatment with OLE (50 mg/kg of diet for 8 weeks) significantly decreased the levels of HDAC2 in both wild-type and TgCRND8 mice and increased H3K9 and H4K5 especially in the transgenic model (Figure 2). This was accompanied by an improvement of synaptic function revealed by restoring high-frequency stimulation-induced long-term potentiation as well as 3-theta burst and high-frequency stimulation-evoked posttetanic potentiation in slices of brains of OLE-treated transgenic mice.

On the other hand, transcription factor EB (TFEB) is a master regulator of lysosomal and autophagic function [81, 82]. Previous studies demonstrated that mTOR-mediated phosphorylation of TFEB in Ser 211 promotes the interaction of TFEB with the 14-3-3 protein and results in a cytoplasmic localization, therefore inhibiting its function as a transcriptional factor [83] (Figure 2). It has been recently shown that activation of calcineurin by lysosomal Ca^{2+} releases binds and dephosphorylates TFEB, thus promoting its nuclear translocation inducing autophagy and lysosomal biogenesis [84]. As we previously mentioned, OLE was able to increase intracellular Ca^{2+} that activates Ca^{2+}/CaMKKβ/AMPK axis [71]. Hence, Ca^{2+} release coming from different organelles might induce autophagic flux independently but possibly, synergistically. Further studies will be necessary to unravel if intracellular Ca^{2+} increase mediated by OLE is able to activate calcineurin and the subsequent TFEB dephosphorylation and activation which induce autophagy (Figure 2).

6. Bioavailability and Effective Dose of OLE

Before presuming an effect of OLE in neurons in humans, it is important to know its bioavailability in the organism once

ingested and if it arrives in its full form or with modifications. In humans, it has been demonstrated that the apparent absorption of olive oil phenols, among which is OLE, ranges between 55 and 66% of the ingested dose [85]. This is performed mainly in the small intestine and in a minor proportion in the colon and, once in the blood, the oleuropein is transported by lipoproteins where it is rapidly distributed [85, 86] (Figure 2). The mechanism of OLE absorption is still unknown, as well as those of other olive oil polyphenols [87]. However, it is known that intestinal absorption and renal clearance of oleuropein and hydroxytyrosol are relatively rapid, reaching their maximum plasma concentration at half an hour after ingestion, followed by a rapid decline culminating in 2.5 hours [86]. The main metabolites that can be found in plasma and urine after oleuropein ingestion are hydroxytyrosol and its conjugates (sulfated and glucuronidated) followed by OLE [86]. In addition, there is a strong individual component regarding the bioavailability and metabolism of oleuropein depending on the gender, being men the most efficient in conjugating this substance, which explains the lower plasma levels compared to the increased levels of conjugated forms of hydroxytyrosol [86]. On the other hand, the absorption of oleuropein is much more efficient if the compound is taken in liquid form rather than ingestion in capsules [86].

In addition, it has been suggested that OLE, being one of the less polar compounds of the olive oil polyphenols, is mostly transformed in hydroxytyrosol either in the gastrointestinal tract before it is absorbed or in the intestinal cells, blood, or liver after its absorption [85]. Nonetheless, it has been recently demonstrated that hydroxytyrosol is able to induce autophagy in chondrocytes after oxidative stress exposure mainly, but not exclusively, by SIRT1 induction [88]. This opens the possibility that not only OLE but also its derivatives are able to induce the protective autophagy induction described in this review.

The autophagy induction by OLE can occur even at low doses. It was demonstrated that high concentrations (50 mg/kg) of a mixture of polyphenols present in olive mill waste water (among which was OLE), as well as medium (12.5 mg/kg) and low concentrations (0.5 mg/kg) of OLE increased autophagic activity in the cortex of TgCRND8 mice [89]. They could also observe that 12.5 mg/kg of OLE or 50 mg/kg of polyphenols mix significantly improved cognitive functions and diminished Aβ deposition of TgCRND8 mice.

