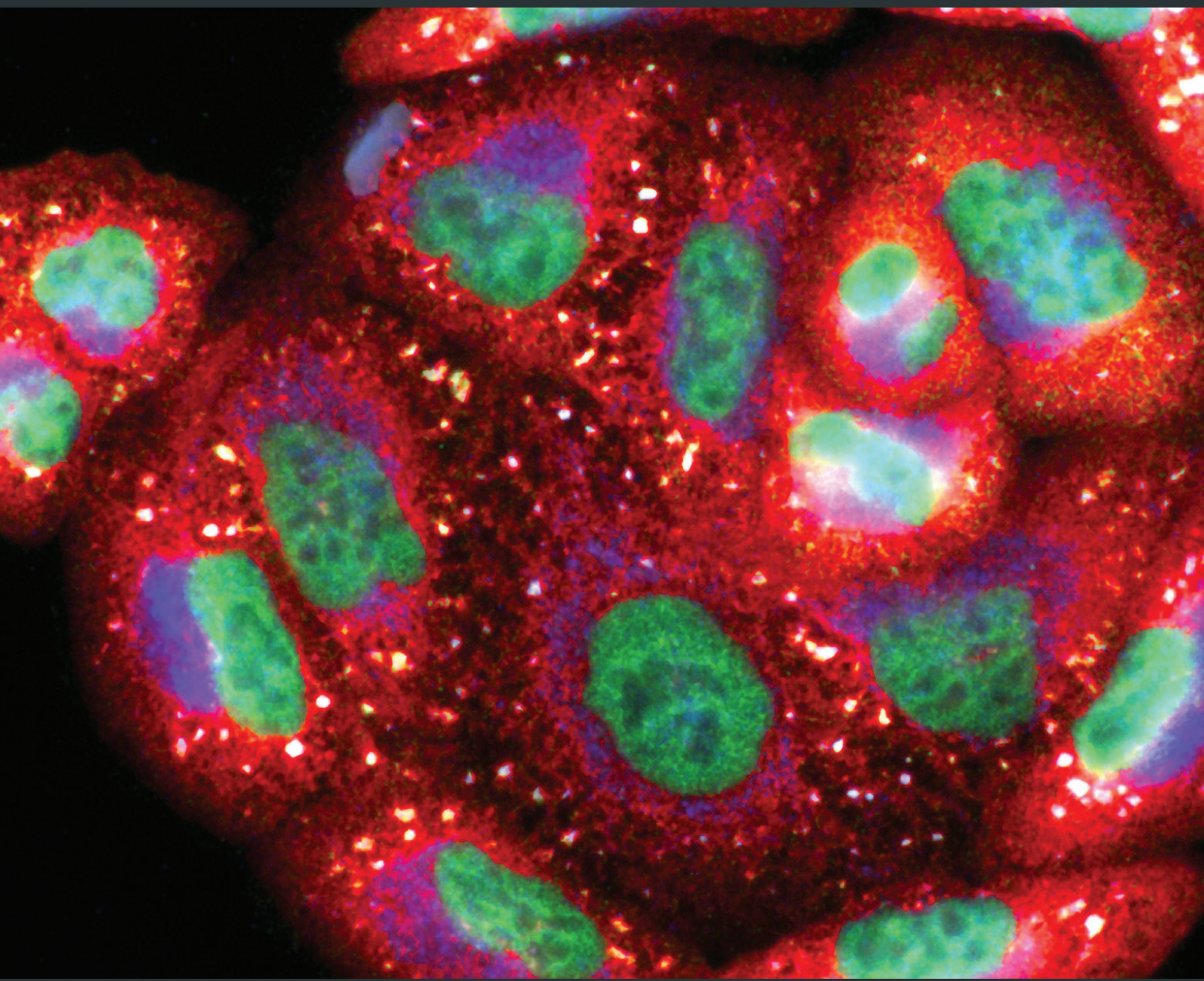
Oxidative Medicine and Cellular Longevity

Natural Products for the Prevention of Oxidative Stress-Related Diseases: Mechanisms and Strategies

Guest Editors: Wei Chen, Zhenquan Jia, Min-Hsiung Pan, and Pon Velayutham Anandh Babu



Natural Products for the Prevention of Oxidative Stress-Related Diseases: Mechanisms and Strategies

Oxidative Medicine and Cellular Longevity

Natural Products for the Prevention of Oxidative Stress-Related Diseases: Mechanisms and Strategies

Guest Editors: Wei Chen, Zhenquan Jia, Min-Hsiung Pan, and Pon Velayutham Anandh Babu

Editorial Board

Contents

Natural Products for the Prevention of Oxidative Stress-Related Diseases: Mechanisms and Strategies
Wei Chen, Zhenquan Jia, Min-Hsiung Pan, and Pon Velayutham Anandh Babu
Volume 2016, Article ID 4628502, 2 pages

A Potential Alternative against Neurodegenerative Diseases: Phytodrugs
Jesús Pérez-Hernández, Víctor Javier Zaldívar-Machorro, David Villanueva-Porras, Elisa Vega-Ávila, and Anahí Chavarría
Volume 2016, Article ID 8378613, 19 pages

Role of Natural Stilbenes in the Prevention of Cancer
J. Antoni Sirerol, María L. Rodríguez, Salvador Mena, Miguel A. Asensi, José M. Estrela, and Angel L. Ortega
Volume 2016, Article ID 3128951, 15 pages

Wild Raspberry Subjected to Simulated Gastrointestinal Digestion Improves the Protective Capacity against Ethyl Carbamate-Induced Oxidative Damage in Caco-2 Cells
Wei Chen, Yang Xu, Lingxia Zhang, Ya Li, and Xiaodong Zheng
Volume 2016, Article ID 3297363, 12 pages

Low-Dose *Aronia melanocarpa* Concentrate Attenuates Paraquat-Induced Neurotoxicity
A. J. Case, D. Agraz, I. M. Ahmad, and M. C. Zimmerman
Volume 2016, Article ID 5296271, 11 pages

Reviews on Mechanisms of *In Vitro* Antioxidant Activity of Polysaccharides
Junqiao Wang, Shuzhen Hu, Shaoping Nie, Qiang Yu, and Mingyong Xie
Volume 2016, Article ID 5692852, 13 pages

Sulforaphane Protects against Cardiovascular Disease via Nrf2 Activation
Yang Bai, Xiaolu Wang, Song Zhao, Chunye Ma, Jiuwei Cui, and Yang Zheng
Volume 2015, Article ID 407580, 13 pages

Biomolecular Modulation of Neurodegenerative Events during Ageing
Marcella Nebbioso, Gianfranco Scarsella, Aloisa Librando, and Nicola Pescosolido
Volume 2015, Article ID 978654, 10 pages

Lico A Enhances Nrf2-Mediated Defense Mechanisms against *t*-BHP-Induced Oxidative Stress and Cell Death via Akt and ERK Activation in RAW 264.7 Cells
Hongming Lv, Hua Ren, Lidong Wang, Wei Chen, and Xinxin Ci
Volume 2015, Article ID 709845, 13 pages

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4628502, 2 pages
http://dx.doi.org/10.1155/2016/4628502

Editorial

Natural Products for the Prevention of Oxidative Stress-Related Diseases: Mechanisms and Strategies

Wei Chen,[1] Zhenquan Jia,[2] Min-Hsiung Pan,[3] and Pon Velayutham Anandh Babu[4]

[1]*Department of Food Science and Nutrition, Zhejiang Key Laboratory for Agro-Food Processing, Zhejiang University, Hangzhou 310058, China*
[2]*Department of Biology, The University of North Carolina at Greensboro, Greensboro, NC 27412, USA*
[3]*Institute of Food Science and Technology, National Taiwan University, Taipei 10617, Taiwan*
[4]*Division of Nutrition, College of Health, University of Utah, Salt Lake City, UT 84112, USA*

Correspondence should be addressed to Wei Chen; zjuchenwei@zju.edu.cn

Received 26 November 2015; Accepted 26 November 2015

Current opinions with respect to the etiology of chronic diseases such as neurodegenerative disease, cardiovascular diseases, and cancer are still controversial. There are multiple factors involved, and oxidative stress that results from the imbalance between reactive oxygen species and antioxidants is a one of the key factors in the development of these chronic diseases. Indeed, experimental and clinical evidences support the casual relationship between oxidative stress and various chronic diseases. Thus, numerous studies are focused on ameliorating these chronic diseases by reducing the oxidative stress. Epidemiological and clinical studies suggest that natural products can combat oxidative stress and reduce the morbidity and mortality associated with chronic diseases. Many natural compounds such as flavonoids are potential antioxidants that protect against reactive oxygen species (ROS) or reactive nitrogen species- (RNS-) induced damage and ameliorate oxidative stress-related diseases, such as neurodegenerative diseases, cardiovascular diseases, inflammatory conditions, and cancer. Although various natural products have been shown to possess potential protective effects against chronic diseases, the bioactivities of large number of natural compounds remain unknown. Therefore, understanding and validating the bioactivities of the natural compounds and the molecular mechanisms involved would provide solid scientific foundation to use the natural compounds for the prevention and treatment of oxidative stress-related diseases. The primary objective of this issue is to highlight the role of some of the natural products that ameliorate oxidative stress and reduce pathological complications associated with chronic diseases. The articles published in this special issue include four reviews and four original research articles that describe the importance of some of the natural products.

The review by J. Pérez-Hernández et al. is focused on the possible role of antioxidant herbal compounds as an alternative source for the treatment of neurodegenerative diseases (ND). The herbal compounds discussed in this review include polyphenols, flavonoids, alkaloids, and other miscellaneous antioxidant compounds from plants. An interesting review article by Y. Bai et al. discusses the cardiovascular beneficial effects of sulforaphane, a sulfur-based isothiocyanate compound in the cruciferous vegetables. Clinical and animal studies showed sulforaphane improved cardiovascular complications through activation of Nrf2. In this review, the authors discuss the possible molecular mechanisms involved in sulforaphane induced Nrf2 activation. J. A. Sirerol et al.'s review is focused on the role of natural compound stilbene in the prevention of cancer. The authors discuss about the studies that support the anticancer effects of stilbene, which also include few clinical trials. In this issue, the review article by J. Wang et al. discusses the latest advancements and up-to-date discoveries on the mechanisms of antioxidant activity of polysaccharides and glycoconjugates derived from natural products.

ROS play a major role in the aging process and significantly contribute to the aging related pathological complications. The antioxidant system is compromised with aging and the antioxidant levels are no longer sufficient to counteract the generation of free radicals. Exogenous antioxidants may be a promising strategy to suppress oxidative stress associated with aging. In this issue, the original research article by M. Nebbioso et al. shows that α-lipoic acid (ALA) and superoxide dismutase (SOD) can counteract senile neurodegenerative deterioration to the retina and optic nerve. This study indicates that the combination of ALA and SOD could reduce oxidative stress and thereby prevent nuclear degradation and the subsequent cell death.

In everyday life, humans are readily exposed to toxicants derived from various sources such as food (e.g., ethyl carbamate), pesticides (e.g., paraquat), and industry (e.g., tert-butyl hydroperoxide). These toxic substances trigger oxidative stress in the biological systems, which consequently lead to acute or chronic diseases. In this issue, W. Chen et al. reported that wild raspberry can improve the protective capacity against ethyl carbamate-induced oxidative damage in Caco-2 cells. Their results demonstrate that raspberry extract subjected to simulated gastrointestinal digestion could improve the cellular antioxidant activity and attenuate ethyl carbamate-induced oxidative damage in Caco-2 cells. A. J. Case et al.'s research article shows that *Aronia melanocarpa* concentrate attenuated paraquat-induced neurotoxicity. In this study, *Aronia* berry concentrate at low doses can restore the homeostatic redox environment of neurons treated with paraquat, while high doses exacerbate the imbalance leading to further cell death. This suggests that moderate levels of *Aronia* berry concentrate may prevent reactive oxygen species-mediated neurotoxicity whereas high doses may have toxic effects. The study by H. Lv et al. shows that Licochalcone A (Lico A) enhances Nrf2-mediated defense mechanisms against tert-butyl hydroperoxide (t-BHP) induced oxidative stress and cell death via Akt and ERK activation in RAW 264.7 cells. Further, their results indicate that Lico A might modulate HO-1 and scavenge ROS via the activation of the PI3K/Akt and ERK/Nrf2 signaling pathways.

In summary, this special issue covers a series of topics addressing the role of natural products as antioxidants counteracting oxidative stress-related chronic diseases. We hope that the articles brought in this special issue not only enrich our understanding of the therapeutic role of natural products on oxidative stress-related disease but also provide promising perspectives on future novel therapeutic agents development.

Wei Chen
Zhenquan Jia
Min-Hsiung Pan
Pon Velayutham Anandh Babu

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8378613, 19 pages
http://dx.doi.org/10.1155/2016/8378613

Review Article

A Potential Alternative against Neurodegenerative Diseases: Phytodrugs

Jesús Pérez-Hernández,[1,2] Víctor Javier Zaldívar-Machorro,[1,3] David Villanueva-Porras,[1,4] Elisa Vega-Ávila,[5] and Anahí Chavarría[1]

[1]*Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, 06726 México, DF, Mexico*
[2]*Posgrado en Biología Experimental, Universidad Autónoma Metropolitana-Iztapalapa, 09340 México, DF, Mexico*
[3]*Programa de Becas Posdoctorales, Universidad Nacional Autónoma de México, 04510 México, DF, Mexico*
[4]*Posgrado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, 04510 México, DF, Mexico*
[5]*División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana, 09340 México, DF, Mexico*

Correspondence should be addressed to Anahí Chavarría; anahi.chavarria@gmail.com

Received 3 July 2015; Revised 2 November 2015; Accepted 5 November 2015

Academic Editor: Anandh B. P. Velayutham

Neurodegenerative diseases (ND) primarily affect the neurons in the human brain secondary to oxidative stress and neuroinflammation. ND are more common and have a disproportionate impact on countries with longer life expectancies and represent the fourth highest source of overall disease burden in the high-income countries. A large majority of the medicinal plant compounds, such as polyphenols, alkaloids, and terpenes, have therapeutic properties. Polyphenols are the most common active compounds in herbs and vegetables consumed by man. The biological bioactivity of polyphenols against neurodegeneration is mainly due to its antioxidant, anti-inflammatory, and antiamyloidogenic effects. Multiple scientific studies support the use of herbal medicine in the treatment of ND; however, relevant aspects are still pending to explore such as metabolic analysis, pharmacokinetics, and brain bioavailability.

1. Introduction

Neurodegenerative diseases (ND) such as Alzheimer's (AD) and Parkinson's disease (PD) and multiple sclerosis (MS) primarily affect the neurons in the human brain and are characterized by deterioration of neurons or myelin sheath, sensory information transmission disruption, movement control, and more [1]. The greatest risk factor for ND is aging, which carries mitochondrial dysfunction, chronic immune-inflammatory response, and oxidative stress [2, 3], the major causes of neuronal damage and death. Nowadays, ND are chronic and incurable conditions whose disabling effects may continue for years or even decades representing an enormous disease load, regarding human suffering and economic cost. The ND are more common and have a disproportionate impact on countries with longer life expectancies and represent the fourth highest source of overall disease burden in the high-income countries. According to the World Health Organization, 37 million people currently have dementia worldwide, and about 50% of them are being affected by AD and this number is expected to grow up to 115.4 million people by 2050 [4].

Recently, a great number of natural medicinal plants have been tested for their therapeutic properties, showing that the raw extracts or isolated pure compounds from them have more effective properties than the whole plant as an alternative for the treatment of ND. These properties are due mainly to the presence of polyphenols (Figure 1), alkaloids (Figure 2), and terpenes (Figure 3(d)), among others, that are micronutrients produced by plants as secondary metabolites [5, 6]. There is substantial evidence (epidemiological studies, animal studies, and human clinical trials) that indicates that polyphenols reduce a wide range of pathologies associated

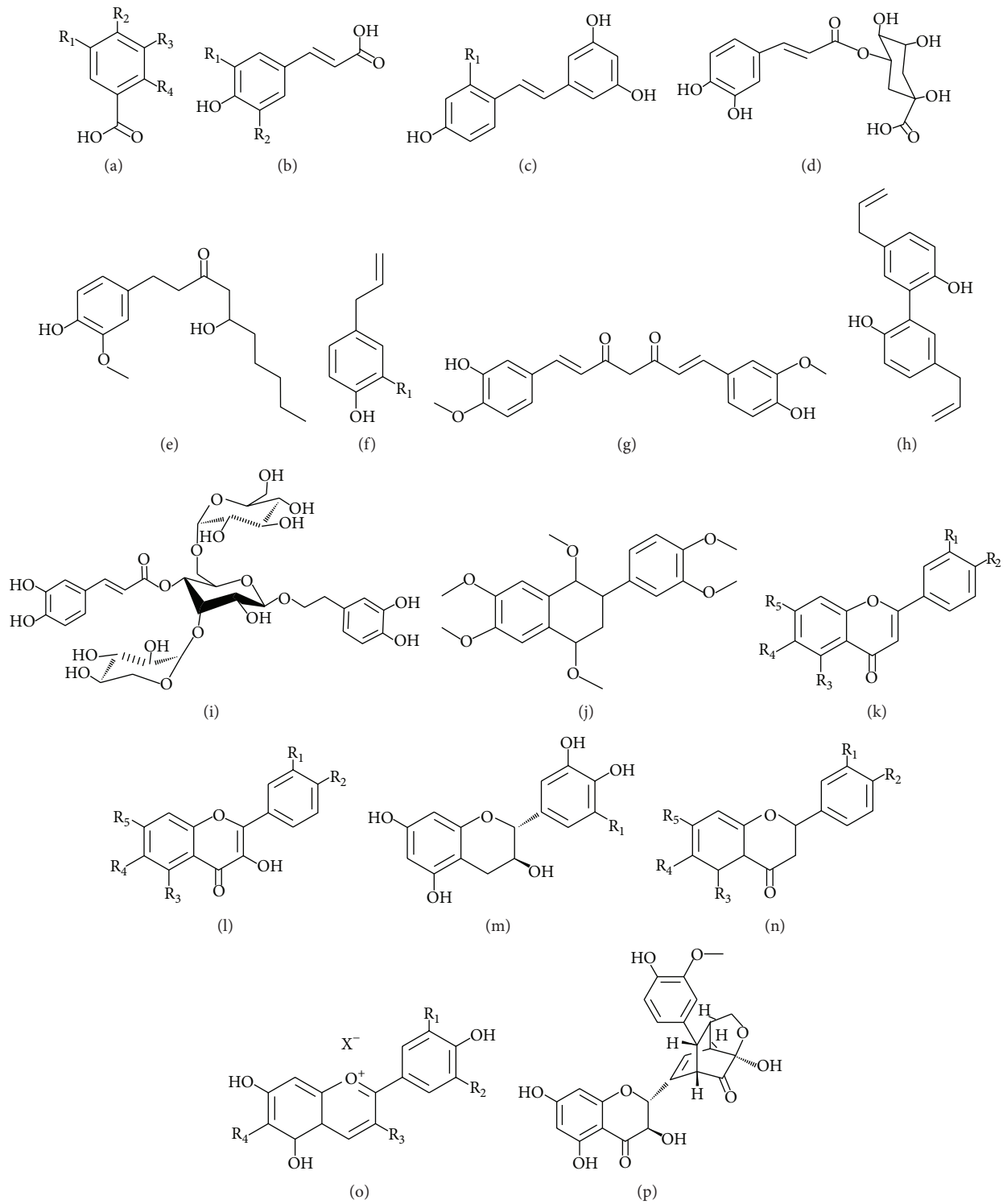


Figure 1: Representative polyphenol compounds. (a) Benzoic acids: *p*-hydroxybenzoic acid $R_1 = R_3 = R_4 = H$, $R_2 = OH$; protocatechuic acid $R_1 = R_4 = H$, $R_2 = R_3 = OH$; gallic acid $R_1 = R_2 = R_3 = OH$, $R_4 = H$; and salicylic acid $R_1 = R_2 = R_3 = H$, $R_4 = OH$. (b) Hydroxycinnamic acids: coumaric acid $R_1 = R_2 = H$; caffeic acid $R_1 = OH$, $R_2 = H$; ferulic acid $R_1 = OMe$, $R_2 = H$; and sinapic acid $R_1 = R_2 = OMe$. (c) Stilbenes: resveratrol $R_1 = H$; oxyresveratrol $R_1 = OH$. (d) Hydroxycinnamoyl ester: chlorogenic acid. (e) Hydroxycinnamoyl derivatives: gingerol; (f) Chavicol $R_1 = H$; eugenol $R_1 = OMe$; (g) curcumin; (h) magnolol; and (i) echinacoside. Flavonoid compounds. (j) Nobiletin; (k) Flavones: apigenin $R_1 = R_4 = H$, $R_2 = R_3 = R_5 = OH$; baicalein $R_1 = R_2 = H$, $R_3 = R_4 = R_5 = OH$; chrysin $R_1 = R_2 = R_4 = H$, $R_3 = R_5 = OH$; and luteolin $R_4 = H$, $R_1 = OMe$, $R_2 = R_3 = R_5 = OH$. (l) Flavonols: kaempferol $R_1 = R_4 = H$, $R_2 = R_3 = R_5 = OH$; quercetin $R_4 = H$, $R_1 = R_2 = R_3 = R_5 = OH$. (m) Flavanols (+)-catechin $R_1 = H$; (+)-gallocatechin $R_1 = OH$. (n) Flavanones: hesperetin $R_4 = H$, $R_1 = R_3 = R_5 = OH$, $R_2 = OMe$; naringenin $R_1 = R_4 = H$, $R_2 = R_3 = R_5 = OH$; pinocembrin $R_1 = R_2 = R_4 = H$, $R_3 = R_5 = OH$. (o) Anthocyanins: aurantinidin $R_1 = R_2 = H$, $R_3 = R_4 = OH$; cyanidin $R_2 = R_4 = H$, $R_1 = R_3 = OH$; pelargonidin $R_1 = R_3 = R_4 = H$, $R_2 = OH$; and peonidin $R_2 = R_4 = H$, $R_1 = OMe$, $R_3 = OH$. (p) Flavonolignans: silydianin.

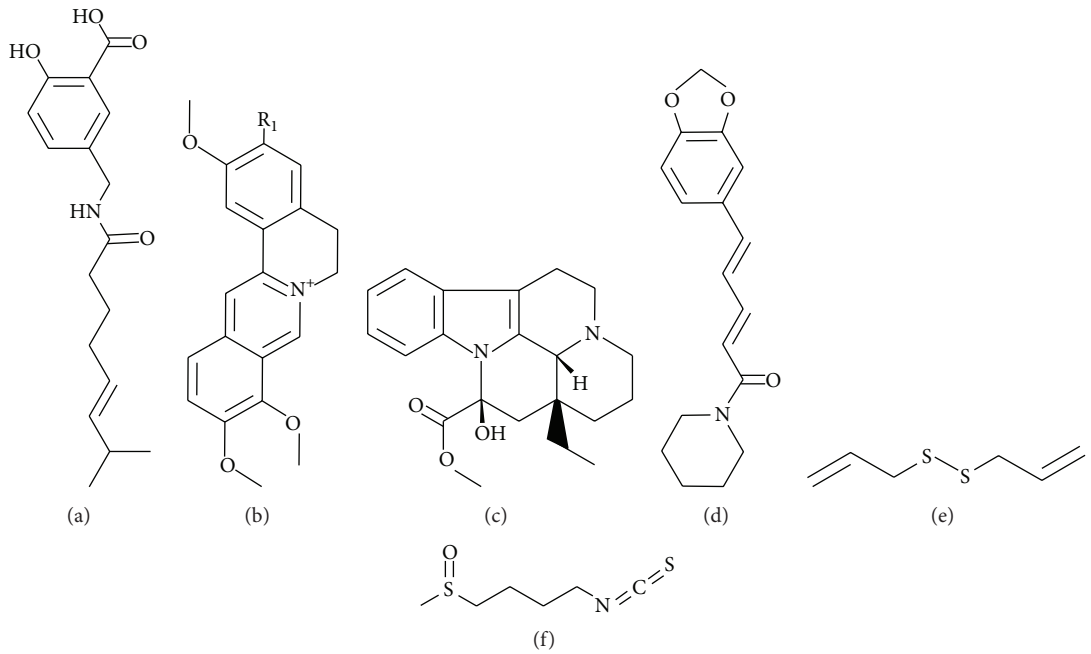


Figure 2: Some alkaloid compounds in plants. (a) Capsaicin; (b) protoberberines: jatrorrhizine R_1 = OH, palmatine R_1 = OMe; (c) vincamine; (d) piperine; (e) diallyl sulfide; and (f) sulphoraphane.

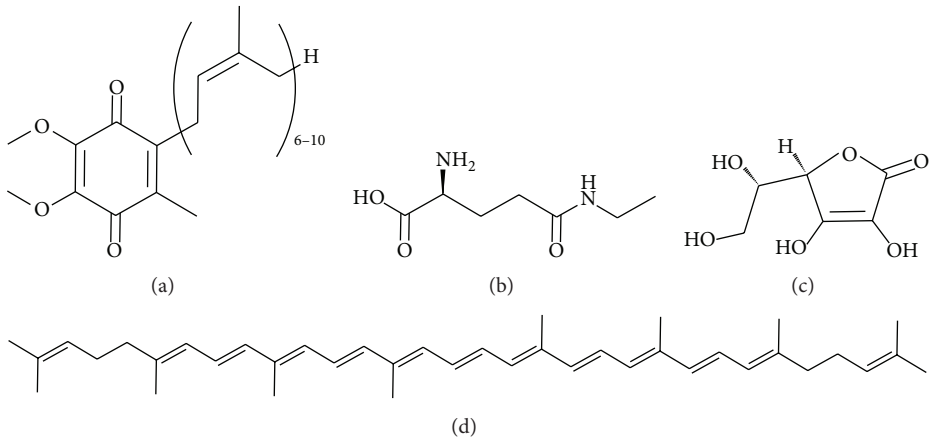


Figure 3: Some miscellaneous antioxidant compounds from plants. (a) Coenzyme Q_{6-10}; (b) l-theanine; (c) ascorbic acid; and (d) lycopene.

with inflammation [7–9]. The main mechanisms of polyphenols include their well-characterized antioxidant effects [10, 11], inhibition of intracellular kinases activity [12], binding to cell surface receptors [13], and modifying cell membrane functions [14]. Also, recently the neuroprotective effects of polyphenols have been described in several models of ND and involve mainly signaling pathways mediators [15], modulation of enzymes in neurotransmission [16, 17], inhibition of neurotoxicity via ionotropic glutamate receptors [18], antiamyloidogenic [19], and anti-inflammatory effects [20]. This review focuses on the plant extracts or compounds isolated from plants that may hold potential in the treatment of the principal ND.

2. Etiology of Neurodegenerative Diseases

ND are incurable and disabling conditions secondary to progressive neuronal loss, which leads to chronic brain damage and neurodegeneration. The etiology of ND is still unknown, although several ND animal models showed associated damage with the blood-brain barrier, protein aggregation, toxin exposure, and mitochondrial dysfunction, which lead to oxidative stress and inflammation, and consequently neuronal death [21].

The blood-brain barrier controls the internal environment of the vertebrate CNS and represents the border between the capillary and the extracellular fluid of CNS neurons and glial cells; it also ensures specific brain homeostasis

allowing adequate neuronal function [22]. Neurovascular changes normally occur as part of aging, but these are more evident in chronic ND [23]. About 20% of blood flow decreases in the aged brain, which associates with reduced protein synthesis [24]. Interestingly, this blood flow reduction is higher in the presence of any ND, which may lead to changes in intracellular pH and accumulation of interstitial lactate and glutamate [23, 25]. These changes are observed in specific brain regions in diseases such as AD, PD, MS among other CNS disorders [25–28].

Abnormal protein aggregation of specific regions and neuronal populations is a common feature among ND. For example, the α-synuclein inclusions in dopaminergic neurons from the substantia nigra are the main histopathological marker in PD [29]. Also, insoluble aggregates of the amyloid beta-peptide (Aβ) and neurofibrils composed of Tau protein are found in AD [30, 31] and hyperphosphorylated Tau aggregation in demyelination areas in MS [32]. Finally, superoxide dismutase 1 (SOD1) aggregations are present in amyotrophic lateral sclerosis (ALS) [33]. The main relevance of protein aggregates is that they lead to mitochondrial dysfunction inducing apoptotic neuronal death.

Redox state imbalance and chronic inflammation, a major cause of cell damage and death, characterize ND [34]. Reactive oxygen species (ROS) are key mediators of cell survival, proliferation, differentiation, and apoptosis [35, 36]. Excessive production of ROS by mitochondria and NADPH oxidase in oxidative stress is usually thought to be responsible for tissue damage associated with inflammation and ND [34, 36–38]. Moreover, many of the well-known inflammatory target proteins, including matrix metalloproteinase-9, cytosolic phospholipase A_2, cyclooxygenase-2, inducible nitric oxide synthase (iNOS), and adhesion molecules, are associated with oxidative stress and induced by proinflammatory factors such as cytokines, peptides, and peroxidants agents [36, 39, 40]. Several studies have shown that ROS act as a critical signaling molecule to trigger inflammatory responses in CNS through the activation of the redox-sensitive transcription factors, including nuclear factor-κB (NF-κB) and activator protein-1 [34, 36–39].

Mitochondrial damage leads to neuronal oxidative damage in ND pathogenesis. ROS and reactive nitrogen species, which are normal byproducts of mitochondrial respiratory chain activity, are mediated by mitochondrial antioxidants such as manganese superoxide dismutase and glutathione peroxidase. In addition to the ROS generation, mitochondria are also involved with life-sustaining functions including adenosine triphosphate synthesis by oxidative phosphorylation, apoptosis, calcium homeostasis, mitochondrial fission and fusion, lipid concentration of the mitochondrial membranes, and the mitochondrial permeability transition. Mitochondrial disease leading to neurodegeneration is likely, at least on some level, to involve all of these functions [41]. In ND several mitochondrial alterations are found like bioenergetics anomalies in the process of oxidative phosphorylation and ATP production, defects of mitochondrial dynamics, increase sensitivity to apoptosis, and accumulation of damaged mitochondria with unstable mitochondrial DNA [2].

The proteins aggregation also plays an important role in mitochondrial dysfunction; for example, the accumulation of mitochondrial Aβ aggregates has been observed both in patients and in transgenic models of AD [42–44]. Additionally, inhibition of mitochondrial complex I occurs in PD patients [45] and the two principal models used for the study of PD. Rotenone—a natural compound used as an insecticide, piscicide, and pesticide—and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)—a neurotoxin precursor of 1-methyl-4-phenylpyridinium (MPP^+), which destroys dopaminergic neurons in the substantia nigra—both act by inhibiting mitochondrial complex I [21]. In ALS, mitochondrial SOD1 enzyme aggregates cause loss of mitochondrial function and induce cellular death by apoptosis [46]. This phenomenon is present in almost all ND and associated with inflammation, which is one of the points of therapeutic interest and study.

The CNS inflammation is dependent on inflammatory mediators produced mainly by glial cells, specifically microglia and CNS macrophages [47]. Microglial activation is crucial in the pathogenesis and the course of PD [48], AD [49], prion disease [50], and MS [51], among others. Uncontrolled microglia activation produces neuronal damage due to overproduction of proinflammatory mediators such as tumor necrosis factor α (TNFα) [52], and nitric oxide (NO), leading to the generation of oxidative stress and apoptotic cell death [48, 52, 53].

3. Main Therapeutic Effects of Plant Extracts

The plant extracts have become interesting candidates as therapeutic agents due to their antioxidant, anti-inflammatory properties, and chemical characteristics derived as follows.

(i) Direct Uptake of Free Radicals. Primarily polyphenols (Figure 1) and alkaloids (Figure 2) function as scavengers due to their multiple phenolic hydroxyl and nitrogen groups, respectively, which act as an electron donor to the aromatic ring. These systems are excellent nucleophiles that readily lose electrons and easily oxidize. Therefore, they can catch free radicals and react with ROS, such as superoxide, peroxyl, hydroxyl radicals, NO, nitrogen dioxide, peroxynitrite, and singlet oxygen [54–56].

(ii) Chelation of the Divalent Cations in Fenton Reactions Involved. Many polyphenol compounds chelate iron cations due to multiple hydrophilic groups and are efficient scavengers because phenolic groups inhibit iron-mediated oxyradical formation like other iron chelators, such as desferrioxamine, 1,10-phenanthroline, and pyridoxal isonicotinoyl hydrazone [57, 58].

(iii) Modulation of Enzymes Associated with Oxidative Stress. ND associate with molecular alterations in cell-signaling pathways that regulate cell proliferation and differentiation, such as the family of mitogen-activated protein kinases (MAPK). Abnormal activation or silencing of the MAPK pathway or its downstream transcription factors can result

in uncontrolled cell growth leading to malignant transformation. Some plant compounds "switch on" or "turn off" the specific signaling molecule(s), depending on the nature of the signaling cascade they target, preventing abnormal cell proliferation and growth [59, 60].

4. Antioxidant and Anti-Inflammatory Properties in Central Nervous System

Flavonoids, a type of polyphenolic compounds found in fruits, vegetables, red wine, and green tea, reduce the risk to developing ND [61]. In 2010, Vuong and colleagues showed that cranberry juice in neuronal cultures significantly increased the activity of antioxidant enzymes such as catalase and SOD1 and protected neurons against H_2O_2 induced cell death, possibly due to the activation of survival pathways dependent from p38 and blocking death pathway associated with MEK1/2 and ERK1/2 [62]. A comparative study of two extracts of *Salvia* species, *S. hydrangea* and *S. macilenta*, also showed strong antioxidant properties, also at high concentrations ($\geq 50\,\mu$g/mL) they can inhibit DNA damage by free radicals. Moreover, these species not only showed no cytotoxic effect in cultured PC12 cells, a cell line derived from a pheochromocytoma obtained from rat adrenal medulla differentiated with neural growth factor, but also protected them from peroxide-induced cell death [63]. Similarly selaginellin, a compound extracted from the plant *Saussurea pulvinata*, showed a neuroprotective effect in a glutamate neurotoxicity model in PC12 cells by trapping ROS and regulating the expression of the *klotho* gene, which has an antiapoptotic role [64].

Ginger, the root of *Zingiber officinale*, an important specie used in the Chinese, Ayurvedic, and Tibia-Unani traditional medicine, has anti-inflammatory [65–67] and antioxidant [68] properties, among others. The hexane fraction of ginger extract and the methanol extract of *Ficus religiosa* sheet significantly decreased the production of NO, prostaglandin E2, IL-1β, IL-6, and TNFα through the inhibition of MAPK and NF-κB in BV2 microglial cell line stimulated with lipopolysaccharide (LPS) [69, 70].

Similarly, the ethanol extract of *Knema laurina* exerted anti-inflammatory and neuroprotective effects in a BV2 microglial cell culture line, in HT-22 hippocampal neurons and in organotypic hippocampal cultures. *Knema laurina* reduced microglial production of NO and IL-6 through the inhibition of ERK1/2 and IKKβ phosphorylation, and the subsequent translocation NF-$\kappa\beta$ in microglial cells [71].

5. Therapeutic Opportunities for Plant Extracts in Central Nervous System Age-Related Changes

It is clear that aging is a critical factor for developing ND and facilitates the microglial promoted proinflammatory environment [72–74] and oxidative stress [75]. Therefore, studying potential drugs that prevent or retard age-related changes has become crucial. Natural antioxidants such as some cocoa derivatives have shown to contain higher flavonoids levels [76]. For example, acticoa, a cocoa-derived polyphenol extract, administered daily orally at 24 mg/kg dose in Wistar rats 15 to 27 months old, improved cognitive performance, increased life expectancy, and preserved free dopamine levels in urine [77]. Another extract with high antioxidant activity is silymarin, a standardized mixture of flavonolignans (Figure 1(p)) extracted from the *Silybum marianum* fruits and seeds [78]. The treatment with 400 mg/kg/day of silymarin during three days increased reduced glutathione (GSH) and SOD activity in the brain of aged rats [79]. Vincamine (Figure 2(c)), a monoterpenoid indole alkaloid purified from the *Vinca minor* plant, has antioxidant activity similar to vitamin E. This compound increased cerebral blood flow, glucose, and oxygen utilization in neural tissue and promoted the rise of dopamine, serotonin, and noradrenaline levels [80]. Also, the treatment of rats with vincamine during 14 days at a daily dose of 15 mg/kg reduced about 50% the brain iron levels, which suggests a beneficial effect in reducing the oxidative stress associated with the iron deposition in ND [81]. Moreover, paeonol, a compound extracted from the *Paeonia suffruticosa* cortex or *Paeonia lactiflora* root, has been ascribed to anti-inflammatory and antioxidant properties. Paeonol effects were tested in a model of neurotoxicity induced with D-galactose injected subcutaneously in aged mice. Paeonol prevented memory loss in this model since it increased acetylcholine and GSH levels and decreased the activity of acetylcholinesterase (AChE) and SOD1 in the hippocampus and cortex, positioning it as a potential drug useful in age-related ND [15]. Also, *Magnolia officinalis* compounds, magnolol (Figure 1(h)) and their isomer honokiol, were tested in a senescence-accelerated prone mice; this compound prevented learning and memory deterioration, as well as acetylcholine deficiency by preserving forebrain cholinergic neurons [18, 82].

6. Plant Compounds Used for Alzheimer's Disease

AD manifests as a progressive cognitive and behavioral disorder and is characterized by an immediate loss of memory secondary to neuronal loss in the limbic and association cortices. This neuronal death results from oxidative stress, neuroinflammation, and abnormal protein deposition [83], leading to a therapeutic opportunity for medicinal plants, which improve AD course principally by modulating A aggregation, AChE activity, oxidative stress, and inflammatory response [84].

Cryptotanshinone is an active component of *Salvia miltiorrhiza* with anti-inflammatory, antioxidant, and antiapoptotic properties [85, 86]. This compound crossed the blood brain barrier and decreased cognitive deficits in male IRC mice with scopolamine-induced cognitive impairments [87]. This compound also provided beneficial effects in patients with ischemia and cerebral infarct [88]. Additionally, cryptotanshinone reduced the Aβ aggregation in brain tissue and improved spatial learning and memory in APP/PS1 transgenic mice by promoting amyloid precursor protein metabolism via α-secretase pathway [89]. Silymarin also showed antiamyloid properties *in vitro*, and its chronic

administration (half a year) significantly reduced the Aβ plaque burden associated with microglial activation, Aβ oligomer formation, and hyperactivity and disturbed behavior in APP transgenic mice [90]. The protective effect of silymarin on Aβ accumulation is attributable to the blockade of its aggregation, not to β-secretase inhibition [89]. The use of *Centella asiatica* in a dementia model in PSAPP mice improved memory retention in rodents [91, 92] and decreased amyloid deposition and the spontaneously Aβ plaque formation [93]. Likewise, the grape seed polyphenolic extract from *Vitis vinifera* attenuated the cognitive impairment observed in aging AD transgenic mice and decreased Aβ plaques deposition in the brains [94]. Nobiletin (Figure 1(j)), a flavonoid purified from *Citrus depressa* plant, prevents memory loss in APP695 transgenic mice and Aβ treated rats. This compound reduces the Aβ plaques amount in the hippocampus [95, 96], probably by reducing the inhibition of protein kinase A and cAMP response element-binding protein phosphorylation signaling cascade [97]. Nobiletin also stimulated long-term potentiation in organotypic hippocampal cultures [98]. Other compounds that can prevent Aβ aggregation by inhibition of the metabolic pathway that generates Aβ plaques are berberine, palmatine, jateorrhizine, epiberberine, coptisine, groenlandicine, and magnoflorine, alkaloids isolated from *Coptis chinensis* rhizome [99]. These compounds also exhibit AChE inhibiting properties [100, 101] and antidepressant effects [59] and enhance cognitive improvements [102]. Also, jateorrhizine (Figure 2(b)) and groenlandicine have significant peroxynitrite scavenging activities, while coptisine and groenlandicine present moderate total ROS inhibitory activities [99].

The ethanol extract from *Cassia obtusifolia* has potential use in AD, which reduced scopolamine-induced memory loss in mice by inhibiting AChE [103]. Similarly, methoxsalen, the main component of the aqueous extract of *Poncirus trifoliata*, inhibited AChE activity reducing memory loss and learning problems associated with a neurotoxicity *in vivo* model induced with trimethyltin [16]. In the AD model induced with ethylcholine aziridinium, which mimics the cholinergic hypofunction present in AD [104], piperine (Figure 2(d)), an alkaloid present in *Piper longum*, lowered the cognitive deficits and the hippocampal neurodegeneration associated with this AD model [105]. These effects could be probable due to its anti-inflammatory [106] and antioxidant activities [71].

The treatment for 5 weeks with L-theanine (Figure 3(b)), an amino acid present in green tea *Camellia sinensis*, significantly decreased memory loss associated with intraventricular Aβ_{1-42} AD model. L-theanine as well reduced cortical and hippocampal neuronal death, also inhibited lipid peroxidation and protein damage, and increased GSH levels, suggesting its potential use in AD prevention and treatment [17]. Also, *Dioscorea opposita* chloroform extract, which has been used to treat memory-related diseases such as AD and others ND, prevented neuronal death, and significantly increased spatial learning and memory improvement, probably due to its antiexcitotoxic and antioxidant effects [107].

Sanmjuanhwan (Sjh), a multiherbal formula from oriental traditional medicine, composed of *Morus alba*, *Lycium chinense*, and *Atractylodes japonica*, showed neuroprotective effects on primary neuronal cultures exposed to Aβ_{25-35}. Sjh increased the expression of antiapoptotic proteins such as Bcl-2 and avoided cytochrome c release and caspase-3 activation [108]. *B. monnieri* and its active components bacoside A, bacopaside I and II, and bacosaponin C [109, 110] have anti-inflammatory, antimicrobial, and antidepressant effects [111–113]. Treatment with *B. monnieri* prevented neuronal death by the inhibition of AChE activity in primary cortical culture pretreated with Aβ_{25-35} [114]. Furthermore, animals and volunteers treated with this plant presented enhanced memory [115–117]. The antioxidant effect of *S*-allyl cysteine (SAC), an amino acid isolated from aged garlic, was tested in the Aβ_{25-35}-AD rat model, showing ROS scavenger activity *in vivo* [118]. Also, in the mice AD dementia model induced with the intracerebroventricular streptozotocin infusion, SAC pretreatment decreased p53 expression, restored Bcl-2 protein expression, reduced, and prevented DNA fragmentation [119].

Mono- and diacetyled cyanidin and peonidin, the purple sweet potato anthocyanins (PSPA; Figure 1(o)) extracted from *Ipomoea batatas*, can easily attract ROS, which has high clinical value as antioxidant therapy in AD and other ND [120, 121]. For example, pretreatment of PC12 cells with PSPA reduced Aβ toxicity preventing lipid peroxidation, caspase-3 activation, and Aβ-induced apoptosis, suggesting a possible use in the treatment of AD [122].

Finally, the use of ginseng, *Panax ginseng*, was evaluated in AD patients, those who received a daily dose 9 g of Korean red ginseng for 12 weeks showed a significant improvement in the AD assessment scale and the clinical dementia rating scale compared to control patients [123].

7. Plant Compounds for Parkinson's Disease Treatment

PD is the second most frequent ND and is primarily a movement disorder characterized by the loss of dopamine-producing neurons in substantia nigra. Activation of neuronal death pathways involves oxidative stress, neuroinflammation, and mitochondrial dysfunction [124].

Green tea extract and its isolated (–)-epigallocatechin-3-gallate polyphenol, as well as ginseng extract, have neuroprotective effects since their use diminished dopaminergic neuron loss in the substantia nigra and oxidative damage in an MPTP and its toxic metabolite MPP^+ in PD animal models [125, 126]. Also, *Chrysanthemum morifolium*, which has antioxidant activity [126], inhibited MPTP-induced cytotoxicity and maintained cell viability of SH-SY5Y cell line, preventing ROS formation, decreasing Bax/Bcl2 ratio and caspase-3 activation [127]. The administration of 20 mg/kg of echinoside, a compound isolated from *Cistanche salsa*, before MPTP intoxication maintained striatal dopamine levels, reduced cell death, significantly increased the tyrosine hydroxylase enzyme expression, and reduced the activation of caspase-3 and caspase-8 expression, thus preventing neuronal death [128]. Likewise, silymarin treatment preserved dopamine levels, diminished the number of apoptotic cells, and preserved dopaminergic neurons in the substantia nigra of MPTP- and 6-hydroxydopamine-intoxicated mice

(6-OHDA) [74, 129–131]. Besides, pelargonidin (Figure 1(o)), an anthocyanidin with neuroprotective effects, reduced the motor deficit and histological damage and prevented lipid peroxidation in the 6-OHDA model [132–134].

In the MPTP-intoxicated model of PD, SAC prevented lipid peroxidation and mitochondrial dysfunction [135], protected the striatum of mice from the morphological alterations with a reduction in TNF-α and iNOS expressions, and further reduction in astrocyte activation [136] and also, at 120 mg/kg dose by five days, partially ameliorated the MPTP-induced striatal and nigral dopamine and tyrosine hydroxylase depletion, attenuated the loss of manganese-dependent superoxide dismutase and heme oxygenase-1 activities, and preserved the protein content of these enzymes [137]. These findings suggest that SAC can exert neuroprotection since the origin of the dopaminergic lesion—at the substantia nigra—not only by using direct antioxidant actions but also through Nrf2 nuclear transactivation and phase 2 enzymes upregulation [137].

The commercial extract of *Anemopaegma mirandum*, a Brazilian tree, and the crude extract of *Valeriana officinalis* increased the viability of SH-SY5Y cells after rotenone exposure [138, 139], while the extract of *Rhus verniciflua* decreased ROS production, preserved the mitochondrial integrity, and decreased the number of apoptotic cells [140]. An extract from *Tripterygium regelii*, a plant with antioxidant properties, reduced oxidative stress-induced cell death through the inhibition of apoptotic cascades, preserved mitochondrial function, and promoted tyrosine hydroxylase expression and brain-derived neurotrophic factor (BDNF) production in H_2O_2 treated SH-SY5Y cells [141]. Also, in the MPP^+-intoxicated SH-SY5Y cells, the orchid increased cell viability, decreased cytotoxicity and ROS production, and prevented caspase-3 activation by diminishing the Bax/Bcl2 ratio [142].

In the same model, the flavonoid luteolin (Figure 1(k))—a compound present in celery, green pepper, pear leaves, and chamomile tea—provided neuroprotection against oxidative stress [143]. Also, luteolin inhibited LPS induced microglial activation, as well as the production of TNFα, NO, and superoxide in a midbrain mixed primary cultures [144]. Pedicularoside A, a glycosylated phenylethanoid isolated from *Buddleja lindleyana*, has anti-inflammatory properties and is a good scavenger of superoxide anions and hydroxyl radicals [145]; it protected against MPP^+-induced death in mixed midbrain primary culture by increasing tyrosine hydroxylase expression and decreasing caspase-3 cleavage [146]. The plant extract from *Uncaria rhynchophylla* decreased cell death and ROS production and increased GSH levels in cultured PC12 cells, while 6-OHDA-induced caspase-3 activation was attenuated preventing cell death and rotational behavior was significantly reduced in the 6-OHDA PD model [147]. The ethyl extract from *Myracrodruon urundeuva* displayed similar properties in mesencephalic cultured cells since it preserved cell viability and attenuated oxidative stress after 6-OHDA exposure [148].

Panax notoginseng (PN) has the property to increase the expression of certain molecules such as nestin and BDNF, promoting neural plasticity and recovery after cerebral ischemia [149, 150]. Also, PN induces the expression of thioredoxin-1, an oxidoreductase with antiapoptotic and cell growth promoter effects [151], reducing MPTP-induced cell death in PC12 cells [152]. Likewise, the root extract of *Withania somnifera* promoted axon and dendrite growth [153, 154] and also increased the levels of SOD1, catalase, and GSH, preventing deficit motor in MPTP-intoxicated animals [155].

The isoflavones daidzin, daidzein, and genistein contained in *Pueraria thomsonii* protected PC12 cells stimulated with 6-OHDA through the inhibition of the caspase-3 activation [156]. Moreover, genistein, a soy phytoestrogen, protected neurons from substantia nigra pars compact and attenuated the rotational behavior in a hemiparkinsonian 6-OHDA model [157]. Interestingly, the administration of *Mucuna pruriens* preceding 6-OHDA intoxication was more efficient than levodopa in controlling motor symptoms, since it restored dopamine and norepinephrine levels in the nigrostriatal track exhibiting a neuroprotective effect [158]. The mechanism of action of *Mucuna pruriens* is not fully understood; however, it has been proposed that increases the mitochondrial complex I activity without affecting the monoamine oxidase B activity, probably due to its high content of NADH and Q-10 coenzyme (Figure 3(a)), and its ability to scavenge ROS [159].

The herbal mixture Toki To (TKT), prepared of ten different plants (*Angelicae Radix*, *Pinelliae Tuber*, *Cinnamomi Cortex*, *Ginseng Radix*, *Magnoliae Cortex*, *Paeoniae Radix*, *Astragali Radix*, *Zanthoxyli fructus*, *Zingiberis siccatum Rhizoma*, and *Glycyrrhizae Radix*), has excellent results against PD [159]. TKT orally administered reduced motor symptoms such as bradykinesia, prevented dopaminergic neurons loss in the substantia nigra, and increased tyrosine hydroxylase and dopamine transporter expression in MPTP-intoxicated mice [159]. Through microarray it was determined that TKT *per se* regulates the expression of serum- and glucocorticoid regulated kinase gene (*sgk*), which are implicated in the PD pathogenesis [159].

Psoralea corylifolia seeds, specifically Δ3,2-hydroxybakuchiol monoterpene, which has been used for years in Chinese medicine for the treatment of cerebral aging and dementia [102, 160], protected SK-N-SH cells from MPP^+ intoxication and prevented the dopaminergic neurons loss in MPTP-intoxicated mice by inhibition of the monoamine transporter [161, 162]. Also it is worth mentioning that *Rosmarinus officinalis*, a plant used as flavoring in Mediterranean cuisine, has antioxidant properties [163]. *Rosmarinus officinalis* inhibits NO production [164] and protects dopaminergic neurons in different degenerative disease models [165–168], probably due to its a high content of polyphenols and terpenes such as carnosol, carnosic acid, and rosmarinic acid and antiapoptotic effects [169].

8. Plant Compounds for Cerebral Ischemia Management

In cerebral ischemia, severe neuronal damage occurs during the reperfusion period due to excitotoxicity, which consists of

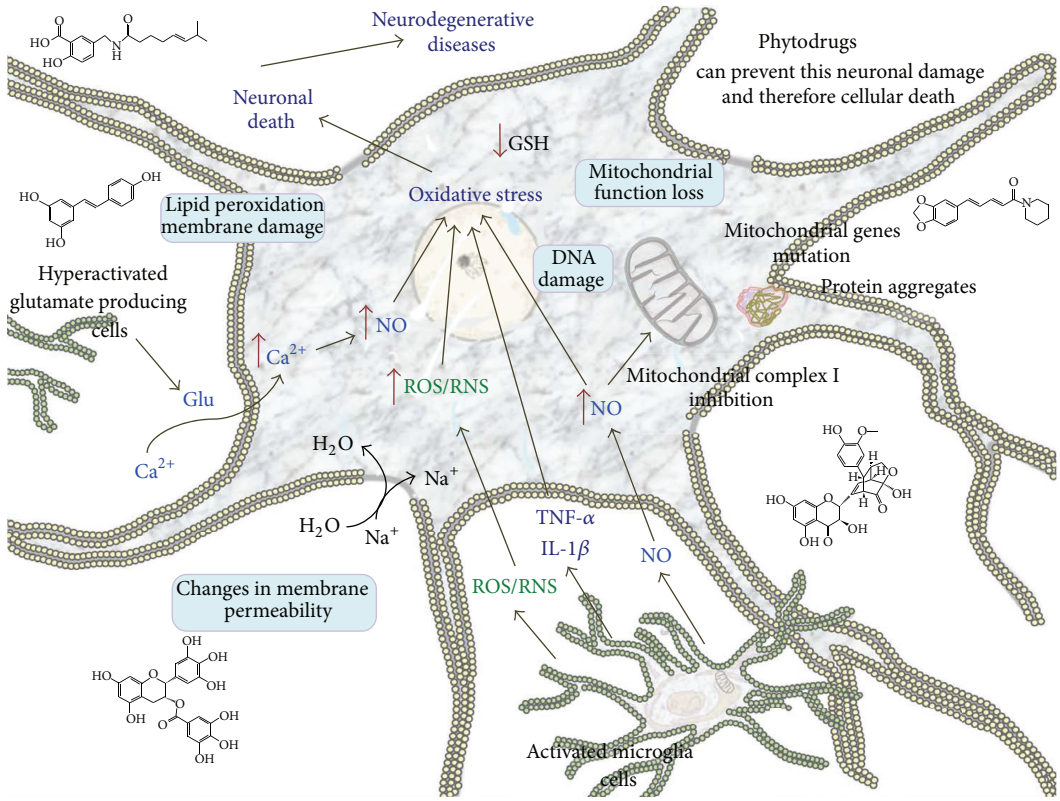


Figure 4: Main neuronal death pathways caused by oxidative stress. Oxidative stress can lead to neuronal death via several mechanisms such as mitochondrial dysfunction, DNA damage, membrane permeability loss, protein aggregation, and apoptosis. Phytodrugs, mainly polyphenols and alkaloids, can prevent this neuronal damage and, therefore, cellular death. Thus, these natural compounds can be used in the treatment of ND and also could serve as models for developing new specific drugs against these pathologies.

an overstimulation of *N*-methyl-D-aspartate (NMDA) receptors leading to glutamate production, which in turn triggers oxidative and inflammatory processes [26]. The intraperitoneal administration of 200 mg/kg of cactus polysaccharides, the active component isolated from *Opuntia dillenii*, prior to the middle cerebral artery occlusion showed neuroprotective effects [170, 171]. *Opuntia dillenii* significantly reduced infarct volume, decreased neuronal loss in the cerebral cortex, and diminished importantly the nitric oxide synthase (NOS) synthesis, which is usually induced during the experimental period of reperfusion and ischemia [171]. Also, oral pretreatment with 30 and 50 mg/kg daily of *Smilacis chinae* rhizome (SCR) methanol extract reduced the histological changes associated with ischemic injury [172]. It is possible that SCR prevented excitotoxicity-induced neuronal death by decreasing ROS generation, similar to the observations made *in vitro* in primary cultures of cortical cells treated with 1 mM NMDA [172]. Additionally, intravenous pretreatment with silymarin reduced infarcted area size, as well as neurological deficits associated with ischemic damage [173]. Also, silymarin inhibited protein expression associated with inflammation such as iNOS, cyclooxygenase-2, myeloperoxidase, the nuclear transcription factor NF-κB, and proinflammatory cytokines like IL-1β and TNFα, avoiding neurodegeneration associated with ischemia [173]. Similarly, SAC administration reduced infarct volume in a rat brain ischemia model [174] and decreased lipid peroxidation to basal levels suggesting SAC beneficial effects in brain ischemia and that the major protective mechanism may be the inhibition of free radical-mediated lipid peroxidation [175].

9. Conclusions

Neurodegenerative diseases (ND) are chronic and progressive conditions, characterized by neuronal loss secondary to oxidative stress and neuroinflammation (Figure 4). Until now ND have no cure and represent high costs for the health system and patients families. Exploring alternative sources for ND therapy has led to set eyes on herbal medicine since most herbal compounds have antioxidant and anti-inflammatory properties. At present, the use of several plants in the treatment of ND is being supported by numerous scientific investigations (the main effects of herbal plants against ND are listed in Table 1). However, information is still missing on relevant aspects such as metabolism, pharmacokinetics, and bioavailability in the brain as well as any changes that they may have in the CNS. Nevertheless, plant compounds or extracts remain interesting therapeutic candidates for ND management.

Table 1: Main biological effects of phytodrugs in neurodegenerative diseases models.

Effect	Plant compound/extract	Model	Disease/condition	Reference
Antiapoptotic and/or cell viability	*Anemopaegma mirandum* extracts.	*In vitro*	Rotenone model in SH-SY5Y cells.	[137]
	Pueraria thomsonii.	*In vitro*	6-OHDA model in PC12 cells.	[155]
	Cistanche salsa.	*In vivo*	MPP^+ mice model.	[127]
	Gastrodia elata extract.	*In vitro*	MPP^+ model in SH-SY5Y cells.	[141]
	Rosmarinus officinalis extract.	*In vitro*	Aβ model in cortical neurons.	[168]
	Chrysanthemum morifolium extract.	*In vitro*	MPP^+ model in SH-SY5Y cells.	[126]
	Panax notoginseng.	*In vitro*	MPTP model in mesencephalic neurons.	[151]
	Piperine (Figure 2(d)).	*In vitro*	Rotenone model in SH-SY5Y cells.	[104]
	L-theanine, from green tea (Figure 3(b)).	*In vitro*	H_2O_2 model in SH-SY5Y cells.	[16]
	Toki-to, mixed medicinal herbs.	*In vitro* *In vivo*	6-OHDA model in PC12 cells. 6-OHDA rat model.	[158]
	Nobiletin, a flavonoid from citrus peels.	*In vitro* *In vivo*	H_2O_2 model in PC12 cells. Rat artery occlusion model.	[95]
	Psoralea corylifolia.	*In vitro* *In vivo*	MPP^+ model in CHO cells and SK-N-SH cells. MPTP mice and rat model.	[161]
	Chrysanthemum morifolium extract.	*In vitro*	MPP^+ model in SH-SY5Y cell.	[126]
	Uncaria rhynchophylla extract.	*In vitro*	Rotenone model in SH-SY5Y cells.	[146]
	Polyphenolic extract from *Vitis vinifera.*	*In vitro*	Rotenone model in SH-SY5Y cells.	[93]
	Withania somnifera extract.	*In vitro* *In vivo*	MPP^+ model in CHO cells and SK-N-SH cells. MPTP mice and rat model.	[154]
	Paeonol from *Paeonia suffruticosa* or *Paeonia lactiflora.*	*In vitro* *In vivo*	MPP^+ model in PC12 cells. MPP^+ mice model.	[14]
	Ipomoea batatas PoirCv.	*In vitro*	Aβ model in PC12 cells.	[121]
	Biotransformed blueberry juice by *Serratia vaccinii bacteria.*	*In vitro*	H_2O_2 model in neuronal cells.	[61]
	Polyphenolic from cocoa.	*In vivo*	Aged rats.	[76]
	Salvia miltiorrhiza.	*In vitro* *In vivo*	Cortical neurons overexpressing APP695. APP/PS1 transgenic mice.	[88]
	Opuntia dillenii.	*In vitro* *In vivo*	NMDA model in cortical neurons. Rat artery occlusion model.	[169]
	Selaginellin from *Saussurea pulvinata.*	*In vivo*	Aβ mice model.	[63]
	Mucuna pruriens.	*In vivo*	APP-SL 7-5 transgenic mice APP695.	[157]
	Urundeuvines A, B, and C chalcones from *Myracrodruon urundeuva.*	*In vivo*	6-OHDA model in mesencephalic cells.	[147]

Table 1: Continued.

Effect	Plant compound/extract	Model	Disease/condition	Reference
	Bacopa monnieri extract.	*In vitro*	Aβ model in cortical neurons.	[113]
	Dioscorea opposita.	*In vitro* *In vivo*	H_2O_2 or glutamate model in cortical neurons. Scopolamine mice model.	[106]
	Nobiletin, flavonoid from citrus peels.	*In vitro* *In vivo*	H_2O_2 model in PC12 cells. Rat artery occlusion model.	[95]
	Opuntia dillenii.	*In vitro* *In vivo*	NMDA model in cortical neurons. Rat artery occlusion model.	[169]
	Pelargonidin (Figure 1(o)).	*In vivo*	Ethylcholine aziridinium ion model (AF64A).	[133]
	Psoralea corylifolia.	*In vitro* *In vivo*	MPP^+ model in CHO cells and SK-N-SH cells. MPTP mice and rat model.	[161]
	Withania somnifera extract.	*In vitro* *In vivo*	MPP^+ model in CHO cells and SK-N-SH cells. MPTP mice and rat model.	[154]
	Paeonol from *Paeonia suffruticosa* or *Paeonia lactiflora.*	*In vitro* *In vivo*	MPP^+ model in PC12 cells. MPP^+ mice model.	[14]
	Pedicularioside A from *Buddleia lindleyana.*	*In vivo*	6-OHDA rat model.	[145]
Cell survival	*Silybum marianum.*	*In vivo*	MPTP mice model.	[172]
	Toki-to, mixed medicinal herbs.	*In vitro* *In vivo*	6-OHDA model in PC12 cells. 6-OHDA rat model.	[158]
	Urundeuvines A, B and C chalcones from *Myracrodruon urundeuva.*	*In vivo*	6-OHDA model in mesencephalic cells.	[147]
	Salvia miltiorrhiza.	*In vitro* *In vivo*	Cortical neurons overexpressing APP695. APP/PS1 transgenic mice.	[88]
	Centella asiatica extract.	*In vivo*	PSAPP mice.	[92]
	Ipomoea batatas PoirCv.	*In vitro*	Aβ model in PC12 cells.	[121]
	Mucuna pruriens.	*In vivo*	APP-SL 7-5 model in transgenic mice APP695.	[157]
	Valeriana officinalis extract.	*In vivo*	Tg2576 transgenic mice.	[138]
	Luteolin.	*In vitro*	LPS model in mesencephalic neuron-glia and microglia cells.	[143]
	Panax notoginseng.	*In vitro*	MPTP model in mesencephalic neuron.	[151]
	Piperine (Figure 2(d)).	*In vitro*	Rotenone model in SH-SY5Y cells.	[104]
	L-theanine, from green tea (Figure 3(b)).	*In vitro*	H_2O_2 model in SH-SY5Y cells.	[16]
	Tripterygium regelii methanolic extract.	*In vitro*	6-OHDA model in mesencephalic cells.	[140]

Table 1: Continued.

Effect	Plant compound/extract	Model	Disease/condition	Reference
Antioxidant	Biotransformed blueberry juice by *Serratia vaccinii bacteria.*	*In vitro*	H_2O_2 model in neuronal cell.	[61]
	Rosmarinus officinalis.	*In vitro* *In vivo*	H_2O_2 or rotenone model in SH-SY5Y cells. Dieldrin model in SN4741 cells. Aged rats.	[166]
	Centella asiatica extract.	*In vivo*	PSAPP mice.	[92]
	Chrysanthemum morifolium extract.	*In vitro*	MPP^+ model in SH-SY5Y cell.	[126]
	Ipomoea batatas PoirCv.	*In vitro*	Aβ model in PC12 cells.	[121]
	Gastrodia elata extract.	*In vitro*	MPP^+ model in SH-SY5Y cells.	[141]
	Nobiletin, flavonoid from citrus peels.	*In vitro* *In vivo*	H_2O_2 model in PC12 cells. Rat artery occlusion model.	[95]
	Opuntia dillenii.	*In vitro* *In vivo*	NMDA model in cortical neurons. Rat artery occlusion model.	[169]
	Methanolic extracts from *Salvia species.*	*In vitro*	Glutamate model in PC12 cells.	[62]
	Toki-to, mixed medicinal herbs.	*In vitro* *In vivo*	6-OHDA model in PC12 cells. 6-OHDA rat model.	[158]
	Tripterygium regelii methanolic extract.	*In vitro*	6-OHDA model in mesencephalic cells.	[140]
	Vincamine from *Vinca minor* (Figure 2(c)).	*In vivo*	Mice.	[80]
	Paeonol from *Paeonia suffruticosa* or *Paeonia lactiflora.*	*In vitro* *In vivo*	MPP^+ model in PC12 cells. MPP^+ mice model.	[14]
	Oxyresveratrol and resveratrol from *Smilacis chinae rhizome* (Figure 1(c)).	*In vivo*	d-galactose mice model.	[171]
	Rhus verniciflua extract.	*In vitro*	H_2O_2 model in PC12 cells.	[139]
	Salvia miltiorrhiza.	*In vitro* *In vivo*	Cortical neurons overexpressing APP695. APP/PS1 transgenic mice.	[88]
	Bacopa monnieri extract.	*In vivo*	Aβ model in cortical neurons.	[113]
	Buddleia lindleyana.	*In vivo*	6-OHDA rat model.	[145]
	Pelargonidin (Figure 1(o)).	*In vivo*	Ethylcholine aziridinium ion model (AF64A).	[133]
	Samjunghwan, multiherbal extract.	*In vivo*	Acute ischemic stroke model.	[107]

Table 1: Continued.

Effect	Plant compound/extract	Model	Disease/condition	Reference
Motor/cognitive improvement	*Silybum marianum.*	*In vivo*	MPTP mice model.	[172]
	Methanolic extracts from *species of Salvia.*	*In vitro*	Glutamate model in PC12 cells.	[62]
	Pedicularioside A from *Buddleia lindleyana.*	*In vivo*	6-OHDA rat model.	[145]
	Paeonol from *Paeonia suffruticosa* or *Paeonia lactiflora.*	*In vitro* *In vivo*	MPP^+ model in PC12 cells. MPP^+ mice model.	[14]
	Oxyresveratrol & resveratrol from *Smilacis chinae rhizome* (Figure 1(c)).	*In vivo*	d-galactose mice model.	[171]
	Magnolia officinalis.	*In vivo*	SAMP8 mice.	[81]
	Cistanche salsa.	*In vivo*	MPP^+ mice model.	[127]
	Polyphenolic compounds extracted from cocoa.	*In vivo*	Aged rats.	[76]
	Luteolin.	*In vitro*	LPS model in mesencephalic neuron-glia and microglia cells.	[143]
	Cassia obtusifolia.	*In vivo*	Scopolamine model. Transient cerebral hypoperfusion model.	[102]
	Dioscorea opposita.	*In vitro* *In vivo*	H_2O_2 or glutamate model in cortical neurons. Scopolamine mice model.	[106]
	Korean red ginseng.	*Clinical trials*	AD patients.	[122]
	Pelargonidin (Figures 1(d) and 1(e)).	*In vivo*	Ethylcholine aziridinium ion model (AF64A).	[133]
	Saussurea pulvinata.	*In vivo*	Aβ mice model.	[63]
	Toki-to, mixed medicinal herbs.	*In vitro* *In vivo*	6-OHDA model in PC12 cells. 6-OHDA rat model.	[158]
	Valeriana officinalis extract.	*In vivo*	Tg2576 transgenic mice.	[138]
	Piperine (Figure 2(d)).	*In vitro*	Rotenone model in SH-SY5Y cells.	[104]
	L-theanine, from green tea (Figure 3(b)).	*In vitro*	H_2O_2 model in SH-SY5Y cells.	[16]
Anti-inflammatory	*Zingiberis Rhizoma* hexane extract.	*In vitro*	LPS model in BV-2 microglia cells.	[69]
	Ficus religiosa leaf.	*In vitro*	LPS model in BV-2 microglia cells.	[68]
	Luteolin.	*In vitro*	LPS model in mesencephalic neuron-glia and microglia cells.	[143]
	Samjunghwan, multiherbal extract.	*In vivo*	Acute ischemic stroke model.	[107]
	Saussurea pulvinata.	*In vivo*	Aβ mice model.	[63]
	Silybum marianum.	*In vivo*	MPTP mice model.	[172]
	Rosmarinus officinalis.	*In vitro* *In vivo*	H_2O_2 or rotenone model in SH-SY5Y cells. Dieldrin model in SN4741 cells. Aged rats.	[166]
	Nobiletin, flavonoid from citrus peels.	*In vitro* *In vivo*	H_2O_2 model in PC12 cells. Rat artery occlusion model.	[95]
	Cassia obtusifolia.	*In vivo*	Scopolamine model. Transient cerebral hypoperfusion model.	[102]
	Methoxsalen from *Poncirus trifoliate.*	*In vivo*	Trimethyltin mice model.	[15]
	Pelargonidin (Figure 1(o)).	*In vivo*	Ethylcholine aziridinium ion model (AF64A).	[133]
	Tripterygium regelii methanolic extract.	*In vitro*	6-OHDA model in mesencephalic cells.	[140]

6-OHDA: 6-hydroxydopamine; Aβ: beta-peptide amyloid aggregation; LPS: lipopolysaccharide; MPP^+:1-methyl-4-phenylpyridinium; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDA: *N*-methyl-D-aspartate.

Abbreviations

AChE: Acetyl cholinesterase
AD: Alzheimer's disease
Aβ: Amyloid beta-peptide
ALS: Amyotrophic lateral sclerosis
Bax: Apoptosis regulator
Bcl-2: B cell lymphoma 2; family of regulator proteins of apoptosis
BDNF: Brain-derived neurotrophic factor
CNS: Central nervous system
CAMP: Cyclic adenosine monophosphate
DNA: Deoxyribonucleic acid
ERK: Extracellular signal-regulated kinase
GSH: Glutathione reduced
H_2O_2: Hydrogen peroxide
IL-1β: Interleukin 1β
IL-6: Interleukin 6
iNOS: Inducible nitric oxide synthase
LPS: Lipopolysaccharide
MAPK: Mitogen-activated protein kinase
MEK: Mitogen/extracellular signal-regulated kinase
MPP^+: 1-Methyl-4-phenylpyridinium
MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS: Multiple sclerosis
NADPH: Reduced form of nicotinamide adenine dinucleotide phosphate
NO: Nitric oxide
NMDA: N-Methyl-D-aspartate
ND: Neurodegenerative diseases
NF-κB: Nuclear factor-κB
Nrf2: Nuclear factor (erythroid-derived 2)-like 2
NOS: Nitric oxide synthase
6-OHDA: 6-Hydroxydopamine-intoxicated mice
PC12: Cell line derived from a pheochromocytoma of the rat adrenal medulla
PD: Parkinson's disease
PN: Panax notoginseng
ROS: Reactive oxygen species
SAC: S-Allyl cysteine
Sjh: Sanmjuanhwan
SCR: Smilacis chinae rhizome
SOD1: Superoxide dismutase 1
TKT: Toki To
TNFα: Tumor necrosis factor α.

Conflict of Interests

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

Authors' Contribution

Jesús Pérez-Hernández and Víctor Javier Zaldívar-Machorro contributed equally to this paper.

Acknowledgments

Funding for this research was provided by the Medical School and grants from Dirección General de Asuntos del Personal Académico (DGAPA; IN217612 and IN222215), National Autonomous University of Mexico (UNAM). Jesús Pérez-Hernández and David Villanueva-Porras are recipient of a fellowship sponsored by the Consejo Nacional de Ciencia y Tecnología (CONACyT) and Víctor Javier Zaldívar-Machorro is recipient of a postdoctoral fellowship sponsored by DGAPA, UNAM.

References

[1] R. Fischer and O. Maier, "Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 610813, 18 pages, 2015.

[2] V. Procaccio, C. Bris, J. M. Chao de la Barca et al., "Perspectives of drug-based neuroprotection targeting mitochondria," *Revue Neurologique*, vol. 170, no. 5, pp. 390–400, 2014.

[3] M. Talarowska, K. Bobińska, M. Zajaczkowska, K.-P. Su, M. Maes, and P. Gałecki, "Impact of oxidative/nitrosative stress and inflammation on cognitive functions in patients with recurrent depressive disorders," *Medical Science Monitor*, vol. 20, pp. 110–115, 2014.

[4] C. Ballard, S. Gauthier, A. Corbett, C. Brayne, D. Aarsland, and E. Jones, "Alzheimer's disease," *The Lancet*, vol. 377, no. 9770, pp. 1019–1031, 2011.

[5] T. B. Joseph, S. W. J. Wang, X. Liu et al., "Disposition of flavonoids via enteric recycling: enzyme stability affects characterization of prunetin glucuronidation across species, organs, and UGT isoforms," *Molecular Pharmaceutics*, vol. 4, no. 6, pp. 883–894, 2007.

[6] S. Ramos, "Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention," *Journal of Nutritional Biochemistry*, vol. 18, no. 7, pp. 427–442, 2007.

[7] G. Chiva-Blanch, M. Urpi-Sarda, R. Llorach et al., "Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial," *The American Journal of Clinical Nutrition*, vol. 95, no. 2, pp. 326–334, 2012.

[8] S. A. Rieder, P. Nagarkatti, and M. Nagarkatti, "Multiple anti-inflammatory pathways triggered by resveratrol lead to amelioration of staphylococcal enterotoxin B-induced lung injury," *British Journal of Pharmacology*, vol. 167, no. 6, pp. 1244–1258, 2012.

[9] J. W. Gatson, M.-M. Liu, K. Abdelfattah et al., "Resveratrol decreases inflammation in the brain of mice with mild traumatic brain injury," *Journal of Trauma and Acute Care Surgery*, vol. 74, no. 2, pp. 470–475, 2013.

[10] C. A. Rice-Evans, N. J. Miller, and G. Paganga, "Structure-antioxidant activity relationships of flavonoids and phenolic acids," *Free Radical Biology and Medicine*, vol. 20, no. 7, pp. 933–956, 1996.

[11] P. Pignatelli, A. Ghiselli, B. Buchetti et al., "Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine," *Atherosclerosis*, vol. 188, no. 1, pp. 77–83, 2006.

[12] B. Wright, L. A. Moraes, C. F. Kemp et al., "A structural basis for the inhibition of collagen-stimulated platelet function by quercetin and structurally related flavonoids," *British Journal of Pharmacology*, vol. 159, no. 6, pp. 1312–1325, 2010.

[13] K. A. Jacobson, S. Moro, J. A. Manthey, P. L. West, and X.-D. Ji, "Interactions of flavones and other phytochemicals with adenosine receptors," *Advances in Experimental Medicine and Biology*, vol. 505, pp. 163–171, 2002.

[14] B. Pawlikowska-Pawlega, W. Ignacy Gruszecki, L. Misiak et al., "Modification of membranes by quercetin, a naturally occurring flavonoid, via its incorporation in the polar head group," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1768, no. 9, pp. 2195–2204, 2007.

[15] S.-Z. Zhong, Q.-H. Ge, R. Qu, Q. Li, and S.-P. Ma, "Paeonol attenuates neurotoxicity and ameliorates cognitive impairment induced by d-galactose in ICR mice," *Journal of the Neurological Sciences*, vol. 277, no. 1-2, pp. 58–64, 2009.

[16] J. K. Kim, H. Bae, M.-J. Kim et al., "Inhibitory effect of poncirus trifoliate on acetylcholinesterase and attenuating activity against trimethyltin-induced learning and memory impairment," *Bioscience, Biotechnology and Biochemistry*, vol. 73, no. 5, pp. 1105–1112, 2009.

[17] T. I. Kim, Y. K. Lee, S. G. Park et al., "l-Theanine, an amino acid in green tea, attenuates β-amyloid-induced cognitive dysfunction and neurotoxicity: reduction in oxidative damage and inactivation of ERK/p38 kinase and NF-κB pathways," *Free Radical Biology and Medicine*, vol. 47, no. 11, pp. 1601–1610, 2009.

[18] D. Y. Chuang, M.-H. Chan, Y. Zong et al., "Magnolia polyphenols attenuate oxidative and inflammatory responses in neurons and microglial cells," *Journal of Neuroinflammation*, vol. 10, article 15, 2013.

[19] K. Ono, Y. Yoshiike, A. Takashima, K. Hasegawa, H. Naiki, and M. Yamada, "Potent anti-amyloidogenic and fibril-destabilizing effects of polyphenols in vitro: implications for the prevention and therapeutics of Alzheimer's disease," *Journal of Neurochemistry*, vol. 87, no. 1, pp. 172–181, 2003.

[20] T. Sergent, N. Piront, J. Meurice, O. Toussaint, and Y.-J. Schneider, "Anti-inflammatory effects of dietary phenolic compounds in an in vitro model of inflamed human intestinal epithelium," *Chemico-Biological Interactions*, vol. 188, no. 3, pp. 659–667, 2010.

[21] J. R. Cannon and J. T. Greenamyre, "Neurotoxic *in vivo* models of Parkinson's disease: recent advances," in *Progress in Brain Research*, vol. 184, chapter 2, pp. 17–33, Elsevier, 2010.

[22] R. F. Haseloff, I. E. Blasig, H.-C. Bauer, and H. Bauer, "In search of the astrocytic factor(s) modulating blood-brain barrier functions in brain capillary endothelial cells in vitro," *Cellular and Molecular Neurobiology*, vol. 25, no. 1, pp. 25–39, 2005.

[23] B. V. Zlokovic, "The blood-brain barrier in health and chronic neurodegenerative disorders," *Neuron*, vol. 57, no. 2, pp. 178–201, 2008.

[24] K.-A. Hossmann, "Viability thresholds and the penumbra of focal ischemia," *Annals of Neurology*, vol. 36, no. 4, pp. 557–565, 1994.

[25] C. T. Drake and C. Iadecola, "The role of neuronal signaling in controlling cerebral blood flow," *Brain and Language*, vol. 102, no. 2, pp. 141–152, 2007.

[26] E. H. Lo, T. Dalkara, and M. A. Moskowitz, "Mechanisms, challenges and opportunities in stroke," *Nature Reviews Neuroscience*, vol. 4, no. 5, pp. 399–415, 2003.

[27] J. Lok, P. Gupta, S. Guo et al., "Cell-cell signaling in the neurovascular unit," *Neurochemical Research*, vol. 32, no. 12, pp. 2032–2045, 2007.

[28] T. R. Melzer, R. Watts, M. R. MacAskill et al., "Arterial spin labelling reveals an abnormal cerebral perfusion pattern in Parkinson's disease," *Brain*, vol. 134, no. 3, pp. 845–855, 2011.

[29] M. G. Spillantini, M. L. Schmidt, V. M.-Y. Lee, J. Q. Trojanowski, R. Jakes, and M. Goedert, "Alpha-synuclein in Lewy bodies," *Nature*, vol. 388, no. 6645, pp. 839–840, 1997.

[30] M. A. Burack, J. Hartlein, H. P. Flores, L. Taylor-Reinwald, J. S. Perlmutter, and N. J. Cairns, "In vivo amyloid imaging in autopsy-confirmed Parkinson disease with dementia," *Neurology*, vol. 74, no. 1, pp. 77–84, 2010.

[31] K. Iqbal and I. Grundke-Iqbal, "Alzheimer's disease, a multifactorial disorder seeking multitherapies," *Alzheimer's and Dementia*, vol. 6, no. 5, pp. 420–424, 2010.

[32] J. M. Anderson, D. W. Hampton, R. Patani et al., "Abnormally phosphorylated tau is associated with neuronal and axonal loss in experimental autoimmune encephalomyelitis and multiple sclerosis," *Brain*, vol. 131, no. 7, pp. 1736–1748, 2008.

[33] B. F. Shaw and J. S. Valentine, "How do ALS-associated mutations in superoxide dismutase 1 promote aggregation of the protein?" *Trends in Biochemical Sciences*, vol. 32, no. 2, pp. 78–85, 2007.

[34] B. Uttara, A. V. Singh, P. Zamboni, and R. T. Mahajan, "Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options," *Current Neuropharmacology*, vol. 7, no. 1, pp. 65–74, 2009.

[35] W. Dröge, "Free radicals in the physiological control of cell function," *Physiological Reviews*, vol. 82, no. 1, pp. 47–95, 2002.

[36] R. Von Bernhardi and J. Eugenín, "Alzheimer's disease: redox dysregulation as a common denominator for diverse pathogenic mechanisms," *Antioxidants and Redox Signaling*, vol. 16, no. 9, pp. 974–1031, 2012.

[37] B. Halliwell, "Oxidative stress and neurodegeneration: where are we now?" *Journal of Neurochemistry*, vol. 97, no. 6, pp. 1634–1658, 2006.

[38] A. Melo, L. Monteiro, R. M. F. Lima, D. M. de Oliveira, M. D. de Cerqueira, and R. S. El-Bachá, "Oxidative stress in neurodegenerative diseases: mechanisms and therapeutic perspectives," *Oxidative Medicine and Cellular Longevity*, vol. 2011, Article ID 467180, 14 pages, 2011.

[39] V. Chiurchiù and M. MacCarrone, "Chronic inflammatory disorders and their redox control: from molecular mechanisms to therapeutic opportunities," *Antioxidants and Redox Signaling*, vol. 15, no. 9, pp. 2605–2641, 2011.

[40] I.-T. Lee and C.-M. Yang, "Role of NADPH oxidase/ROS in pro-inflammatory mediators-induced airway and pulmonary diseases," *Biochemical Pharmacology*, vol. 84, no. 5, pp. 581–590, 2012.

[41] S. DiMauro and E. A. Schon, "Mitochondrial disorders in the nervous system," *Annual Review of Neuroscience*, vol. 31, pp. 91–123, 2008.

[42] J. W. Lustbader, M. Cirilli, C. Lin et al., "ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease," *Science*, vol. 304, no. 5669, pp. 448–452, 2004.

[43] C. Caspersen, N. Wang, J. Yao et al., "Mitochondrial Aβ: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease," *The FASEB Journal*, vol. 19, no. 14, pp. 2040–2041, 2005.

[44] M. Manczak, T. S. Anekonda, E. Henson, B. S. Park, J. Quinn, and P. H. Reddy, "Mitochondria are a direct site of Aβ accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression," *Human Molecular Genetics*, vol. 15, no. 9, pp. 1437–1449, 2006.

[45] W. D. Parker Jr., S. J. Boyson, and J. K. Parks, "Abnormalities of the electron transport chain in idiopathic Parkinson's disease," *Annals of Neurology*, vol. 26, no. 6, pp. 719–723, 1989.

[46] P. Shi, J. Gal, D. M. Kwinter, X. Liu, and H. Zhu, "Mitochondrial dysfunction in amyotrophic lateral sclerosis," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1802, no. 1, pp. 45–51, 2010.

[47] W. J. Streit and C. A. Kincaid-Colton, "The brain's immune system," *Scientific American*, vol. 273, no. 5, pp. 54–61, 1995.

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

[49] E. G. McGeer and P. L. McGeer, "The role of anti-inflammatory agents in Parkinson's disease," *CNS Drugs*, vol. 21, no. 10, pp. 789–797, 2007.

[50] P. Eikelenboom, C. Bate, W. A. Van Gool et al., "Neuroinflammation in Alzheimer's disease and prion disease," *Glia*, vol. 40, no. 2, pp. 232–239, 2002.

[51] P. Sanders and J. De Keyser, "Janus faces of microglia in multiple sclerosis," *Brain Research Reviews*, vol. 54, no. 2, pp. 274–285, 2007.

[52] R. M. Locksley, N. Killeen, and M. J. Lenardo, "The TNF and TNF receptor superfamilies: integrating mammalian biology," *Cell*, vol. 104, no. 4, pp. 487–501, 2001.

[53] R. E. Huie and S. Padmaja, "The reaction of no with superoxide," *Free Radical Research*, vol. 18, no. 4, pp. 195–199, 1993.

[54] E. Skrzydlewska, J. Ostrowska, R. Farbiszewski, and K. Michalak, "Protective effect of green tea against lipid peroxidation in the rat liver, blood serum and the brain," *Phytomedicine*, vol. 9, no. 3, pp. 232–238, 2002.

[55] T. Yokozawa, T. Nakagawa, and K. Kitani, "Antioxidative activity of green tea polyphenol in cholesterol-fed rats," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 12, pp. 3549–3552, 2002.

[56] H. Negishi, J.-W. Xu, K. Ikeda, M. Njelekela, Y. Nara, and Y. Yamori, "Black and green tea polyphenols attenuate blood pressure increases in stroke-prone spontaneously hypertensive rats," *Journal of Nutrition*, vol. 134, no. 1, pp. 38–42, 2004.

[57] A. C. Mello-Filho and R. Meneghini, "Iron is the intracellular metal involved in the production of DNA damage by oxygen radicals," *Mutation Research*, vol. 251, no. 1, pp. 109–113, 1991.

[58] M. Bhattacharya, P. Ponka, P. Hardy et al., "Prevention of postasphyxia electroretinal dysfunction with a pyridoxal hydrazone," *Free Radical Biology and Medicine*, vol. 22, no. 1-2, pp. 11–16, 1997.

[59] Ling Dong Kong, C. H. K. Cheng, and Ren Xiang Tan, "Monoamine oxidase inhibitors from rhizoma of *Coptis chinensis*," *Planta Medica*, vol. 67, no. 1, pp. 74–76, 2001.

[60] E. D. Owuor and A.-N. T. Kong, "Antioxidants and oxidants regulated signal transduction pathways," *Biochemical Pharmacology*, vol. 64, no. 5-6, pp. 765–770, 2002.

[61] A. Scalbert, I. T. Johnson, and M. Saltmarsh, "Polyphenols: antioxidants and beyond," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, supplement, pp. 215S–217S, 2005.

[62] T. Vuong, C. Matar, C. Ramassamy, and P. S. Haddad, "Biotransformed blueberry juice protects neurons from hydrogen peroxide-induced oxidative stress and mitogen-activated protein kinase pathway alterations," *British Journal of Nutrition*, vol. 104, no. 5, pp. 656–663, 2010.

[63] S. Asadi, A. Ahmadiani, M. A. Esmaeili, A. Sonboli, N. Ansari, and F. Khodagholi, "In vitro antioxidant activities and an investigation of neuroprotection by six *Salvia* species from Iran: a comparative study," *Food and Chemical Toxicology*, vol. 48, no. 5, pp. 1341–1349, 2010.

[64] C.-J. Wang, C.-P. Hu, K.-P. Xu et al., "Protective effect of selaginellin on glutamate-induced cytotoxicity and apoptosis in differentiated PC12 cells," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 381, no. 1, pp. 73–81, 2010.

[65] N. Mascolo, R. Jain, S. C. Jain, and F. Capasso, "Ethnopharmacologic investigation of ginger (*Zingiber officinale*)," *Journal of Ethnopharmacology*, vol. 27, no. 1-2, pp. 129–140, 1989.

[66] R. Grzanna, L. Lindmark, and C. G. Frondoza, "Ginger—an herbal medicinal product with broad anti-inflammatory actions," *Journal of Medicinal Food*, vol. 8, no. 2, pp. 125–132, 2005.

[67] R. C. Lantz, G. J. Chen, M. Sarihan, A. M. Sólyom, S. D. Jolad, and B. N. Timmermann, "The effect of extracts from ginger rhizome on inflammatory mediator production," *Phytomedicine*, vol. 14, no. 2-3, pp. 123–128, 2007.

[68] Y. Masuda, H. Kikuzaki, M. Hisamoto, and N. Nakatani, "Antioxidant properties of gingerol related compounds from ginger," *BioFactors*, vol. 21, no. 1–4, pp. 293–296, 2004.

[69] H. W. Jung, H. Y. Son, C. Van Minn, Y. H. Kim, and Y.-K. Park, "Methanol extract of ficus leaf inhibits the production of nitric oxide and proinflammatory cytokines in LPS-stimulated microglia via the MAPK pathway," *Phytotherapy Research*, vol. 22, no. 8, pp. 1064–1069, 2008.

[70] H. W. Jung, C.-H. Yoon, K. M. Park, H. S. Han, and Y.-K. Park, "Hexane fraction of Zingiberis Rhizoma Crudus extract inhibits the production of nitric oxide and proinflammatory cytokines in LPS-stimulated BV2 microglial cells via the NF-kappaB pathway," *Food and Chemical Toxicology*, vol. 47, no. 6, pp. 1190–1197, 2009.

[71] I. Häke, S. Schönenberger, J. Neuman et al., "Neuroprotection and enhanced neurogenesis by extract from the tropical plant *Knema laurina* after inflammatory damage in living brain tissue," *Journal of Neuroimmunology*, vol. 206, no. 1-2, pp. 91–99, 2009.

[72] D. Davalos, J. Grutzendler, G. Yang et al., "ATP mediates rapid microglial response to local brain injury in vivo," *Nature Neuroscience*, vol. 8, no. 6, pp. 752–758, 2005.

[73] L. Fetler and S. Amigorena, "Brain under surveillance: the microglia patrol," *Science*, vol. 309, no. 5733, pp. 392–393, 2005.

[74] J. Pérez-H, C. Carrillo-S, E. García, G. Ruiz-Mar, R. Pérez-Tamayo, and A. Chavarría, "Neuroprotective effect of silymarin in a MPTP mouse model of Parkinson's disease," *Toxicology*, vol. 319, no. 1, pp. 38–43, 2014.

[75] D. Metodiewa and C. Kośka, "Reactive oxygen species and reactive nitrogen species: relevance to cyto(neuro)toxic events and neurologic disorders. An overview," *Neurotoxicity Research*, vol. 1, no. 3, pp. 197–233, 1999.

[76] G. E. Adamson, S. A. Lazarus, A. E. Mitchell et al., "HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 10, pp. 4184–4188, 1999.

[77] J.-F. Bisson, A. Nejdi, P. Rozan, S. Hidalgo, R. Lalonde, and M. Messaoudi, "Effects of long-term administration of a cocoa polyphenolic extract (Acticoa powder) on cognitive performances in aged rats," *British Journal of Nutrition*, vol. 100, no. 1, pp. 94–101, 2008.

[78] N. Sangeetha, S. Aranganathan, and N. Nalini, "Silibinin ameliorates oxidative stress induced aberrant crypt foci and lipid

peroxidation in 1, 2 dimethylhydrazine induced rat colon cancer," *Investigational New Drugs*, vol. 28, no. 3, pp. 225–233, 2010.

[79] F. Galhardi, K. Mesquita, J. M. Monserrat, and D. M. Barros, "Effect of silymarin on biochemical parameters of oxidative stress in aged and young rat brain," *Food and Chemical Toxicology*, vol. 47, no. 10, pp. 2655–2660, 2009.

[80] M. Miyamoto, T. H. Murphy, R. L. Schnaar, and J. T. Coyle, "Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line," *Journal of Pharmacology and Experimental Therapeutics*, vol. 250, no. 3, pp. 1132–1140, 1989.

[81] A.-H. A. Fayed, "Brain trace element concentration of rats treated with the plant alkaloid, Vincamine," *Biological Trace Element Research*, vol. 136, no. 3, pp. 314–319, 2010.

[82] N. Matsui, K. Takahashi, M. Takeichi et al., "Magnolol and honokiol prevent learning and memory impairment and cholinergic deficit in SAMP8 mice," *Brain Research*, vol. 1305, pp. 108–117, 2009.

[83] N. C. Berchtold and C. W. Cotman, "Evolution in the conceptualization of dementia and Alzheimer's disease: Greco-Roman period to the 1960s," *Neurobiology of Aging*, vol. 19, no. 3, pp. 173–189, 1998.

[84] G. Perry, A. D. Cash, and M. A. Smith, "Alzheimer disease and oxidative stress," *Journal of Biomedicine and Biotechnology*, vol. 2, no. 3, pp. 120–123, 2002.

[85] S. Y. Kim, T. C. Moon, H. W. Chang, K. H. Son, S. S. Kang, and H. P. Kim, "Effects of tanshinone I isolated from *Salvia miltiorrhiza* Bunge on arachidonic acid metabolism and in vivo inflammatory responses," *Phytotherapy Research*, vol. 16, no. 7, pp. 616–620, 2002.

[86] E.-J. Park, Y.-Z. Zhao, Y.-C. Kim, and D. H. Sohn, "PF2401-SF, standardized fraction of *Salvia miltiorrhiza* and its constituents, tanshinone I, tanshinone IIA, and cryptotanshinone, protect primary cultured rat hepatocytes from bile acid-induced apoptosis by inhibiting JNK phosphorylation," *Food and Chemical Toxicology*, vol. 45, no. 10, pp. 1891–1898, 2007.

[87] D. H. Kim, S. J. Jeon, J. W. Jung et al., "Tanshinone congeners improve memory impairments induced by scopolamine on passive avoidance tasks in mice," *European Journal of Pharmacology*, vol. 574, no. 2-3, pp. 140–147, 2007.

[88] J. D. Adams, R. Wang, J. Yang, and E. J. Lien, "Preclinical and clinical examinations of *Salvia miltiorrhiza* and its tanshinones in ischemic conditions," *Chinese Medicine*, vol. 1, article 3, 2006.

[89] Z. Mei, F. Zhang, L. Tao et al., "Cryptotanshinone, a compound from Salvia miltiorrhiza modulates amyloid precursor protein metabolism and attenuates β-amyloid deposition through upregulating α-secretase in vivo and in vitro," *Neuroscience Letters*, vol. 452, no. 2, pp. 90–95, 2009.

[90] N. Murata, K. Murakami, Y. Ozawa et al., "Silymarin attenuated the amyloid beta plaque burden and improved behavioral abnormalities in an Alzheimer's disease mouse model," *Bioscience, Biotechnology and Biochemistry*, vol. 74, no. 11, pp. 2299–2306, 2010.

[91] Y. K. Gupta, M. H. V. Kumar, and A. K. Srivastava, "Effect of *Centella asiatica* on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats," *Pharmacology Biochemistry and Behavior*, vol. 74, no. 3, pp. 579–585, 2003.

[92] M. H. Veerendra Kumar and Y. K. Gupta, "Effect of different extracts of *Centella asiatica* on cognition and markers of oxidative stress in rats," *Journal of Ethnopharmacology*, vol. 79, no. 2, pp. 253–260, 2002.

[93] M. Dhanasekaran, L. A. Holcomb, A. R. Hitt et al., "*Centella asiatica* extract selectively decreases amyloid β levels in hippocampus of Alzheimer's disease animal model," *Phytotherapy Research*, vol. 23, no. 1, pp. 14–19, 2009.

[94] J. Wang, L. Ho, W. Zhao et al., "Grape-derived polyphenolics prevent Aβ oligomerization and attenuate cognitive deterioration in a mouse model of Alzheimer's disease," *Journal of Neuroscience*, vol. 28, no. 25, pp. 6388–6392, 2008.

[95] A. Nakajima, T. Yamakuni, M. Haraguchi et al., "Nobiletin, a citrus flavonoid that improves memory impairment, rescues bulbectomy-induced cholinergic neurodegeneration in mice," *Journal of Pharmacological Sciences*, vol. 105, no. 1, pp. 122–126, 2007.

[96] H. Onozuka, A. Nakajima, K. Matsuzaki et al., "Nobiletin, a citrus flavonoid, improves memory impairment and Aβ pathology in a transgenic mouse model of Alzheimer's disease," *Journal of Pharmacology and Experimental Therapeutics*, vol. 326, no. 3, pp. 739–744, 2008.

[97] H. Nagase, N. Omae, A. Omori et al., "Nobiletin and its related flavonoids with CRE-dependent transcription-stimulating and neuritegenic activities," *Biochemical and Biophysical Research Communications*, vol. 337, no. 4, pp. 1330–1336, 2005.

[98] K. Matsuzaki, Kenichi 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.

[99] H. A. Jung, B.-S. Min, T. Yokozawa, J.-H. Lee, Y. S. Kim, and J. S. Choi, "Anti-Alzheimer and antioxidant activities of coptidis rhizoma alkaloids," *Biological and Pharmaceutical Bulletin*, vol. 32, no. 8, pp. 1433–1438, 2009.

[100] D. K. Kim, K. T. Lee, N.-I. Baek et al., "Acetylcholinesterase inhibitors from the aerial parts of *Corydalis speciosa*," *Archives of Pharmacal Research*, vol. 27, no. 11, pp. 1127–1131, 2004.

[101] C. H. Park, S.-H. Kim, W. Choi et al., "Novel anticholinesterase and antiamnesic activities of dehydroevodiamine, a constituent of *Evodia rutaecarpa*," *Planta Medica*, vol. 62, no. 5, pp. 405–409, 1996.

[102] H. M. Shaheen, M.-T. Hsieh, B. H. Ali et al., "The ameliorating effects of the cognitive-enhancing Chinese herbs on scopolamine-induced amnesia in rats," *Phytotherapy Research*, vol. 14, no. 5, pp. 375–377, 2000.

[103] D. H. Kim, B. H. Yoon, Y. W. Kim et al., "The seed extract of *Cassia obtusifolia* ameliorates learning and memory impairments induced by scopolamine or transient cerebral hypoperfusion in mice," *Journal of Pharmacological Sciences*, vol. 105, no. 1, pp. 82–93, 2007.

[104] A. Fischer and I. Hanin, "Potential animal models for senile dementia of Alzheimer's type, with emphasis on AF64A-induced cholinotoxicity," *Annual Review of Pharmacology and Toxicology*, vol. 26, pp. 161–181, 1986.

[105] P. Chonpathompikunlert, J. Wattanathorn, and S. Muchimapura, "Piperine, the main alkaloid of Thai black pepper, protects against neurodegeneration and cognitive impairment in animal model of cognitive deficit like condition of Alzheimer's disease," *Food and Chemical Toxicology*, vol. 48, no. 3, pp. 798–802, 2010.

[106] S. K. Gupta, P. Bansal, R. K. Bhardwaj, and T. Velpandian, "Comparative anti-nociceptive, anti-inflammatory and toxicity profile of nimesulide vs nimesulide and piperine combination," *Pharmacological Research*, vol. 41, no. 6, pp. 657–662, 2000.

[107] M. H. Yang, K. D. Yoon, Y.-W. Chin et al., "Neuroprotective effects of *Dioscorea opposita* on scopolamine-induced memory impairment in in vivo behavioral tests and in vitro assays," *Journal of Ethnopharmacology*, vol. 121, no. 1, pp. 130–134, 2009.

[108] H. G. Kim, M. S. Ju, H. Park et al., "Evaluation of Samjunghwan, a traditional medicine, for neuroprotection against damage by amyloid-beta in rat cortical neurons," *Journal of Ethnopharmacology*, vol. 130, no. 3, pp. 625–630, 2010.

[109] C.-C. Hou, S.-J. Lin, J.-T. Cheng, and F.-L. Hsu, "Bacopaside III, bacopasaponin G, and bacopasides A, B, and C from *Bacopa monniera*," *Journal of Natural Products*, vol. 65, no. 12, pp. 1759–1763, 2002.

[110] M. Deepak, G. K. Sangli, P. C. Arun, and A. Amit, "Quantitative determination of the major saponin mixture bacoside A in *Bacopa monnieri* by HPLC," *Phytochemical Analysis*, vol. 16, no. 1, pp. 24–29, 2005.

[111] K. Sairam, M. Dorababu, R. K. Goel, and S. K. Bhattacharya, "Antidepressant activity of standardized extract of *Bacopa monniera* in experimental models of depression in rats," *Phytomedicine*, vol. 9, no. 3, pp. 207–211, 2002.

[112] S. Channa, A. Dar, S. Anjum, M. Yaqoob, and Atta-ur-Rahman, "Anti-inflammatory activity of *Bacopa monniera* in rodents," *Journal of Ethnopharmacology*, vol. 104, no. 1-2, pp. 286–289, 2006.

[113] P. K. Chaudhuri, R. Srivastava, S. Kumar, and S. Kumar, "Phytotoxic and antimicrobial constituents of *Bacopa monnieri* and *Holmskioldia sanguinea*," *Phytotherapy Research*, vol. 18, no. 2, pp. 114–117, 2004.

[114] N. Limpeanchob, S. Jaipan, S. Rattanakaruna, W. Phrompittayarat, and K. Ingkaninan, "Neuroprotective effect of *Bacopa monnieri* on beta-amyloid-induced cell death in primary cortical culture," *Journal of Ethnopharmacology*, vol. 120, no. 1, pp. 112–117, 2008.

[115] C. Stough, J. Lloyd, J. Clarke et al., "The chronic effects of an extract of *Bacopa monniera* (Brahmi) on cognitive function in healthy human subjects," *Psychopharmacology*, vol. 156, no. 4, pp. 481–484, 2001.

[116] A. Das, G. Shanker, C. Nath, R. Pal, S. Singh, and H. K. Singh, "A comparative study in rodents of standardized extracts of *Bacopa monniera* and *Ginkgo biloba*: anticholinesterase and cognitive enhancing activities," *Pharmacology Biochemistry and Behavior*, vol. 73, no. 4, pp. 893–900, 2002.

[117] S. Roodenrys, D. Booth, S. Bulzomi, A. Phipps, C. Micallef, and J. Smoker, "Chronic effects of Brahmi (*Bacopa monnieri*) on human memory," *Neuropsychopharmacology*, vol. 27, no. 2, pp. 279–281, 2002.

[118] F. Pérez-Severiano, R. Salvatierra-Sánchez, M. Rodríguez-Pérez et al., "S-Allylcysteine prevents amyloid-beta peptide-induced oxidative stress in rat hippocampus and ameliorates learning deficits," *European Journal of Pharmacology*, vol. 489, no. 3, pp. 197–202, 2004.

[119] H. Javed, M. M. Khan, A. Khan et al., "S-allyl cysteine attenuates oxidative stress associated cognitive impairment and neurodegeneration in mouse model of streptozotocin-induced experimental dementia of Alzheimer's type," *Brain Research*, vol. 1389, pp. 133–142, 2011.

[120] M. Philpott, K. S. Gould, C. Lim, and L. R. Ferguson, "*In situ* and *in vitro* antioxidant activity of sweetpotato anthocyanins," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 6, pp. 1511–1513, 2004.

[121] L. E. Steed and V.-D. Truong, "Anthocyanin content, antioxidant activity, and selected physical properties of flowable purple-fleshed sweetpotato purees," *Journal of Food Science*, vol. 73, no. 5, pp. S215–S221, 2008.

[122] J. Ye, X. Meng, C. Yan, and C. Wang, "Effect of purple sweet potato anthocyanins on β-amyloid-mediated PC-12 cells death by inhibition of oxidative stress," *Neurochemical Research*, vol. 35, no. 3, pp. 357–365, 2010.

[123] J.-H. Heo, S.-T. Lee, K. Chu et al., "An open-label trial of Korean red ginseng as an adjuvant treatment for cognitive impairment in patients with Alzheimer's disease," *European Journal of Neurology*, vol. 15, no. 8, pp. 865–868, 2008.

[124] G. Fiskum, A. Starkov, B. M. Polster, and C. Chinopoulos, "Mitochondrial mechanisms of neural cell death and neuroprotective interventions in Parkinson's disease," *Annals of the New York Academy of Sciences*, vol. 991, pp. 111–119, 2003.

[125] Y. Levites, O. Weinreb, G. Maor, M. B. H. Youdim, and S. Mandel, "Green tea polyphenol (−)-epigallocatechin-3-gallate prevents *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurodegeneration," *Journal of Neurochemistry*, vol. 78, no. 5, pp. 1073–1082, 2001.

[126] J. Van Kampen, H. Robertson, T. Hagg, and R. Drobitch, "Neuroprotective actions of the ginseng extract G115 in two rodent models of Parkinson's disease," *Experimental Neurology*, vol. 184, no. 1, pp. 521–529, 2003.

[127] I. S. Kim, S. Koppula, P.-J. Park et al., "*Chrysanthemum morifolium* Ramat (CM) extract protects human neuroblastoma SH-SY5Y cells against MPP+-induced cytotoxicity," *Journal of Ethnopharmacology*, vol. 126, no. 3, pp. 447–454, 2009.

[128] X. Geng, X. Tian, P. Tu, and X. Pu, "Neuroprotective effects of echinacoside in the mouse MPTP model of Parkinson's disease," *European Journal of Pharmacology*, vol. 564, no. 1–3, pp. 66–74, 2007.

[129] M. Geed, D. Garabadu, A. Ahmad, and S. Krishnamurthy, "Silibinin pretreatment attenuates biochemical and behavioral changes induced by intrastriatal MPP^+ injection in rats," *Pharmacology Biochemistry and Behavior*, vol. 117, pp. 92–103, 2014.

[130] R. Haddadi, A. M. Nayebi, S. Farajniya, S. E. Brooshghalan, and H. Sharifi, "Silymarin improved 6-OHDA-induced motor impairment in hemi-parkisonian rats: behavioral and molecular study," *DARU Journal of Pharmaceutical Sciences*, vol. 22, no. 1, article 38, 2014.

[131] N. K. Singhal, G. Srivastava, D. K. Patel, S. K. Jain, and M. P. Singh, "Melatonin or silymarin reduces maneb- and paraquat-induced Parkinson's disease phenotype in the mouse," *Journal of Pineal Research*, vol. 50, no. 2, pp. 97–109, 2011.

[132] S. K. Abraham, N. Schupp, U. Schmid, and H. Stopper, "Antigenotoxic effects of the phytoestrogen pelargonidin chloride and the polyphenol chlorogenic acid," *Molecular Nutrition and Food Research*, vol. 51, no. 7, pp. 880–887, 2007.

[133] D. Vauzour, G. Ravaioli, K. Vafeiadou, A. Rodriguez-Mateos, C. Angeloni, and J. P. E. Spencer, "Peroxynitrite induced formation of the neurotoxins 5-S-cysteinyl-dopamine and DHBT-1: implications for Parkinson's disease and protection by polyphenols," *Archives of Biochemistry and Biophysics*, vol. 476, no. 2, pp. 145–151, 2008.

[134] M. Roghani, A. Niknam, M.-R. Jalali-Nadoushan, Z. Kiasalari, M. Khalili, and T. Baluchnejadmojarad, "Oral pelargonidin exerts dose-dependent neuroprotection in 6-hydroxydopamine rat model of hemi-parkinsonism," *Brain Research Bulletin*, vol. 82, no. 5-6, pp. 279–283, 2010.

[135] E. Garcia, D. Limon, V. Perez-De La Cruz et al., "Lipid peroxidation, mitochondrial dysfunction and neurochemical and

behavioural deficits in different neurotoxic models: protective role of S-allylcysteine," *Free Radical Research*, vol. 42, no. 10, pp. 892–902, 2008.

[136] E. García, J. Villeda-Hernández, J. Pedraza-Chaverrí, P. D. Maldonado, and A. Santamaría, "S-allylcysteine reduces the MPTP-induced striatal cell damage via inhibition of pro-inflammatory cytokine tumor necrosis factor-α and inducible nitric oxide synthase expressions in mice," *Phytomedicine*, vol. 18, no. 1, pp. 65–73, 2010.

[137] E. García, R. Santana-Martínez, C. A. Silva-Islas et al., "S-allyl cysteine protects against MPTP-induced striatal and nigral oxidative neurotoxicity in mice: participation of Nrf2," *Free Radical Research*, vol. 48, no. 2, pp. 159–167, 2014.

[138] D. V. G. de Andrade, D. M. de Oliveria, G. Barreto et al., "Effects of the extract of *Anemopaegma mirandum* (Catuaba) on Rotenone-induced apoptosis in human neuroblastomas SH-SY5Y cells," *Brain Research*, vol. 1198, pp. 188–196, 2008.

[139] D. M. de Oliveria, G. Barreto, D. V. G. de Andrade et al., "Cytoprotective effect of valeriana officinalis extract on an in vitro experimental model of parkinson disease," *Neurochemical Research*, vol. 34, no. 2, pp. 215–220, 2009.

[140] K. Sapkota, S. Kim, S.-E. Park, and S.-J. Kim, "Detoxified extract of *Rhus verniciflua* stokes inhibits rotenone-induced apoptosis in human dopaminergic cells, SH-SY5Y," *Cellular and Molecular Neurobiology*, vol. 31, no. 2, pp. 213–223, 2011.

[141] B.-S. Choi, K. Sapkota, S. Kim, H. J. Lee, H.-S. Choi, and S.-J. Kim, "Antioxidant activity and protective effects of *Tripterygium regelii* extract on hydrogen peroxide-induced injury in human dopaminergic cells, SH-SY5Y," *Neurochemical Research*, vol. 35, no. 8, pp. 1269–1280, 2010.

[142] H. An, I. S. Kim, S. Koppula et al., "Protective effects of *Gastrodia elata* Blume on MPP+-induced cytotoxicity in human dopaminergic SH-SY5Y cells," *Journal of Ethnopharmacology*, vol. 130, no. 2, pp. 290–298, 2010.

[143] S. S. Kang, J. Y. Lee, Y. K. Choi, G. S. Kim, and B. H. Han, "Neuroprotective effects of flavones on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblostoma cells," *Bioorganic and Medicinal Chemistry Letters*, vol. 14, no. 9, pp. 2261–2264, 2004.

[144] H.-Q. Chen, Z.-Y. Jin, X.-J. Wang, X.-M. Xu, L. Deng, and J.-W. Zhao, "Luteolin protects dopaminergic neurons from inflammation-induced injury through inhibition of microglial activation," *Neuroscience Letters*, vol. 448, no. 2, pp. 175–179, 2008.

[145] P. Wang, J. Kang, R. Zheng et al., "Scavenging effects of phenylpropanoid glycosides from Pedicularis on superoxide anion and hydroxyl radical by the spin trapping method(95)02255-4," *Biochemical Pharmacology*, vol. 51, no. 5, pp. 687–691, 1996.

[146] Y.-Y. Li, J.-H. Lu, Q. Li, Y.-Y. Zhao, and X.-P. Pu, "Pedicularioside A from *Buddleia lindleyana* inhibits cell death induced by 1-methyl-4-phenylpyridinium ions (MPP^+) in primary cultures of rat mesencephalic neurons," *European Journal of Pharmacology*, vol. 579, no. 1–3, pp. 134–140, 2008.

[147] J. S. Shim, H. G. Kim, M. S. Ju, J. G. Choi, S. Y. Jeong, and M. S. Oh, "Effects of the hook of *Uncaria rhynchophylla* on neurotoxicity in the 6-hydroxydopamine model of Parkinson's disease," *Journal of Ethnopharmacology*, vol. 126, no. 2, pp. 361–365, 2009.

[148] H. V. Nobre-Júnior, R. A. Oliveira, F. D. Maia et al., "Neuroprotective effects of chalcones from *Myracrodruon urundeuva* on 6-hydroxydopamine-induced cytotoxicity in rat mesencephalic cells," *Neurochemical Research*, vol. 34, no. 6, pp. 1066–1075, 2009.

[149] Y. Wang, Y. Feng, Q. Fu, and L. Li, "Panax notoginsenoside Rb1 ameliorates Alzheimer's disease by upregulating brain-derived neurotrophic factor and downregulating Tau protein expression," *Experimental and Therapeutic Medicine*, vol. 6, no. 3, pp. 826–830, 2013.

[150] S. Y. Cheon, K. J. Cho, J. E. Lee et al., "Cerebroprotective effects of red ginseng extract pretreatment against ischemia-induced oxidative stress and apoptosis," *International Journal of Neuroscience*, vol. 123, no. 4, pp. 269–277, 2013.

[151] J. Bai, H. Nakamura, Y.-W. Kwon et al., "Critical roles of thioredoxin in nerve growth factor-mediated signal transduction and neurite outgrowth in PC12 cells," *The Journal of Neuroscience*, vol. 23, no. 2, pp. 503–509, 2003.

[152] F.-C. Luo, S.-D. Wang, K. Li, H. Nakamura, J. Yodoi, and J. Bai, "Panaxatriol saponins extracted from *Panax notoginseng* induces thioredoxin-1 and prevents 1-methyl-4-phenylpyridinium ion-induced neurotoxicity," *Journal of Ethnopharmacology*, vol. 127, no. 2, pp. 419–423, 2010.

[153] T. Kuboyama, C. Tohda, J. Zhao, N. Nakamura, M. Hattori, and K. Komatsu, "Axon- or dendrite-predominant outgrowth induced by constituents from Ashwagandha," *NeuroReport*, vol. 13, no. 14, pp. 1715–1720, 2002.

[154] C. Tohda, T. Kuboyama, and K. Komatsu, "Search for natural products related to regeneration of the neuronal network," *NeuroSignals*, vol. 14, no. 1-2, pp. 34–45, 2005.

[155] S. RajaSankar, T. Manivasagam, and S. Surendran, "Ashwagandha leaf extract: a potential agent in treating oxidative damage and physiological abnormalities seen in a mouse model of Parkinson's disease," *Neuroscience Letters*, vol. 454, no. 1, pp. 11–15, 2009.

[156] C.-M. Lin, R.-D. Lin, S.-T. Chen et al., "Neurocytoprotective effects of the bioactive constituents of *Pueraria thomsonii* in 6-hydroxydopamine (6-OHDA)-treated nerve growth factor (NGF)-differentiated PC12 cells," *Phytochemistry*, vol. 71, no. 17-18, pp. 2147–2156, 2010.

[157] T. Baluchnejadmojarad, M. Roghani, M. R. J. Nadoushan, and M. Bagheri, "Neuroprotective effect of genistein in 6-hydroxydopamine hemi-parkinsonian rat model," *Phytotherapy Research*, vol. 23, no. 1, pp. 132–135, 2009.

[158] B. V. Manyam, M. Dhanasekaran, and T. A. Hare, "Neuroprotective effects of the antiparkinson drug *Mucuna pruriens*," *Phytotherapy Research*, vol. 18, no. 9, pp. 706–712, 2004.

[159] R. Sakai, Y. Irie, T. Murata, A. Ishige, N. Anjiki, and K. Watanabe, "Toki-to protects dopaminergic neurons in the substantia nigra from neurotoxicity of MPTP in mice," *Phytotherapy Research*, vol. 21, no. 9, pp. 868–873, 2007.

[160] Y. H. Choi, C. H. Yon, K. S. Hong et al., "In vitro BACE-1 inhibitory phenolic components from the seeds of *Psoralea corylifolia*," *Planta Medica*, vol. 74, no. 11, pp. 1405–1408, 2008.

[161] G. Zhao, S.-Y. Zang, X.-W. Zheng, X.-H. Zhang, and L.-H. Guo, "Bakuchiol analogs inhibit monoamine transporters and regulate monoaminergic functions," *Biochemical Pharmacology*, vol. 75, no. 9, pp. 1835–1847, 2008.

[162] G. Zhao, X.-W. Zheng, G.-W. Qin, Y. Gai, Z.-H. Jiang, and L.-H. Guo, "In vitro dopaminergic neuroprotective and in vivo antiparkinsonian-like effects of Δ^3,2-hydroxybakuchiol isolated from *Psoralea corylifolia* (L.)," *Cellular and Molecular Life Sciences*, vol. 66, no. 9, pp. 1617–1629, 2009.

[163] S. Cheung and J. Tai, "Anti-proliferative and antioxidant properties of rosemary *Rosmarinus officinalis*," *Oncology Reports*, vol. 17, no. 6, pp. 1525–1531, 2007.

[164] A.-H. Lo, Y.-C. Liang, S.-Y. Lin-Shiau, C.-T. Ho, and J.-K. Lin, "Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-κB in mouse macrophages," *Carcinogenesis*, vol. 23, no. 6, pp. 983–991, 2002.

[165] S.-J. Kim, J.-S. Kim, H.-S. Cho et al., "Carnosol, a component of rosemary (*Rosmarinus officinalis* L.) protects nigral dopaminergic neuronal cells," *NeuroReport*, vol. 17, no. 16, pp. 1729–1733, 2006.

[166] H. J. Lee, H.-S. Cho, E. Park et al., "Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis," *Toxicology*, vol. 250, no. 2-3, pp. 109–115, 2008.

[167] J. A. Park, S. Kim, S.-Y. Lee et al., "Beneficial effects of carnosic acid on dieldrin-induced dopaminergic neuronal cell death," *NeuroReport*, vol. 19, no. 13, pp. 1301–1304, 2008.

[168] S. J. Posadas, V. Caz, C. Largo et al., "Protective effect of supercritical fluid rosemary extract, *Rosmarinus officinalis*, on antioxidants of major organs of aged rats," *Experimental Gerontology*, vol. 44, no. 6-7, pp. 383–389, 2009.

[169] S.-E. Park, S. Kim, K. Sapkota, and S.-J. Kim, "Neuroprotective effect of *Rosmarinus officinalis* extract on human dopaminergic cell line, SH-SY5Y," *Cellular and Molecular Neurobiology*, vol. 30, no. 5, pp. 759–767, 2010.

[170] X. Huang, Q. Li, Y. Zhang et al., "Neuroprotective effects of cactus polysaccharide on oxygen and glucose deprivation induced damage in rat brain slices," *Cellular and Molecular Neurobiology*, vol. 28, no. 4, pp. 559–568, 2008.

[171] X. Huang, Q. Li, H. Li, and L. Guo, "Neuroprotective and antioxidative effect of cactus polysaccharides in vivo and in vitro," *Cellular and Molecular Neurobiology*, vol. 29, no. 8, pp. 1211–1221, 2009.

[172] Y. B. Ju, O. C. Soon, S.-H. Choi et al., "Neuroprotective effect of *Smilacis chinae* rhizome on NMDA-induced neurotoxicity in vitro and focal cerebral ischemia in vivo," *Journal of Pharmacological Sciences*, vol. 106, no. 1, pp. 68–77, 2008.

[173] Y.-C. Hou, K.-T. Liou, C.-M. Chern et al., "Preventive effect of silymarin in cerebral ischemia-reperfusion-induced brain injury in rats possibly through impairing NF-κB and STAT-1 activation," *Phytomedicine*, vol. 17, no. 12, pp. 963–973, 2010.

[174] Y. Numagami, S. Sato, and S. T. Ohnishi, "Attenuation of rat ischemic brain damage by aged garlic extracts: a possible protecting mechanism as antioxidants," *Neurochemistry International*, vol. 29, no. 2, pp. 135–143, 1996.

[175] Y. Numagami and S. T. Ohnishi, "S-allylcysteine inhibits free radical production, lipid peroxidation and neuronal damage in rat brain ischemia," *Journal of Nutrition*, vol. 131, no. 3, pp. 1100S–1105S, 2001.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3128951, 15 pages
http://dx.doi.org/10.1155/2016/3128951

Review Article

Role of Natural Stilbenes in the Prevention of Cancer

J. Antoni Sirerol, María L. Rodríguez, Salvador Mena, Miguel A. Asensi, José M. Estrela, and Angel L. Ortega

Department of Physiology, Faculty of Pharmacy, University of Valencia, Avenida Vicente Andrés Estellés s/n, Burjassot 46100, Valencia, Spain

Correspondence should be addressed to Angel L. Ortega; angel.ortega@uv.es

Received 3 July 2015; Revised 3 October 2015; Accepted 5 October 2015

Academic Editor: Zhenquan Jia

Natural stilbenes are an important group of nonflavonoid phytochemicals of polyphenolic structure characterized by the presence of a 1,2-diphenylethylene nucleus. Stilbenes have an extraordinary potential for the prevention and treatment of different diseases, including cancer, due to their antioxidant, cell death activation, and anti-inflammatory properties which associate with low toxicity under *in vivo* conditions. This review aims to discuss various approaches related to their mechanisms of action, pharmacological activities in animal models and humans, and potential chemoprevention in clinical studies. The biological activity of natural stilbenes is still incompletely understood. Furthermore, after administration to animals or humans, these molecules are rapidly metabolized. Thus pharmacokinetics and/or activities of the natural structures and their metabolites may be very different. Novel drug formulations have been postulated in order to improve stability and bioavailability, to minimize side effects, and to facilitate interaction with their domains in target proteins. These pharmacological improvements should lead stilbenes to become effective candidates as anticancer drugs.

1. Introduction

Despite the fact that the total European population comprises just one-ninth of the world's population, the percentage of the global burden of cancer in Europe is of approximately 25% [1]. Recent epidemiological research estimates that approx. 1,323,000 and 585,000 deaths were caused by cancer in the European Union and the United States, respectively, in 2014 [2, 3]. At the beginning of 21st century cancer was the second cause of death only preceded by cardiovascular diseases and followed by diseases derived from complications associated with diabetes and chronic respiratory diseases [4]. This tendency has been changing with time and nowadays cancer exceeds the cardiovascular diseases mortality rate in some advanced countries, possibly due to improvements in patient care, more effective therapies, and awareness of the population to acquire healthier life style [3, 5, 6]. In consequence, considerable attention has been focused on chemoprevention as an alternative approach to the control of cancer.

Multiple evidences suggest that oxidative stress induced by reactive oxygen species (ROS) is closely related to multistage carcinogenesis [7]. ROS are the most abundant free radicals in cells and have been related with a number of tissue/organ injuries. Oxidative stress is caused by an imbalance between ROS production and the biological system's ability to neutralize or remove ROS by specific scavengers and the antioxidant enzymatic machinery. Thus, oxidative stress can cause protein, lipid, and DNA damage and thereby modulate/trigger initiation, promotion, and progression of cancer [8]. In this sense, antioxidants are defined as compounds that can delay, inhibit, or prevent the oxidative damage by scavenging free radicals and diminishing oxidative stress [9].

During the last 20 years the interest in phytochemicals of polyphenolic structure has grown considerably. Natural polyphenols are plant secondary metabolites generated through the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), with two or more phenolic rings, and being devoid of any nitrogen-based functional group in their basic structure [10]. They are produced by plants to protect themselves against stressing situations such as excessive ultraviolet (UV) irradiation, heat exposition, insects attacks, and fungus or bacterial infections [10]. Over 8,000 different phenolic compounds have been identified in the plant kingdom. Natural polyphenols are abundant in fruits,

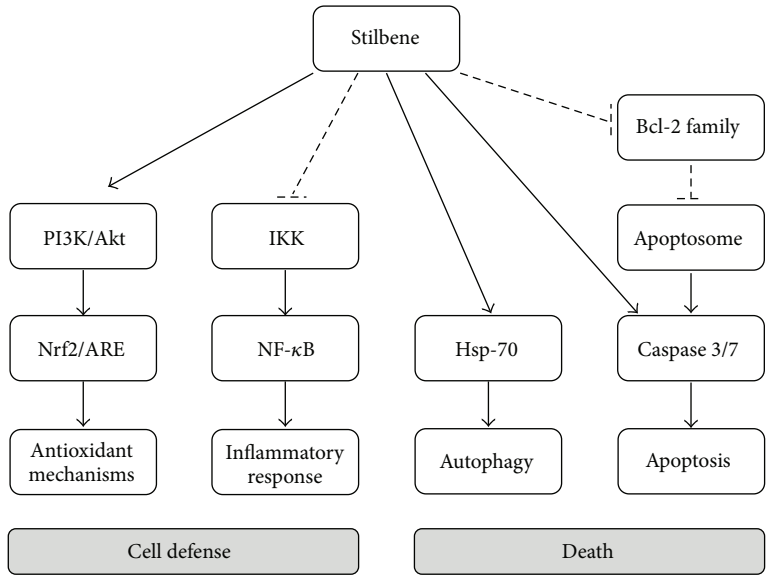


Figure 1: Anticarcinogenic mechanisms induced by major stilbenes.

vegetables, whole grains, and foods and beverages derived from them, such as chocolate, wine, olive oil, or tea, thus becoming the most important among all phytochemicals present in the human diet [11]. Natural polyphenols have received increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as cancer [12, 13]. Indeed, potential anticancer properties have been suggested for various polyphenols, including, for example, green tea polyphenols, grape seed proanthocyanidins, resveratrol, silymarin, curcumin, quercetin, luteolin, and genistein [14, 15]. Although the chemopreventive effects of natural polyphenols are mainly due to their antioxidant activity, mechanistic studies suggest that, in addition, they have multiple intracellular targets (Figure 1) [7].

Natural stilbenes are a group of polyphenols characterized by the presence of a 1,2-diphenylethylene nucleus [16, 17]. There are more than 400 natural stilbenes [16], however they are present in a limited and heterogeneous group of plant families since the key enzyme involved in stilbene biosynthesis, stilbene synthase, is not ubiquitously expressed [17]. Since the original research by Jang et al. where a stilbene, resveratrol (Resv), was shown as a potent chemopreventive agent [18], these compounds have awakened the interest of the scientific community involved in anticancer drug development.

This review will focus on stilbenes and their potential as antioxidants and chemopreventive agents, thus including their molecular targets and signaling pathways; evidences from clinical trials for its toxicity, bioavailability, and benefit in humans; and biological improvements based on the development of analogs.

2. Cancer Chemopreventive Role of Natural Stilbenes

Cancer development is a progressive multistep process started with initial driver mutations (initiation) and followed by promotion and progression that ultimately lead to malignancy. Administration and consumption of agents to prevent, inhibit, or delay carcinogenesis are gathered in the global concept of chemoprevention [7, 19]. Stilbenes have shown ability to reduce the incidence of tumorigenesis by interfering with molecular events at all steps, that is, initiation, promotion, and progression stages of carcinogenesis. The limited distribution of the stilbenes in the plant kingdom led anticancer studies to focus on a reduced number of compounds [20]. With similarities and particularities, the number of targets and mechanisms where they are involved paved the way to their protective or therapeutic effects against cancer.

2.1. Resveratrol. Resv (3,4′,5-trihydroxy stilbene) was originally identified as a phytoalexin by Langcake and Pryce [21]. This natural stilbene has been found in at least 185 plant species [17] and is present in foods and beverages derived from them such as, for example, mulberries, peanuts, grapes, and red wine [18]. Its potential anticancer activity was originally reported by Jang et al. [18] and more than 2,000 references may be found in PubMed crossing Resv and cancer, thus showing the great interests in their chemopreventive and chemotherapeutic properties. In fact, Resv has undergone *in vitro* and *in vivo* carcinogenesis assays for many types of cancers, that is, breast [22], lung [23], colon [24], skin (nonmelanoma skin cancer and melanoma) [25], prostate [26], ovarian [27], liver [28], oral cavities [29], thyroid [30], and leukemia [31].

The chemopreventive properties of Resv have been associated with its antioxidant activity since it was first published that its anticancer activity, affecting all steps in the carcinogenesis process, was linked to the inhibition of cyclooxygenase 2 (COX-2) [18].

Up to now three different COX isoforms have been described: COX-1, expressed in normal tissue, participating in tissue homeostasis; COX-2, overexpressed in case of

inflammation or neoplasia development; and COX-3, a variant of COX-1 [32]. The important role of COX-2 in the progression of tumorigenesis is supported by studies that show an elevated level of the enzyme in premalignant and malignant tissue, which is accompanied by a decrease in the rate of survival of cancer patients [33, 34] and is a bad prognostic factor [35, 36]. Clinical trials have shown that COX-2 inhibitors may be a good strategy to prevent the development of colonic adenomas and potentially carcinomas [32]. However, the clinical efficacy of COX-2 inhibitors in the prevention of cancer has been challenged due to higher cardiovascular risks [37]. In this scenario, the use of natural compounds without toxic effects and demonstrated efficacy as potential COX-2 inhibitors, such as Resv, is of particular interest.

It has also been described that prostaglandins, produced by COX activity, are able to enhance cancer development and progression acting as tumor promoters or carcinogens [36, 38]. In fact, an increase in prostaglandin synthesis has important effects on carcinogen metabolism, tumor cell proliferation, and metastatic potential [39, 40] and may affect tumor growth in both humans and experimental animals. Thus inhibition of prostaglandin synthesis has been investigated to prevent tumor development [38, 40, 41].

Different authors have confirmed that Resv inhibits COX-2 expression and decreases prostaglandin E2 (PGE(2)) production. In this regard, Cianciulli et al. described that Resv downregulates COX-2 and PGE(2) in a concentration dependent fashion in the human intestinal cell line Caco-2 treated with lipopolysaccharide and that this mechanism may be related to NF-κB inhibition [42]. NF-κB is an inducible transcription factor strongly linked to inflammatory and immune responses and associated with oncogenesis [43]. Different stimuli may activate the release and translocation of NF-κB to the nucleus (e.g., those activating some membrane receptors (B cell receptor or tumor necrosis factor receptors) or several extracellular stimuli (inflammatory cytokines, viral and bacterial infections, oxidative and DNA-damaging agents, UV light, and osmotic shock)), where the transcription factor binds to promoter regions of genes encoding proinflammatory inducible enzymes such as COX-2, iNOS, and other inflammatory-related proteins [44, 45]. These anti-inflammatory effects of Resv have been also observed in a variety of cell lines, such as HeLa cells, Jurkat, RAW 264.7 macrophage, or U-937 cells [46, 47], and in *in vivo* experiments in rodents [48].

In addition to suppressing LPS-induced NF-κB-dependent COX-2 activation, Resv also activates AMPK [42, 47, 49] which effectively prevents tumorigenesis [29]. Thus, these mechanisms, at least in part, support the chemopreventive role of this stilbene.

On the other hand, studies on the redox status and functionality of the antioxidant machinery show the ability of Resv as a potent chemoprotector in different *in vivo* models of cancer development. Administration to rats of the potent hepatotoxic carcinogen azoxymethane (AOM) induced a potent oxidative unbalance triggered by glutathione (GSH) depletion, lipid peroxidation, and increased NO levels in the liver. All these effects were partially reversed by Resv administration [50]. Moreover, Resv acts as an antioxidant, at nutritionally relevant concentrations, by inducing the expression of superoxide dismutase (SOD) and catalase through a mechanism involving phosphatase and tensin homologue (PTEN)/protein kinase B (PKB) signaling pathway [51]. PTEN is a tumor suppressor gene and its expression is commonly decreased or lost in a large number of cancers of high frequency. The protein encoded by this gene is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and its main role is to dephosphorylate phosphoinositide substrates. So, it negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and acts as a tumor suppressor by negatively regulating the PKB signaling pathway. Inhibition of phosphatidylinositol 3-kinase (PI3K)/PKB pathway by PTEN has been associated with upregulation of SOD, GSH peroxidase, and catalase activities [52]. We confirmed these antioxidant properties of Resv in a mouse model. The pretreatment of mouse skin with Resv decreased several ultraviolet B radiation- (UVB-) induced oxidative events in a dose-dependent manner. Resv administration restored GSH levels, SOD, GSH peroxidase, and catalase activities to control values (mice without UVB irradiation) [53].

Despite scientific advances regarding the biological effects of Resv, our understanding of its anticancer mechanisms is far from a complete understanding. There are numerous evidences showing the capability of this polyphenol to induce programed cell death in different types of cancer. Proapoptotic stimulation by Resv has been associated with cell cycle alterations [54–56], caspase induction [54, 55, 57, 58], downregulation of Bcl-2, Bcl-xL, Survivin, and XIAP levels [59], and upregulation of Bax levels [58, 59], Bak, PUMA, Noxa, P21, Bim, TRAIL-R1/DR4, and TRAIL-R2/DR5 [59, 60]. Interestingly, a number of these effects may be correlated with P53 activation [55, 57–59]. For instance, Resv and piceatannol increased the cytoplasmic concentration of calcium in MDA-MB-231 human breast cancer cells, which induced the activation of P53 and the transcription of different proapoptotic genes [60]. Moreover, treatment of mutant P53 prostate cancer DU145 cells with Resv induced phosphorylation of the tumor suppressor which restored wild-type P53 DNA binding [61, 62] and P53 acetylation [63], activating proapoptotic events.

2.2. Pterostilbene. Pterostilbene (3,5-dimethoxy-4′-hydroxystilbene; Pter) is a natural analog of Resv, but with higher bioavailability [64, 65]. Due to its close structural similarity Pter possesses significant antioxidant activity *in vitro* in comparison with Resv [66, 67] and a clear clinical potential in different diseases [68]. Moreover, Pter has been reported to have cancer chemopreventive properties in different *in vitro* and *in vivo* experiments and other Resv-like health benefits. In these experiments, Pter was shown to inhibit growth, adhesion, and metastatic growth and to be an active apoptotic agent [68–71]. These effects have been shown in different types of cancers such as breast cancer [54, 68, 72–74], lung cancer [54, 75, 76], stomach cancer [68], prostate cancer [77], pancreatic cancer [78], melanoma [54], or colon carcinoma [54].

As it occurs with Resv, the antioxidant properties of Pter may also contribute to cancer chemoprevention. Rimando et al. [66] demonstrated that the antioxidant activity of Pter inhibits carcinogen-induced preneoplastic lesions in a mouse mammary organ culture model. Later, Chiou et al. [79] demonstrated that Pter is more potent than Resv in preventing AOM-induced colon tumorigenesis via activation of the Nrf2-mediated antioxidant signaling pathway. In the same experimental model, Pter decreased the expression of inflammatory genes, such as iNOS and COX-2 [80, 81]. Similarly, in HaCaT immortalized human keratinocytes Pter increased Nrf2 translocation into the nucleus and expression of Nrf2-dependent (oxidative stress related) molecules, thus further supporting the role of Nrf2 as a central regulator in the chemoprevention effect elicited by Pter [53]. Furthermore, in cultured HT-29 colon cancer cells the cytokine induction of the p38-activating transcription factor 2 pathway was markedly inhibited by the polyphenol compared to other anti-inflammatory pathways, such as NF-κB, Janus-activated kinase-signal transducer and activator of transcription (JAK-STAT), extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase, and PI3K [80]. That inhibition was associated with iNOS and COX-2 reduction, suggesting that p38 mitogen-activated protein kinase cascade is a key signal transduction pathway for the anti-inflammatory action of Pter [80].

Pter has also been found as potent as Resv in inhibiting NF-κB, AP-1, COX-2, and iNOS in a 12-O-tetradecanoylphorbol-13-acetate (TPA) induced mouse skin carcinogenesis model [82]. Moreover, Pter induces the expression of PTEN in prostate cancer decreasing the levels of miR-17, miR-20a, and miR-106b. The effect in restoring both PTEN mRNA and protein levels was lower for Resv [83], thus suggesting that Pter might show higher *in vivo* activity due to the substitution of hydroxyl by methoxy groups. In this context, we have recently published that, in an UVB-induced mouse skin carcinogenesis model, Pter is clearly superior to Resv in preventing acute and chronic skin damage [53]. In this study we demonstrated that the anticarcinogenic effect associated with a Pter-induced maintenance of skin antioxidant defenses (i.e., GSH levels, catalase, superoxide, and GSH peroxidase activities) and a reduction of UVB-induced oxidative damage on proteins, DNA, and lipids [53].

In addition, numerous studies have corroborated that Pter is an efficient anticancer agent acting on multiple signal transduction pathways. In the AOM-induced colon carcinogenesis model in rats, Pter, administered in the diet, decreased formation of aberrant crypt foci [81, 84]; transcriptional activation of iNOS and COX-2; GSK-3b phosphorylation and Wnt/b-catenin signaling; expression of VEGF, cyclin D1, and MMPs; activation of Ras, PI3K/PKB, and EGFR signaling pathways [84]; and mucosal levels of the proinflammatory cytokines, TNF-α, IL-1b, and IL-4 [85] and reduced the nuclear presence of phospho-p65 [85]. Moreover, McCormack et al. [86] showed the inhibitory effect of Pter on leptin-stimulated breast cancer *in vitro* through reduction of cell proliferation and JAK/STAT3 signaling. After that, microarray analysis of Pter-treated pancreatic cancer cells revealed upregulation of proapoptosis genes and altered levels of phosphorylated STAT3, MnSOD antioxidant activity, cytochrome C, and Smac/DIABLO [78]. Moreover Liu et al. have recently reported the ability of Pter to inhibit JAK2/STAT3 signaling downregulating the expression of STAT3 target genes, including the antiapoptotic proteins Bcl-xL and Mcl-1, and leading to upregulation of mitochondrial apoptosis pathway-related proteins (Bax, Bak, cytosolic cytochrome c, and cleaved caspase 3) and cyclin-dependent kinase inhibitors such as p21 and p27 in osteosarcoma [87].

The chemopreventive role of Pter is not limited to its antioxidant and anti-inflammatory properties or the cell death induction by apoptosis. It has been suggested that this stilbene may induce cell death, also, by autophagy [73, 76, 77, 88, 89]. However, the initial observations were based on accumulation of LC3II and autophagosomes, which is not a clear evidence of autophagic cell death [90, 91]. In fact, autophagosomes and LC3II accumulation are not significantly associated with active autophagy [54, 92]. Recently we have shown that Pter-induced tumor autophagy is an hsp70-dependent lysosomal membrane permeabilization mechanism [54].

The traditional cancer progression model has been rewritten in the last years highlighting the importance of tumor heterogeneity in chemo/radio-resistance development and relapse after treatment. In this sense, cancer stem cells (CSC) have emerged as a highly tumorigenic cell pool displaying properties of normal stem cells such as their ability to self-renew, to form differentiated progeny, and to generate a heterogeneous lineage of all types of cancer cells within a tumor, thus turning into a very attractive anticancer target [93–95]. In this context, it has been described that Pter and Resv can promote expression and activity of Argonaute-2, a central RNA interference (RNAi) component, which inhibits breast cancer stem-like cell characteristics by increasing the expression of a number of tumor-suppressive miRNAs (including miR-16, miR-141, miR-143, and miR-200c) [96]. Pter suppressed not only the generation of CSC but the metastatic potential in different experimental models [97, 98]. Under the influence of tumor-associated macrophages, which promote tumor growth and progression, Pter was shown to modulate epithelial-to-mesenchymal transition signaling pathways [97]. In addition, this stilbene was able to prevent the enrichment of CD133(+) hepatoma CSCs under irradiation [98].

2.3. Piceatannol and Pinosylvin. Piceatannol (trans-3,5,3′,4′-tetrahydroxystilbene) is a hydroxylated analog of Resv found in a variety of plant sources including, for example, grapes, peanut, passion fruit, and white tea. Although less studied, piceatannol has health-promoting effects similar to Resv [99–101]. Li et al. [102] showed that the anticancer properties of piceatannol may be attributed to its prooxidant properties, which in the presence of copper (Cu)(II) induces formation of the hydroxyl radical through the Haber Weiss and Fenton reactions and DNA breakage. In fact, there are authors that propose that the anticancer action of plant polyphenols involves, in part, mobilization of endogenous copper and its consequent prooxidant action [103].

Paradoxically, in accordance with its origin and structure, piceatannol also shows similar activities as those indicated

for Resv such as the antioxidant activity, although mediated by different pathways. Piceatannol inhibits NF-κB activation by H_2O_2, phorbol 12-myristate 13-acetate, LPS, okadaic acid, and ceramide [104]. Moreover, piceatannol inhibits TNF-induced Iκ-Ba phosphorylation, p65 phosphorylation, and p65 nuclear translocation. These effects, also observed under Resv treatment, suggest a crucial role of the hydroxyl group in positions 3 and 4′ [104]. It has also been described that piceatannol reduces the expression of iNOS, decreasing the NO production, and COX-2 in LPS-stimulated RAW 264.7 cells and BV2 microglia cells [105, 106]. Piceatannol is also able to increase heme oxygenase-1 expression and protein levels in human breast epithelial MCF10A cells. The underlying mechanism involves stimulation of Nrf-2 release from Keap1, nucleus translocation, and direct binding of the transcriptional factor to the antioxidant response element, leading to an enhancement of heme oxygenase-1 expression [107].

Regarding pinosylvin (3,5-dihydroxy-trans-stilbene), a pine antifungal and antibacterial stilbene, its chemopreventive activity may be also attributed to its antioxidant and anti-inflammatory activity. In fact, pinosylvin, like Resv or piceatannol, inhibited the production of PGE_2 in LPS-induced RAW 264.7 cells, thereby inhibiting the expression of COX-2 [108]. Later, in the same cellular model it was shown that pinosylvin is also able to inhibit iNOS expression [109].

3. *In Vivo* Toxicity of Resveratrol and Pterostilbene

3.1. Resveratrol. An initial starting point in the safety evaluation of a naturally occurring food substance is its natural intake. The daily intake of dietary Resv is mainly from the consumption of wine and grapes and foods derived from them. This intake of up to 2 mg/day is relatively low in comparison to the level of safe oral intake that is derived from oral preclinical studies with ResVida, a high purity trans-Resv formulation [110]. This compound, commercialized by DSM Nutritional Products Ltd., obtained GRAS (Generally Regarded As Safe) designation by the U.S. Food and Drug Administration (FDA) in 2008, with his Allowable Daily Intake (ADI) being 450 mg/day [110]. The ADI was based on no-observed-adverse-effect-levels (NOAELs) of 750 mg/kg bw/day in rats on a 13-week developmental toxicity study by the dietary route and a standard safety margin of 100 [111]. Although it has been also described, in studies by gavage, that Resv caused toxicity in the kidney and bladder after 4-week treatment in rats, this was at very high dosages (2.000–3.000 mg/kg bw/day) [112].

Regarding Resv's toxicity versus time, six-month studies in rat and rabbit models showed no significant increase in toxicity in comparison to the 4-week studies [110]. Kinetic data from the DSM 13-week toxicity study support the expectation of no increase in toxicity with longer term intake [111]. About Resv genotoxic activity, short-term studies based on the Ames test showed that this compound does not have genotoxic activity *in vivo*, but experimental details are too limited to evaluate the data in full [111].

Only a small number of clinical trials using Resv as a single-agent, and formulated as a medicinal product, have formally addressed and reported on safety and tolerability [113–117]. No serious adverse event was detected in all these studies. Adverse events were mild and only lasted for a few days. The most common toxicity was gastrointestinal, particularly diarrhea, nausea, and abdominal pain, but also frontal headache and rash occurred in some patients. A sequential dose study of Resv at repeated daily doses of up to 5 g (0.5, 1.0, 2.5, and 5.0 g) for 29 days in healthy volunteers was performed. The results of these clinical, biochemical, and hematological analyses showed that Resv administration is safe, although at the 2.5 g and 5 g dose levels it caused reversible gastrointestinal symptoms such as diarrhea, nausea, or flatulence in some individuals.

It is worthwhile to mention that a phase II clinical trial (https://www.clinicaltrials.gov/), sponsored by Glaxo-SmithKline in patients with multiple myeloma to assess the safety and activity of SRT501 (a micronized formulation of Resv), was terminated due to safety concerns after kidney damage (cast nephropathy) developed in some patients. In this trial, a high dose of 5 g SRT501/day was administered orally for 20 consecutive days. This dose of Resv was significantly higher than that used in the safety study mentioned above for ResVida [110]. Nevertheless cast nephropathy is a condition closely associated with multiple myeloma, so the finding in this study is of doubtful significance outside of this disease condition.

3.2. Pterostilbene. The toxicity of Pter, after intravenous administration to xenografted mice, has been assessed in several studies involving the treatment of colorectal cancer [118], prostate cancer [119], and melanoma [69]. The doses and time of administration were 20 mg/kg and 30 mg/kg per day during 23 days [118]; 50 mg/kg per day during 4 weeks [119]; and 20 mg/kg during 10 days [69]. In all these studies, Pter was found therapeutically effective and pharmacologically safe because it showed no organ-specific or systemic toxicity.

Regarding oral administration, Ruiz et al. [120] published in 2009 a study in which they evaluated the toxicity of Pter at high doses in healthy mice. For this purpose, mice were fed during 28 days at doses of 30, 300, and 3000 mg/kg body weight/day of Pter. These daily doses did not cause mortality during the experimental period at any dose, but the red blood cell number and hematocrit increased after Pter administration compared to control groups. However, histopathological examination and evaluation of biochemical parameters revealed no alterations regarding clinical signs or organ weight at any dose [120].

Chromadex Inc. (Irvine, CA) achieved GRAS status for its ingredient pTeroPure-branded Pter (http://www.fda.gov/) in 2011. The ADI for pTeroPure is up to 30 mg/kg per day for food use (https://chromadex.com/NewsEventDetail.aspx?Aid=510). Data from the first clinical trial on Pter (Effect of Pter on Cholesterol, Blood Pressure and Oxidative Stress, https://www.clinicaltrials.gov/, conducted at the University of Mississippi Medical Center) were released in 2012. It was concluded that oral administration of 125 mg of Pter twice per day was well-tolerated because there were no

statistically significant adverse drug reactions on hepatic, renal, or glucose markers based on biochemical analysis [121]. Despite these observations, more rigorous studies are needed before dietary/therapeutic dosages can be standardized for different applications.

4. Pharmacokinetics of Stilbenes

Stilbenes, as the majority of phenolic compounds, have low bioavailability which limits their potential benefits for health [122]. The bioavailability depends on the route of administration but also relies on their absorption and metabolism. Those factors are mainly determined by the chemical structure of the compound (degree of glycosylation/acylation, their basic structure, conjugation with other phenolics, molecular size, degree of polymerization, solubility, etc.) [11, 123]. That is the reason why bioavailability may greatly differ among the many different (even closely related) phenolic compounds.

4.1. Resveratrol. Many concerns regarding Resv effectiveness *in vivo* arise from its low bioavailability and short half-life. According to Asensi et al. [124], after intravenous administration to rabbits of 20 mg of Resv/Kg its highest concentration in plasma was 42.8 ± 4.4 μM 5 min after administration. But, because of its rapid metabolism and short half-life (14.4 min), this concentration decreased very rapidly at 60 min to 0.9 ± 0.2 μM. After oral administration of the same dose the highest concentration in plasma within the first 5 min was lower (2-3 μM), thus indicating the higher limitations on bioavailability linked to the oral intake. Similar results were reported by others in humans [125, 126].

However Resv is highly absorbed after oral administration (about 75% of the dose administered to humans) mainly by transepithelial diffusion [127]. Therefore, its low bioavailability is caused by its rapid and extensive first-pass metabolism in the intestine and liver. Once metabolized, Resv is excreted through the urine and feces although some conjugated metabolites can be also reabsorbed by enterohepatic recirculation (Figure 2) [126, 128].

The main metabolites of Resv are produced through three metabolic pathways: glucuronic and sulfate conjugation of the 3 and 4′ phenolic groups (phase II metabolites) and hydrogenation of the aliphatic double bound. The latter has been suggested to be produced by intestinal microflora [126, 127, 129] (Figure 2). Up to nearly 20 Resv-derived metabolites have been described in plasma, urine, and some tissues [115, 126, 130–134]. Among these metabolites there are mono- and diglucuronides; monosulfates, disulfates and trisulfates; and sulfoglucuronides, as well as equivalent conjugations of the hydrogenated Resv.

In plasma, the major circulating metabolites of Resv are phase II conjugates, being the most abundant Resv-3-sulfate in humans [115, 116, 131]. In contrast, in rats and pigs is Resv-3-glucuronide the main metabolite. In both cases the plasma concentrations of Resv metabolites are much higher than the concentration of the parent molecule [128, 135].

The efficacy of the Resv metabolites is still under debate. Emerging data suggests that Resv conjugates have anticancer activity *in vitro*. The biological effects of those metabolites appear to be reduced when compared to Resv in some studies [136, 137] and similar in others [138–140]. However, although Resv glucuronides have some biological effects, no cytotoxic activity against cancer cell lines has been demonstrated. Only one study has reported cytotoxic activity of glucuronide metabolites but only when administered as a mixture of them [141]. Nevertheless, a common hypothesis is that as it has been reported for other compounds [142, 143], these metabolites could undergo deconjugation, releasing the parent compound (Figure 2). Consequently, the glucuronide and sulfate conjugates of Resv may provide a pool from which active Resv can be released [129]. This hypothesis has been proved recently for Resv sulfate conjugates in mouse [144] although it is uncertain (very unlikely in fact) that Resv deconjugation may release sufficient effective levels, in terms of real biological activity, under *in vivo* conditions.

The biological activity of the Resv metabolite dihydroResv is also incompletely understood. In some *in vitro* studies it exerts an antiproliferative effect in tumor and normal cell lines but less potent than Resv [67, 139]. On the other hand, a recent study *in vitro* shows potent antiproliferative effects on hormone-sensitive breast cancer cells [145]. However, since the dihydroResv metabolite is mainly formed in the colon, it might be expected that it contributes to chemopreventive effects at that site [127]. In fact, sulfate and glucuronide conjugates of dihydroResv have been found in cecum, colon, and rectum of the pig [130] and in the colon of the rat [129].

Recently, the detection of Resv and its derived metabolites has been reported in colorectal tissue of patients after oral treatment with Resv [117]. The major metabolites found in tumor and normal tissue were phase II metabolites (glucuronides, sulfates, and sulfoglucuronides). The highest concentration was detected for Resv-sulfoglucuronide. However, the possible concurrency of dihydroResv and derived conjugates was not explored [117].

4.2. Pterostilbene. Pter, as a natural occurring dimethoxy analog of Resv, has a more favorable pharmacokinetics profile [64, 65, 70]. On one hand, as Pter has less hydroxyl groups (only one instead of three in Resv), it is less susceptible to conjugation metabolism and, therefore, is predicted to have a longer half-life [146, 147]. On the other hand, the dimethoxy structure enhances its lipophilicity thus increasing membrane permeability and improving its bioavailability [64, 146, 148].

Similarly to Resv, the major Pter metabolites found in mouse plasma and urine are phase II conjugates: Pter glucuronide, Pter sulfate, monodemethylated Pter glucuronide, monodemethylated Pter sulfate, monohydroxylated Pter, monohydroxylated Pter glucuronide, monohydroxylated Pter sulfate, and monohydroxylated Pter glucuronide sulfate [64, 70, 149]. Nevertheless, there is no evidence of the presence of a Pter hydrogenated form or the equivalent phase II conjugations of this. Those metabolites have been reported to be recycled by enterohepatic recirculation as it has previously been reported for Resv (Figure 2) [70]. No studies are available at the moment on the possible biological activity of Pter metabolites.

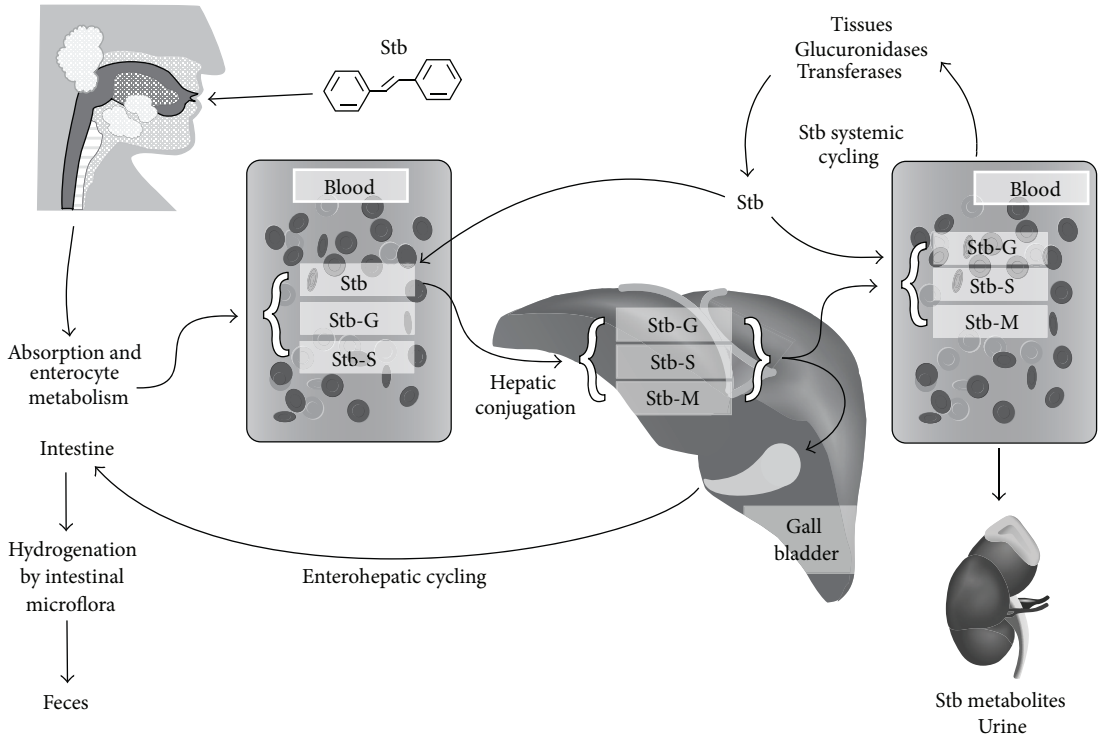


Figure 2: General metabolic pathways of the major stilbenes.