Considering a variety of EVOO specially enriched in OLE as is Seggianese oil, an Italian olive oil whose OLE content is above 30% of total secoiridoids of which are ranging 619 ± 128 mg/L [90], the amount of OLE is over 185.7 mg/L in this EVOO variety. The daily recommendation of EVOO consumption in the Mediterranean diet is 25–50 ml [91] that would represent between 4.6 and 9.3 mg of OLE using Seggianese oil. Taking into consideration the differences of weight between mice (20 g) and humans (e.g., 60 Kg), the daily concentration of OLE would be the equivalent to 1.5–3.1 μg/day in mice. The OLE daily intake at which autophagy induction was achieved in the study by Pantano et al. was 0.5 mg/kg of food [89]. Considering that mice model eats 3–5 g/day (http://www.researchdiets.com), this would correspond to a dose of 1.5–2.5 μg/day of OLE. As we calculated before, this amount of OLE was equivalent to the daily intake of Seggianese oil. The minimum dose in the study by Pantano et al. that achieved functional recovery and reduced amyloid beta burden was 12.5 mg/Kg of food [89] that would correspond to 37.5–62.5 μg/day of OLE. This amount exceeds the OLE quantity guaranteed by the daily intake of EVOO and this may indicate that the effect of EVOO might not be enough in advanced stages of the disease. However, EVOO contains many other components with potential beneficial effects over health and cognitive improvement apart from OLE. Therefore, further studies are necessary to determine the minimum active dose of OLE and the potential benefit of EVOO intake.

In addition, administration of 50 mg/Kg OLE in the diet was safe and none of the TgCRND8 mice involved in the experiment died or suffered any side effects due to high intake of OLE [70]. Moreover, the presence of several foods with substantial levels of polyphenols is one of the main explanations for the healthy properties of the Mediterranean and Asian diets [92, 93]. Taking in consideration all these studies, we can deduce that high doses of polyphenols might not be harmful to humans. However, specific studies of OLE intake in humans are necessary to rule out its possible toxicity.

Many open questions remain regarding the action of OLE once ingested and whether it is able to reach the neurons and exert its function. Furthermore, the determination of these metabolites' bioactivity and the levels at which OLE can become toxic needs to be analyzed. Regarding the possible effect of OLE in humans, further studies are necessary to determine OLE stability in human gastric fluids and blood. Additionally, it is important to unravel whether OLE is absorbed and is able to cross the blood-brain barrier unmodified. One important limitation for these studies is that purified OLE is not available for human consumption so far. Notwithstanding, there are many ongoing studies that analyze the beneficial effects of EVOO consumption in humans; from those, it might be possible to extrapolate conclusions about OLE effect. With this aim, it will be necessary to determine the exact concentration of OLE in a concrete EVOO variety and supplement volunteers with an exact daily dose. This would allow to estimate the concentration of OLE and its derivatives in blood and cerebrospinal fluid. However, being EVOO a mixture of many polyphenols, which most of them generate similar derivatives, this determination might be inexact. Moreover, as several polyphenols and derivatives may have similar beneficial effects on health, it would be difficult to attribute this outcome to one single compound. It is mandatory to clarify if the autophagy induction mediated by OLE depends on its unmodified form or on its conversion in hydroxytyrosol. This issue would be easier to achieve in vitro by studying autophagy induction after treatment of human cells, such as SH-SY5Y, with equivalent doses of OLE or hydroxytyrosol and evaluating their stability during experimental conditions.

Finally, unless we could acquire purified OLE for human consumption, the conclusions obtained from these studies

will be merely speculative and should be carefully supported by equivalent experiments in animals where purified OLE and EVOO can be compared.

In summary, the cognitive improvement developed in animal models of Alzheimer's disease, such as TgCRND8 mice [70], indicates that supplementation of the diet with OLE may have beneficial effects in slowing cognitive decline in these patients. This clearly indicates that either directly OLE or its derivatives are able to cross the blood-brain barrier and develop their neuroprotective function in the brain, where a decrease in protein aggregates and a significant activation of autophagy were observed.

7. Conclusions

Polyphenols are known to be the substances responsible for the neuroprotective properties attributed to the Asian and Mediterranean diets, rich in foods that contain a large amount of these compounds of plant origin [50]. We have focused our attention in OLE, one of the polyphenols abundant in EVOO and one of the bases of the Mediterranean diet [94].