After intravenous administration in mouse of Pter and Resv, either of them reaches their highest concentrations within the first 5 minutes. However, while for Resv this concentration decreased very rapidly to 1 μM within the first 60 minutes, Pter remains longer in plasma reaching the 1 μM concentration in 480 minutes [69, 124]. From these data, the calculated half-life for Pter is 6-7 times longer than for Resv [69, 124]. Similar results have been reported in recent studies in rats [65, 70].

Regarding oral bioavailability, it has been reported that in rats it is greater for Pter (80%) than for Resv (20%) [64]. Also in this study, as it has been reported by others [150], the major metabolite in plasma is Pter sulfate, with its levels being higher than those of the parent compound. In addition, plasma levels of Pter and Pter sulfate after oral administration were greater than Resv and Resv sulfate, respectively, whereas levels of Resv glucuronide were higher than Pter glucuronide. Another issue to point out is that after Pter administration Resv was not detectable, indicating that Pter is not a prodrug of Resv [64].

Interestingly, a recent study reports that levels of Pter and its main metabolite, Pter sulfate, are higher in tissues than in blood, meaning that they accumulate in tissues where Pter conjugates may act as a source of the natural compound. This observation has logical implications for the *in vivo* bioactivity of Pter because it may explain the paradox of Pter biological activity despite its low plasma concentrations [150]. Levels of Pter sulfate were higher than levels of Pter in every organ except the brain, where levels of the parent compound were higher. This observation has a particular interest given the reports of the biological activity of Pter on the central nervous system [150].

4.3. Piceatannol and Pinosylvin. These stilbenes, after intravenous administration in rats, are distributed into tissues and highly extracted by the liver where they undergo extensive glucuronidation. All are predominantly eliminated via nonurinary routes and as they have short half-lives, their estimate oral bioavailability is poor as it is the case for Resv and Pter [151–155].

The major metabolic pathways for piceatannol are glucuronidation and sulfation, as it occurs for Resv and Pter, but also methylation [153]. In contrast to piceatannol, methylated metabolites have not been found in rat plasma after treatment with Resv [153, 156]. Piceatannol conjugates could be also recycled by enterohepatic recirculation as the other stilbenes (Figure 2) [157].

A remarkable finding is that piceatannol can be metabolized into another stilbene, the isorhapontigenin, suggesting that piceatannol could exhibit additional biological functions [153]. Compared to Resv piceatannol may have a higher metabolic stability and similar beneficial effects [100, 101, 153]. In fact, it has been suggested that anticancer properties of Resv may be due to its metabolism to piceatannol by the cytochrome P450 enzyme CYP1B1, suggesting that Resv may act as a source or prodrug of piceatannol [158].

Glucuronidation has been described as the major conjugation pathway of pinosylvin. However, interestingly, two minor oxidized metabolites of this polyphenol have been detected: Z- and E-Resv [151, 154]. Structurally, pinosylvin,

compared to Resv, lacks the 4′-hydroxyl group while it retains the 3-hydroxyl moiety which has been identified as a major target of phase II conjugation reactions. Furthermore, the absence of the 4′-hydroxyl group in pinosylvin may have enhanced its binding to first-pass metabolic enzymes. Thus, due to its extensive metabolism, compared to Resv, pinosylvin has lower oral bioavailability [155].

5. Analyses of Structure-Activity Relationships to Improve the Effectiveness of Stilbenes

Generally, the main problem regarding the use of polyphenols is the partial knowledge of their mechanisms of action [159] and their low bioavailability [124], which as mentioned before is determined by their chemical structure [160–162]. These features regulate both absorption and excretion of phenolic compounds. As an example, 0.3% of the intake of anthocyanins is excreted by urine, compared to 43% of isoflavones, thus reflecting the potential importance of the chemical structure [163].

There are a high number of works in which the structure-activity relationships (SARs) of polyphenols are studied. These studies intend to figure out, using structural analogs, which modifications may confer increased resistance to oxidation of the polyphenols [164], improving the interaction with domains of the target proteins [159] and finally increasing the pharmacokinetics properties [165]. Theoretically, part of these changes may help to direct certain polyphenols to target tissues [166, 167].

The main changes in structural analogs affect the number and position of hydroxylated and methylated groups, which also influence their metabolism. In fact, polyphenols metabolized to their secondary metabolites may even have more activity. However, there are critical residues for the functional groups that are directly linked to the activity; for example, hydroxylation at C4 in Resv analogues is critical to its function in *in vitro* studies [165]. Structure-activity studies have revealed that increasing the number of OH groups at their ortho position on the phenol ring of stilbenes could increase the free radical scavenging capacity, the cytotoxic activity, and the anti-inflammatory effects of these compounds [100, 168]. In fact, polyhydroxylated analogs of Resv as hexahydroxystilbene turned out to be more potent and specific inhibitors of COX-2 activity than Resv both *in vivo* and *in vitro* [168, 169]. Moreover, this analog, showing higher antiradical activity, also induces apoptosis at concentrations than the parent compound [168].

Nevertheless, in animal studies, the 3,4,5,4′-tetramethoxystilbene (DMU-212), wherein the C4-OH is blocked by methylation, possesses stronger antiproliferative properties in human colon cancer cells than Resv, possibly, because these methylated groups, by slowing excretion, could provide better plasma levels [159]. Another example is pinosylvin. Pinosylvin differs from Resv in lacking one hydroxyl at C4′, which makes it more lipophilic but losing its antioxidant activity. Nevertheless, once inside the cell, it recovers the antioxidant activity [170]. The methoxylated analogs have higher lipophilicity, which may favor their entry into cells and confer more resistance to degradation, thus improving pharmacokinetics [54, 171]. However, the number of methoxy and hydroxyl groups must be under equilibrium. The hydroxyl groups confer more solubility, which allows a better interaction with proteins [166], whereas the methoxylated group confer resistance to degradation although an excessive number of methoxylated groups may impair the interaction with the target protein [165]. Pter, with two methyl groups, a trans-3,4′-dihydroxy-2′,3′,5-trimethoxystilbene with higher anticancer effects than Resv both *in vitro* and *in vivo* [54, 172].

Polyphenols exhibit excellent healthy and therapeutic properties to treat various diseases, including a broad spectrum of actions involved in a large number of targets and very low toxicity properties. Manipulation of the polyphenolic structure can improve its bioavailability and activity. DMU-212 is an example of a more lipophilic structural analog of Resv capable of crossing the blood-brain barrier [173]. These successes show that modifying the polyphenolic structures we may be able to exploit their properties improving its activity and action.

6. Clinical Trials

Natural stilbenes have been used in traditional medicine. Resv, piceatannol, and Pter are examples of stilbenes synthesized by several types of plants in response to a variety of stress conditions [79]. Starting on their implication on the known "French paradox" (which associates red wine consumption and lower coronary heart disease), several clinical and pharmacometric studies on Resv have been performed in the last years. Even though most pharmacometric studies of Resv in humans show that its plasma concentrations are below the effectiveness range indicated by *in vitro* assays, it may show interesting effects *in vivo*. Boocock et al. [115] found out that administration of a single dose of Resv (5.0 g) rendered a peak plasma concentration of 2.4 nmol/mL, which is only slightly below the required concentration *in vitro* to show chemopreventive properties. Most clinical studies on Resv and cancer have been performed in colorectal cancer patients, possibly because oral administration may facilitate reaching higher concentrations of this stilbene in tumors located along the gastrointestinal tract. For example, a clinical assay by Patel et al. showed that 0.5 g and 1.0 g doses of Resv were able to significantly reduce colorectal cell proliferation [117]. Howells et al. [174] assayed micronized Resv SRT501 in colorectal cancer patients with hepatic metastases, who had not received therapeutic intervention for their cancer within 6 weeks of study commencement and had a life expectancy of less than 3 months. They concluded that SRT501 administration during 21 days was safe, although some individuals suffered some adverse effects like nausea or diarrhea. The study accomplished by Noguer et al. [175] showed that alcohol-free red wine consumption can increase our antioxidant enzyme activities (SOD, catalase, and GSH reductase). This assay demonstrated that alcohol-free red wine may improve the health of people suffering oxidative stress related diseases.

At present, despite the promising anticancer properties elicited by Pter, there is only a clinical trial performed at the University of Mississippi. In this clinical trial researchers

assessed the effects of Pter in cholesterol, blood pressure, and oxidative stress. They showed that Pter is able to improve these aforementioned parameters under safe conditions (ClinicalTrials.gov Identifier NCT01267227). Clinical trials on specific anticancer effects are expected to be performed in the next future.

7. Conclusions

The identification of protective molecules without side effects should be a main objective in the fight against cancer. Experimental *in vitro* and *in vivo* studies, and a few clinical trials, show evidences about the effectivity of stilbenes as anticancer agents, both in the form of nutritional supplements or functional foods and as potential anticancer drugs. This group of polyphenols show a very low toxicity and, although having multiple molecular targets, act on different protective and common pathways usually altered in a great number of tumors. This is important since it suggests that natural stilbenes may be more prone for their use as anticarcinogens. The capability to prevent carcinogenesis includes inhibition of inflammation, oxidative stress, and cancer cell proliferation and using tightly regulated cell death mechanisms. Due to the complexity and number of cellular processes involved more studies must be done to fully understand how stilbenes may be used to avoid the development of cancer. Moreover, due to their low concentration in food and their rapid metabolism and excretion in the body mammals, improvements in delivery systems, stability, and solubility are necessary in order to make their use in clinical settings as chemopreventive drugs possible.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgment

This work was supported by grant from the MICINN (SAF2012-31565).

References

[1] J. Ferlay, E. Steliarova-Foucher, J. Lortet-Tieulent et al., "Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012," *European Journal of Cancer*, vol. 49, no. 6, pp. 1374–1403, 2013.

[2] M. Malvezzi, P. Bertuccio, F. Levi, C. La Vecchia, and E. Negri, "European cancer mortality predictions for the year 2014," *Annals of Oncology*, vol. 25, no. 8, pp. 1650–1656, 2014.

[3] R. Siegel, J. Ma, Z. Zou, and A. Jemal, "Cancer statistics, 2014," *CA Cancer Journal for Clinicians*, vol. 64, no. 1, pp. 9–29, 2014.

[4] S. B. Jones, "Cancer in the developing world: a call to action," *British Medical Journal*, vol. 319, no. 7208, pp. 505–508, 1999.

[5] M. Pereira, B. Peleteiro, S. Capewell, K. Bennett, A. Azevedo, and N. Lunet, "Changing patterns of cardiovascular diseases and cancer mortality in Portugal, 1980–2010.," *BMC Public Health*, vol. 12, article 1126, 2012.

[6] R. Siegel, D. Naishadham, and A. Jemal, "Cancer statistics for Hispanics/Latinos, 2012," *CA Cancer Journal for Clinicians*, vol. 62, no. 5, pp. 283–298, 2012.

[7] N. J. Kang, S. H. Shin, H. J. Lee, and K. W. Lee, "Polyphenols as small molecular inhibitors of signaling cascades in carcinogenesis," *Pharmacology & Therapeutics*, vol. 130, no. 3, pp. 310–324, 2011.

[8] S. Mena, A. Ortega, and J. M. Estrela, "Oxidative stress in environmental-induced carcinogenesis," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 674, no. 1-2, pp. 36–44, 2009.

[9] B. N. Ames, M. K. Shigenaga, and T. M. Hagen, "Oxidants, antioxidants, and the degenerative diseases of aging," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 17, pp. 7915–7922, 1993.

[10] S. Quideau, D. Deffieux, C. Douat-Casassus, and L. Pouységu, "Plant polyphenols: chemical properties, biological activities, and synthesis," *Angewandte Chemie—International Edition*, vol. 50, no. 3, pp. 586–621, 2011.

[11] L. Bravo, "Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance," *Nutrition Reviews*, vol. 56, no. 11, pp. 317–333, 1998.

[12] S. M. Poulose, N. Thangthaeng, M. G. Miller, and B. Shukitt-Hale, "Effects of pterostilbene and resveratrol on brain and behavior," *Neurochemistry International*, vol. 89, pp. 227–233, 2015.

[13] J. Dai and R. J. Mumper, "Plant phenolics: extraction, analysis and their antioxidant and anticancer properties," *Molecules*, vol. 15, no. 10, pp. 7313–7352, 2010.

[14] Y. H. Li, Y. B. Niu, Y. Sun et al., "Role of phytochemicals in colorectal cancer prevention," *World Journal of Gastroenterology*, vol. 21, no. 31, pp. 9262–9272, 2015.

[15] J. A. Nichols and S. K. Katiyar, "Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms," *Archives of Dermatological Research*, vol. 302, no. 2, pp. 71–83, 2010.

[16] T. Shen, X.-N. Wang, and H.-X. Lou, "Natural stilbenes: an overview," *Natural Product Reports*, vol. 26, no. 7, pp. 916–935, 2009.

[17] C. Rivière, A. D. Pawlus, and J.-M. Mérillon, "Natural stilbenoids: distribution in the plant kingdom and chemotaxonomic interest in Vitaceae," *Natural Product Reports*, vol. 29, no. 11, pp. 1317–1333, 2012.

[18] M. Jang, L. Cai, G. O. Udeani et al., "Cancer chemopreventive activity of resveratrol, a natural product derived from grapes," *Science*, vol. 275, no. 5297, pp. 218–220, 1997.

[19] M. B. Sporn, "Carcinogenesis and cancer: different perspectives on the same disease," *Cancer Research*, vol. 51, part 1, no. 23, pp. 6215–6218, 1991.

[20] M. Asensi, A. Ortega, S. Mena, F. Feddi, and J. M. Estrela, "Natural polyphenols in cancer therapy," *Critical Reviews in Clinical Laboratory Sciences*, vol. 48, no. 5-6, pp. 197–216, 2011.

[21] P. Langcake and R. J. Pryce, "The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury," *Physiological Plant Pathology*, vol. 9, no. 1, pp. 77–86, 1976.

[22] A. Chatterjee, A. Ronghe, B. Singh, N. K. Bhat, J. Chen, and H. K. Bhat, "antioxidants exhibit chemopreventive characteristics through the regulation of CNC b-Zip transcription factors in estrogen-induced breast carcinogenesis," *Journal of Biochemical and Molecular Toxicology*, vol. 28, no. 12, pp. 529–538, 2014.

[23] A. Malhotra, P. Nair, and D. K. Dhawan, "Study to evaluate molecular mechanics behind synergistic chemo-preventive effects of curcumin and resveratrol during lung carcinogenesis," *PLoS ONE*, vol. 9, no. 4, Article ID e93820, 2014.

[24] F. Mazué, D. Delmas, G. Murillo, D. Saleiro, E. Limagne, and N. Latruffe, "Differential protective effects of red wine polyphenol extracts (RWEs) on colon carcinogenesis," *Food & Function*, vol. 5, no. 4, pp. 663–670, 2014.

[25] J. H. Back, Y. Zhu, A. Calabro et al., "Resveratrol-mediated downregulation of rictor attenuates autophagic process and suppresses UV-induced skin carcinogenesis," *Photochemistry and Photobiology*, vol. 88, no. 5, pp. 1165–1172, 2012.

[26] T.-C. Hsieh, C.-J. Yang, C.-Y. Lin, Y.-S. Lee, and J. M. Wu, "Control of stability of cyclin D1 by quinone reductase 2 in CWR22Rv1 prostate cancer cells," *Carcinogenesis*, vol. 33, no. 3, pp. 670–677, 2012.

[27] C. Lin, D. R. Crawford, S. Lin et al., "Inducible COX-2-dependent apoptosis in human ovarian cancer cells," *Carcinogenesis*, vol. 32, no. 1, pp. 19–26, 2011.

[28] D. Rajasekaran, J. Elavarasan, M. Sivalingam et al., "Resveratrol interferes with N-nitrosodiethylamine-induced hepatocellular carcinoma at early and advanced stages in male Wistar rats," *Molecular Medicine Reports*, vol. 4, no. 6, pp. 1211–1217, 2011.

[29] S. Shrotriya, A. Tyagi, G. Deep et al., "Grape seed extract and resveratrol prevent 4-nitroquinoline 1-oxide induced oral tumorigenesis in mice by modulating AMPK activation and associated biological responses," *Molecular Carcinogenesis*, vol. 54, no. 4, pp. 291–300, 2015.

[30] H. J. Kang, Y.-K. Youn, M.-K. Hong, and L. S. Kim, "Antiproliferation and redifferentiation in thyroid cancer cell lines by polyphenol phytochemicals," *Journal of Korean Medical Science*, vol. 26, no. 7, pp. 893–899, 2011.

[31] M.-F. Tsan, J. E. White, J. G. Maheshwari, and G. Chikkappa, "Anti-leukemia effect of resveratrol," *Leukemia & Lymphoma*, vol. 43, no. 5, pp. 983–987, 2002.

[32] S. Temraz, D. Mukherji, and A. Shamseddine, "Potential targets for colorectal cancer prevention," *International Journal of Molecular Sciences*, vol. 14, no. 9, pp. 17279–17303, 2013.

[33] D. Wang and R. N. Dubois, "The role of COX-2 in intestinal inflammation and colorectal cancer," *Oncogene*, vol. 29, no. 6, pp. 781–788, 2010.

[34] K. Mosalpuria, C. Hall, S. Krishnamurthy et al., "Cyclooxygenase-2 expression in non-metastatic triple-negative breast cancer patients," *Molecular and Clinical Oncology*, vol. 2, no. 5, pp. 845–850, 2014.

[35] D. Wang, X.-Z. Guo, H.-Y. Li, J.-J. Zhao, X.-D. Shao, and C.-Y. Wu, "Prognostic significance of cyclooxygenase-2 protein in pancreatic cancer: a meta-analysis," *Tumor Biology*, vol. 35, no. 10, pp. 10301–10307, 2014.

[36] A. Fernández-Alvarez, C. Llorente-Izquierdo, R. Mayoral et al., "Evaluation of epigenetic modulation of cyclooxygenase-2 as a prognostic marker for hepatocellular carcinoma," *Oncogenesis*, vol. 1, article e23, 2012.

[37] C. Roubille, J. Martel-Pelletier, J.-M. Davy, B. Haraoui, and J.-P. Pelletier, "Cardiovascular adverse effects of anti-inflammatory drugs," *Anti-Inflammatory & Anti-Allergy Agents in Medicinal Chemistry*, vol. 12, no. 1, pp. 55–67, 2013.

[38] J. Jiao, T.-O. Ishikawa, D. S. Dumlao et al., "Targeted deletion and lipidomic analysis identify epithelial cell COX-2 as a major driver of chemically induced skin cancera," *Molecular Cancer Research*, vol. 12, no. 11, pp. 1677–1688, 2014.

[39] L. J. Marnett, "Aspirin and the potential role of prostaglandins in colon cancer," *Cancer Research*, vol. 52, no. 20, pp. 5575–5589, 1992.

[40] G. N. Levy, "Prostaglandin H synthases, nonsteroidal anti-inflammatory drugs, and colon cancer," *The FASEB Journal*, vol. 11, no. 4, pp. 234–247, 1997.

[41] A. Kitasato, T. Kuroki, T. Adachi et al., "A selective cyclooxygenase-2 inhibitor (etodolac) prevents spontaneous biliary tumorigenesis in a hamster bilioenterostomy model," *European Surgical Research*, vol. 52, no. 1-2, pp. 73–82, 2014.

[42] A. Cianciulli, R. Calvello, P. Cavallo, T. Dragone, V. Carofiglio, and M. A. Panaro, "Modulation of NF-kappaB activation by resveratrol in LPS treated human intestinal cells results in downregulation of PGE2 production and COX-2 expression," *Toxicology in Vitro*, vol. 26, no. 7, pp. 1122–1128, 2012.

[43] S. A. Schwartz, A. Hernandez, and B. Mark Evers, "The role of NF-κB/IκB proteins in cancer: implications for novel treatment strategies," *Surgical Oncology*, vol. 8, no. 3, pp. 143–153, 1999.

[44] P. Viatour, M.-P. Merville, V. Bours, and A. Chariot, "Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation," *Trends in Biochemical Sciences*, vol. 30, no. 1, pp. 43–52, 2005.

[45] K. J. Campbell and N. D. Perkins, "Regulation of NF-kappaB function," *Biochemical Society Symposium*, vol. 73, pp. 165–180, 2006.

[46] K. P. L. Bhat and J. M. Pezzuto, "Cancer chemopreventive activity of resveratrol," *Annals of the New York Academy of Sciences*, vol. 957, pp. 210–229, 2002.

[47] C. O. Yi, B. T. Jeon, H. J. Shin et al., "Resveratrol activates AMPK and suppresses LPS-induced NF-kappaB-dependent COX-2 activation in RAW 264.7 macrophage cells," *Anatomy & Cell Biology*, vol. 44, no. 3, pp. 194–203, 2011.

[48] A. Bishayee, A. Waghray, K. F. Barnes et al., "Suppression of the inflammatory cascade is implicated in resveratrol chemoprevention of experimental hepatocarcinogenesis," *Pharmaceutical Research*, vol. 27, no. 6, pp. 1080–1091, 2010.

[49] Y. Han, C. Jiang, J. Tang et al., "Resveratrol reduces morphine tolerance by inhibiting microglial activation via AMPK signalling," *European Journal of Pain*, vol. 18, no. 10, pp. 1458–1470, 2014.

[50] S. Gürocak, E. Karabulut, N. Karadag, D. Ozgor, N. Ozkeles, and A. Bay Karabulut, "Preventive effects of resveratrol against azoxymethane induced damage in rat liver," *Asian Pacific Journal of Cancer Prevention*, vol. 14, no. 4, pp. 2367–2370, 2013.

[51] M. Inglés, J. Gambini, M. G. Miguel et al., "PTEN mediates the antioxidant effect of resveratrol at nutritionally relevant concentrations," *BioMed Research International*, vol. 2014, Article ID 580852, 6 pages, 2014.

[52] H. Akca, A. Demiray, M. Aslan, I. Acikbas, and O. Tokgun, "Tumour suppressor PTEN enhanced enzyme activity of GPx, SOD and catalase by suppression of PI3K/AKT pathway in non-small cell lung cancer cell lines," *Journal of Enzyme Inhibition and Medicinal Chemistry*, vol. 28, no. 3, pp. 539–544, 2013.

[53] J. A. Sirerol, F. Feddi, S. Mena et al., "Topical treatment with pterostilbene, a natural phytoalexin, effectively protects hairless mice against UVB radiation-induced skin damage and carcinogenesis," *Free Radical Biology and Medicine*, vol. 85, pp. 1–11, 2015.

[54] S. Mena, M. L. Rodríguez, X. Ponsoda, J. M. Estrela, M. Jäättela, and A. L. Ortega, "Pterostilbene-induced tumor cytotoxicity: a lysosomal membrane permeabilization-dependent mechanism," *PLoS ONE*, vol. 7, no. 9, Article ID e44524, 2012.

[55] F. Casanova, J. Quarti, D. C. Ferraz da Costa, C. A. Ramos, J. L. Da Silva, and E. Fialho, "Resveratrol chemosensitizes breast cancer cells to melphalan by cell cycle arrest," *Journal of Cellular Biochemistry*, vol. 113, no. 8, pp. 2586–2596, 2012.

[56] X. D. Yu, J. L. Yang, W. L. Zhang, and D. X. Liu, "Resveratrol inhibits oral squamous cell carcinoma through induction of apoptosis and G2/M phase cell cycle arrest," *Tumor Biology*, 2015.

[57] A. A. Gokbulut, E. Apohan, and Y. Baran, "Resveratrol and quercetin-induced apoptosis of human 232B4 chronic lymphocytic leukemia cells by activation of caspase-3 and cell cycle arrest," *Hematology*, vol. 18, no. 3, pp. 144–150, 2013.

[58] R. Frazzi, R. Valli, I. Tamagnini, B. Casali, N. Latruffe, and F. Merli, "Resveratrol-mediated apoptosis of hodgkin lymphoma cells involves SIRT1 inhibition and FOXO3a hyperacetylation," *International Journal of Cancer*, vol. 132, no. 5, pp. 1013–1021, 2013.

[59] S. Shankar, Q. Chen, I. Siddiqui, K. Sarva, and R. K. Srivastava, "Sensitization of TRAIL-resistant LNCaP cells by resveratrol (3, 4′, 5 tri-hydroxystilbene): molecular mechanisms and therapeutic potential," *Journal of Molecular Signaling*, vol. 2, article 7, 2014.

[60] P. R. van Ginkel, M. B. Yan, S. Bhattacharya, A. S. Polans, and J. D. Kenealey, "Natural products induce a G protein-mediated calcium pathway activating p53 in cancer cells," *Toxicology and Applied Pharmacology*, 2015.

[61] H.-Y. Lin, A. Shih, F. B. Davis et al., "Resveratrol induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line," *Journal of Urology*, vol. 168, no. 2, pp. 748–755, 2002.

[62] N. Singh, M. Nigam, V. Ranjan, R. Sharma, A. K. Balapure, and S. K. Rath, "Caspase mediated enhanced apoptotic action of cyclophosphamide- and resveratrol-treated MCF-7 cells," *Journal of Pharmacological Sciences*, vol. 109, no. 4, pp. 473–485, 2009.

[63] L. Kai, S. K. Samuel, and A. S. Levenson, "Resveratrol enhances p53 acetylation and apoptosis in prostate cancer by inhibiting MTA1/NuRD complex," *International Journal of Cancer*, vol. 126, no. 7, pp. 1538–1548, 2010.

[64] I. M. Kapetanovic, M. Muzzio, Z. Huang, T. N. Thompson, and D. L. McCormick, "Pharmacokinetics, oral bioavailability, and metabolic profile of resveratrol and its dimethylether analog, pterostilbene, in rats," *Cancer Chemotherapy and Pharmacology*, vol. 68, no. 3, pp. 593–601, 2011.

[65] H.-S. Lin, B.-D. Yue, and P. C. Ho, "Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study," *Biomedical Chromatography*, vol. 23, no. 12, pp. 1308–1315, 2009.

[66] A. M. Rimando, M. Cuendet, C. Desmarchelier, R. G. Mehta, J. M. Pezzuto, and S. O. Duke, "Cancer chemopreventive and antioxidant activities of pterostilbene, a naturally occurring analogue of resveratrol," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 12, pp. 3453–3457, 2002.

[67] L. A. Stivala, M. Savio, F. Carafoli et al., "Specific structural determinants are responsible for the antioxidant activity and the cell cycle effects of resveratrol," *The Journal of Biological Chemistry*, vol. 276, no. 25, pp. 22586–22594, 2001.

[68] D. McCormack and D. McFadden, "A review of pterostilbene antioxidant activity and disease modification," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 575482, 15 pages, 2013.

[69] P. Ferrer, M. Asensi, R. Segarra et al., "Association between pterostilbene and quercetin inhibits metastatic activity of B16 melanoma," *Neoplasia*, vol. 7, no. 1, pp. 37–47, 2005.

[70] C. M. Remsberg, J. A. Yáñez, Y. Ohgami, K. R. Vega-Villa, A. M. Rimando, and N. M. Davies, "Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity," *Phytotherapy Research*, vol. 22, no. 2, pp. 169–179, 2008.

[71] M. Tolomeo, S. Grimaudo, A. Di Cristina et al., "Pterostilbene and 3′-hydroxypterostilbene are effective apoptosis-inducing agents in MDR and BCR-ABL-expressing leukemia cells," *International Journal of Biochemistry and Cell Biology*, vol. 37, no. 8, pp. 1709–1726, 2005.

[72] J. A. Alosi, D. E. McDonald, J. S. Schneider, A. R. Privette, and D. W. McFadden, "Pterostilbene inhibits breast cancer in vitro through mitochondrial depolarization and induction of caspase-dependent apoptosis," *Journal of Surgical Research*, vol. 161, no. 2, pp. 195–201, 2010.

[73] Y. Wang, L. Ding, X. Wang et al., "Pterostilbene simultaneously induces apoptosis, cell cycle arrest and cyto-protective autophagy in breast cancer cells," *American Journal of Translational Research*, vol. 4, no. 1, pp. 44–51, 2012.

[74] D. Moon, D. McCormack, D. McDonald, and D. McFadden, "Pterostilbene induces mitochondrially derived apoptosis in breast cancer cells in vitro," *Journal of Surgical Research*, vol. 180, no. 2, pp. 208–215, 2013.

[75] J. G. Schneider, J. A. Alosi, D. E. McDonald, and D. W. McFadden, "Pterostilbene Inhibits Lung Cancer Through Induction of Apoptosis1," *Journal of Surgical Research*, vol. 161, no. 1, pp. 18–22, 2010.

[76] R.-J. Chen, S.-J. Tsai, C.-T. Ho et al., "Chemopreventive effects of pterostilbene on urethane-induced lung carcinogenesis in mice via the inhibition of EGFR-mediated pathways and the induction of apoptosis and autophagy," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 46, pp. 11533–11541, 2012.

[77] A. Chakraborty, N. Gupta, K. Ghosh, and P. Roy, "In vitro evaluation of the cytotoxic, anti-proliferative and anti-oxidant properties of pterostilbene isolated from *Pterocarpus marsupium*," *Toxicology in Vitro*, vol. 24, no. 4, pp. 1215–1228, 2010.

[78] D. E. McCormack, P. Mannal, D. McDonald, S. Tighe, J. Hanson, and D. McFadden, "Genomic analysis of pterostilbene predicts its antiproliferative effects against pancreatic cancer in vitro and in vivo," *Journal of Gastrointestinal Surgery*, vol. 16, no. 6, pp. 1136–1143, 2012.

[79] Y.-S. Chiou, M.-L. Tsai, K. Nagabhushanam et al., "Pterostilbene is more potent than resveratrol in preventing azoxymethane (AOM)-induced colon tumorigenesis via activation of the NF-E2-related factor 2 (Nrf2)-mediated antioxidant signaling pathway," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 6, pp. 2725–2733, 2011.

[80] S. Paul, A. M. Rimando, H. J. Lee, Y. Ji, B. S. Reddy, and N. Suh, "Anti-inflammatory action of pterostilbene is mediated through the p38 mitogen-activated protein kinase pathway in colon cancer cells," *Cancer Prevention Research*, vol. 2, no. 7, pp. 650–657, 2009.

[81] N. Suh, S. Paul, X. Hao et al., "Pterostilbene, an active constituent of blueberries, suppresses aberrant crypt foci formation

in the azoxymethane-induced colon carcinogenesis model in rats," *Clinical Cancer Research*, vol. 13, no. 1, pp. 350–355, 2007.

[82] M. Cichocki, J. Paluszczak, H. Szaefer, A. Piechowiak, A. M. Rimando, and W. Baer-Dubowska, "Pterostilbene is equally potent as resveratrol in inhibiting 12-O-tetradecanoylphorbol-13-acetate activated NFκB, AP-1, COX-2, and iNOS in mouse epidermis," *Molecular Nutrition and Food Research*, vol. 52, supplement 1, pp. S62–S70, 2008.

[83] S. Dhar, A. Kumar, A. M. Rimando, X. Zhang, and A. S. Levenson, "Resveratrol and pterostilbene epigenetically restore PTEN expression by targeting oncomiRs of the miR-17 family in prostate cancer," *Oncotarget*, vol. 6, no. 29, pp. 27214–27226, 2015.

[84] Y.-S. Chiou, M.-L. Tsai, Y.-J. Wang et al., "Pterostilbene inhibits colorectal aberrant crypt foci (ACF) and colon carcinogenesis via suppression of multiple signal transduction pathways in azoxymethane-treated mice," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 15, pp. 8833–8841, 2010.

[85] S. Paul, A. J. de Castro, H. J. Lee et al., "Dietary intake of pterostilbene, a constituent of blueberries, inhibits the beta-catenin/p65 downstream signaling pathway and colon carcinogenesis in rats," *Carcinogenesis*, vol. 31, no. 7, pp. 1272–1278, 2010.

[86] D. McCormack, J. Schneider, D. McDonald, and D. McFadden, "The antiproliferative effects of pterostilbene on breast cancer in vitro are via inhibition of constitutive and leptin-induced Janus kinase/signal transducer and activator of transcription activation," *The American Journal of Surgery*, vol. 202, no. 5, pp. 541–544, 2011.

[87] Y. Liu, L. Wang, Y. Wu et al., "Pterostilbene exerts antitumor activity against human osteosarcoma cells by inhibiting the JAK2/STAT3 signaling pathway," *Toxicology*, vol. 304, pp. 120–131, 2013.

[88] R.-J. Chen, C.-T. Ho, and Y.-J. Wang, "Pterostilbene induces autophagy and apoptosis in sensitive and chemoresistant human bladder cancer cells," *Molecular Nutrition and Food Research*, vol. 54, no. 12, pp. 1819–1832, 2010.

[89] A. Chakraborty, N. Bodipati, M. K. Demonacos, R. Peddinti, K. Ghosh, and P. Roy, "Long term induction by pterostilbene results in autophagy and cellular differentiation in MCF-7 cells via ROS dependent pathway," *Molecular and Cellular Endocrinology*, vol. 355, no. 1, pp. 25–40, 2012.

[90] N. Mizushima and T. Yoshimori, "How to interpret LC3 immunoblotting," *Autophagy*, vol. 3, no. 6, pp. 542–545, 2007.

[91] D. J. Klionsky, H. Abeliovich, P. Agostinis et al., "Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes," *Autophagy*, vol. 4, no. 2, pp. 151–175, 2008.

[92] K. Siedlecka-Kroplewska, A. Jozwik, W. Boguslawski et al., "Pterostilbene induces accumulation of autophagic vacuoles followed by cell death in HL60 human leukemia cells," *Journal of Physiology and Pharmacology*, vol. 64, no. 5, pp. 545–556, 2013.

[93] J. A. Magee, E. Piskounova, and S. J. Morrison, "Cancer stem cells: impact, heterogeneity, and uncertainty," *Cancer Cell*, vol. 21, no. 3, pp. 283–296, 2012.

[94] L. V. Nguyen, R. Vanner, P. Dirks, and C. J. Eaves, "Cancer stem cells: an evolving concept," *Nature Reviews Cancer*, vol. 12, no. 2, pp. 133–143, 2012.

[95] J. E. Visvader and G. J. Lindeman, "Cancer stem cells: current status and evolving complexities," *Cell Stem Cell*, vol. 10, no. 6, pp. 717–728, 2012.

[96] K. Hagiwara, N. Kosaka, Y. Yoshioka, R.-U. Takahashi, F. Takeshita, and T. Ochiya, "Stilbene derivatives promote Ago2-dependent tumour-suppressive microRNA activity," *Scientific Reports*, vol. 2, article 314, 2012.

[97] K.-K. Mak, A. T. H. Wu, W.-H. Lee et al., "Pterostilbene, a bioactive component of blueberries, suppresses the generation of breast cancer stem cells within tumor microenvironment and metastasis via modulating NF-κB/microRNA 448 circuit," *Molecular Nutrition and Food Research*, vol. 57, no. 7, pp. 1123–1134, 2013.

[98] C.-M. Lee, Y.-H. Su, T.-T. Huynh et al., "BlueBerry isolate, pterostilbene, functions as a potential anticancer stem cell agent in suppressing irradiation-mediated enrichment of hepatoma stem cells," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 258425, 9 pages, 2013.

[99] T.-C. Hsieh, D. J. Bennett, Y.-S. Lee, E. Wu, and J. M. Wu, "In silico and biochemical analyses identify quinone reductase 2 as a target of piceatannol," *Current Medicinal Chemistry*, vol. 20, no. 33, pp. 4195–4202, 2013.

[100] M. Murias, W. Jäger, N. Handler et al., "Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship," *Biochemical Pharmacology*, vol. 69, no. 6, pp. 903–912, 2005.

[101] H. Piotrowska, M. Kucinska, and M. Murias, "Biological activity of piceatannol: leaving the shadow of resveratrol," *Mutation Research/Reviews in Mutation Research*, vol. 750, no. 1, pp. 60–82, 2012.

[102] Z. Li, X. Yang, S. Dong, and X. Li, "DNa breakage induced by piceatannol and copper(II): mechanism and anticancer properties," *Oncology Letters*, vol. 3, no. 5, pp. 1087–1094, 2012.

[103] A. S. Azmi, S. H. Bhat, and S. M. Hadi, "Resveratrol-Cu(II) induced DNA breakage in human peripheral lymphocytes: implications for anticancer properties," *FEBS Letters*, vol. 579, no. 14, pp. 3131–3135, 2005.

[104] K. Ashikawa, S. Majumdar, S. Banerjee, A. C. Bharti, S. Shishodia, and B. B. Aggarwal, "Piceatannol inhibits TNF-induced NF-κB activation and NF-κB-mediated gene expression through suppression of IκBα kinase and p65 phosphorylation," *Journal of Immunology*, vol. 169, no. 11, pp. 6490–6497, 2002.

[105] S. Islam, F. Hassan, M. M. Mu et al., "Piceatannol prevents lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nuclear factor (NF)-kappaB activation by inhibiting IkappaB kinase (IKK)," *Microbiology and Immunology*, vol. 48, no. 10, pp. 729–736, 2004.

[106] C.-Y. Jin, D.-O. Moon, K.-J. Lee et al., "Piceatannol attenuates lipopolysaccharide-induced NF-κB activation and NF-κB-related proinflammatory mediators in BV2 microglia," *Pharmacological Research*, vol. 54, no. 6, pp. 461–467, 2006.

[107] H.-H. Lee, S.-A. Park, I. Almazari, E.-H. Kim, H.-K. Na, and Y.-J. Surh, "Piceatannol induces heme oxygenase-1 expression in human mammary epithelial cells through activation of ARE-driven Nrf2 signaling," *Archives of Biochemistry and Biophysics*, vol. 501, no. 1, pp. 142–150, 2010.

[108] E.-J. Park, H.-Y. Min, Y.-H. Ahn, C.-M. Bae, J.-H. Pyee, and S. K. Lee, "Synthesis and inhibitory effects of pinosylvin derivatives on prostaglandin E2 production in lipopolysaccharide-induced mouse macrophage cells," *Bioorganic and Medicinal Chemistry Letters*, vol. 14, no. 23, pp. 5895–5898, 2004.

[109] E.-J. Park, H.-Y. Min, H.-J. Chung, Y.-H. Ahn, J.-H. Pyee, and S. K. Lee, "Pinosylvin suppresses LPS-stimulated inducible nitric oxide synthase expression via the MyD88-independent, but

TRIF-dependent downregulation of IRF-3 signaling pathway in mouse macrophage cells," *Cellular Physiology and Biochemistry*, vol. 27, no. 3-4, pp. 353–362, 2011.

[110] J. A. Edwards, M. Beck, C. Riegger, and J. Bausch, "Safety of resveratrol with examples for high purity, trans-resveratrol, resVida," *Annals of the New York Academy of Sciences*, vol. 1215, pp. 131–137, 2011.

[111] L. D. Williams, G. A. Burdock, J. A. Edwards, M. Beck, and J. Bausch, "Safety studies conducted on high-purity trans-resveratrol in experimental animals," *Food and Chemical Toxicology*, vol. 47, no. 9, pp. 2170–2182, 2009.

[112] J. A. Crowell, P. J. Korytko, R. L. Morrissey, T. D. Booth, and B. S. Levine, "Resveratrol-associated renal toxicity," *Toxicological Sciences*, vol. 82, no. 2, pp. 614–619, 2004.

[113] L. Almeida, M. Vaz-da-Silva, A. Falcão et al., "Pharmacokinetic and safety profile of trans-resveratrol in a rising multiple-dose study in healthy volunteers," *Molecular Nutrition and Food Research*, vol. 53, supplement 1, pp. S7–S15, 2009.

[114] C. La Porte, N. Voduc, G. Zhang et al., "Steady-state pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (Ethanol) in healthy human subjects," *Clinical Pharmacokinetics*, vol. 49, no. 7, pp. 449–454, 2010.

[115] D. J. Boocock, G. E. S. Faust, K. R. Patel et al., "Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent," *Cancer Epidemiology Biomarkers and Prevention*, vol. 16, no. 6, pp. 1246–1252, 2007.

[116] V. A. Brown, K. R. Patel, M. Viskaduraki et al., "Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics, and effect on the insulin-like growth factor axis," *Cancer Research*, vol. 70, no. 22, pp. 9003–9011, 2010.

[117] K. R. Patel, V. A. Brown, D. J. L. Jones et al., "Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients," *Cancer Research*, vol. 70, no. 19, pp. 7392–7399, 2010.

[118] S. Priego, F. Feddi, P. Ferrer et al., "Natural polyphenols facilitate elimination of HT-29 colorectal cancer xenografts by chemoradiotherapy: a Bcl-2- and superoxide dismutase 2-dependent mechanism," *Molecular Cancer Therapeutics*, vol. 7, no. 10, pp. 3330–3342, 2008.

[119] K. Li, S. J. Dias, A. M. Rimando et al., "Pterostilbene acts through metastasis-associated protein 1 to inhibit tumor growth, progression and metastasis in prostate cancer," *PLoS ONE*, vol. 8, no. 3, Article ID e57542, 2013.

[120] M. J. Ruiz, M. Fernández, Y. Picó et al., "Dietary administration of high doses of pterostilbene and quercetin to mice is not toxic," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 8, pp. 3180–3186, 2009.

[121] D. M. Riche, C. L. McEwen, K. D. Riche et al., "Analysis of safety from a human clinical trial with pterostilbene," *Journal of Toxicology*, vol. 2013, Article ID 463595, 5 pages, 2013.

[122] S. Gao and M. Hu, "Bioavailability challenges associated with development of anti-cancer phenolics," *Mini-Reviews in Medicinal Chemistry*, vol. 10, no. 6, pp. 550–567, 2010.

[123] F. Visioli, C. A. De La Lastra, C. Andres-Lacueva et al., "Polyphenols and human health: a prospectus," *Critical Reviews in Food Science and Nutrition*, vol. 51, no. 6, pp. 524–546, 2011.

[124] M. Asensi, I. Medina, A. Ortega et al., "Inhibition of cancer growth by resveratrol is related to its low bioavailability," *Free Radical Biology and Medicine*, vol. 33, no. 3, pp. 387–398, 2002.

[125] D. M. Goldberg, J. Yan, and G. J. Soleas, "Absorption of three wine-related polyphenols in three different matrices by healthy subjects," *Clinical Biochemistry*, vol. 36, no. 1, pp. 79–87, 2003.

[126] T. Walle, F. Hsieh, M. H. DeLegge, J. E. Oatis Jr., and U. K. Walle, "High absorption but very low bioavailability of oral resveratrol in humans," *Drug Metabolism and Disposition*, vol. 32, no. 12, pp. 1377–1382, 2004.

[127] T. Walle, "Bioavailability of resveratrol," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 9–15, 2011.

[128] J.-F. Marier, P. Vachon, A. Gritsas, J. Zhang, J.-P. Moreau, and M. P. Ducharme, "Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model," *Journal of Pharmacology and Experimental Therapeutics*, vol. 302, no. 1, pp. 369–373, 2002.

[129] I. Alfaras, M. Emilia Juan, and J. M. Planas, "Trans-resveratrol reduces precancerous colonic lesions in dimethylhydrazine-treated rats," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 13, pp. 8104–8110, 2010.

[130] M. Azorín-Ortuño, M. J. Yáñez-Gascón, F. Vallejo et al., "Metabolites and tissue distribution of resveratrol in the pig," *Molecular Nutrition & Food Research*, vol. 55, no. 8, pp. 1154–1168, 2011.

[131] A. Burkon and V. Somoza, "Quantification of free and protein-bound trans-resveratrol metabolites and identification of trans-resveratrol-C/O-conjugated diglucuronides—two novel resveratrol metabolites in human plasma," *Molecular Nutrition and Food Research*, vol. 52, no. 5, pp. 549–557, 2008.

[132] M. Rotches-Ribalta, C. Andres-Lacueva, R. Estruch, E. Escribano, and M. Urpi-Sarda, "Pharmacokinetics of resveratrol metabolic profile in healthy humans after moderate consumption of red wine and grape extract tablets," *Pharmacological Research*, vol. 66, no. 5, pp. 375–382, 2012.

[133] C. Andres-Lacueva, M. T. MacArulla, M. Rotches-Ribalta et al., "Distribution of resveratrol metabolites in liver, adipose tissue, and skeletal muscle in rats fed different doses of this polyphenol," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 19, pp. 4833–4840, 2012.

[134] E. Wenzel, T. Soldo, H. Erbersdobler, and V. Somoza, "Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats," *Molecular Nutrition and Food Research*, vol. 49, no. 5, pp. 482–494, 2005.

[135] M. Azorín-Ortuño, M. J. Yañéz-Gascón, F. J. Pallarés et al., "Pharmacokinetic study of trans-resveratrol in adult pigs," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 20, pp. 11165–11171, 2010.

[136] M. Miksits, K. Wlcek, M. Svoboda et al., "Antitumor activity of resveratrol and its sulfated metabolites against human breast cancer cells," *Planta Medica*, vol. 75, no. 11, pp. 1227–1230, 2009.

[137] J. Hoshino, E.-J. Park, T. P. Kondratyuk et al., "Selective synthesis and biological evaluation of sulfate-conjugated resveratrol metabolites," *Journal of Medicinal Chemistry*, vol. 53, no. 13, pp. 5033–5043, 2010.

[138] C. E. Storniolo and J. J. Moreno, "Resveratrol metabolites have an antiproliferative effect on intestinal epithelial cancer cells," *Food Chemistry*, vol. 134, no. 3, pp. 1385–1391, 2012.

[139] D.-L. Lu, D.-J. Ding, W.-J. Yan et al., "Influence of glucuronidation and reduction modifications of resveratrol on its biological activities," *ChemBioChem*, vol. 14, no. 9, pp. 1094–1104, 2013.

[140] E. Polycarpou, L. B. Meira, S. Carrington, E. Tyrrell, H. Modjtahedi, and M. A. Carew, "Resveratrol 3-O-d-glucuronide

and resveratrol 4′-O-d-glucuronide inhibit colon cancer cell growth: evidence for a role of A3 adenosine receptors, cyclin D1 depletion, and G1 cell cycle arrest," *Molecular Nutrition and Food Research*, vol. 57, no. 10, pp. 1708–1717, 2013.

[141] V. Aires, E. Limagne, A. K. Cotte, N. Latruffe, F. Ghiringhelli, and D. Delmas, "Resveratrol metabolites inhibit human metastatic colon cancer cells progression and synergize with chemotherapeutic drugs to induce cell death," *Molecular Nutrition and Food Research*, vol. 57, no. 7, pp. 1170–1181, 2013.

[142] H. Sakamoto, H. Yokota, R. Kibe, Y. Sayama, and A. Yuasa, "Excretion of bisphenol A-glucuronide into the small intestine and deconjugation in the cecum of the rat," *Biochimica et Biophysica Acta*, vol. 1573, no. 2, pp. 171–176, 2002.

[143] H. Kong, Y. Kim, Y. Lee et al., "Sulfate-conjugated methylprednisolone: evaluation as a colon-specific methylprednisolone prodrug and comparison with sulfate-conjugated prednisolone and dexamethasone," *Journal of Drug Targeting*, vol. 17, no. 2, pp. 159–167, 2009.

[144] K. R. Patel, C. Andreadi, R. G. Britton et al., "Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence," *Science Translational Medicine*, vol. 5, no. 205, Article ID 205ra133, 2013.

[145] A. A. Gakh, N. Y. Anisimova, M. V. Kiselevsky et al., "Dihydro-resveratrol—a potent dietary polyphenol," *Bioorganic and Medicinal Chemistry Letters*, vol. 20, no. 20, pp. 6149–6151, 2010.

[146] S. C. M. Yeo, P. C. Ho, and H.-S. Lin, "Pharmacokinetics of pterostilbene in Sprague-Dawley rats: the impacts of aqueous solubility, fasting, dose escalation, and dosing route on bioavailability," *Molecular Nutrition and Food Research*, vol. 57, no. 6, pp. 1015–1025, 2013.

[147] R. W. Dellinger, A. M. G. Garcia, and F. L. Meyskens Jr., "Differences in the glucuronidation of resveratrol and pterostilbene: altered enzyme specificity and potential gender differences," *Drug Metabolism and Pharmacokinetics*, vol. 29, no. 2, pp. 112–119, 2014.

[148] J. M. Estrela, A. Ortega, S. Mena, M. L. Rodriguez, and M. Asensi, "Pterostilbene: biomedical applications," *Critical Reviews in Clinical Laboratory Sciences*, vol. 50, no. 3, pp. 65–78, 2013.

[149] X. Shao, X. Chen, V. Badmaev, C.-T. Ho, and S. Sang, "Structural identification of mouse urinary metabolites of pterostilbene using liquid chromatography/tandem mass spectrometry," *Rapid Communications in Mass Spectrometry*, vol. 24, no. 12, pp. 1770–1778, 2010.

[150] M. Azzolini, M. La Spina, A. Mattarei, C. Paradisi, M. Zoratti, and L. Biasutto, "Pharmacokinetics and tissue distribution of pterostilbene in the rat," *Molecular Nutrition and Food Research*, vol. 58, no. 11, pp. 2122–2132, 2014.

[151] K. A. Roupe, J. A. Yáñez, X. W. Teng, and N. M. Davies, "Pharmacokinetics of selected stilbenes: rhapontigenin, piceatannol and pinosylvin in rats," *Journal of Pharmacy and Pharmacology*, vol. 58, no. 11, pp. 1443–1450, 2006.

[152] K. Roupe, X. W. Teng, X. Fu, G. G. Meadows, and N. M. Davies, "Determination of piceatannol in rat serum and liver microsomes: pharmacokinetics and phase I and II biotransformation," *Biomedical Chromatography*, vol. 18, no. 8, pp. 486–491, 2004.

[153] Y. Setoguchi, Y. Oritani, R. Ito et al., "Absorption and metabolism of piceatannol in rats," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 12, pp. 2541–2548, 2014.

[154] K. Roupe, S. Halls, and N. M. Davies, "Determination and assay validation of pinosylvin in rat serum: application to drug metabolism and pharmacokinetics," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 38, no. 1, pp. 148–154, 2005.

[155] S. C. M. Yeo, W. Luo, J. Wu, P. C. Ho, and H.-S. Lin, "Quantification of pinosylvin in rat plasma by liquid chromatography-tandem mass spectrometry: application to a pre-clinical pharmacokinetic study," *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, vol. 931, pp. 68–74, 2013.

[156] M. Miksits, M. Sulyok, R. Schuhmacher, T. Szekeres, and W. Jäger, "In-vitro sulfation of piceatannol by human liver cytosol and recombinant sulfotransferases," *Journal of Pharmacy and Pharmacology*, vol. 61, no. 2, pp. 185–191, 2009.

[157] H.-S. Lin, C. Tringali, C. Spatafora, C. Wu, and P. C. Ho, "A simple and sensitive HPLC-UV method for the quantification of piceatannol analog trans-3,5,3′,4′-tetramethoxystilbene in rat plasma and its application for a pre-clinical pharmacokinetic study," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 51, no. 3, pp. 679–684, 2010.

[158] G. A. Potter, L. H. Patterson, E. Wanogho et al., "The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1b1," *British Journal of Cancer*, vol. 86, no. 5, pp. 774–778, 2002.

[159] S. Sale, R. G. Tunstall, K. C. Ruparelia, G. A. Potter, W. P. Steward, and A. J. Gescher, "Comparison of the effects of the chemopreventive agent resveratrol and its synthetic analog trans 3,4,5,4′-tetramethoxystilbene (DMU-212) on adenoma development in the Apc^{Min+} mouse and cyclooxygenase-2 in human-derived colon cancer cells," *International Journal of Cancer*, vol. 115, no. 2, pp. 194–201, 2005.

[160] J. Correa-Betanzo, E. Allen-Vercoe, J. McDonald, K. Schroeter, M. Corredig, and G. Paliyath, "Stability and biological activity of wild blueberry (*Vaccinium angustifolium*) polyphenols during simulated *in vitro* gastrointestinal digestion," *Food Chemistry*, vol. 165, pp. 522–531, 2014.

[161] S. Karakaya, "Bioavailability of phenolic compounds," *Critical Reviews in Food Science and Nutrition*, vol. 44, no. 6, pp. 453–464, 2004.

[162] B. B. Aggarwal, A. Bhardwaj, R. S. Aggarwal, N. P. Seeram, S. Shishodia, and Y. Takada, "Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies," *Anticancer Research*, vol. 24, no. 5, pp. 2783–2840, 2004.

[163] C. Manach, A. Mazur, and A. Scalbert, "Polyphenols and prevention of cardiovascular diseases," *Current Opinion in Lipidology*, vol. 16, no. 1, pp. 77–84, 2005.

[164] C. Manach, A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez, "Polyphenols: food sources and bioavailability," *American Journal of Clinical Nutrition*, vol. 79, no. 5, pp. 727–747, 2004.

[165] Y.-W. Shi, C.-P. Wang, L. Liu et al., "Antihyperuricemic and nephroprotective effects of resveratrol and its analogues in hyperuricemic mice," *Molecular Nutrition and Food Research*, vol. 56, no. 9, pp. 1433–1444, 2012.

[166] T. Bohn, "Dietary factors affecting polyphenol bioavailability," *Nutrition Reviews*, vol. 72, no. 7, pp. 429–452, 2014.

[167] T. T. H. Duong, B. Chami, A. C. McMahon et al., "Pre-treatment with the synthetic antioxidant T-butyl bisphenol protects cerebral tissues from experimental ischemia reperfusion injury," *Journal of Neurochemistry*, vol. 130, no. 6, pp. 733–747, 2014.

[168] T. Szekeres, P. Saiko, M. Fritzer-Szekeres, B. Djavan, and W. Jäger, "Chemopreventive effects of resveratrol and resveratrol

derivatives," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 89–95, 2011.

[169] M. Murias, N. Handler, T. Erker et al., "Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship," *Bioorganic & Medicinal Chemistry*, vol. 12, no. 21, pp. 5571–5578, 2004.

[170] T. Perečko, V. Jančinová, K. Drábiková, R. Nosál', and J. Harmatha, "Structure-efficiency relationship in derivatives of stilbene. Comparison of resveratrol, pinosylvin and pterostilbene," *Neuroendocrinology Letters*, vol. 29, no. 5, pp. 802–805, 2008.

[171] S.-Y. Kang, J. K. Lee, O. Choi et al., "Biosynthesis of methylated resveratrol analogs through the construction of an artificial biosynthetic pathway in *E. coli*," *BMC Biotechnology*, vol. 14, article 67, 2014.

[172] G. Wang, X. Guo, H. Chen et al., "A resveratrol analog, phoyunbene B, induces G2/M cell cycle arrest and apoptosis in HepG2 liver cancer cells," *Bioorganic and Medicinal Chemistry Letters*, vol. 22, no. 5, pp. 2114–2118, 2012.

[173] S. Sale, R. D. Verschoyle, D. Boocock et al., "Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue trans 3,4,5,4′-tetramethoxystilbene," *British Journal of Cancer*, vol. 90, no. 3, pp. 736–744, 2004.

[174] L. M. Howells, D. P. Berry, P. J. Elliott et al., "Phase I randomized, double-blind pilot study of micronized resveratrol (SRT501) in patients with hepatic metastases—safety, pharmacokinetics, and pharmacodynamics," *Cancer Prevention Research*, vol. 4, no. 9, pp. 1419–1425, 2011.

[175] M. A. Noguer, A. B. Cerezo, E. D. Navarro, and M. C. Garcia-Parrilla, "Intake of alcohol-free red wine modulates antioxidant enzyme activities in a human intervention study," *Pharmacological Research*, vol. 65, no. 6, pp. 609–614, 2012.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3297363, 12 pages
http://dx.doi.org/10.1155/2016/3297363

Research Article

Wild Raspberry Subjected to Simulated Gastrointestinal Digestion Improves the Protective Capacity against Ethyl Carbamate-Induced Oxidative Damage in Caco-2 Cells

Wei Chen,[1] Yang Xu,[1] Lingxia Zhang,[1] Ya Li,[2] and Xiaodong Zheng[1]

[1]*Department of Food Science and Nutrition, Zhejiang Key Laboratory for Agro-Food Processing, Zhejiang University, Hangzhou 310058, China*
[2]*College of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310035, China*

Correspondence should be addressed to Wei Chen; zjuchenwei@zju.edu.cn and Xiaodong Zheng; xdzheng@zju.edu.cn

Received 3 July 2015; Accepted 14 September 2015

Academic Editor: Hassan Obied

Ethyl carbamate (EC), a probable human carcinogen, occurs widely in many fermented foods. Previous studies indicated that EC-induced cytotoxicity was associated with oxidative stress. Wild raspberries are rich in polyphenolic compounds, which possess potent antioxidant activity. This study was conducted to investigate the protective effect of wild raspberry extracts produced before (RE) and after *in vitro* simulated gastrointestinal digestion (RD) on EC-induced oxidative damage in Caco-2 cells. Our primary data showed that ethyl carbamate could result in cytotoxicity and genotoxicity in Caco-2 cells and raspberry extract after digestion (RD) may be more effective than that before digestion (RE) in attenuating toxicity caused by ethyl carbamate. Further investigation by fluorescence microscope revealed that RD may significantly ameliorate EC-induced oxidative damage by scavenging the overproduction of intracellular reactive oxygen species (ROS), maintaining mitochondrial function and preventing glutathione (GSH) depletion. In addition, HPLC-ESI-MS results showed that the contents of identified polyphenolic compounds (esculin, kaempferol O-hexoside, and pelargonidin O-hexoside) were remarkably increased after digestion, which might be related to the better protective effect of RD. Overall, our results demonstrated that raspberry extract undergoing simulated gastrointestinal digestion may improve the protective effect against EC-induced oxidative damage in Caco-2 cells.

1. Introduction

Ethyl carbamate (EC) was initially identified as a carcinogen to animals in the 1940s and subsequently classified as a group 2A carcinogen that is probably carcinogenic to humans by IARC, a World Health Organization's International Agency for Research on Cancer [1]. It was noticeable that ethyl carbamate was detected in many fermented foods, particularly in yeast breads and alcoholic beverages [2]. Generally, the formation of EC in those fermented foods was based on the reaction between ethanol and nitrogen-containing compounds such as urea and citrulline [3]. Fermented foods, due to its good taste, have become an important part of human diets. However, recent studies carried out in mice have revealed that frequent exposure to EC may cause genotoxicity and cytotoxicity and even leads to cancer development [4–6]. Therefore, potential harm of EC on human health cannot be neglected.

Recently, accumulating evidence unveils that EC-induced toxicity is associated with cellular oxidative stress. For example, Chun et al. suggested that the excessive production of intracellular ROS induced by EC may create a persistent oxidative stress environment promoting lung epithelial cells transformation, thereby resulting in cancer development [7]. Polyphenols exist widely in foodstuffs including vegetables and fruits [8] and are capable of preventing oxidative damage-related diseases [9]. Therefore, increasing attentions have been paid to explore naturally occurring antioxidants derived from vegetables and fruits for protection against EC-induced toxicity.

Raspberry is well known to contain massive phytochemicals such as flavones, flavonols, anthocyanins, and phenolic acids [10], which could ameliorate cellular oxidative stress [9]. It is noticeable that foodstuff will pass through gastrointestinal tract and be exposed to gastrointestinal condition

before providing specific health benefits. As a consequence, some functional components may be transformed into other compounds with varied bioactivity [11]. Although aforementioned studies have addressed that raspberry extract possessed potent antioxidant activity, the simulated gastrointestinal condition should be taken into consideration to evaluate the real effect of raspberry extract *in vitro*. Therefore, our study aims to elucidate the protective effect of raspberry subjected to *in vitro* simulated gastrointestinal digestion on EC-induced oxidative stress in Caco-2 cells.

2. Materials and Methods

2.1. Materials and Reagents. Wild raspberry fruits (*Rubus hirsutus* Thunb.) were gathered from Siming Mountain in Zhejiang Province, China. Fresh fruits were washed with distilled water and the water absorbed on the surface of fruits was then dried in dust free environment at room temperature. After that, the fruits were screened through maturity, size, and integrity and then stored at −80°C in a refrigerator prior to use. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), dihydroethidium (DHE), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Nonyl Acridine Orange (NAO), Rhodamine 123 (Rh123), Hoechst 33258, Folin & Ciocalteu's phenol reagent, pepsin, pancreatin, and bile salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). Total superoxide dismutase assay kit and cell lysis buffer were purchased from Beyotime Institute of Biotechnology, Ltd. (Shanghai, China). All other reagents used were of analytical grade.

2.2. In Vitro Digestion. The *in vitro* digestion method previously described by Faller et al. [12] was modified and then used for simulating gastrointestinal condition in this work. Briefly, 20 g of wild raspberry fruit was homogenized and diluted to a volume of 20 mL with distilled water. For the gastric digestion stage, 5 M HCL was added to adjust pH value to 2, with the porcine pepsin (6000 units) added subsequently. The mixture was then incubated at 37°C in a shaking water bath for 1.5 h at 100 rpm. For the intestinal digestion stage, the pH of mixture undergoing gastric digestion was adjusted to 6.5 with 1 M sodium bicarbonate and then 5 mL of pancreatin was added (consisting of 25 mg/mL porcine bile salts and 4 mg/mL trypsin). Subsequently, pH was immediately adjusted to 7.4 using 1 M sodium hydroxide. Thereafter, the mixture was incubated at 37°C in a shaking water bath for 2 h at 100 rpm. At the end of incubation, the mixture was diluted to a volume of 25 mL with distilled water and then centrifuged at 5000 rpm for 6 min. Supernatant was collected, named as raspberry digesta (RD). The wild raspberry fruit without *in vitro* digestion was named as raspberry extract (RE). Both RE and RD were stored in −80°C for further investigation.

2.3. Chemical Characterization

2.3.1. Total Phenolics Determination. Total phenolic content was determined using Folin-Ciocalteau method. Briefly, 0.1 mL RE or RD solution diluted with 0.5 mL distilled water was mixed with 0.1 mL Folin-Ciocalteau reagent and then shaked in a water bath for 5 min. After that, 0.2 mL sodium carbonate was added to the mixture and then diluted to a final volume of 1 mL with distilled water before incubating at room temperature for 2 h. The absorbance of the mixture was measured at 760 nm. Gallic acid was used as a standard and results were expressed as mg of gallic acid equivalents (GAE) per 100 g fresh weight.

2.3.2. Total Flavonoids Determination. The colorimetric assay described by Zhishen et al. [13] was used to determine the total flavonoids content. Briefly, 0.04 mL sodium carbonate (5%, m/v), 0.5 mL RD solution, and 0.5 mL distilled water were mixed and then incubated for 5 min. After that, 0.04 mL aluminium nitrate (10%, m/v) was added and diluted to a final volume of 1 mL with distilled water. The mixture was incubated at room temperature for 15 min and its absorbance was measured at 510 nm. Rutin was used as a standard and the total flavonoids content was expressed as mg of rutin equivalents (RE) per 100 g fresh weight.

2.3.3. Phenolic Compounds Identification by HPLC-ESI-MS. Before analyzing by Waters UPLC system equipped with Promosil C18 column (4.6 × 250 mm, 5 μm) and a Triple-TOF Mass Spectrometry System (AB SCIEX, Triple-TOF 5600 plus Framingham, USA), wild raspberry extracts produced before and after *in vitro* digestion were filtered through 0.45 μm membrane. The eluent consisted of 0.1% formic acid aqueous solution (A) and methanol (B), and the elution process was performed by the following linear gradient: from 95% to 85.8% A for 20 min, from 85.8% to 40% A for 50 min, from 40% to 95% for 10 min, and then isocratic elution for 5 min. with the inject volume of 10 μL and flow rate of 0.8 mL/min. The MS conditions were listed as follows: detection was performed in negative ion modes at a temperature of 550°C and voltage of 4.5 KV, the scan range (m/z) ranged from 100 to 2000, and the UV detector set at 260 nm. Identification was based on the ion molecular mass, MS^2 and UV-visible spectra data. Through the peak area, the content of identified compounds in raspberry extracts was compared.

2.4. Cell Culture. Human Caco-2 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Caco-2 cells were cultured in RPMI 1640 medium (Gibco) containing 10% of the new calf serum, 100 units/mL penicillin, and 100 units/mL streptomycin and incubated in a humidified incubator with 5% CO_2 at 37°C.

For the control group, Caco-2 cells were incubated in the absence of EC, RE, and RD. For the EC group, Caco-2 cells were incubated only with EC for 24 h. For the RE group, Caco-2 cells were pretreated with RE for 2 h and then incubated with EC for 24 h. For the RD group, Caco-2 cells were pretreated with RD for 2 h and then incubated with EC for 24 h.

2.5. Cell Viability Assay. Cell viability was measured by the MTT method as previously described [14]. Briefly, the cells were seeded into 96-well cell culture plates, with the concentration of 5×10^3 cells/well. Cells, which were pretreated

with RE or RD for 2 h after incubation in cell culture plate for 24 h, were then cultured with ethyl carbamate (62.5 mM) for another 24 h. Subsequently, cells were incubated with MTT (0.5 mg/mL) for 4 h, and the generated formazan precipitate was dissolved with 150 μL of DMSO. Finally, the absorbance was measured at 490 nm using a Tecan infinite M200 microplate reader.

2.6. Determination of Intracellular Reactive Oxygen Species

2.6.1. DCF Fluorescence Assay. The fluorescent reaction that transmit nonfluorescent 2′,7′-dichlorofluorescein diacetate (H_2DCFDA, Invitrogen) to fluorescent 2′,7′-dichlorofluorescein (DCF) was used to detect the intracellular reactive oxygen species [15]. Caco-2 cells were seeded into 12-well cell culture plates at a concentration of 1×10^5 cells/well and cultured for 24 h. After that, cells were pretreated with RD (2 mg/mL) or RE (2 mg/mL) for 2 h and then incubated with ethyl carbamate (62.5 mM) for 24 h. Subsequently, cells were washed with PBS, collected and incubated with 10 μM DCFH-DA at 37°C for 30 min, and then washed with PBS again and immediately determined by fluorescence microscope. The results were expressed as mean DCF fluorescence intensity calculated by image analysis software ImageProPlus 6.0 (Media Cybernetics, Inc.) from six different microscopic fields.

2.6.2. DHE Fluorescence Assay. Intracellular superoxide anion radicals ($O_2^{\cdot-}$) were analyzed using DHE fluorescence assay [16]. In brief, Caco-2 cells were subjected to the same treatment procedure mentioned in Section 2.6.1. The collected cells were incubated with 10 μM DHE at 37°C for 30 min and then washed with PBS and immediately analyzed by fluorescence microscope. The results were expressed as mean DHE fluorescence intensity calculated by image analysis software ImageProPlus 6.0 from six different microscopic fields.

2.7. Determination of Cellular Glutathione (GSH). Cellular glutathione was determined based on a previously described method with slight modifications [16]. Briefly, Caco-2 cells were treated according to the process mentioned in Section 2.6.1. Then cells were collected and incubated with 50 μM NDA at 37°C for 30 min. After incubation with the fluorescence probe, cells were washed with PBS and analyzed by fluorescence microscope. The results were expressed as mean NDA fluorescence intensity calculated by image analysis software ImageProPlus 6.0 from six different microscopic fields.

2.8. Detection of Mitochondrial Membrane Potential (MMP). Mitochondrial membrane potential (MMP) was measured using the method described by Chen et al. with some slight modifications [14, 17]. Briefly, cells were treated as the same process mentioned in Section 2.6.1. The collected cells were incubated with 10 μg/mL RH123 at 37°C for 30 min. Then the cells were washed with PBS and immediately measured using fluorescence microscope. The results were expressed as mean RH123 fluorescence intensity calculated by image analysis software ImageProPlus 6.0 from six different microscopic fields.

2.9. Detection of Mitochondrial Membrane Lipid Peroxidation. According to the method previously described with some slight modifications [18], mitochondrial membrane lipid peroxidation was detected. Briefly, after treatment, the collected cells were incubated with 10 μM of NAO at 37°C for 30 min, washed with PBS, and then detected by fluorescence microscope. The results were expressed as mean NAO fluorescence intensity calculated by image analysis software ImageProPlus 6.0 from six different microscopic fields.

2.10. Detection of Cell Nucleus Stained with Hoechst 33258. Hoechst 33258, a DNA-bound fluorescence dye, was used to observe the cell nucleus morphology according to the method with some modification [19]. Briefly, after treatment, the collected cells were incubated with 10 μM of Hoechst 33258 at 37°C for 30 min. Then cells were washed with PBS and analyzed by fluorescence microscope.

2.11. Statistical Analysis. All experiments were carried out at least three times. The results were expressed as mean ± standard deviations (SD) and analyzed by one-way ANOVA using SPSS (version 19.0). $p < 0.05$ was considered to be significant.

3. Results and Discussion

3.1. Effect of RD on EC-Induced Cytotoxicity and Genotoxicity in Caco-2 Cells. Cytotoxicity and genotoxicity induced by ethyl carbamate towards human cells have been reported previously [7, 20]. In this work, MTT assay and Caco-2 cells model were employed to observe the cytotoxicity induced by EC. As shown in Figures 1(a) and 1(b), after incubation with 62.5 mM EC for 24 h, the cell viability was remarkably decreased to 74.10% ± 2.22% compared with that of control group (its cell viability is considered as 100%), which was in accordance with the results observed in RAW 264.7 cells by Chun et al. [7]. Therefore, Caco-2 cells model can be properly used to investigate the effect of RE or RD on EC-induced cytotoxicity. Before incubating with EC, Caco-2 cells were pretreated with RE (2 mg/mL) or RD (2 mg/mL) for 2 h. As we can see from Figure 1(b), the cell viability of RD pretreatment was increased by 23% compared with that of EC group, whereas that of RE pretreatment was only increased by 5.87%, which indicated that RD may effectively ameliorate EC-induced cytotoxicity. In addition, considering the genotoxicity generated by dietary EC exposure, the minor groove binder Hoechst 33258, which is known to sensitively bind the adenine-thymine rich sites of DNA, was used to detect the genotoxicity induced in Caco-2 cells and to evaluate the effect of RE or RD on EC-induced genotoxicity. It could be observed from Figure 1(c) that most of nucleus in EC group contained small bright blue dots representing chromatin condensation or nuclear fragmentation compared with control group [21], whereas RD treatment group showed

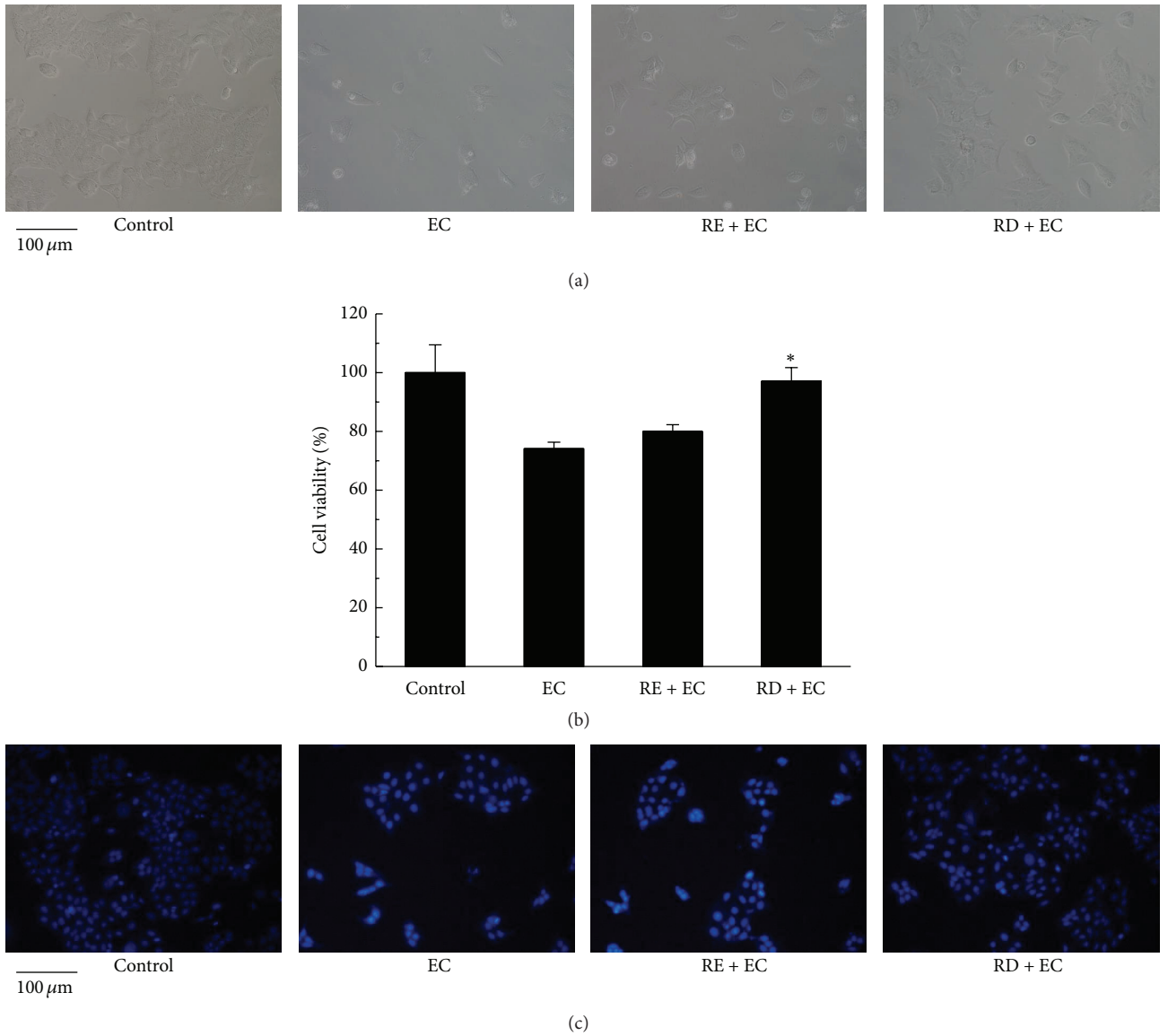


Figure 1: Effect of RD on EC-induced toxicity in Caco-2 cells. Caco-2 cells were incubated with 62.5 mM EC for 24 h in the presence or absence of RE (2 mg/mL) or RD (2 mg/mL). (a) Cell morphological image of Caco-2 cells. (b) The quantitative data of cell viability and results were expressed as mean percent (mean ± standard deviations). (c) Nuclear staining of Caco-2 cells with Hoechst 33258. $^{*}P < 0.05$ represents significant difference compared with EC group.

few bright blue dots, which demonstrated that RD pretreatment may exert a better performance in suppressing the toxicity to DNA induced by EC. Previous study unveiled that EC was metabolized to vinyl carbamate and then to vinyl carbamate epoxide *in vivo*; the latter can directly react with DNA, resulting in the DNA alkylation and the nucleic-acid adducts formation [22]. In our results, EC-induced DNA damage was ameliorated by treatment with RD. The possible explanation of this protective effect may be attributed to the reaction occurring between the metabolites and raspberry digesta.

3.2. RD Suppressed the Production of EC-Induced Reactive Oxygen Species. It was reported that EC-induced toxicity was related to the generation of ROS in lung epithelial cells [7]. On the basis of our result that EC could cause cytotoxicity in Caco-2 cell, we next studied whether EC could induce ROS overproduction in Caco-2 cells by incubation with DCFH-DA. The result displayed in Figures 2(a) and 2(b) showed that the DCF fluorescence intensity of EC group was increased to 392.76% compared with control group (its fluorescence intensity was considered as 100%), which indicated that large amount of ROS was accumulated in Caco-2 cells after EC treatment. Subsequently, we explored whether the overproduction of ROS could be scavenged by raspberry digesta (RD). As expected, comparing with that of EC group (392.76%), a sharp decrease of fluorescence intensity was observed in RE group and RD group,

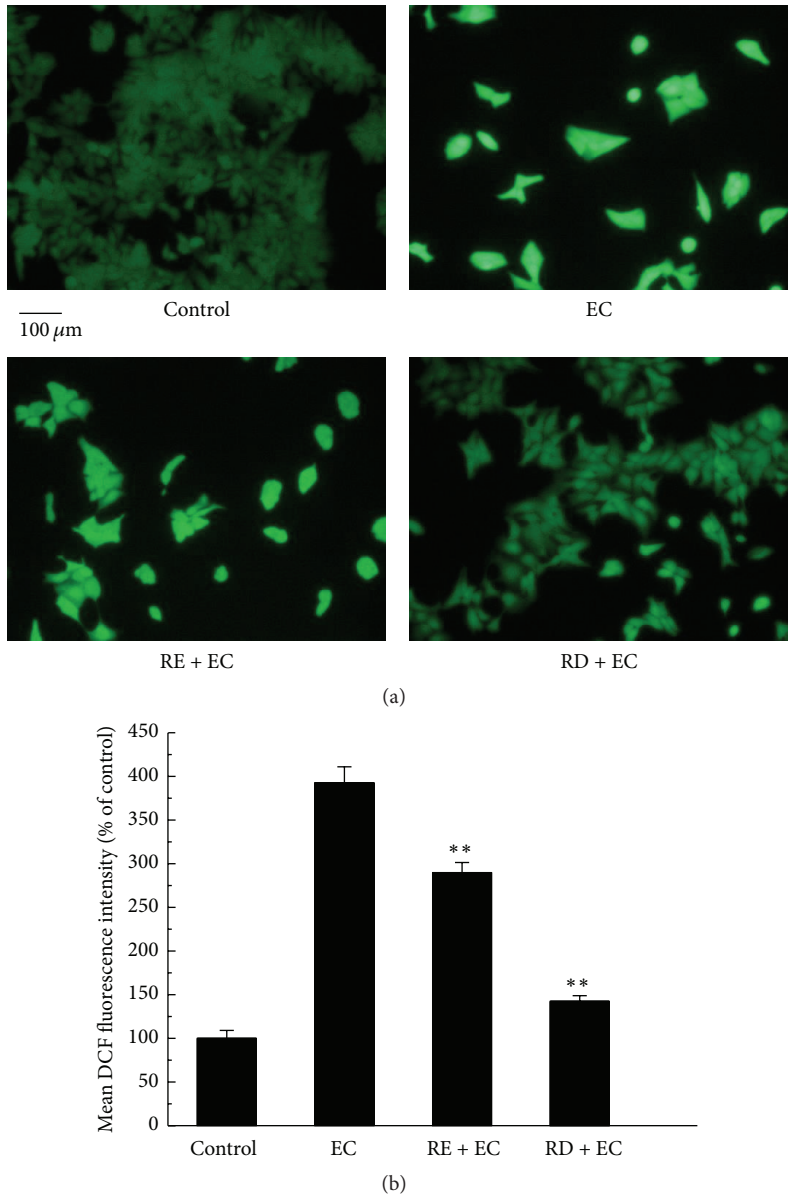


Figure 2: Effect of RD on EC-induced ROS generation in Caco-2 cells. (a) After treatment with 62.5 mM EC in the presence or absence of RE (2 mg/mL) or RD (2 mg/mL) for 24 h, cells were collected and incubated with 10 μM of DCF at 37°C for 30 min; then cells were washed with PBS and evaluated by fluorescence microscope. (b) The quantitative data of panel (a) and results were expressed as mean DCF fluorescence intensity (mean ± standard deviations). $^{*}P < 0.05$ represents significant difference compared with EC group.

with the mean fluorescence intensity declining to 289.83% and 142.40%, respectively. In addition, further study (DHE staining experiment) was employed to examine whether EC could induce the generation of intracellular superoxide anion radicals ($O_2^{\cdot-}$). Similar results were found and presented in Figures 3(a) and 3(b), in which Caco-2 cells pretreated with RE and RD significantly decreased the DHE fluorescence intensity to 215.88% and 120.41%, respectively, compared with solely EC-treated group (its fluorescence intensity reached 264.68%). The reason contributing to these phenomena may be the release of some bioactive phenolic components after *in vitro* digestion [23], since the structure of phenolic compounds that hydroxyl groups linked to phenolic rings were considered to donate electrons and neutralize reactive oxygen species [24]. As we detected that the total phenolic content was increased from 188.43 mg GAE/100 g to 254.60 mg GAE/100 g and the total flavonoid content increased from 78.30 mg RE/100 g to 103.32 mg RE/100 g after *in vitro* digestion. In conclusion, RD may be more effective than RE in terms of scavenging intracellular ROS and superoxide anion radicals in Caco-2 cells.

3.3. RD Improved EC-Induced Abrogation of Intracellular Glutathione (GSH). Reduced glutathione (GSH) is a key

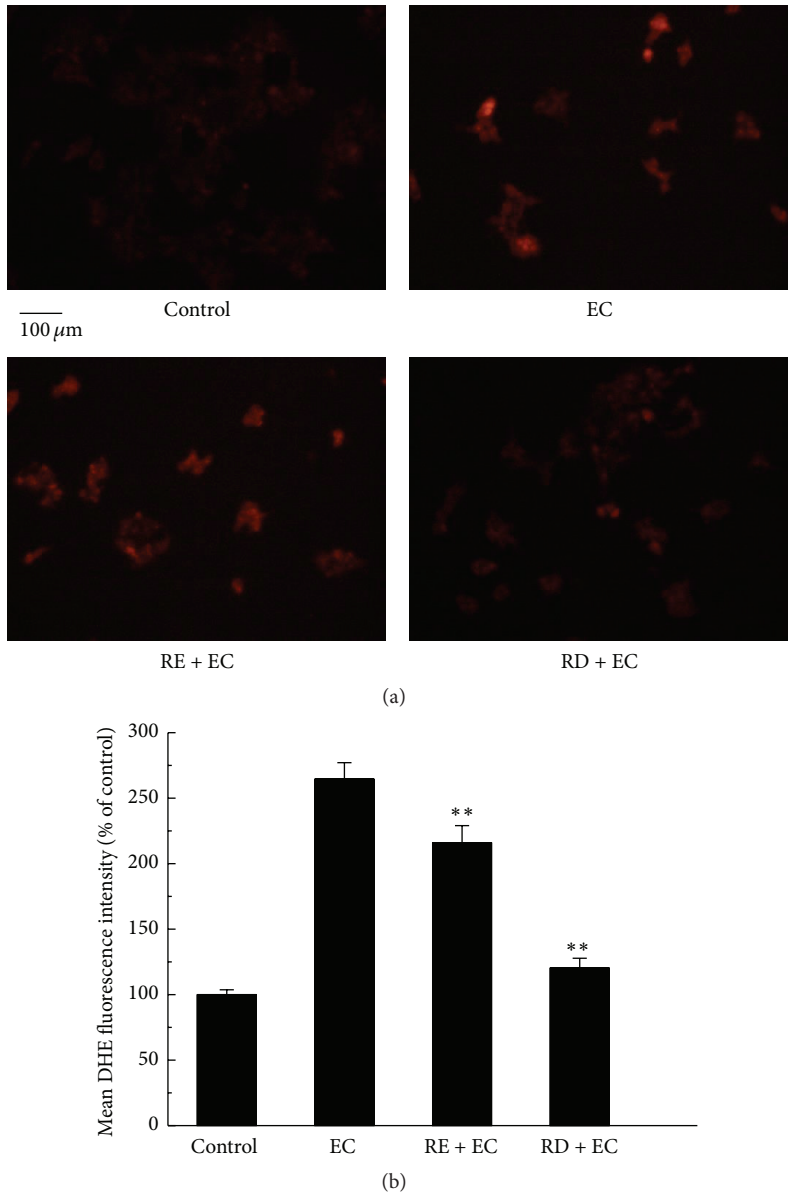


Figure 3: Effect of RD on EC-induced superoxide anion radicals generation in Caco-2 cells. (a) After treatment with 62.5 mM EC in the presence or absence of RE (2 mg/mL) or RD (2 mg/mL) for 24 h, cells were collected and incubated with 10 μM of DHE at 37°C for 30 min; then cells were washed with PBS and evaluated by fluorescence microscope. (b) The quantitative data of panel (a) and results were expressed as mean DHE fluorescence intensity (mean ± standard deviations). $^{*}P < 0.05$ represents significant difference compared with EC group.

nonenzymatic antioxidant and plays an important role in cellular redox reaction [25]. In several studies, GSH has been identified as an effectively protective agent against oxidative stress [26]. Based on the observation that RD could effectively scavenge intracellular ROS, we thus further investigated the role of RD on intracellular GSH content in the presence or absence of EC. In the present study, cellular GSH level was detected using NDA fluorescence probe. As shown in Figures 4(a) and 4(b), an evident depletion of GSH was observed in EC treatment group, whereas RE and RD pretreatment could effectively attenuate the abrogation of GSH induced by EC. The fluorescence intensities of EC, RE, and RD group were 30.35%, 35.54%, and 70.81%, respectively, compared with that of control group. Together, these results revealed that RD may afford protection against EC-induced glutathione (GSH) depletion.

3.4. RD Inhibited EC-Induced Oxidative Damage to Mitochondrial Membrane. Previous reports have revealed that generation of ROS was associated with mitochondrial membrane potential (MMP) collapse [27]. Due to EC-induced accumulation of intracellular ROS, we examined whether EC could cause mitochondrial dysfunction using RH123 fluorescence probe. As expected, the mean fluorescence

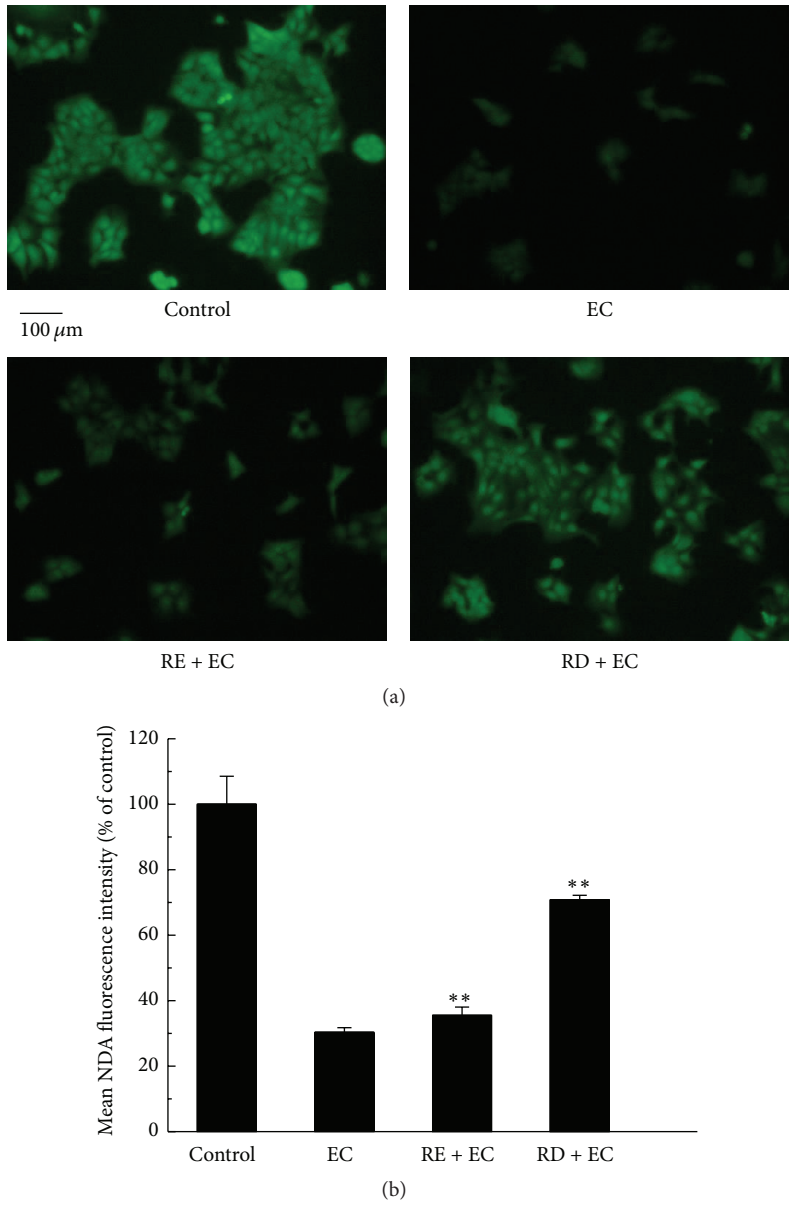


Figure 4: Effect of RD on EC-induced oxidative stress to GSH antioxidant systems in Caco-2 cells. (a) After treatment with 62.5 mM EC in the presence or absence of RE (2 mg/mL) or RD (2 mg/mL) for 24 h. Then cells were collected and incubated with 50 μM NDA at 37°C for 30 min and evaluated by fluorescence microscope. (b) The quantitative data of panel (a) and results were expressed as mean NDA fluorescence intensity (mean ± standard deviations). $^{*}P < 0.05$ represents significant difference compared with EC group.

intensity decreased to 34.49% compared with that of control group (Figures 5(a) and 5(b)), indicating that mitochondrial membrane potential was collapsed in exposure to EC (62.5 mM). We then explored the protective effect of RE or RD on mitochondrial membrane. As shown in Figures 5(a) and 5(b), a potent protective effect of RD was found against MMP collapse triggered by EC, with the mean fluorescence intensity increasing to 79.30% compared with that of EC group, whereas RE treatment only conferred a weak protection (its fluorescence intensity was 38.24%), suggesting that RD may be better than RE in preventing EC-induced mitochondrial membrane impairment.

In addition, it is reported that overproduction of ROS can result in mitochondrial membrane lipid peroxidation [28]. Thus, we further studied the effect of RD on suppressing EC-induced lipid peroxidation in Caco-2 cells. NAO, a fluorescence probe, was designed to detect cardiolipin which is a mitochondrial membrane lipid component and would be oxidized in the presence of ROS. As shown in Figures 6(a) and 6(b), without RE or RD pretreatment, massive cardiolipins were oxidized in exposure to EC, with the NAO fluorescence intensity declining to 45.46% compared with that of control group. Nevertheless, after treatment with RE or RD for 2 h, the result turned out to be that their fluorescence intensity

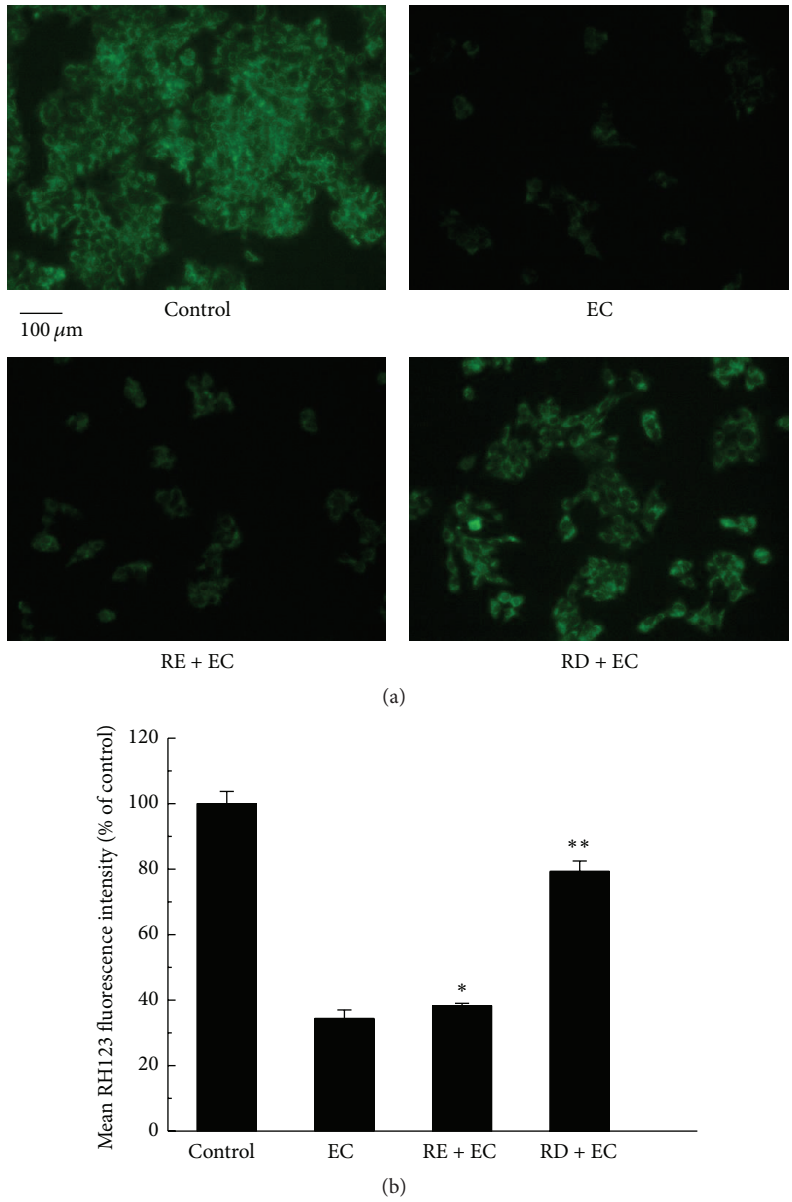


Figure 5: Effect of RD on EC-induced oxidative damage to mitochondrial membrane in Caco-2 cells. (a) After treatment with 62.5 mM EC in the presence or absence of RE (2 mg/mL) or RD (2 mg/mL) for 24 h, Caco-2 cells were incubated with 10 μg/mL RH123 for 30 min and then adopted to fluorescence microscope analysis. (b) The quantitative data of panel (a) and results were expressed as mean RH123 fluorescence intensity (mean ± standard deviations). $^{*}P < 0.05$ represents significant difference compared with EC group.

increased to 55.47% and 84.70%, respectively, compared with that of EC group (45.46%). On the basis of these results, it can be concluded that RD may afford protection against EC-induced oxidative damage to mitochondrial membrane.

3.5. Identification of Phenolic Compounds in RE and RD. The aforementioned results revealed that EC could result in oxidative stress damage in Caco-2 cells, whereas RD were more effective than RE in attenuating EC-induced oxidative damage. The possible explanation for this phenomenon may be due to structural modification or release of biological active components which were entrapped in food matrix after *in vitro* simulated gastrointestinal digestion [12, 23]. Therefore, HPLC-ESI-MS was employed to analyze the composition of raspberry extracts produced before and after *in vitro* simulated gastrointestinal digestion. HPLC chromatograms displayed the major changes between RE and RD extracts in terms of the content of identified compounds. As we can see from Figures 7(a) and 7(b), the contents of three identified compounds were increased significantly after *in vitro* digestion. According to their MS and MS^2 data, these compounds were identified as esculin (compound 1) [29], kaempferol O-hexoside (compound 2) [30], and pelargonidin O-hexoside (compound 3) [31], respectively. Berries are rich

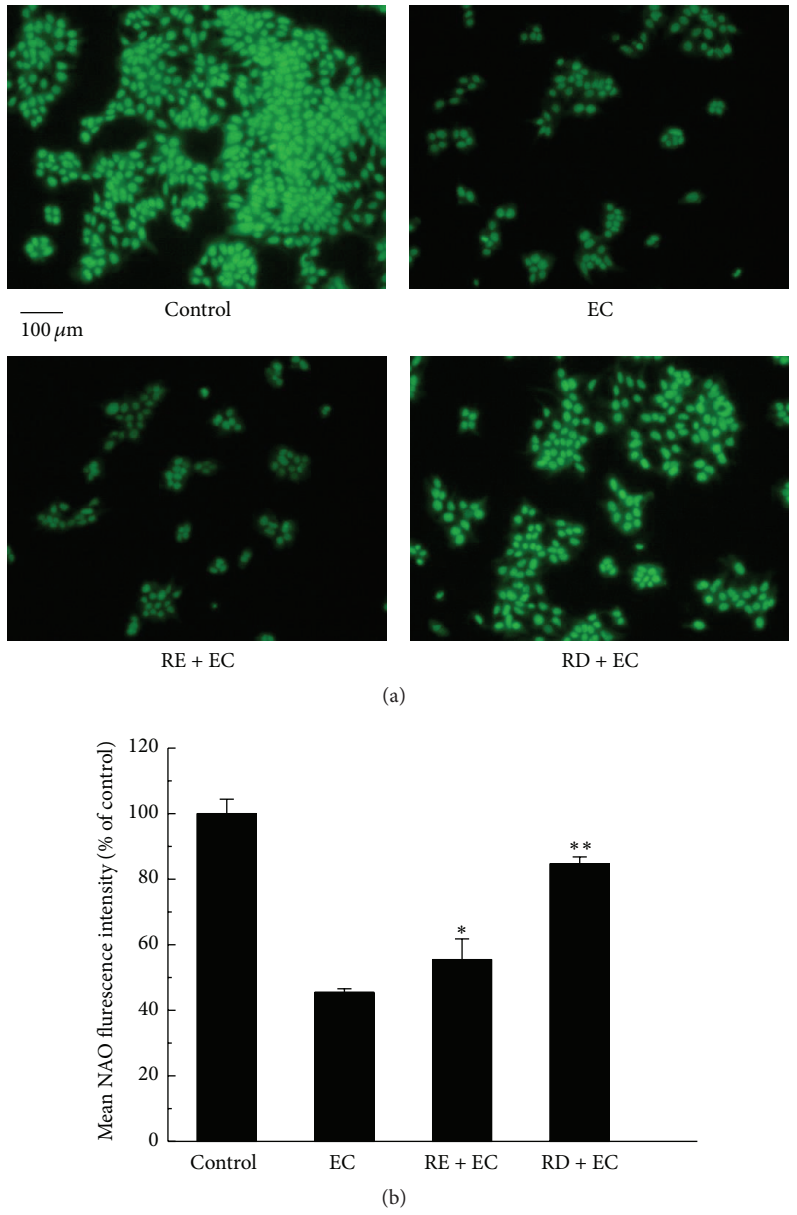


Figure 6: Effect of RD on EC-induced oxidative damage to mitochondrial membrane in Caco-2 cells. (a) After treatment with 62.5 mM EC in the presence or absence of RE (2 mg/mL) or RD (2 mg/mL) for 24 h, Caco-2 cells were incubated with 10 μM NAO for 30 min and subsequently adopted to fluorescence microscope analysis. (b) The quantitative data of panel (a) and results were expressed as mean NAO fluorescence intensity (mean ± standard deviations). $^{*}p < 0.05$ represents significant difference compared with EC group.

in phytochemicals, such as anthocyanins, flavonoids, and various phenolic acids, which can provide potent protection against oxidative damage [32, 33]. Kaempferol O-hexoside and pelargonidin O-hexoside are common phytochemicals in berries; they can effectively remove intracellular ROS and increase the total antioxidant activity [8, 34]. Besides, esculin was a novel compound identified in the wild raspberry extract and has been reported to be effective in protecting cells against DNA damage triggered by oxidative stress and scavenging ROS levels [35]. Flavonoids and anthocyanins are mainly located in the vacuoles of cells. Under the simulated gastrointestinal digestion condition, the extreme pH and the enzymatic digestion may fully break down raspberry matrix and vacuoles [36], which leads to the release of these compounds completely. Moreover, some reports considered that the formation of glycoside was increased after digestion probably due to the partial digestion of the dietary fiber present in the matrix [37], which may be beneficial for the formation of anthocyanins including compound 3 (pelargonidin O-hexoside). Overall, we concluded that the better performance of RD in suppressing EC-induced oxidative damage might be partially attributed to these three compounds.

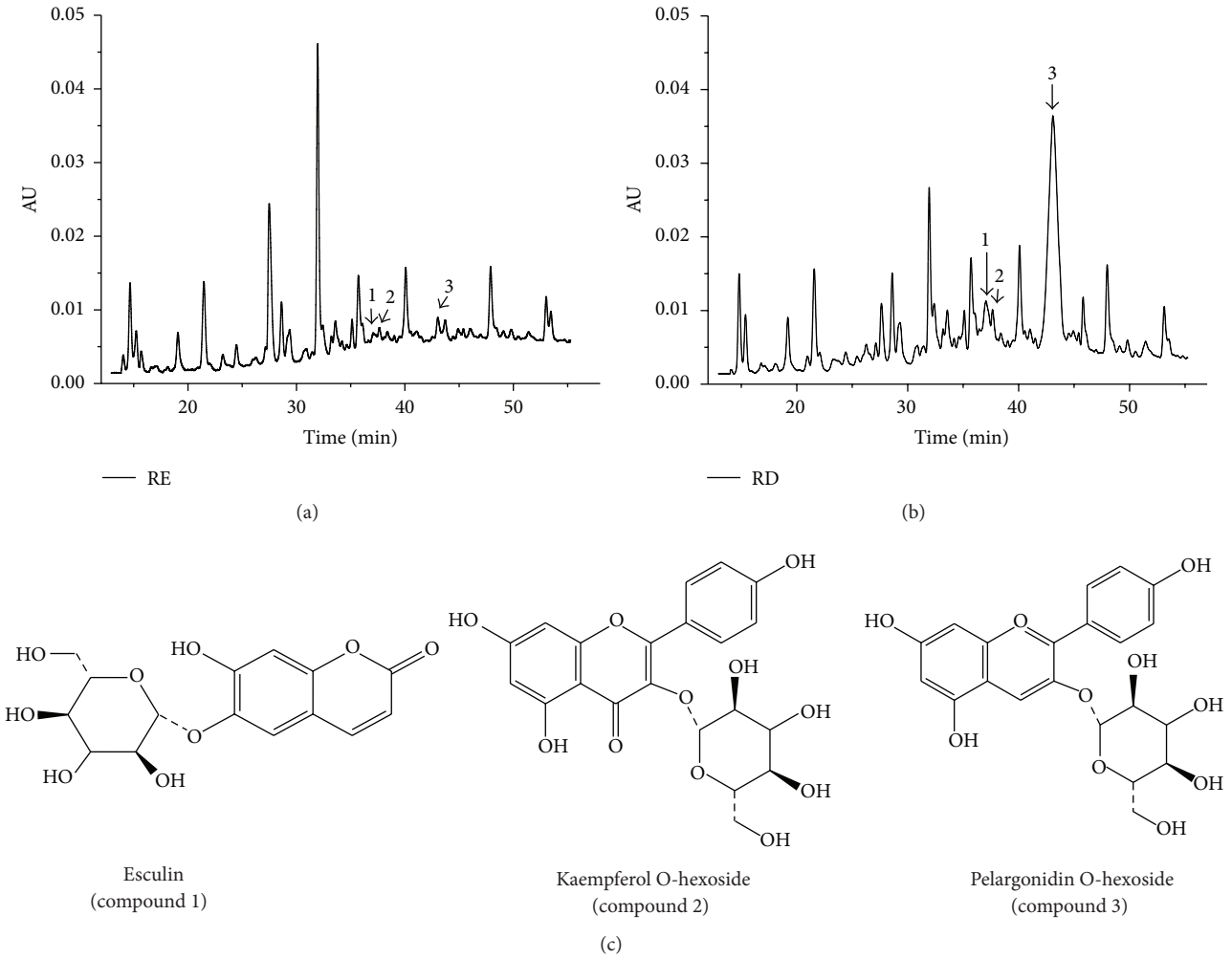


Figure 7: HPLC chromatograms of raspberry extracts produced before and after *simulated* gastrointestinal digestion. (a) Chromatogram of RE. (b) Chromatogram of RD. (c) Chemical structures of identified compounds.

4. Conclusion

The present study, for the first time, revealed that EC induced excessive production of intracellular ROS, accelerated GSH depletion and caused mitochondrial dysfunction, which resulted in disturbed cellular redox balance and oxidative damage in Caco-2 cells. Further investigation indicated that *in vitro* simulated gastrointestinal may enhance the ability of wild raspberry extract to suppress EC-induced oxidative stress in Caco-2 cells by scavenging intracellular ROS, preventing GSH depletion, as well as maintaining mitochondrial membrane potential. Moreover, LC-MS results showed that the contents of identified compounds (esculin, kaempferol O-hexoside, and pelargonidin O-hexoside) were significantly increased after *in vitro* digestion, which might be associated with a better biological activity of RD in ameliorating EC-induced oxidative stress. Together, our study indicated that raspberry undergoinggastrointestinal digestion improved its bioactivity, which might have implication for preventing EC-caused health problem.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

This work was supported by Grants from National Key Technology R&D Program of China (no. 2012BAD33B08), Qianjiang Scholars Program of Zhejiang Province (no. 2013R10044), Zhejiang Provincial Natural Science Foundation of China (no. LY13C200003), the Foundation of Fuli Institute of Food Science, Zhejiang University (2013), and the Foundation of Food Science and Engineering, the most important discipline of Zhejiang province (JYTSP20142012).

References

[1] D. W. Lachenmeier, F. Kanteres, T. Kuballa, M. G. López, and J. Rehm, "Ethyl carbamate in alcoholic beverages from Mexico (tequila, mezcal, bacanora, sotol) and Guatemala (cuxa): market

survey and risk assessment," *International Journal of Environmental Research and Public Health*, vol. 6, no. 1, pp. 349–360, 2009.

[2] U. Hoffler, D. Dixon, S. Peddada, and B. I. Ghanayem, "Inhibition of urethane-induced genotoxicity and cell proliferation in CYP2E1-null mice," *Mutation Research—Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 572, no. 1-2, pp. 58–72, 2005.

[3] D. W. Lachenmeier, M. C. P. Lima, I. C. C. Nóbrega et al., "Cancer risk assessment of ethyl carbamate in alcoholic beverages from Brazil with special consideration to the spirits cachaça and tiquira," *BMC Cancer*, vol. 10, article 266, 2010.

[4] A. K. Bauer, H.-Y. Cho, L. Miller-DeGraff et al., "Targeted deletion of Nrf2 reduces urethane-induced lung tumor development in mice," *PLoS ONE*, vol. 6, no. 10, Article ID e26590, 2011.

[5] T. Nomura, "Urethan (ethyl carbamate) as a cosolvent of drugs commonly used parenterally in humans," *Cancer Research*, vol. 35, no. 10, pp. 2895–2899, 1975.

[6] E. P. O'Donnell, L. K. Zerbe, L. D. Dwyer-Nield, L. R. Kisley, and A. M. Malkinson, "Quantitative analysis of early chemically-induced pulmonary lesions in mice of varying susceptibilities to lung tumorigenesis," *Cancer Letters*, vol. 241, no. 2, pp. 197–202, 2006.

[7] S.-H. Chun, Y.-N. Cha, and C. Kim, "Urethane increases reactive oxygen species and activates extracellular signal-regulated kinase in RAW 264.7 macrophages and A549 lung epithelial cells," *Archives of Pharmacal Research*, vol. 36, no. 6, pp. 775–782, 2013.

[8] C. L. L. Saw, Y. Guo, A. Y. Yang et al., "The berry constituents quercetin, kaempferol, and pterostilbene synergistically attenuate reactive oxygen species: involvement of the Nrf2-ARE signaling pathway," *Food and Chemical Toxicology*, vol. 72, pp. 303–311, 2014.

[9] W. Chen, H. Su, Z. Huang, L. Feng, and H. Nie, "Neuroprotective effect of raspberry extract by inhibiting peroxynitrite-induced DNA damage and hydroxyl radical formation," *Food Research International*, vol. 49, no. 1, pp. 22–26, 2012.

[10] P. Zafrilla, F. Ferreres, and F. A. Tomás-Barberán, "Effect of processing and storage on the antioxidant ellagic acid derivatives and flavonoids of red raspberry (*Rubus idaeus*) jams," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 8, pp. 3651–3655, 2001.

[11] B. Cerdá, J. C. Espín, S. Parra, P. Martínez, and F. A. Tomás-Barberán, "The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans," *European Journal of Nutrition*, vol. 43, no. 4, pp. 205–220, 2004.

[12] A. L. K. Faller, E. Fialho, and R. H. Liu, "Cellular antioxidant activity of *Feijoada* whole meal coupled with an in vitro digestion," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 19, pp. 4826–4832, 2012.

[13] J. Zhishen, T. Mengcheng, and W. Jianming, "The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals," *Food Chemistry*, vol. 64, no. 4, pp. 555–559, 1999.

[14] W. Chen, Z. Zhao, L. Li et al., "Hispolon induces apoptosis in human gastric cancer cells through a ROS-mediated mitochondrial pathway," *Free Radical Biology and Medicine*, vol. 45, no. 1, pp. 60–72, 2008.

[15] K. L. Wolfe and H. L. Rui, "Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 22, pp. 8896–8907, 2007.

[16] O. Orwar, H. A. Fishman, N. E. Ziv, R. H. Scheller, and R. N. Zare, "Use of 2,3-naphthalenedicarboxaldehyde derivatization for single-cell analysis of glutathione by capillary electrophoresis and histochemical localization by fluorescence microscopy," *Analytical Chemistry*, vol. 67, no. 23, pp. 4261–4268, 1995.

[17] W. Chen, L. Feng, H. Nie, and X. Zheng, "Andrographolide induces autophagic cell death in human liver cancer cells through cyclophilin D-mediated mitochondrial permeability transition pore," *Carcinogenesis*, vol. 33, no. 11, pp. 2190–2198, 2012.

[18] D. Trachootham, Y. Zhou, H. Zhang et al., "Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by β-phenylethyl isothiocyanate," *Cancer Cell*, vol. 10, no. 3, pp. 241–252, 2006.

[19] M.-C. Kang, K.-N. Kim, W. A. J. P. Wijesinghe, X. Yang, G. Ahn, and Y.-J. Jeon, "Protective effect of polyphenol extracted from Ecklonia cava against ethanol induced oxidative damage in vitro and in zebrafish model," *Journal of Functional Foods*, vol. 6, no. 1, pp. 339–347, 2014.

[20] R. M. Balansky and S. De Flora, "Chemoprevention by N-acetylcysteine of urethane-induced clastogenicity and lung tumors in mice," *International Journal of Cancer*, vol. 77, no. 2, pp. 302–305, 1998.

[21] L. N. Cariddi, M. C. Sabini, F. M. Escobar et al., "*In vitro* and *in vivo* cytogenotoxic effects of hot aqueous extract of *Achyrocline satureioides* (Lam.) DC," *BioMed Research International*, vol. 2015, Article ID 270973, 12 pages, 2015.

[22] P.-G. Forkert, "Mechanisms of lung tumorigenesis by ethyl carbamate and vinyl carbamate," *Drug Metabolism Reviews*, vol. 42, no. 2, pp. 355–378, 2010.

[23] H. Huang, Y. Sun, S. Lou, and X. Ye, "*In vitro* digestion combined with cellular assay to determine the antioxidant activity in Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) fruits: a comparison with traditional methods," *Food Chemistry*, vol. 146, pp. 363–370, 2014.

[24] J. B. Harborne, "Nature, distribution and function of plant flavonoids," *Progress in Clinical and Biological Research*, vol. 213, pp. 15–24, 1986.

[25] S. Shila, M. Subathra, M. A. Devi, and C. Panneerselvam, "Arsenic intoxication-induced reduction of glutathione level and of the activity of related enzymes in rat brain regions: reversal by dl-α-lipoic acid," *Archives of Toxicology*, vol. 79, no. 3, pp. 140–146, 2005.

[26] B. D'Autréaux and M. B. Toledano, "ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 10, pp. 813–824, 2007.

[27] E. Trushina and C. T. McMurray, "Oxidative stress and mitochondrial dysfunction in neurodegenerative diseases," *Neuroscience*, vol. 145, no. 4, pp. 1233–1248, 2007.

[28] R. Preeta and R. R. Nair, "Stimulation of cardiac fibroblast proliferation by cerium: a superoxide anion-mediated response," *Journal of Molecular and Cellular Cardiology*, vol. 31, no. 8, pp. 1573–1580, 1999.

[29] D. Mišić, B. Šiler, U. Gašić et al., "Simultaneous UHPLC/DAD/(+/−)HESI-MS/MS analysis of phenolic acids and nepetalactones in methanol extracts of nepeta species: a possible application in chemotaxonomic studies," *Phytochemical Analysis*, vol. 26, no. 1, pp. 72–85, 2015.

[30] H. Olsen, K. Aaby, and G. I. A. Borge, "Characterization and quantification of flavonoids and hydroxycinnamic acids in curly kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica*) by HPLC-DAD-ESI-MSn," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 7, pp. 2816–2825, 2009.

[31] F. Lopes-Da-Silva, S. de Pascual-Teresa, J. Rivas-Gonzalo, and C. Santos-Buelga, "Identification of anthocyanin pigments in strawberry (cv Camarosa) by LC using DAD and ESI-MS detection," *European Food Research and Technology*, vol. 214, no. 3, pp. 248–253, 2002.

[32] G. Borges, A. Degeneve, W. Mullen, and A. Crozier, "Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 7, pp. 3901–3909, 2010.

[33] C. C. Neto, "Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases," *Molecular Nutrition & Food Research*, vol. 51, no. 6, pp. 652–664, 2007.

[34] K. H. Han, T. Kitano-Okada, J. M. Seo et al., "Characterisation of anthocyanins and proanthocyanidins of adzuki bean extracts and their antioxidant activity," *Journal of Functional Foods*, vol. 14, pp. 692–701, 2015.

[35] T. Kaneko, S. Tahara, and F. Takabayashi, "Suppression of lipid hydroperoxide-induced oxidative damage to cellular DNA by esculetin," *Biological and Pharmaceutical Bulletin*, vol. 26, no. 6, pp. 840–844, 2003.

[36] J. M. Hernández-Hierro, N. Quijada-Morín, L. Martínez-Lapuente et al., "Relationship between skin cell wall composition and anthocyanin extractability of *Vitis vinifera* L. cv. Tempranillo at different grape ripeness degree," *Food Chemistry*, vol. 146, pp. 41–47, 2014.

[37] N. Ortega, J. Reguant, M.-P. Romero, A. Macià, and M.-J. Motilva, "Effect of fat content on the digestibility and bioaccessibility of cocoa polyphenol by an *in vitro* digestion model," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 13, pp. 5743–5749, 2009.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5296271, 11 pages
http://dx.doi.org/10.1155/2016/5296271

Research Article

Low-Dose *Aronia melanocarpa* Concentrate Attenuates Paraquat-Induced Neurotoxicity

A. J. Case,[1] D. Agraz,[1] I. M. Ahmad,[2] and M. C. Zimmerman[1,3]

[1]*Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE 68198-5850, USA*
[2]*College of Allied Health Professions, University of Nebraska Medical Center, Omaha, NE 68198, USA*
[3]*Redox Biology Center, University of Nebraska Lincoln, Lincoln, NE 68588, USA*

Correspondence should be addressed to M. C. Zimmerman; mczimmerman@unmc.edu

Received 25 June 2015; Revised 26 August 2015; Accepted 30 August 2015

Academic Editor: Wei Chen

Herbicides containing paraquat may contribute to the pathogenesis of neurodegenerative disorders such as Parkinson's disease. Paraquat induces reactive oxygen species-mediated apoptosis in neurons, which is a primary mechanism behind its toxicity. We sought to test the effectiveness of a commercially available polyphenol-rich *Aronia melanocarpa* (aronia berry) concentrate in the amelioration of paraquat-induced neurotoxicity. Considering the abundance of antioxidants in aronia berries, we hypothesized that aronia berry concentrate attenuates the paraquat-induced increase in reactive oxygen species and protects against paraquat-mediated neuronal cell death. Using a neuronal cell culture model, we observed that low doses of aronia berry concentrate protected against paraquat-mediated neurotoxicity. Additionally, low doses of the concentrate attenuated the paraquat-induced increase in superoxide, hydrogen peroxide, and oxidized glutathione levels. Interestingly, high doses of aronia berry concentrate increased neuronal superoxide levels independent of paraquat, while at the same time decreasing hydrogen peroxide. Moreover, high-dose aronia berry concentrate potentiated paraquat-induced superoxide production and neuronal cell death. In summary, aronia berry concentrate at low doses restores the homeostatic redox environment of neurons treated with paraquat, while high doses exacerbate the imbalance leading to further cell death. Our findings support that moderate levels of aronia berry concentrate may prevent reactive oxygen species-mediated neurotoxicity.

1. Introduction

Neurodegeneration is a hallmark of numerous neurological disorders such as age-related dementia, Alzheimer's disease, and Parkinson's disease [1]. While several etiologies have been identified leading to the loss of neurons, one possible contributing factor is contact with environmental toxins [2]. A major source of these poisons in rural farming areas is insecticides and herbicides, and exposure to these has been suggested as a major risk factor for neurological diseases such as Parkinson's disease [3, 4]. One commonly used compound in herbicides is paraquat (PQ), and extensive research has demonstrated a direct link between neurotoxicity and PQ contact [5–7]. PQ is a known redox cycling agent that impacts complex I activity of the mitochondria, increases superoxide ($O_2^{\bullet-}$) production, and decreases endogenous antioxidant capacity leading to increased neurotoxicity through apoptosis [8, 9]. Several studies have examined the effects of single antioxidant supplementation in the amelioration of PQ-induced neurotoxicity [10–12], but to date it remains unclear how combinations of small molecule antioxidants gained through dietary or nutritional means affect this toxin-mediated neuron loss.

Aronia melanocarpa, also known as black chokeberries or simply aronia berries, are small, dark, cherry-like berries belonging to the plant family Rosaceae [13]. Aronia berries are native to Eastern Europe and the Eastern United States but have recently become cultivated in large quantities by Midwest farmers. The berries have garnered much attention by the general public due to their significantly high quantity of polyphenols, in particular anthocyanins and flavonoids, which are estimated at 2-3 times greater amounts than in comparable berries [14, 15]. Polyphenols, such as resveratrol and quercetin, have been shown to possess significant

antioxidant properties by both directly scavenging reactive oxygen species (ROS) and inducing cellular antioxidant systems to help combat oxidative environments [15]. Aronia berries are no exception, and a widespread literature exists examining the potential beneficial effect of aronia berries on diseases including hypercholesterolemia, cancer, diabetes, and inflammation [16–19]. However, the vast majority of these studies only examine enriched extracts of the polyphenols from aronia berries and not the effects of the whole berry or berry concentrate in the disease models. Moreover, a dearth of studies exists examining the potential beneficial effects of aronia berries on diseases affecting the nervous system.

Herein, we tested the hypothesis that polyphenolic-rich aronia berry concentrate (AB) has antioxidant protective effects against ROS-induced neurotoxicity by PQ. Utilizing a neuronal cell culture model, we indeed demonstrate AB protects against PQ-induced cellular toxicity and an increase in ROS. However, we show that only low doses of AB demonstrate this protective effect, while high doses potentiate the negative effects elicited by PQ. Overall, this work suggests a proper balance of prooxidants and antioxidants are required for normal neuronal homeostasis, and moderate levels of AB shift the balance in favor of neuronal survival following PQ exposure.

2. Materials and Methods

2.1. Cell Culture and Reagents. NG108-15 neuroblastoma cells (ATCC #HB-12317) were cultured and maintained in RPMI 1640 (Gibco #11875-093, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals #S11150, Lawrenceville, GA) and 1% penicillin/streptomycin (Gibco #15140-122, Grand Island, NY). As per manufacturer's instructions for human consumption, the aronia berry concentrate (Superberries/Mae's Health and Wellness-Westin Foods, Omaha, NE) was diluted to the drinking concentration (1 : 16 in culture media) prior to making serial working dilutions. Paraquat (Sigma-Aldrich #36541, St. Louis, MO) was diluted in double-distilled water and filter-sterilized prior to use. Cells were plated (200,000 cells/60 mm dish) 24 hours prior to counting or treatment at 0 hours. For AB + PQ experiments, AB was started at 0 hours and PQ was started at 24 hours; pretreatment was performed to examine the protective effects of AB to PQ toxicity. Media were made fresh and changed daily.

2.2. Growth Curves and Apoptosis Assays. For growth curve analyses, cells were washed twice to remove unattached dead cells. Remaining live and attached cells were scrape harvested, isolated by centrifugation, and counted using size exclusion on a Beckman Coulter counter [20]. Apoptotic fraction of live cells was performed on the same cell population using the Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Molecular Probes #V13241, Grand Island, NY) as per manufacturer's instructions [21]. Briefly, freshly isolated cells were incubated with an Alexa Fluor 488-conjugated annexin V antibody as well as propidium iodide (PI). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at 488 nm excitation and 535 and 610 emission for annexin V and PI, respectively. Apoptotic fraction was considered as cells that were annexin V positive, while remaining are PI negative.

2.3. Superoxide Quantification. Cells were resuspended in serum-free, phenol red-free RPMI 1640 (Gibco #11835-030, Grand Island, NY) with 10 μM dihydroethidium (DHE; VWR #101447-534, Chicago, IL) for measuring total cellular $O_2^{\bullet-}$ levels or with 10 μM MitoSOX (Life Technologies #M36008, Grand Island, NY) to measure mitochondrial-specific $O_2^{\bullet-}$ levels and incubated for 30 min at 37°C. Following this, cells were immediately centrifuged at 4°C and resuspended in ice-cold serum-free, phenol red-free media. Cells were analyzed immediately on a LSRII Green Laser flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at 488 nm excitation and 610 nm emission and quantified using FlowJo cytometric analysis software (Tree Star, Ashland, OR) [20].

2.4. Hydrogen Peroxide (H_2O_2) Quantification. Replication-deficient recombinant adenoviruses (Ad5-CMV) encoding either HyPer Cyto (Cytoplasm-targeted HyPer construct; Evrogen #FP941, Moscow, Russia) or HyPer-Mito (Mitochondria-targeted HyPer construct, Evrogen #FP942, Moscow, Russia) were purchased from the University of Iowa Viral Vector Core Facility (Iowa City, IA). After plating, cells were transduced with 100 multiplicity of infection (MOI; transduction efficiency measured at 95.4% ± 3.2% by flow cytometry with negligible toxicity) of respective virus for 24 hours in serum-free media prior to treatment with AB or PQ. Following treatment, cells were analyzed immediately on a LSRII Green Laser flow cytometer at 488 nm excitation and 509 nm emission and quantified using FlowJo cytometric analysis software [22].

2.5. Western Blotting Analysis. Immunoblotting was performed on whole cell lysates. Samples were separated on 10% denaturing gels, followed by a transfer to nitrocellulose membranes. After blocking in 5% milk in Tris-Buffered Saline and Tween 20, membranes were incubated with primary antibody (copper/zinc superoxide dismutase, CuZnSOD, 1 : 1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA; manganese superoxide dismutase, MnSOD, 1 : 1000 dilution, Upstate Biotech/Millipore, Billerica, MA; catalase, 1 : 1000 dilution, Abcam, Cambridge, MA; NADPH oxidase 2, Nox2, 1 : 500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA; NADPH oxidase 4, Nox4, 1 : 500 dilution, Novus Biologicals, Littleton, CO; β-actin, 1 : 1000 dilution, Sigma-Aldrich, St. Louis, MO) overnight at 4°C. Following washout of primary antibody, membranes were incubated with secondary antibody (1 : 10,000, Thermo Scientific, Rockford, IL) for 1 hour at room temperature. After addition of chemiluminescence substrate (SuperSignal West Femto, Thermo Scientific, Rockford, IL), images were acquired on a UVP Bioimaging System (UVP LLC, Upland, CA) [23].

2.6. Antioxidant Activity Gels. Activity gels were run utilizing whole cell lysates. Samples were separated on 12% nondenaturing gels with ammonium persulfate used as

the polymerization catalyst in the running gel and riboflavin-light in the stacking gel. Gels were prerun for one hour at 4°C prior to sample loading. For superoxide dismutase activity, the gel was stained in a solution containing 2.43 mM nitroblue tetrazolium, 28 mM tetramethylethylenediamine, and 25 μM riboflavin-5′-phosphate for 20 minutes at room temperature protected from light. Following this incubation, the gel was rinsed thrice with double-distilled water and allowed to expose under fluorescent light. For catalase activity, the gel was first allowed to incubate in a 0.003% H_2O_2 solution for 10 minutes prior to staining with 2% ferric chloride and 2% potassium ferricyanide. Gel images were obtained by scanning using a Brother MFC-8870DW scanner [24].

2.7. Glutathione Assay. Oxidized (GSSG) as well as reduced (GSH) glutathione was measured using the GSSG/GSH Quantification kit (Dojindo Molecular Technologies #G257-10, Rockville, MD) as per manufacturer's instructions. Briefly, the assay is based on the glutathione dependent reduction of 5,5′-dithiobis-2-nitrobenzoic acid to 5-mercapto-2-nitrobenzoic acid (λ_{max}: 415 nm). Absorbance was measured at 415 nm using a SpectraMax M5 multimode plate reader (Molecular Devices, Sunnyvale, CA) [25].

2.8. Statistics. Data are presented as mean ± standard error of the mean (SEM). For two group comparisons, Student's t-test was used. For multiple group comparisons, one-way ANOVA followed by Newman-Keuls posttest was used. GraphPad Prism 5.0 statistical and graphing software was used for all analyses. Differences were considered significant at $p < 0.05$.

3. Results

3.1. AB Protects Neurons from PQ-Induced Cell Death. PQ is a well-established neurotoxin known to induce neuron cell death by ROS-mediated apoptosis [26]. To identify an appropriate dose of PQ required to induce neurotoxicity in our neuronal cell culture model, we performed growth curves in the presence of increasing amounts of PQ and identified the IC_{50} of PQ to be approximately 50 μM (Figure 1(a)). Additionally, to understand if AB alone had any effects on cellular viability we exposed cells to increasing concentrations of AB in 10-fold serial dilutions (Figure 1(b)). Only the highest dose tested (i.e., 1 : 10 AB) demonstrated significant toxicity to the cells and thus was not used in further studies. Last, to identify if AB had any effect on attenuating PQ-induced neurotoxicity, we treated cells with various dilutions of AB with 50 μM PQ (Figure 1(c), left panel). Interestingly, only the lowest concentrations of AB (i.e., 1 : 1000 and 1 : 10000) demonstrated significant rescuing effects on the PQ-treated cells. In contrast, the highest concentration of AB (i.e., 1 : 100) potentiated the PQ-induced cell death at 72 hours. Furthermore, low doses of AB decreased, while high doses of AB exacerbated the apoptotic fraction of PQ-treated NG108-15 cells (Figure 1(c), right panel). Taken together, these data suggest that lower doses of AB have protective effects against PQ-induced neurotoxicity.

3.2. PQ-Induced Increase in $O_2^{\bullet-}$ Levels Is Attenuated by Low-Dose AB. The primary and direct ROS generated by PQ is $O_2^{\bullet-}$. We first measured total cellular $O_2^{\bullet-}$ utilizing the $O_2^{\bullet-}$-sensitive probe DHE (Figure 2(a)). As expected, PQ alone increased DHE oxidation roughly 2-fold. Interestingly, low-dose AB significantly attenuated the PQ-induced increase in $O_2^{\bullet-}$ levels, while high-dose AB exacerbated this response. In addition, high-dose AB alone significantly increased DHE oxidation in the absence of PQ. Next, because PQ is known to play a role in the direct generation of mitochondrial-localized $O_2^{\bullet-}$, we measured mitochondrial-specific $O_2^{\bullet-}$ levels using MitoSOX Red (Figure 2(b)). Similar to what we observed with total cellular $O_2^{\bullet-}$ levels, PQ alone also significantly increased mitochondrial $O_2^{\bullet-}$ levels. Low-dose AB moderately decreased these levels, but these differences were not statistically significant. Additionally, high-dose AB alone increased mitochondrial $O_2^{\bullet-}$ levels and once again intensified PQ-induced mitochondrial $O_2^{\bullet-}$. In summary, these data suggest that low, but not high, doses of AB may have antioxidant effects that reduce the PQ-induced increase in neuronal $O_2^{\bullet-}$ levels.

3.3. AB Alters Steady-State Cellular H_2O_2 Levels. $O_2^{\bullet-}$ is a short lived species that is spontaneously and enzymatically (by superoxide dismutases) converted to H_2O_2 [27]. To assess intracellular H_2O_2 levels, we utilized fluorescent proteins that increase in fluorescence when oxidized specifically by H_2O_2 (i.e., HyPer) [22]. First, using a cytoplasm-targeted HyPer (HyPer Cyto) we observed a dose-dependent decrease in cytoplasmic H_2O_2 levels with increased concentration of AB alone (Figure 3(a)). PQ treatment led to a small but significant increase in cytoplasmic H_2O_2 levels, and this response was attenuated with increasing doses of AB. Neither PQ nor AB had any effect on mitochondrial-localized H_2O_2 levels as measured by the mitochondrial-targeted HyPer construct (Hyper Mito; Figure 3(b)). These data suggest that AB has potent H_2O_2 scavenging effects under both normal, nonoxidative stress and PQ-induced oxidative stress conditions.

3.4. AB Has a Minimal Effect on Prooxidant and Antioxidant Enzyme and Activity Levels. The decrease in ROS observed by the addition of AB may be due to direct scavenging of ROS or by the alteration of endogenous antioxidant or prooxidant enzyme systems. First, we performed western blot analyses on whole cell lysates and observed no significant changes in the protein levels of cytoplasmic CuZnSOD, mitochondrial MnSOD, or the peroxisomal H_2O_2 removing enzyme catalase (Figure 4(a)). Because polyphenolic compounds like those found in AB have been shown to activate the sirtuin class of enzymes [28], which may alter the activity of endogenous antioxidant enzymes [29], we further examined antioxidant enzyme activities for both SOD and catalase and observed no significant differences in any treatment group (Figure 4(b)). In addition to exploring endogenous antioxidant systems, we also investigated the prooxidant NADPH oxidase (Nox) family of enzymes, which contribute to the production and steady-state levels of cellular $O_2^{\bullet-}$ and H_2O_2 levels. Examining the catalytic subunits of the two major Nox

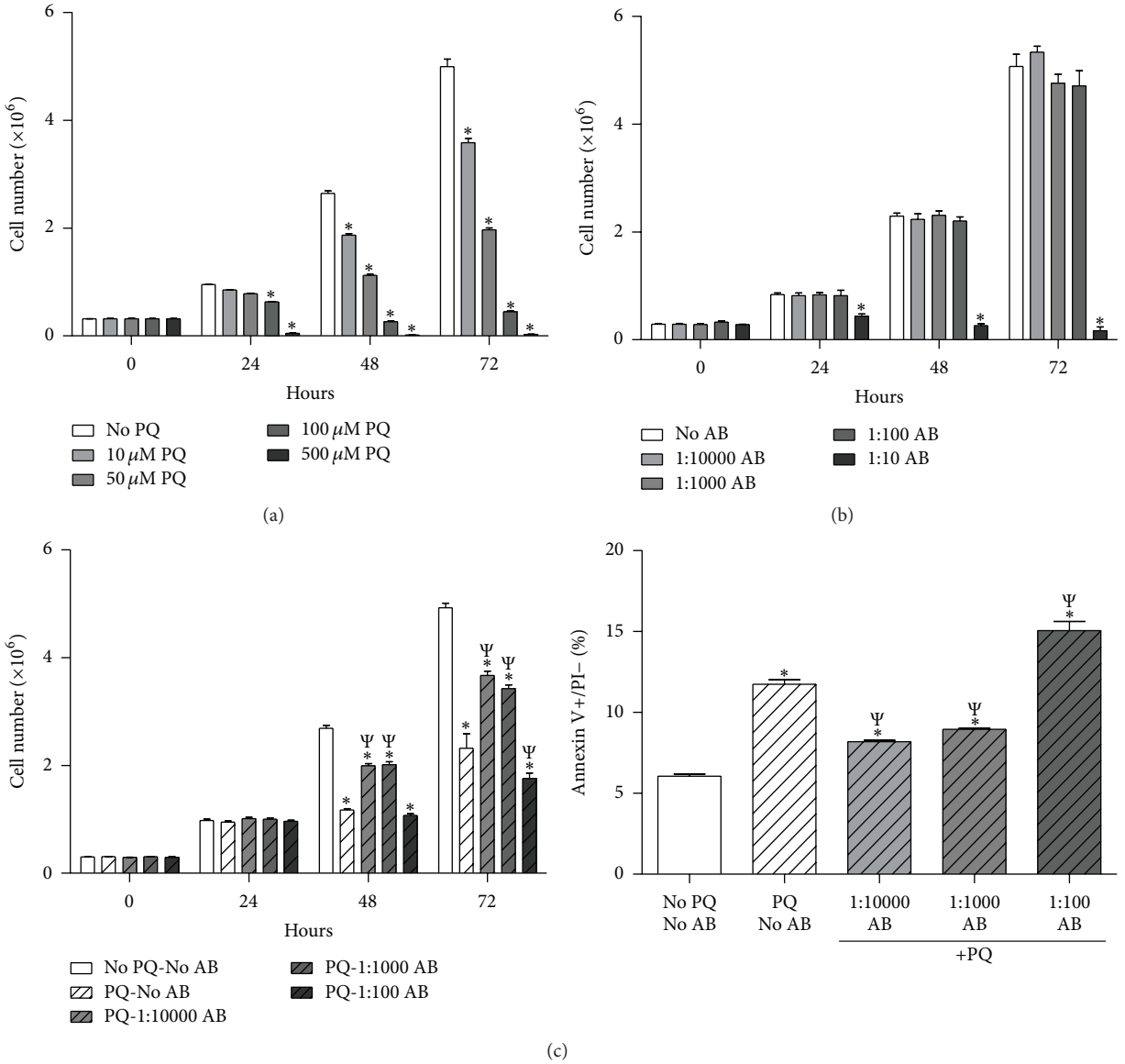


Figure 1: AB attenuates PQ-induced neurotoxicity. NG108-15 cells were treated with various doses of PQ, AB, or AB + PQ for 72 hours. (a) Growth curve of NG108-15 cells with increasing doses of PQ. IC_{50} of PQ calculated at approximately 50 μM. $N = 6$. (b) Growth curve of NG108-15 cells with increasing doses of AB. 1 : 10 AB demonstrated significant toxicity and thus was not used in further studies. $N = 6$. (c) Left panel, growth curve of NG108-15 cells with 50 μM of PQ along with various doses of AB. AB was added at 0 hours, while PQ was added at 24 hours after plating. $N = 6$. Right panel, analysis of apoptotic NG108-15 cells with 50 μM of PQ along with various doses of AB. Apoptotic fraction was defined as annexin V positive and propidium iodide (PI) negative. $N = 4$. $^{*}p < 0.05$ versus No PQ or AB; $^{\Psi}p < 0.05$ versus PQ-No AB.

enzymes found in neurons (i.e. Nox2 and Nox4) we observed a substantial reduction in the amount of immunoreactivity for Nox2 with high-dose AB independent of PQ treatment (Figure 4(a)), but no changes were observed with lower doses. Taken together, while high-dose AB appears to have an effect on Nox2 levels, overall, AB does not appear to have a significant impact on the endogenous antioxidant or prooxidant enzyme systems in our neuronal cell culture model.

3.5. PQ-Induced Oxidized Glutathione Is Significantly Reduced with Low-Dose AB. In addition to antioxidant enzyme systems, the cell is home to numerous small molecule antioxidant systems. The most abundant small molecule antioxidant system in the cell is glutathione, which may be cycled between a reduced and oxidized state depending on the redox environment of the cell and has shown incredible importance in attenuating ROS-induced neurotoxicity [8, 30]. When examining GSH in our neuronal cell culture model, we observed no significant changes in any treatment group (Figure 5(a)). In contrast, when measuring GSSG we observed that PQ alone increased GSSG roughly 4-fold compared to control neurons. Moreover, low-dose AB

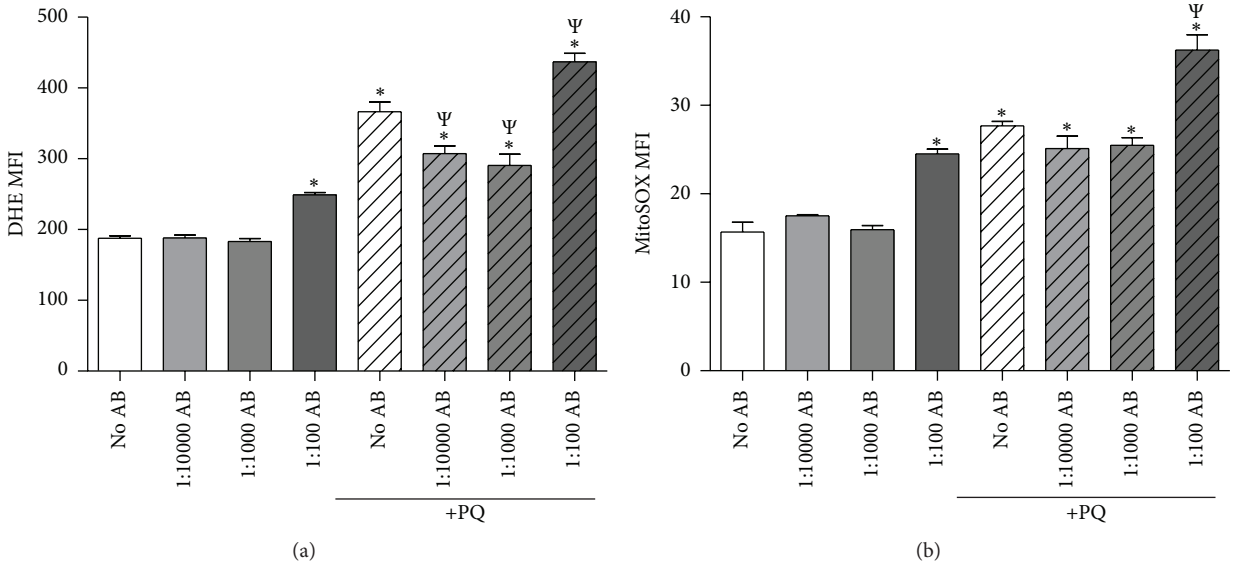


Figure 2: Low-dose AB decreases PQ-induced increase in $O_2^{\bullet-}$ levels. NG108-15 cells were treated with 50 μM PQ with various doses of AB. AB was added 24 hours prior to PQ; cells were analyzed 48 hours after PQ administration. (a) Total cellular $O_2^{\bullet-}$ levels measured by dihydroethidium (DHE) oxidation and flow cytometry. $N = 4$. (b) Mitochondrial-specific $O_2^{\bullet-}$ levels measured by MitoSOX oxidation and flow cytometry. MFI = mean fluorescence intensity. $N = 4$. $^{*}p < 0.05$ versus No AB; $^{\Psi}p < 0.05$ versus PQ-No AB.

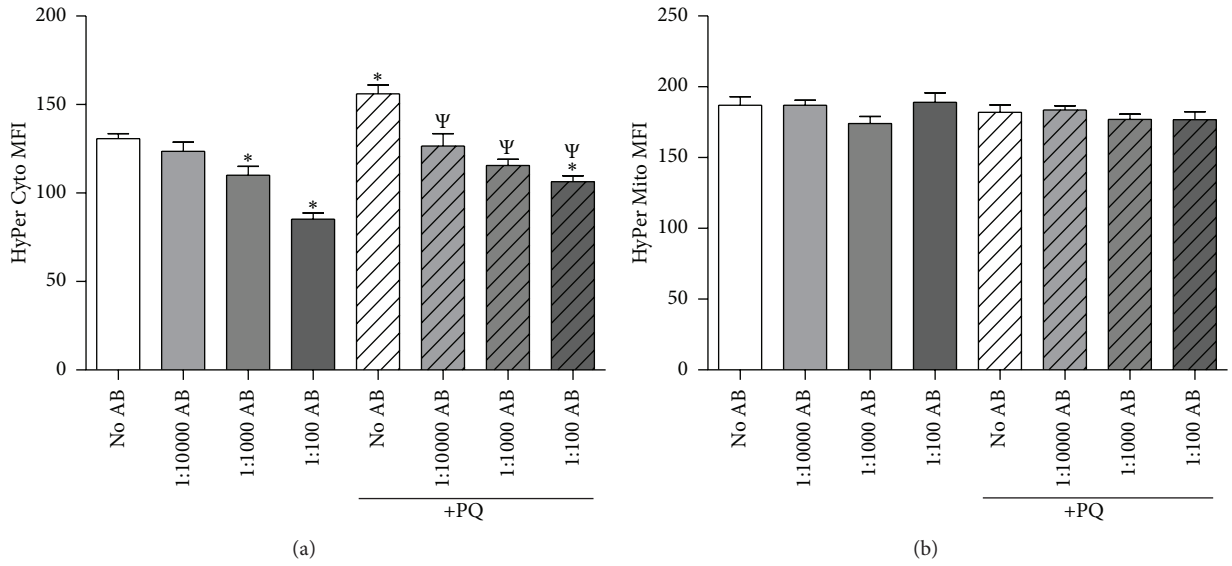


Figure 3: AB decreases cellular H_2O_2 in a dose-dependent fashion. NG108-15 cells were treated with 50 μM PQ with various doses of AB. AB was added 24 hours prior to PQ; cells were analyzed 48 hours after PQ administration. (a) Cytoplasmic H_2O_2 levels measured by a cytoplasmic-targeted H_2O_2-sensitive fluorescent protein (HyPer Cyto). $N = 4$. (b) Mitochondrial H_2O_2 levels measured by a mitochondria-targeted H_2O_2-sensitive fluorescent protein (HyPer Mito). MFI = mean fluorescence intensity. $N = 4$. $^{*}p < 0.05$ versus No AB; $^{\Psi}p < 0.05$ versus PQ-No AB.

attenuated the PQ-elevated GSSG levels back to control levels, while high-dose AB had no significant change on GSSG levels in PQ-treated cells (Figure 5(b)). Overall, these findings support our $O_2^{\bullet-}$ and H_2O_2 data (Figures 2 and 3) and together strongly suggest that low-dose AB decreases levels of ROS, attenuates oxidative stress, and inhibits neurotoxicity following PQ exposure.

4. Discussion

Of the neurodegenerative diseases, Parkinson's disease is highly associated with oxidative stress induced by environmental factors such as herbicide (i.e., PQ) exposure [31]. While the exact cause of Parkinson's disease remains elusive, numerous studies have elucidated excess ROS production to

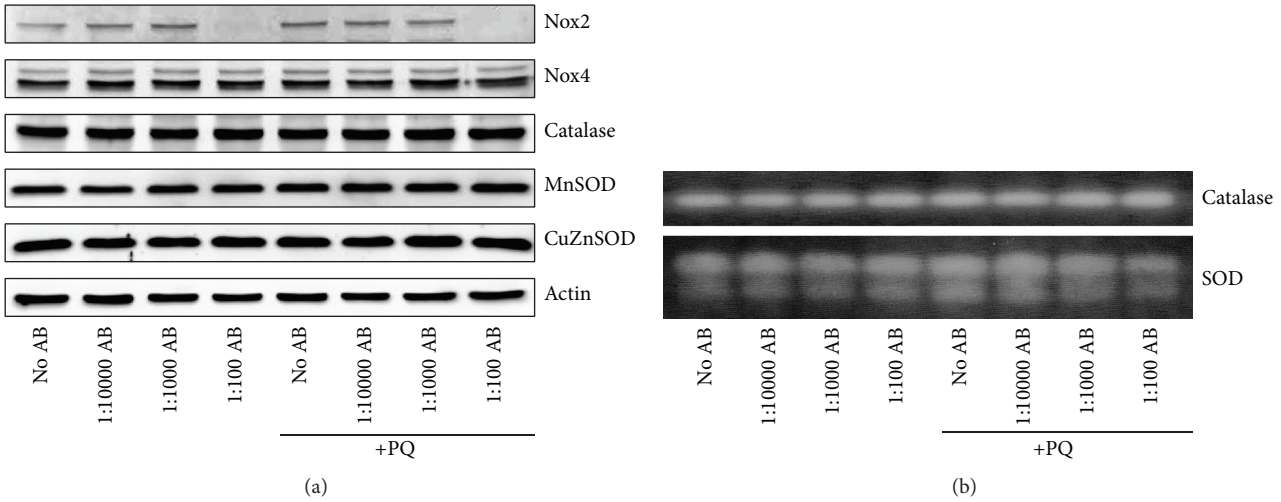


Figure 4: High-dose AB decreases Nox2 expression but does not affect other cellular antioxidant or prooxidant enzyme systems. NG108-15 cells were treated with 50 μM PQ with various doses of AB. AB was added 24 hours prior to PQ; cells were analyzed 48 hours after PQ administration. (a) Western blot analysis of the major neuronal prooxidant enzymes NADPH oxidases 2 and 4 (Nox2 and Nox4) and antioxidant enzymes catalase, manganese superoxide dismutase (MnSOD), and copper/zinc superoxide dismutase (CuZnSOD). (b) In-gel activity assay for catalase and SOD demonstrating no change with any treatment course. Images are representative of 4 separate experiments; with the exception of Nox2, no significant changes were observed upon quantification.

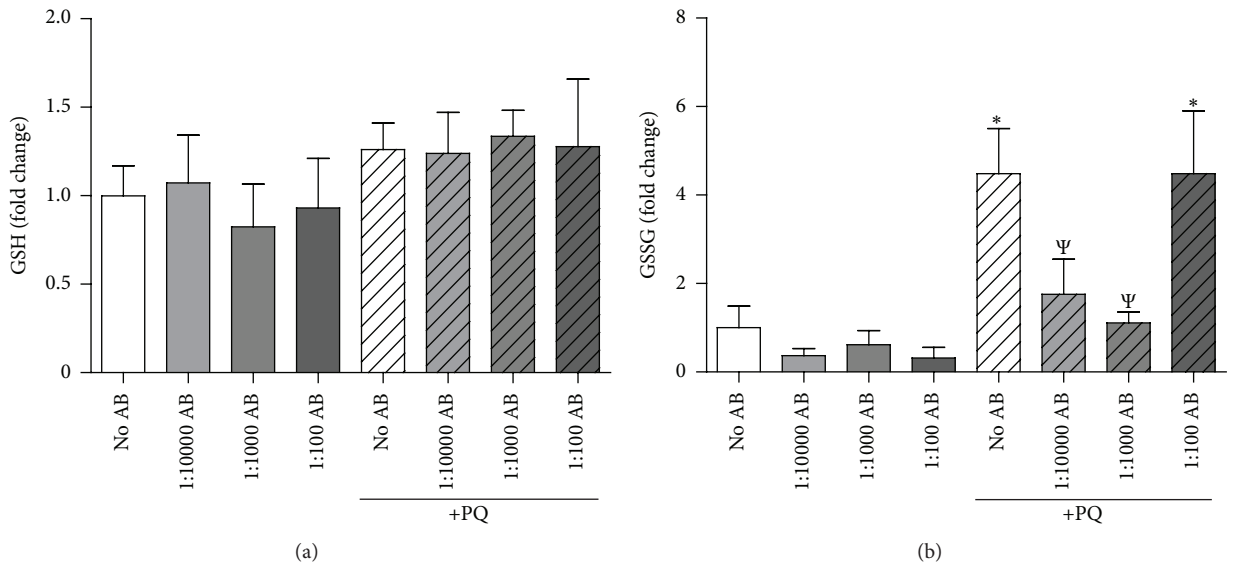


Figure 5: Low-dose AB rescues neurons from PQ-mediated increases in oxidized glutathione (GSSG). NG108-15 cells were treated with 50 μM PQ with various doses of AB. AB was added 24 hours prior to PQ; cells were analyzed 48 hours after PQ administration. (a) Relative levels of reduced glutathione (GSH). $N = 3$. (b) Relative levels of oxidized glutathione (GSSG). $N = 3$. $^{*}p < 0.05$ versus No AB; $^{\Psi}p < 0.05$ versus PQ-No AB.

be a potential mechanism in the loss of critical dopaminergic neurons in the substantia nigra in the brain [32]. A primary source of intraneuronal ROS, more specifically $O_2^{\bullet-}$, implicated to be involved in the disease is complex I of mitochondria [33]. Complex I inhibitors (which are also found in pesticides and herbicides) such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) create a backup of electrons in the mitochondrial respiratory chain, which further leak onto molecular oxygen generating $O_2^{\bullet-}$ and induce oxidative stress [34]. Interestingly, PQ possesses a similar structure to MPTP and has also been demonstrated to interact with complex I to generate reactive radical species [35]. Herein, we confirm these findings by demonstrating that mitochondrial $O_2^{\bullet-}$ is indeed increased in NG108-15 cells treated with PQ. Intriguingly, we observed no change in mitochondrial H_2O_2 levels, which suggests a predominantly 1 electron transfer to generate primarily $O_2^{\bullet-}$. Moreover, low doses of AB were able to significantly attenuate this

increase in mitochondrial oxidative stress, which translated to a more reducing cellular environment as evidenced by lower DHE oxidation as well as decreased levels of oxidized glutathione. In contrast, high doses of AB could not rescue the PQ-induced oxidative stress and exacerbated some of the effects. These findings warrant examination into the specific components of the AB concentrate to elucidate potential molecules that could exacerbate redox cycling reactions in a dose-dependent manner.

There are currently limited medical therapies for the treatment of neurodegenerative diseases. On the contrary, a breadth of evidence exists suggesting dietary intake of polyphenols may have beneficial effects in counteracting neurological disorders. For example, consumption of red wine, which is known to possess high levels of polyphenols, may reduce the incidence of neurological disorders [36, 37]. Other studies have demonstrated intake of polyphenol-rich foods may preserve cognitive function, delay the onset, or even reduce the risk of neurodegenerative diseases like age-related dementia or Alzheimer's disease [38–40]. However, it remains controversial if the beneficial effects of polyphenol-rich diets are actually acting in the brain, as it is not clear if polyphenols cross the blood brain barrier [41]. Polyphenols have been reported to be poorly absorbed by the intestines, rapidly excreted, and exist in low concentrations in systemic circulation [42, 43], which further argues for a potential limited role in the brain. In contrast, several investigations have concluded that low concentrations of polyphenols do in fact cross the blood brain barrier under both experimental *in situ* conditions and after *in vivo* dietary consumption of polyphenol-rich foods [44–47]. In the present study, we identified that only low concentrations of AB provided a protective role against ROS-induced neuron cell death caused by PQ. With the understanding that only small amounts of polyphenols may reach the brain after dietary consumption of polyphenol-rich foods, our data support a beneficial and antioxidant effect of these molecules in low concentrations and the possible protection against neuron cell death.