In the present work, we have summarized all the works that have demonstrated that OLE reduced symptoms of AD and cognitive impairment [68, 70, 71, 89]. Several studies have proposed that OLE mechanism of action associated to this cognitive improvement was by autophagy induction that has been also shown to reduce amyloid aggregates [50, 68–71, 77, 89]. Furthermore, OLE does not show side effect (cell death or apoptosis), as is the case of some polyphenols such as curcumin [48], nor neurodegeneration as is the case of prolonged treatment with rapamycin [32]. This, together with its ability to fight cytotoxicity derived from the accumulation of Aβ and reduce inflammation derived from the activation of astrocytes and microglia are responsible for the decrease in cognitive impairment in TgCRND8 mice. Unfortunately, there are no evidences of OLE benefits in humans due to the complexity of these studies. But extrapolating the results of the studies that related adherence to the Mediterranean diet [40] or olive oil consumption [42] with the decrease in the prevalence of neurodegenerative diseases, the cognitive improvement in AD mice model [70] and the results in human SH-SY5Y neuroblastoma cells [71], we can conclude that OLE consumption might be useful for delaying cognitive impairment in humans.

The data presented in our study confirm that OLE is a compound capable of inducing autophagy in both in vitro and in vivo models and that this leads to an improvement in cognitive impairment as well as in β amyloid and Tau aggregation. Therefore, based on studies on the consumption of EVOO and adherence to the Mediterranean diet, rich in polyphenols including OLE, we can hypothesize OLE would be useful to prevent and lessen symptoms associated with AD. However, more studies are needed to test the effects of OLE in humans, in terms of metabolic pathways and bioavailability, as well as to demonstrate the effects of OLE on gene expression.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the Ministerio de Economía y Competitividad (SAF 2011 program, project 24841) from Spain and the Centro de Investigación en Red de Enfermedades Neurodegenerativas (CIBERNED, ISCIII, JA). The AECC Scientific Foundation has financed Ricardo Gargini. The authors would like to specially acknowledge Gladys Sanchez Gonzalez for English editing.

References

[1] K. Blennow and H. Hampel, "CSF markers for incipient Alzheimer's disease," *The Lancet Neurology*, vol. 2, no. 10, pp. 605–613, 2003.

[2] A. Wimo, L. Jönsson, J. Bond, M. Prince, B. Winblad, and Alzheimer Disease International, "The worldwide economic impact of dementia 2010," *Alzheimers Dement*, vol. 9, no. 1, pp. 1–11.e3, 2013.

[3] N. D. Barnard, A. I. Bush, A. Ceccarelli et al., "Dietary and lifestyle guidelines for the prevention of Alzheimer's disease," *Neurobiology of Aging*, vol. 35, Supplement 2, pp. S74–S78, 2014.

[4] M. G. Spillantini and M. Goedert, "Tau pathology and neurodegeneration," *The Lancet Neurology*, vol. 12, no. 6, pp. 609–622, 2013.

[5] Y. W. Zhang, R. Thompson, H. Zhang, and H. Xu, "APP processing in Alzheimer's disease," *Molecular Brain*, vol. 4, no. 1, p. 3, 2011.

[6] R. J. Castellani, R. K. Rolston, and M. A. Smith, "Alzheimer disease," *Disease-a-Month*, vol. 56, no. 9, pp. 484–546, 2010.

[7] A. Rostagno, J. L. Holton, T. Lashley, T. Revesz, and J. Ghiso, "Cerebral amyloidosis: amyloid subunits, mutants and phenotypes," *Cellular and Molecular Life Sciences*, vol. 67, no. 4, pp. 581–600, 2010.

[8] Y. K. Chuah, R. Basir, H. Talib, T. H. Tie, and N. Nordin, "Receptor for advanced glycation end products and its involvement in inflammatory diseases," *International Journal of Inflammation*, vol. 2013, Article ID 403460, 15 pages, 2013.

[9] V. I. Korolchuk, F. M. Menzies, and D. C. Rubinsztein, "Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems," *FEBS Letters*, vol. 584, no. 7, pp. 1393–1398, 2010.

[10] D. J. Klionsky, "Autophagy: from phenomenology to molecular understanding in less than a decade," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 11, pp. 931–937, 2007.