The use of antioxidants as therapeutics is controversial due to an extensive list of failed clinical trials in an array of diseases. Based on this, it is easy to conclude that antioxidants are not sufficient in ameliorating disease, but numerous variables must be taken into account when assessing the efficacy of these trials. The first variable to consider is dosage. It is commonly presumed in medicine that if a positive dose response to a drug is achieved at low concentrations then high concentrations will produce an even more favorable outcome, but this is not always found to be true. For example, in 2002 a phase II, double blind, randomized, and placebo controlled clinical trial was performed on the potential effectiveness of coenzyme Q_{10} in slowing the progression of Parkinson's disease [48]. A negative correlation was observed with increasing dose of coenzyme Q_{10} (ranging from 300 to 1200 mg/day) and progression of the disease, which thus prompted researchers to investigate even higher doses of coenzyme Q_{10} in Parkinson's disease. In 2007, another phase II, double blind, randomized, and placebo controlled study was performed utilizing doses of coenzyme Q_{10} ranging from 2400 to 4000 mg/day and found no significant improvement with any dose on the diminution of progression of Parkinson's disease [49]. The conclusion drawn from this study was that coenzyme Q_{10} provided no benefit over placebo in Parkinson's disease due to the fact that high doses could not replicate what was seen in the lower dose clinical trial. Another example of dosage discrepancies involves the use of vitamin E for therapy in Alzheimer's or Parkinson's disease patients. Three separate clinical trials utilizing vitamin E supplements (ranging from 800 to 2000 IU/day) found no significant impact or even worsening of the severity of Alzheimer's or Parkinson's disease progression [50–52]. However, three separate studies utilizing vitamin E administration through means of dietary intake (ranging from 5 to 15 mg/day in foods naturally containing higher levels of vitamin E) showed positive benefits in slowing the progression of both diseases [53–55]. Similar to what was observed with coenzyme Q_{10}, it appears that lower doses (and possibly vehicle of administration) are possibly more efficacious than higher doses when examining the effects of antioxidants. In our study presented here, we observe a similar phenomenon where only low-dose AB ameliorated PQ-induced neurotoxicity, while higher doses exacerbated the phenotype. This nonlinear regression between antioxidant dosage and disease outcome may explain the subjective failure of antioxidant clinical trials and warrants further investigation into the potential mechanisms leading to the nonmonotonic response.

Another significant variable in the outcome of antioxidant therapy is the timing of administration. The majority of clinical trials focus on the treatment of patients that have already been diagnosed with a major disease, and as such assessing the preventative capabilities of antioxidants is already past due. Conversely, numerous retrospective analyses have examined the potential for dietary intake of antioxidants in altering the risk of developing neurodegenerative disorders like Alzheimer's disease. For example, it has been shown that diets rich in fruits and vegetables reduce cognitive decline and the risk for Alzheimer's disease later in life [56, 57]. Additionally, in the aforementioned Rotterdam study it was observed that intake of vitamin E in the form of food (not supplements) also reduced the incidence of dementia [54]. These studies suggest that antioxidants serve as preventative measures as opposed to reactive measures against neurological disorders. Herein, we present evidence that supports this hypothesis as we show pretreatment of neurons with AB for 24 hours prior to PQ administration protects neurons from ROS-induced cell death. Performing the converse experiment in which AB was administered at the same time or 24 hours after PQ treatment did not produce any observable beneficial response (data not shown). Taken together, antioxidant supplementation through dietary intake appears to play a greater role in the prevention of neurological diseases as opposed to their treatment.

The last major variable to consider when assessing the efficacy of antioxidants in the treatment of diseases is the specific ROS that is being targeted. ROS are often considered a homogenous group of substances that are harmful to the cell, but this view overlooks the vast complexity of the redox environment. ROS are diverse with some being free radicals, possessing charges, or participating in one or two

electron oxidation/reduction reactions depending on the structure of the specific species [58]. Additionally, not all ROS cause "oxidative stress," which is defined as irreversible damage to cellular components, but many ROS participate in controlled, regulated, and reversible modifications to cellular constituents that lead to redox-mediated signaling pathways [59]. For example, H_2O_2 oxidizes reduced cysteines in proteins creating reversible adducts that may alter the shape and function of a protein, thus making the protein redox responsive [60]. In contrast, $O_2^{\bullet-}$ is a poor oxidant but reacts readily with iron-sulfur cluster containing enzymes reversibly affecting their activity and contributing to redox-mediated cellular signaling [61]. With the understanding that ROS-mediated reactions are unique and diverse, it becomes clear that the use of a generalized antioxidant that may scavenge several ROS at once (or potentially a ROS that is not highly relevant in the disease state) may not prove to be efficacious or even deleterious. In our data set, we demonstrate that the primary ROS produced by PQ is $O_2^{\bullet-}$, and this has been shown by others as well [26]. Low doses of AB demonstrated the ability to significantly attenuate PQ-induced $O_2^{\bullet-}$ in neurons, yet, high doses potentiated the production. Moreover, high dose of AB appeared to significantly reduce the amount of steady-state H_2O_2 in neurons even in the absence of PQ suggesting that high dosage of antioxidants altered normal redox signaling within the cells or even created a reductive stress upon the cells [62]. In summary, it appears that low, but not high, dose of AB restores the homeostatic redox environment and decreases cellular death caused by the PQ-induced $O_2^{\bullet-}$-mediated oxidative stress.

Next, we observed an interesting phenomenon that Nox2 protein was virtually absent in neurons treated with high doses of AB (independent of PQ treatment). Polyphenols have been demonstrated to attenuate Nox activity in various models, but their role in regulating actual protein levels is unclear [63–65]. Our data suggest that AB may be interfering with the normal expression of Nox2, but it is unclear at this time if this occurs at the transcriptional, posttranscriptional, translational, or posttranslational level. Furthermore, the Nox2 catalytic subunit of the Nox complex is also known as gp91phox due to the fact that the 55 kDa protein becomes heavily glycosylated causing it to run on a western blot at approximately 91 kDa [66]. Polyphenols have been shown to interfere with and reduce the amount of advanced end glycation products observed in several disease states [67–69], which raises the question if these small molecules also play a role in modifying normal cellular glycosylation of proteins. Our data suggest AB plays a significant role in the downregulation of Nox2, and further investigation is warranted into the mechanism of this process.

Finally, our study does possess some potential limitations. First, due to proprietary reasons we are limited in the understanding of the exact constituents and concentrations of the commercially available AB concentrate. Additionally, while the dilutions we utilized did produce favorable outcomes, further biodistribution studies are needed to understand if the optimal concentrations we observed translate *in vivo*. Next, our use of a neuronal cell line may not perfectly mimic the effects on primary neurons. However, NG108-15 cells divide and grow in a highly differentiated manner, which increases their likelihood to react like primary neurons in an *in vitro* setting. Lastly, treatment of neuronal cells *in vitro* with AB does not take into account *in vivo* variables such as absorption and biotransformation that may alter the AB components and exposure to neurons in a living system. Upon consumption, polyphenols may be oxidized by liver enzymes and the digestive microbiota, which could ultimately change the structure and function of these molecules once they have reached a target organ. While our current studies do not address the potential alterations digestion may have on the AB, we believe the data presented herein show significant preliminary promise for AB in the amelioration of ROS-induced neurotoxicity. With these promising results, we are currently investigating the ability of AB to attenuate neurological dysfunction *in vivo* utilizing various animal models of neurodegeneration. These models will allow for a deeper understanding regarding AB bioavailability to neurons of the central nervous system, and if concentrations are able to reach levels necessary for the attenuation of oxidative stress-mediated neurological disease.

Abbreviations

PQ: Paraquat
$O_2^{\bullet-}$: Superoxide
ROS: Reactive oxygen species
AB: Aronia berry concentrate
DHE: Dihydroethidium
H_2O_2: Hydrogen peroxide
CuZnSOD: Copper/zinc superoxide dismutase
MnSOD: Manganese superoxide dismutase
GSH: Reduced glutathione
GSSG: Oxidized glutathione
Nox: NADPH oxidase.

Conflict of Interests

No conflict of interests exists for all authors.

Authors' Contribution

A. J. Case, D. Agraz, and M. C. Zimmerman designed research; A. J. Case, D. Agraz, and I. M. Ahmad conducted research; A. J. Case, D. Agraz, and I. M. Ahmad analyzed data; A. J. Case and M. C. Zimmerman wrote the paper; M. C. Zimmerman had primary responsibility for final content. All authors read and approved the final paper.

Acknowledgments

The authors thank Superberries/Mae's Health and Wellness LLC/Westin Foods for providing the aronia berry concentrate used for these studies. This work was supported by institutional and departmental funds awarded to M. C. Zimmerman.

References

[1] A. Xie, J. Gao, L. Xu, and D. Meng, "Shared mechanisms of neurodegeneration in Alzheimer's disease and Parkinson's disease," *BioMed Research International*, vol. 2014, Article ID 648740, 8 pages, 2014.

[2] M. T. Baltazar, R. J. Dinis-Oliveira, M. de Lourdes Bastos, A. M. Tsatsakis, J. A. Duarte, and F. Carvalho, "Pesticides exposure as etiological factors of Parkinson's disease and other neurodegenerative diseases—a mechanistic approach," *Toxicology Letters*, vol. 230, no. 2, pp. 85–103, 2014.

[3] C. Freire and S. Koifman, "Pesticide exposure and Parkinson's disease: epidemiological evidence of association," *NeuroToxicology*, vol. 33, no. 5, pp. 947–971, 2012.

[4] C. M. Tanner, F. Kame, G. W. Ross et al., "Rotenone, paraquat, and Parkinson's disease," *Environmental Health Perspectives*, vol. 119, no. 6, pp. 866–872, 2011.

[5] F. Wang, R. Franco, M. Skotak, G. Hu, and N. Chandra, "Mechanical stretch exacerbates the cell death in SH-SY5Y cells exposed to paraquat: mitochondrial dysfunction and oxidative stress," *NeuroToxicology*, vol. 41, pp. 54–63, 2014.

[6] B. Thrash, S. Uthayathas, S. S. Karuppagounder, V. Suppiramaniam, and M. Dhanasekaran, "Paraquat and maneb induced neurotoxicity," *Proceedings of the Western Pharmacology Society*, vol. 50, pp. 31–42, 2007.

[7] R. Franco, S. Li, H. Rodriguez-Rocha, M. Burns, and M. I. Panayiotidis, "Molecular mechanisms of pesticide-induced neurotoxicity: relevance to Parkinson's disease," *Chemico-Biological Interactions*, vol. 188, no. 2, pp. 289–300, 2010.

[8] W. Yang and E. Tiffany-Castiglioni, "The bipyridyl herbicide paraquat produces oxidative stress-mediated toxicity in human neuroblastoma SH-SY5Y cells: relevance to the dopaminergic pathogenesis," *Journal of Toxicology and Environmental Health Part A*, vol. 68, no. 22, pp. 1939–1961, 2005.

[9] W. Yang and E. Tiffany-Castiglioni, "Paraquat-induced apoptosis in human neuroblastoma SH-SY5Y cells: involvement of p53 and mitochondria," *Journal of Toxicology and Environmental Health Part A*, vol. 71, no. 4, pp. 289–299, 2008.

[10] S. Medina-Leendertz, M. Paz, M. Mora et al., "Longterm melatonin administration alleviates paraquat mediated oxidative stress in *Drosophila melanogaster*," *Investigacion Clinica*, vol. 55, pp. 352–364, 2014.

[11] I. Ahmad, S. Shukla, A. Kumar et al., "Biochemical and molecular mechanisms of *N*-acetyl cysteine and silymarin-mediated protection against maneb- and paraquat-induced hepatotoxicity in rats," *Chemico-Biological Interactions*, vol. 201, no. 1–3, pp. 9–18, 2013.

[12] Z. Wang, Y. Xiao, W. Chen, K. Tang, and L. Zhang, "Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in Arabidopsis," *Journal of Integrative Plant Biology*, vol. 52, no. 4, pp. 400–409, 2010.

[13] S. E. Kulling and H. M. Rawel, "Chokeberry (Aronia melanocarpa)—a review on the characteristic components and potential health effects," *Planta Medica*, vol. 74, no. 13, pp. 1625–1634, 2008.

[14] X. Wu, L. Gu, R. L. Prior, and S. McKay, "Characterization of anthocyanins and proanthocyanidins in some cultivars of Ribes, Aronia, and Sambucus and their antioxidant capacity," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 26, pp. 7846–7856, 2004.

[15] X. Wu, G. R. Beecher, J. M. Holden, D. B. Haytowitz, S. E. Gebhardt, and R. L. Prior, "Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 11, pp. 4069–4075, 2006.

[16] D. Rugină, Z. Sconţa, L. Leopold, A. Pintea, A. Bunea, and C. Socaciu, "Antioxidant activities of chokeberry extracts and the cytotoxic action of their anthocyanin fraction on hela human cervical tumor cells," *Journal of Medicinal Food*, vol. 15, no. 8, pp. 700–706, 2012.

[17] B. Kim, C. S. Ku, T. X. Pham et al., "Aronia melanocarpa (chokeberry) polyphenol-rich extract improves antioxidant function and reduces total plasma cholesterol in apolipoprotein E knockout mice," *Nutrition Research*, vol. 33, no. 5, pp. 406–413, 2013.

[18] B. Qin and R. A. Anderson, "An extract of chokeberry attenuates weight gain and modulates insulin, adipogenic and inflammatory signalling pathways in epididymal adipose tissue of rats fed a fructose-rich diet," *The British Journal of Nutrition*, vol. 108, no. 4, pp. 581–587, 2012.

[19] K. Ohgami, I. Ilieva, K. Shiratori et al., "Anti-inflammatory effects of aronia extract on rat endotoxin-induced uveitis," *Investigative Ophthalmology & Visual Science*, vol. 46, no. 1, pp. 275–281, 2005.

[20] A. J. Case and M. C. Zimmerman, "Redox-regulated suppression of splenic T-lymphocyte activation in a model of sympathoexcitation," *Hypertension*, vol. 65, no. 4, pp. 916–923, 2015.

[21] A. J. Case, J. L. McGill, L. T. Tygrett et al., "Elevated mitochondrial superoxide disrupts normal T cell development, impairing adaptive immune responses to an influenza challenge," *Free Radical Biology and Medicine*, vol. 50, no. 3, pp. 448–458, 2011.

[22] V. V. Belousov, A. F. Fradkov, K. A. Lukyanov et al., "Genetically encoded fluorescent indicator for intracellular hydrogen peroxide," *Nature Methods*, vol. 3, no. 4, pp. 281–286, 2006.

[23] A. J. Case, S. Li, U. Basu, J. Tian, and M. C. Zimmerman, "Mitochondrial-localized NADPH oxidase 4 is a source of superoxide in angiotensin II-stimulated neurons," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 305, no. 1, pp. H19–H28, 2013.

[24] S. Li, A. J. Case, R.-F. Yang, H. D. Schultz, and M. C. Zimmerman, "Over-expressed copper/zinc superoxide dismutase localizes to mitochondria in neurons inhibiting the angiotensin II-mediated increase in mitochondrial superoxide," *Redox Biology*, vol. 2, no. 1, pp. 8–14, 2013.

[25] I. M. Ahmad, M. Y. Abdalla, N. Aykin-Burns et al., "2-Deoxyglucose combined with wild-type p53 overexpression enhances cytotoxicity in human prostate cancer cells via oxidative stress," *Free Radical Biology and Medicine*, vol. 44, no. 5, pp. 826–834, 2008.

[26] J. S. Bus and J. E. Gibson, "Paraquat: model for oxidant-initiated toxicity," *Environmental Health Perspectives*, vol. 55, pp. 37–46, 1984.

[27] I. Fridovich, "Superoxide dismutases," *Annual Review of Biochemistry*, vol. 44, pp. 147–159, 1975.

[28] D. A. Sinclair and L. Guarente, "Small-molecule allosteric activators of sirtuins," *Annual Review of Pharmacology and Toxicology*, vol. 54, pp. 363–380, 2014.

[29] O. Ozden, S.-H. Park, H.-S. Kim et al., "Acetylation of MnSOD directs enzymatic activity responding to cellular nutrient status or oxidative stress," *Aging*, vol. 3, no. 2, pp. 102–107, 2011.

[30] J. S. Bains and C. A. Shaw, "Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death," *Brain Research Reviews*, vol. 25, no. 3, pp. 335–358, 1997.

[31] J. Blesa, I. Trigo-Damas, A. Quiroga-Varela, and V. R. Jackson-Lewis, "Oxidative stress and Parkinson's disease," *Frontiers in Neuroanatomy*, vol. 9, article 91, 2015.

[32] J. A. Obeso, M. C. Rodriguez-Oroz, C. G. Goetz et al., "Missing pieces in the Parkinson's disease puzzle," *Nature Medicine*, vol. 16, no. 6, pp. 653–661, 2010.

[33] A. H. Schapira, "Mitochondria in the aetiology and pathogenesis of Parkinson's disease," *The Lancet Neurology*, vol. 7, no. 1, pp. 97–109, 2008.

[34] J. Blesa and S. Przedborski, "Parkinson's disease: animal models and dopaminergic cell vulnerability," *Frontiers in Neuroanatomy*, vol. 8, article 155, 2014.

[35] T. Fukushima, K. Yamada, A. Isobe, K. Shiwaku, and Y. Yamane, "Mechanism of cytotoxicity of paraquat. I. NADH oxidation and paraquat radical formation via complex I," *Experimental and Toxicologic Pathology*, vol. 45, no. 5-6, pp. 345–349, 1993.

[36] J.-M. Orgogozo, J.-F. Dartigues, S. Lafont et al., "Wine consumption and dementia in the elderly: a prospective community study in the Bordeaux area," *Revue Neurologique*, vol. 153, no. 3, pp. 185–192, 1997.

[37] T. Truelsen, D. Thudium, M. Gronbaek, and Copenhagen City Heart Study, "Amount and type of alcohol and risk of dementia: the Copenhagen City Heart Study," *Neurology*, vol. 59, pp. 1313–1319, 2002.

[38] D. Commenges, V. Scotet, S. Renaud, H. Jacqmin-Gadda, P. Barberger-Gateau, and J.-F. Dartigues, "Intake of flavonoids and risk of dementia," *European Journal of Epidemiology*, vol. 16, no. 4, pp. 357–363, 2000.

[39] L. Letenneur, C. Proust-Lima, A. Le Gouge, J. F. Dartigues, and P. Barberger-Gateau, "Flavonoid intake and cognitive decline over a 10-year period," *American Journal of Epidemiology*, vol. 165, no. 12, pp. 1364–1371, 2007.

[40] Q. Dai, A. R. Borenstein, Y. Wu, J. C. Jackson, and E. B. Larson, "Fruit and vegetable juices and Alzheimer's disease: the Kame Project," *American Journal of Medicine*, vol. 119, no. 9, pp. 751–759, 2006.

[41] S. Schaffer and B. Halliwell, "Do polyphenols enter the brain and does it matter? Some theoretical and practical considerations," *Genes & Nutrition*, vol. 7, no. 2, pp. 99–109, 2012.

[42] B. Halliwell, "Dietary polyphenols: good, bad, or indifferent for your health?" *Cardiovascular Research*, vol. 73, no. 2, pp. 341–347, 2007.

[43] S. B. Lotito and B. Frei, "Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon?" *Free Radical Biology and Medicine*, vol. 41, no. 12, pp. 1727–1746, 2006.

[44] K. A. Youdim, M. Z. Qaiser, D. J. Begley, C. A. Rice-Evans, and N. J. Abbott, "Flavonoid permeability across an in situ model of the blood-brain barrier," *Free Radical Biology and Medicine*, vol. 36, no. 5, pp. 592–604, 2004.

[45] S. Passamonti, U. Vrhovsek, A. Vanzo, and F. Mattivi, "Fast access of some grape pigments to the brain," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 18, pp. 7029–7034, 2005.

[46] W. Kalt, J. B. Blumberg, J. E. McDonald et al., "Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 3, pp. 705–712, 2008.

[47] P. E. Milbury and W. Kalt, "Xenobiotic metabolism and berry flavonoid transport across the blood-brain barrier," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 7, pp. 3950–3956, 2010.

[48] C. W. Shults, D. Oakes, K. Kieburtz et al., "Effects of coenzyme Q10 in early Parkinson disease: evidence of slowing of the functional decline," *Archives of Neurology*, vol. 59, no. 10, pp. 1541–1550, 2002.

[49] NINDS NET-PD Investigators, "A randomized clinical trial of coenzyme Q10 and GPI-1485 in early Parkinson disease," *Neurology*, vol. 68, no. 1, pp. 20–28, 2007.

[50] R. C. Petersen, R. G. Thomas, M. Grundman et al., "Vitamin E and donepezil for the treatment of mild cognitive impairment," *The New England Journal of Medicine*, vol. 352, no. 23, pp. 2379–2388, 2005.

[51] I. Shoulson, S. Fahn, D. Oakes et al., "Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease," *The New England Journal of Medicine*, vol. 328, no. 3, pp. 176–183, 1993.

[52] A. Lloret, M.-C. Badía, N. J. Mora, F. V. Pallardó, M.-D. Alonso, and J. Viña, "Vitamin E paradox in Alzheimer's disease: it does not prevent loss of cognition and may even be detrimental," *Journal of Alzheimer's Disease*, vol. 17, no. 1, pp. 143–149, 2009.

[53] M. C. Morris, D. A. Evans, C. C. Tangney et al., "Relation of the tocopherol forms to incident Alzheimer disease and to cognitive change," *The American Journal of Clinical Nutrition*, vol. 81, no. 2, pp. 508–514, 2005.

[54] E. E. Devore, F. Grodstein, F. J. A. van Rooij et al., "Dietary antioxidants and long-term risk of dementia," *Archives of Neurology*, vol. 67, no. 7, pp. 819–825, 2010.

[55] M. C. de Rijk, M. M. B. Breteler, J. H. den Breeijen et al., "Dietary antioxidants and Parkinson disease. The Rotterdam Study," *Archives of Neurology*, vol. 54, no. 6, pp. 762–765, 1997.

[56] J. H. Kang, A. Ascherio, and F. Grodstein, "Fruit and vegetable consumption and cognitive decline in aging women," *Annals of Neurology*, vol. 57, no. 5, pp. 713–720, 2005.

[57] M. C. Morris, D. A. Evans, C. C. Tangney, J. L. Bienias, and R. S. Wilson, "Associations of vegetable and fruit consumption with age-related cognitive change," *Neurology*, vol. 67, no. 8, pp. 1370–1376, 2006.

[58] V. I. Lushchak, "Free radicals, reactive oxygen species, oxidative stress and its classification," *Chemico-Biological Interactions*, vol. 224, pp. 164–175, 2014.

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

[60] S. G. Rhee, "H_2O_2, a necessary evil for cell signaling," *Science*, vol. 312, no. 5782, pp. 1882–1883, 2006.

[61] I. Fridovich, "Superoxide anion radical O_2^-, superoxide dismutases, and related matters," *The Journal of Biological Chemistry*, vol. 272, no. 30, pp. 18515–18517, 1997.

[62] J. S. Teodoro, A. P. Rolo, and C. M. Palmeira, "The NAD ratio redox paradox: why does too much reductive power cause oxidative stress?" *Toxicology Mechanisms and Methods*, vol. 23, no. 5, pp. 297–302, 2013.

[63] N. A. Al-Awwadi, C. Araiz, A. Bornet et al., "Extracts enriched in different polyphenolic families normalize increased cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 1, pp. 151–157, 2005.

[64] N. Ryszawa, A. Kawczyńska-Drózdz, J. Pryjma et al., "Effects of novel plant antioxidants on platelet superoxide production and aggregation in atherosclerosis," *Journal of Physiology and Pharmacology*, vol. 57, no. 4, pp. 611–626, 2006.

[65] L. K. Sarna, N. Wu, S.-Y. Hwang, Y. L. Siow, and O. Karmin, "Berberine inhibits NADPH oxidase mediated superoxide anion production in macrophages," *Canadian Journal of Physiology and Pharmacology*, vol. 88, no. 3, pp. 369–378, 2010.

[66] F. R. M. Laurindo, T. L. S. Araujo, and T. B. Abrahão, "Nox NADPH oxidases and the endoplasmic reticulum," *Antioxidants & Redox Signaling*, vol. 20, no. 17, pp. 2755–2775, 2014.

[67] T. Yunoki, K. Deguchi, Y. Omote et al., "Anti-oxidative nutrient rich diet protects against acute ischemic brain damage in rats," *Brain Research*, vol. 1587, no. 1, pp. 33–39, 2014.

[68] I.-M. Liu, T.-F. Tzeng, S.-S. Liou, and C. J. Chang, "*Angelica acutiloba* root alleviates advanced glycation end-product-mediated renal injury in streptozotocin-diabetic rats," *Journal of Food Science*, vol. 76, no. 7, pp. H165–H174, 2011.

[69] A. A. Maruf, H. Lip, H. Wong, and P. J. O'Brien, "Protective effects of ferulic acid and related polyphenols against glyoxal- or methylglyoxal-induced cytotoxicity and oxidative stress in isolated rat hepatocytes," *Chemico-Biological Interactions*, vol. 234, pp. 96–104, 2015.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5692852, 13 pages
http://dx.doi.org/10.1155/2016/5692852

Review Article

Reviews on Mechanisms of *In Vitro* Antioxidant Activity of Polysaccharides

Junqiao Wang, Shuzhen Hu, Shaoping Nie, Qiang Yu, and Mingyong Xie

State Key Laboratory of Food Science and Technology, Nanchang University, 235 Nanjing East Road, Nanchang, Jiangxi 330047, China

Correspondence should be addressed to Shaoping Nie; nie68@sina.com

Received 9 May 2015; Revised 29 July 2015; Accepted 6 August 2015

Academic Editor: Zhenquan Jia

It is widely acknowledged that the excessive reactive oxygen species (ROS) or reactive nitrogen species (RNS) induced oxidative stress will cause significant damage to cell structure and biomolecular function, directly or indirectly leading to a number of diseases. The overproduction of ROS/RNS will be balanced by nonenzymatic antioxidants and antioxidant enzymes. Polysaccharide or glycoconjugates derived from natural products are of considerable interest from the viewpoint of potent *in vivo* and *in vitro* antioxidant activities recently. Particularly, with regard to the *in vitro* antioxidant systems, polysaccharides are considered as effective free radical scavenger, reducing agent, and ferrous chelator in most of the reports. However, the underlying mechanisms of these antioxidant actions have not been illustrated systematically and sometimes controversial results appeared among various literatures. To address this issue, we summarized the latest discoveries and advancements in the study of antioxidative polysaccharides and gave a detailed description of the possible mechanisms.

1. Introduction

Polysaccharide is a high molecular weight polymer, consisting of at least ten monosaccharides mutually joined by glycosidic linkages. The glycosyl moiety of hemiacetal or hemiketal, together with the hydroxyl group of another sugar unit, formed the glycosidic linkages [1]. Unlike protein and nucleic acid, the structure of polysaccharide is far more complicated based on the differences in composition of monosaccharide residues, glycosidic linkages, sequence of sugar units, degrees of polymerization, and branching point. Apart from those, other factors, such as differences of cultivars, origins, and batches, or even extraction methods and fraction procedures are evidenced to have significant influence on the physicochemical and structural properties of polysaccharides. Owing to the rapid development of modern analytical techniques, the identification of polysaccharide structures is becoming more and more feasible and convenient.

In recent years, researches have confirmed that polysaccharides from natural products possess wide-ranging beneficial therapeutic effects and health-promoting properties. Specifically, seaweed-derived polysaccharides, such as alginate, fucoidan, carrageenan, laminaran, and agar [2], are widely distributed in biomedical and biological applications [3–8], for example, tissue engineering, drug delivery, wound healing, and biosensor due to their biocompatibility and availability. Fungal polysaccharides, derived from *G. frondosa*, *L. edodes*, oyster mushroom, as well as *Ganoderma*, *Flammulina*, *Cordyceps*, *Coriolus*, and *Pleurotus*, and so forth, are demonstrated to have multiple bioactivities [9–15], including immunomodulating, anticancer, antimicrobial, hypocholesterolemic, and hypoglycemic effects. Bacterial polysaccharides, including extracellular polysaccharides, loosely associated with bacterium, capsular polysaccharides, tightly bound to bacteria surface, and lipopolysaccharides, always anchored to cell surface by lipid, are nontoxic natural biopolymers and provide extensive applications in areas such as pharmacology, nutraceutical, functional food, cosmeceutical, herbicides, and insecticides [16–18]. Consequently, there is growing interest in further pursuing the potential bioactivities of diverse polysaccharides. In particular, most of these polysaccharides emerged as an important agent of antioxidants, both *in vitro* and *in vivo*. Recently, the polysaccharide

is reported as a kind of effective free radical scavenger and antioxidants, playing a critical role in protecting against oxidation damage in living organisms. On the other hand, many diseases, such as asthma, chronic obstructive pulmonary disease, inflammation, diabetes, myocardial infarction, and cardiovascular diseases, are reported to associate with oxidative stress [19–23]. This paper aims to review the recent advancements in analyzing antioxidative polysaccharides and summarize the possible mechanisms so as to better utilize the biopolymer.

2. Types of *In Vitro* Antioxidant Assays

Many different *in vitro* models have been introduced to evaluate the antioxidant activities so as to assess an antioxidant that would be useful for food and biological system [24, 25]. Generally, methods for determining antioxidant activities could be classified into two major groups: hydrogen atom transfer (HAT) based methods and single electron transfer (SET) based methods according to their reaction mechanisms [26, 27]. The HAT based methods usually measure the ability of quench free radical by hydrogen donation, that is, oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), inhibition of induced low-density lipoprotein (LDL) oxidation, total oxyradical scavenging capacity assay, and so forth. On the other hand, SET based methods detect the ability of transferring one electron to reduce any compound, including metals, carbonyls, and radicals, and result in a change in color when this compound is reduced, such as Trolox equivalence antioxidant capacity (TEAC) assay, ferric ion reducing antioxidant power (FRAP) assay, and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging. Other assays, for example, superoxide radical scavenging, hydrogen peroxide scavenging, and singlet oxygen quenching, evaluate the scavenging ability for oxidants.

3. Factors That Influence the Antioxidant Activity of Polysaccharides

Recently, natural materials are proved to be a highly promising source of antioxidants, since a wide range of bioactive constituents derived from them, such as flavonoids, polyphenols, sterols, peptides [28], polysaccharides, and others [29–33], have been reported to possess strong antioxidant abilities. Screening bioactive compounds from natural materials based on antioxidant potentials is widely adopted at present. Ray et al. [34] employed DPPH-scavenging-guided fractionation with silica gel column chromatography to separate potent fractions from methanolic extract of *Aloe vera* L. gel. Hossain et al. [35] obtained three fractions with high activities from the marjoram based on the results of DPPH and ferric ion reducing antioxidant power assays.

Previously, polysaccharides and polysaccharide-complex extracted from many natural sources, including higher plants, fungi, marine flora, and fauna, are of considerable interest from the viewpoint of multipharmacological activities and potential advancement towards food, nutraceuticals, and pharmaceutical industry [9, 36–39]. However, despite the great antioxidant potentials of polysaccharides exerted, their underlying mechanism is not systematically elucidated. As a result, the following sections summarize the current understanding of possible antioxidant mechanisms of polysaccharides.

3.1. Polysaccharide Conjugates. Natural polysaccharides do not always exist singly but conjugate with other components, such as amino acid, protein, lipids, and nucleic acids residues, and sometimes the polysaccharide conjugates act as a whole in isolation [40]. For example, cereal polysaccharides were reported to be associated with a certain amount of phenolic compounds [41] and tea polysaccharides were mostly glycoconjugates in which a protein carries carbohydrate chain covalently linked with a polypeptide backbone [42]. The formation of polysaccharide-polyphenol conjugates would be mediated by either H-bonding or hydrophobic interactions, and for polysaccharide-protein conjugates it may be by the existence of hydrophobic cavities and crevasses [43].

Several studies have postulated that the protein or peptide moiety in polysaccharide is responsible for part of radical scavenging effect. As mentioned in a report by Liu et al. [44], the content of protein in polysaccharide extracts appeared to contribute a direct scavenging effect on superoxide and hydroxyl radicals. Lentinan and *Schizophyllum* with only trace amount of protein exhibited negligible scavenging effect towards superoxide radicals, whereas polysaccharide-protein complexes extracted from mushrooms such as *Ganoderma* and *Grifola*, with lower polysaccharide/protein ratios, were more favorable for the scavenging function. Similarly, Huang et al. [45] demonstrated that the protein-free fractions ($P_{1/5}$ and $P_{2/5}$) separated from fermentation medium of *Cordyceps sinensis* did not show any antioxidant properties, while the fraction (P_5) with high amount of protein exhibited remarkable activity. In their previous study, TEAC value was discovered to correlate with the protein content of exopolysaccharide fractions as well [46]. Liu et al. [47] also supposed that the superoxide radical scavenging effect of crude polysaccharide from *Athyrium multidentatum* (Doll.) Ching (CPA) depended on the amount of peptides presented as a form of polysaccharide-peptide complex in CPA. Moreover, the antioxidant activity of polysaccharide-protein complexes from three mushrooms, *G. frondosa*, *Coriolus versicolor*, and *L. edodes*, attained by ultrasound-assisted extraction was generally higher than conventional hot-water method, which probably attributed to the fact that ultrasound treatment resulted in an increase of protein content in polysaccharides [48]. Zhang et al. [49] isolated three extracts (EXT-A, EXT-B, and EXT-C) from paddlefish cartilage by single alkali method, microwave-assisted alkali without or with deproteinization, respectively. Result showed that EXT-B, containing predominantly protein (87.9%), exhibited noticeable antioxidant potentials with TEAC value of 118.5 μmol Trolox/g sample and FRAP value of 107.7 μmol Fe^{2+}/g sample, while EXT-C with total sugar content of 99.0% showed little activity, indicating that the protein constituents in the extracts play a dominant role.

The further amino acid composition analysis showed that EXB-1 was abundant in tyrosine, glycine, and glutamic acid, and this investigation was performed to clarify the speculation, since the antioxidant action of protein or peptide molecules has been proved to be related to their amino acids, such as tyrosine, methionine, histidine, lysine, and tryptophan, which were capable of donating protons to electron-deficient radicals [50–52].

On the contrary, in some cases, lack of correlation between the protein content and the FRAP value was also noticed, possibly attributing to the functional groups of protein (such as -SH) which are less sensitive to FRAP assay [46].

Phenolic compounds, especially phenolic acids, play an important role in the overall radical scavenging ability of xylans and xylooligosaccharides from the wheat bran [53, 54]. Hromádková et al. [55] pointed out that both protein and phenolic compounds contributed to the radical scavenging effects of xylans, and the protein-free fraction displayed the highest hydroxyl radical scavenging ability indicating the distinct role of phenolic acids. A study [56] revealed that the antioxidant activities of all polysaccharide fractions from three mushrooms (*L. edodes, G. frondosa*, and *T. versicolor*) were significantly correlated with the total phenolic and protein content according to three *in vitro* assessments, including TEAC, FRAP, and ferrous ion chelating activity assay. However, no significant correlation was observed between the total sugar content and any of tested antioxidant assays. The results were similar to a study carried out by Wang et al. [57] that the neutral content was not apparently correlated with DPPH and FRAP antioxidant actions of polysaccharides from oolong tea. Furthermore, purified polysaccharide fractions, free of phenolics and proteins, hardly showed significant antioxidant activities. Indeed, polysaccharide-polyphenol residues have been demonstrated to have noticeable antioxidant functions in many reports. Li et al. [58] found no statistical difference in scavenging linoleic acid radicals between the polysaccharides from *Lycium barbarum* fruits and the positive control (BHT). The coupled oxidation of β-carotene and linoleic acid developed free radicals, which oxidize unsaturated β-carotene molecules, leading to the discoloration of the system. In this model, the proposed mechanism in hindering β-carotene oxidation could be attributed to the polyphenolic-associated polysaccharide neutralizing the free radicals. In DPPH radical scavenging assay, the polysaccharide showed pronounced antioxidant ability as well, possibly attributing to polyphenolic-associated polysaccharide fraction formed between high molecular weight phenolics and polysaccharides.

However, not all the conjugated moiety of polysaccharides was responsible for antioxidant power. After removing polyphenols, the tea polysaccharide conjugate from low grade green tea was found to possess strong antioxidant properties based on the results of free radical scavenging and lipid peroxidation inhibitory effect [59]. Likewise, Wang et al. [60] evidenced that the DPPH radical scavenging effect of another tea polysaccharide fraction (TPS1) was beyond 90%, close to that of ascorbic acid, although both of the protein and polyphenol content were relatively low in TPS1, suggesting other factors such as carboxyl group other than polyphenol compounds that are of concern. In order to determine the molecular interactions between tea polyphenols and oat β-glucan, Wu et al. [61] prepared complex and physical mixture of oat β-glucan and tea polyphenols, further using four *in vitro* antioxidant evaluations (DPPH radical, hydroxyl radical, superoxide radical, and reducing power) to compare the activity among tea polyphenols, β-glucan, their complex, and physical mixture. Results showed that the complex had the strongest effect against superoxide radical, whereas the mixture had the strongest hydroxyl radical scavenging effect in the concentration of 0.5–2.5 mg/mL. With regard to reducing power assay, no synergistic effect was found between tea polyphenols and β-glucan, but it was observed in DPPH scavenging assay when β-glucan was combined with tea polyphenols at low concentration (<0.05 mg/mL). However, when tea polyphenol was used at a high concentration (0.09 mg/mL), it was changed to antagonistic effect in scavenging DPPH radical. The inconsistent antioxidant outcomes of tea polyphenols and oat β-glucan complex might be dependent on its structure and provided dose, as well as the strong hydrogen bonds between them.

Ferulic acid, a kind of phenolic acid and a strong antioxidant, was shuttled to wall matrices via attachment to structural polysaccharides. Feruloylation, in certain cases, occurs on the arabinose or galactose side chains of pectin polysaccharides and influences their chemical properties. The attachment of ferulic acid is covalently via an ester linkage formed between carboxylic acid group and the primary hydroxyl at carbon-5 position of α-L-arabinofuranosyl residues [62, 63]. Some researchers [64, 65] obtained feruloyl oligosaccharide (FH) released from wheat bran insoluble dietary fiber by xylanases and found that FH could inhibit 91.7% of erythrocyte hemolysis induced by peroxyl radicals and retard the hemolytic initiation for more than 120 min under *in vitro* condition at a concentration of 4 mg/mL.

Therefore, the content of total phenolic or protein compounds conjugated in the polysaccharide extracts might explain their high antioxidant potentials.

3.2. Polysaccharide Mixture. In many reports, crude polysaccharide extracts exhibited notable antioxidant activity, but after further fractionation, the final purified polysaccharide showed moderate or low activity. It seemed that other antioxidant substances contained in the crude polysaccharide extract, such as pigments, flavones, peptide, protein, and polyphenol, might contribute to the antioxidant activity [13, 66]. Wang et al. [67] investigated the role of tea polyphenol (EGCG) in crude polysaccharide extracts from tea leaves (TPS) in the view of antioxidant ability. Results showed that the crude TPS exhibited strong antioxidant functions, whereas the further purified TPS fractions were hardly effective. But in the presence of EGCG, the reducing power and DPPH radical scavenging ability of TPS fractions were obviously enhanced. Meanwhile, the same results were also observed in dextran-EGCG system, indicating EGCG caused a synergistic increase in the antioxidant activity and tea polyphenol was the major antioxidant in the crude TPS.

Mu et al. [68] illustrated the existence of protein and pigment would influence the scavenging effect of both water-soluble and alkali-soluble crude polysaccharides from *Inonotus obliquus*. Wei et al. [69] purified an acidic polysaccharide from *Prunella vulgaris* Linn., of which scavenging abilities against DPPH and hydroxyl radical were significantly lower than crude polysaccharide, possibly ascribed to other antioxidants, such as flavones and pigments contained in the crude polysaccharide extracts. Lin et al. [70] compared the antioxidant properties of different polysaccharide fractions isolated from *Lycium barbarum* Linnaeus, including crude polysaccharide (CP), crude extract of polysaccharide (CE), deproteinated polysaccharide (DP), and deproteinated and dialyzed polysaccharide (DDP), as well as four purified fractions (one neutral and three acidic polysaccharides, named as LBPN, LBPa1, LBPa2, and LBPa3, resp.). In their study, it was suggested that the inhibition effect of superoxide and hydroxyl radical by hydroxyl groups in polysaccharides was minor due to lacking phenolic-type structure which was essential for scavenging free radicals. Many other factors, such as molecular weight, galacturonic acid, and other chemical components in polysaccharide fractions, were also supposed to play a role in their antioxidant activities. Crude and purified polysaccharide, obtained from *G. atrum*, was compared in terms of DPPH scavenging ability and self-oxidation of 1,2,3-phentriol. Although the high concentration of purified polysaccharide, PSG-1, showed noticeable antioxidant ability, it was much lower than the crude polysaccharide, probably attributing to other constituents contained in crude polysaccharides extracts, such as proteins, amino acids, peptides, cellulose, phytosterol, ascorbic acid, thiamine, nucleotide, nicotinic acid, organic acids, and microelements [71].

3.3. Polysaccharide Chelating Metal. It is worth noting that one mechanism of antioxidant activity is to inhibit the generation of free radicals by chelating ions such as ferrous and copper instead of directly scavenging them. Transition metal ions could catalyze the generation of extremely reactive hydroxyl radicals from superoxide and hydrogen peroxide, known as Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+}OH^- + OH^{\cdot}$), especially ferrous ion, which is the most effective prooxidant in the food system [72]. Two polysaccharide fractions (GAPS-1 and SAPS-1) from *A. barbadensis* Miller were isolated and purified. The hydroxyl radical scavenging activity of GAPS-1 was significantly higher than SAPS-1. Meanwhile, GAPS-1 had a higher chelating ability against ferrous ion, indicating that the chelating effect might impart polysaccharides capable of antioxidant potentials [73]. A similar correlation was also revealed by Li et al. [74] investigating an extracellular polysaccharide from *N. commune*.

Generally, the structure of compounds containing more than one of the following functional groups, that is, -OH, -SH, -COOH, $-PO_3H_2$, -C=O, $-NR_2$, -S-, and -O-, is in favor of chelating ability [75]. Therefore, presence of uronic acid and sulfate groups appeared to be essential in demonstrating the chelating ability of polysaccharides. Chang et al. [76] illustrated that the larger the content of galacturonic acid in polysaccharide, the higher the ability of chelating ferrous ion. Fan et al. [66] fractionated four polysaccharides from the leaves of *Ilex latifolia* Thunb. by DEAE cellulose-52 chromatography (ILPS1, ILPS2, ILPS3, and ILPS4) with ILPS4 having the highest contents of sulfuric radical (3.7%) and uronic acid (23.2%). Results showed that IC_{50} of ferrous chelating activity for ILPS4 was 1965 ± 8.1 μg/mL, while for other fractions the abilities were 4.7%, 11.3%, and 46.7%, respectively. This observation confirmed that the chelating effect might partly be due to the presence of functional groups such as carboxyl group and sulfuric radical in the polysaccharide structure. However, the ferrous ion chelating effect of a carboxymethylated polysaccharide (C-GLP) from *G. lucidum* was weak as compared to EDTA [77]. The reason was probably attributed to its structural features unsuitability for chelating metal ion, as the chelating ability of ferrous was dependent upon hydroxyl numbers and the hydroxyl substitution in the ortho position [78].

3.4. Metal Ions-Enriched Polysaccharide. Selenium (Se) is an essential trace element for nutrition of a capital importance in the human biology. Se does not directly act as a ROS/RNS scavenger but is a cofactor of selenoprotein, for example, glutathione peroxidase, which exerts various antioxidant activities *in vivo*. Confirmed by FT-IR and NMR spectra, the selenylation modification by H_2SeO_3/HNO_3 method predominantly happened at the C-6 position of polysaccharides and a distinct decrease of molecular weight was also induced due to the acid environment of selenized reaction. Additionally, it was proposed that the combination of Se in polysaccharides was possibly in the form of selenyl group (-SeH) or selenoacid ester [79]. Wei et al. [80] synthesized a series of selenylated polysaccharide from *Radix hedysari* (Se-RHP), the content of Se ranging from 1.04 to 3.29 mg/g and the molecular weight decreasing from 62.7 kDa to 27.7 kDa, which showed better scavenging activity and reducing power in contrast to the native RHP.

Likewise, Se-containing derivatives from *Artemisia sphaerocephala* [81] and *Potentilla anserina* L. [82] have been acknowledged to improve the antioxidant activity compared to the native polysaccharides. The proposed mechanism might be involved in changes of conformation structure of polysaccharides and emerged the increasing amount of hydroxyl group, resulting in an influence on the antioxidant activity.

Further analysis on polysaccharide obtained from Se-enriched materials confirmed the important role of Se in enhancing the antioxidant potentials of polysaccharides. As evidenced by the results of Yu et al. [83], polysaccharide from Se-enriched green tea presented significant higher antioxidant capacity than that from regular green tea. In addition, all polysaccharides isolated from Se-enriched *G. lucidum* were more effective on attenuating the production of superoxide radicals [84]. Mao et al. [85] revealed that although there was no significant difference of polysaccharide content and molecular weight of each Se-enriched *G. frondosa* polysaccharide (Se-GP) fraction and the corresponding GP, except for the Se content, Se-GPs were a more effective scavenger

(against DPPH, ABTS, and hydroxyl radicals), especially for hydroxyl radical, reaching 71.32% at a concentration of 2 mg/mL. On the other hand, selenium-polysaccharide synthesized by adding selenium chloride oxide ($SeCl_2O$) also exhibited a higher total antioxidant capacity, superoxide radical, and hydroxyl radical scavenging effect as reported by Guo et al. [86].

In addition to Se, iron was also evidenced to correlate with the antioxidant actions of polysaccharides. Abu et al. [87] found that ascophyllan and fucoidan naturally contain certain amount of iron and other metal elements. After treatment with EDTA and subsequent dialysis, most of the metal elements except Mg were removed from these two polysaccharides, especially more than 90% of ferrous ions. EDTA treatment leads to a significant increase of ferrous chelating efficiency for both of the two polysaccharides with different extents and the effect may possibly depend on the inherently existent Fe levels in the polysaccharides.

3.5. Chemical Modification. Chemical modifications, such as sulfation, carboxymethylation, phosphorylation, benzoylation, acetylation, and $NaIO_4$ oxidation, are evidenced to influence the antioxidant activity of polysaccharides to some extent. It is widely accepted that chemical modifications could enhance the antioxidant activity of polysaccharides, for example, sulfated polysaccharide from fresh persimmon (*Diospyros kaki* L.) fruit [88], *Tremella fuciformis* [89], acetylated, phosphorylated, and benzoylated levan-type exopolysaccharide from *Paenibacillus polymyxa* EJS-3 [90], phosphorylated polysaccharide from *Radix hedysari* [91], and acetylated and benzoylated derivatives from *Ulva pertusa* [92], which exhibited obviously stronger scavenging activity and/or reducing power than the unmodified polysaccharides. One mechanism is that the introduction of these substitution groups into polysaccharide molecules leads to weaker dissociation energy of hydrogen bond. Therefore, the hydrogen donating ability of polysaccharide derivatives was increased. Another mechanism is speculated to activate the abstraction of the anomeric carbon. On the other hand, the chemical modification is sometimes accompanied with a decrease of molecular weight, hence improving the antioxidant potentials of polysaccharides. Among the derivatives, the sulfate polysaccharide is commonly reported as a stronger antioxidant, which is partly due to its ordered, extended structure. The sulfated polysaccharide usually traps free radicals in an electrostatic manner since the sulfate groups usually generate a highly acidic environment and the sulfur substitution may also weaken hydrogen bond interactions between polysaccharides.

An algal sulfated polysaccharide, fucoidan, extracted from *Laminaria japonica* has been fully studied on various molecular modification derivatives. Six low molecular fucoidan derivatives (sulfated DFPS, acetylated ADF, benzoylated PHDF, phosphorylated PDF1 and PDF2, and aminated NDF) were all exhibiting potent antioxidant potentials. PHDF had the strongest radical scavenging abilities and DFPS had the highest reducing power [93]. Feng et al. [94] found unsulfated lentinan nearly detected antioxidant capacity, but when it was sulfated by either conventional heating or novel microwave radiation, the antioxidant effect was considerably enhanced, indicating the positive correlation between antioxidant effects and introduction of the sulfate group. Chen et al. [95] prepared four phosphorylated polysaccharides (POP1-p) from *Portulaca oleracea* L. and compared the antioxidant activity with the native POP1. They found that POP1-p had stronger scavenging effect on hydroxide radical, superoxide radical, and DPPH radical, as well as a higher ferrous ions chelating ability and reducing power.

Types of substitution groups and degrees of substitution (DS) appeared to have an effect on the physicochemical properties and conformation of native polysaccharides, such as molecular weight, polarity, solubility, and charge density. DS may also affect the activity through interruption of inter- and intramolecular hydrogen bonds. Chen et al. [96] found that not only did the total sugar content of acetylated and carboxymethylated derivatives decrease significantly, but also its molecular weight was reduced in contrast to native *G. atrum* polysaccharide. Liu et al. [97] proved that sulfation effectively improved the water solubility and bile acid-binding capacities of a water-insoluble polysaccharide from *G. lucidum* (GLP). Furthermore, ^{13}C NMR results showed that C-2, C-4, and C-6 position might be partially substituted, and C-4 was the most reactive. It was probably due to its special structure features and the influence of steric hindrance.

A linear relationship between the degree of substitution and antioxidant potentials was not always observed, suggesting high DS was not necessary for antioxidant behavior. Xie et al. [98] revealed that antioxidant activity of sulfated CP with a highest DS of 0.55 was not as effective as derivatives with middle DS (0.42 and 0.06). However, the influence of DS was still disputable, as a high DS could enhance the antioxidant activity evidenced in many reports. Yan et al. [99] pointed out that the sulfation of exopolysaccharide, produced by *Cordyceps sinensis* fungus (Cs-HK1), occurred most frequently at hydroxyl groups of C-6 and caused a conformation change from random coils or aggregates to single helices in aqueous solution. The antioxidant activity of the sulfated derivatives for hydroxyl radical and ABTS radical scavenging effect was significantly enhanced with increasing DS and reducing molecular weight. Wang et al. [100] showed that C-6 substitution was predominately in phosphorylated derivatives of galactomannan (PGG) from guar gum according to ^{13}C NMR analysis and PGG with high DS achieved a higher radical scavenging effect and stronger chelating ability than PGG with lower DS. Jung et al. found that DPPH radical scavenging ability of polysaccharide from *Pleurotus eryngii* was improved with increasing degree of sulfation [101]. This finding was also consistent with the report that high degree of sulfated substitution (0.90) was more effective than that of low DS (0.43) in scavenging DPPH [102]. Another study also revealed a positive relationship between the degrees of acetylated substitution and scavenging effects against DPPH and superoxide radical, as well as reducing power [103].

It is worth pointing out that the reducing power of polysaccharide was nearly lost after phosphorylated modification in some studies [104, 105]. This may be because

the negative charged phosphorylate groups were effective at some specific sites of residues in certain polysaccharides, but they were weakened at others.

3.6. Structural Features of Polysaccharide. It is widely believed that the bioactivity of polysaccharides is affected by their structure characteristics, such as chemical composition, molecular mass, types of glycosidic linkage, and conformation. Differences in origin materials, extraction procedures, and even drying technologies that influence the physiochemical properties, structure, or conformation of polysaccharides will lead to differences in antioxidant activity, speculating their possible relationships [106–109]. Specifically, a correlation between molecular weight and radical scavenging activity was well documented [48, 57] and a similar observation in uronic acid content was reported as well in several reports [110, 111]. Additionally, it is suggested that the overall radical scavenging ability was associated with the number of hydroxyl or amino groups in polysaccharide molecules such as chitosan [112].

Molecular weight was one of the most important structural features of polysaccharide. A number of reports suggested that the antioxidant potency is mainly associated with molecular weight of polysaccharides. It was supposed that polysaccharides with low molecular weights would have more reductive hydroxyl group terminals (on per unit mass basis) to accept and eliminate the free radicals. Liu et al. [113] obtained two low molecular weight polysaccharides (GLP_L1 and GLP_L2) from *G. lucidum* and investigated the antioxidant activity of these two polysaccharides. Results showed that both GLP_L1 and GLP_L2 are effective radical scavenger and ferrous chelator. Xing et al. [114] reported that scavenging effect against superoxide radical of low molecular weight chitosan (9 kDa) was more potent than that of the high molecular one (760 kDa). The possible mechanism may be related to the structure characteristics of chitosan, which contained two hydrogen groups and one amino group in each monomer unit. High molecular weight chitosan has a more compact structure, resulting in stronger intramolecular hydrogen bond and thus making the hydrogen and amino groups restricted. By a stepwise fractionated precipitation with ethanol at a final concentration of 40%, 60%, and 80%, Zha et al. [115] obtained three polysaccharides from rice bran with a molecular weight ranging from 1.2×10^5 to 6.3×10^6 Da (PW1), 3.5×10^4 to 7.4×10^4 Da (PW2), and 5.3×10^3 to 2.3×10^4 Da (PW3), respectively. Results showed that PW3 exhibited the best potentials of reducing power, chelating metal ion, and scavenging abilities against DPPH and ABTS radical among three fractions, revealing that a relative low molecular weight fraction had high antioxidant abilities. Likewise, similar findings were also reported on other plant derived polysaccharides or extracellular polysaccharides [116–119].

Many techniques and methods (physically, chemically, or enzymatically) were used for degrading polysaccharides, resulting in a reduction of molecular weight, proved to influence the antioxidant activities as well. Feng et al. [120] revealed that a γ-ray treated chitosan had more pronounced antioxidant properties without changes in its backbone structure except for a decrease of molecular weight. Two well-known seaweeds polysaccharides, fucoidan and laminarin, were shown to increase the DPPH radical scavenging activity and reducing power after γ-ray irradiation, which result in the decrease of molecular weight and increase of carboxyl and carbonyl groups and double bonds [121].

Ultrasonic treatment, another method for degradation of polysaccharide, was also shown to enhance hydroxyl and superoxide anion radical scavenging capacity, chelating iron ion ability, and reducing power, possibly attributing to the decreased molecular weight and increased sulfate groups [122]. Zhang et al. [123] chemically degraded a polysaccharide from *Enteromorpha linza* using combination of ascorbic acid and H_2O_2 in order to obtain a lower molecular weight fraction, possessing higher hydroxyl scavenging effect and reducing power. Sun et al. [124] investigated the microwave-degraded polysaccharides from *Porphyridium cruentum* and found that high molecular weight polysaccharides had no obvious antioxidant activity, whereas the low molecular weight fragments showed strong scavenging effect on free radicals. And this microwave treatment did not apparently change the chemical components of the polysaccharide confirmed by physicochemical analysis.

However, inconsistent findings were also described. Cheng et al. [125] investigated the antioxidant potentials of polysaccharides from *Epimedium acuminatum*. Fractions with higher molecular weight displayed better antioxidant actions with regard to hydroxyl radical, H_2O_2-induced hemolysis inhibition, and lipid peroxidation. Additionally, Kardošová and Machová [126] revealed the effect of molecular weight was not significant based on similar antioxidant levels of polysaccharides and oligosaccharides.

Generally, the acidic polysaccharides, which contained a certain amount of uronic acid, were potent antioxidants [127, 128]. Therefore, uronic acid is considered to be another important indicator reflecting the antioxidant activity of the polysaccharides. It is supposed that the presence of electrophilic groups like keto or aldehyde in acidic polysaccharide facilitates the liberation of hydrogen from O-H bond. Evaluation of β-carotene linoleate emulsion ability, DPPH radical scavenging effect, and FRAP on glucuronic acid, galacturonic acid, and polygalacturonic acid was carried out in order to conclusively confirm the role of uronic acid in antioxidant potency [129]. Results showed that all the three kinds of uronic acid exhibited strong antioxidant effect in the order of polygalacturonic acid > glucuronic acid > galacturonic acid, indicating degree/nature of polymerization may impart the activity. But other compounds that also contained carboxylic group, such as formic, acetic, propionic, butyric, succinic, and citric acids, showed very low effect. The carbonyl group in the above acid was in an open chain, while in phenolic acid or uronic acid it was attached to a ring molecule. Li et al. [130] reported that two polysaccharide fractions (ZSP3c and ZSP4b) attained from *Zizyphus Jujuba* cv. *Jinsixiaozao* with the higher uronic acid content (25.5% and 29.0%, resp.) showed stronger free radical scavenging activities than ZSP1b containing no uronic acid. Different drying methods, such as hot air drying, vacuum drying, and freeze drying,

will influence the antioxidant activity of polysaccharides. Among them, freeze drying was an appropriated and effective method to yield polysaccharides with higher free radical scavenging ability, reducing power and Fe^{2+}-chelating ability, and the different contents of uronic acid might partly be involved in [131, 132]. Therefore, the antioxidant properties of polysaccharides might due to, in part, the presence of uronic acid.

Among various antioxidative molecules, sulfated polysaccharides effectively scavenge free radicals, bind metal ion catalysts to inhibit the continuous production of radicals, and protect against lipid peroxidation. Polysaccharide obtained from marine algae is a kind of native sulfated polysaccharide displaying considerable antioxidant activities. Yang et al. [133] compared the antioxidant activity of sulfated polysaccharide from *Corallina officinalis* and its desulfated derivatives. Results showed that the native sulfated polysaccharides possessed more excellent radical scavenging activity and reducing power than the desulfated fractions. The reduced antioxidant capacity after desulfated treatment was also evidenced on sulfated polysaccharides from *Undaria pinnatifida* [134]. Apart from molecular modification treatment, the native sulfated polysaccharide obtained from *Laminaria japonica* was demonstrated to be an effective antioxidant, partly related to the sulfate groups in the polysaccharide although other factors, that is, molar ratio of sulfate/(fucose or total sugar), molecular weight, could not be ignored as well [135]. Analysis of four sulfated polysaccharide fractions with different molecular weights prepared from *Ulva pertusa* Kjellm. demonstrated that lower molecular weight polysaccharide fractions, presented higher number of reducing and nonreducing ends, showed the stronger reducing power [136]. Pectic acids, known as polygalacturonic acids, showed extremely the highest reducing power among the tested polysaccharides, including chitosans and alginates with low and high molecular weights. Consequently, certain structural characteristics specific to the polysaccharides other than molecular weight might be responsible for the reducing power. Chitosan is a deacetylation product of chitin, which was naturally existent in the shells of crabs, shrimp, and krill. Both hydroxyl group and amino groups in chitosan backbone affect its antioxidant ability [137].

Except for the polysaccharide with anionic or cationic functional groups, such as chitosan, sulfated or phosphorylated glucans, most carbohydrates are not a class of potent antioxidant, significantly weaker than the synthetic antioxidants such as BHT, Trolox, and pyrrolidine dithiocarbamate. Chen et al. [138] have demonstrated that only polysaccharide in a polyelectrolyte form exhibited powerful antioxidant activities, that is, agar with sulfate group and chitosan with amino group, but not starch which only had hydroxyl group. Rao and Muralikrishna [129] revealed that neither glucose nor soluble starch, laminarin, showed any antioxidant activity according to the results of emulsion assay, even at very high concentration of 2 mg/mL. β-glucans from yeast cell walls appeared to exhibit a low antioxidant activity compared to other cell wall fractions, that is, protein [139, 140]. Another report [141] revealed that all yeast mannans (derived from *S. cerevisiae*, *C. dubliniensis*, *C. tropicalis*, *C. albicans* ser A, and *C. albicans* ser B) and commercial β-glucans (laminarin, lichenan, and curdlan) were weak DPPH and hydroxyl radical scavenger and poor Fe^{2+}-chelator with the most effective one chelated only about 13.1% of Fe^{2+}.

One proposed model for free radical scavenging effect was to subtract the anomeric hydrogen from carbohydrates by free radicals and combine it to form a neutral molecular [142], and then the generated alkoxyl radical promoted the intramolecular hydrogen abstraction reaction which triggers spirocyclization reaction to terminate the reaction of radical chain [143–145]. Further verification experiments to confirm the model, however, are not conducted.

On the other hand, the definite role of monosaccharide or glycosidic linkages in antioxidant activity of polysaccharide remained confused. Lo et al. [146] investigated the relationship between antioxidant properties of polysaccharides and monosaccharide or glycosyl linkages using four conventional antioxidant models (conjugated diene, reducing power, DPPH scavenging, and ferrous ions chelating) on multiple linear regression analysis (MLRA). Results revealed that compositions and ratios of monosaccharide as well as types of glycosyl linkages would be of concern in modulating the antioxidant properties. Specifically, rhamnose and mannose showed positive coefficients in all the four MLRA models. Meanwhile, glycosyl linkages, specifically arabinose $1 \rightarrow 4$ and mannose $1 \rightarrow 2$ of the side chain, were significantly related to the reducing power, whereas glucose $1 \rightarrow 6$ and arabinose $1 \rightarrow 4$ were closely in relation to DPPH radical scavenging effect. Tsiapali et al. [147] pointed out that a portion of antioxidant ability of the carbohydrate appeared to correlate with the monosaccharide composition rather than types of intrachain linkages, molecular weight, or degree of branching, since either dextrose or mannose showed weaker free radical scavenging ability than the polymer. It was interesting to find that the polymer had better radical scavenging effect than either of the monosaccharides, suggesting that the polymeric structure conferred additional activity of the carbohydrates. Meng et al. [148] adopted Pearson correlation analysis test and linear regression analysis to explore the relationship between the monosaccharide composition of polysaccharides and the antioxidant activity. Results showed that the antioxidant activity was significantly correlated with the content of mannose ($P < 0.01$) and glucose ($P < 0.05$), whereas galactose was not correlated ($P > 0.05$). Furthermore, both the contents of monosaccharide were observed to have high correlation coefficients concerning radical scavenging activity with mannose the positive ($r = 0.942$) and glucose the negative ($r = -0.905$).

4. Conclusion and Perspective

According to the extensive *in vitro* antioxidant studies, the polysaccharide is indeed an effective antioxidant. The underlying mechanism is, however, uncertain as the relationships between antioxidant activity and physicochemical properties or structural features are not comprehensively elucidated and confirmed. Besides, it is worth noting that conflicting results are observed in comparison with a number of literatures,

since different sources, extraction methods, and even drying procedures can influence the evaluated polysaccharides. On the other hand, limited information is available on antioxidant activity of high-purity polysaccharides, and therefore other antioxidant substances, for instance, protein, peptide, and polyphenol, that may always be retained in polysaccharides in a form of either conjugation or mixture should be taken into account. Overall, the antioxidant potentials of polysaccharides are not determined by a single factor but a combination of several related factors.

In the future, more studies should be concentrated on the exact mechanism of *in vitro* antioxidant activities of polysaccharides themselves and design some experiments if pure carbohydrates possess *in vitro* antioxidative capacity. Based on the various antioxidant mechanisms, different antioxidative evaluations, such as DPPH radical scavenging, hydroxyl radical scavenging, ABTS radical scavenging, reducing power, and chelating ability, should be adopted to assess the respective influences of carbohydrates, especially their structural features. With regard to the intensive researches on natural antioxidants, the properties of polysaccharides from natural products useful for the antioxidant activity and healthy benefits should be clearly elucidated, and more specific research, therefore, on this topic is imperative.

Conflict of Interests

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

Acknowledgments

The financial support from National Natural Science Foundation of China for Excellent Young Scholars (31422042), the Program for New Century Excellent Talents in University (NCET-12-0749), the Project of Science and Technology of Jiangxi Provincial Education Department (KJLD13004), and Research Project of State Key Laboratory of Food Science and Technology (SKLF-ZZA-201301) is gratefully acknowledged.

References

[1] S. W. Cui, *Food Carbohydrates: Chemistry, Physical Properties, and Applications*, CRC Press, 2005.

[2] P. Laurienzo, "Marine polysaccharides in pharmaceutical applications: an overview," *Marine Drugs*, vol. 8, no. 9, pp. 2435–2465, 2010.

[3] J. Venkatesan, B. Lowe, S. Anil et al., "Seaweed polysaccharides and their potential biomedical applications," *Starch—Stärke*, vol. 67, no. 5-6, pp. 381–390, 2015.

[4] J. F. A. Valente, T. A. M. Valente, P. Alves, P. Ferreira, A. Silva, and I. J. Correia, "Alginate based scaffolds for bone tissue engineering," *Materials Science and Engineering C*, vol. 32, no. 8, pp. 2596–2603, 2012.

[5] T. H. Silva, A. Alves, E. G. Popa et al., "Marine algae sulfated polysaccharides for tissue engineering and drug delivery approaches," *Biomatter*, vol. 2, no. 4, pp. 278–289, 2012.

[6] E. G. Popa, R. L. Reis, and M. E. Gomes, "Seaweed polysaccharide-based hydrogels used for the regeneration of articular cartilage," *Critical Reviews in Biotechnology*, pp. 1–14, 2014.

[7] L. Li, R. Ni, Y. Shao, and S. Mao, "Carrageenan and its applications in drug delivery," *Carbohydrate Polymers*, vol. 103, no. 1, pp. 1–11, 2014.

[8] A. Srivastava, A. Srivastava, A. Srivastava, and P. Chandra, "Marine biomaterials in therapeutics and diagnostic," in *Springer Handbook of Marine Biotechnology*, pp. 1247–1263, Springer, Berlin, Germany, 2015.

[9] I. Giavasis, "Bioactive fungal polysaccharides as potential functional ingredients in food and nutraceuticals," *Current Opinion in Biotechnology*, vol. 26, pp. 162–173, 2014.

[10] I. Giavasis, "Production of microbial polysaccharides for use in food," in *Microbial Production of Food Ingredients, Enzymes and Nutraceuticals*, pp. 413–468, Woodhead Publishing, Cambridge, UK, 2013.

[11] S. Nie, H. Zhang, W. Li, and M. Xie, "Current development of polysaccharides from *Ganoderma*: isolation, structure and bioactivities," *Bioactive Carbohydrates and Dietary Fibre*, vol. 1, no. 1, pp. 10–20, 2013.

[12] S. Nie, S. W. Cui, M. Xie, A. O. Phillips, and G. O. Phillips, "Bioactive polysaccharides from *Cordyceps sinensis*: isolation, structure features and bioactivities," *Bioactive Carbohydrates and Dietary Fibre*, vol. 1, no. 1, pp. 38–52, 2013.

[13] C. Wu, X. Wang, H. Wang et al., "Extraction optimization, isolation, preliminary structural characterization and antioxidant activities of the cell wall polysaccharides in the petioles and pedicels of Chinese herbal medicine Qian (*Euryale ferox* Salisb.)," *International Journal of Biological Macromolecules*, vol. 64, pp. 458–467, 2014.

[14] L. Ren, C. Perera, and Y. Hemar, "Antitumor activity of mushroom polysaccharides: a review," *Food & Function*, vol. 3, no. 11, pp. 1118–1130, 2012.

[15] E. Vamanu, "Biological activities of the polysaccharides produced in submerged culture of two edible *Pleurotus ostreatus* mushrooms," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 565974, 8 pages, 2012.

[16] U. U. Nwodo, E. Green, and A. I. Okoh, "Bacterial exopolysaccharides: functionality and prospects," *International Journal of Molecular Sciences*, vol. 13, no. 11, pp. 14002–14015, 2012.

[17] F. Freitas, V. D. Alves, and M. A. M. Reis, "Advances in bacterial exopolysaccharides: from production to biotechnological applications," *Trends in Biotechnology*, vol. 29, no. 8, pp. 388–398, 2011.

[18] F. Freitas, V. D. Alves, and M. A. Reis, "Bacterial polysaccharides: production and applications in cosmetic industry," in *Polysaccharides*, pp. 1–24, Springer, 2014.

[19] H. D. Scheibmeir, K. Christensen, S. H. Whitaker, J. Jegaethesan, R. Clancy, and J. D. Pierce, "A review of free radicals and antioxidants for critical care nurses," *Intensive and Critical Care Nursing*, vol. 21, no. 1, pp. 24–28, 2005.

[20] I. Olmez and H. Ozyurt, "Reactive oxygen species and ischemic cerebrovascular disease," *Neurochemistry International*, vol. 60, no. 2, pp. 208–212, 2012.

[21] K. Sugamura and J. F. Keaney Jr., "Reactive oxygen species in cardiovascular disease," *Free Radical Biology and Medicine*, vol. 51, no. 5, pp. 978–992, 2011.

[22] D. P. Rosanna and C. Salvatore, "Reactive oxygen species, inflammation, and lung diseases," *Current Pharmaceutical Design*, vol. 18, no. 26, pp. 3889–3900, 2012.

[23] I. Afanas'ev, "ROS and RNS signaling in heart disorders: could antioxidant treatment be successful?" *Oxidative Medicine and Cellular Longevity*, vol. 2011, Article ID 293769, 13 pages, 2011.

[24] B. Halliwell, R. Aeschbach, J. Löliger, and O. I. Aruoma, "The characterization of antioxidants," *Food and Chemical Toxicology*, vol. 33, no. 7, pp. 601–617, 1995.

[25] C. Sánchez-Moreno, "Review: methods used to evaluate the free radical scavenging activity in foods and biological systems," *Food Science and Technology International*, vol. 8, no. 3, pp. 121–137, 2002.

[26] İ. Gülçin, "Antioxidant activity of food constituents: an overview," *Archives of Toxicology*, vol. 86, no. 3, pp. 345–391, 2012.

[27] J. B. L. Tan and Y. Y. Lim, "Critical analysis of current methods for assessing the in vitro antioxidant and antibacterial activity of plant extracts," *Food Chemistry*, vol. 172, pp. 814–822, 2015.

[28] J. Sun, H. He, and J. X. Bi, "Novel antioxidant peptides from fermented mushroom *Ganoderma lucidum*," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 21, pp. 6646–6652, 2004.

[29] F. Shahidi and Y. Zhong, "Novel antioxidants in food quality preservation and health promotion," *European Journal of Lipid Science and Technology*, vol. 112, no. 9, pp. 930–940, 2010.

[30] P. A. S. White, R. C. M. Oliveira, A. P. Oliveira et al., "Antioxidant activity and mechanisms of action of natural compounds isolated from lichens: a systematic review," *Molecules*, vol. 19, no. 9, pp. 14496–14527, 2014.

[31] R. da Silva Marineli, É. A. Moraes, S. A. Lenquiste, A. T. Godoy, M. N. Eberlin, and M. R. Maróstica Jr., "Chemical characterization and antioxidant potential of Chilean chia seeds and oil (*Salvia hispanica* L.)," *LWT—Food Science and Technology*, vol. 59, no. 2, pp. 1304–1310, 2014.

[32] A. López, G.-A. Javier, J. Fenoll, P. Hellín, and P. Flores, "Chemical composition and antioxidant capacity of lettuce: comparative study of regular-sized (Romaine) and baby-sized (Little Gem and Mini Romaine) types," *Journal of Food Composition and Analysis*, vol. 33, no. 1, pp. 39–48, 2014.

[33] X.-Z. Liu, S.-P. Nie, W. J. Li et al., "Triterpenoids content and antioxidant activity of neutral components from *Ganoderma atrum*," *Journal of Nanchang University (Engineering & Technology)*, vol. 4, p. 8, 2011.

[34] A. Ray, S. D. Gupta, and S. Ghosh, "Isolation and characterization of potent bioactive fraction with antioxidant and UV absorbing activity from *Aloe barbadensis* Miller gel," *Journal of Plant Biochemistry and Biotechnology*, vol. 22, no. 4, pp. 483–487, 2013.

[35] M. B. Hossain, G. Camphuis, I. Aguiló-Aguayo, N. Gangopadhyay, and D. K. Rai, "Antioxidant activity guided separation of major polyphenols of marjoram (*Origanum majorana* L.) using flash chromatography and their identification by liquid chromatography coupled with electrospray ionization tandem mass spectrometry," *Journal of Separation Science*, vol. 37, no. 22, pp. 3205–3213, 2014.

[36] I. Wijesekara, R. Pangestuti, and S.-K. Kim, "Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae," *Carbohydrate Polymers*, vol. 84, no. 1, pp. 14–21, 2011.

[37] A. Jiménez-Escrig, E. Gomez-Ordonez, and P. Rupérez, "Seaweed as a source of novel nutraceuticals: sulfated polysaccharides and peptides," *Advances in Food and Nutrition Research*, vol. 64, no. 64, pp. 325–337, 2011.

[38] M. Zhang, S. W. Cui, P. C. K. Cheung, and Q. Wang, "Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity," *Trends in Food Science and Technology*, vol. 18, no. 1, pp. 4–19, 2007.

[39] A. Zong, H. Cao, and F. Wang, "Anticancer polysaccharides from natural resources: a review of recent research," *Carbohydrate Polymers*, vol. 90, no. 4, pp. 1395–1410, 2012.

[40] H. Chen, M. Zhang, Z. Qu, and B. Xie, "Antioxidant activities of different fractions of polysaccharide conjugates from green tea (*Camellia Sinensis*)," *Food Chemistry*, vol. 106, no. 2, pp. 559–563, 2008.

[41] P. S. Thondre, L. Ryan, and C. J. K. Henry, "Barley β-glucan extracts as rich sources of polyphenols and antioxidants," *Food Chemistry*, vol. 126, no. 1, pp. 72–77, 2011.

[42] S.-P. Nie and M.-Y. Xie, "A review on the isolation and structure of tea polysaccharides and their bioactivities," *Food Hydrocolloids*, vol. 25, no. 2, pp. 144–149, 2011.

[43] C. M. G. C. Renard, A. Baron, S. Guyot, and J.-F. Drilleau, "Interactions between apple cell walls and native apple polyphenols: quantification and some consequences," *International Journal of Biological Macromolecules*, vol. 29, no. 2, pp. 115–125, 2001.

[44] F. Liu, V. E. C. Ooi, and S. T. Chang, "Free radical scavenging activities of mushroom polysaccharide extracts," *Life Sciences*, vol. 60, no. 10, pp. 763–771, 1997.

[45] Q.-L. Huang, K.-C. Siu, W.-Q. Wang, Y.-C. Cheung, and J.-Y. Wu, "Fractionation, characterization and antioxidant activity of exopolysaccharides from fermentation broth of a *Cordyceps sinensis* fungus," *Process Biochemistry*, vol. 48, no. 2, pp. 380–386, 2013.

[46] P. H. Leung, S. Zhao, K. P. Ho, and J. Y. Wu, "Chemical properties and antioxidant activity of exopolysaccharides from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1," *Food Chemistry*, vol. 114, no. 4, pp. 1251–1256, 2009.

[47] D. Liu, J. Sheng, Z. Li et al., "Antioxidant activity of polysaccharide fractions extracted from *Athyrium multidentatum* (Doll.) Ching," *International Journal of Biological Macromolecules*, vol. 56, pp. 1–5, 2013.

[48] Y.-C. Cheung, K.-C. Siu, Y.-S. Liu, and J.-Y. Wu, "Molecular properties and antioxidant activities of polysaccharide-protein complexes from selected mushrooms by ultrasound-assisted extraction," *Process Biochemistry*, vol. 47, no. 5, pp. 892–895, 2012.

[49] L. Zhang, S. Zhao, S. Xiong, Q. Huang, and S. Shen, "Chemical structure and antioxidant activity of the biomacromolecules from paddlefish cartilage," *International Journal of Biological Macromolecules*, vol. 54, no. 1, pp. 65–70, 2013.

[50] R. J. Elias, S. S. Kellerby, and E. A. Decker, "Antioxidant activity of proteins and peptides," *Critical Reviews in Food Science and Nutrition*, vol. 48, no. 5, pp. 430–441, 2008.

[51] A. Minelli, I. Bellezza, S. Grottelli, and F. Galli, "Focus on cyclo(His-Pro): history and perspectives as antioxidant peptide," *Amino Acids*, vol. 35, no. 2, pp. 283–289, 2008.

[52] A. R. Pérez, M. T. Iglesias, E. Pueyo, M. González, and C. De Lorenzo, "Amino acid composition and antioxidant capacity of Spanish honeys," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 2, pp. 360–365, 2007.

[53] Z. Hromádková, Z. Košťálová, and A. Ebringerová, "Comparison of conventional and ultrasound-assisted extraction of phenolics-rich heteroxylans from wheat bran," *Ultrasonics Sonochemistry*, vol. 15, no. 6, pp. 1062–1068, 2008.

[54] B. R. Veenashri and G. Muralikrishna, "*In vitro* anti-oxidant activity of xylo-oligosaccharides derived from cereal and millet brans—a comparative study," *Food Chemistry*, vol. 126, no. 3, pp. 1475–1481, 2011.

[55] Z. Hromádková, B. S. Paulsen, M. Polovka, Z. Košťálová, and A. Ebringerová, "Structural features of two heteroxylan polysaccharide fractions from wheat bran with anti-complementary and antioxidant activities," *Carbohydrate Polymers*, vol. 93, no. 1, pp. 22–30, 2013.

[56] K. C. Siu, X. Chen, and J. Y. Wu, "Constituents actually responsible for the antioxidant activities of crude polysaccharides isolated from mushrooms," *Journal of Functional Foods*, vol. 11, pp. 548–556, 2014.

[57] C. Wang, Y. Chen, M. Hu, J. Ding, C. Xu, and R. Wang, "*In vitro* antioxidant activities of the polysaccharides from *Tricholoma lobayense*," *International Journal of Biological Macromolecules*, vol. 50, no. 3, pp. 534–539, 2012.

[58] X. M. Li, X. L. Li, and A. G. Zhou, "Evaluation of antioxidant activity of the polysaccharides extracted from *Lycium barbarum* fruits in vitro," *European Polymer Journal*, vol. 43, no. 2, pp. 488–497, 2007.

[59] H. Chen, M. Zhang, and B. Xie, "Components and antioxidant activity of polysaccharide conjugate from green tea," *Food Chemistry*, vol. 90, no. 1-2, pp. 17–21, 2005.

[60] Y. Wang, Z. Yang, and X. Wei, "Antioxidant activities potential of tea polysaccharide fractions obtained by ultra filtration," *International Journal of Biological Macromolecules*, vol. 50, no. 3, pp. 558–564, 2012.

[61] Z. Wu, J. Ming, R. Gao et al., "Characterization and antioxidant activity of the complex of tea polyphenols and oat β-glucan," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 19, pp. 10737–10746, 2011.

[62] R. D. Hatfield, J. Ralph, and J. H. Grabber, "Cell wall cross-linking by ferulates and diferulates in grasses," *Journal of the Science of Food and Agriculture*, vol. 79, no. 3, pp. 403–407, 1999.

[63] T. Ishii, "Structure and functions of feruloylated polysaccharides," *Plant Science*, vol. 127, no. 2, pp. 111–127, 1997.

[64] X. Yuan, J. Wang, H. Yao, and F. Chen, "Free radical-scavenging capacity and inhibitory activity on rat erythrocyte hemolysis of feruloyl oligosaccharides from wheat bran insoluble dietary fiber," *LWT—Food Science and Technology*, vol. 38, no. 8, pp. 877–883, 2005.

[65] X. Yuan, J. Wang, and H. Yao, "Antioxidant activity of feruloylated oligosaccharides from wheat bran," *Food Chemistry*, vol. 90, no. 4, pp. 759–764, 2005.

[66] J. Fan, Z. Wu, T. Zhao et al., "Characterization, antioxidant and hepatoprotective activities of polysaccharides from *Ilex latifolia* Thunb," *Carbohydrate Polymers*, vol. 101, no. 1, pp. 990–997, 2014.

[67] Y. Wang, Y. Zhao, K. Andrae-Marobela, H. Okatch, and J. Xiao, "Tea polysaccharides as food antioxidants: an old woman's tale?" *Food Chemistry*, vol. 138, no. 2-3, pp. 1923–1927, 2013.

[68] H. Mu, A. Zhang, W. Zhang, G. Cui, S. Wang, and J. Duan, "Antioxidative properties of crude polysaccharides from *Inonotus obliquus*," *International Journal of Molecular Sciences*, vol. 13, no. 7, pp. 9194–9206, 2012.

[69] M. Wei, S. L. Xiong, H. Jin et al., "Isolation and antioxidant activity of water-soluble acidic polysaccharide from *Prunella vulgaris* Linn," *Food Science*, vol. 1, article 022, 2010.

[70] C. L. Lin, C. C. Wang, S. C. Chang, B. S. Inbaraj, and B. H. Chen, "Antioxidative activity of polysaccharide fractions isolated from *Lycium barbarum* Linnaeus," *International Journal of Biological Macromolecules*, vol. 45, no. 2, pp. 146–151, 2009.

[71] Y. Chen, M.-Y. Xie, S.-P. Nie, C. Li, and Y.-X. Wang, "Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*," *Food Chemistry*, vol. 107, no. 1, pp. 231–241, 2008.

[72] R. Yamauchi, Y. Tatsumi, M. Asano, K. Kato, and Y. Ueno, "Effect of metal salts and fructose on the autoxidation of methyl linoleate in emulsions," *Agricultural and Biological Chemistry*, vol. 52, no. 3, pp. 849–850, 1988.

[73] L. Chun-hui, W. Chang-hai, X. Zhi-liang, and W. Yi, "Isolation, chemical characterization and antioxidant activities of two polysaccharides from the gel and the skin of *Aloe barbadensis* Miller irrigated with sea water," *Process Biochemistry*, vol. 42, no. 6, pp. 961–970, 2007.

[74] H. Li, J. Xu, Y. Liu et al., "Antioxidant and moisture-retention activities of the polysaccharide from *Nostoc commune*," *Carbohydrate Polymers*, vol. 83, no. 4, pp. 1821–1827, 2011.

[75] I. Gülçin, "Antioxidant and antiradical activities of L-carnitine," *Life Sciences*, vol. 78, no. 8, pp. 803–811, 2006.

[76] S. C. Chang, B. Y. Hsu, and B. H. Chen, "Structural characterization of polysaccharides from *Zizyphus jujuba* and evaluation of antioxidant activity," *International Journal of Biological Macromolecules*, vol. 47, no. 4, pp. 445–453, 2010.

[77] J. Xu, W. Liu, W. Yao, X. Pang, D. Yin, and X. Gao, "Carboxymethylation of a polysaccharide extracted from *Ganoderma lucidum* enhances its antioxidant activities *in vitro*," *Carbohydrate Polymers*, vol. 78, no. 2, pp. 227–234, 2009.

[78] H. Wang, X. D. Gao, G. C. Zhou, L. Cai, and W. B. Yao, "*In vitro* and *in vivo* antioxidant activity of aqueous extract from *Choerospondias axillaris* fruit," *Food Chemistry*, vol. 106, no. 3, pp. 888–895, 2008.

[79] H. Xu and K. Huang, *Selenium: Its Chemistry, Biochemistry and Application in Life Science*, Huazhong Science and Technology University Press, Wuhan, China, 1994.

[80] D. Wei, T. Chen, M. Yan et al., "Synthesis, characterization, antioxidant activity and neuroprotective effects of selenium polysaccharide from *Radix hedysari*," *Carbohydrate Polymers*, vol. 125, pp. 161–168, 2015.

[81] J. Wang, B. Zhao, X. Wang, J. Yao, and J. Zhang, "Synthesis of selenium-containing polysaccharides and evaluation of antioxidant activity *in vitro*," *International Journal of Biological Macromolecules*, vol. 51, no. 5, pp. 987–991, 2012.

[82] B. Zhao, J. Zhang, J. Yao, S. Song, Z. Yin, and Q. Gao, "Selenylation modification can enhance antioxidant activity of *Potentilla anserina* L. polysaccharide," *International Journal of Biological Macromolecules*, vol. 58, pp. 320–328, 2013.

[83] F. Yu, J. Sheng, J. Xu, X. An, and Q. Hu, "Antioxidant activities of crude tea polyphenols, polysaccharides and proteins of selenium-enriched tea and regular green tea," *European Food Research and Technology*, vol. 225, no. 5-6, pp. 843–848, 2007.

[84] L. Zhao, G. Zhao, M. Du, Z. Zhao, L. Xiao, and X. Hu, "Effect of selenium on increasing free radical scavenging activities of polysaccharide extracts from a Se-enriched mushroom species of the genus *Ganoderma*," *European Food Research and Technology*, vol. 226, no. 3, pp. 499–505, 2008.

[85] G. Mao, Y. Zou, W. Feng et al., "Extraction, preliminary characterization and antioxidant activity of Se-enriched Maitake polysaccharide," *Carbohydrate Polymers*, vol. 101, no. 1, pp. 213–219, 2014.

[86] Y. Guo, D. Pan, H. Li, Y. Sun, X. Zeng, and B. Yan, "Antioxidant and immunomodulatory activity of selenium exopolysaccharide produced by *Lactococcus lactis* subsp. *lactis*," *Food Chemistry*, vol. 138, no. 1, pp. 84–89, 2013.

[87] R. Abu, Z. Jiang, M. Ueno, T. Okimura, K. Yamaguchi, and T. Oda, "*In vitro* antioxidant activities of sulfated polysaccharide ascophyllan isolated from *Ascophyllum nodosum*," *International Journal of Biological Macromolecules*, vol. 59, pp. 305–312, 2013.

[88] Y. Zhang, X. Lu, Z. Fu, Z. Wang, and J. Zhang, "Sulphated modification of a polysaccharide obtained from fresh persimmon (*Diospyros kaki* L.) fruit and antioxidant activities of the sulphated derivatives," *Food Chemistry*, vol. 127, no. 3, pp. 1084–1090, 2011.

[89] Q. Wu, C. Zheng, Z.-X. Ning, and B. Yang, "Modification of low molecular weight polysaccharides from *Tremella fuciformis* and their antioxidant activity *in vitro*," *International Journal of Molecular Sciences*, vol. 8, no. 7, pp. 670–679, 2007.

[90] J. Liu, J. Luo, H. Ye, and X. Zeng, "Preparation, antioxidant and antitumor activities *in vitro* of different derivatives of levan from endophytic bacterium *Paenibacillus polymyxa* EJS-3," *Food and Chemical Toxicology*, vol. 50, no. 3-4, pp. 767–772, 2012.

[91] D. Wei, W. Cheng, Y. Wei, and L. Zhang, "Phosphorylated modification and *in vitro* antioxidant activity of *Radix hedysari* polysaccharide," *Glycoconjugate Journal*, vol. 29, no. 4, pp. 167–172, 2012.

[92] H. Qi, Q. Zhang, T. Zhao, R. Hu, K. Zhang, and Z. Li, "*In vitro* antioxidant activity of acetylated and benzoylated derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta)," *Bioorganic & Medicinal Chemistry Letters*, vol. 16, no. 9, pp. 2441–2445, 2006.

[93] J. Wang, F. Wang, Q. Zhang, Z. Zhang, X. Shi, and P. Li, "Synthesized different derivatives of low molecular fucoidan extracted from *Laminaria japonica* and their potential antioxidant activity *in vitro*," *International Journal of Biological Macromolecules*, vol. 44, no. 5, pp. 379–384, 2009.

[94] Y. Feng, W. Li, X. Wu, L. He, and S. Ma, "Rapid and efficient microwave-assisted sulfate modification of lentinan and its antioxidant and antiproliferative activities *in vitro*," *Carbohydrate Polymers*, vol. 82, no. 3, pp. 605–612, 2010.

[95] T. Chen, L. Zhu, X. Liu et al., "Synthesis and antioxidant activity of phosphorylated polysaccharide from *Portulaca oleracea* L. with $H_3PW_{12}O_{40}$ immobilized on polyamine functionalized polystyrene bead as catalyst," *Journal of Molecular Catalysis A: Chemical*, vol. 342-343, pp. 74–82, 2011.

[96] Y. Chen, H. Zhang, Y. Wang, S. Nie, C. Li, and M. Xie, "Acetylation and carboxymethylation of the polysaccharide from *Ganoderma atrum* and their antioxidant and immunomodulating activities," *Food Chemistry*, vol. 156, pp. 279–288, 2014.

[97] W. Liu, H. Wang, W. Yao, X. Gao, and L. Yu, "Effects of sulfation on the physicochemical and functional properties of a water-insoluble polysaccharide preparation from *Ganoderma lucidum*," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 6, pp. 3336–3341, 2010.

[98] J. Xie, Z. Wang, M. Shen et al., "Sulfated modification, characterization and antioxidant activities of polysaccharide from *Cyclocarya paliurus*," *Food Hydrocolloids*, 2015.

[99] J.-K. Yan, W.-Q. Wang, H.-L. Ma, and J.-Y. Wu, "Sulfation and enhanced antioxidant capacity of an exopolysaccharide produced by the medicinal fungus *Cordyceps sinensis*," *Molecules*, vol. 18, no. 1, pp. 167–177, 2012.

[100] J. Wang, T. Yang, J. Tian et al., "Synthesis and characterization of phosphorylated galactomannan: the effect of DS on solution conformation and antioxidant activities," *Carbohydrate Polymers*, vol. 113, pp. 325–335, 2014.

[101] H. Y. Jung, I. Y. Bae, S. Lee, and H. G. Lee, "Effect of the degree of sulfation on the physicochemical and biological properties of *Pleurotus eryngii* polysaccharides," *Food Hydrocolloids*, vol. 25, no. 5, pp. 1291–1295, 2011.

[102] X. Wang, Z. Zhang, Z. Yao, M. Zhao, and H. Qi, "Sulfation, anticoagulant and antioxidant activities of polysaccharide from green algae *Enteromorpha linza*," *International Journal of Biological Macromolecules*, vol. 58, pp. 225–230, 2013.

[103] Y. Song, Y. Yang, Y. Zhang et al., "Effect of acetylation on antioxidant and cytoprotective activity of polysaccharides isolated from pumpkin (*Cucurbita pepo*, lady godiva)," *Carbohydrate Polymers*, vol. 98, no. 1, pp. 686–691, 2013.

[104] Y. He, M. Ye, L. Jing et al., "Preparation, characterization and bioactivities of derivatives of an exopolysaccharide from *Lachnum*," *Carbohydrate Polymers*, vol. 117, pp. 788–796, 2015.

[105] Z. Zhang, Q. Zhang, J. Wang, X. Shi, H. Song, and J. Zhang, "*In vitro* antioxidant activities of acetylated, phosphorylated and benzoylated derivatives of porphyran extracted from *Porphyra haitanensis*," *Carbohydrate Polymers*, vol. 78, no. 3, pp. 449–453, 2009.

[106] L. Ma, H. Chen, W. Zhu, and Z. Wang, "Effect of different drying methods on physicochemical properties and antioxidant activities of polysaccharides extracted from mushroom *Inonotus obliquus*," *Food Research International*, vol. 50, no. 2, pp. 633–640, 2013.

[107] H. Chen, Z. Qu, L. Fu, P. Dong, and X. Zhang, "Physicochemical properties and antioxidant capacity of 3 polysaccharides from green tea, oolong tea, and black tea," *Journal of Food Science*, vol. 74, no. 6, pp. C469–C474, 2009.

[108] S. Shen, H. Cheng, X. Li et al., "Effects of extraction methods on antioxidant activities of polysaccharides from camellia seed cake," *European Food Research and Technology*, vol. 238, no. 6, pp. 1015–1021, 2014.

[109] X. Gou, Q. Wang, G. Gao et al., "Effects of extraction methods on antioxidant activities of polysaccharides from the *Curcuma phaeocaulis* rhizomes," *Asian Journal of Agricultural and Food Sciences*, vol. 2, no. 4, 2014.

[110] I. Mateos-Aparicio, C. Mateos-Peinado, A. Jiménez-Escrig, and P. Rupérez, "Multifunctional antioxidant activity of polysaccharide fractions from the soybean byproduct okara," *Carbohydrate Polymers*, vol. 82, no. 2, pp. 245–250, 2010.

[111] H.-H. Sun, W.-J. Mao, Y. Chen et al., "Isolation, chemical characteristics and antioxidant properties of the polysaccharides from marine fungus *Penicillium* sp. F23-2," *Carbohydrate Polymers*, vol. 78, no. 1, pp. 117–124, 2009.

[112] Z. Guo, R. Xing, S. Liu et al., "The synthesis and antioxidant activity of the Schiff bases of chitosan and carboxymethyl chitosan," *Bioorganic & Medicinal Chemistry Letters*, vol. 15, no. 20, pp. 4600–4603, 2005.

[113] W. Liu, H. Wang, X. Pang, W. Yao, and X. Gao, "Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*," *International Journal of Biological Macromolecules*, vol. 46, no. 4, pp. 451–457, 2010.

[114] R. Xing, S. Liu, Z. Guo et al., "Relevance of molecular weight of chitosan and its derivatives and their antioxidant activities *in vitro*," *Bioorganic & Medicinal Chemistry*, vol. 13, no. 5, pp. 1573–1577, 2005.

[115] X.-Q. Zha, J.-H. Wang, X.-F. Yang et al., "Antioxidant properties of polysaccharide fractions with different molecular

mass extracted with hot-water from rice bran," *Carbohydrate Polymers*, vol. 78, no. 3, pp. 570–575, 2009.

[116] S. Guo, W. Mao, Y. Han et al., "Structural characteristics and antioxidant activities of the extracellular polysaccharides produced by marine bacterium *Edwardsiella tarda*," *Bioresource Technology*, vol. 101, no. 12, pp. 4729–4732, 2010.

[117] X. Yang, R. Wang, S. Zhang et al., "Polysaccharides from *Panax japonicus* C.A. Meyer and their antioxidant activities," *Carbohydrate Polymers*, vol. 101, no. 1, pp. 386–391, 2014.

[118] M. Şen, "Effects of molecular weight and ratio of guluronic acid to mannuronic acid on the antioxidant properties of sodium alginate fractions prepared by radiation-induced degradation," *Applied Radiation and Isotopes*, vol. 69, no. 1, pp. 126–129, 2011.

[119] J. K. Yan, L. Li, Z. M. Wang, P. H. Leung, W. Q. Wang, and J. Y. Wu, "Acidic degradation and enhanced antioxidant activities of exopolysaccharides from *Cordyceps sinensis* mycelial culture," *Food Chemistry*, vol. 117, no. 4, pp. 641–646, 2009.

[120] T. Feng, Y. Du, J. Li, Y. Hu, and J. F. Kennedy, "Enhancement of antioxidant activity of chitosan by irradiation," *Carbohydrate Polymers*, vol. 73, no. 1, pp. 126–132, 2008.

[121] J.-I. Choi, H.-J. Kim, J.-H. Kim et al., "Application of gamma irradiation for the enhanced physiological properties of polysaccharides from seaweeds," *Applied Radiation and Isotopes*, vol. 67, no. 7-8, pp. 1277–1281, 2009.

[122] C. Zhou, Y. Wang, H. Ma, and R. He, "Effect of ultrasonic degradation on *in vitro* antioxidant activity of polysaccharides from *Porphyra yezoensis* (Rhodophyta)," *Food Science and Technology International*, vol. 14, no. 6, pp. 479–486, 2008.

[123] Z. Zhang, X. Wang, X. Mo, and H. Qi, "Degradation and the antioxidant activity of polysaccharide from *Enteromorpha linza*," *Carbohydrate Polymers*, vol. 92, no. 2, pp. 2084–2087, 2013.

[124] L. Sun, C. Wang, Q. Shi, and C. Ma, "Preparation of different molecular weight polysaccharides from *Porphyridium cruentum* and their antioxidant activities," *International Journal of Biological Macromolecules*, vol. 45, no. 1, pp. 42–47, 2009.

[125] H. Cheng, S. Feng, S. Shen et al., "Extraction, antioxidant and antimicrobial activities of *Epimedium acuminatum* Franch. polysaccharide," *Carbohydrate Polymers*, vol. 96, no. 1, pp. 101–108, 2013.

[126] A. Kardošová and E. Machová, "Antioxidant activity of medicinal plant polysaccharides," *Fitoterapia*, vol. 77, no. 5, pp. 367–373, 2006.

[127] H. Wu, T. Min, X. Li et al., "Physicochemical properties and antioxidant activities of acidic polysaccharides from wampee seeds," *International Journal of Biological Macromolecules*, vol. 59, pp. 90–95, 2013.

[128] S. H. Al-Sheraji, A. Ismail, M. Y. Manap, S. Mustafa, R. M. Yusof, and F. A. Hassan, "Purification, characterization and antioxidant activity of polysaccharides extracted from the fibrous pulp of *Mangifera pajang* fruits," *LWT—Food Science and Technology*, vol. 48, no. 2, pp. 291–296, 2012.

[129] R. S. P. Rao and G. Muralikrishna, "Water soluble feruloyl arabinoxylans from rice and ragi: changes upon malting and their consequence on antioxidant activity," *Phytochemistry*, vol. 67, no. 1, pp. 91–99, 2006.

[130] J. Li, Y. Liu, L. Fan, L. Ai, and L. Shan, "Antioxidant activities of polysaccharides from the fruiting bodies of *Zizyphus Jujuba* cv. *Jinsixiaozao*," *Carbohydrate Polymers*, vol. 84, no. 1, pp. 390–394, 2011.

[131] L. Fan, J. Li, K. Deng, and L. Ai, "Effects of drying methods on the antioxidant activities of polysaccharides extracted from *Ganoderma lucidum*," *Carbohydrate Polymers*, vol. 87, no. 2, pp. 1849–1854, 2012.

[132] S. Wu, F. Li, S. Jia et al., "Drying effects on the antioxidant properties of polysaccharides obtained from *Agaricus blazei* Murrill," *Carbohydrate Polymers*, vol. 103, no. 1, pp. 414–417, 2014.

[133] Y. Yang, D. Liu, J. Wu, Y. Chen, and S. Wang, "*In vitro* antioxidant activities of sulfated polysaccharide fractions extracted from *Corallina officinalis*," *International Journal of Biological Macromolecules*, vol. 49, no. 5, pp. 1031–1037, 2011.

[134] T. Hu, D. Liu, Y. Chen, J. Wu, and S. Wang, "Antioxidant activity of sulfated polysaccharide fractions extracted from *Undaria pinnitafida in vitro*," *International Journal of Biological Macromolecules*, vol. 46, no. 2, pp. 193–198, 2010.

[135] J. Wang, Q. Zhang, Z. Zhang, H. Song, and P. Li, "Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from *Laminaria japonica*," *International Journal of Biological Macromolecules*, vol. 46, no. 1, pp. 6–12, 2010.

[136] H. Qi, T. Zhao, Q. Zhang, Z. Li, Z. Zhao, and R. Xing, "Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (Chlorophyta)," *Journal of Applied Phycology*, vol. 17, no. 6, pp. 527–534, 2005.

[137] A. Wan, Q. Xu, Y. Sun, and H. Li, "Antioxidant activity of high molecular weight chitosan and N,O-quaternized chitosans," *Journal of Agricultural and Food Chemistry*, vol. 61, no. 28, pp. 6921–6928, 2013.

[138] S. K. Chen, M. L. Tsai, J. R. Huang, and R. H. Chen, "*In vitro* antioxidant activities of low-molecular-weight polysaccharides with various functional groups," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 7, pp. 2699–2704, 2009.

[139] S. C. Jaehrig, S. Rohn, L. W. Kroh et al., "*In vitro* potential antioxidant activity of $(1 \rightarrow 3)$, $(1 \rightarrow 6)$-β-d-glucan and protein fractions from *Saccharomyces cerevisiae* cell walls," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 12, pp. 4710–4716, 2007.

[140] S. C. Jaehrig, S. Rohn, L. W. Kroh et al., "Antioxidative activity of $(1 \rightarrow 3)$, $(1 \rightarrow 6)$-β-d-glucan from *Saccharomyces cerevisiae* grown on different media,," *LWT—Food Science and Technology*, vol. 41, no. 5, pp. 868–877, 2008.

[141] E. Machová and S. Bystrický, "Antioxidant capacities of mannans and glucans are related to their susceptibility of free radical degradation," *International Journal of Biological Macromolecules*, vol. 61, pp. 308–311, 2013.

[142] B.-W. Zhu, D.-Y. Zhou, T. Li et al., "Chemical composition and free radical scavenging activities of a sulphated polysaccharide extracted from abalone gonad (*Haliotis Discus Hannai* Ino)," *Food Chemistry*, vol. 121, no. 3, pp. 712–718, 2010.

[143] C. G. Francisco, R. Freire, A. J. Herrera, I. Peréz-Martín, and E. Suárez, "Intramolecular 1, 5-versus 1, 6-hydrogen abstraction reaction promoted by alkoxy radicals in carbohydrate models," *Organic Letters*, vol. 4, no. 11, pp. 1959–1961, 2002.

[144] C. G. Francisco, A. J. Herrera, and E. Suárez, "Intramolecular hydrogen abstraction reaction promoted by alkoxy radicals in carbohydrates. Synthesis of chiral 2,7-dioxabicyclo[2.2.1]heptane and 6,8-dioxabicyclo[3.2.1]octane ring systems," *The Journal of Organic Chemistry*, vol. 67, no. 21, pp. 7439–7445, 2002.

[145] A. Martin, J. A. Salazar, and E. Suárez, "Synthesis of chiral spiroacetals from carbohydrates," *The Journal of Organic Chemistry*, vol. 61, no. 12, pp. 3999–4006, 1996.

[146] T. C.-T. Lo, C. A. Chang, K.-H. Chiu, P.-K. Tsay, and J.-F. Jen, "Correlation evaluation of antioxidant properties on the monosaccharide components and glycosyl linkages of polysaccharide with different measuring methods," *Carbohydrate Polymers*, vol. 86, no. 1, pp. 320–327, 2011.

[147] E. Tsiapali, S. Whaley, J. Kalbfleisch, H. E. Ensley, I. W. Browder, and D. L. Williams, "Glucans exhibit weak antioxidant activity, but stimulate macrophage free radical activity," *Free Radical Biology and Medicine*, vol. 30, no. 4, pp. 393–402, 2001.

[148] L. Meng, S. Sun, R. Li, Z. Shen, P. Wang, and X. Jiang, "Antioxidant activity of polysaccharides produced by *Hirsutella* sp. and relation with their chemical characteristics," *Carbohydrate Polymers*, vol. 117, pp. 452–457, 2015.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 407580, 13 pages
http://dx.doi.org/10.1155/2015/407580

Review Article

Sulforaphane Protects against Cardiovascular Disease via Nrf2 Activation

Yang Bai,[1,2] Xiaolu Wang,[2] Song Zhao,[3] Chunye Ma,[1] Jiuwei Cui,[4] and Yang Zheng[1]

[1] *The Cardiac Surgery Department, The First Hospital of Jilin University, Changchun 130021, China*
[2] *The Jilin Province People's Hospital, Changchun 130021, China*
[3] *The Spine Surgery Department, The First Hospital of Jilin University, Changchun 130021, China*
[4] *Cancer Center, The First Hospital of Jilin University, Changchun 130021, China*

Correspondence should be addressed to Jiuwei Cui; cuijiuwei@yahoo.com and Yang Zheng; zhengyang@jlu.edu.cn

Received 10 January 2015; Revised 20 April 2015; Accepted 28 April 2015

Academic Editor: Anandh B. P. Velayutham

Cardiovascular disease (CVD) causes an unparalleled proportion of the global burden of disease and will remain the main cause of mortality for the near future. Oxidative stress plays a major role in the pathophysiology of cardiac disorders. Several studies have highlighted the cardinal role played by the overproduction of reactive oxygen or nitrogen species in the pathogenesis of ischemic myocardial damage and consequent cardiac dysfunction. Isothiocyanates (ITC) are sulfur-containing compounds that are broadly distributed among cruciferous vegetables. Sulforaphane (SFN) is an ITC shown to possess anticancer activities by both *in vivo* and epidemiological studies. Recent data have indicated that the beneficial effects of SFN in CVD are due to its antioxidant and anti-inflammatory properties. SFN activates NF-E2-related factor 2 (Nrf2), a basic leucine zipper transcription factor that serves as a defense mechanism against oxidative stress and electrophilic toxicants by inducing more than a hundred cytoprotective proteins, including antioxidants and phase II detoxifying enzymes. This review will summarize the evidence from clinical studies and animal experiments relating to the potential mechanisms by which SFN modulates Nrf2 activation and protects against CVD.

1. Introduction

Cardiovascular disease (CVD) is a class of diseases that involve the heart or blood vessels, such as ischemic heart disease, hypertensive heart disease, and peripheral artery disease. Generally, CVD is related to hypertension, atherosclerosis, obesity, or diabetes [1]. CVD underlies an unparalleled proportion of the global disease burden and it will remain the main cause of mortality for the near future [2]. Although our understanding of the pathobiology and treatment of atherosclerosis and CVD has advanced considerably over the past 100 years and therapies continue to evolve, the global problem we face in alleviating the enormous burden of CVD is complex and multidimensional [3]. CVD is now increasingly prevalent in developing countries, as the increased affluence of industrialized societies leads to abundant calorie-dense food, access to labor-saving devices that reduce physical activity, and ready availability of tobacco products. Traditional cultures based on consumption of grains, fruits, and vegetables along with heavy physical activity and a less stressful life are being replaced by more intense urban cultures [4]. CVD is also the biggest killer in the western world, despite significant advances in the understanding of the relevant molecular mechanisms [5]. It is in the public interest to identify effective approaches to reducing the impact of CVD in both developed and developing regions [6]. Oxidative stress plays a major role in the pathophysiology of cardiac disorders [7]. High levels of oxidative stress resulting from increased cardiac generation of reactive oxygen species (ROS) are thought to contribute to contractile and endothelial dysfunction, myocyte apoptosis and necrosis, and extracellular matrix remodeling in the heart [8]. There are two types of small molecular antioxidant involved in cellular protection against oxidative and electrophile toxicities; direct antioxidants are redox-active and short-lived, necessitating replenishment or regeneration, and may also evoke prooxidant effects, while indirect antioxidants may or may not be redox-active, are not consumed, have long half-lives, and are

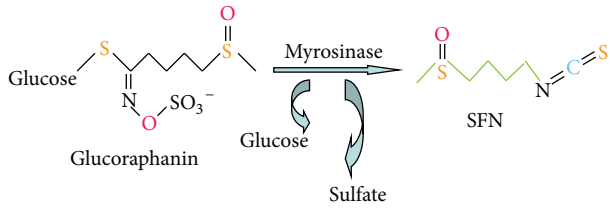


Figure 1: Glucoraphanin is the major glucosinolate in broccoli. Under neutral conditions, Grn is hydrolyzed by myrosinase to yield glucose, sulfate, and sulforaphane (SFN).

unlikely to evoke prooxidant effects [9]. Therefore, current research focuses on the characterization and validation of health-promoting functional foods that specifically target endogenous antioxidant defenses. Epidemiological studies have indicated that cruciferous vegetable intake correlated with a decreased risk for CVD. Because broccoli is a cruciferous vegetable and contains isothiocyanates (ITC), which can produce the redox-regulated cardioprotective protein, thioredoxin (Trx), it was reasoned that consumption of broccoli could be beneficial to the heart [10]. Sulforaphane (SFN) is by far the most widely studied and best characterized ITC [11].

2. Structural Features and Biological Activity of SFN

SFN [1-isothiocyanate-(4R)-(methylsulfinyl) butane] [12] is the hydrolysis product of glucoraphanin (Grn), the main glucosinolate (GLS) in broccoli [13, 14]. GLS (β-thioglucose N-hydroxysulfates) are relatively stable biosynthetic precursors along with SFN [15]. GLS exist in association with but structurally segregated from the myrosinase enzyme (β-thioglucoside glucohydrolase); this is released upon plant cell injury (e.g., by chewing) and cleaves GLS to produce SFN, sulfate, and glucose [16, 17] (Figure 1). Ahn et al. [18] showed that 24 synthetic sulfoxythiocarbamate analogs that retained the important structural features of SFN analogs, demonstrating efficacy for phase II enzyme induction in a range of cell lines. In order to eliminate chemicals, drug-metabolizing enzymes initially metabolize the compounds (phase I reaction) and then detoxify electrophiles and oxidants (phase II reaction). A group of heterogeneous enzymes carry out the phase II reactions including glutathione-S-transferase (GST), epoxide hydrolase, UDP-glucuronosyltransferase and sulfotransferase, and NAD(P)H: quinone oxidoreductase 1 (NQO1). The antioxidant systems of phase II reactions can detoxify ROS directly [19, 20]. GST catalyzes the conjugation of glutathione (GSH) with electrophilic compounds making them more water soluble and facilitating their removal from the body. It is well known that ITC-GSH conjugates are exported out to extracellular. This continous conjugation and efflux of the conjugate can cause intracellular GSH level to drop. ITC can also induce GST scavenge ROS [21]. Shibata et al. examined the effect of sulfhydryl molecules on cellular responses induced by 6-methylsulfinylhexyl isothiocyanate (6-HITC), an analog of SFN isolated from broccoli. Significant induction of heme oxygenase-1 (HO-1) was observed when cells were treated with 6-HITC, even in the presence of GSH [22]. It was also demonstrated that the main mechanism of action of SFN involves regulation of the nuclear factor erythroid-derived 2- (NF-E2) related factor 2- (Nrf2-) antioxidant response element (ARE) pathway; this in turn upregulates the expression of a range of antioxidant enzymes including HO-1, NQO1, GST, γ-glutamyl cysteine ligase (GCL), and glutathione reductase (GR) [23]. Therefore, SFN has been considered to act as an indirect antioxidant and a highly potent inducer of phase II cytoprotective enzymes. The resultant detoxification of electrophiles and oxidants can protect against carcinogens, oxidative stress, and inflammation.

3. Nrf2 Signaling Pathway and Antioxidant Mechanism

3.1. Nrf2-Kelch-Like Chicken Erythroid-Derived Cap "n" Collar Homology (ECH) Factor-Associated Protein 1- (Keap1) ARE Signaling Pathway. Nrf2, which belongs to the cap "n" collar family of basic region-leucine zipper (bZIP) transcription factors, plays a central role in the regulation of basal and inducible expression of genes encoding phase II cytoprotective enzymes. Nrf2 has six highly conserved protein regions called Nrf2-ECH domains, Neh1 to Neh6. At its C terminus, Nrf2 has a bZIP structure enabling dimerization with small musculoaponeurotic fibrosarcoma virus (Maf) proteins and for binding to the ARE. At its N terminus, Nrf2 has a Neh2 domain (amino acid residues 1–95) that is highly conserved between species [24]. Neh3–5 contains residues important for Nrf2 transcriptional activity. Neh6 functions as a degron and mediates nuclear Nrf2 degradation [25]. In the absence of oxidative stress, Nrf2 pathway activity is suppressed by the sequestration and degradation of Nrf2 in the cytoplasm. However, in the presence of oxidative stress, Nrf2 translocates to the nucleus.

Keap1 is a cysteine-rich negative regulator of Nrf2. McMahon et al. reported that Keap1 controlled the total cellular level of Nrf2 protein in the RL34 nontransformed rat liver cell line. In the absence of oxidative stress, Nrf2 interacts with Keap1 and induces more rapid proteasomal degradation than that observed in the presence of oxidative stress. Keap1 negatively regulates Nrf2 function by controlling its subcellular location. Specifically, Keap1 sequesters the bZIP protein of Nrf2 in the cytoplasm, leading to low expression of ARE-driven genes. During oxidative stress, a signal that involves phosphorylation and/or redox modification is transduced to the Keap1-Nrf2 complex leading to its disruption, facilitating nuclear translocation of Nrf2 [26].

The Keap1 protein consists of distinct structural domains including the N terminal Broad complex, Tramtrack, and bric-a-brac (BTB) domains, the central intervening region (IVR), the double glycine repeat (DGR) domain where Nrf2 binding occurs, and the C terminal region [27]. Three important cysteine residues have been identified within Keap1: C151, within the BTB domain, and C273 and C288, within the

IVR. C273 and C288 coordinate a zinc ion and are crucial for maintaining the structural integrity required for Keap1 to associate with Nrf2. It is believed that oxidative modification of these two cysteine residues releases the zinc ion and, consequently, Nrf2 ubiquitination is abolished [28].

Keap1-Nrf2 interactions are mediated via the high affinity ETGE motif "hinge" site within the Nrf2 Neh2 domain and the lower affinity DLG motif "latch" site. Under normal cellular conditions, Nrf2 first interacts with the Keap1 dimer through the ETGE hinge and subsequently with the cullin-3- (Cul3) ring box 1 (Rbx1) complex via the DLG latch motif, which leads to the ubiquitination and degradation of Nrf2. During cellular stress or pharmacologic induction, the hinge and the latch of Nrf2 may be disrupted by changes to Keap1 homodimer formation [49] (Figure 2). The available evidence indicates that Nrf2 activators (such as SFN) may block Nrf2 ubiquitination by altering Keap1 conformation by interacting with the thiol groups of specific cysteine residues within Keap1 [49].

The ARE is a *cis*-acting enhancer sequence, also referred to as the electrophile response element (EpRE), with a core nucleotide sequence of 5′-RTGACNNNGC-3′; this element regulates the basal and inducible expression of more than 200 genes [50]. Transcriptional activation of ARE-mediated genes requires Nrf2 heterodimerization with other bZIP proteins, including Jun (c-Jun, Jun-D, and Jun-B) and small Maf (MafG, MafK, and MafF) proteins. In addition, the Nrf2 gene contains two ARE-like promoter sequences, indicating that Nrf2 autoregulation could sustain ARE-mediated gene expression [51]. Furthermore, ARE inducers can inactivate Keap1 by inducing a conformational rearrangement of this protein; this facilitates Nrf2 nuclear translocation and further activation of ARE-mediated transcription of protective genes, in association with bZIP proteins.

3.2. Nrf2 Antioxidant Mechanism. Nrf2 plays pivotal roles in coordinating the antioxidant response and maintaining redox homeostasis. Nrf2 expression is normally suppressed but is strikingly induced under conditions of oxidative stress. Li et al. provided a mechanistic explanation for the translational control of Nrf2 by oxidative stress [52]. An internal-ribosomal-entry site (IRES) was identified within the 5′-untranslated region of human Nrf2 mRNA. The Nrf2-IRES contains a highly conserved 18S rRNA binding site that is required for internal initiation. This Nrf2-IRES also contains a hairpin structured inhibitory element located upstream of the rRNA binding site. Deletion of this inhibitory element remarkably enhanced translation. Significantly, treatment of cells with hydrogen peroxide and SFN further stimulated IRES-mediated Nrf2 translation [52]. Thimmulappa et al. also elucidated another mechanism underlying the translational control of Nrf2 by comparing the transcriptional profiles of small intestines from wild-type ($Nrf2^{+/+}$) and knock-out ($Nrf2^{-/-}$) mice treated orally with vehicle or SFN (9 μM/day) for 1 week. This study identified numerous genes regulated by Nrf2, including previously reported Nrf2-regulated genes (NQO1, GST, UDP-glucuronosyltransferases, and epoxide hydrolase) and a number of genes that were not previously known to be regulated by Nrf2 [53]. Furthermore, Nioi et al. characterized the NQO1 5′-upstream region in murine $Nrf2^{+/+}$ and $Nrf2^{-/-}$ embryonic fibroblasts and showed that Nrf2 was essential for both the constitutive expression of NQO1 and its induction by SFN. Electrophoretic mobility-shift and chromatin immunoprecipitation assays revealed that low levels of Nrf2 were constitutively associated with the NQO1 ARE; following exposure to SFN, substantially greater quantities of Nrf2 were recruited to the ARE as a heterodimer with the small Maf protein [54]. These findings demonstrated the key role played by Nrf2 in mediating detoxification and/or antioxidant effects, thereby protecting cells from genotoxic damage.

3.3. Nrf2 Nuclear Localization. Nrf2 is the key transcription factor regulating the antioxidant response and its site of action is within the nucleus. Li et al. identified a nuclear localization signal (NLS) within the basic region of Nrf2 and a nuclear exporting signal (NES) within the bZIP domain [55]. They also characterized a new functional NES within the transactivation (TA) domain of Nrf2. A variety of oxidants, including SFN, effectively induced nuclear translocation of a green fluorescent protein-tagged Nrf2 segment. The discovery of multiple NLS/NES motifs in Nrf2 and the redox sensitivity of NESTA imply that Nrf2 may transduce oxidative signals into the nucleus, consequently initiating antioxidant gene transcription [55]. Nrf2 is known to promote the transcription of many cytoprotective genes and is a major drug target for the prevention of cancers and other diseases. Li et al. demonstrated that while the activation of Nrf2 by prototypical chemical activators, including SFN, was mediated solely by inhibition of its ubiquitination, such inhibition occurred predominantly in the nucleus [56]. Pickering et al. further proved that increasing the cellular capacity to degrade oxidized proteins required increased cellular levels of Nrf2, translocation of Nrf2 from the cytoplasm to the nucleus, and maximal increases in proteolytic capacity [57].

We have previously employed immunofluorescent staining to identify SFN-stimulated nuclear translocation of Nrf2 in diabetic cardiomyopathy (DCM), resulting in the upregulation of the expression of Nrf2 and its downstream genes [47]. Exogenous chemical activators of Nrf2 have often been suggested to inhibit Nrf2 ubiquitination and degradation, thereby increasing its nuclear translocation [56]. These findings provided significant new insights into Nrf2 activation. On the other hand, the oxidative stress or antioxidants can also induce the expression of intracellular kinases, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and protein kinase C (PKC); as a result, these kinases phosphorylate Nrf2 to produce p-Nrf2, affecting nuclear-cytoplasmic trafficking of Nrf2 or modulating Nrf2 protein stability [58]. Therefore, the ability to adapt to acute oxidative stress by interrupting the cytoplasmic degradation of Nrf2 and promoting its nuclear translocation is an important component of cellular defense mechanisms.

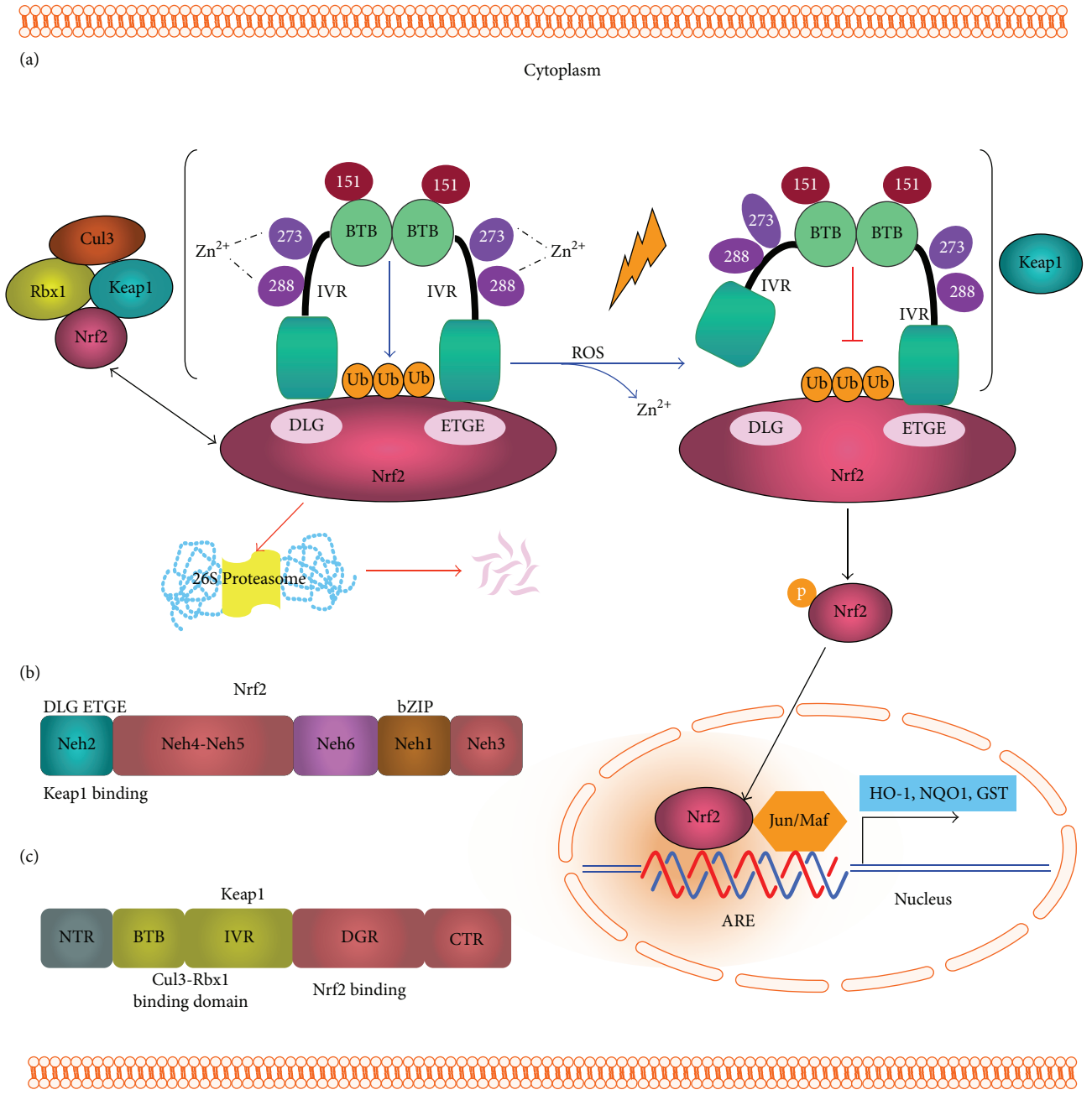


Figure 2: The Kelch-like chicken erythroid-derived cap "n" collar homology factor-associated protein 1- (Keap1-) NF-E2-related factor 2- (Nrf2) antioxidant response element (ARE) signaling pathway. (a) Keap1-Nrf2 interactions are mediated via the high affinity ETGE motif "hinge" site and the lower affinity DLG motif "latch" site within the Nrf2 Neh2 domain. Under normal cellular conditions, Nrf2 first interacts with the Keap1 dimer through the ETGE hinge and subsequently with the cullin-3- (Cul3-) ring box 1 (Rbx1) complex via the DLG latch motif, which leads to the ubiquitination and degradation of Nrf2. During cellular stress, the hinge and the latch of Nrf2 may be disrupted by changes to Keap1 homodimer formation and translocation to nucleus. (b) The structure of Nrf2, including Neh1–6 domains. (c) Keap1 is composed of distinct structural domains, including the N terminal region (NTR), Broad complex, Tramtrack, and bric-a-brac (BTB) domain, intervening region (IVR), double glycine repeat (DGR; Nrf2 binding region), and C terminal region (CTR).

In summary, the Nrf2 Neh2 domain binds with the region between the BTB and DGR Kelch repeat domains of actin-bound Keap1. Exposure of cells to ARE inducers results in the dissociation of Nrf2 from Keap1 and facilitates translocation of Nrf2 to the nucleus, where it heterodimerizes with a small Maf protein and binds to ARE, resulting in the transcriptional activation of target genes [59]. Therefore, the Keap1-Nrf2-ARE pathway represents one of the most important cellular defense mechanisms against oxidative stress and xenobiotic damage. Transcriptional activation of ARE-regulated phase II

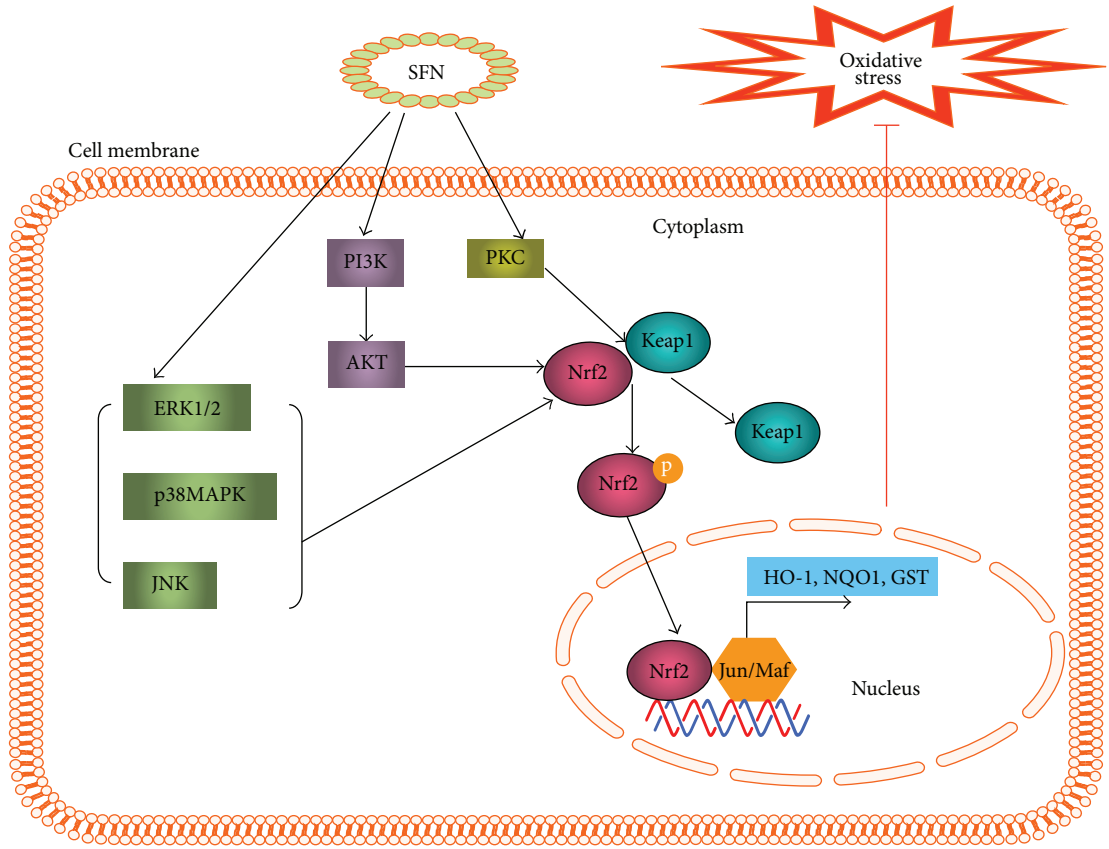


Figure 3: Sulforaphane (SFN) activation of Nrf2 signaling. SFN activates three mitogen-activated protein kinases (MAPKs): extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38; these may stimulate Nrf2 activation. SFN may also activate protein kinase C (PKC), which directly phosphorylates Nrf2. Alternatively SFN activates Nrf2 through phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT). Ultimately, Nrf2 phosphorylation triggers nuclear translocation and induction of antioxidant-response element- (ARE-) mediated gene transcription, reducing oxidative stress.

detoxification and antioxidant genes by Nrf2 is considered to represent the prime mechanism involved in the antioxidant effects of SFN.

4. SFN-Mediated Activation of Nrf2 Signal Transduction Pathways

4.1. Direct Effects of SFN on Nrf2 via Related Signaling Pathways. As SFN induces Nrf2 accumulation due to inhibition of proteasomal degradation of the bZIP protein [26], it has attracted interest for the promotion of human health and wellbeing [12]. SFN is an electrophile that can react with protein thiols to form thionoacyl adducts; this is believed to affect the critical Keap1 cysteine residues and block Nrf2 polyubiquitination and degradation, resulting in Nrf2-Keap1-ARE signaling and translocation Nrf2 to nucleus. Our research group also found that SFN induced significantly higher Nrf2 levels expression levels in the cardiac cell nuclei [47].

4.2. Indirect Effects of SFN on Nrf2 via Related Signaling Pathways. The Nrf2-Keap1-ARE signaling pathway can be modulated by several upstream kinases including MAPK, PI3K, and PKC [60]. SFN may affect the activity of a variety of intracellular kinases that phosphorylate Nrf2 to produce p-Nrf2, altering nuclear-cytoplasmic trafficking of Nrf2 or modulating Nrf2 protein stability [59, 60]. Previous studies have reported that Nrf2 activation is potentiated by its phosphorylation of Nrf2 serine 40 [15]. Leoncini et al. used cultured rat cardiomyocytes to analyze the signaling pathways involved in the protective effects of SFN. This study investigated the ability of SFN to activate protein kinase B (AKT) and extracellular signal-regulated kinases 1 and 2 signaling pathways; these are implicated in cardiac cell survival and also increase Nrf2 phosphorylation and ARE binding. Using specific inhibitors, this study demonstrated that the PI3K/AKT pathway mediated the influence of SFN on the expression and activity of GR, GST, Trx reductase, and NQO1 (analyzed by western blot and spectrophotometric assay, resp.); these effects modulated Nrf2 binding and phosphorylation, resulting in a cytoprotective effect against oxidative damage [61] (Figure 3).

4.3. Long-Term SFN-Mediated Effects. Although absorbed SFN is cleared from the body within a few hours, some findings demonstrated that ingestion of broccoli or SFN

induced long-term protection against free radical-induced damage. In order to explore the cause of this phenomenon, Bergström et al. studied the kinetics of the SFN-induced Nrf2 response in astrocytes, which is a cell type known to be highly involved in the defense against oxidative stress in the brain [62]. The results of this analysis showed that 4 h SFN exposure induced Nrf2-dependent increases in NQO1 and HO-1 mRNAs that persisted for 24 h, and the levels of the corresponding proteins remained elevated for over 48 h. In addition, peroxide-clearing activity and glutathione levels were elevated for more than 20 h, resulting in an increased resistance to superoxide-induced cell damage [62]. In cultured rat neonatal cardiomyocytes, Angeloni et al. also showed that SFN induced long-term decreases in intracellular ROS production, increased cell viability, and decreased DNA fragmentation, accompanied by the induction of antioxidants and phase II enzymes [63]. Our research group has shown that diabetic mice treated with SFN for three months showed a reduced incidence of DCM at the end of the treatment (3 months) and also after 6 months [47]. These findings indicate that short-term stimulation of the Nrf2 pathway by SFN results in a long-term elevation of endogenous antioxidant effects.

Taken together, these reports indicate that SFN can induce phase II enzyme expression via either direct or indirect activation of Nrf2-Keap1-ARE signaling, which is very important for our understanding of the mechanisms underlying the effects of SFN on Nrf2 activity.

5. SFN-Mediated Protection from Hypertension

The prevalence of hypertension increases markedly with age. It is one of the major causes of CVD, and prevention of hypertension may play a crucial role in protesting against CVD. Oxidative stress caused by reduced production of nitric oxide and/or increased production of ROS (mainly superoxide) may promote endothelial dysfunction. Therefore, increased oxidative stress represents one possible driver of the increased prevalence of hypertension [64]. Noyan-Ashraf et al. demonstrated that a diet containing broccoli sprouts high in Grn (Grn+) decreased oxidative stress and associated problems in male spontaneously hypertensive stroke-prone rats (SHRsp). As one of Grn key metabolites, SFN was also found to improve blood pressure [30].

Females on a Grn+ diet also showed decreased oxidative stress and associated problems, such as hypertension, as compared with females administered a control diet. Oxidative stress in pregnant female rats can affect fetal determinants of adult health and the offspring of pregnant females administered the Grn+ diet also had lower blood pressure and less tissue inflammation in adulthood; regardless of their subsequent diet, offspring of mothers fed a Grn+ diet had the best health outcomes [29].

Regarding the mechanism underlying SFN-mediated protection from hypertension, there is evidence that SFN-induced upregulation of phase II enzymes reduces the oxidative stress experienced by the vascular smooth muscle cell (VSMC), particularly in SHR. Wu and Juurlink found that, in the pathogenesis of hypertension, basal levels of cellular GSH, GR, and glutathione peroxidase (GPX) were significantly lower in SMC from SHRsp than in those from normotensive Wistar-Kyoto rats. SFN (0.05–1 μM) induced significant and concentration-dependent increases in the levels of GSH and HO-1 protein, in the activities of GR and GPX, and in VSMC from both rat strains [32]. In a subsequent study, Wu et al. further demonstrated that SFN significantly increased GSH content, decreased levels of oxidized GSH, decreased protein nitrosylation, and increased activities of GR and GPX; these changes correlated with improved endothelial-dependent relaxation of the aorta and significantly lower blood pressure [33]. Although these studies did not directly measure Nrf2 expression and function, the observed effects were most likely due to the upregulation of Nrf2 expression and function induced by SFN.

6. SFN-Mediated Protection from Atherosclerosis

Atherosclerosis is found in a range of CVD, including myocardial infarction, stroke, and peripheral artery disease; these remain major causes of morbidity and mortality globally [65, 66]. It is generally accepted that shear stress plays a key role in the development of early atherosclerosis [67]. Atherosclerotic lesions are preferentially located at the outer walls of the arterial branches and curvatures, where the local flow is disturbed; steady laminar blood flow (s-flow) and sustained high shear stress can modulate the expression of genes and proteins that protect against atherosclerosis in endothelial cells (EC) [68]. Dekker et al. identified Krüppel-like factor 2 (KLF2) as a transcription factor specifically induced by s-flow in EC. Nrf2 was identified as a shear-induced transcription factor that is responsible for antioxidant gene expression. It was also shown that both of these transcription factors were induced in s-flow regions of the vasculature *in vivo*. KLF2 increased the nuclear localization of Nrf2 and the combined actions of these two factors constituted about 70% of the s-flow-induced endothelial gene expression [68]. Zakkar et al. also investigated whether Nrf2 regulated arterial EC activation. En face staining revealed that Nrf2 was activated in EC at an atheroprotected region of the murine aorta but was present in an inactive form in EC at an atherosusceptible site. Treatment with SFN activated Nrf2 at the susceptible site in wild-type but not $Nrf2^{-/-}$ animals, indicating that it suppressed EC activation via Nrf2 [35].

Atherosclerosis is also associated with long-term inflammatory disease of the arterial wall. Increased expression of cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) is associated with increased proliferation of VSMC, leading to increased neointimal or atherosclerotic lesion formation. Kim et al. investigated the effect of SFN on the expression of VCAM-1 in cultured mouse VSMC. Pretreatment of VSMC for 2 h with SFN (1–5 μg/mL) dose-dependently inhibited TNF-α-induced protein expression of VCAM-1. SFN also suppressed TNF-α-induced production of intracellular ROS [34]. Kwon et al. further investigated

the effects of SFN on VSMC proliferation and neointimal formation in a carotid artery injury model and found that SFN inhibited TNF-α-induced VCAM-1 mRNA and protein expression in VSMC [36]. These *in vivo* and *in vitro* animal studies indicated that SFN had beneficial effects, suppressing inflammation within the atherosclerotic lesion. To investigate the effects of SFN on endothelial inflammatory gene expression in human aortic EC (HAEC), Chen et al. demonstrated that TNF-α-induced VCAM-1 mRNA and protein levels in HAEC were suppressed after treatment of HAEC with SFN (1–4 μM) for 1 h, which suggested that SFN may be useful as a therapeutic agent for the treatment of inflammatory diseases [37].

In addition to these inflammatory mechanisms, endothelial lipase (EL) is also involved in atherosclerosis-EL is a member of the triacylglycerol lipase family and it decreases the levels of high-density lipoprotein (HDL) cholesterol. Kivelä et al. demonstrated that SFN inhibited TNF-α-mediated induction of EL in human umbilical vein EC (HUVEC), indicating that SFN may have a beneficial effect on HDL cholesterol levels; this would reduce the risk for atherosclerosis [38]. Oxidized low-density lipoprotein (oxLDL) contributes to atherogenesis through multiple mechanisms. Huang et al. reported that treatment with SFN (0–10 μM) reversed oxLDL-induced ROS production by inducing dose-dependent increases in HO-1 levels, expression of GCL catalytic and modifier subunits, intracellular glutathione content, and ARE-luciferase reporter activity [69]. Collectively, these results indicated that SFN modified inflammation, EL activity, and oxLDL levels, all of which play important roles in atherosclerosis.

Obesity is associated with metabolic disorder, which is another risk factor for atherosclerosis and CVD. Choi et al. investigated whether SFN prevented high-fat diet- (HFD-) induced obesity in C57BL/6N mice [70]. Mice were fed with a normal diet (ND), HFD, or HFD plus 0.1% SFN for 6 weeks. Food efficiency ratios and body weights were lower in the HFD-SFN-fed mice than in the HFD mice. SFN attenuated HFD-induced visceral adiposity, adipocyte hypertrophy, and fat accumulation in the liver. Serum total cholesterol and leptin and liver triglyceride levels were lower in HFD-SFN mice than in HFD mice. These results suggested that SFN may induce antiobesity effects by inhibiting adipogenesis [70].

Platelet aggregation plays an important role in arterial thrombosis, which leads to atherosclerosis and CVD. Chuang et al. proved that SFN inhibited human platelet aggregation and reduced thrombus formation under flow conditions [71]. Oh et al. also found that SFN inhibited platelet aggregation dose-dependently. A comparison of platelet activators revealed that collagen was most inhibited by SFN, which markedly decreased collagen-induced glycoprotein IIb/IIIa activation and thromboxane A2 formation *in vitro*. SFN also reduced collagen and epinephrine-induced pulmonary embolism but did not affect prothrombin time *in vivo*; this indicated that SFN might be a potential antithrombotic drug [72, 73].

Taken together, the above animal and human studies indicate that SFN-medicated activation of Nrf2 reduces EC activation at atherosusceptible sites, regulates migration and proliferation of VSMC, decreases the expression of inflammatory factors, prevents HFD-induced obesity, and has antithrombic effect. These findings indicate that SFN may provide a potential therapeutic agent for the prevention of atherosclerosis, thereby reducing the risk for CVD.

7. SFN Protects from Cardiac Ischemia-Reperfusion (I/R) Injury

It has been demonstrated that I/R injury is crucial in the pathology of major CVD, such as stroke and myocardial infarction [74]. Mukherjee et al. [10, 75] fed a group of rats with broccoli by oral gavage. After 30 days, the rats were sacrificed and their isolated perfused hearts were made ischemic for 30 min, followed by 2 h reperfusion. Treatment with broccoli provided significant cardioprotection, as evidenced by improved postischemic ventricular function, reduced myocardial infarct size, and decreased cardiomyocyte apoptosis along with significant reduction of cytochrome c release and procaspase 3 activity. These protective effects were found to be associated with the enhanced activity of Nrf2 [10, 75]. Similarly, subsequent studies showed that SFN protected against cardiac I/R injury. They found that SFN treatment attenuated I/R-induced decreases in Mn-superoxide dismutase, catalase, and HO-1 levels [39, 76]. The protective effects of SFN against I/R injury may be partly mediated through the Nrf2-Keap1-ARE antioxidant pathway.

8. SFN Protects from Diabetes and Diabetes-Induced Complications

8.1. SFN Protects from Diabetes. Diabetes has reached epidemic levels worldwide. Increased levels of ROS, mainly produced by the NADPH oxidase family, have been identified in the pathogenesis of experimental and clinical diabetes [77, 78]. Patients with diabetes are two to four times more likely to develop CVD than those without diabetes; indeed CVD is the leading cause of premature death and disability in patients with diabetes [79]. de Souza et al. evaluated the effect of SFN in streptozotocin- (STZ-) induced diabetes. Male Wistar rats were given water or 0.1, 0.25, or 0.5 mg/kg SFN by oral gavage before injection of STZ (80 mg/kg). Diabetic animals had elevated levels of serum triacylglycerols, urea, and creatinine, and all SFN doses were able to reverse these alterations [40]. Pretreatment with SFN also blocked the development of type 1 diabetes in STZ-treated mice and inhibited cytokine-induced β-cell damage [41].

SFN protection against oxidative stress-induced β-cell damage or death was also observed under other experimental conditions. For instance, islet cell transplantation is often limited by poor transplanted β-cell survival. SFN was found to significantly prolong the survival time of these cells by protecting them against transplantation-induced oxidative damage [80]. Based on the above studies, we can conclude that SFN had protective effects on the pancreas and this may have a potential clinical application in improving the condition of this organ in hemodynamically unstable donors.

In view of the fact that the etiologic factors involved in the development of type 2 diabetes are complicated and multifaceted, including obesity, systemic chronic inflammation, hepatic steatosis, hyperlipidemia, and insulin resistance, Xu et al. have investigated the role of Nrf2 in the prevention of hepatic steatosis, insulin resistance, obesity, and type 2 diabetes. Obesity and lipid accumulation in white adipose tissue were significantly decreased in HFD-treated Keap1-knockdown mice, which showed increased Nrf2 expression and function, as compared with C57Bl/6J wild-type mice [81]. In addition, compared with the Lep(ob/ob) mice, a common model of type 2 diabetes model, Lep(ob/ob)-Keap1-knockdown mice had increased Nrf2 activity and decreased lipid accumulation, smaller adipocytes, and reduced lipogenic gene expression [81]. Adipocyte differentiation is triggered by adipocyte hyperplasia, which leads to obesity. Choi et al. [31] demonstrated that SFN decreased the accumulation of lipid droplets and triglycerides in these adipocytes in 3T3-L1 cells and also inhibited the proliferation of adipocytes. These results suggested that insulin resistance, metabolic syndrome, and diabetes could be prevented by SFN-mediated effects on Nrf2 expression and function.

8.2. SFN Protects against Diabetes-Induced Complications. Diabetes complications are associated with micro- and macrovascular complications, and the major complications include nephropathy, retinopathy, neuropathy, DCM, and accelerated atherosclerosis. Currently, high glucose-driven production of ROS intermediates and inflammatory damage are recognized contributors to complications of diabetes. A systemic review stated that supplementation with broccoli sprouts containing high levels of SFN increased plasma total antioxidant capacity and decreased the oxidative stress index, lipid peroxidation, serum triglycerides, oxLDL/LDL-cholesterol ratio, serum insulin, insulin resistance, and serum high-sensitive C-reactive protein *in vivo*. [82]. The pathological state is defined as metabolic syndrome, which is a risk factor for diabetes and the complications of diabetic cardiac-cerebral vascular disease. Therefore, alleviation of oxidative and inflammation status may protect from diabetes and diabetes-induced complications.

Diabetic nephropathy is the most common cause of end-stage kidney disease worldwide and is associated with increased morbidity and mortality in patients with both type 1 and type 2 diabetes [83]. Diabetic nephropathy results in premature death from accelerated CVD and various other complications [79]. It was also shown that the dual antioxidant and anti-inflammatory activities of SFN effectively activated Nrf2 and reversed the various deficits observed in experimental diabetic neuropathy [44]. Zheng et al. studied $Nrf2^{+/+}$ and $Nrf2^{-/-}$ with STZ-induced diabetes. SFN was administered 2 weeks after STZ injection and metabolic indices and renal structure and function were assessed at 18 weeks. SFN significantly attenuated common symptoms of metabolic disorder that are associated with diabetes in $Nrf2^{+/+}$ mice but not in $Nrf2^{-/-}$ mice, indicating that SFN operated via specific activation of the Nrf2 pathway. Furthermore, SFN improved renal performance and minimized pathological changes in the glomeruli of STZ-$Nrf2^{+/+}$ mice [42]. Cui et al. also demonstrated that SFN injection (0.5 mg/kg daily for 3 months) prevented diabetic nephropathy in a type 1 diabetic mouse model induced by multiple low doses of STZ [43].

Xue et al. assessed whether activation of Nrf2 by SFN in human microvascular EC (HMEC-1) prevented metabolic dysfunction in hyperglycemia. HMEC-1 cells were incubated with media containing low and high glucose concentrations (5 and 30 mM, resp.). SFN induced nuclear translocation of Nrf2 and increased ARE-linked gene expression. Hyperglycemia increased the formation of ROS, an effect that was linked to mitochondrial dysfunction and prevented by SFN [45]. Miao et al. demonstrated significant increases in oxidative stress and inflammation in the aorta in a type 1 STZ-induced diabetic model at both 3 and 6 months, with fibrotic responses after 6 months of diabetes. SFN completely prevented these pathogenic diabetic changes and also significantly upregulated the expression of Nrf2 and its downstream antioxidants [46].

Diabetes-induced cardiomyopathy is one of the major types of CVD. Our research group investigated whether SFN prevented DCM. Type 1 diabetes was induced in FVB mice by multiple intraperitoneal injections of low doses of STZ. Hyperglycemic and age-matched control mice were treated with or without 0.5 mg/kg SFN (Sigma, L-SFN, 2.8 μmol/kg) daily, five days/week for 3 months, and then kept until 6 months after the induction of diabetes. At 3 and 6 months of diabetes, SFN significantly prevented diabetes-induced hypertension and cardiac dysfunction and also prevented diabetes-induced cardiac hypertrophy and fibrosis. SFN almost completely prevented diabetes-induced cardiac oxidative damage and inflammation. SFN upregulated Nrf2 expression and transcriptional activity, as evidenced by increased Nrf2 nuclear accumulation and phosphorylation, as well as increased mRNA and protein expression of Nrf2 downstream antioxidants. These results suggested that DCM could be prevented by SFN via upregulation of Nrf2 expression and transcriptional activity [47]. Zhang et al. investigated the effects of SFN on type 2 diabetes-induced cardiac lipid accumulation and, subsequently, DCM. SFN treatment significantly attenuated cardiac remodeling and dysfunction, significantly inhibited cardiac lipid accumulation as measured by Oil Red O staining, and improved cardiac inflammation, oxidative stress, and fibrosis [48].

Based on these improvements, SFN-induced activation of Nrf2 counteracted glucose-induced ROS and inflammation, protecting against diabetes and diabetic complications.

9. Prospects on the Clinical Application of SFN in the Prevention of CVD

It has been shown that SFN produced protective effects against CVD *in vitro* and animal studies [29, 34, 47, 75]. However, there are no systematic clinical studies in identifying the optimal consumption level of SFN in humans. Herein, based on the current results of the studies, the dose and application form of SFN and its rationalities are discussed to provide basis for further CVD prevention clinical trials.

Table 1: Summary of *in vivo* and *in vitro* studies of sulforaphane- (SFN-) mediated protection against cardiovascular disease (CVD).

CVD	Model	SFN dose	Biomarker modulation	Reference
Hypertension	Female SHRsp rats on the Grn+ and Grn diets (1st generation), and the offspring (2nd generation)	0.9 mg SFN in 200 mg air-dried broccoli sprouts	Female SHRsp on a Grn+ diet had decreased hypertension. Their offspring had lower blood pressure in adulthood, regardless of diet, and the best health outcomes	[29]
	Female SHRsp rats, oral gavage of SFN in corn oil	1.77 mg/kg	Mean arterial pressure was 20% higher in vehicle-treated SHRsp and SFN administration to SHRsp improved blood pressure	[30]
	3T3-L1 preadipocytes, cultured with SFN	20 μM for 6 days	SFN inhibited early-stage adipocyte differentiation	[31]
	Isolated aortic SMCs from SHRsp rats or controls	0.05–1 μM for 24 h	SFN induced concentration-dependent increases in cellular GSH levels and HO-1 protein content and decreased oxidative stress	[32]
	Male SHRsp and Sprague-Dawley rats on control, Grn+, and Grn− diets	0.9 mg SFN in 200 mg air-dried broccoli sprouts	SHRsp fed a Grn+ diet had a major improvement in the cardiovascular and kidney tissues and reduced hypertension	[33]
Atherosclerosis	Mouse VSMCs incubated with TNF-α (10 ng/mL) for 4 and 8 h	Pretreatment with 5, 15, and 25 μM for 2 h	SFN dose-dependently inhibited TNF-α-induced protein expression of VCAM-1 and intracellular ROS, which may have beneficial effects on inflammation within the atherosclerotic lesion	[34]
	Male C57BL/6 or Nrf2$^{-/-}$ mice aortic EC were stained at susceptible and protected sites	Pretreatment with 5 mg/kg for 4 h or 24 h	SFN activation of Nrf2 reduced endothelial activation at atherosusceptible sites	[35]
	Confluent HUVEC exposed to unidirectional laminar shear for 24 h	1 μM, 24 h	SFN inhibited TNF-α-induced VCAM-1 expression and prevented VSMC proliferation	
	Sprague-Dawley rats subjected to carotid artery balloon injury; VSMCs were exposed to TNF-α	5 μM	SFN attenuated neointima formation after balloon injury and intima area ratio and stenosis	[36]
	HAEC treated with TNF-α (100 U/mL) for 4 h	1–4 μM pretreatment of HAECs for 1 h	SFN suppressed TNF-α-induced VCAM-1 protein expression, suggesting that SFN may be a useful treatment for inflammatory diseases	[37]
	HUVEC treated with 0–40 ng/mL TNF-α for 24 h or with 10 ng/mL TNF-α for 0–48 h	10 μM pretreatment for 1 h	SFN inhibited TNF-α-mediated induction of endothelial lipase in HUVEC, indicating that SFN may have a beneficial effect on HDL cholesterol levels	[38]
I/R	Ischemia in isolated perfused Langendorff Sprague-Dawley rat hearts	0.5 mg/kg daily i.p. pretreatment for 3 days before ischemia	SFN significantly improved coronary flow and reduced I/R-induced increases in LDH level and infarct size, showing that SFN protected against I/R injury	[39]
Diabetes	Male Wistar rats injected with STZ (80 mg/kg)	Oral pretreatment with 0.1, 0.25, or 0.5 mg/kg	All SFN doses reduced levels of triacylglycerol, urea, and creatinine, cholesterol, alanine, and aspartate aminotransferase levels	[40]
	RIN cells treated with IL-1β (2 ng/mL) and IFN-γ (100 U/mL) to produce H_2O_2	2.5–10 μM pretreatment for 3 h	Pretreatment with SFN resulted in concentration-dependent protection against the toxic effect of cytokines, with increased survival of RIN cells	[41]
Diabetic nephropathy	Eight-week-old mice received STZ (50 mg/kg) i.p. for 5 consecutive days	Pretreatment with 12.5 mg/kg i.p. three times a week for 16 weeks	SFN significantly attenuated common metabolic disorder symptoms, improved renal performance, and minimized pathological alterations in the glomerulus of STZ-Nrf2$^{+/+}$ mice	[42]
	Mice were injected i.p. with 50 mg/kg STZ daily for 5 days	Subcutaneous injection of 0.5 mg/kg, five days per week, for 3 months	SFN prevented diabetes-induced renal inflammation and oxidative stress and also prevented renal structural changes and fibrosis	[43]
Diabetic neuropathy	Diabetic neuropathy was induced in rats using STZ	Administration of 0.5 and 1 mg/kg six weeks after diabetes	SFN reduced NF-κB expression and IκB kinase phosphorylation, along with abrogation of inducible nitric oxide synthase, cyclooxygenase-2 expression, and TNF-α and IL-6 levels	[44]
Diabetic angiopathy	HMEC-1 cultured in high glucose medium (30 mM)	4 μM for 6–48 h	Multiple pathways of biochemical dysfunction in HMEC-1 cells induced by hyperglycemia were reversed by SFN	[45]
DCM	Mice were injected with 50 mg/kg STZ i.p. daily for 5 days	0.5 mg/kg subcutaneous injection daily for 3 months after STZ-induced diabetes	Diabetes induced significant increases in oxidative stress and inflammation in the aorta at both 3 and 6 months, and fibrotic response at 6 months. SFN completely prevented these pathogenic changes	[46]
	Mice were injected with 50 mg/kg STZ i.p. daily for 5 days	0.5 mg/kg subcutaneous injection daily for 3 months after STZ-induced diabetes	SFN significantly reduced hypertension and cardiac dysfunction at both 3 and 6 months and also prevented cardiac hypertrophy and fibrosis. SFN also almost completely prevented cardiac oxidative damage and inflammation	[47]
	Mice were fed a high-fat diet for 3 months, then treated with 100 mg/kg STZ i.p. to induce T2DM	0.5 mg/kg subcutaneous injection daily five days a week for 4 months	SFN significantly inhibited cardiac lipid accumulation improved cardiac inflammation oxidative stress and fibrosis induced by T2DM	[48]

Notes. EC: endothelial cells. Grn: glucoraphanin. GSH: glutathione. HMEC: human microvascular endothelial cells. HUVEC: human umbilical vein endothelial cells. IL: interleukin. i.p.: intraperitoneal injection. I/R: ischemia-reperfusion. i.v.: intravenous injection. RIN cells: rat pancreatic β-cell line RINm5F. SHRsp: spontaneously hypertensive stroke-prone rats. SMC: smooth muscle cell. STZ: streptozocin. TNF-α: tumor necrosis factor-α. VSMC: vascular smooth muscle cell. T2DM: type 2 diabetes mellitus. VCAM: vascular cell adhesion molecule. HAEC: human aortic endothelial cells. DCM: diabetic cardiomyopathy.