[11] F. M. Menzies, A. Fleming, and D. C. Rubinsztein, "Compromised autophagy and neurodegenerative diseases," *Nature Reviews Neuroscience*, vol. 16, no. 6, pp. 345–357, 2015.

[12] S. Alers, A. S. Loffler, S. Wesselborg, and B. Stork, "Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks," *Molecular and Cellular Biology*, vol. 32, no. 1, pp. 2–11, 2012.

[13] R. A. Nixon, "The role of autophagy in neurodegenerative disease," *Nature Medicine*, vol. 19, no. 8, pp. 983–997, 2013.

[14] Y. Kabeya, N. Mizushima, T. Ueno et al., "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing," *The EMBO Journal*, vol. 19, no. 21, pp. 5720–5728, 2000.

[15] T. Johansen and T. Lamark, "Selective autophagy mediated by autophagic adapter proteins," *Autophagy*, vol. 7, no. 3, pp. 279–296, 2011.

[16] T. H. Clausen, T. Lamark, P. Isakson et al., "p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy," *Autophagy*, vol. 6, no. 3, pp. 330–344, 2010.

[17] I. Novak, V. Kirkin, D. G. McEwan et al., "Nix is a selective autophagy receptor for mitochondrial clearance," *EMBO Reports*, vol. 11, no. 1, pp. 45–51, 2010.

[18] D. J. Klionsky, K. Abdelmohsen, A. Abe et al., "Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition)," *Autophagy*, vol. 12, no. 1, pp. 1–222, 2016.

[19] Z. Yang and D. J. Klionsky, "Permeases recycle amino acids resulting from autophagy," *Autophagy*, vol. 3, no. 2, pp. 149-150, 2007.

[20] H. Pi, M. Li, L. Tian, Z. Yang, Z. Yu, and Z. Zhou, "Enhancing lysosomal biogenesis and autophagic flux by activating the transcription factor EB protects against cadmium-induced neurotoxicity," *Scientific Reports*, vol. 7, article 43466, 2017.

[21] A. Zare-Shahabadi, E. Masliah, G. V. Johnson, and N. Rezaei, "Autophagy in Alzheimer's disease," *Reviews in the Neurosciences*, vol. 26, no. 4, pp. 385–395, 2015.

[22] F. Pickford, E. Masliah, M. Britschgi et al., "The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid β accumulation in mice," *The Journal of Clinical Investigation*, vol. 118, no. 6, pp. 2190–2199, 2008.

[23] P. Martín-Maestro, R. Gargini, G. Perry, J. Avila, and V. García-Escudero, "PARK2 enhancement is able to compensate mitophagy alterations found in sporadic Alzheimer's disease," *Human Molecular Genetics*, vol. 25, no. 4, pp. 792–806, 2016.

[24] J. Lu, L. He, C. Behrends et al., "NRBF2 regulates autophagy and prevents liver injury by modulating Atg14L-linked phosphatidylinositol-3 kinase III activity," *Nature Communications*, vol. 5, p. 3920, 2014.

[25] C. Yang, C. Z. Cai, J. X. Song et al., "NRBF2 is involved in the autophagic degradation process of APP-CTFs in Alzheimer disease models," *Autophagy*, vol. 13, no. 12, pp. 2028–2040, 2017.

[26] S. M. Son, E. S. Jung, H. J. Shin, J. Byun, and I. Mook-Jung, "Aβ-induced formation of autophagosomes is mediated by RAGE-CaMKKβ-AMPK signaling," *Neurobiology Aging*, vol. 33, no. 5, pp. 1006.e11–1006.e23, 2012.

[27] V. Garcia-Escudero et al., "Deconstructing mitochondrial dysfunction in Alzheimer disease," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 162152, 13 pages, 2013.

[28] J. Cooper-Knock, J. Kirby, L. Ferraiuolo, P. R. Heath, M. Rattray, and P. J. Shaw, "Gene expression profiling in human neurodegenerative disease," *Nature Reviews Neurology*, vol. 8, no. 9, pp. 518–530, 2012.

[29] R. X. Santos, S. C. Correia, X. Wang et al., "A synergistic dysfunction of mitochondrial fission/fusion dynamics and mitophagy in Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 20, Supplement 2, pp. S401–S412, 2010.