Since SFN can be found in brassicates like broccoli, it will be reasonable to have a diet containing broccoli to prevent CVD in the studied group. But what amounts of SFN are contained in the brassicates? Saha et al. reported that extraction of 100–200 mg broccoli powder using a series of purification steps provided a reasonable dose of SFN (23.5 μmol) [84]. Cramer et al. showed that incubation in water at room temperature for 24 h with the addition of 0.8 U myrosinase fresh broccoli sprouts produced 1.69 μmol SFN/g fresh weight on average, as determined by bench hydrolysis [85].

As these were taken orally, the dose of SFN was also influenced by its bioavailability in the body. SFN bioavailability from dietary sources is a critical determinant of its efficacy in humans. Atwell et al. evaluated SFN absorption from a myrosinase-treated broccoli sprout extract. Subjects consumed fresh broccoli sprouts or the extract, each providing 200 μmol SFN daily, as a single dose and as two 100 μmol doses taken 12 h apart. The study showed that 12 h dosing was associated with higher plasma SFN metabolite levels at later time points than those achieved by 24 h dosing. This indicated that the dietary form and dosing schedule of SFN may influence its absorption and efficacy in human [86].

Then, what amount of SFN should be suggested to consume in order to achieve the levels for prevention of CVD? It has shown that 10 μM SFN can protect cardiomyocytes *in vitro* and that 0.5 mg/kg (2.8 μmol/kg) SFN could prevent CVD in STZ induced diabetes mice. According to the conversion formula between mice and human [87, 88], the consumption of SFN in human is supposed to be 0.05 mg/kg (0.28 μmol/kg) per day. That is, if a man is with the body weight of 70 kg, the dose of SFN will be 19.6 μmol per day, and the consumption of fresh broccoli dose will be about 12 g per day. It is much lower than the dose (150 μmol per day [89]) used in the cancer chemoprevention trial without showing any toxic effects [90]. Therefore, it implies that SFN will be much well-tolerated if it is used clinically in the prevention of CVD.

Collectively, a diet containing SFN, such as brassicates, could prevent CVD, and the required consumption of SFN is easy to get and shows harmless. It is worthwhile to perform clinical trials to study the effects and optimal dose of SFN in prevention of CVD.

10. Conclusions

Oxidative stress plays a major role in the pathophysiology of cardiac disorders. SFN found in cruciferous vegetables is an indirect antioxidant that can activate Nrf2 and its downstream target genes to induce antioxidant effects. Animal and human experiments have identified substantial SFN-mediated protection from a range of CVD, including hypertension, atherosclerosis, I/R injury, diabetes, and diabetic complications (Table 1). The findings presented in this review indicate that SFN, a phytochemical isolated from extracts of an edible plant with a presumed low level of toxicity, protects against CVD. SFN could therefore contribute to the prevention of CVD.

Conflict of Interests

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

Acknowledgments

The work of the authors was supported in part by the National Science Foundation of China (81300186, to Yang Bai; 81370318 to Yang Zheng; and 30901702 to Jiuwei Cui) and Ministry of Education Key Project of Science and Technology (311015, to Jiuwei Cui).

References

[1] M. Rafieian-Kopaei, M. Setorki, M. Doudi, A. Baradaran, and H. Nasri, "Atherosclerosis: process, indicators, risk factors and new hopes," *International Journal of Preventive Medicine*, vol. 5, no. 8, pp. 927–946, 2014.

[2] J. M. Nunez-Cordoba and M. A. Martinez-Gonzalez, "Antioxidant vitamins and cardiovascular disease," *Current Topics in Medicinal Chemistry*, vol. 11, no. 14, pp. 1861–1869, 2011.

[3] J. C. Kovacic and V. Fuster, "From treating complex coronary artery disease to promoting cardiovascular health: therapeutic transitions and challenges, 2010–2020," *Clinical Pharmacology & Therapeutics*, vol. 90, no. 4, pp. 509–518, 2011.

[4] R. V. Luepker, "Cardiovascular disease: rise, fall, and future prospects," *Annual Review of Public Health*, vol. 32, pp. 1–3, 2011.

[5] F. C. McGillicuddy, H. P. Moll, S. Farouk, S. M. Damrauer, C. Ferran, and M. P. Reilly, "Translational studies of A20 in atherosclerosis and cardiovascular disease," in *The Multiple Therapeutic Targets of A20*, Advances in Experimental Medicine and Biology, pp. 83–101, Springer, New York, NY, USA, 2014.

[6] R. Lindblom, K. Ververis, S. Tortorella, and T. Karagiannis, "The early life origin theory in the development of cardiovascular disease and type 2 diabetes," *Molecular Biology Reports*, vol. 42, no. 4, pp. 791–797, 2015.

[7] J. B. Halter, N. Musi, F. McFarland Horne et al., "Diabetes and cardiovascular disease in older adults: current status and future directions," *Diabetes*, vol. 63, no. 8, pp. 2578–2589, 2014.

[8] S. Ichihara, "The pathological roles of environmental and redox stresses in cardiovascular diseases," *Environmental Health and Preventive Medicine*, vol. 18, no. 3, pp. 177–184, 2013.

[9] A. T. Dinkova-Kostova and P. Talalay, "Direct and indirect antioxidant properties of inducers of cytoprotective proteins," *Molecular Nutrition & Food Research*, vol. 52, supplement 1, pp. S128–S138, 2008.

[10] S. Mukherjee, H. Gangopadhyay, and D. K. Das, "Broccoli: a unique vegetable that protects mammalian hearts through the redox cycling of the thioredoxin superfamily," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 2, pp. 609–617, 2008.

[11] C. Fimognari, M. Lenzi, and P. Hrelia, "Interaction of the isothiocyanate sulforaphane with drug disposition and metabolism: pharmacological and toxicological implications," *Current Drug Metabolism*, vol. 9, no. 7, pp. 668–678, 2008.

[12] S. Boddupalli, J. R. Mein, S. Lakkanna, and D. R. James, "Induction of phase 2 antioxidant enzymes by broccoli sulforaphane: perspectives in maintaining the antioxidant activity of vitamins A, C, and E," *Frontiers in Genetics*, vol. 3, no. 7, 2012.

[13] E.-S. Hwang and E. H. Jeffery, "Induction of quinone reductase by sulforaphane and sulforaphane N-acetylcysteine conjugate in murine hepatoma cells," *Journal of Medicinal Food*, vol. 8, no. 2, pp. 198–203, 2005.

[14] S. K. Ghawi, L. Methven, and K. Niranjan, "The potential to intensify sulforaphane formation in cooked broccoli (*Brassica oleracea var. italica*) using mustard seeds (*Sinapis alba*)," *Food Chemistry*, vol. 138, no. 2-3, pp. 1734–1741, 2013.

[15] T. W. Kensler, P. A. Egner, A. S. Agyeman et al., "Keap1-Nrf2 signaling: a target for cancer prevention by sulforaphane," *Topics in Current Chemistry*, vol. 329, pp. 163–178, 2013.

[16] Y. Zhang, R. H. Kolm, B. Mannervik, and P. Talalay, "Reversible conjugation of isothiocyanates with glutathione catalyzed by human glutathione transferases," *Biochemical and Biophysical Research Communications*, vol. 206, no. 2, pp. 748–755, 1995.

[17] S. S. Hecht, "Chemoprevention of cancer by isothiocyanates, modifiers of carcinogen metabolism," *The Journal of Nutrition*, vol. 129, no. 3, pp. 768S–774S, 1999.

[18] Y.-H. Ahn, Y. Hwang, H. Liu et al., "Electrophilic tuning of the chemoprotective natural product sulforaphane," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 21, pp. 9590–9595, 2010.

[19] Q. Ma and X. He, "Molecular basis of electrophilic and oxidative defense: promises and perils of Nrf2," *Pharmacological Reviews*, vol. 64, no. 4, pp. 1055–1081, 2012.

[20] P. Jancova, P. Anzenbacher, and E. Anzenbacherova, "Phase II drug metabolizing enzymes," *Biomedical Papers*, vol. 154, no. 2, pp. 103–116, 2010.

[21] P. Gupta, B. Kim, S. Kim, and S. K. Srivastava, "Molecular targets of isothiocyanates in cancer: recent advances," *Molecular Nutrition & Food Research*, vol. 58, no. 8, pp. 1685–1707, 2014.

[22] T. Shibata, Y. Kimura, A. Mukai et al., "Transthiocarbamoylation of proteins by thiolated isothiocyanates," *The Journal of Biological Chemistry*, vol. 286, no. 49, pp. 42150–42161, 2011.

[23] C. E. Guerrero-Beltrán, M. Calderón-Oliver, J. Pedraza-Chaverri, and Y. I. Chirino, "Protective effect of sulforaphane against oxidative stress: recent advances," *Experimental and Toxicologic Pathology*, vol. 64, no. 5, pp. 503–508, 2012.

[24] K. Itoh, K. I. Tong, and M. Yamamoto, "Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles," *Free Radical Biology & Medicine*, vol. 36, no. 10, pp. 1208–1213, 2004.

[25] E. Bhakkiyalakshmi, D. Sireesh, P. Rajaguru, R. Paulmurugan, and K. M. Ramkumar, "The emerging role of redox-sensitive Nrf2-Keap1 pathway in diabetes," *Pharmacological Research*, vol. 91, pp. 104–114, 2014.

[26] M. McMahon, K. Itoh, M. Yamamoto, and J. D. Hayes, "Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression," *The Journal of Biological Chemistry*, vol. 278, no. 24, pp. 21592–21600, 2003.

[27] K. T. Turpaev, "Keap1-Nrf2 signaling pathway: Mechanisms of regulation and role in protection of cells against toxicity caused by xenobiotics and electrophiles," *Biochemistry*, vol. 78, no. 2, pp. 111–126, 2013.

[28] W. Hur and N. S. Gray, "Small molecule modulators of antioxidant response pathway," *Current Opinion in Chemical Biology*, vol. 15, no. 1, pp. 162–173, 2011.

[29] M. H. Noyan-Ashraf, L. Wu, R. Wang, and B. H. J. Juurlink, "Dietary approaches to positively influence fetal determinants of adult health," *The FASEB Journal*, vol. 20, no. 2, pp. 371–373, 2006.

[30] G. V. K. Senanayake, A. Banigesh, L. Wu, P. Lee, and B. H. J. Juurlink, "The dietary phase 2 protein inducer sulforaphane can normalize the kidney epigenome and improve blood pressure in hypertensive rats," *American Journal of Hypertension*, vol. 25, no. 2, pp. 229–235, 2012.

[31] K.-M. Choi, Y.-S. Lee, D.-M. Sin et al., "Sulforaphane inhibits mitotic clonal expansion during adipogenesis through cell cycle arrest," *Obesity*, vol. 20, no. 7, pp. 1365–1371, 2012.

[32] L. Wu and B. H. J. Juurlink, "The impaired glutathione system and its up-regulation by sulforaphane in vascular smooth muscle cells from spontaneously hypertensive rats," *Journal of Hypertension*, vol. 19, no. 10, pp. 1819–1825, 2001.

[33] L. Wu, M. H. N. Ashraf, M. Facci et al., "Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 18, pp. 7094–7099, 2004.

[34] J.-Y. Kim, H.-J. Park, S. H. Um et al., "Sulforaphane suppresses vascular adhesion molecule-1 expression in TNF-α-stimulated mouse vascular smooth muscle cells: involvement of the MAPK, NF-κB and AP-1 signaling pathways," *Vascular Pharmacology*, vol. 56, no. 3-4, pp. 131–141, 2012.

[35] M. Zakkar, K. van der Heiden, L. A. Luong et al., "Activation of Nrf2 in endothelial cells protects arteries from exhibiting a proinflammatory state," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 11, pp. 1851–1857, 2009.

[36] J.-S. Kwon, H. Joung, Y. S. Kim et al., "Sulforaphane inhibits restenosis by suppressing inflammation and the proliferation of vascular smooth muscle cells," *Atherosclerosis*, vol. 225, no. 1, pp. 41–49, 2012.

[37] X.-L. Chen, G. Dodd, and C. Kunsch, "Sulforaphane inhibits TNF-α-induced activation of p38 MAP kinase and VCAM-1 and MCP-1 expression in endothelial cells," *Inflammation Research*, vol. 58, no. 8, pp. 513–521, 2009.

[38] A. M. Kivelä, P. I. Mäkinen, H.-K. Jyrkkänen et al., "Sulforaphane inhibits endothelial lipase expression through NF-κB in endothelial cells," *Atherosclerosis*, vol. 213, no. 1, pp. 122–128, 2010.

[39] C. S. Piao, S. Gao, G.-H. Lee et al., "Sulforaphane protects ischemic injury of hearts through antioxidant pathway and mitochondrial K_{ATP} channels," *Pharmacological Research*, vol. 61, no. 4, pp. 342–348, 2010.

[40] C. G. de Souza, J. A. Sattler, A. M. De Assis, A. Rech, M. L. S. Perry, and D. O. Souza, "Metabolic effects of sulforaphane oral treatment in streptozotocin-diabetic rats," *Journal of Medicinal Food*, vol. 15, no. 9, pp. 795–801, 2012.

[41] M.-Y. Song, E.-K. Kim, W.-S. Moon et al., "Sulforaphane protects against cytokine- and streptozotocin-induced β-cell damage by suppressing the NF-κB pathway," *Toxicology and Applied Pharmacology*, vol. 235, no. 1, pp. 57–67, 2009.

[42] H. Zheng, S. A. Whitman, W. Wu et al., "Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy," *Diabetes*, vol. 60, no. 11, pp. 3055–3066, 2011.

[43] W. Cui, Y. Bai, X. Miao et al., "Prevention of diabetic nephropathy by sulforaphane: possible role of Nrf2 upregulation and activation," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 821936, 12 pages, 2012.

[44] G. Negi, A. Kumar, and S. S. Sharma, "Nrf2 and NF-κB modulation by sulforaphane counteracts multiple manifestations of diabetic neuropathy in rats and high glucose-induced changes," *Current Neurovascular Research*, vol. 8, no. 4, pp. 294–304, 2011.

[45] M. Xue, Q. Qian, A. Adaikalakoteswari, N. Rabbani, R. Babaei-Jadidi, and P. J. Thornalley, "Activation of NF-E2-related factor-2 reverses biochemical dysfunction of endothelial cells induced by hyperglycemia linked to vascular disease," *Diabetes*, vol. 57, no. 10, pp. 2809–2817, 2008.

[46] X. Miao, B. Yang, W. Sun et al., "Sulforaphane prevention of diabetes-induced aortic damage was associated with the up-regulation of Nrf2 and its down-stream antioxidants," *Nutrition & Metabolism*, vol. 9, no. 1, article 84, 2012.

[47] Y. Bai, W. Cui, Y. Xin et al., "Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation," *Journal of Molecular and Cellular Cardiology*, vol. 57, no. 1, pp. 82–95, 2013.

[48] Z. Zhang, S. Wang, S. Zhou et al., "Sulforaphane prevents the development of cardiomyopathy in type 2 diabetic mice probably by reversing oxidative stress-induced inhibition of LKB1/AMPK pathway," *Journal of Molecular and Cellular Cardiology C*, vol. 77, pp. 42–52, 2014.

[49] T. W. Kensler, P. A. Egner, A. S. Agyeman et al., "Keap1-Nrf2 signaling: a target for cancer prevention by sulforaphane," *Topics in Current Chemistry*, vol. 329, pp. 163–177, 2013.

[50] T. Ichikawa, J. Li, C. J. Meyer, J. S. Janicki, M. Hannink, and T. Cui, "Dihydro-CDDO-trifluoroethyl amide (dh404), a novel Nrf2 activator, suppresses oxidative stress in cardiomyocytes," *PLoS ONE*, vol. 4, no. 12, Article ID e8391, 2009.

[51] E. E. Vomhof-DeKrey and M. J. Picklo, "The Nrf2-antioxidant response element pathway: a target for regulating energy metabolism," *The Journal of Nutritional Biochemistry*, vol. 23, no. 10, pp. 1201–1206, 2012.

[52] W. Li, N. Thakor, E. Y. Xu et al., "An internal ribosomal entry site mediates redox-sensitive translation of Nrf2," *Nucleic Acids Research*, vol. 38, no. 3, pp. 778–788, 2009.

[53] R. K. Thimmulappa, K. H. Mai, S. Srisuma, T. W. Kensler, M. Yamamoto, and S. Biswal, "Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray," *Cancer Research*, vol. 62, no. 18, pp. 5196–5203, 2002.

[54] P. Nioi, M. McMahon, K. Itoh, M. Yamamoto, and J. D. Hayes, "Identification of a novel NRF2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence," *Biochemical Journal*, vol. 374, no. 2, pp. 337–348, 2003.

[55] W. Li, S.-W. Yu, and A.-N. T. Kong, "Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain," *The Journal of Biological Chemistry*, vol. 281, no. 37, pp. 27251–27263, 2006.

[56] Y. Li, J. D. Paonessa, and Y. Zhang, "Mechanism of chemical activation of Nrf2," *PLoS ONE*, vol. 7, no. 4, Article ID e35122, 2012.

[57] A. M. Pickering, R. A. Linder, H. Zhang, H. J. Forman, and K. J. A. Davies, "Nrf2-dependent induction of proteasome and Pa28$\alpha\beta$ regulator are required for adaptation to oxidative stress," *The Journal of Biological Chemistry*, vol. 287, no. 13, pp. 10021–10031, 2012.

[58] Y.-S. Keum, "Regulation of the Keap1/Nrf2 system by chemopreventive sulforaphane: implications of posttranslational modifications," *Annals of the New York Academy of Sciences*, vol. 1229, no. 1, pp. 184–189, 2011.

[59] S. Magesh, Y. Chen, and L. Hu, "Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents," *Medicinal Research Reviews*, vol. 32, no. 4, pp. 687–726, 2012.

[60] X. Yu and T. Kensler, "Nrf2 as a target for cancer chemoprevention," *Mutation Research*, vol. 591, no. 1-2, pp. 93–102, 2005.

[61] E. Leoncini, M. Malaguti, C. Angeloni, E. Motori, D. Fabbri, and S. Hrelia, "Cruciferous vegetable phytochemical sulforaphane affects phase II enzyme expression and activity in rat cardiomyocytes through modulation of Akt signaling pathway," *Journal of Food Science*, vol. 76, no. 7, pp. H175–H181, 2011.

[62] P. Bergström, H. C. Andersson, Y. Gao et al., "Repeated transient sulforaphane stimulation in astrocytes leads to prolonged Nrf2-mediated gene expression and protection from superoxide-induced damage," *Neuropharmacology*, vol. 60, no. 2-3, pp. 343–353, 2011.

[63] C. Angeloni, E. Leoncini, M. Malaguti, S. Angelini, P. Hrelia, and S. Hrelia, "Modulation of phase II enzymes by sulforaphane: implications for its cardioprotective potential," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 12, pp. 5615–5622, 2009.

[64] Y. Higashi, Y. Kihara, and K. Noma, "Endothelial dysfunction and hypertension in aging," *Hypertension Research*, vol. 35, no. 11, pp. 1039–1047, 2012.

[65] H. Sillesen and V. Fuster, "Predicting coronary heart disease: from framingham risk score to ultrasound bioimaging," *The Mount Sinai Journal of Medicine*, vol. 79, no. 6, pp. 654–663, 2012.

[66] S. Murakami, "Taurine and atherosclerosis," *Amino Acids*, vol. 46, no. 1, pp. 73–80, 2014.

[67] F. Gijsen, A. van der Giessen, A. van der Steen, and J. Wentzel, "Shear stress and advanced atherosclerosis in human coronary arteries," *Journal of Biomechanics*, vol. 46, no. 2, pp. 240–247, 2013.

[68] P. Nigro, J.-I. Abe, and B. C. Berk, "Flow shear stress and atherosclerosis: a matter of site specificity," *Antioxidants and Redox Signaling*, vol. 15, no. 5, pp. 1405–1414, 2011.

[69] C.-S. Huang, A.-H. Lin, C.-T. Liu et al., "Isothiocyanates protect against oxidized LDL-induced endothelial dysfunction by upregulating Nrf2-dependent antioxidation and suppressing NFκB activation," *Molecular Nutrition and Food Research*, vol. 57, no. 11, pp. 1918–1930, 2013.

[70] K.-M. Choi, Y.-S. Lee, W. Kim et al., "Sulforaphane attenuates obesity by inhibiting adipogenesis and activating the AMPK pathway in obese mice," *The Journal of Nutritional Biochemistry*, vol. 25, no. 2, pp. 201–207, 2014.

[71] W.-Y. Chuang, P.-H. Kung, C.-Y. Kuo, and C.-C. Wu, "Sulforaphane prevents human platelet aggregation through inhibiting the phosphatidylinositol 3-kinase/Akt pathway," *Thrombosis and Haemostasis*, vol. 109, no. 6, pp. 1120–1130, 2013.

[72] C.-H. Oh, J.-I. Shin, S. J. Mo, S.-J. Yun, S.-H. Kim, and Y.-H. Rhee, "Antiplatelet activity of L-sulforaphane by regulation of platelet activation factors, glycoprotein IIb/IIIa and thromboxane A2," *Blood Coagulation and Fibrinolysis*, vol. 24, no. 5, pp. 498–504, 2013.

[73] T. Jayakumar, W.-F. Chen, W.-J. Lu et al., "A novel antithrombotic effect of sulforaphane via activation of platelet adenylate cyclase: ex vivo and in vivo studies," *The Journal of Nutritional Biochemistry*, vol. 24, no. 6, pp. 1086–1095, 2013.

[74] P. W. M. Kleikers, K. Wingler, J. J. R. Hermans et al., "NADPH oxidases as a source of oxidative stress and molecular target in ischemia/reperfusion injury," *Journal of Molecular Medicine*, vol. 90, no. 12, pp. 1391–1406, 2012.

[75] H. Masutani, R. Otsuki, Y. Yamaguchi et al., "Fragrant unsaturated aldehydes elicit activation of the keap1/Nrf2 system

leading to the upregulation of thioredoxin expression and protection against oxidative stress," *Antioxidants & Redox Signaling*, vol. 11, no. 5, pp. 949–962, 2009.

[76] Z. Li, U. Galli, L. E. Becker et al., "Sulforaphane protects hearts from early injury after experimental transplantation," *Annals of Transplantation*, vol. 18, no. 1, pp. 558–566, 2013.

[77] M. Sedeek, A. C. Montezano, R. L. Hebert et al., "Oxidative stress, nox isoforms and complications of diabetes-potential targets for novel therapies," *Journal of Cardiovascular Translational Research*, vol. 5, no. 4, pp. 509–518, 2012.

[78] B. Li, S. Liu, L. Miao, and L. Cai, "Prevention of diabetic complications by activation of Nrf2: diabetic cardiomyopathy and nephropathy," *Experimental Diabetes Research*, vol. 2012, Article ID 216512, 7 pages, 2012.

[79] L. Gnudi and J. Karalliedde, "Beat it early: putative renoprotective haemodynamic effects of oral hypoglycaemic agents," *Nephrology Dialysis Transplantation*, 2015.

[80] E. Sołowiej, J. Sołowiej, T. Kasprzycka-Guttman, and W. Rowiński, "Application of sulforaphane—does it lead to improvement of islet graft survival after warm and/or cold ischemia," *Annals of Transplantation*, vol. 9, no. 3, pp. 68–71, 2004.

[81] J. Xu, S. R. Kulkarni, A. C. Donepudi, V. R. More, and A. L. Slitt, "Enhanced Nrf2 activity worsens insulin resistance, impairs lipid accumulation in adipose tissue, and increases hepatic steatosis in leptin-deficient mice," *Diabetes*, vol. 61, no. 12, pp. 3208–3218, 2012.

[82] Z. Bahadoran, P. Mirmiran, and F. Azizi, "Potential efficacy of broccoli sprouts as a unique supplement for management of type 2 diabetes and its complications," *Journal of Medicinal Food*, vol. 16, no. 5, pp. 375–382, 2013.

[83] Y. Ding and M. E. Choi, "Autophagy in diabetic nephropathy," *The Journal of Endocrinology*, vol. 224, no. 1, pp. R15–R30, 2015.

[84] S. Saha, W. Hollands, B. Teucher et al., "Isothiocyanate concentrations and interconversion of sulforaphane to erucin in human subjects after consumption of commercial frozen broccoli compared to fresh broccoli," *Molecular Nutrition and Food Research*, vol. 56, no. 12, pp. 1906–1916, 2012.

[85] J. M. Cramer, M. Teran-Garcia, and E. H. Jeffery, "Enhancing sulforaphane absorption and excretion in healthy men through the combined consumption of fresh broccoli sprouts and a glucoraphanin-rich powder," *The British Journal of Nutrition*, vol. 107, no. 9, pp. 1333–1338, 2012.

[86] L. L. Atwell, A. Hsu, C. P. Wong et al., "Absorption and chemopreventive targets of sulforaphane in humans following consumption of broccoli sprouts or a myrosinase-treated broccoli sprout extract," *Molecular Nutrition & Food Research*, vol. 59, no. 3, pp. 424–433, 2015.

[87] K. Wojcikowski and G. Gobe, "Animal studies on medicinal herbs: predictability, dose conversion and potential value," *Phytotherapy Research*, vol. 28, no. 1, pp. 22–27, 2014.

[88] O. L. Blanchard and J. M. Smoliga, "Translating dosages from animal models to human clinical trials—revisiting body surface area scaling," *The FASEB Journal*, vol. 29, no. 5, pp. 1629–1634, 2015.

[89] P. A. Egner, J. G. Chen, J. B. Wang et al., "Bioavailability of sulforaphane from two broccoli sprout beverages: results of a short-term, cross-over clinical trial in Qidong, China," *Cancer Prevention Research*, vol. 4, no. 3, pp. 384–395, 2011.

[90] K. P. Latté, K.-E. Appel, and A. Lampen, "Health benefits and possible risks of broccoli—an overview," *Food and Chemical Toxicology*, vol. 49, no. 12, pp. 3287–3309, 2011.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 978654, 10 pages
http://dx.doi.org/10.1155/2015/978654

Research Article

Biomolecular Modulation of Neurodegenerative Events during Ageing

Marcella Nebbioso,[1] Gianfranco Scarsella,[2] Aloisa Librando,[1] and Nicola Pescosolido[3]

[1]*Department of Sense Organs, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy*
[2]*Department of Biology and Biotechnology Charles Darwin, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy*
[3]*Department of Cardiovascular, Respiratory, Nephrology, Geriatric, and Anesthetic Sciences, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy*

Correspondence should be addressed to Marcella Nebbioso; marcella.nebbioso@uniroma1.it

Received 2 March 2015; Accepted 26 April 2015

Academic Editor: Zhenquan Jia

The objective is to assess the modulation of retinal and optic nerve degenerative events induced by the combination of α-lipoic acid (ALA) and superoxide dismutase (SOD) in an animal model of ageing. For this study, 24 male Wistar-Harlan strain rats were left to age for up to 24 months. One group of rats was subjected to a diet supplemented with ALA and SOD for 8 weeks, while another group was used as a positive control and not subjected to any dietary treatment. To assess the cytoprotective effects of the antioxidants, a morphological analysis was carried out on sections of retina and optic nerve head, stained with haematoxylin-eosin, followed by an analysis of the modifications to nuclear DNA detected by the TUNEL technique. The lipid peroxidation assay was used to assess the damage induced by oxidative stress at cell membrane level. The molecules involved in apoptosis mediated by oxidative stress, such as caspase-3 and inducible nitric oxide synthase, were also assayed by immunolocalization and western blot. ALA and SOD are able to counteract senile neurodegenerative deterioration to the retina and optic nerve. Indeed, the combination of these antioxidant molecules can reduce oxidative stress levels and thus prevent both nuclear degradation and subsequent cell death.

1. Introduction

The term oxidative stress was first coined in 1989 by Sies who defined it as an imbalance between the production of free radical species and antioxidant defence systems [1]. In physiological conditions, the inside of the cell has a negative charge owing to an array of enzymes and molecules that counterbalance its production. Moreover, the cells of all aerobic organisms produce most of their chemical energy by consuming oxygen in their mitochondria. Mitochondria are thus the main place where intracellular oxygen consumption is the major source of reactive oxygen species (ROS) formation [1, 2]. Alongside ROS, there is a family of free radicals that affects the type of nitrogen known as reactive nitrogen species (RNS), which includes nitric oxide (NO). The enzyme responsible for the production of NO in cells is nitric oxide synthase (NOS), present in three isoforms. It is capable of catalysing the conversion of the amino acid L-arginine into L-citrulline, with the release of NO [3]. Two of the three isoforms of NOS, the constitutively expressed brain and endothelial NOS, are dependent on concentrations of calcium and calmodulin and generate limited amounts of NO. The third isoform of NOS, also known as inducible nitric oxide synthase (iNOS), is calcium independent, not found in the tissues under normal conditions, and produces such high amounts of NO that it induces cytotoxicity [3, 4]. The expression of iNOS was detected in optic nerve astrocytes in patients with glaucoma, a disease that causes increased intraocular pressure (IOP) and ischaemia of the optic nerve [3]. As the body grows older, in accordance with the theory of ageing, normal antioxidant levels are no longer sufficient to counteract the generation of free radicals (FR) [5]. The increase in FR causes molecular damage, some of it being irreversible that accumulates in tissues with age [5, 6]. Nohl

and Hegner (1978) were the first to observe that there is greater production of superoxide radical in the liver of 19-month-old than in that of 6-month-old rats [7].

It has been shown that retinal tissue is particularly susceptible to oxidative damage. Indeed, it is one of the most metabolically active tissues in the body, contains an abundant number of mitochondria, and is subjected to constant photochemical stress [8]. For these reasons, ocular nerve cells are able to produce high concentrations of radical species which, if not sufficiently neutralized, can cause multiple incidents of oxidative damage to the hydrocarbon chains of unsaturated fatty acids, to the amino acid residues of proteins, to the nitrogenous bases of nucleic acids, and to carbohydrates [8].

In extreme cases, all this can be followed by alteration and functional impairment of all the structures, leading to cell death. The death of a significant number of retinal ganglion cells (RGCs) also leads to a reduction in the number of axons forming the optic nerve. The evolution of this phenomenon causes functional deterioration of the optic nerve, which eventually results in neurosensory visual deficit [9].

To counteract this oxidative damage, the body has at its disposal a series of antioxidants that, even at very low concentrations, are able to delay or inhibit oxidation of the substrate [10]. One of the major enzyme systems involved in this defence is superoxide dismutase (SOD), a ubiquitous enzyme that plays a key role in the defence mechanisms against the ROS and RNS produced during cellular metabolism [11]. Some foods containing SOD include melon, wheat sprouts, maize, and soybeans. SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. In mammals, there are three isoforms of SOD expressed by cells: copper- and zinc-containing SOD (CuZnSOD or SOD1) is a homodimer primarily localized to the cytoplasm; manganese SOD (MnSOD or SOD2) is a homotetramer localized exclusively in the mitochondrial matrix and is found in multiple organisms; and extracellular SOD (ECSOD or SOD3) shares significant amino acid homology with CuZnSOD (40–60%) and contains both copper and zinc in its active site but is localized to the extracellular region of the cell. Each isoform is a product of distinct genes and with distinct subcellular locations, but catalyses the same reaction [11].

Several studies using knockout mice have shown that mitochondrial MnSOD (mitSOD) is essential for survival. Indeed, mice lacking this isoform exhibit various symptoms such as damage to the myocardium and liver, lipid peroxidation, and mitochondrial dysfunction and do not survive for more than three weeks. Conversely, where the cytosolic Cu/ZnSOD isoform is missing, mice appear normal and exhibit abnormalities only after trauma [12].

Conversely, increased SOD production in transgenic or mutant strains of *Caenorhabditis elegans* and *Drosophila melanogaster* leads to increased tolerance to oxidative stress and therefore longevity [13, 14].

A second line of defence is also formed by endogenous antioxidant compounds, including α-lipoic acid (ALA), a water-fat-soluble vitamin derivative, isolated for the first time in 1951 from bovine liver [15]. Humans can only synthesize small amounts of ALA from fatty acids and cysteine, so they need exogenous substances [16–18]. ALA is found primarily in animal sources such as liver, heart, muscle, and kidney. To a lesser extent it is also present in fruits and vegetables: broccoli, spinach, tomatoes, potatoes, and rice bran [16–18]. ALA is regarded as a scavenger of free radicals; it has two oxidized or reduced thiol groups and is able to neutralize various reactive species of ROS and RNS. ALA also has the ability to greatly increase intracellular glutathione (GSH) levels, as well as hepatic levels of ascorbate, which conversely decline with age [19, 20].

Based on these considerations, we decided to undertake an experimental study to evaluate the ability of ALA and SOD to modulate the physiological phenomena of ocular neurodegeneration linked to ageing and caused in large part by the damaging effects of the production and accumulation of free radicals. To this end, the study used an experimental animal model in order to reproduce the biochemical and histopathological framework typical of cellular ageing.

2. Materials and Methods

2.1. Animals and Treatments. All experimental procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC), Italian Health Ministry guidelines, and EU Directive 2010/63/EU for animal experiments.

Twenty-four male Wistar-Harlan rats, aged between 20 and 24 months (centenarian rats) with an average weight of 300 to 350 g, were divided into two groups. The first group of rats was pretreated with the same diet for eight weeks, supplemented with ALA and SOD, while the second group was used as a positive control and was not subjected to any supplementary treatment. The dosage of the product per kilogram of body weight was equal to 8.57 mg/day for ALA and 2 IU/day for SOD, that is, corresponding to the human diet, equal to 600 mg/day of ALA and 140 IU/day of SOD (ALA600 SOD, Alfa Wassermann, Bologna, Italy). The negative control group was made up of 12 young male rats of the same strain, aged between 4 and 6 months, and with an average weight of 200 to 250 g, fed normally. The animals were then sacrificed by carotid bleeding, after which the eyeballs were removed.

2.2. Experimental Procedures. The degenerative process was assessed using morphological techniques, immunohistochemistry, western blot, and lipid peroxidation (LPO) assays. The aim was to find morphological, nuclear, and cell membrane damage both at retinal and at optic nerve level. Cytotoxicity and apoptotic markers were also assessed.

2.3. Molecular Analysis of Tissue Sections and of Retina Total Protein Extracts: Morphology Techniques and Sample Preparation. The eyes were enucleated, the corneas were rapidly cut vertically, and the crystalline lens and vitreous humour were removed. We then carried out the following procedures on the residual tissues: fixation in paraformaldehyde 4% for 6 hours at 4°C; rapid wash with 1X PBS pH 7.4; immersion in a 30% sucrose solution in 1X PBS pH 7.4 at 4°C overnight; and

embedding in Killik embedding medium (Bio-Optica, Milan, Italy) in order to perform the cryostat cut into 10 μm sections, horizontally, to allow longitudinal observation of the optic nerve. The sections were placed on microscope slides and stored at −20°C.

In order to observe their morphology, the samples were stained with haematoxylin-eosin. The sections, left to dry at room temperature, were subjected to the following treatments: rehydration with decreasing alcohol (100°, 95°, and 75°) for 7.5 minutes; distilled water for 5 minutes; haematoxylin 0.1% for 1 minute (to stain the chromatin); running water for 10 minutes; distilled water for 2 minutes; eosin for 45 seconds (to stain cytoplasmic components); distilled water for 2 minutes; dehydration with increasing alcohol (75°, 95°, and 100°) for 7.5 minutes; and mounting of the slides with glycerol-PBS 1 : 3. Finally, the tissue sections were observed under an optical microscope and photos taken using computerized image acquisition.

2.4. TUNEL Assay. Tissue sections with a thickness of 10 μm were subjected to TUNEL technique (Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling). It consists of *in situ* labelling of DNA fragments by fluoresceinated nucleotide conjugation using terminal deoxynucleotidyl transferase, iNOS, and caspase-3. At an advanced stage of the apoptosis cascade, the cell presents extensive DNA degradation which leads to the production of molecules 180–200 bp in length and their multiples.

The sections, left to dry at room temperature for 30 min, were subjected to the following treatments: rehydration through a descending alcohol series (100°, 95°, and 75°, 2 min each); incubation with protease 20 μg/mL in Triton X-100 to 0.1% at 37°C for 30 min; washes with 1X PBS for 2 min; incubation with the TUNEL reagent (*In Situ* Cell Death Detection Kit, AP, ROCHE), containing 1/10 in volume of terminal deoxynucleotidyl transferase and the remaining fraction of nucleotides labelled with a fluorescent chromophore, 10 μL per section at 37°C for 1 h. On each slide, one of the sections was always used as the negative control, that is, incubated only with the nucleotides but without terminal transferase in order to detect any nonspecific staining of the sections; washes with 1X PBS for 5 min; and mounting of the slides with Eukitt. The observations were made under a fluorescence microscope and pictures taken by computerized image acquisition.

2.5. Lipid Peroxidation (LPO) Assay. The LPO-586 assay was used to assess the presence of oxidative stress at cell membrane level. This technique is based on the reaction of two molecules of the chromogenic reagent, N-methyl-2-phenylindole (R1), with one molecule of malondialdehyde (MDA) at 45° to form a stable chromophore with maximum absorbance at 586 nm. LPO-586 is a detection kit distributed by *Bioxytech*. Normally, in this kit there is an *insert system* to avoid several experimental problems. The retinas were collected from the enucleated eyes, weighed, transferred to a glass tube with 230 μL of *lysis buffer*, and homogenized in a glass-glass *Potter homogenizer* to carry out the LPO assay. The samples were then subjected to three freeze-thaw cycles and then added to 650 μL of diluted R1 reagent and then 150 μL of HCl (12 N). Subsequently, the samples were incubated at 45°C for 60 minutes and centrifuged at 15000 ×g for 10 minutes. The supernatant was collected in a cuvette to measure absorbance at 586 nm. The blank sample was prepared in the same manner, but with the addition of acetonitrile (75%) and diluent (25%) and not diluted R1. Standard data were used to calculate the absorbance of each sample by subtracting the blank value from the values measured in the spectrophotometer at 586 nm. The MDA concentration was calculated from the absorbance value by linear regression analysis.

2.6. Fluorescence Immunolocalization. This method was used to locate iNOS and caspase-3 in the retina and optic nerve head. iNOS is one of the main molecules induced in the case of tissue damage, while caspase-3 is the protease responsible for the final stages of the apoptotic cascade. To detect iNOS and caspase-3 expression we used primary polyclonal anti-iNOS and anti-caspase-3 antibodies (Santa Cruz) produced in rabbits (diluted to 1 : 50 and 1 : 100, resp.) while the secondary antibodies used were goat anti-rabbit IgG Alexa Fluor antibodies (Molecular Probes) that are green-fluorescing. The sections, left to dry at room temperature for 30 min, were subjected to various treatments: washes in 1X PBS for 5 min; postfixation with alcohol at increasing volumes (70°, 95°, and 100°) for 2 min; washes in 1X PBS for 5 min; block with serum (NRS or NGS), 10% in PBS, 1 h in a moist chamber; incubation with the primary antibody diluted in 1X PBS and BSA 1% for 1 h; fast washes in 1X PBS; incubation with the secondary antibody diluted 1 : 200 in 1X PBS and 1% of the serum of the animal in which the antibody was produced, for 30 min at room temperature; washes in 1X PBS for 5 min; and mounting of the slides with Eukitt. The observations were made under a fluorescence microscope and pictures taken by computerized image acquisition.

2.7. Western Blotting and Immunodetection. Western blotting was used for the identification and quantization of iNOS and caspase-3 on retinal protein extracts. The results were normalized by locating α-tubulin, constitutively expressed in the cells. The Bradford assay was then used to extract protein from animal tissues and to estimate their concentration, by measuring the absorbance at 595.0 nm of IGg, at known increasing concentrations, to which the Bradford reagent was added. Measuring the absorbance of these standards provided a calibration line, on which the protein sample concentration was calculated. The proteins were then subjected to electrophoretic separation on SDS-PAGE (SDS-*Polyacrylamide Gel Electrophoresis*). After weighing the tissues, they were immersed in lysis buffer, and the protein was extracted. The test-tubes were kept for 10 min on ice both before and after centrifugation, performed twice at 13000 rpm for 5 seconds. Subsequently, the supernatant was collected to be used in the study.

Solutions and Gels. Upper gel buffer 4x is as follows: Tris/Cl, pH 6.8, 0.5 M, and SDS 0.4%. *Lower gel buffer 4x* is as follows:

Tris/Cl, pH 8.8, 1.5 M, and SDS 0.4%. *Stacking gel* is as follows: upper gel buffer 2.5 mL H_2O 6.6 mL, acrylamide solution 30% 1.1 mL, APS (ammonium persulfate) 10% 100 μL, and TEMED (tetramethylethylenediamine) 10 μL. *Resolving gel* (*12%*) is as follows: lower gel buffer 2.5 mL, H_2O 4.1 mL, acrylamide solution 30% 4.1 mL, APS 10% 50 μL, and TEMED 5 μL. *Loading buffer* (*10 mL*) is as follows: 18% glycerol, 2% SDS, 2.5% bromophenol blue, 5% β-mercaptoethanol, 2.5 mL stacking gel buffer 4x, and H_2O to volume. *Gel running buffer 1x* is as follows: 25 mM Tris base (3 g/L), 192 mM glycine (14.4 g/L), and 0.1% SDS, all titrated to pH 8.3.

2.8. Transfer of Proteins to Nitrocellulose. The transfer took place by pressing the gel against a nitrocellulose filter in a constant 300 mA electric field. The filter was then washed with T-TBS 1X 0.1% and after saturation of nonspecific bonds (T-TBS 1X + milk 5%), the proteins were probed with the primary antibody capable of specifically detecting those of interest. The same antibodies were used as for the immunolocalization of iNOS and caspase-3. Normalization was performed by recognizing α-tubulin (goat, Santa Cruz) and the anti-tubulin antibody was diluted 1 : 1000, while all others were diluted 1 : 500. During incubation, each antibody was added to a solution of T-TBS and dry milk 5% for one hour. After several washes with T-TBS, carried out following absorption of the primary antibody, incubation with the secondary antibody was performed. The secondary antibodies used for caspase-3 and iNOS (*anti-rabbit* IgG, *Jackson*) and α-tubulin (*anti-goat IgG, Jackson*) were used at a dilution of 1 : 5000. Finally, detection by electrochemiluminescence (ECL) was performed.

Solutions and Products. Blotting buffer 1X is as follows: 25 mM Tris base (3 g/L), 192 mM glycine (14.4 g/L), 20% methanol. *T-TBS 1X 0.1%*, 5 mM Tris/Cl pH 7.4, 15 mM NaCl, and 0.1% Tween 20. *Blocking buffer 1x* is as follows: T-TBS 1X 0.1% and dry milk 5% final.

2.9. Statistical Analysis. All the experiments were repeated at least four times. Statistical analysis of results was made by the ANOVA test. Significativity of result was evaluated by Student's t-test. Followed by Student's t-test, p values < 0.05 were considered significant.

3. Results

3.1. Morphology of the Retina and Optic Nerve Head. Cryostat sections of experimental and control rat eyes were stained with haematoxylin-eosin. Any changes in the astrocyte cells were observed, as well as any morphological changes to the retinal layers and changes to the nuclear chromatin.

Tissue sections from the eyes of young rats not subjected to treatment showed no abnormalities (Figure 1(a)) but were used to highlight the alterations in the optic nerve head of aged rats.

In tissue sections from the eyes of control aged rats not treated with antioxidants, there was a reduction in neural tissue due to apoptosis of RGCs and loss of optic nerve axons and apoptosis of astrocytes (Figure 1(b)). The main alteration it was possible to highlight was the thinning of the optic nerve head following ageing.

In the tissue sections from aged rats treated with ALA- and SOD-based antioxidants, a smaller papillary loss and a good columnar shape typical of astrocyte cells were highlighted (Figure 1(c)).

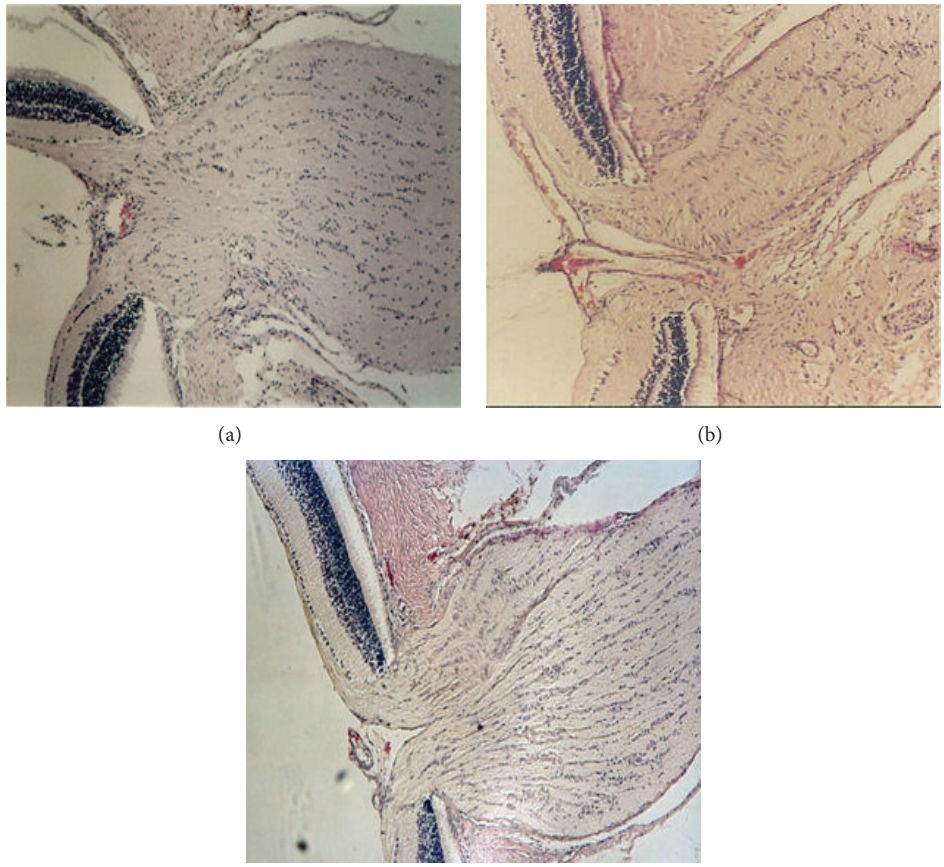
3.2. Study of Modifications due to DNA Degradation (TUNEL Assay). This technique labels the nucleus of cells in an advanced stage of death, where endonucleases have fragmented the DNA. Nuclei with degraded DNA showed greater fluorescence than nuclei in which the degenerative process was absent, because the terminal transferase specifically incorporates fluorescent nucleotides onto the free $3'$OH ends of fragmented DNA (Figures 2(a) and 2(b)). Indeed, in the sections of guinea-pig eye pretreated with ALA and SOD (Figure 2(c)), the assay was not very positive, due to reduced degradation of DNA caused by the cytoprotective effect of the antioxidants both on the astrocyte cells and on the RGCs.

3.3. Study of Modifications to Cell Membranes (LPO-586 Assay). The oxidative stress induced an increase in membrane damage. The extent of membrane damage was assessed by measuring the production of MDA, a widely recognized marker of lipoperoxidation whose production occurs in cells as a direct result of damage to the membrane structure and function. Samples obtained from aged rats showed very high cytoplasmic MDA concentrations with extensive lipoperoxidative damage. By contrast, treatment with ALA and SOD reduced levels of all apoptosis markers, as well as improving overall homeostatic response and limiting apoptotic phenomena (Figure 3).

3.4. Immunolocalization of iNOS and Caspase-3. There was no immunolocalization of iNOS in juvenile rats, while the cells of normal-fed aged rats had high iNOS expression (Figures 4(a) and 4(c′)). High cytotoxic levels of NO were then detected. Conversely, immunofluorescence was reduced in the sections of aged rats fed with ALA and SOD, indicating reduced expression of iNOS (Figures 4(c) and 4(c′)). This would confirm that this enzyme is activated in the presence of damage caused by ageing, as in our model (Figures 4(b), 4(c), 4(b′), and 4(c′)).

Similarly, no significant activation of caspase-3 was observed in juvenile rats (Figures 5(a) and 5(c′)). Conversely, significant expression of caspase-3 was found (indicating suffering and therefore apoptotic activation and the consequent cell death induced by ageing) in sections of eyes from untreated aged rats (Figures 5(b) and 5(b′)), whereas in rats fed with ALA and SOD there was a marked decrease in the expression of this protease (Figures 5(c) and 5(c′)).

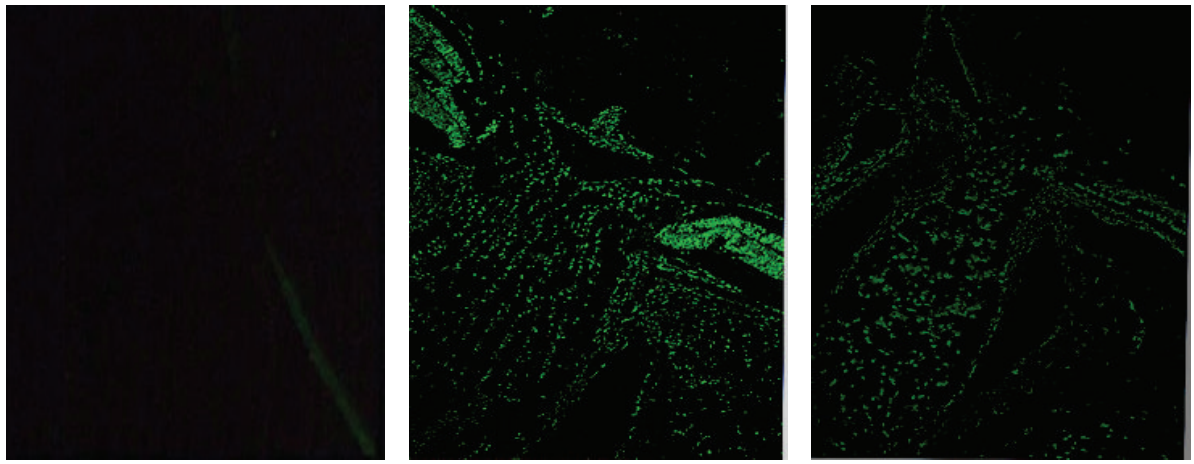
3.5. Western Blot Analysis of iNOS and Caspase-3. The iNOS band was less present, as confirmed by western blot in aged rats pretreated with ALA and SOD (Figure 6), as seen previously via immunolocalization. As expected, a decrease in caspase-3 expression was also observed in retinal extracts



(a) (b)

(c)

Figure 1: Morphology of the retina and optic nerve head stained with haematoxylin-eosin. (a) Section of the optic nerve head of a young rat with no obvious abnormalities (6 months, negative control). (b) Section of the optic nerve head of an untreated aged rat (24 months, positive control). The main alteration it was possible to highlight was the increased excavation of the optic nerve head. (c) Section of the optic nerve head of an aged rat after oral supplementation with α-lipoic acid (ALA) and superoxide dismutase (SOD) (24 months, positive control). Here, a smaller papillary excavation and a good columnar shape typical of astrocyte cells were highlighted compared to the section in panel (b).



(a) (b) (c)

Figure 2: Modifications to nuclear DNA (*TUNEL assay*). (a) Section of the optic nerve head of a young rat with no obvious abnormalities (6 months, negative control). (b) Section of the optic nerve head of an untreated aged rat (24 months, positive control). Nuclei with degraded DNA showed greater fluorescence than nuclei in which the degenerative process was absent. (c) Section of the optic nerve head of an aged rat after oral supplementation with α-lipoic acid (ALA) and superoxide dismutase (SOD) (24 months, positive control). In this case, the assay was not positive, due to reduced degradation of DNA caused by the cytoprotective effect of the antioxidants.

Table 1: The inducible nitric oxide synthase (iNOS) and caspase-3 bands in the different samples were subjected to quantization by electrochemiluminescence (ECL) and the values obtained were expressed as a percentage.

Bands	(A) Aged rats Untreated	(B) Aged rats ALA + SOD treated	(C) Young rats Controls	(A)-(B) p	(B)-(C) p
iNOS	100%	26 ± 4	6 ± 2	≤**0.01***	≤ 0.1
Caspase-3	100%	23 ± 3	8 ± 2	≤**0.01***	≤ 0.1

Mean values ± SD. Significativity of the results was considered with * p values < 0.05.

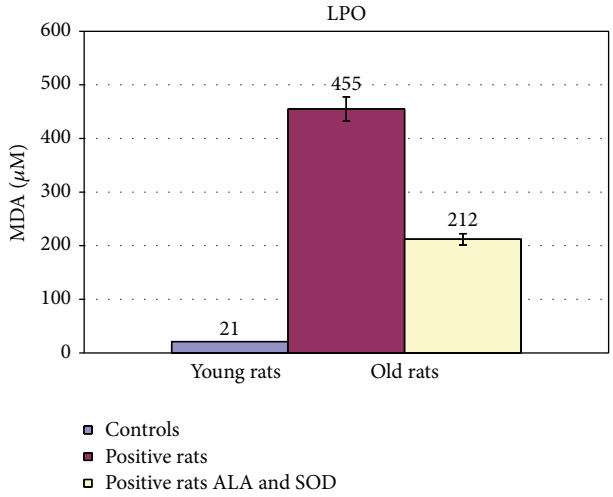


Figure 3: Level of membrane lipoperoxidation (LPO) in retina samples and role of α-lipoic acid (ALA) and superoxide dismutase (SOD) in the control of apoptosis. Bars report the level of malondialdehyde (MDA) evaluated as indicated in the following legend for each bar. The values represent mean ± S.E.M. Controls = young rats (untreated); positive rats = aged rats (untreated); positive rats ALA + SOD = aged rats after treatment with ALA and SOD.

subjected to antioxidant treatment, indicating that the reduction in cell death was due to lower activation of the apoptotic death programme (Figure 6). Normalization was performed using the expression of α-tubulin, constitutively expressed in the cells. In both cases, the bands obtained from the different samples were subjected to quantization by ECL and the values obtained were expressed as percentages (Figure 7 and Table 1).

4. Discussion

The primary aim of our work was to use an experimental animal model to reproduce and assess the histological and therefore biochemical phenomena of cell ageing. Secondarily, we investigated the changes of antioxidant molecules, such as ALA and SOD, on the harmful effect of the free radicals that accumulate over the normal biological processes of life.

From the various parameters considered, we observed that the antioxidant combination of ALA and SOD, administered for 8 weeks to aged rats, would be able to counteract the degenerative events associated with ageing. In fact, our assays seem to confirm that older and therefore damaged tissues have significant iNOS expression. Once NO is produced, it is capable of causing irreversible damage to tissues [21–23]. The toxicity of iNOS has been found in several inflammatory and degenerative eye diseases such as multiple sclerosis, Parkinson's disease, and Alzheimer's disease [22, 23]. Studies of iNOS overexpression in mice have shown that increased NO levels would be able to cause the apoptotic death of retinal photoreceptors [24]. Furthermore, our study would seem to highlight the fact that a reduction in NO, through the use of antioxidants, could provide significant therapeutic benefits against the retinal degeneration induced by oxidative stress due to natural causes, such as ageing. Presumably, the positive effect could also follow a cell degeneration with various pathological causes such as inflammatory, traumatic, or metabolic disorders [17, 18, 22, 25, 26].

Furthermore, the increased stability of mitochondrial membranes, as demonstrated by the reduction in the LPO reaction induced by free radicals, would help reduce the release of proapoptotic factors responsible for inducing the programmed cell death process.

Interestingly, in some studies, it has been found that changes in mitochondrial membrane permeability can lead to opening of transition pores (PTPs) in the liver and brain of aged rats. This would lead to the subsequent release of cytochrome c [27]. Cytochrome c, when released into the cytoplasm, interacts with Apaf-1 to form a protein complex called the apoptosome, which promotes activation of caspase and therefore of the apoptotic death cascade [28].

Going forward, Kokoszka et al. have shown that chronic oxidative stress in mice leads to a partial deficiency in MnSOD which would entail an increase in the sensitization of mitochondrial PTPs and consequently induce apoptosis [29]. Indeed, SOD, as well as MnSOD in particular, plays a major role in maintaining normal mitochondrial function and structural integrity [30]. It has been found that mutant mice totally lacking MnSOD suffer from severe mitochondrial defects, mainly in the brain and heart [12, 31, 32].

Furthermore, differential expressions have been shown to exist between the Bcl-2 family of proteins and the caspase family during ageing in the rat brain [25, 33]. Data confirm these studies, indicating that aged rats would show a marked expression of caspase-3, an executioner protease known to take part in the final stages of the apoptotic cascade [34]. Thus, the oral administration of ALA and SOD would also reduce the activation of this protease, thereby preventing the apoptotic death of retinal cells and the consequent loss of axons forming the optic nerve.

ALA is regarded as one of the most important antioxidants and therefore has an equally significant therapeutic

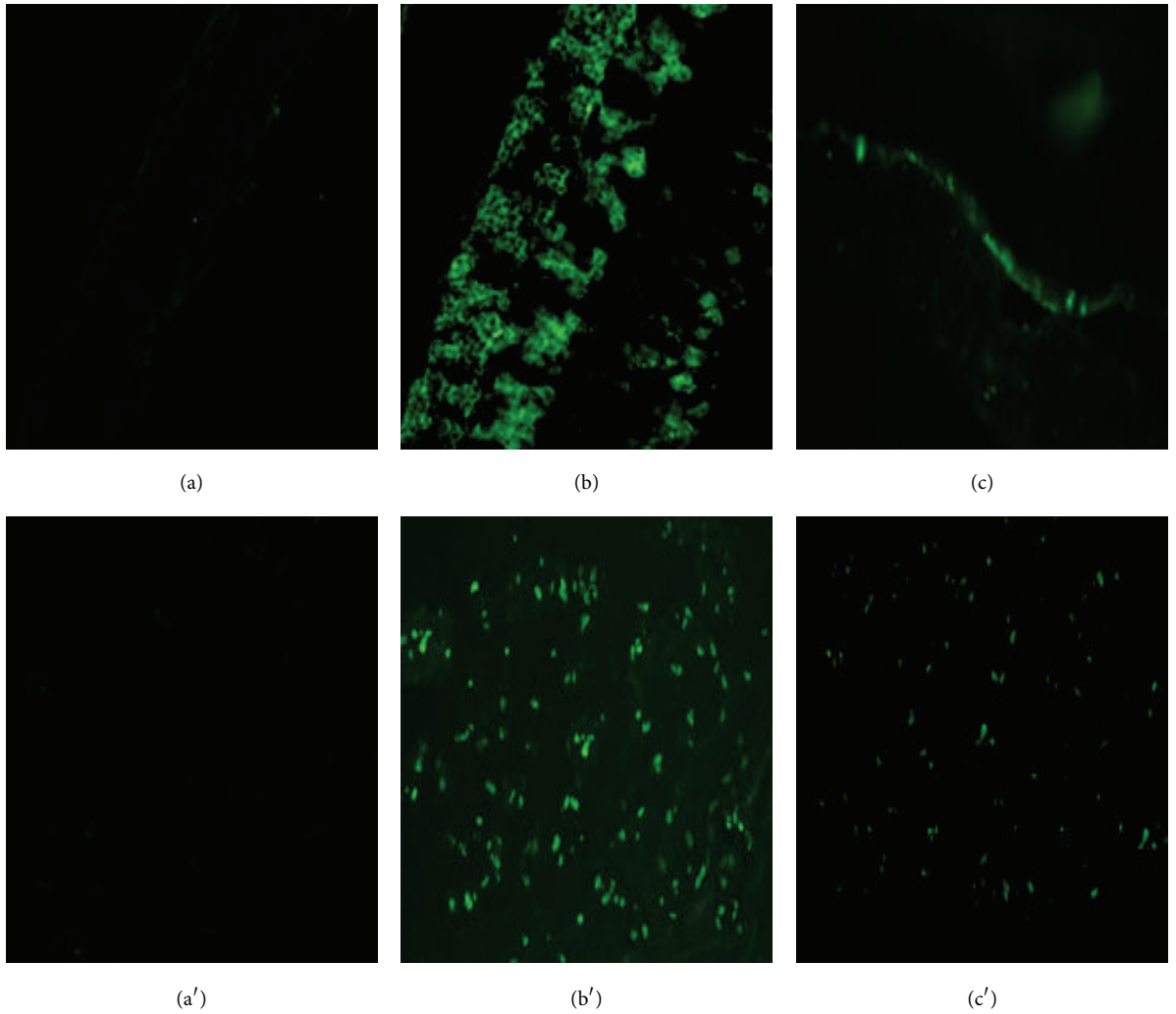


Figure 4: Inducible nitric oxide synthase (iNOS) immunolocalization in the sections of the optic nerve head (a/b/c) and in the retina (a′/b′/c′). ((a) and (a′)) Young rat without iNOS immunolocalization (6 months, negative control). ((b) and (b′)) Aged rat fed normally with high iNOS expression in cells. ((c) and (c′)) Aged rat after oral supplementation of α-lipoic acid (ALA) and superoxide dismutase (SOD) with reduced immunofluorescence, indicating lower iNOS expression.

value in pathological conditions related to the overproduction of radicals with oxidative capacity. Furthermore, its reduced form, dihydrolipoic acid, reacts by neutralizing such radicals as superoxide, hydroxyl radicals, hypochlorous acid, peroxides, and singlet oxygen [35, 36].

Some Authors have observed that ALA reduces the markers of apoptosis and oxidative stress more significantly than SOD. But these effects seem particularly relevant when treatment is administered concomitantly with ALA and SOD, probably due to the presence of synergistic mechanisms of action. Mainly, SOD acts in the extracellular environment, and conversely ALA also acts in intracellular level [4, 14, 18, 26].

5. Conclusion

In summary, this study appears to show that, during the RGC ageing process, axons in the optic nerve cells and astrocytes are subjected to an array of degenerative damage, due mainly to an increase in oxidative stress levels. The damage would no longer be countered by the normal antioxidant defence systems which, as is known, decline with age. Oral administration of ALA and SOD would provide a number of significant benefits against the retinal degeneration mediated by oxidative stress. This is due to a reduction in DNA fragmentation and iNOS expression caused by antioxidants' ability to counteract the high levels of free radicals typical of ageing. This would thus improve the condition of treated eye nerve tissue compared with control cases. Furthermore, the lower LPO assay obtained from the antioxidant combination would improve membrane integrity, thus protecting retinal cells and optic nerve fibres from tissue death.

Ultimately, further research will be required in order to gain a better understanding of the different ways in which antioxidants act, not only in advanced stages of life but also in the course of pathological events with inflammatory, tumoral, traumatic, and dystrophic causes.

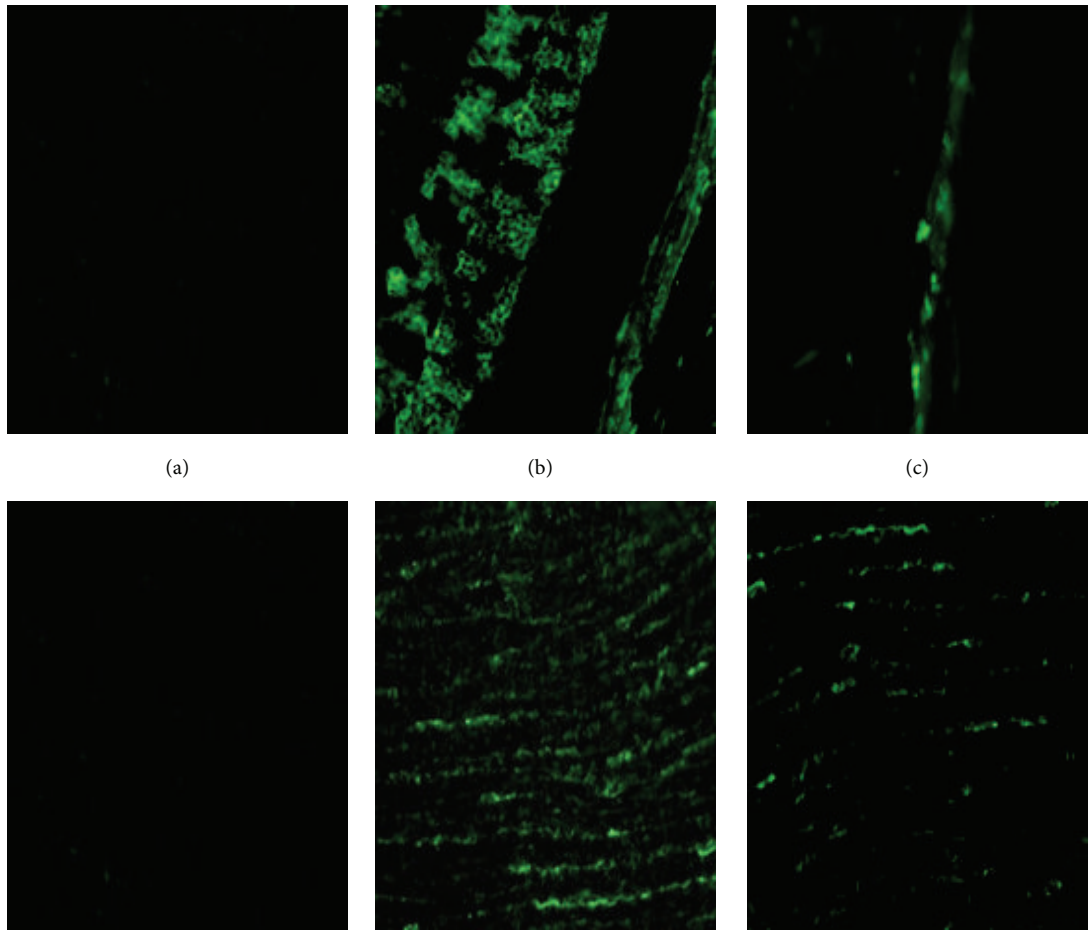


Figure 5: Caspase-3 immunolocalization in the sections of the optic nerve head (a/b/c) and in the retina (a′/b′/c′). ((a) and (a′)) Young rat without caspase-3 immunolocalization (6 months, negative control). ((b) and (b′)) Aged rat fed normally with high caspase-3 expression. ((c) and (c′)) Aged rat after oral supplementation of α-lipoic acid (ALA) and superoxide dismutase (SOD) with reduced immunofluorescence, indicating lower caspase-3 expression.

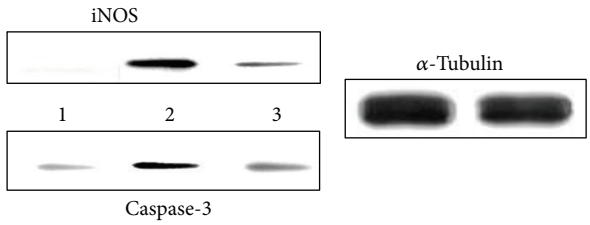


Figure 6: Western blot analysis of inducible nitric oxide synthase (iNOS) and caspase-3. (1) Young rats. (2) Untreated aged rats: increased presence of iNOS and caspase-3 bands. (3) Treated aged rats: iNOS and caspase-3 bands are less frequent.

Abbreviations

ALA: α-Lipoic acid
SOD: Superoxide dismutase
LPO: Lipid peroxidation
iNOS: Inducible nitric oxide synthase
ROS: Reactive oxygen species
RNS: Reactive nitrogen species
RGCs: Retinal ganglion cells
NO: Nitric oxide
NOS: Nitric oxide synthase
IOP: Intraocular pressure
FR: Rree radicals
CuZnSOD or SOD1: Copper- and zinc-containing SOD
MnSOD or SOD2: Manganese SOD
ECSOD or SOD3: Extracellular SOD
mitSOD: Mitochondrial MnSOD
GSH: Glutathione
TUNEL: Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling
RI: Methyl-2-phenylindole
MDA: Malondialdehyde
SDS-PAGE: SDS-polyacrylamide gel electrophoresis

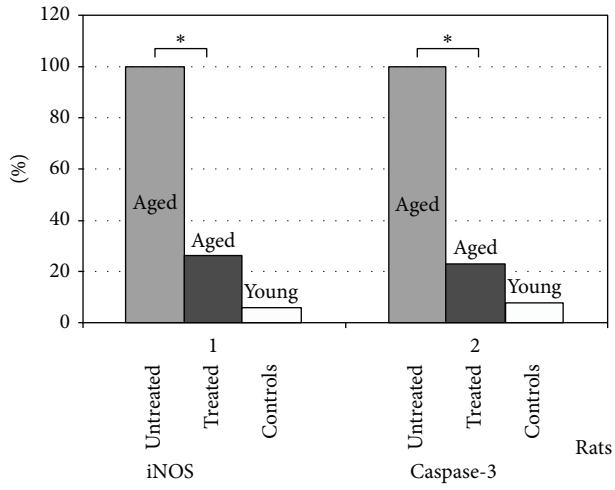


Figure 7: Quantization of the inducible nitric oxide synthase (iNOS) and caspase-3 bands obtained by western blot. The iNOS and caspase-3 were less present in aged rats pretreated with antioxidants indicating that the reduction in cell death was due to lower activation of tissue damage and apoptotic death.

APS: Ammonium persulfate
TEMED: Tetramethylethylenediamine
ECL: Electrochemiluminescence
PTPs: Permeability of transition pores.

Disclosure

This submission has not been published anywhere previously and it is not simultaneously being considered for any other publication.

Conflict of Interests

The authors have no proprietary interest in any materials or methods described within this paper.

Authors' Contribution

Marcella Nebbioso wrote the paper. Gianfranco Scarsella performed the study. Aloisa Librando and Nicola Pescosolido designed the research.

Acknowledgments

This research was supported by the following grant: Alfa Wassermann S.p.A., Bologna, Italy.

References

[1] H. Sies, "Strategies of antioxidant defense," *European Journal of Biochemistry*, vol. 215, no. 2, pp. 213–219, 1993.

[2] B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, UK, 4th edition, 2007.

[3] B. Liu and A. H. Neufeld, "Nitric oxide synthase-2 in human optic nerve head astrocytes induced by elevated pressure in vitro," *Archives of Ophthalmology*, vol. 119, no. 2, pp. 240–245, 2001.

[4] M. Nebbioso, G. Scarsella, M. Tafani, and N. Pescosolido, "Mechanisms of ocular neuroprotection by antioxidant molecules in animal models," *Journal of Biological Regulators and Homeostatic Agents*, vol. 27, no. 1, pp. 197–209, 2013.

[5] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *The Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.

[6] M. Nebbioso, A. Pascarella, C. Cavallotti, and N. Pescosolido, "Monoamine oxidase enzymes and oxidative stress in the rat optic nerve: age-related changes," *International Journal of Experimental Pathology*, vol. 93, no. 6, pp. 401–405, 2012.

[7] H. Nohl and D. Hegner, "Do mitochondria produce oxygen radicals *in vivo*?" *European Journal of Biochemistry*, vol. 82, no. 2, pp. 563–567, 1978.

[8] S. G. Jarrett, H. Lin, B. F. Godley, and M. E. Boulton, "Mitochondrial DNA damage and its potential role in retinal degeneration," *Progress in Retinal and Eye Research*, vol. 27, no. 6, pp. 596–607, 2008.

[9] N. L. Barnett, D. V. Pow, and N. D. Bull, "Differential perturbation of neuronal and glial glutamate transport systems in retinal ischaemia," *Neurochemistry International*, vol. 39, no. 4, pp. 291–299, 2001.

[10] B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford, UK, 2nd edition, 1989.

[11] A. K. Holley, V. Bakthavatchalu, J. M. Velez-Roman, and D. K. St. Clair, "Manganese superoxide dismutase: guardian of the powerhouse," *International Journal of Molecular Sciences*, vol. 12, no. 10, pp. 7114–7162, 2011.

[12] Y. Li, T.-T. Huang, E. J. Carlson et al., "Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase," *Nature Genetics*, vol. 11, no. 4, pp. 376–381, 1995.

[13] J. R. Vanfleteren, "Oxidative stress and ageing in *Caenorhabditis elegans*," *Biochemical Journal*, vol. 292, no. 2, pp. 605–608, 1993.

[14] I. Reveillaud, A. Niedzwiecki, K. G. Bensch, and J. E. Fleming, "Expression of bovine superoxide dismutase in *Drosophila melanogaster* augments resistance of oxidative stress," *Molecular and Cellular Biology*, vol. 11, no. 2, pp. 632–640, 1991.

[15] L. J. Reed, B. G. de Busk, I. C. Gunsalus, and C. S. Hornberger Jr., "Crystalline α-lipoic acid: a catalytic agent associated with pyruvate dehydrogenase," *Science*, vol. 114, no. 2952, pp. 93–94, 1951.

[16] L. Packer, K. Kraemer, and G. Rimbach, "Molecular aspects of lipoic acid in the prevention of diabetes complications," *Nutrition*, vol. 17, no. 10, pp. 888–895, 2001.

[17] S. D. Wollin and P. J. H. Jones, "α-lipoic acid and cardiovascular disease," *Journal of Nutrition*, vol. 133, no. 11, pp. 3327–3330, 2003.

[18] M. Nebbioso, F. Pranno, and N. Pescosolido, "Lipoic acid in animal models and clinical use in diabetic retinopathy," *Expert Opinion on Pharmacotherapy*, vol. 14, no. 13, pp. 1829–1838, 2013.