[30] N. C. Berchtold, D. H. Cribbs, P. D. Coleman et al., "Gene expression changes in the course of normal brain aging are sexually dimorphic," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 40, pp. 15605–15610, 2008.

[31] A. Caccamo, S. Majumder, A. Richardson, R. Strong, and S. Oddo, "Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-β, and Tau: effects on cognitive impairments," *The Journal of Biological Chemistry*, vol. 285, no. 17, pp. 13107–13120, 2010.

[32] X. Zhang, L. Li, S. Chen et al., "Rapamycin treatment augments motor neuron degeneration in $SOD1^{G93A}$ mouse model of amyotrophic lateral sclerosis," *Autophagy*, vol. 7, no. 4, pp. 412–425, 2011.

[33] P. J. Khandelwal, A. M. Herman, H. S. Hoe, G. W. Rebeck, and C. E. H. Moussa, "Parkin mediates beclin-dependent autophagic clearance of defective mitochondria and ubiquitinated Aβ in AD models," *Human Molecular Genetics*, vol. 20, no. 11, pp. 2091–2102, 2011.

[34] I. Lonskaya, A. R. Shekoyan, M. L. Hebron, N. Desforges, N. K. Algarzae, and C. E. Moussa, "Diminished parkin solubility and co-localization with intraneuronal amyloid-β are associated with autophagic defects in Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 33, no. 1, pp. 231–247, 2013.

[35] D. S. Yang, P. Stavrides, P. S. Mohan et al., "Reversal of autophagy dysfunction in the TgCRND8 mouse model of Alzheimer's disease ameliorates amyloid pathologies and memory deficits," *Brain*, vol. 134, no. 1, pp. 258–277, 2011.

[36] S. Majumder, A. Richardson, R. Strong, and S. Oddo, "Inducing autophagy by rapamycin before, but not after, the formation of plaques and tangles ameliorates cognitive deficits," *PLoS One*, vol. 6, no. 9, article e25416, 2011.

[37] H. Soininen, A. Solomon, P. J. Visser et al., "24-month intervention with a specific multinutrient in people with prodromal Alzheimer's disease (LipiDiDiet): a randomised, double-blind, controlled trial," *The Lancet Neurology*, vol. 16, no. 12, pp. 965–975, 2017.

[38] C. Dussaillant, G. Echeverría, I. Urquiaga, N. Velasco, and A. Rigotti, "Evidencia actual sobre los beneficios de la dieta mediterránea en salud," *Revista Médica de Chile*, vol. 144, no. 8, pp. 1990–1997, 2016.

[39] C. Feart, C. Samieri, and P. Barberger-Gateau, "Mediterranean diet and cognitive function in older adults," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 13, no. 1, pp. 14–18, 2010.

[40] N. Scarmeas, Y. Stern, M. X. Tang, R. Mayeux, and J. A. Luchsinger, "Mediterranean diet and risk for Alzheimer's disease," *Annals of Neurology*, vol. 59, no. 6, pp. 912–921, 2006.

[41] C. Féart, C. Samieri, V. Rondeau et al., "Adherence to a Mediterranean diet, cognitive decline, and risk of dementia," *JAMA*, vol. 302, no. 6, pp. 638–648, 2009.

[42] C. Berr, F. Portet, I. Carriere et al., "Olive oil and cognition: results from the three-city study," *Dementia and Geriatric Cognitive Disorders*, vol. 28, no. 4, pp. 357–364, 2009.

[43] A. H. Abuznait, H. Qosa, B. A. Busnena, K. A. el Sayed, and A. Kaddoumi, "Olive-oil-derived oleocanthal enhances β-amyloid clearance as a potential neuroprotective mechanism against Alzheimer's disease: in vitro and in vivo studies," *ACS Chemical Neuroscience*, vol. 4, no. 6, pp. 973–982, 2013.

[44] S. A. Farr, T. O. Price, L. J. Dominguez et al., "Extra virgin olive oil improves learning and memory in SAMP8 mice," *Journal of Alzheimer's Disease*, vol. 28, no. 1, pp. 81–92, 2012.

[45] H. Qosa, L. A. Mohamed, Y. S. Batarseh et al., "Extra-virgin olive oil attenuates amyloid-β and tau pathologies in the brains of TgSwDI mice," *The Journal of Nutritional Biochemistry*, vol. 26, no. 12, pp. 1479–1490, 2015.

[46] C. Valls-Pedret, R. M. Lamuela-Raventós, A. Medina-Remón et al., "Polyphenol-rich foods in the Mediterranean diet are associated with better cognitive function in elderly subjects at high cardiovascular risk," *Journal of Alzheimer's Disease*, vol. 29, no. 4, pp. 773–782, 2012.

[47] V. Pitozzi, M. Jacomelli, M. Zaid et al., "Effects of dietary extra-virgin olive oil on behaviour and brain biochemical parameters in ageing rats," *British Journal of Nutrition*, vol. 103, no. 11, pp. 1674–1683, 2010.

[48] K. Pallauf and G. Rimbach, "Autophagy, polyphenols and healthy ageing," *Ageing Research Reviews*, vol. 12, no. 1, pp. 237–252, 2013.

[49] R. Tsao, "Chemistry and biochemistry of dietary polyphenols," *Nutrients*, vol. 2, no. 12, pp. 1231–1246, 2010.

[50] M. Stefani and S. Rigacci, "Beneficial properties of natural phenols: highlight on protection against pathological conditions associated with amyloid aggregation," *BioFactors*, vol. 40, no. 5, pp. 482–493, 2014.

[51] N. Hasima and B. Ozpolat, "Regulation of autophagy by polyphenolic compounds as a potential therapeutic strategy for cancer," *Cell Death & Disease*, vol. 5, no. 11, article e1509, 2014.

[52] H. Lewandowska, M. Kalinowska, W. Lewandowski, T. M. Stępkowski, and K. Brzóska, "The role of natural polyphenols in cell signaling and cytoprotection against cancer development," *The Journal of Nutritional Biochemistry*, vol. 32, pp. 1–19, 2016.

[53] G. Gossner, M. Choi, L. Tan et al., "Genistein-induced apoptosis and autophagocytosis in ovarian cancer cells," *Gynecologic Oncology*, vol. 105, no. 1, pp. 23–30, 2007.

[54] A. B. Kunnumakkara, P. Anand, and B. B. Aggarwal, "Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins," *Cancer Letters*, vol. 269, no. 2, pp. 199–225, 2008.

[55] E. Maioli, L. Greci, K. Soucek et al., "Rottlerin inhibits ROS formation and prevents NFκB activation in MCF-7 and HT-29 cells," *Journal of Biomedicine and Biotechnology*, vol. 2009, Article ID 742936, 7 pages, 2009.

[56] B. Ozpolat, U. Akar, K. Mehta, and G. Lopez-Berestein, "PKCδ and tissue transglutaminase are novel inhibitors of autophagy in pancreatic cancer cells," *Autophagy*, vol. 3, no. 5, pp. 480–483, 2007.

[57] M. Sandri, "FOXOphagy path to inducing stress resistance and cell survival," *Nature Cell Biology*, vol. 14, no. 8, pp. 786–788, 2012.

[58] C. Soler-Rivas, J. C. Espín, and H. J. Wichers, "Oleuropein and related compounds," *Journal of the Science of Food and Agriculture*, vol. 80, no. 7, pp. 1013–1023, 2000.

[59] F. Gutierrez-Rosales, M. P. Romero, M. Casanovas, M. J. Motilva, and M. I. Mínguez-Mosquera, "Metabolites involved in oleuropein accumulation and degradation in fruits of *Olea europaea* L.: Hojiblanca and Arbequina varieties," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 24, pp. 12924–12933, 2010.

[60] R. Mateos, A. Cert, M. C. Pérez-Camino, and J. M. García, "Evaluation of virgin olive oil bitterness by quantification of secoiridoid derivatives," *Journal of the American Oil Chemists' Society*, vol. 81, no. 1, pp. 71–75, 2004.

[61] M. Esti, L. Cinquanta, and E. La Notte, "Phenolic compounds in different olive varieties," *Journal of Agricultural and Food Chemistry*, vol. 46, no. 1, pp. 32–35, 1998.