[19] E. Busse, G. Zimmer, B. Schopohl, and B. Kornhuber, "Influence of α-lipoic acid on intracellular glutathione *in vitro* and *in vivo*," *Arzneimittel-Forschung/Drug Research*, vol. 42, no. 6, pp. 829–831, 1992.

[20] J. Lykkesfeldt and B. N. Ames, "Ascorbic acid recycling in rat hepatocytes as measurement of antioxidant capacity: decline with age," *Methods in Enzymology*, vol. 299, pp. 83–88, 1998.

[21] P. Kubes, "Inducible nitric oxide synthase: a little bit of good in all of us," *Gut*, vol. 47, no. 1, pp. 6–9, 2000.

[22] G. S. Wu and N. A. Rao, "Photoreceptor mitochondrial oxidative stress in uveitis," *Expert Review of Ophthalmology*, vol. 3, no. 3, pp. 299–310, 2008.

[23] J. R. Steinert, T. Chernova, and I. D. Forsythe, "Nitric oxide signaling in brain function, dysfunction, and dementia," *Neuroscientist*, vol. 16, no. 4, pp. 435–452, 2010.

[24] G. S. Wu, M. Jiang, Y.-H. Liu, Y. Nagaoka, and N. A. Rao, "Phenotype of transgenic mice overexpressed with inducible nitric oxide synthase in the retina," *PLoS ONE*, vol. 7, no. 8, Article ID e43089, 2012.

[25] Z.-Q. Xiao, L. Moragoda, R. Jaszewski, J. A. Hatfield, S. E. G. Fligiel, and A. P. N. Majumdar, "Aging is associated with increased proliferation and decreased apoptosis in the colonic mucosa," *Mechanisms of Ageing and Development*, vol. 122, no. 15, pp. 1849–1864, 2001.

[26] F. Bertolotto and A. Massone, "Combination of alpha lipoic acid and superoxide dismutase leads to physiological and symptomatic improvements in diabetic neuropathy," *Drugs in R and D*, vol. 12, no. 1, pp. 29–34, 2012.

[27] M. Mather and H. Rottenberg, "Aging enhances the activation of the permeability transition pore in mitochondria," *Biochemical and Biophysical Research Communications*, vol. 273, no. 2, pp. 603–608, 2000.

[28] G. Paradies, G. Petrosillo, V. Paradies, and F. M. Ruggiero, "Oxidative stress, mitochondrial bioenergetics, and cardiolipin in aging," *Free Radical Biology and Medicine*, vol. 48, no. 10, pp. 1286–1295, 2010.

[29] J. E. Kokoszka, P. Coskun, L. A. Esposito, and D. C. Wallace, "Increased mitochondrial oxidative stress in the Sod2 (+/−) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 5, pp. 2278–2283, 2001.

[30] R. Corniola, Y. Zou, D. Leu, J. R. Fike, and T.-T. Huang, "Paradoxical Relationship between Mn Superoxide Dismutase Deficiency and Radiation-Induced Cognitive Defects," *PLoS ONE*, vol. 7, no. 11, Article ID e49367, 2012.

[31] S. L. H. Ong, H. Vohra, Y. Zhang, M. Sutton, and J. A. Whitworth, "The effect of alpha-lipoic acid on mitochondrial superoxide and glucocorticoid-induced hypertension," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 517045, 9 pages, 2013.

[32] T.-T. Huang, M. Naeemuddin, S. Elchuri et al., "Genetic modifiers of the phenotype of mice deficient in mitochondrial superoxide dismutase," *Human Molecular Genetics*, vol. 15, no. 7, pp. 1187–1194, 2006.

[33] S. Shimohama, H. Tanino, and S. Fujimoto, "Differential expression of rat brain caspase family proteins during development and aging," *Biochemical and Biophysical Research Communications*, vol. 289, no. 5, pp. 1063–1066, 2001.

[34] N. Pescosolido, A. Barbato, A. Pascarella, R. Giannotti, M. Genzano, and M. Nebbioso, "Role of protease-inhibitors in ocular diseases," *Molecules*, vol. 19, no. 12, pp. 20557–20569, 2014.

[35] P. Atukeren, S. Aydin, E. Uslu, M. K. Gumustas, and U. Cakatay, "Redox homeostasis of albumin in relation to alpha-lipoic acid and dihydrolipoic acid," *Oxidative Medicine and Cellular Longevity*, vol. 3, no. 3, pp. 206–213, 2010.

[36] J. Liu, "The effects and mechanisms of mitochondrial nutrient α-lipoic acid on improving age-associated mitochondrial and cognitive dysfunction: an overview," *Neurochemical Research*, vol. 33, no. 1, pp. 194–203, 2008.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 709845, 13 pages
http://dx.doi.org/10.1155/2015/709845

Research Article

Lico A Enhances Nrf2-Mediated Defense Mechanisms against *t*-BHP-Induced Oxidative Stress and Cell Death via Akt and ERK Activation in RAW 264.7 Cells

Hongming Lv,[1,2] Hua Ren,[1] Lidong Wang,[1] Wei Chen,[2] and Xinxin Ci[1]

[1] *Institute of Translational Medicine, Department of Ophthalmology, The First Hospital, Jilin University, Changchun 130001, China*
[2] *Key Laboratory of Zoonosis Research, Ministry of Education, College of Animal Science and Veterinary Medicine, Jilin University, 5333 Xi'an Road, Changchun 130062, China*

Correspondence should be addressed to Xinxin Ci; cixinxin@gmail.com

Received 22 December 2014; Revised 28 January 2015; Accepted 7 February 2015

Academic Editor: Zhenquan Jia

Licochalcone A (Lico A) exhibits various biological properties, including anti-inflammatory and antioxidant activities. In this study, we investigated the antioxidative potential and mechanisms of Lico A against *tert*-butyl hydroperoxide- (*t*-BHP-) induced oxidative damage in RAW 264.7 cells. Our results indicated that Lico A significantly inhibited *t*-BHP-induced cytotoxicity, apoptosis, and reactive oxygen species (ROS) generation and reduced glutathione (GSH) depletion but increased the glutamate-cysteine ligase modifier (GCLM) subunit and the glutamate-cysteine ligase catalytic (GCLC) subunit genes expression. Additionally, Lico A dramatically upregulated the antioxidant enzyme heme oxygenase 1 (HO-1) and nuclear factor erythroid 2-related factor 2 (Nrf2), which were associated with inducing Nrf2 nuclear translocation, decreasing Keap1 protein expression and increasing antioxidant response element (ARE) promoter activity. Lico A also obviously induced the activation of serine/threonine kinase (Akt) and extracellular signal-regulated kinase (ERK), but PI3K/Akt and ERK inhibitors treatment displayed clearly decreased levels of LicoA-induced Nrf2 nuclear translocation and HO-1 expression, respectively. Furthermore, Lico A treatment markedly attenuated *t*-BHP-induced oxidative damage, which was reduced by treatment with PI3K/Akt, ERK, and HO-1 inhibitors. Therefore, Lico A might have a protective role against *t*-BHP-induced cytotoxicity by modulating HO-1 and by scavenging ROS via the activation of the PI3K/Akt and ERK/Nrf2 signaling pathways.

1. Introduction

Various severe imbalances in the systems involved in generating and scavenging reactive oxygen species (ROS) could induce oxidative damage [1]. Moreover, oxidative stress plays a vital role in the mechanisms of various diseases, including aging, cancer, and inflammation [2]. Under oxidative stress conditions, various cells, including macrophages, have developed their own defensive mechanisms to counteract ROS generation via the induction of intracellular phase II enzymes, such as heme oxygenase-1 [3]. Increasing evidence has shown that natural products such as dietary phytochemicals exert protective effects by not only scavenging ROS but also inducing the de novo expression of antioxidant genes [4].

Reduced glutathione (GSH) is not only the most abundant thiol antioxidant in cells, but also a key intracellular antioxidant in mammals, which involves in scavenging free radicals, maintaining redox status, and inhibiting cell apoptosis and is regulated by glutamate-cysteine ligase (GCL) [5–7]. GCL, a rate-limiting enzyme of GSH biosynthesis, is a heterodimer which consisted of the glutamate-cysteine ligase modifier (GCLM) subunit and the glutamate-cysteine ligase catalytic (GCLC) subunit [8, 9]. In addition, heme oxygenase-1 (HO-1), which is a significant antioxidant gene, plays a crucial role in maintaining the cellular redox homeostasis against oxidative stress [10]. Previous reports have shown that HO-1 could catalyze the oxidative degradation of heme to biliverdin, which is then reduced to produce bilirubin, which

is a potent antioxidant [11]. Importantly, GCLC, GCLM, and HO-1 genes expression are regulated by nuclear factor-erythroid 2 related factor 2 (Nrf2). Nrf2 plays a critical role in protecting cells against oxidative stress via regulating antioxidant responses [12, 13]. Under normal conditions, the inactive form of Nrf2 is bound to Keap1 (Kelch-like ECH-associated protein 1, also known as a repressor of Nrf2) in the cytoplasm. Under stress conditions, Nrf2 dissociates from Keap1, translocates into the nucleus, and binds to the antioxidant response element (ARE), which results in the expression of several antioxidant and detoxification genes, including HO-1 [14]. Interestingly, the mechanism by which Nrf2 is released from the Keap1-Nrf2 complex remains to be demonstrated. However, recently it is reported that several signal transduction pathways, which are protein kinase C (PKC) [15, 16], phosphatidylinositol 3-kinase (PI3K) [17], and mitogen-activated protein kinase (MAPK) pathways [18], may also play a significant role in involving in the regulation of Nrf2 nuclear translocation. Flavonoids, which are naturally occurring bioactive compounds, are extensively distributed in natural products such as vegetables, fruits, and many medicinal plants [19]. Flavonoids, particularly flavonols, possess a range of pharmaceutical activities, such as anti-inflammatory, antioxidant, hepatoprotective, antiviral, and anticarcinogenic activities [20, 21]. Licochalcone A (Lico A), which is one of the primary flavonoids isolated from the root of the Xinjiang licorice *Glycyrrhiza inflate* [22], has various biological activities, including anti-inflammatory, antioxidant, antitumorigenic, and antimicrobial activities [23, 24]. However, the cytoprotective effect of Lico A against oxidative stress damage has not yet been demonstrated in RAW 264.7 cells. Accordingly, we investigated the cytoprotective effect of Lico A and the mechanism related to oxidative stress in *tert*-butyl hydroperoxide- (*t*-BHP-) induced RAW 264.7 cells.

2. Materials and Methods

2.1. Reagents and Chemical. Licochalcone A (Lico A), purity >98%, used in the experiments was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *tert*-butyl hydroperoxide (*t*-BHP), dimethyl sulfoxide (DMSO), U0126, SB203580, SP600125, and LY294002 (specific inhibitors of the ERK1/2, p38, JNK1/2, and PI3K/Akt, resp.) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin and streptomycin, fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen-Gibco (Grand Island, NY). Antibodies against Nrf2, HO-1, Keap1, Akt, phospho-Akt, phospho-extracellular signal-regulated kinase (ERK), ERK, phospho-c-Jun NH2-terminal kinase (JNK), JNK, phospho-p38, p38, Lamin B, and β-actin were purchased from Cell Signaling (Boston, MA, USA) or Abcam (Cambridge, MA, USA). The horseradish peroxidase- (HRP-) conjugated anti-rabbit or anti-mouse IgG were obtained from Proteintech (Boston, MA, USA). Tin protoporphyrin IX (SnPP IX, HO-1 inhibitor) was purchased from Calbiochem (La Jolla, CA, USA). The control siRNA and Nrf2 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Faststart Universal SYBR Green Master was purchased from Roche (Basel, Switzerland). PrimeScript RT-PCR kit was purchased from Takara (Dalian, China). In addition, GSH test kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Cell Culture and Cell Treatment. The RAW 264.7 mouse macrophage cell line, purchased from the China Cell Line Bank (Beijing China), was grown in DMEM medium supplemented with 3 mM glutamine, 10% foetal bovine serum (FBS), and 100 U/mL of penicillin and 100 U/mL of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. In all experiments, cells were allowed to acclimate for 24 h before any treatments.

2.3. Cell Viability Assay. According to the manufacturer's instructions, cell viability was evaluated by MTT assay. RAW 264.7 cells were seeded in 96-well plates at the concentration of 3×10^4 cells/well. After 24 h, cells were treated with *t*-BHP only, Lico A and *t*-BHP, or Lico A, *t*-BHP, and selective inhibitors at an indicated concentration and time. Then, the cells were added with MTT (5 mg/mL) and incubated for another 4 h, the supernatant was removed, and DMSO was added to each well to lyse the cells. The absorbance of MTT was measured at 570 nm.

2.4. Quantification of Apoptotic and Necrosis Cells. RAW 264.7 cells were seeded into 12-well plates (5×10^5 cells/well) for 24 h incubation, and then were exposed to various concentrations of Lico A for 18 h and subjected to *t*-BHP (10 μM) for additional 3 h. Subsequently, cells were washed twice with ice-cold PBS, collected, and centrifuged at 1500 rpm/min for 5 min, 4°C. Next, cells were subjected to Hoechst 33342 and propidium iodide staining and the percentage of apoptosis and necrosis were determined using flow cytometry (LSR II Flow Cytometer; BD Biosciences, San Jose, CA, USA).

2.5. Detection of Intracellular ROS Levels. To measure intracellular ROS production, RAW 264.7 cells were grown in 24-well plates (1×10^5 cells/well) for 24 h incubation and then recovered in serum-free DMEM for 6 h; the cells were then preincubated with various concentrations of Lico A for 18 h. Next, the cells were stained with 50 μM of DCFH-DA for 1 h and subsequently incubated with *t*-BHP (10 μM) for 30 min to induce the ROS generation. DCF fluorescence intensities were measured in a multidetection reader (Bio-Tek Instruments Inc.) at an excitation and emission wavelength of 485 nm and 535 nm, respectively.

2.6. Measurement of Intracellular GSH Levels. To measure intracellular reduced glutathione (GSH) levels, RAW 264.7 cells were grown in 6-well plates (1×10^6 cells/well) for 24 h incubation, and then the cells were exposed to various concentrations of Lico A for 18 h and subsequently subjected to *t*-BHP (10 μM) for 3 h. According to the manufacturer's instructions, the level of intracellular of GSH was quantified using a commercially available GSH test kit (Nanjing

Jiancheng Bioengineering Institute, Nanjing, China). The absorbance was measured at 405 nm using a microplate reader (Bio-Tek Instruments Inc.).

2.7. Total RNA Extraction and qPCR. Total RNA from cells was isolated using Trizol reagent according to the procedure described by the manufacturer. After the concentration of RNA was determined by spectrophotometer, 1 μg of RNA was transformed into cDNA using Prime-Script RT-PCR kit (Takara). The following PCR primer sequences (forward and reverse, resp.) were used: GCLC: 5′-ACG GCT GCT ACG ACA ACG GCC CTC-3′ and 5′-ACC CAG CGG TGC AAA CTC CGC GC-3′; GCLM: 5′-TCC TCT CGA AGA GGG CGT GTC CAG-3′ and 5′-AGG GAG G GA AGG AAG GGA GGG AG-3′; β-actin: 5′-TCT GTG TGG ATT GTG GCT CTA-3′ and 5′-CTG CTT GCT GAT CCA CAT CTG-3′. PCR reactions were carried out using the SYBR green working solution and quantitatively measured with the Applied Biosystems 7300 real-time PCR system and software (Applied Biosystems, Carlsbad, CA, USA). The following thermal cycler parameters were used: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. Gene expression changes were calculated by the comparative Ct method and the values were analyzed by normalizing with β-actin mRNA expression.

2.8. Western Blot Analysis. The RAW 264.7 mouse macrophage cell line (1×10^6 cells/well in 6-well plate) was washed twice with ice-cold PBS, collected, and centrifuged at 6000 rpm/min for 5 min, 4°C. Then, the cells were lysed in a RIPA with protease and phosphatase inhibitors for 30 min. The protein concentrations were measured using a BCA protein assay kit (Beyotime, China). An equal amount of protein (40 μg) for each sample was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and then electrophoretically transferred onto a polyvinylidene difluoride membranes (PVDF), which was purchased from Bio-Rad (Hercules, CA). The membrane was blocked with blocking solution (5% (w/v) nonfat dry milk) for 2 h and followed by an overnight incubation at 4°C with specific primary antibody, including Keap1, Nrf2, HO-1, p-Akt/Akt, p-JNK/JNK, p-ERK/ERK, p-p38/p38, Lamin B, and β-actin. Next day, after thoroughly washing with TBST for three times, the membrane was incubated for an additional 2 h with a peroxidase conjugated secondary antibody at room temperature and followed by ECL detection (Millipore corporation, Billerca, MA, USA). β-actin and Lamin B were used as loading controls for whole, cytosolic, and nuclear cell proteins, respectively. Band intensities were quantified by using ImageJ gel analysis software. The experiments were repeated three times for each experimental condition.

2.9. Preparation of Nuclear and Cytosolic Fractions. The nuclear and the cytoplasmic extracts were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL, USA), in accordance with the manufacturer's instructions. All steps were carried out on ice or at 4°C unless stated otherwise.

2.10. Nrf2-siRNA Transfection. For Nrf2-siRNA transfection, RAW 264.7 cells were grown in 6-well plates (2×10^5 cells/well) until the confluence of cells reached approximately 50%. Then, Nrf2-siRNA or Nrf2-negative control siRNA was transiently transfected into the cells using siRNA transfection reagent lipofectamine 2000 in accordance with the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA). After 6 h, the transfected cells were treated with Lico A for 18 h and followed by lysis buffer for Western blot analysis.

2.11. ARE Promoter Activity. RAW 264.7 cells were grown in 24-well plates (2×10^5 cells/well) until the confluence of cells reached approximately 50%. According to the manufacturer's protocol of Invitrogen (Carlsbad, CA, USA), pRL-TK and pGL-ARE plasmids were transfected into cells using Lipofectamine 2000. After Lico A (3.7 μM) treatment for different periods, we used a dual-luciferase reporter assay system (Dual-Glo Luciferase Assay System) for detecting and analyzing ARE-driven promoter activity.

2.12. Statistical Analysis. All results were expressed as means ± SEM of three independent experiments. Differences between mean values of normally distributed data were analyzed using two-tailed Student's t-test. Statistical significance was accepted when $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Lico A Protected against t-BHP-Induced Cytotoxicity and Reduced Apoptosis Percentage in RAW 264.7 Cells. t-BHP is commonly used to induce oxidative stress in biological systems. The viability of RAW 264.7 cells decreased in a dose-dependent manner after 24 h of incubation with t-BHP. However, a significant difference between the control and t-BHP-stimulated group was observed only at 10 μM t-BHP (Figure 1(a)). Therefore, we chose 10 μM t-BHP as the treatment dose to induce obvious oxidative injury. Moreover, the RAW 264.7 cells were pretreated with various concentrations of Lico A (1.85, 3.7 and 7.4 μM) for 18 h and subsequently exposed to t-BHP (10 μM) for 3 h to investigate the protective effects of Lico A on these cells. Our results suggested that Lico A significantly protected these cells against t-BHP-induced oxidative cytotoxicity (Figure 1(b)). Furthermore, t-BHP also markedly induced cell death through increasing the percentage of apoptosis and necrosis in total cells, and Lico A effectively decreased t-BHP-induced cell apoptosis at concentrations of 1.85, 3.7, and 7.4 μM, whereas Lico A could not attenuate t-BHP-induced necrosis cells (Figure 1(c)).

3.2. Lico A Inhibited t-BHP-Induced ROS Production and GSH Depletion and Enhanced GCLC and GCLM Expression in RAW 264.7 Cells. Due to t-BHP elevating ROS generation to induce cell oxidative damage, we investigated that Lico A protected against t-BHP-induced oxidative injury

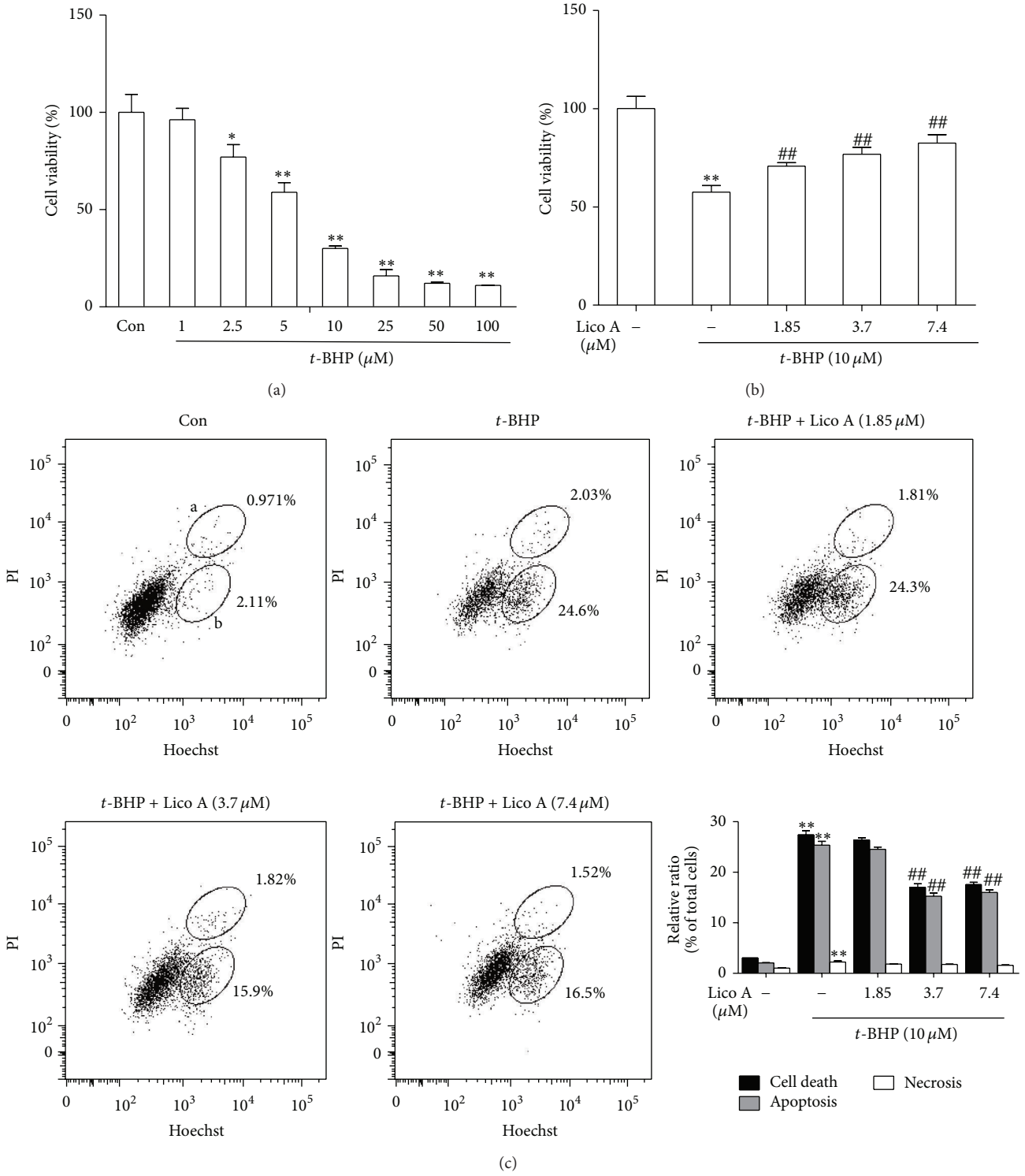


Figure 1: Effects of Lico A on *t*-BHP-induced RAW 264.7 cell cytotoxicity, apoptosis, and necrosis. (a) RAW 264.7 cells were treated by 0, 1, 2.5, 10, 25, 50, and 100 μM *t*-BHP for 24 h. (b) Cells were pretreated with Lico A (1.85, 3.7 and 7.4 μM) for 18 h, subsequently exposed to *t*-BHP (10 μM) for 3 h. Cell viability after *t*-BHP exposure was measured by MTT assay. (c) Cells were exposed to various concentrations of Lico A for 18 h and subsequently subjected to *t*-BHP (10 μM) for 3 h. The percentage of cell apoptosis and necrosis were determined using flow cytometry. (a and b) represent necrosis and apoptosis, respectively. All results were expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the *t*-BHP group.

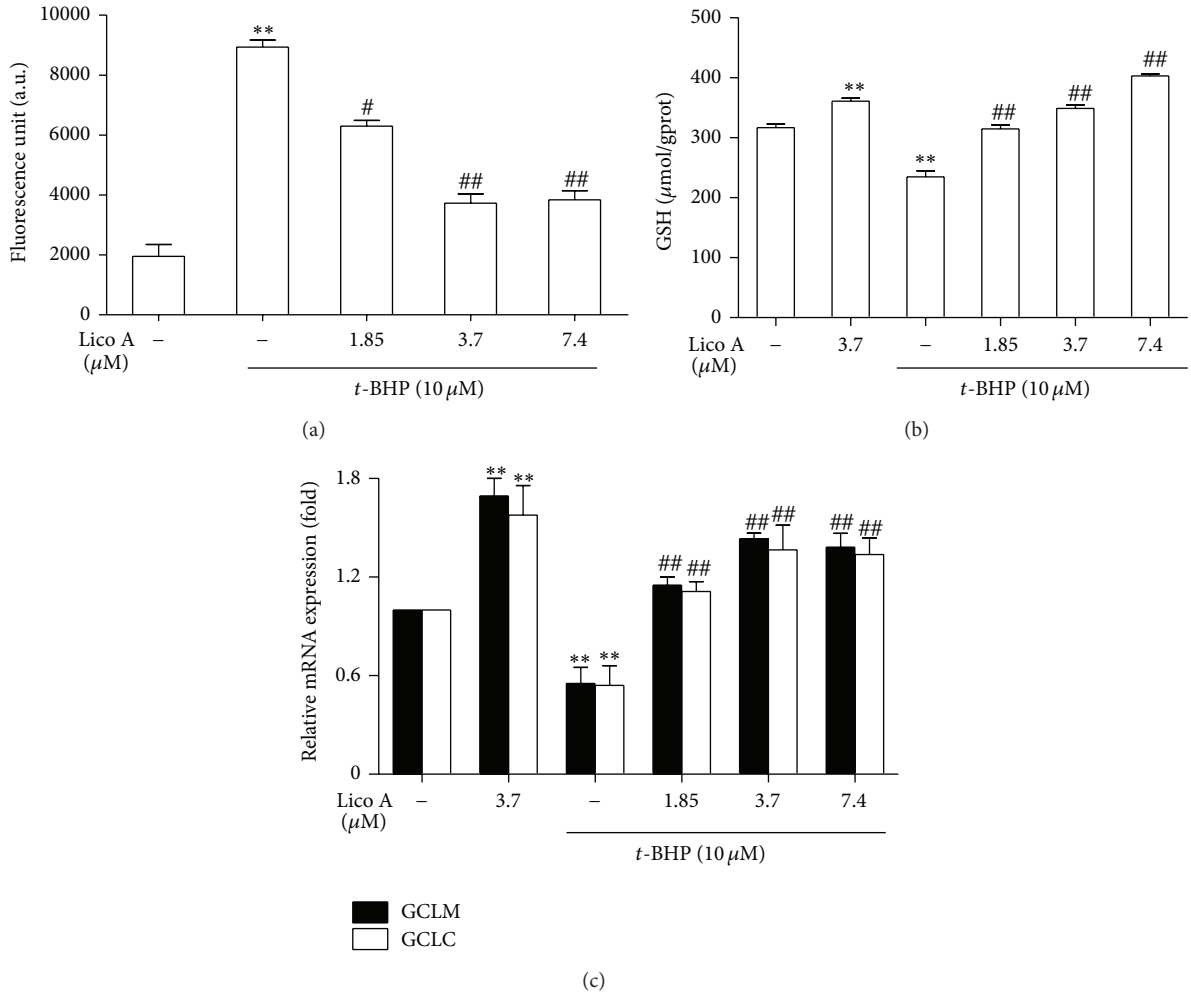


Figure 2: Effects of Lico A on *t*-BHP-induced ROS generation, GSH levels, and GCLM and GCLC genes expression in RAW 264.7 cells. RAW 264.7 cells were pretreated with or without Lico A for 18 h and then were exposed to *t*-BHP for additional 3 h. (a) Effect of Lico A on *t*-BHP-induced ROS generation in RAW 264.7 cells. The ROS generation was determined in accordance with the Experimental Section. (b) Effect of Lico A on *t*-BHP-induced GSH depletion was determined using a commercial GSH test kit. (c) Effects of Lico A on GCLM and GCLC genes expression. Total RNA was extracted from RAW 264.7 cells and genes expression was quantified using real-time PCR. All results were expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the *t*-BHP group.

via inhibiting intracellular ROS production in RAW 264.7 cells. In this study, *t*-BHP treatment markedly increased ROS production, which was inhibited by Lico A treatment (Figure 2(a)). In addition, GSH is recognized to be a vital antioxidant, which protects against *t*-BHP-induced oxidative injury. In fact, GCLC and GCLM are closely associated with the expression of GSH level. Hence, we examined GSH contents as well as GCLC and GCLM expression in *t*-BHP-exposed cells pretreated with or without Lico A. Our results showed that *t*-BHP treatment considerably enhanced GSH depletion, whereas Lico A treatment significantly decreased the depletion of GSH *t*-BHP-induced and increased the expression of GCLC and GCLM (Figures 2(b) and 2(c)).

3.3. Lico A Upregulated HO-1 Protein Expression in RAW 264.7 Cells. Because HO-1 is an essential component of the cellular defense against oxidative stress, we examined whether Lico A-induced HO-1 expression increased the resistance of RAW 264.7 cells to oxidative injury. RAW 264.7 cells were treated with Lico A for 18 h to determine the most effective concentration for increasing HO-1 protein expression (Figures 3(a) and 3(b)). The cells were treated with Lico A (3.7 μM) for different periods to determine the optimal exposure period for enhancing HO-1 protein expression (Figures 3(c) and 3(d)). Our results showed that exposure to 3.7 μM Lico A for 18 h dramatically upregulated HO-1 protein expression in RAW 264.7 cells.

3.4. Lico A Enhanced Nrf2 Protein Expression and ARE Activation and Increased Keap1 Degradation in RAW 264.7 Cells. Nrf2 regulates the antioxidant responses via transcriptionally activating the HO-1 gene expression. In addition,

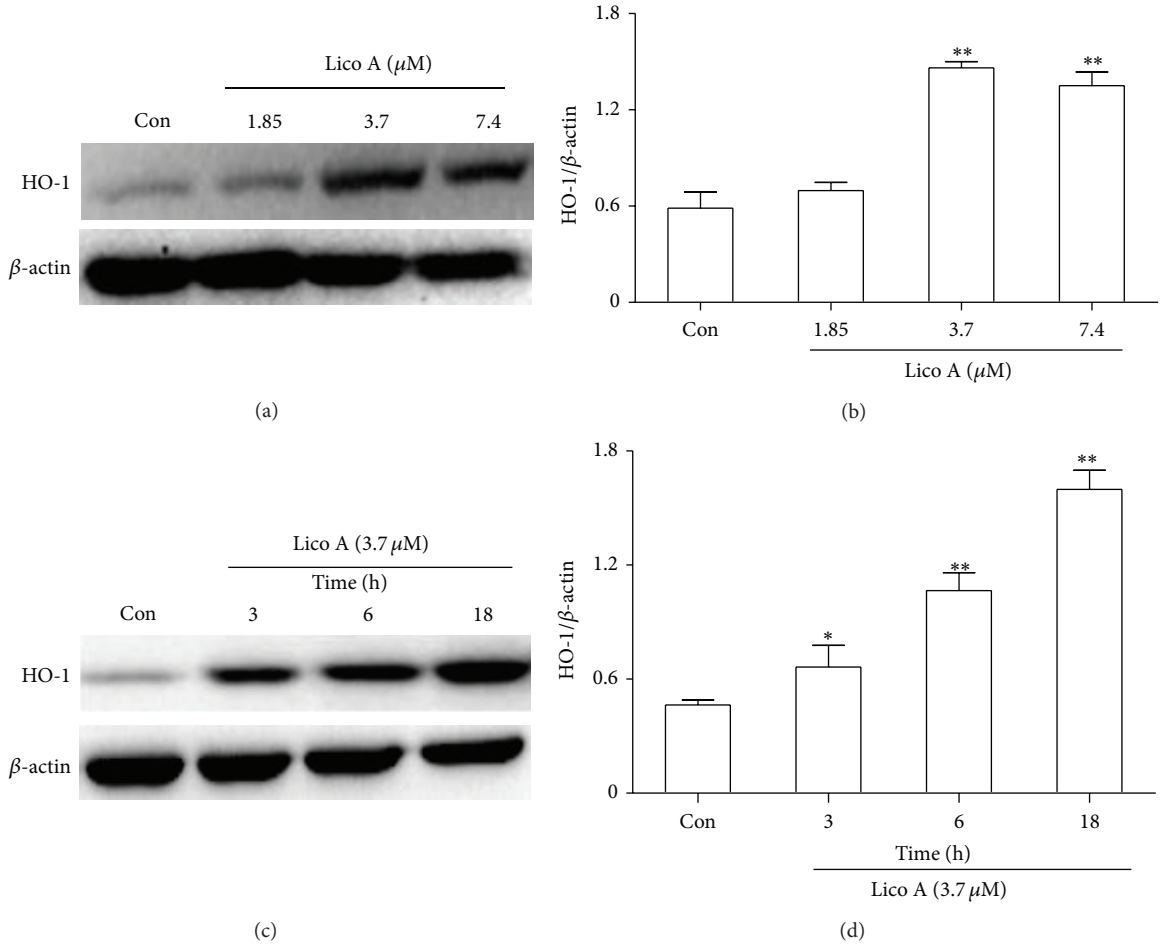


Figure 3: Effects of Lico A on HO-1 protein expression in RAW 264.7 cells. (a) Cells were treated with increasing doses of Lico A (1.85, 3.7, and 7.4 μM) for 18 h, and (c) cells were treated with Lico A (3.7 μM) indicated time periods. Protein expression of HO-1 was determined by Western blot analysis. (b and d) Quantification of HO-1 protein expression was performed by densitometric analysis and β-actin acted as an internal control. All results were expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group.

Keap1 negatively regulates Nrf2 through inhibiting the Nrf2 activation. Consequently, we examined whether Lico A could induce Nrf2 activation and Keap1 degradation in association with HO-1 upregulation. RAW 264.7 cells were treated with Lico A (1.85, 3.7 and 7.4 μM) for 18 h, and then total protein was extracted from the cells for Western blot analysis. The results showed that 3.7 μM Lico A significantly increased the total protein expression of Nrf2 and the degradation of Keap1 (Figures 4(a) and 4(b)). Thus, we furthermore examined whether 3.7 μM Lico A could lead to a decrease in the cytoplasmic levels and a concomitant increase in the nuclear levels of Nrf2 in a time-dependent manner (Figures 4(c) and 4(d)). In addition, due to the increased Nrf2 expression in the nucleus is required for ARE activation, the ARE-luciferase plasmid was transiently transfected into the cells and then were exposed to Lico A, as well as changes in luciferase activity were used as a measure of ARE activation. The results of this assay suggested that Lico A also markedly increased ARE-driven luciferase activity in a time-dependent manner (Figure 4(e)).

3.5. Lico A Increased Nrf2-Mediated HO-1 Protein Expression in RAW 264.7 Cells. Several previous reports showed that Nrf2 is essential for HO-1 regulation. Accordingly, we attempted to investigate whether the upregulation of HO-1 expression is mediated by Nrf2. The role of Nrf2 in Lico A-induced HO-1 expression was confirmed using siRNA to knockdown Nrf2. Control or Nrf2 siRNA was transiently transfected into RAW 264.7 cells, and then Nrf2 and HO-1 protein expression was measured by Western blot analysis. The data demonstrated that Nrf2 siRNA markedly inhibited total Nrf2 and HO-1 protein expression to a similar extent when compared with the negative control (Figures 5(a) and 5(b)). Additionally, to further investigate whether Lico A-induced HO-1 protein expression is mediated by Nrf2, we measured HO-1 expression after Nrf2 siRNA transfection. Our studies indicated that Lico A-increased HO-1 protein expression significantly decreased in Nrf2 siRNA-transfected cells, whereas the same amount of nonspecific control siRNA did not affect HO-1 expression in RAW 264.7 cells (Figures 5(c) and 5(d)). These results further provided a support that

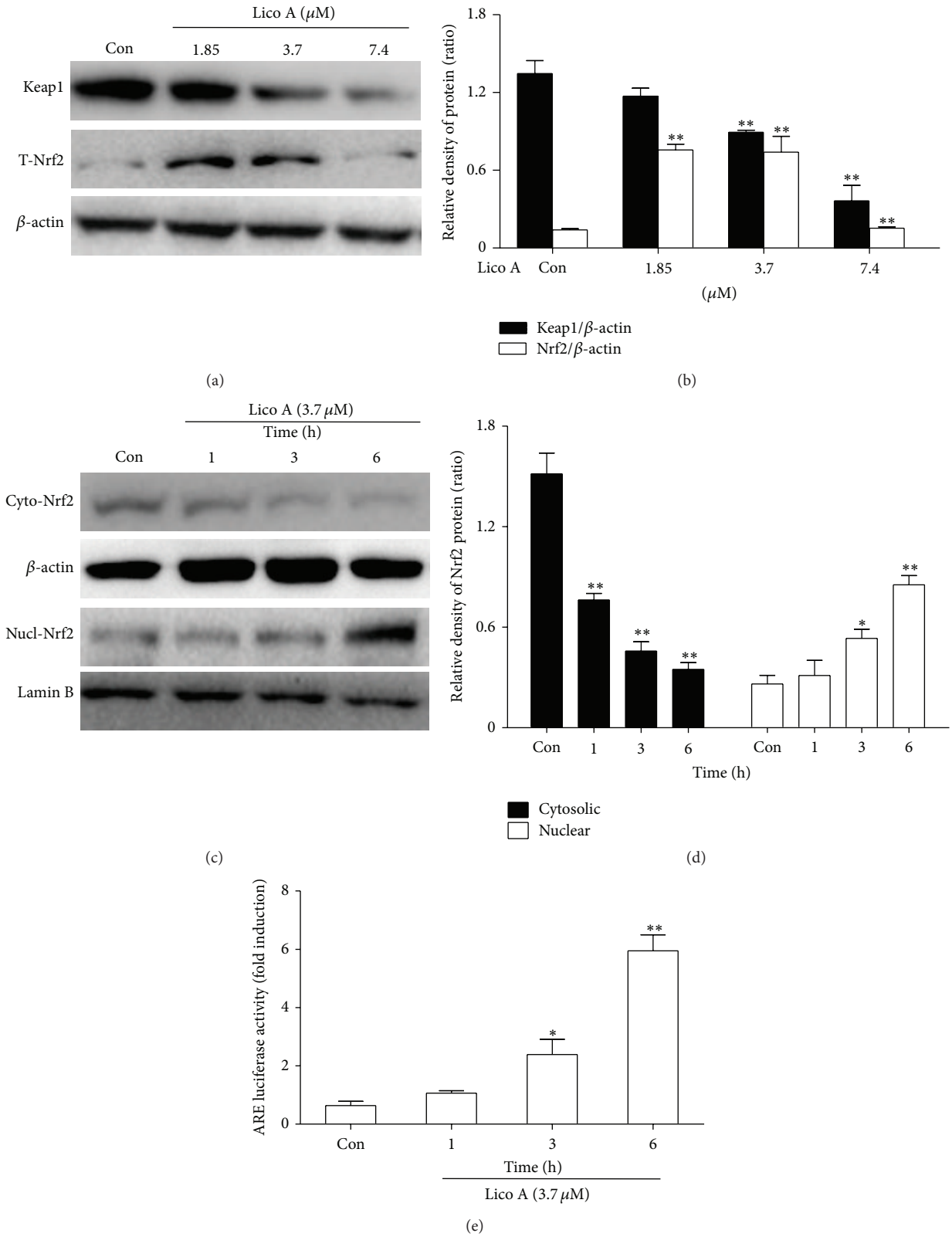


Figure 4: Effects of Lico A on the Keap1/Nrf2/ARE signaling pathway in RAW264.7 cells. (a) Cells were treated with different concentration of Lico A (1.85, 3.7 and 7.4 μM) for 18 h, and the total protein were measured by Western blot analysis. (c) Cells were treated with Lico A (3.7 μM) indicated time periods, and the nuclear and cytoplasmic levels of Nrf2 were examined by Western blot analysis. (b and d) The relative density of protein was performed by densitometric analysis; β-actin and Lamin B acted as an internal control, respectively. (e) The luciferase plasmids pGL-ARE and pRL-TK was transiently transfected into cells for 24 h and subsequently exposed to 3.7 μM Lico A for the indicated periods. ARE luciferase activity was detected by a dual-luciferase reporter assay system. All results were expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group.

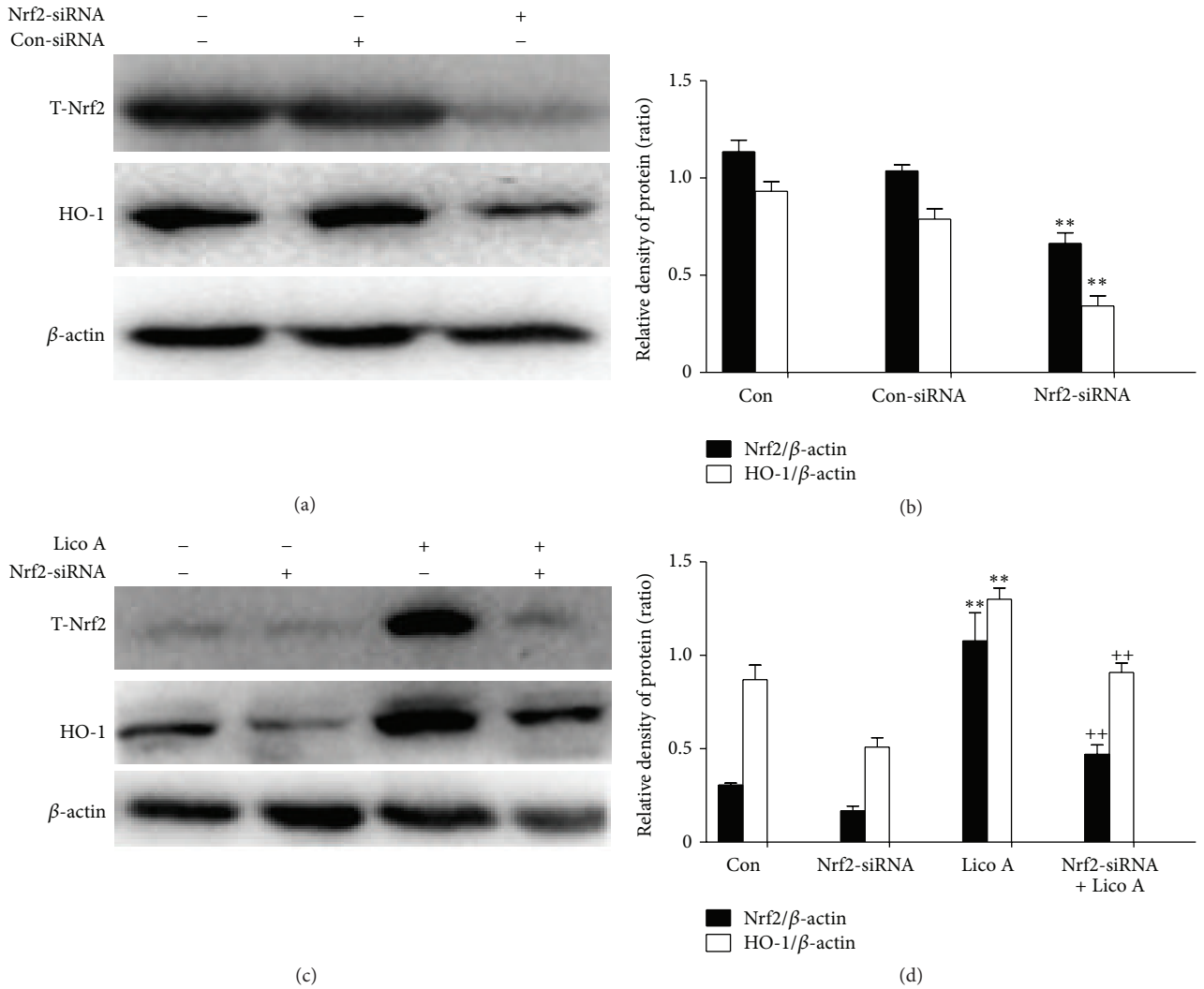


Figure 5: Effects of Nrf2-siRNA transfection on Lico A-induced HO-1 protein expression in RAW 264.7 cells. (a) Nrf2-siRNA or Nrf2-negative control siRNA was transfected into cells for 24 h and was collected; proteins were detected by Western blot analysis. (c) Nrf2 mediates Lico A-induced HO-1 protein expression. Nrf2-siRNA or Nrf2-negative control siRNA were transfected into cells for 6 h and then were treated with Lico A (3.7 μM) for 18 h. (b and d) The relative density of protein was performed by densitometric analysis and β-actin acted as an internal control. All results were expressed as means ± SEM of three independent experiments. $^{**}P < 0.01$ versus the control group; $^{++}P < 0.01$ versus the Lico A group.

the upregulation of HO-1 expression is mediated primarily through the transcriptional activator Nrf2.

3.6. Lico A Activated the PI3K/Akt and MAPK Pathways in RAW 264.7 Cells. Recent reports have suggested that several signal transduction pathways, such as the PI3K and MAPK pathways are involved in the regulation of Nrf2 nuclear translocation. ERK and c-Jun N-terminal kinase (JNK) positively regulate the Nrf2 pathway whereas p38 MAPK exerts both positive and negative regulations [8, 25]. They all belong to members of MAPK family. Therefore, we examined the activation of PI3K/Akt and MAPK pathway in RAW 264.7 cells. RAW 264.7 cells were exposed to Lico A (1.85, 3.7, and 7.4 μM) for 18 h, and then total protein was extracted from these cells for Western blot analysis using specific antibodies. The results indicated that 3.7 μM Lico A clearly increased Akt and ERK phosphorylation in RAW 264.7 cells. In contrast, the phosphorylation of p38 and JNK was not activated at these three concentrations of Lico A (Figure 6).

3.7. Lico A Modulated HO-1 Expression and Nrf2 Nuclear Translocation via Akt and ERK Activation in RAW 264.7 Cells. To further determine the upstream signaling pathway involved in Lico A-mediated Nrf2 activation and HO-1 induction, we investigated the effects of LY294002 and U0126, which are specific inhibitors of the PI3K/Akt and ERK pathways, respectively, on Nrf2 nuclear translocation and HO-1 protein expression. RAW 264.7 cells were treated with either LY294002 (20 μM) or U0126 (10 μM) for 6 h and then exposed to Lico A (3.7 μM) for 18 h. We found

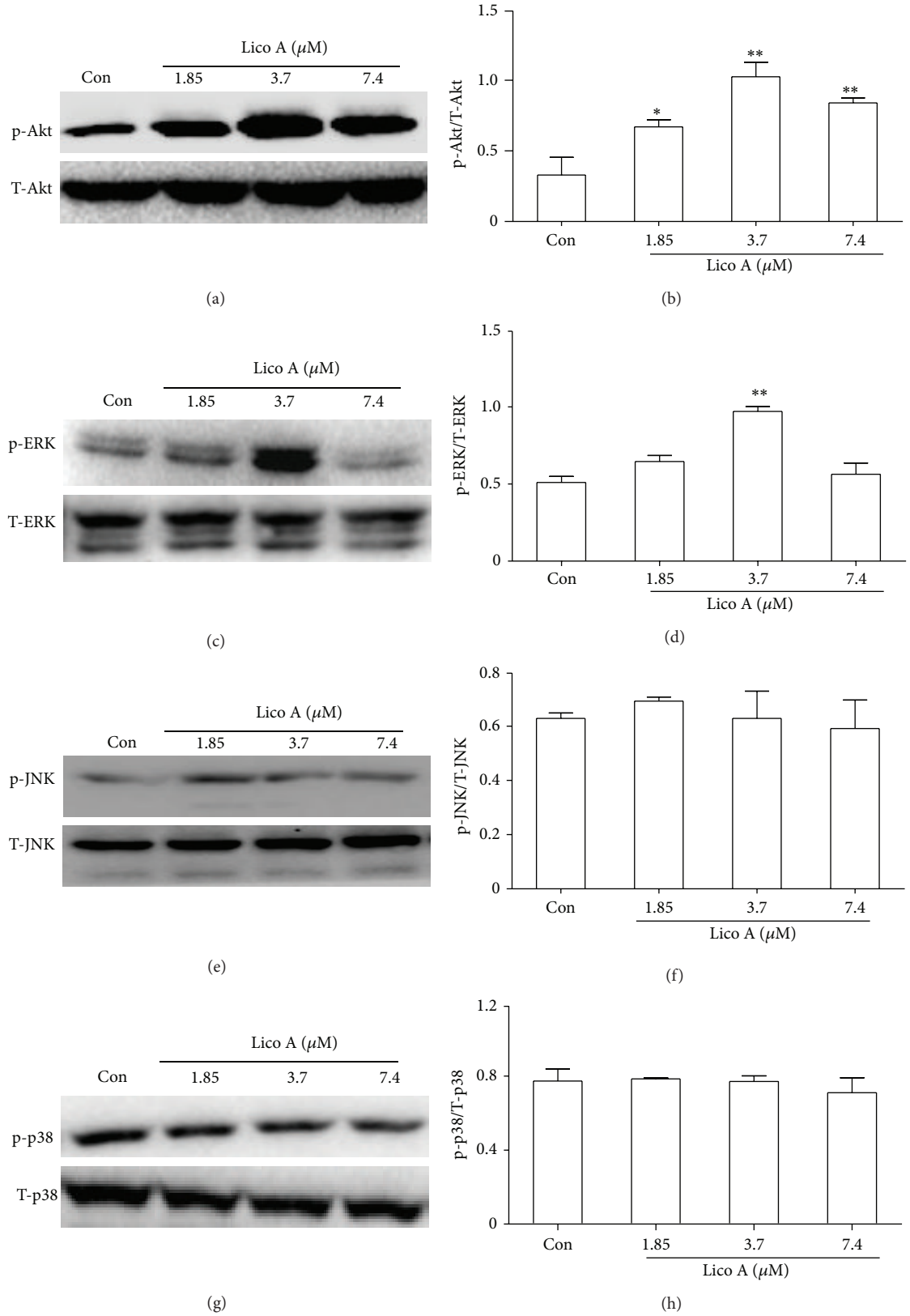


Figure 6: Effects of Lico A on the activation of the PI3K/Akt and MAPK pathways in RAW 264.7 cells. Cells were treated with increasing doses of Lico A (1.85, 3.7 and 7.4 μM) for 18 h, and whole cell lysates were prepared and detected by Western blot analysis for phosphorylated and total Akt, ERK, JNK, and p38 protein expression. (b, d, f, and h) Quantification of induction of PI3K/Akt and MAPKs phosphorylation were performed by densitometric analysis and its unphosphorylated forms acted as an internal control, respectively. All results were expressed as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group.

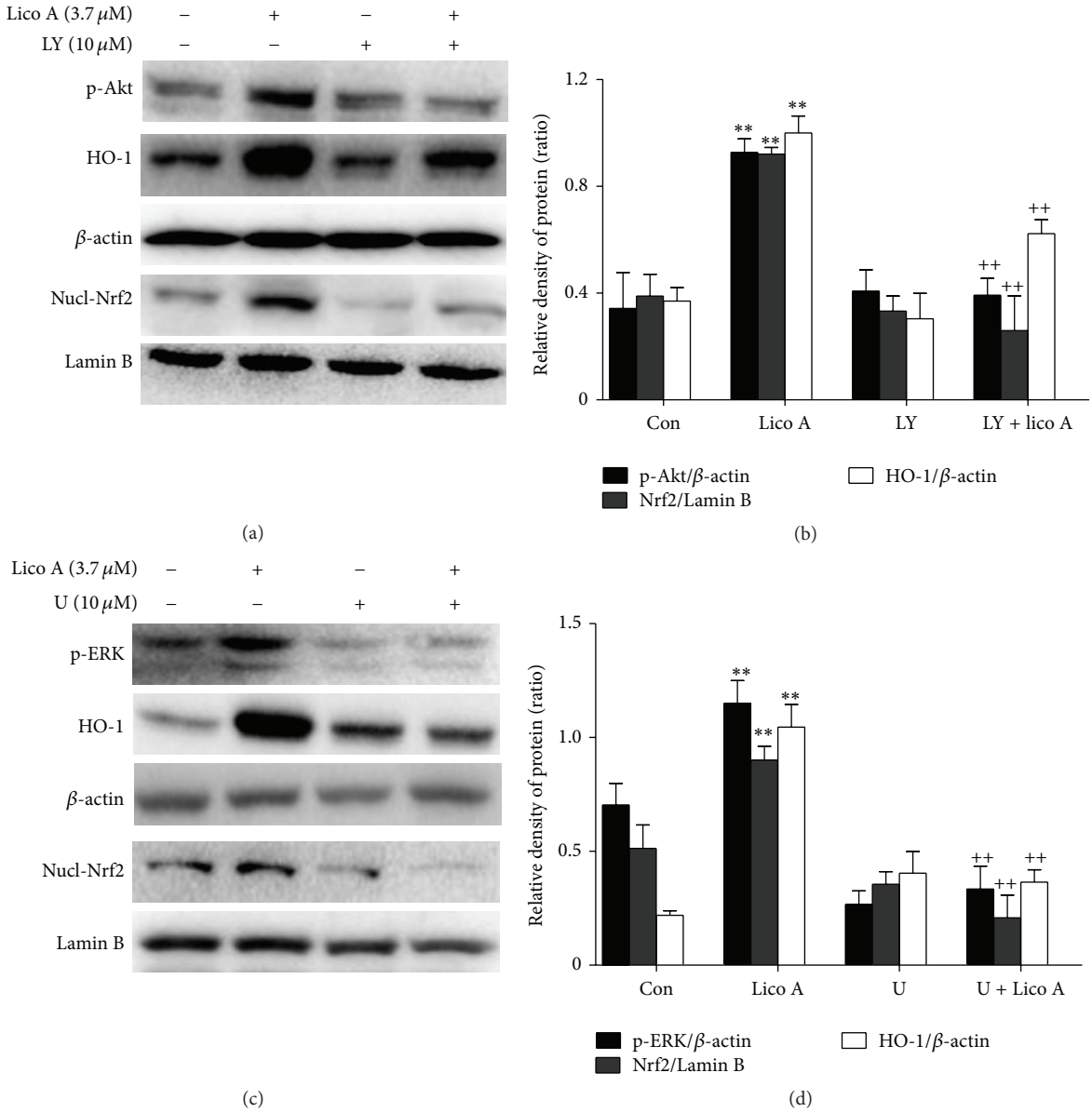


Figure 7: Effects of Lico A-induced Akt and ERK activation on HO-1 expression and Nrf2 nuclear translocation. Cells were pretreated with LY294002 (10 μM) and U0126 (10 μM) for 6 h and then were exposed to Lico A (3.7 μM) for 18 h. The whole cells lysates were examined by Western blot analysis with anti-HO-1 and anti-β-actin antibodies, and nuclear extracts were subjected to Western blot analysis with anti-Nrf2 and anti-Lamin B antibodies. (b and d) The relative density of protein was performed by densitometric analysis; β-actin and Lamin B acted as an internal control, respectively. All results were expressed as means ± SEM of three independent experiments. $^{**}P < 0.01$ versus the control group; $^{++}P < 0.01$ versus the Lico A group.

that Lico A-mediated Nrf2 activation and HO-1 induction were dramatically inhibited by PI3K/Akt and ERK kinase inhibitors, respectively (Figure 7). These investigations suggested that Lico A modulated Nrf2 nuclear translocation and HO-1 expression via the activation of PI3K/Akt and ERK signaling in RAW 264.7 cells.

3.8. Lico A Alleviated Cellular Injury by Upregulating Nrf2 and HO-1 via Akt and ERK Activation in RAW 264.7 Cells. Based on the above outcomes, we hypothesized that the protective effects of Lico A against *t*-BHP-induced oxidative stress result from the induction of antioxidant genes, such as HO-1 and its transcription factor Nrf2. Furthermore, we hypothesized that the PI3K/Akt and MAPK pathways, which are upstream signaling pathways, are involved in Lico A-mediated Nrf2 activation and HO-1 induction. Thus, RAW 264.7 cells were pretreated with LY294002 (PI3K/Akt inhibitor, 20 μM), U0126 (ERK inhibitor, 10 μM), SB203580 (p38 inhibitor, 10 μM), SP600125 (JNK inhibitor, 40 μM), or SnPP (HO-1 inhibitor, 40 μM) for 6 h, respectively, and then treated with Lico A (3.7 μM) for 18 h. Next, the cells were

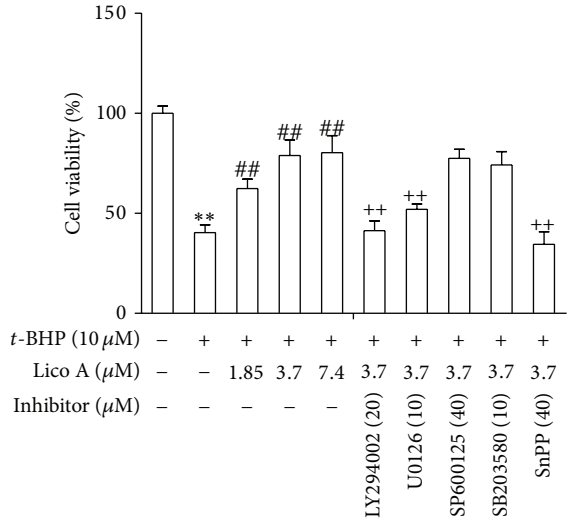


Figure 8: Effects of Lico A-induced Akt and ERK pathway activation on *t*-BHP-induced cytotoxicity in RAW 264.7 cells. Cells were pretreated with or without LY294002 (20 μM), U0126 (10 μM), SB203580 (10 μM), SP600125 (40 μM), and SnPP (40 μM), respectively, for 6 h and treated with Lico A for 18 h. Then, cells were exposed to *t*-BHP (10 μM) for 10 h. Cell viability after *t*-BHP exposure was measured by MTT assay. All results were expressed as means ± SEM of three independent experiments. $^{**}P < 0.01$ versus the control group; $^{\#\#}P < 0.01$ versus the *t*-BHP group; $^{++}P < 0.01$ versus the Lico A (3.7 μM) + *t*-BHP group.

exposed to 10 μM *t*-BHP for 10 h to determine cell viability. Our results suggested that Lico A pretreatment significantly increased cell viability compared with that of *t*-BHP-treated cells. In contrast, this effect was partially inhibited in the presence of ERK, PI3K/Akt, and HO-1 inhibitors, whereas JNK and p38 inhibitors could not inhibit this effect (Figure 8). This result showed that Lico A induced HO-1 expression via the activation of Akt, ERK, and Keap1/Nrf2/ARE signaling pathways in RAW 264.7 cells.

4. Discussion and Conclusion

Excessive exposure to reactive oxygen species (ROS) causes oxidative stress. Additionally, excessive ROS production inflicts damage on essential cellular macromolecules including lipids, proteins, and DNA; this damage results in several human diseases, such as inflammation, cancer, atherosclerosis, rheumatoid arthritis, and neurodegenerative diseases [26]. Therefore, ROS clearance and oxidative stress inhibition may play essential roles in preventing numerous diseases. Various natural products, particularly flavonoids, possess multiple cytoprotective effects through free radical scavenging activity [27]. Lico A, which is a flavonoid, possesses radical-scavenging and antioxidant effects [24]. However, the mechanism underlying the biological effects of Lico A in RAW 264.7 cells remains unclear. Our present study aimed to investigate whether Lico A has the ability to induce GSH, GCLC, and GCLM enhancement, to modulate HO-1 induction and Nrf2 nuclear translocation and to protect against *t*-BHP-induced oxidative damage and cell death via ERK and Akt activation in RAW 264.7 cells.

Previous reports suggested that *t*-BHP exposure could not only lead to cell death via inducing apoptosis but also result in oxidative stress via increasing ROS formation. ROS are associated with cell damage and with chronic disease development [28, 29]. Additionally, it is reported that the overproduction of GCL, which comprised of GCLC and GCLM, enhances total GSH contents and protects against H_2O_2-induced cell death in human granulose tumour cells [30]. On the other hand, GSH, which is a nonenzymatic antioxidant, cofactor, or coenzyme, plays an essential role in directly involving in the production and clearance of ROS [31]. For example, previous reports showed that antcin C reduced the depletion of GSH levels in the *t*-BHP-exposed HepG2 cells and mice liver tissues [32]. Hence, the aim of this study was to evaluate the ability of the antioxidant Lico A to reduce oxidant-induced cellular damage in RAW 264.7 cells. Our experimental results showed that *t*-BHP-induced RAW 264.7 cells displayed significantly decreased cell viability in a dose-dependent manner; the viability of RAW 264.7 cells treated with 10 μM *t*-BHP decreased up to 36.72% compared with the control group (Figure 1(a)). However, Lico A pretreatment markedly enhanced cell viability and inhibited *t*-BHP-induced cell apoptosis (Figures 1(b) and 1(c)). Furthermore, increased ROS production and decreased GSH levels are closely associated with apoptosis [33]. In present study, we found that Lico A significantly reduced ROS formation and GSH depletion as well as enhanced GCLC and GCLM genes expression in the *t*-BHP-stimulated RAW 264.7 cells (Figure 2).

Increasing evidence suggests that the cytoprotective properties of antioxidants are generally related to their ability to induce antioxidative enzymes, such as HO-1. HO-1, which is an enzyme that is essential for heme degradation, has been recognized as an important cellular defense mechanism against various stresses, including oxidative stress [11, 34]. Our results indicated that different concentrations and exposure periods of Lico A treatment markedly increased HO-1 induction in RAW 264.7 cells (Figure 3). Furthermore, Keap1/Nrf2/ARE signaling plays a crucial role in protecting cells against endogenous and exogenous stresses [35]. The transcriptional activation of Nrf2 is dependent on the rate of nuclear translocation, followed by the disassociation of Nrf2 from cytoplasmic Keap1, which leads to the induction of some cytoprotective proteins, including HO-1, GCLC, and GCLM [36, 37]. In this study, we found that Lico A treatment increased Nrf2 protein expression and decreased Keap1 protein expression in total cell lysates (Figures 4(a) and 4(b)). In addition, Lico A treatment markedly promoted the nuclear translocation of Nrf2, which was directly proportional to the decrease in Nrf2 in the cytoplasm (Figures 4(c) and 4(d)). Nrf2 is released from Keap1 and is translocated to the nucleus, where Nrf2 binds to ARE in the promoter region of its target genes, thereby inducing many cytoprotective genes and antioxidative enzymes [38]. As shown in Figure 4(e), Lico A treatment significantly enhanced ARE luciferase activity in a time-dependent manner, which indicated the inducing ability of Lico A on various ARE-regulated genes, such as HO-1.

However, transient transfection with Nrf2 siRNA partially abolished Lico A-induced HO-1 expression (Figure 5), which suggested that Lico A induced HO-1 protein expression via the Keap1/Nrf2/ARE signaling pathway in RAW 264.7 cells.

Many previous reports have suggested that the PI3K/Akt and MAPK pathways play a key role in regulating HO-1 expression and Nrf2-dependent transcription [39, 40]. The aim of the present experiment was to investigate a possible role, Lico A-induced HO-1 expression which was the activation of the PI3K/Akt and MAPK pathways. Our results showed that Lico A induced HO-1 expression via activating the PI3K/Akt and ERK pathways, whereas JNK and p38 MAPK signaling molecules did not affect HO-1 expression (Figure 6). Moreover, MAPK and PI3K/Akt are candidate upstream signaling pathways for Nrf2-related HO-1 regulation in RAW 264.7 cells [41]. This study used specific inhibitors of the PI3K/Akt and ERK pathways to further investigate whether the activation of the PI3K/Akt and ERK MAPK signaling pathways was a required event for HO-1 expression and Nrf2 nuclear translocation. As shown in Figure 7, the addition of U0126 and LY294002 significantly abolished Lico A-induced HO-1 protein expression and Nrf2 nuclear translocation. These results indicated that the PI3K/Akt and ERK pathways are important for Lico A-induced HO-1 expression and Nrf2 nuclear translocation. Furthermore, cell viability decreased significantly when Lico A treatment was combined with PI3K/Akt, ERK, and HO-1 inhibitors in *t*-BHP-induced RAW 264.7 cells (Figure 8). These results suggested that Lico A induced HO-1 expression via the activation of Akt, ERK, and Keap1/Nrf2/ARE signaling in RAW 264.7 cells.

In conclusion, the present study demonstrated that Lico A could protect RAW 264.7 cells via the suppression of *t*-BHP-induced oxidative damage, apoptosis, ROS production, and GSH depletion as well as the enhancement of GCLC and GCLM genes expression. Furthermore, Lico A could not only induce Nrf2 nuclear translocation, which is upstream of Lico A-induced antioxidant gene expression, but also activate Akt and ERK phosphorylation. Moreover, the PI3K/Akt and ERK pathways are associated with Lico A-induced Nrf2 nuclear translocation, HO-1 expression, and cytoprotection. This study provides biological evidence supporting the application of Lico A in the treatment of oxidative stress-induced disorders.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content of this paper.

Authors' Contribution

Hongming Lv and Hua Ren contributed equally to this work.

Acknowledgment

This work supported by a grant from the Natural Science Foundation of Jilin (no. 20150520050JH).

References

[1] 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.

[2] L. H. Breimer, "Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage," *Molecular Carcinogenesis*, vol. 3, no. 4, pp. 188–197, 1990.

[3] Q. Ma, "Role of Nrf2 in oxidative stress and toxicity," *Annual Review of Pharmacology and Toxicology*, vol. 53, pp. 401–426, 2013.

[4] Y.-J. Surh, J. K. Kundu, H.-K. Na, and J.-S. Lee, "Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals," *The Journal of Nutrition*, vol. 135, no. 12, supplement, pp. 2993S–3001S, 2005.

[5] M. Kobayashi and M. Yamamoto, "Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation," *Antioxidants and Redox Signaling*, vol. 7, no. 3-4, pp. 385–394, 2005.

[6] L. D. Deleve and N. Kaplowitz, "Importance and regulation of hepatic glutathione," *Seminars in Liver Disease*, vol. 10, no. 4, pp. 251–266, 1990.

[7] D. P. Jones, "Redox potential of GSH/GSSG couple: assay and biological significance," *Methods in Enzymology*, vol. 348, article 11, pp. 93–112, 2002.

[8] V. R. Biljak, L. Rumora, I. Čepelak et al., "Glutathione cycle in stable chronic obstructive pulmonary disease," *Cell Biochemistry and Function*, vol. 28, no. 6, pp. 448–453, 2010.

[9] N. S. Gould, E. Min, S. Gauthier, R. J. Martin, and B. J. Day, "Lung glutathione adaptive responses to cigarette smoke exposure," *Respiratory Research*, vol. 12, article 133, 2011.

[10] X. Shi and B. Zhou, "The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos," *Toxicological Sciences*, vol. 115, no. 2, pp. 391–400, 2010.

[11] C. D. Ferris, S. R. Jaffrey, A. Sawa et al., "Haem oxygenase-1 prevents cell death by regulating cellular iron," *Nature Cell Biology*, vol. 1, no. 3, pp. 152–157, 1999.

[12] M. Mani, S. Khaghani, T. G. Mohammadi et al., "Activation of Nrf2-antioxidant response element mediated glutamate cysteine ligase expression in hepatoma cell line by homocysteine," *Hepatitis Monthly*, vol. 13, no. 5, Article ID e8394, p. e8394, 2013.

[13] L. Baird and A. T. Dinkova-Kostova, "The cytoprotective role of the Keap1-Nrf2 pathway," *Archives of Toxicology*, vol. 85, no. 4, pp. 241–272, 2011.

[14] T. W. Kensler, N. Wakabayashi, and S. Biswal, "Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway," *Annual Review of Pharmacology and Toxicology*, vol. 47, pp. 89–116, 2007.

[15] H.-C. Huang, T. Nguyen, and C. B. Pickett, "Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 23, pp. 12475–12480, 2000.

[16] S. Numazawa, M. Ishikawa, A. Yoshida, S. Tanaka, and T. Yoshida, "Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress," *American Journal of Physiology—Cell Physiology*, vol. 285, no. 2, pp. C334–C342, 2003.

[17] K. Nakaso, H. Yano, Y. Fukuhara, T. Takeshima, K. Wada-Isoe, and K. Nakashima, "PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in

human neuroblastoma cells," *FEBS Letters*, vol. 546, no. 2-3, pp. 181–184, 2003.

[18] A.-N. T. Kong, E. Owuor, R. Yu et al., "Induction of xenobiotic enzymes by the map kinase pathway and the antioxidant or electrophile response element (ARE/EpRE)," *Drug Metabolism Reviews*, vol. 33, no. 3-4, pp. 255–271, 2001.

[19] R. A. Dixon and C. L. Steele, "Flavonoids and isoflavonoids—a gold mine for metabolic engineering," *Trends in Plant Science*, vol. 4, no. 10, pp. 394–400, 1999.

[20] M. G. L. Hertog, E. J. M. Feskens, P. C. H. Hollman, M. B. Katan, and D. Kromhout, "Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly study," *The Lancet*, vol. 342, no. 8878, pp. 1007–1011, 1993.

[21] R. J. Williams, J. P. E. Spencer, and C. Rice-Evans, "Flavonoids: antioxidants or signalling molecules?" *Free Radical Biology & Medicine*, vol. 36, no. 7, pp. 838–849, 2004.

[22] T. Hatano, H. Kagawa, T. Yasuhara, and T. Okuda, "Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects," *Chemical and Pharmaceutical Bulletin*, vol. 36, no. 6, pp. 2090–2097, 1988.

[23] H.-S. Kwon, J. H. Park, D. H. Kim et al., "Licochalcone a isolated from licorice suppresses lipopolysaccharide-stimulated inflammatory reactions in RAW264.7 cells and endotoxin shock in mice," *Journal of Molecular Medicine*, vol. 86, no. 11, pp. 1287–1295, 2008.

[24] H. Haraguchi, H. Ishikawa, K. Mizutani, Y. Tamura, and T. Kinoshita, "Antioxidative and superoxide scavenging activities of retrochalcones in *Glycyrrhiza inflata*," *Bioorganic & Medicinal Chemistry*, vol. 6, no. 3, pp. 339–347, 1998.

[25] J. Pi, Y. Bai, J. M. Reece et al., "Molecular mechanism of human Nrf2 activation and degradation: role of sequential phosphorylation by protein kinase CK2," *Free Radical Biology and Medicine*, vol. 42, no. 12, pp. 1797–1806, 2007.

[26] S. Reuter, S. C. Gupta, M. M. Chaturvedi, and B. B. Aggarwal, "Oxidative stress, inflammation, and cancer: how are they linked?" *Free Radical Biology and Medicine*, vol. 49, no. 11, pp. 1603–1616, 2010.

[27] H. Oh, D.-H. Kim, J.-H. Cho, and Y.-C. Kim, "Hepatoprotective and free radical scavenging activities of phenolic petrosins and flavonoids isolated from *Equisetum arvense*," *Journal of Ethnopharmacology*, vol. 95, no. 2-3, pp. 421–424, 2004.

[28] S. Amoroso, A. D'Alessio, R. Sirabella, G. Di Renzo, and L. Annunziato, "Ca^{2+}-independent caspase-3 but not Ca^{2+}-dependent caspase-2 activation induced by oxidative stress leads to SH-SY5Y human neuroblastoma cell apoptosis," *Journal of Neuroscience Research*, vol. 68, no. 4, pp. 454–462, 2002.

[29] M. S. Cooke, M. D. Evans, M. Dizdaroglu, and J. Lunec, "Oxidative DNA damage: mechanisms, mutation, and disease," *The FASEB Journal*, vol. 17, no. 10, pp. 1195–1214, 2003.

[30] M. M. Cortes-Wanstreet, E. Giedzinski, C. L. Limoli, and U. Luderer, "Overexpression of glutamate-cysteine ligase protects human COV434 granulosa tumour cells against oxidative and γ-radiation-induced cell death," *Mutagenesis*, vol. 24, no. 3, pp. 211–224, 2009.

[31] D. M. Townsend, K. D. Tew, and H. Tapiero, "The importance of glutathione in human disease," *Biomedicine and Pharmacotherapy*, vol. 57, no. 3, pp. 145–155, 2003.

[32] M. G. Vani, K. J. S. Kumar, J.-W. Liao et al., "Antcin C from *Antrodia cinnamomea* protects liver cells against free radical-induced oxidative stress and apoptosis *in vitro* and *in vivo* through nrf2-dependent mechanism," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 296082, 17 pages, 2013.

[33] S. Bhattacharya, R. Gachhui, and P. C. Sil, "Hepatoprotective properties of kombucha tea against TBHP-induced oxidative stress via suppression of mitochondria dependent apoptosis," *Pathophysiology*, vol. 18, no. 3, pp. 221–234, 2011.

[34] L. E. Otterbein, M. P. Soares, K. Yamashita, and F. H. Bach, "Heme oxygenase-1: unleashing the protective properties of heme," *Trends in Immunology*, vol. 24, no. 8, pp. 449–455, 2003.

[35] R. K. Thimmulappa, C. Scollick, K. Traore et al., "Nrf2-dependent protection from LPS induced inflammatory response and mortality by CDDO-Imidazolide," *Biochemical and Biophysical Research Communications*, vol. 351, no. 4, pp. 883–889, 2006.

[36] T. L. Adair-Kirk, J. J. Atkinson, G. L. Griffin et al., "Distal airways in mice exposed to cigarette smoke: Nrf2-regulated genes are increased in Clara cells," *The American Journal of Respiratory Cell and Molecular Biology*, vol. 39, no. 4, pp. 400–411, 2008.

[37] Y. P. Hwang, J. H. Choi, J. M. Choi, Y. C. Chung, and H. G. Jeong, "Protective mechanisms of anthocyanins from purple sweet potato against tert-butyl hydroperoxide-induced hepatotoxicity," *Food and Chemical Toxicology*, vol. 49, no. 9, pp. 2081–2089, 2011.

[38] T. Nguyen, P. J. Sherratt, H.-C. Huang, C. S. Yang, and C. B. Pickett, "Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element: degradation of Nrf2 by the 26 S proteasome," *The Journal of Biological Chemistry*, vol. 278, no. 7, pp. 4536–4541, 2003.

[39] L. M. Zipper and R. T. Mulcahy, "Inhibition of ERK and p38 MAP Kinases inhibits binding of Nrf2 and induction of GCS genes," *Biochemical and Biophysical Research Communications*, vol. 278, no. 2, pp. 484–492, 2000.

[40] P. Gong, B. Hu, and A. I. Cederbaum, "Diallyl sulfide induces heme oxygenase-1 through MAPK pathway," *Archives of Biochemistry and Biophysics*, vol. 432, no. 2, pp. 252–260, 2004.

[41] J. Kim, Y.-N. Cha, and Y.-J. Surh, "A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders," *Mutation Research*, vol. 690, no. 1-2, pp. 12–23, 2010.