[62] M. P. Carrera-González, M. J. Ramírez-Expósito, M. D. Mayas, and J. M. Martínez-Martos, "Protective role of oleuropein and its metabolite hydroxytyrosol on cancer," *Trends in Food Science & Technology*, vol. 31, no. 2, pp. 92–99, 2013.

[63] E. Fuentes and I. Palomo, "Antiplatelet effects of natural bioactive compounds by multiple targets: food and drug interactions," *Journal of Functional Foods*, vol. 6, no. 6, Supplement C, pp. 73–81, 2014.

[64] L. Rubió, A. Serra, A. Macià, C. Piñol, M. P. Romero, and M. J. Motilva, "*In vivo* distribution and deconjugation of hydroxytyrosol phase II metabolites in red blood cells: a potential new target for hydroxytyrosol," *Journal of Functional Foods*, vol. 10, Supplement C, pp. 139–143, 2014.

[65] M. V. Sepporta, R. Fuccelli, P. Rosignoli et al., "Oleuropein inhibits tumour growth and metastases dissemination in ovariectomised nude mice with MCF-7 human breast tumour xenografts," *Journal of Functional Foods*, vol. 8, Supplement C, pp. 269–273, 2014.

[66] A. Daccache, C. Lion, N. Sibille et al., "Oleuropein and derivatives from olives as Tau aggregation inhibitors," *Neurochemistry International*, vol. 58, no. 6, pp. 700–707, 2011.

[67] L. Diomede, S. Rigacci, M. Romeo, M. Stefani, and M. Salmona, "Oleuropein aglycone protects transgenic *C. elegans* strains expressing Aβ42 by reducing plaque load and motor deficit," *PLoS One*, vol. 8, no. 3, article e58893, 2013.

[68] I. Luccarini, C. Grossi, S. Rigacci et al., "Oleuropein aglycone protects against pyroglutamylated-3 amyloid-ß toxicity: biochemical, epigenetic and functional correlates," *Neurobiology of Aging*, vol. 36, no. 2, pp. 648–663, 2015.

[69] F. Casamenti, C. Grossi, S. Rigacci, D. Pantano, I. Luccarini, and M. Stefani, "Oleuropein aglycone: a possible drug against degenerative conditions. In vivo evidence of its effectiveness against Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 45, no. 3, pp. 679–688, 2015.

[70] C. Grossi, S. Rigacci, S. Ambrosini et al., "The polyphenol oleuropein aglycone protects TgCRND8 mice against Aß plaque pathology," *PLoS One*, vol. 8, no. 8, article e71702, 2013.

[71] S. Rigacci, C. Miceli, C. Nediani et al., "Oleuropein aglycone induces autophagy *via* the AMPK/mTOR signalling pathway: a mechanistic insight," *Oncotarget*, vol. 6, no. 34, pp. 35344–35357, 2015.

[72] V. Vingtdeux, L. Giliberto, H. Zhao et al., "AMP-activated protein kinase signaling activation by resveratrol modulates amyloid-β peptide metabolism," *The Journal of Biological Chemistry*, vol. 285, no. 12, pp. 9100–9113, 2010.

[73] J. Zhou, B. L. Farah, R. A. Sinha et al., "Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, stimulates hepatic autophagy and lipid clearance," *PLoS One*, vol. 9, no. 1, article e87161, 2014.

[74] R. T. Marquez and L. Xu, "Bcl-2:Beclin 1 complex: multiple, mechanisms regulating autophagy/apoptosis toggle switch,"

American Journal of Cancer Research, vol. 2, no. 2, pp. 214–221, 2012.

[75] R. C. Russell, Y. Tian, H. Yuan et al., "ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase," *Nature Cell Biology*, vol. 15, no. 7, pp. 741–750, 2013.

[76] F. Ng and B. L. Tang, "Sirtuins' modulation of autophagy," *Journal of Cellular Physiology*, vol. 228, no. 12, pp. 2262–2270, 2013.

[77] I. Luccarini, D. Pantano, P. Nardiello et al., "The polyphenol oleuropein aglycone modulates the PARP1-SIRT1 interplay: an *in vitro* and *in vivo* study," *Journal of Alzheimer's Disease*, vol. 54, no. 2, pp. 737–750, 2016.

[78] S. Chung, H. Yao, S. Caito, J.w. Hwang, G. Arunachalam, and I. Rahman, "Regulation of SIRT1 in cellular functions: role of polyphenols," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 79–90, 2010.

[79] L. Adwan and N. H. Zawia, "Epigenetics: a novel therapeutic approach for the treatment of Alzheimer's disease," *Pharmacology & Therapeutics*, vol. 139, no. 1, pp. 41–50, 2013.

[80] S. M. Henning, P. Wang, C. L. Carpenter, and D. Heber, "Epigenetic effects of green tea polyphenols in cancer," *Epigenomics*, vol. 5, no. 6, pp. 729–741, 2013.

[81] M. Sardiello, M. Palmieri, A. di Ronza et al., "A gene network regulating lysosomal biogenesis and function," *Science*, vol. 325, no. 5939, pp. 473–477, 2009.

[82] C. Settembre, C. di Malta, V. A. Polito et al., "TFEB links autophagy to lysosomal biogenesis," *Science*, vol. 332, no. 6036, pp. 1429–1433, 2011.

[83] C. Settembre, R. Zoncu, D. L. Medina et al., "A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB," *The EMBO Journal*, vol. 31, no. 5, pp. 1095–1108, 2012.

[84] D. L. Medina, S. di Paola, I. Peluso et al., "Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB," *Nature Cell Biology*, vol. 17, no. 3, pp. 288–299, 2015.

[85] M. N. Vissers, P. L. Zock, A. J. C. Roodenburg, R. Leenen, and M. B. Katan, "Olive oil phenols are absorbed in humans," *The Journal of Nutrition*, vol. 132, no. 3, pp. 409–417, 2002.

[86] M. de Bock, E. B. Thorstensen, J. G. B. Derraik, H. V. Henderson, P. L. Hofman, and W. S. Cutfield, "Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (*Olea europaea* L.) leaf extract," *Molecular Nutrition & Food Research*, vol. 57, no. 11, pp. 2079–2085, 2013.

[87] S. H. Omar, "Oleuropein in olive and its pharmacological effects," *Scientia Pharmaceutica*, vol. 78, no. 2, pp. 133–154, 2010.

[88] S. Cetrullo, S. D'Adamo, S. Guidotti, R. M. Borzì, and F. Flamigni, "Hydroxytyrosol prevents chondrocyte death under oxidative stress by inducing autophagy through sirtuin 1-dependent and -independent mechanisms," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1860, no. 6, pp. 1181–1191, 2016.

[89] D. Pantano, I. Luccarini, P. Nardiello, M. Servili, M. Stefani, and F. Casamenti, "Oleuropein aglycone and polyphenols from olive mill waste water ameliorate cognitive deficits and neuropathology," *British Journal of Clinical Pharmacology*, vol. 83, no. 1, pp. 54–62, 2017.

[90] M. J. Oliveras-López, M. Innocenti, C. Giaccherini, F. Ieri, A. Romani, and N. Mulinacci, "Study of the phenolic composition of spanish and italian monocultivar extra virgin olive oils: distribution of lignans, secoiridoidic, simple phenols and flavonoids," *Talanta*, vol. 73, no. 4, pp. 726–732, 2007.

[91] S. Cicerale, L. J. Lucas, and R. S. J. Keast, "Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil," *Current Opinion in Biotechnology*, vol. 23, no. 2, pp. 129–135, 2012.

[92] D. Del Rio, A. Rodriguez-Mateos, J. P. Spencer, M. Tognolini, G. Borges, and A. Crozier, "Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases," *Antioxidants & Redox Signaling*, vol. 18, no. 14, pp. 1818–1892, 2013.

[93] S. Rigacci and M. Stefani, "Nutraceuticals and amyloid neurodegenerative diseases: a focus on natural phenols," *Expert Review of Neurotherapeutics*, vol. 15, no. 1, pp. 41–52, 2015.

[94] F. Márquez-Sandoval, M. Bulló, B. Vizmanos, P. Casas-Agustench, and J. Salas-Salvadó, "Un patrón de alimentación saludable: la dieta mediterránea tradicional," *Antropo*, vol. 16, pp. 11–22, 2008.